Chapter 4

The effect of Cisplatin and Combination treatment

4. The effect of cisplatin and combination treatment

4.1. Introduction

4.1.1. Current therapy for cancer treatment

Introduced in section 1.2.1., cancer treatment has been centred around the use of chemotherapeutic agents since their beneficial use in the 1940s (Gilman 1963, Chabner and Roberts 2005). Since the pioneering work with nitrogen mustard several key agents surfaced including paclitaxel and the camptothecins (Gilman 1963, Chabner and Roberts 2005, Caley and Jones 2012, Galmarini *et al.* 2012). However, of the new drugs that entered to clinical trial during the 1970s to 1990s only 10% of those achieved Food and Drug Administration (FDA) approval (Chabner and Roberts 2005). The most effective use of chemotherapy now is along with surgery either as an adjuvant or neo-adjuvant (Section 1.2.2.) (Caley and Jones 2012, Galmarini *et al.* 2012).

4.1.2. Cisplatin's role as a chemotherapeutic agent

The discovery of cisplatin's beneficial anti-tumour effect in the 1970s and its availability for clinical practice later in that decade had major advantages to several cancer treatments (Section 1.2.2.3.) (Chabner and Roberts 2005, Martins *et al.* 2008, Florea and Busselberg 2011). Cisplatin and other platinum compounds damage tumours through inhibiting DNA synthesis by interacting with the crosslinking mechanism (Jordan and Carmo-Fonseca 2000, Florea and Busselberg 2011). Furthermore, cisplatin has been shown to interact with the mitochondria directly in inducing apoptosis (Yang *et al.* 2006, Martins *et al.* 2008, Marullo *et al.* 2013, Santin *et al.* 2013). This interaction has been seen in increased ROS levels, Ca^{2+} activity alteration and in p53 targeting (Jordan and Carmo-Fonseca 2000, Kawai *et al.* 2006, Bragado *et al.* 2007, Florea and Busselberg 2011, Marullo *et al.* 2013). However, there are many side effects to cisplatin

use including, nephrotoxicity, nausea and vomiting, neurotoxicity and decreased blood cell count which were expanded upon in section 1.2.2.3. (Martins *et al.* 2008, Florea and Busselberg 2011, Galluzzi *et al.* 2012).

4.1.3. The effect of combining two chemotherapeutic agents in cancer therapy

Treatment involving more than one chemotherapeutic agent is seen in most cancer treatment (Section 1.2.2.2.) (Chabner and Roberts 2005, Florea and Busselberg 2011, Caley and Jones 2012, Al-Taweel *et al.* 2014). The treatment of cancer with a combination of agents has been conventional since the pioneering work with ALL (Frei *et al.* 1965, Chabner and Roberts 2005). Combining cisplatin with other agents can enhance the effect of chemotherapy in breast, ovarian and other cancers (Balmaña *et al.* 2014, van Maldegem *et al.* 2015). When combining agents for chemotherapy it is fundamental that each agent can induce cancer cell death when used as a single treatment (Caley and Jones 2012). As seen in section 3. following CBD treatment there was a reduction in cancer cell growth. By examining cisplatin's effect on breast cancer cell viability the potential for increased cell death when combining these two agents is great.

4.2. Aims of this study

- To investigate the mode of action of cisplatin in cancer and control cells.

- Examine the potential of CBD and cisplatin in enhancing cell death when combined.

4.3. The effect of cisplatin and combination treatment on cell viability

4.3.1. Cisplatin treatment's effect on cancer cell's viability

Cisplatin was used as a positive cell death inducer for MDA-MB231 and doses were selected based on previously published data from 50 μM to 150 μM for 24 hours (Section 2.6.3.) (Arisan *et al.* 2010). There was a significant reduction of cell viability (Section 2.7.2.) with all treatment of cisplatin (50 μM cisplatin, 48.11 ± 0.008 %.; 100 μM cisplatin, 41.03 ± 0.131 %; 150 μM cisplatin, 27.13 ± 0.053 %) (p<0.001) (Figure 4.1.).

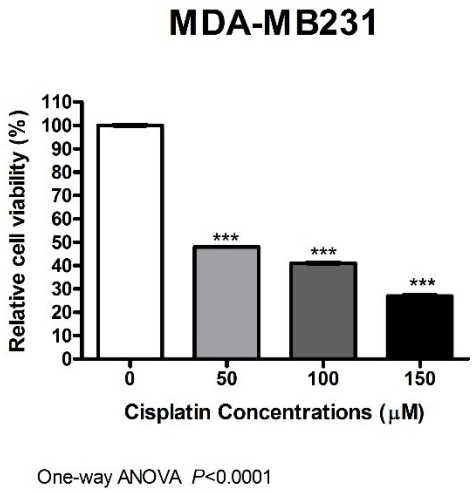

Figure 4.1. The effect of cisplatin on cell viability following 24 hour treatment in cancer cells.
MDA-MB231 cells were treated with cisplatin for 24 hours and consequently assayed for cell viability using an MTT assay. Data shown is repeated 3 times with 5 technical replicates per plate. Data is represented as mean ± SEM. *** p<0.001 *vs* Untreated.

4.3.2. The effect of cisplatin treatment in control cells

The effect of cisplatin was also examined on control cells, where a significant reduction in cell viability was observed; 50 μM cisplatin (90.03 ± 0.283 %), 100 μM cisplatin (37.11 ± 0.078 %), 150 μM cisplatin (26.16 ± 0.086 %) (p<0.001) (Figure 4.2.).

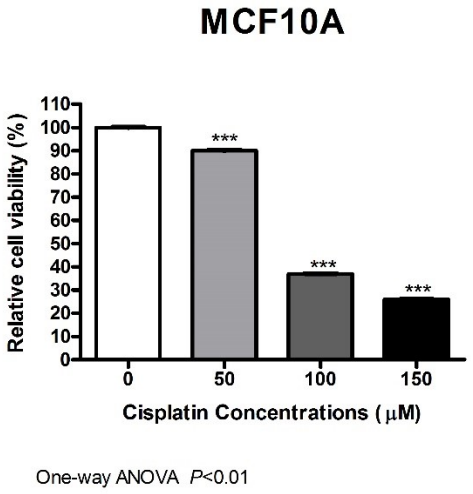

Figure 4.2. Control cell viability following cisplatin treatment for 24 hours.
MCF10A cells were treated with cisplatin for 24 4hours and consequently assayed for cell death using an MTT assay. Data shown is repeated 3 times with 5 technical replicates per plate. Data is represented as mean ± SEM. *** p<0.001 *vs* Untreated.

4.3.3. The effect of CBD and cisplatin combined as a treatment in cancer cells

Based on previous CBD and cisplatin results obtained with cancer and control cells (Sections 3.4.1, 3.4.2., 4.3.1. and 4.3.2.), concentrations of 1 μM CBD and 100 μM cisplatin were chosen to test the potential effect of a combination therapy (Section 2.6.4.). This combination treatment in cancer cells led to a significant reduction in cell viability (72.67 ± 1.453 %) (p<0.01) (Figure 4.3.) using an MTT cell viability assay (Section 2.7.2.). However, the decrease observed with combination was not as pronounced as cisplatin on its own, leading to a suggested “protective” effect (p<0.05).

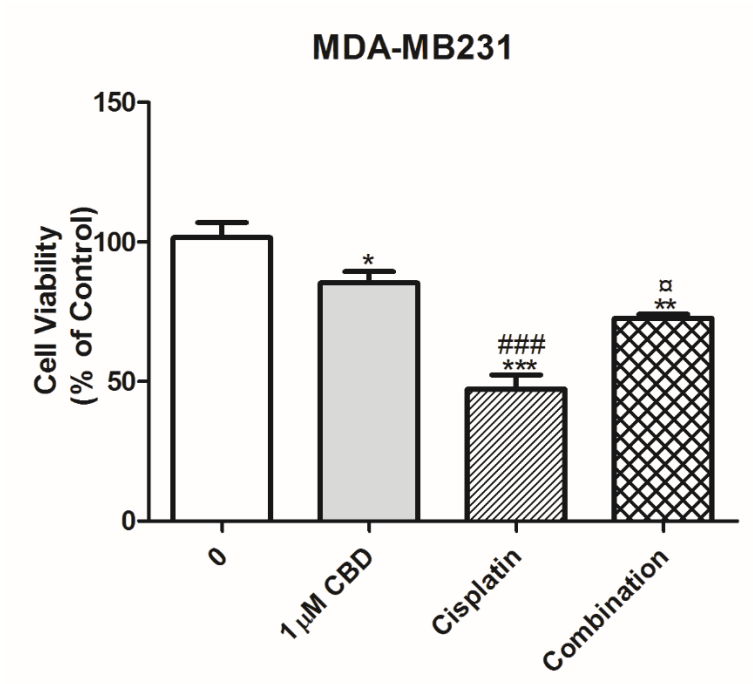

Figure 4.3. The effect of combining CBD and cisplatin on cancer cell viability following 24 hours of treatment.
The combination treatment of 1 μM CBD and 100 μM cisplatin was tested on MDA-MB231 cells and compared to both CBD and cisplatin treatments alone. Data shown is repeated 3 times with 5 technical replicates per plate. Data is represented as mean ± SEM. *p<0.05, **p<0.01, *** p<0.001 *vs* Untreated; ### p<0.001 *vs* 1 μM CBD; ¤ p<0.05 *vs* Cisplatin.

Cell viability was also assessed using a lactate dehydrogenase assay (Section 2.7.3.). There was no change in lactate dehydrogenase concentration following treatment with 1 μM CBD alone (Figure 4.4.). No change was observed following cisplatin (138.6 ± 8.57 %) or combination (192.5 ± 31.46 %) treatments, though there was a trend to increase compared to untreated levels.

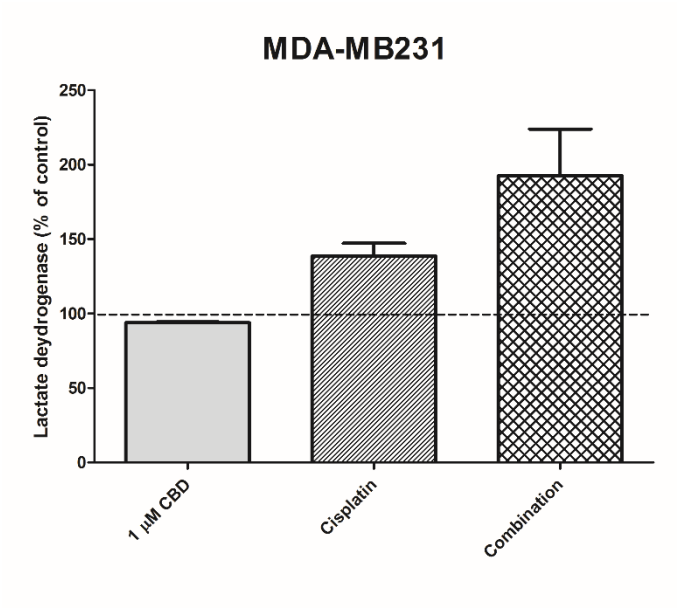

Figure 4.4. Lactate dehydrogenase concentration following treatment with CBD, cisplatin and combination in MDA-MB231 cells.
Cancer cells were tested for lactate dehydrogenase concentration following 1 µM CBD, 100 µM cisplatin and combination treatment for 24 hours. Concentration levels were calculated based on untreated cell levels which is represented by the dashed line. Data shown is repeated 3-5 times with 5 technical replicates per plate. The data was normalised to cell viability. Data is represented as mean ± SEM. ** $p<0.01$ *vs* Untreated.

4.3.4. The effect of combination of CBD and cisplatin treatment in the viability of control cells for 24 hours

The same combination treatment was examined on control cells (Section 2.6.4.). Combination treatment led to a reduction in cell viability compared to untreated cells (70.67 ± 5.457 %) ($p<0.05$) and to 1 µM CBD alone ($p<0.05$) (Figure 4.5.). However, the "protective" effect previously observed with cancer cells was again seen when compared to cisplatin treatment alone ($p<0.05$).

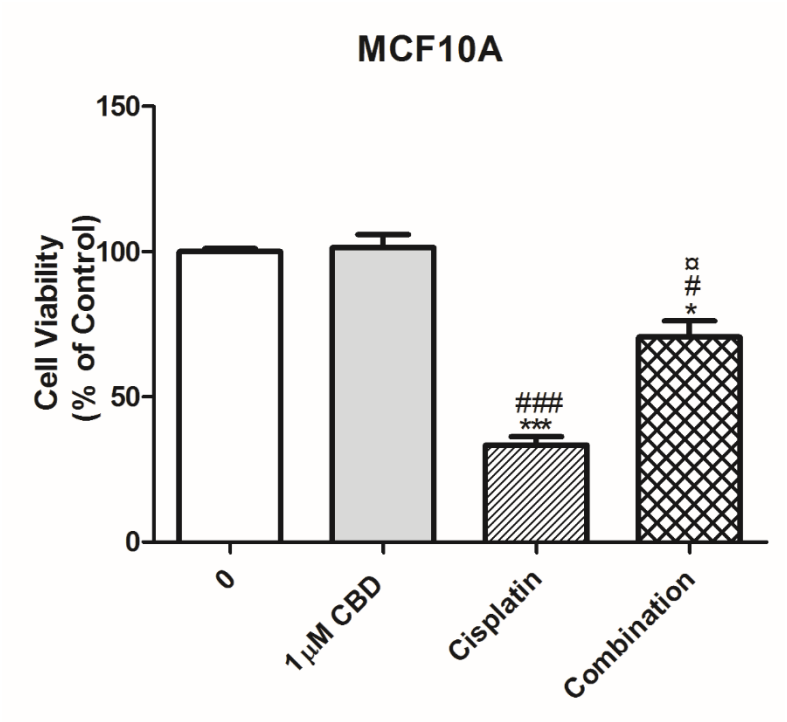

Figure 4.5. Cell Viability of control cells following treatment with CBD, cisplatin and combination for 24 hours.
The combination treatment was examined with MCF10A cells and compared to both CBD and cisplatin treatments alone. Data shown is repeated 3 times with 5 technical replicates per plate. Data is represented as mean ± SEM. *p<0.05, *** p<0.001 *vs* Untreated; # p<0.05, ### p<0.001 *vs* 1 μM CBD; ¤ p<0.05 *vs* Cisplatin.

Cell viability was also assessed using a lactated dehydrogenase assay (Section 2.7.3.). No change was observed in lactate dehydrogenase concentration following CBD treatment, however, a significant increase was observed with both cisplatin (232.81 ± 4.846 %) and combination treatments (147.72 ± 3.330 %) ($p<0.05$) (Figure 4.6.). Cisplatin was also significantly different to CBD ($p<0.001$) and combination ($p<0.001$) treatments. Combination treatment was also significantly increased compared to CBD treatment ($p<0.01$).

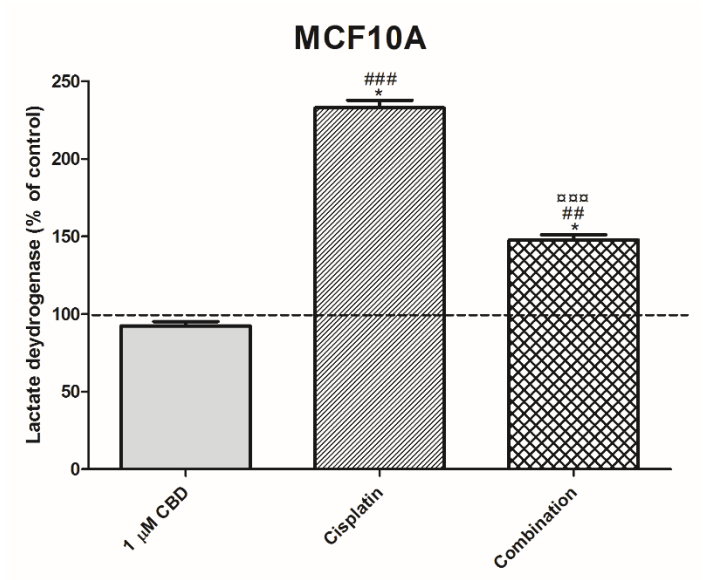

Figure 4.6. Lactate dehydrogenase concentration following CBD, cisplatin and combination treatments in control cells.
Control cells lactate dehydrogenase concentration following 1 μM CBD, 100 μM cisplatin and combination treatment for 24 hours was assessed. Lactate dehydrogenase levels were calculated based on untreated cell levels which is represented by the dashed line. Data shown is repeated 3-5 times with 5 technical replicates per plate. The data was normalised to cell viability. Data is represented as mean ± SEM. * $p<0.05$ *vs* Untreated; ## $p<0.01$, ### $p<0.001$ *vs* CBD; ¤¤¤ $p<0.001$ *vs* Cisplatin.

4.4. Cisplatin and combination treatment's effect on AMPK activity in the cell

AMPK activity levels were measured in cancer and control cells when treated with cisplatin or in combination. AMPK as previously mentioned has a major role in tumour progression (Section 1.1.3. and section 3.5.).

4.4.1. The effect of cisplatin treatment and combination treatment in cancer cell's AMPK activity

AMPK activity was measured following 1 μM CBD, 100 μM cisplatin and combination as described in section 2.8.. MDA-MB231 AMPK activity showed no change following treatment with 1 μM CBD, 100 μM cisplatin or combination compared to untreated cell levels (Figure 4.7.).

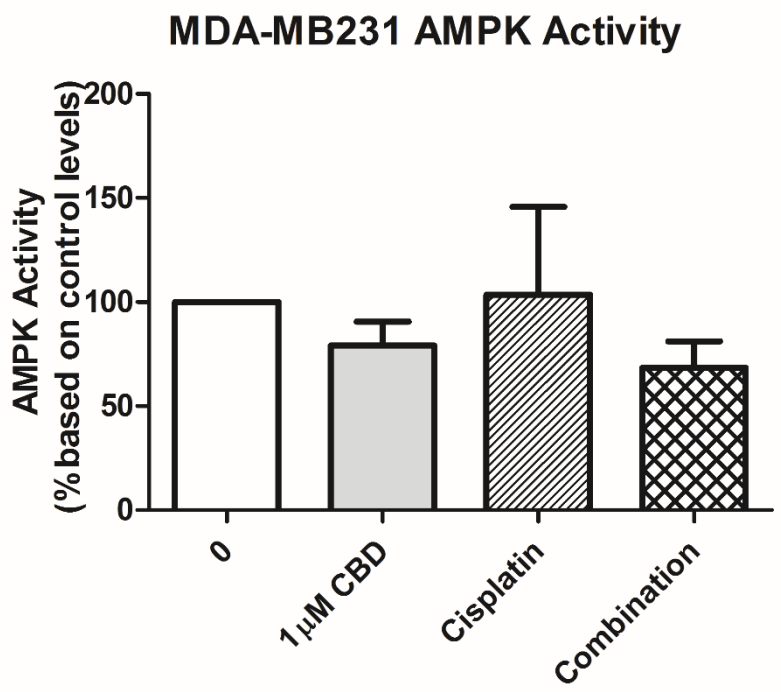

Figure 4.7. MDA-MB231 AMPK activity following CBD, cisplatin and combination treatment.
MDA-MB231 cells were treated with 1 μM CBD, 100 μM cisplatin and a combination of those treatments for 24 hours. The cells were harvested for AMPK and activity analysed. Data shown is repeated 3 - 5 times. Data is represented as mean ± SEM.

4.4.2. The effect of cisplatin treatment and combination treatment on control cell's AMPK activity

Control cell's AMPK activity also showed no difference following treatment with 1 μM CBD or 100 μM cisplatin compared to untreated cell levels (Figure 4.8.). There was however a significant increase in AMPK activity when cells were treated in combination (115.13 ± 3.215 %) ($p<0.05$).

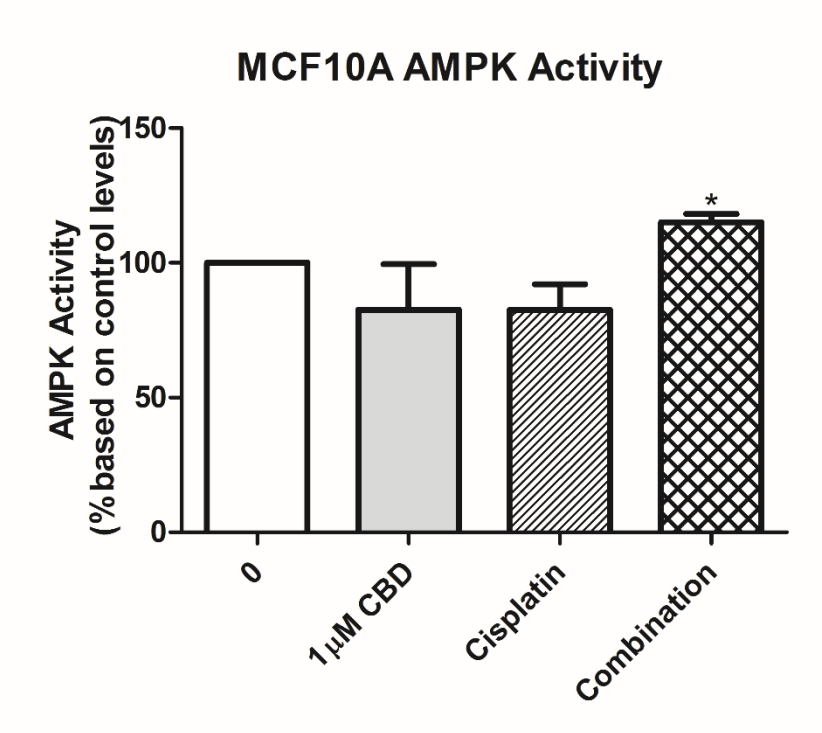

Figure 4.8. The effect of cisplatin treatment and combination treat on control cell's AMPK activity levels.
Control cells were treated with 1 μM CBD, 100 μM cisplatin and a combination of those treatments for 24 hours. The cells were harvested for AMPK and activity analysed after treatment. Data shown is repeated 3 - 5 times. Data is represented as mean ± SEM. * $p<0.05$ *vs* Untreated.

4.5. ROS levels measured following cisplatin treatment and combination treatment

Changes in ROS, important regulators of many cellular pathways, were observed when cancer and normal cells were treated with CBD (Section 3.6.). Cisplatin induces cell death through increased cellular stress due to amplified ROS generation (Section 1.2.2.3.) (Florea and Busselberg 2011, Prevarskaya *et al.* 2011). Determining if the cancer cells respond accordingly is important in order to understand the mechanism behind the combination's effect on both cancer and control cells.

4.5.1. The effect of cisplatin treatment and combination treatment on cancer cell's ROS levels

MDA-MB231 cancer cells showed a significant elevation in ROS levels using a DCFDA assay (Section 2.9.) following treatment with 1 μM CBD (previously reported in section 3.6.1.) (1.11 ± 0.039 A.U.) ($p<0.05$) (Figure 4.9.). Cisplatin treatment resulted in a significant increase in ROS levels compared to untreated cells (7.11 ± 0.150 A.U.) ($p<0.001$) and CBD treated cells ($p<0.001$). Combination therapy again showed a significant increase in ROS compared to untreated cells (1.89 ± 0.069 A.U.) ($p<0.001$) and 1 μM CBD ($p<0.01$). Although, the increase observed was significantly decreased compared to cisplatin treatment ($p<0.001$).

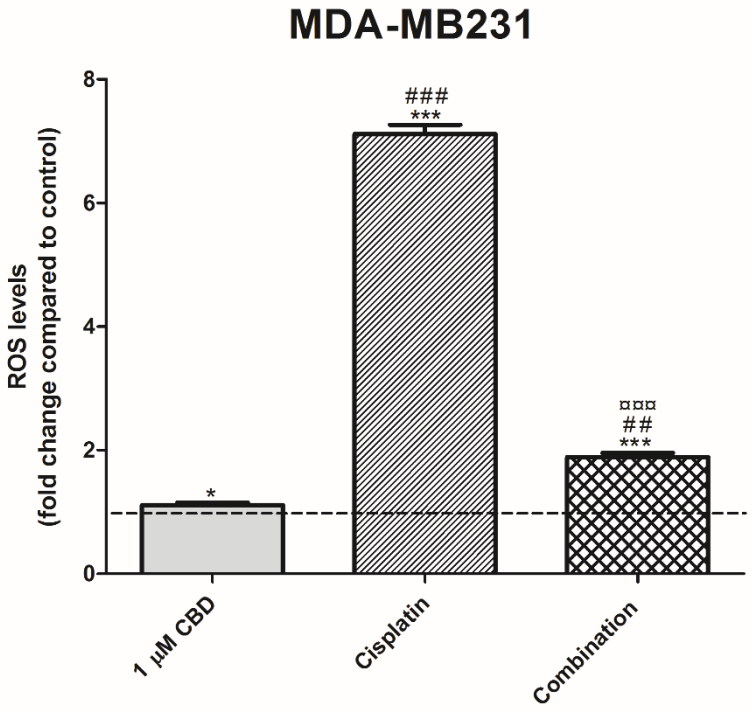

Figure 4.9. The effect of CBD, cisplatin and combination treatment in ROS levels of MDA-MB231 cells.
ROS levels were measured with MDA-MB231 cells following 1 µM CBD, 100 µM cisplatin and a combination of those treatments for 24 hours. The media was analysed after these treatments. Data shown is repeated 3 times with 5 technical replicates per plate. The dashed line represents ROS levels of untreated cells. Data is represented as mean ± SEM. * $p<0.05$, *** $p<0.001$ *vs* Untreated; ## $p<0.01$, ### $p<0.001$ *vs* CBD; ¤¤¤ $p<0.001$ *vs* Cisplatin.

4.5.2. The effect of cisplatin treatment and combination treatment in control cell's ROS levels

No difference in ROS levels were detected following 1 µM CBD treatment (Figure 4.10.). However, a significant increase in ROS levels was observed following treatment with cisplatin, similar to that observed in cancer cells (section 4.5.1.) (7.15 ± 0.927 A.U.) ($p<0.01$). When control cells were treated with combination therapy there was no change in ROS levels compared untreated cells.

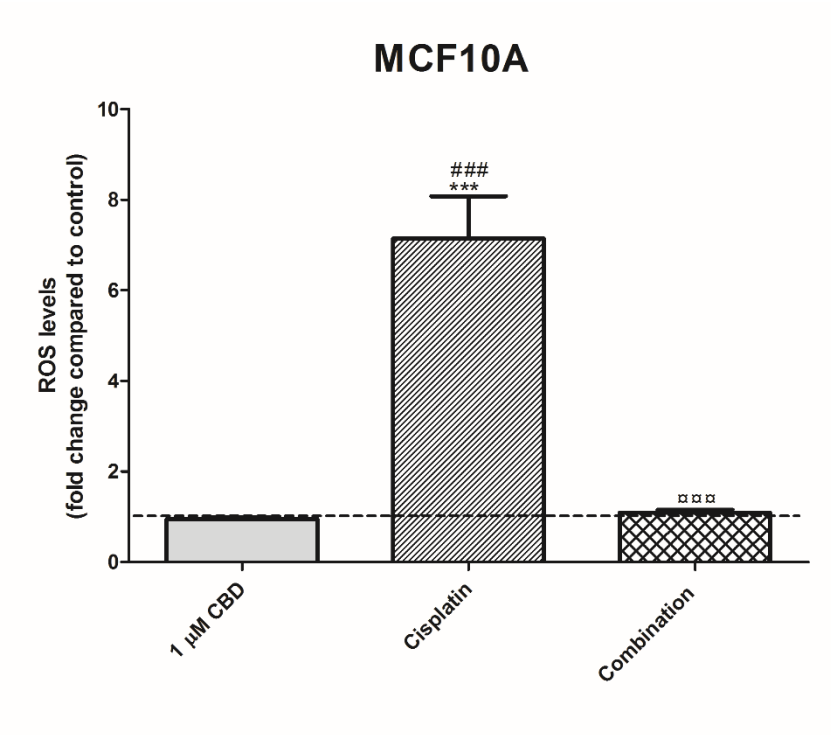

Figure 4.10. The effect of CBD, cisplatin and combination treatment in ROS levels in control cells.
Control cell's ROS levels were analysed in the media following 1 µM CBD, 100 µM cisplatin and combination treatment. Data shown is repeated 3 times with 5 technical replicates per plate. The dashed line represents ROS levels of untreated cells. Data is represented as mean ± SEM. *** $p<0.001$ *vs* Untreated; ### $p<0.001$ *vs* CBD; ¤¤¤ $p<0.001$ *vs* Cisplatin.

4.6. Cisplatin treatment and combination treatment's effect on cellular Ca^{2+} activity

As previously mentioned Ca^{2+} regulation is important when investigating modulators of cell death (Section 1.1.3.3.). An upregulation of Ca^{2+} was observed following CBD treatment in section 3.7. for both cell types. Therefore it was important to investigate whether cisplatin and combination treatment could also have an effect on Ca^{2+} levels.

4.6.1. The levels of Ca^{2+} in cancer cells was measured following cisplatin treatment and combination treatment

Ca^{2+} concentration levels were measured following cisplatin and combination treatment by using a Fluo-4 Direct™ Calcium Assay (Section 2.10.). An increase in calcium levels was observed following CBD treatment compared to untreated cells (1.18 ± 0.038 A.U.) ($p<0.001$) (Figure 4.11.). 100 μM cisplatin treatment also showed an increase in calcium levels (1.52 ± 0.157 A.U.) compared to untreated cells ($p<0.05$) and to CBD treatment levels ($p<0.05$). However, following combination treatment (0.72 ± 0.086 A.U.) a significant decrease in calcium levels was observed compared to untreated calcium levels ($p<0.05$), CBD ($p<0.01$) and cisplatin treatment ($p<0.01$).

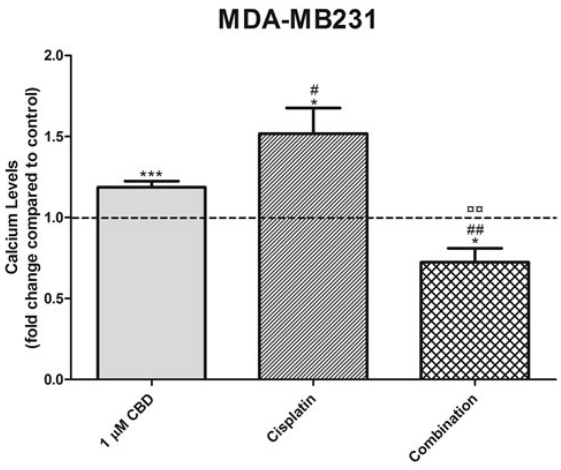

Figure 4.11. The effect of CBD, cisplatin and combination treatment on calcium levels in MDA-MB231 cells.
MDA-MB231 cells were tested for any changes in Ca^{2+} levels following 24 hours of 1 µM CBD, 100 µM cisplatin and combination treatments. Data shown is repeated 3 times with 5 technical replicates per plate. The dashed line represents calcium levels of untreated cells. Data is represented as mean ± SEM. * $p<0.05$, *** $p<0.001$ *vs* Untreated; # $p<0.05$, ## $p<0.01$ *vs* CBD; ¤¤¤ $p<0.001$ *vs* Cisplatin.

4.6.2. The effect of cisplatin treatment and combination treatment in Ca^{2+} levels in control cells

There was a significant increase in Ca^{2+} levels following 1 µM CBD treatment (1.12 ± 0.018 A.U.) ($p<0.01$) compared to untreated cells (Figure 4.12.). Cisplatin also showed an increase in Ca^{2+} levels (4.91 ± 0.457 A.U.) compared to untreated cells ($p<0.01$) and CBD alone ($p<0.01$). Following combination treatment, an increase in Ca^{2+} levels (3.25 ± 0.303 A.U.) was observed compared to untreated cells ($p<0.01$) but the Ca^{2+} levels were significantly reduced compared to cisplatin treatment alone ($p<0.001$).

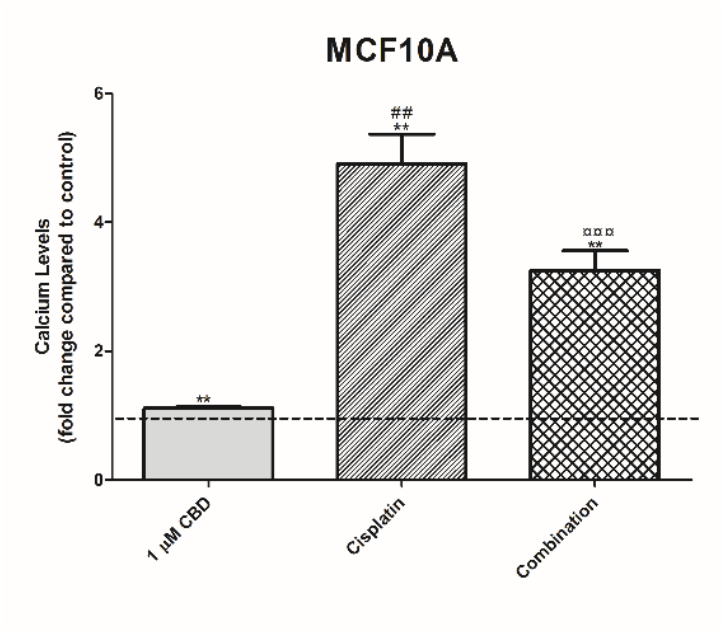

Figure 4.12. The effect of CBD, cisplatin and combination treatment on calcium levels in control cells.
Control cells were analysed for any changes in Ca^{2+} levels following 24 hours of 1 µM CBD, 100 µM cisplatin and combination treatment. Data shown is repeated 3 times with 5 technical replicates per plate. The dashed line represents calcium levels of untreated cells. Data is represented as mean ± SEM. ** $p<0.01$ *vs* Untreated; ## $p<0.01$ *vs* CBD; ¤¤¤ $p<0.001$ *vs* Cisplatin.

4.7. The Mitochondria's function was assessed following cisplatin treatment and combination treatment.

The importance of the mitochondria was highlighted in section 1.1.3.1. and 1.3.4. and was investigated following CBD treatment in section 3.8.. The differences observed here following cisplatin and combination treatment with AMPK, ROS and calcium pointed to the importance of understanding the role of the mitochondria further.

4.7.1. The effect of cisplatin treatment and combination treatment on the mitochondria's function in cancer cells

Mitochondrial analysis was carried out as in section 3.8. using a Seahorse Bioanalyser to investigate mitochondrial respiration (Section 2.11.). As already reported in section 3.7.1. an increase in basal mitochondrial respiration was observed following treatment with 1 μM CBD (411.1 ± 28.19 pMoles/min) (Untreated; 323.1 ± 41.95 pMoles/min) ($p<0.05$) (Figure 4.13.A). However, no change in basal mitochondrial respiration was observed with cisplatin or combination treatment. Non-mitochondrial respiration showed a reduced capacity with 1 μM CBD compared to untreated cells (279.6 ± 31.92 pMoles/min) (Untreated; 499.1 ± 99.19 pMoles/min) ($p<0.05$) (Figure 4.13.B.). This reduction was also observed following treatment with 100 μM cisplatin (175.0 ± 25.29 pMoles/min) ($p<0.001$) and the combination treatment (229.9 ± 61.53 pMoles/min) ($p<0.01$).

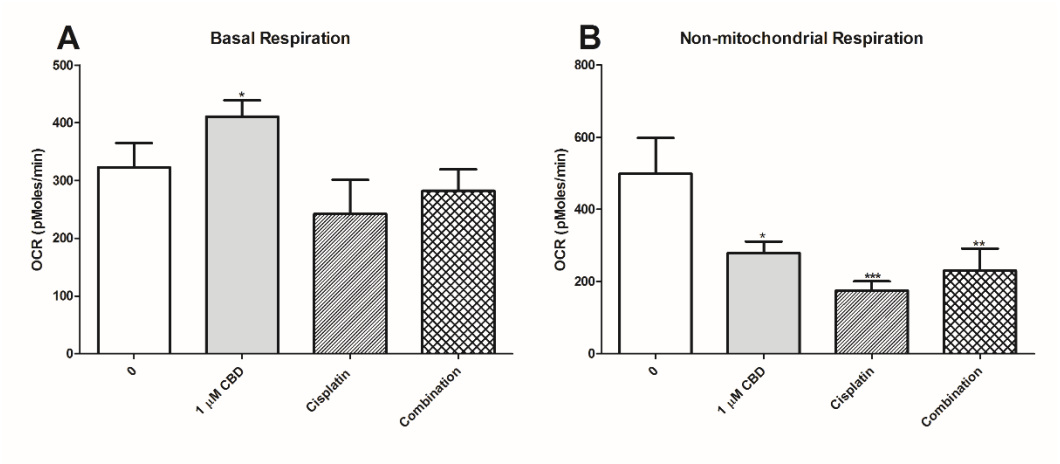

Figure 4.13. The effect of CBD, cisplatin and combination treatment on mitochondrial respiration and non-mitochondrial respiration in cancer cells.
MDA-MB231 cells **(A)** mitochondrial respiration and **(B)** non-mitochondrial respiration was analysed compared to untreated cells. This was carried out following 1 μM CBD, 100 μM cisplatin and combination treatment for 24 hours. Data shown is repeated 3-4 times with 4 technical replicates per plate. The data was normalised to protein level. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs* Untreated.

Alterations within mitochondrial functions were also assessed. An increase in relative ATP production levels was observed when the cells were treated with 1 μM CBD (358.2 ± 17.30 pMoles/min) (Untreated; 243.5 ± 36.93 pMoles/min) ($p<0.01$) (Figure 4.14.A.). No changes in ATP was observed following treatment with cisplatin or with combination treatment. Although, a significant difference was observed between CBD and cisplatin treated cells ($p<0.05$). There was no change in the cells proton leak following treatment with 1 μM CBD, cisplatin or combination treatment (Figure 4.14.B). The cells maximal respiration potential was significantly increased following treatment with 1 μM CBD (625.5± 70.17 pMoles/min) compared to untreated cells (406.2 ± 45.56 pMoles/min) ($p<0.05$), cisplatin ($p<0.01$) and combination treatment ($p<0.01$) (Figure 4.14.C.). Following combination treatment there was a significant reduction in the cell's maximal respiration (319.8 ± 34.27 pMoles/min) compared to untreated cells ($p<0.05$). However, there was no change following cisplatin treatment. There was no change in the spare respiration capacity of the cell following treatment with 1 μM CBD (Figure 4.14.D.). However, cisplatin treatment showed a significant reduction in the spare

respiration capacity (58.9 ± 22.82 pMoles/min) compared to untreated cells (149.5 ± 34.56 pMoles/min) ($p<0.01$). Furthermore, no change was observed following the combination treatment.

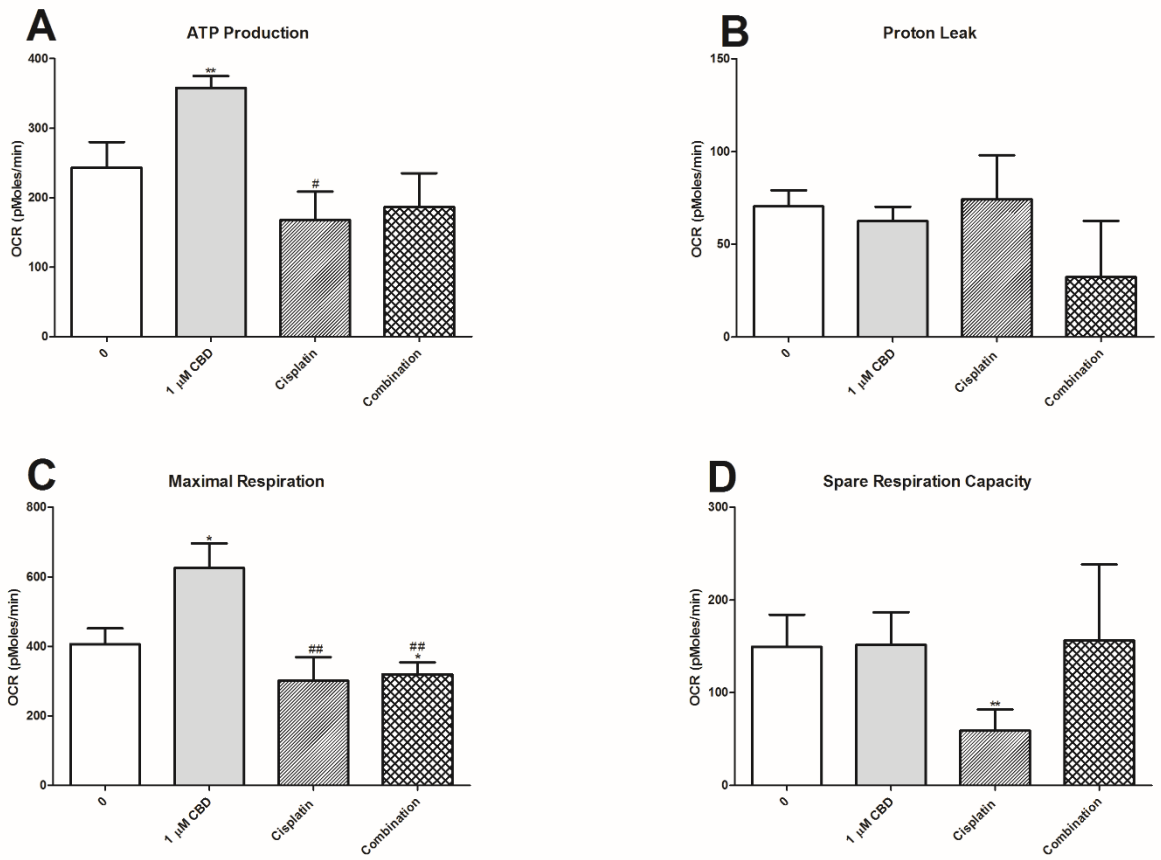

Figure 4.14. Mitochondrial functional analysis following CBD, cisplatin and combination treatment on MDA-MB231 cells.
When MDA-MB231 cells were treated with 1 μM CBD, 100 μM cisplatin and combination treatment their mitochondrial function was tested directly afterwards to observe any potential alteration. **(A)** ATP production levels, **(B)** proton leak, **(C)** maximal respiration and **(D)** spare respiration capacity was analysed. Data shown is repeated 3-4 times with 4 technical replicates per plate. The data was normalised to protein level. Data is represented as mean ± SEM. * $p<0.05$, **$p<0.01$ *vs* Untreated; # $p<0.05$, ## $p<0.01$ *vs* CBD.

4.7.2. The effect of cisplatin treatment and combination treatment on the mitochondrial function of control cells

Control cells basal mitochondrial respiration was unchanged following treatment with 1 μM CBD compared to the untreated cells but 100 μM cisplatin showed a significant reduction (295.8 ± 36.86 pMoles/min) compared to untreated cells (508.4 ± 56.75 pMoles/min) ($p<0.001$) (Figure 4.15.A.). No change was observed following the combination treatment. The corresponding non-mitochondrial respiration showed a significant increase in OCR when the control cells were treated with 1 μM CBD (430.9 ± 18.69 pMoles/min) compared to untreated cells (348.1 ± 64.52 pMoles/min) ($p<0.05$) (Figure 4.15.B.). There was a significant reduction in non-mitochondrial OCR when the cells were treated with cisplatin (208.1 ± 31.48 pMoles/min) compared to untreated cells ($p<0.01$) and CBD alone ($p<0.05$). There was no alteration when cells were treated with combination treatment.

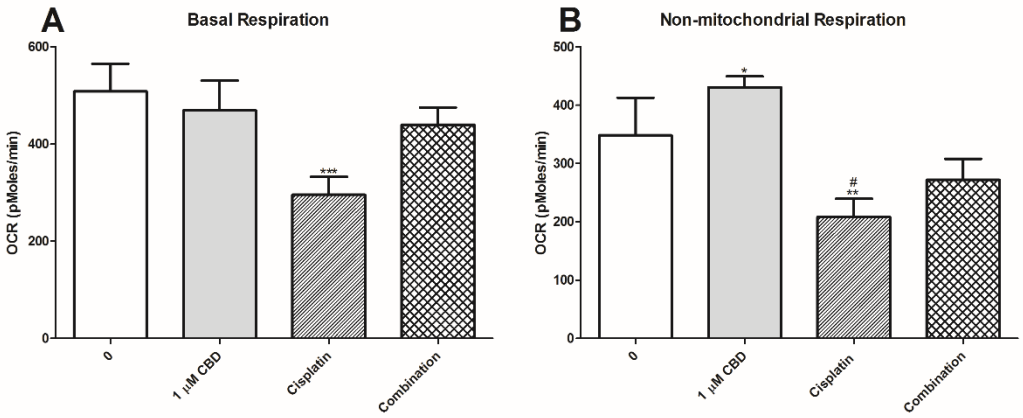

Figure 4.15. The effect of mitochondrial and non-mitochondrial respiration following treatment with cisplatin and combination treatment in control cells.
The alteration in **(A)** mitochondrial respiration and **(B)** non-mitochondrial respiration with control cells was analysed following 1 μM CBD, 100 μM cisplatin and combination treatments for 24 hours. Data shown is repeated 3-4 times with 4 technical replicates per plate. The data was normalised to protein level. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs* Untreated; # $p<0.05$ *vs* CBD.

The function of the mitochondria was also analysed and showed that there was no change in ATP production levels following treatment with CBD, cisplatin or in combination compared to untreated cells (Figure 4.16.A.). However, a significant reduction in proton leak was observed following 1 μM CBD (26.7 ± 4.26 pMoles/min) ($p<0.001$), cisplatin (64.4 ± 11.14 pMoles/min) ($p<0.001$) and combination (91.9 ± 16.72 pMoles/min) ($p<0.001$) treatments compared to untreated cells (184.8 ± 23.21 pMoles/min) (Figure 4.16.B.). There was also a significant difference in proton leak capacity between CBD treatment alone and the combination treatment ($p<0.05$). A significant reduction in the cell's maximal respiration capacity was observed following cisplatin treatment ($p<0.05$) (499.7 ± 101.03 pMoles/min) compared to untreated cells (751.7 ± 189.53 pMoles/min) (Figure 4.16.C.). There was no change in maximal respiration following combination treatment. The control cell's spare respiration capacity showed no significant change following cisplatin or combination treatment compared to untreated cells (Figure 4.16.D.).

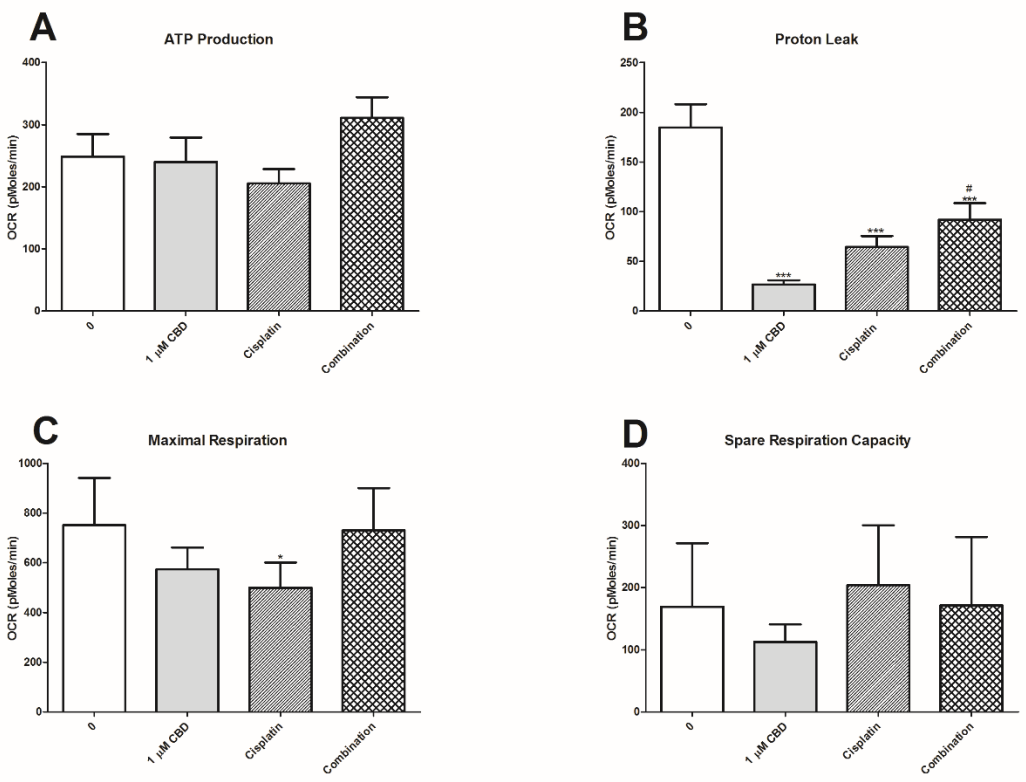

Figure 4.16. The effect of CBD, cisplatin and combination treatment on mitochondrial functional in control cells.
Control cells were treated with 1 μM CBD, 100 μM cisplatin and combination treatment prior to their mitochondrial function was tested to observe any potential alteration. **(A)** ATP production levels, **(B)** proton leak, **(C)** maximal respiration and **(D)** spare respiration capacity was analysed. Data shown is repeated 3-4 times with 4 technical replicates per plate. The data was normalised to protein level. Data is represented as mean ± SEM. * $p<0.05$, *** $p<0.001$ *vs* Untreated; # $p<0.05$ *vs* CBD.

4.8. The effect of cisplatin treatment and combination treatment on glycolysis in the cell

The alteration of the glycolytic cycle was important to understand in the investigation of cisplatin and combination's mode of action as cancer cells alter glycolytic activity (Section 1.1.3.). CBD treatment on its own was shown to alter the "Warburg Effect" switching the cancer cells from glycolytic respiration to mitochondrial respiration (Section 3.8. and section 3.9.). Exploring the role of glycolysis in the cell is an important step in the investigation of cisplatin and combination's mode of action.

4.8.1. The effect of cisplatin treatment and combination treatment on cancer cell's glycolytic functions

The cell's glycolytic function was assessed using the Seahorse Bioanalyser's glycotest (Section 2.11.). Following CBD treatment (section 3.8.1.), an increase in glycolysis was observed with 1 μM CBD (54.2 ± 4.127 mpH/min) compared to untreated cells (35.4 ± 2.47 mpH/min) ($p<0.001$) (Figure 4.17.A.). However, a significant decrease in glycolysis was observed following treatment with cisplatin (19.9 ± 1.30 mpH/min) ($p<0.001$) and combination (17.2 ± 3.55 mpH/min) ($p<0.01$). Both cisplatin and combination treatment was also significantly reduced compared to CBD treated cells ($p<0.001$). The glycolytic capacity of the cell was unchanged following CBD treatment however there was a significant reduction with both cisplatin (23.4 ± 2.34 mpH/min) ($p<0.001$) and combination treatment (23.1 ± 5.06 mpH/min) ($p<0.001$) compared to untreated cells (73.4 ± 6.45 mpH/min) (Figure 4.17.B.). Again, these differences were significantly reduced compared to CBD treatment alone ($p<0.001$). The cells glycolytic reserve was unchanged following CBD treatment but was reduced with cisplatin (2.2 ± 1.86 mpH/min) and combination treatment (8.5 ± 2.24 mpH/min) (Untreated, 42.5 ± 7.30 mpH/min) ($p<0.001$) (Figure 4.17.C.). Cisplatin treatment was significantly reduced compared to CBD treatment also ($p<0.05$). The non-glycolytic acidification rate of the

cancer cells was significantly reduced following 1 μM CBD treatment (7.8 ± 1.24 mpH/min) ($p<0.001$) compared to untreated cells (16.4 ± 1.81 mpH/min) and cisplatin treatment ($p<0.05$) (Figure 4.17.D.). However, there was no change when the cells were treated with cisplatin or combination treatment.

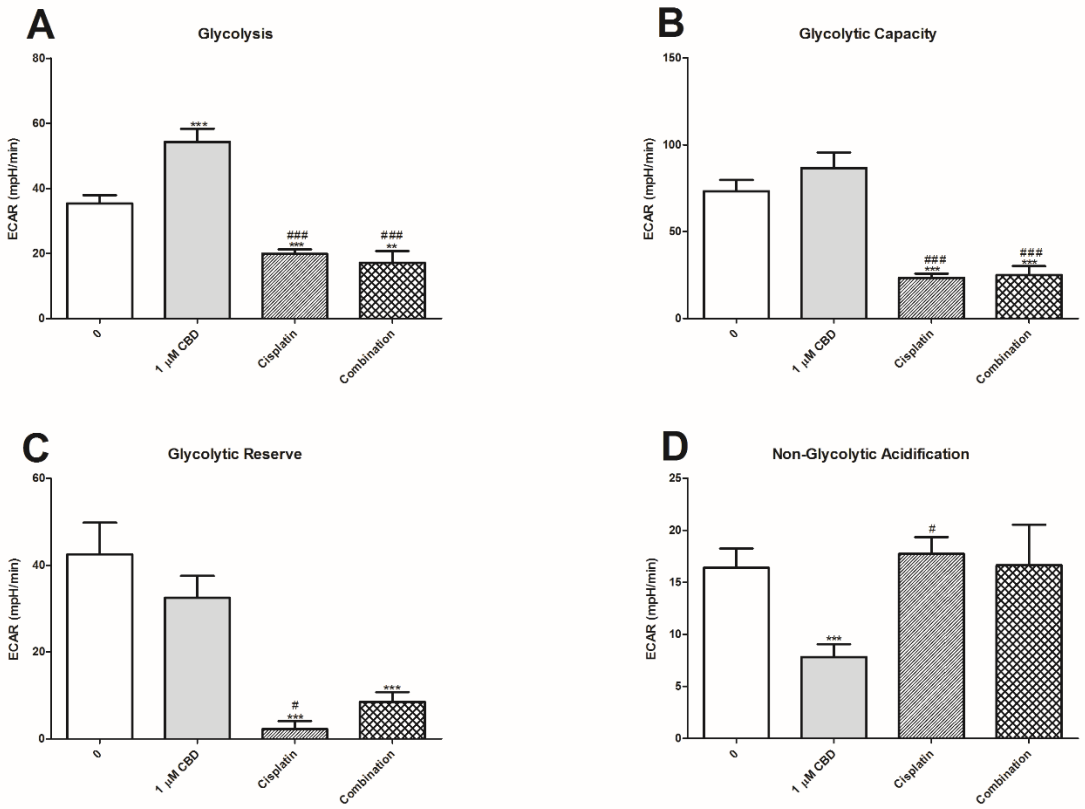

Figure 4.17. The effect of CBD, cisplatin and combination treatment on the glycolysis of MDA-MB231 cells.
Following treatment with 1 μM CBD, 100 μM cisplatin and combination for 24 hours the cells glycolytic activity was measured. **(A)** Glycolysis, **(B)** glycolytic capacity, **(C)** glycolytic reserve and **(D)** non-glycolytic acidification rate were examined. Data shown is repeated 3-4 times with 4 technical replicates per plate. The data was normalised to protein level. Data is represented as mean ± SEM. ** $p<0.01$, *** $p<0.001$ *vs* Untreated; # $p<0.05$. ### $p<0.001$ *vs* CBD.

4.8.2. The effect of cisplatin treatment and combination treatment in control cell's glycolytic functions

An increase in control glycolysis levels was observed following treatment with 1 μM CBD (56.7 ± 3.11 mpH/min) ($p<0.01$) (Untreated, 40.4 ± 2.99 mpH/min) (Figure 4.18.A.). However, a decrease in glycolysis was observed following cisplatin (5.2 ± 0.42 mpH/min) ($p<0.001$) and combination treatment (12.2 ± 1.62 mpH/min) ($p<0.001$) compared to untreated cells. Both treatments were also significantly reduced compared to CBD treatment alone ($p<0.001$). The control cells glycolytic capacity was reduced following CBD (98.1 ± 11.41 mpH/min) ($p<0.01$), cisplatin (46.6 ± 3.34 mpH/min) and combination treatments (57.3 ± 4.04 mpH/min) ($p<0.001$) (Untreated, 123.5 ± 6.34 mpH/min) (Figure 4.18.B.). Again, compared to CBD treatment alone there was a significant reduction in cisplatin ($p<0.001$) and combination ($p<0.05$) glycolytic capacity levels. A significant decrease in glycolytic reserve was observed following 1 μM CBD (48.5 ± 5.53 mpH/min) ($p<0.01$), cisplatin (42.1 ± 3.23 mpH/min) ($p<0.001$) and combination treatment (44.2 ± 2.67 mpH/min) ($p<0.001$) compared to untreated cells (83.1 ± 7.19 mpH/min) (Figure 4.18.C.). Control cell's non-glycolytic acidification rate was significantly reduced with 1 μM CBD (7.0 ± 0.59 mpH/min) ($p<0.001$) and combination treatment (9.6 ± 0.85 mpH/min) ($p<0.01$) (Untreated, 13.2 ± 0.71 mpH/min) (Figure 4.18.D.). There was no change following cisplatin treatment but a significant difference was observed between CBD and cisplatin treatment alone ($p<0.05$).

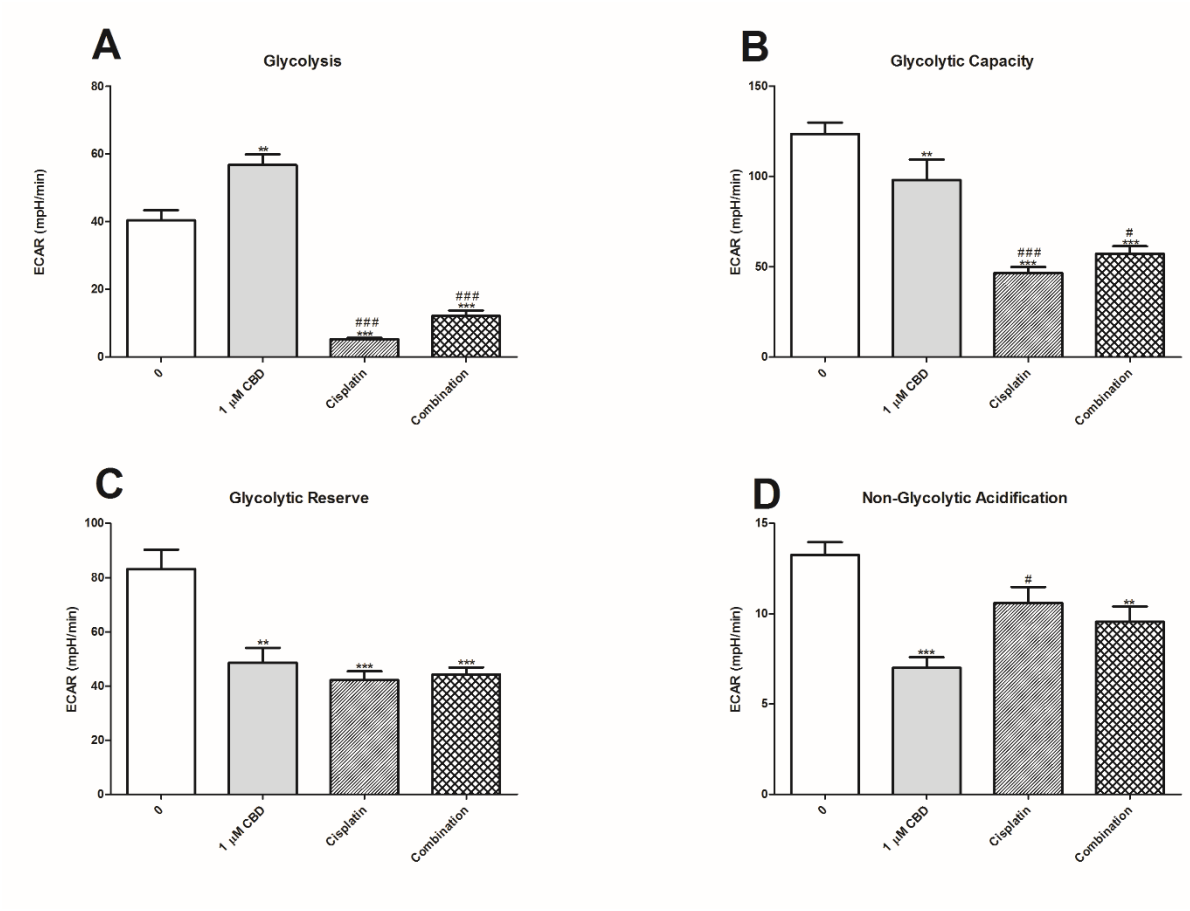

Figure 4.18. The effect of CBD, cisplatin and combination treatment on the glycolytic regulation of control cell.
Following 1 µM CBD, 100 µM cisplatin and the combination treatment the cells were directly assayed for any alterations in the glycolytic pathway. **(A)** Glycolysis, **(B)** glycolytic capacity, **(C)** glycolytic reserve and **(D)** non-glycolytic acidification rate were observed. Data shown is repeated 3-4 times with 4 technical replicates per plate. The data was normalised to protein level. 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.

4.9. The mitochondrial receptor, VDAC1's concentration following cisplatin treatment and combination treatment

The VDAC1 receptor is known to play a pivotal role in the response of mitochondria to cellular stress (Section 1.4.4.) (Madesh and Hajnóczky 2001, Shoshan-Barmatz *et al.* 2010, Tewari *et al.* 2015). Both cisplatin and CBD have been shown to increase VDAC1 activity (Rimmerman *et al*. 2013, Yang *et al*. 2006). VDAC1 concentrations levels were analysed using western blot (Section 2.14.).

4.9.1. The effect of cisplatin treatment and combination treatment on VDAC1 levels in cancer cells

An increase in VDAC1 protein levels was seen following treatment with 1 μM CBD (1.45 ± 0.111 R.I) ($p<0.05$) (Figure 4.19.). No difference in VDAC1 expression levels were observed between 100 μM cisplatin and untreated cells. However, levels of VDAC1 were significantly lowered following combination treatment (0.28 ± 0.039 R.I.) compared to the control ($p<0.001$), CBD ($p<0.01$) and 100 μM cisplatin ($p<0.05$).

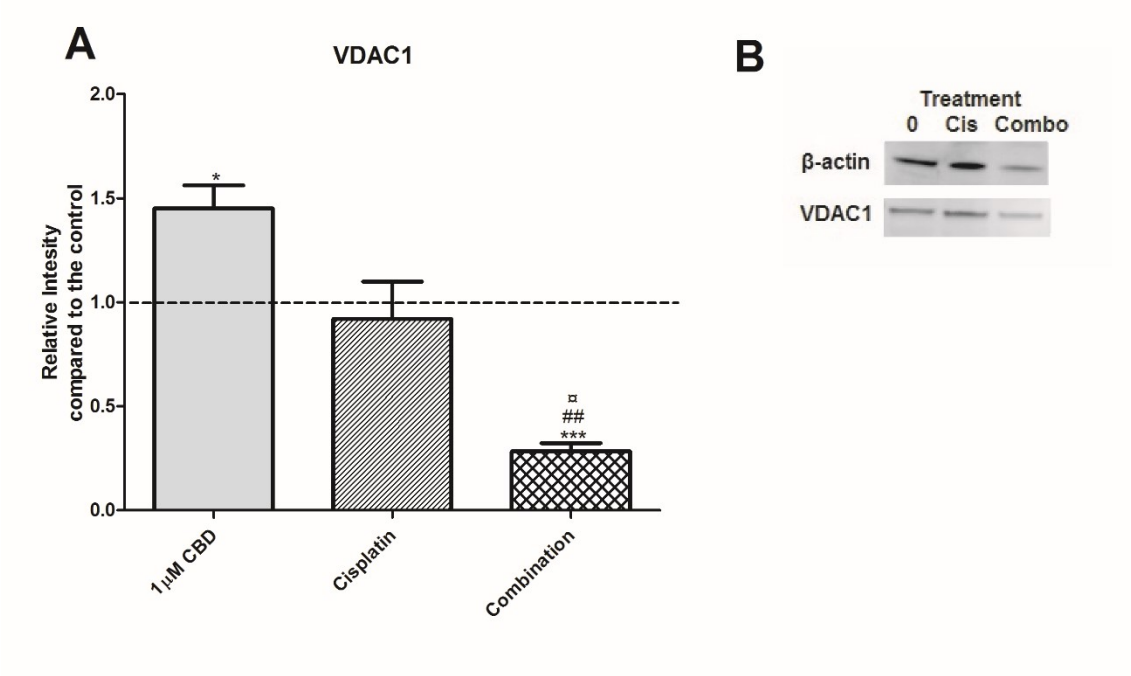

Figure 4.19. The effect of CBD, cisplatin and combination treatment on VDAC1 protein levels in cancer cells.
(A) MDA-MB231 cells following 1 μM CBD, 100 μM cisplatin and combination treatment for 24 hours. The cells were then harvested and tested for VDAC1 protein expression and compared to untreated levels. Data shown is repeated 3 - 5 times with the dashed line representing untreated cells. **(B)** Western blot of relevant graphs with untreated (0) and control of β-actin. Data is represented as mean ± SEM. *** $p<0.001$ *vs* Untreated; ## $p<0.01$ *vs* 1 μM CBD; ¤ $p<0.05$ *vs* Cisplatin.

4.9.2. Cisplatin treatment and combination treatment's effect on VDAC1 levels in control cells

A significant reduction in VDAC1 protein levels was observed following 1 μM CBD (0.79 ± 0.022 R.I.) ($p<0.001$), 100 μM cisplatin (0.37 ± 0.048 R.I.) ($p<0.001$) and combination (0.38 ± 0.083 R.I.) ($p<0.01$) treatment compared to untreated cells (Figure 4.20.).

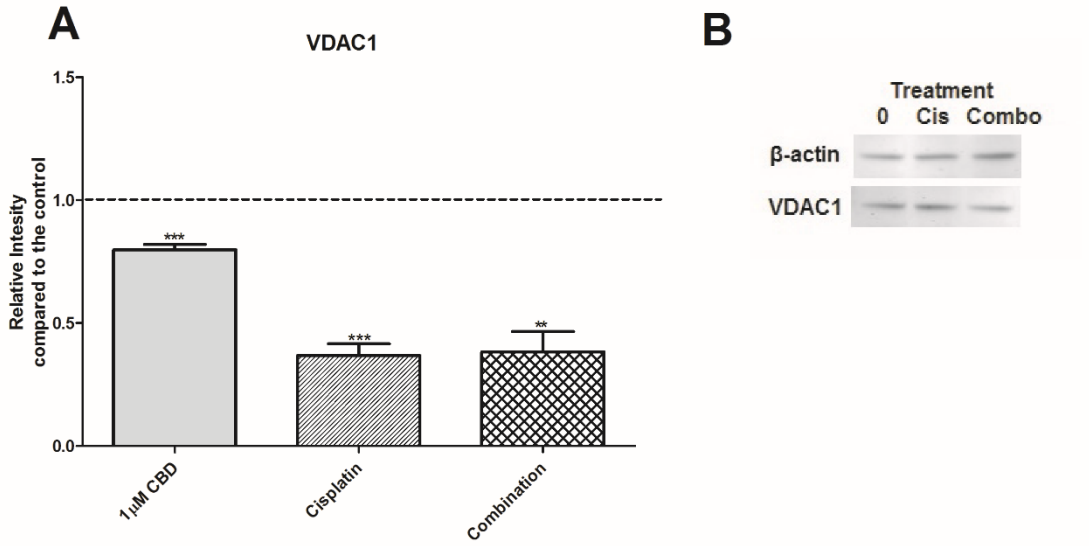

Figure 4.20. CBD, cisplatin and combination treatment's effect on control cell's VDAC1 protein levels.
(A) Control cells following 1 μM CBD, 100 μM cisplatin and combination treatment for 24 hours. The cells were harvested and examined for VDAC1 protein expression and compared to untreated levels. Data shown is repeated 3 - 5 times with the dashed line representing untreated VDAC1 levels. **(B)** Western blot of relevant graphs with untreated (0) and control of β-actin. Data is represented as mean ± SEM. **$p<0.01$, *** $p<0.001$ *vs* Untreated.

4.10. Cisplatin treatment and combination treatment's effect on cellular genetics

Cisplatin is known to damage the DNA cross linking mechanism of cells and hence affect genetic control (Section 1.2.2.3.) (Takahara *et al.* 1995, Arisan *et al.* 2010). Following CBD treatment there was an alteration in the gene expression of key pathways that regulate cell death (p53 (Section 1.4.3.) and Nrf2 (Section 1.1.3.4.1.)) and cell growth (AKT) (Section 3.11.). By using qPCR and normalising to the relevant housekeeping gene (Section 2.12.) the same pathways as in section 3.11. were analysed following cisplatin and combination treatment.

4.10.1. The effect of cisplatin treatment and combination treatment on cancer cell's gene expression

4.10.1.1. Cisplatin treatment and combination treatments' effect on the p53 pathway's gene expression in cancer cells

As mentioned previously in section 3.11.1.1. the pro-apoptotic p53 pathway was analysed along with its regulators HDAC1 and SirT1.

There was a significant reduction in HDAC1 expression following treatment with 1 μM CBD (0.32 ± 0.049 R.I.) ($p<0.001$) and combination (0.46 ± 0.041 R.I.) ($p<0.01$) compared to untreated levels (Figure 4.21.A.). A trend to increased HDAC1 gene expression levels was observed following cisplatin treatment.

SirT1 expression was increased following 1 μM CBD treatment (586.02 ± 140.211 R.I.) compared to untreated cells ($p<0.05$) (Figure 4.21.B.). No change was observed following cisplatin or combination treatment compared to untreated cell levels, however, they were both significantly different to CBD treatment alone ($p<0.001$).

A significant increase in the gene expression levels of p53 was observed following 1 μM CBD treatment (1.53 ± 0.103 R.I.) ($p<0.05$) (Figure 4.21.C.). No change in p53 expression was shown following treatment with cisplatin or combination.

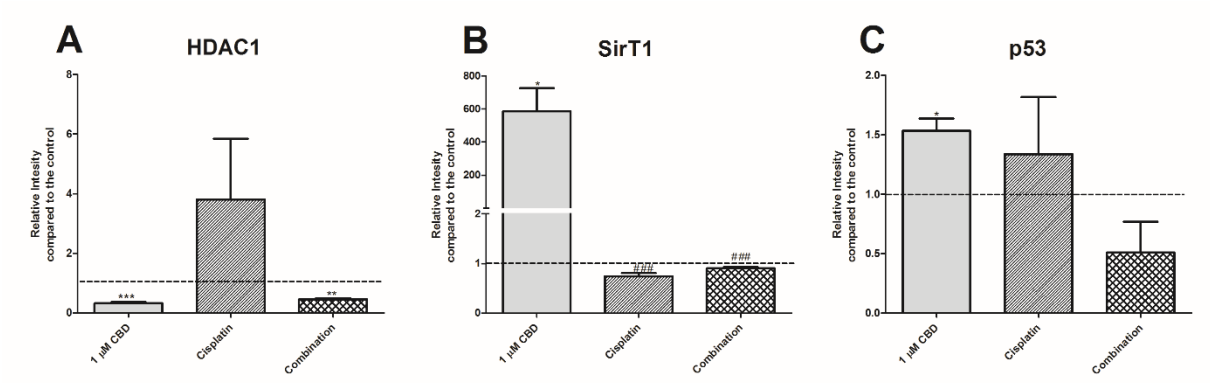

Figure 4.21. The effect of CBD, cisplatin and combination treatment on the gene expression levels of the p53 pathway in MDA-MB231 cells.
Cancer cells were harvested for genetic expression analysis on the p53 pathway following 24 hours of 1 μM CBD, 100 μM cisplatin and combination treatment. **(A)** HDAC1, **(B)** SirT1 and **(C)** p53 expression were examined. The dashed line represents untreated expression levels. Data shown is repeated 3-5 times. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ *vs* Untreated; ## $p<0.01$ *vs* CBD.

4.10.1.2. The effect of cisplatin treatment and combination treatment on the Nrf2 pathway's gene expression in cancer cells

Cisplatin and combination treatment increased ROS levels compare to untreated cells (Section 4.5.1.. As previously mentioned in section 1.1.3.4.1. and 3.11.1.2. increased ROS leads to increased Nrf2 activity. Important regulators of the Nrf2 pathway were also examined as in section 3.11.1.2..

The expression of MAPk was significantly increased following 1 μM CBD treatment (112.11 ± 5.412 R.I.) ($p<0.01$) (Figure 4.22.A.). Furthermore, the increase in MAPk expression with CBD was significantly increased compared to cisplatin and combination treatment ($p<0.001$). A significant increase in MAPκ gene expression levels

were observed following cisplatin treatment (7.56 ± 0.447 R.I.) compared to untreated cells (p<0.01). However, no change in MAPk expression was observed following combination treatment.

There was a significant increase in Nrf2 gene expression following 1 μM CBD treatment (2.49 ± 0.289 R.I.) (p<0.05) (Figure 4.22.B.). No change was observed following treatment with cisplatin and combination, but they did show a difference compared to CBD treatment alone (p<0.05).

IL-6 gene expression was also increased following treatment with 1 μM CBD (10.99 ± 1.849 R.I.) (p<0.05) (Figure 4.22.C.). But again there was no difference when the cells were treated with cisplatin and combination.

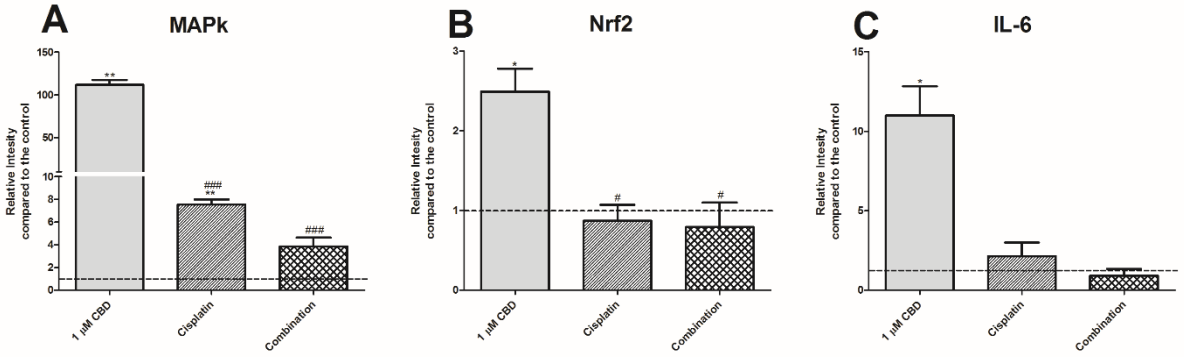

Figure 4.22. The effect of CBD, cisplatin and combination treatment on the gene expression of the cancer cell's Nrf2 pathway.
Cancer cell's Nrf2 pathway was examined following 24 hours of 1 μM CBD, 100 μM cisplatin and combination treatment. **(A)** MAPk, **(B)** Nrf2 and **(C)** IL-6 expression were examined. The dashed line represents the untreated expression level. Data shown is repeated 3-5 times. Data is represented as mean ± SEM. * p<0.05, ** p<0.01 *vs* Untreated; # p<0.05, ### p<0.001 *vs* CBD.

4.10.1.3. The AKT pathway's gene expression response to cisplatin treatment and combination treatment in cancer cells

Following the reduction in cell viability with cisplatin and combination treatment in section 4.3. investigating the regulation of cell growth was important as previously seen in section 3.11.1.3..

No change in AKT expression was observed following 1 μM CBD and cisplatin treatment (Figure 4.23.A.). A significant reduction was shown following combination treatment (0.49 ± 0.047 R.I.) ($p<0.01$). Interestingly, combination treatment was also significantly reduced compared to CBD treatment ($p<0.01$).

mTOR expression was significantly increased following treatment with 1 μM CBD (2.55 ± 0.303 R.I.) ($p<0.05$), however, no change was observed following cisplatin or combination treatments (Figure 4.23.B.).

NFkB expression showed a significant increase following treatment with 1 μM CBD (2.40 ± 0.221 R.I.) ($p<0.05$) which was also significant to cisplatin and combination treatments ($p<0.001$) (Figure 4.23.C.). No change in NFkB gene expression was observed following treatment with cisplatin or combination.

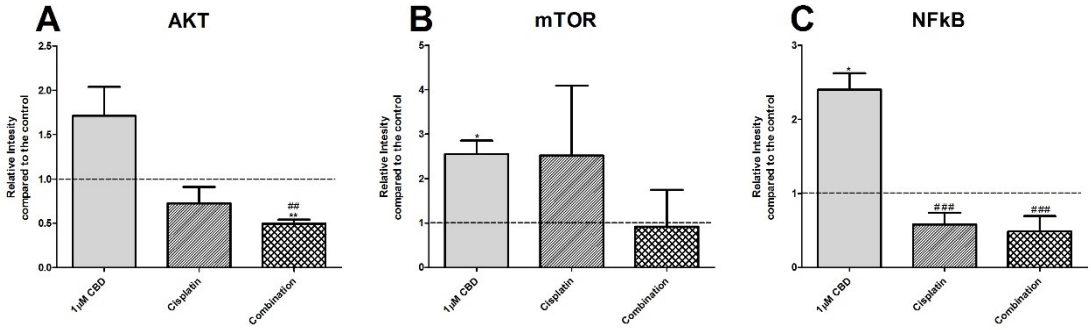

Figure 4.23. The effect of CBD, cisplatin and combination treatment on the gene expression of the AKT pathway in MDA-MB231 cells.
Cancer cell's AKT pathway was examined following 24 hours of 1 μM CBD, 100 μM cisplatin and combination treatment. **(A)** AKT, **(B)** mTOR and **(C)** NFkB expression was examined. The dashed line represents the untreated expression levels. Data shown is repeated 3-5 times. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated; # $p<0.05$, ### $p<0.001$ *vs* CBD.

4.10.1.4. The effect of cisplatin treatment and combination treatment in cancer cell's FOXO1 gene expression

FOXO1 was also analysed further following cisplatin and combination treatment due to its role in suppressing cellular oxidative stress and cancer growth (Section 3.11.1.4.).

FOXO1 expression levels were significantly increased following treatment with 1 µM CBD (33.79 ± 9.896 R.I.) ($p<0.05$) (Figure 4.24.). A significant reduction of FOXO1 was observed following treatment with cisplatin (0.08 ± 0.029 R.I.) ($p<0.01$) and combination (0.46 ± 0.091 R.I.) ($p<0.05$) compared to untreated cells and CBD treatment alone ($p<0.05$).

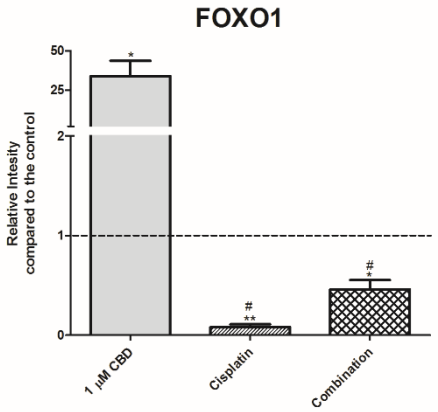

Figure 4.24. The effect of CBD, cisplatin and combination treatment on the gene expression of FOXO1 in MDA-MB231 cells.
Cancer cells FOXO1 was analysed following 24 hours of 1 µM CBD, 1000 µM cisplatin and combination treatment. The dashed line represents untreated expression levels. Data shown is repeated 3-5 times. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated; # $p<0.05$ *vs* CBD.

4.10.2. Cisplatin treatment and combination treatment's effect on control cell's gene expression

4.10.2.1. The effect of cisplatin treatment and combination treatment on the p53 pathway in control cells

An increase in HDAC1 gene expression was observed following 1 μM CBD treatment (2.28 ± 0.313 R.I.) compared to untreated levels ($p<0.05$) (Figure 4.25.A.). There was a trend to increase following treatment with cisplatin and combination.

No change in SirT1 gene expression was observed following 1 μM CBD, cisplatin and combination treatment (Figure 4.25.B.).

p53 gene expression was unchanged following treatment with 1 μM CBD and combination (Figure 4.25.C.). However, when treated with cisplatin a significant increase in p53 expression was observed (2.84 ± 0.662 R.I.) compared to untreated cells ($p<0.05$), CBD ($p<0.01$) and combination ($p<0.05$).

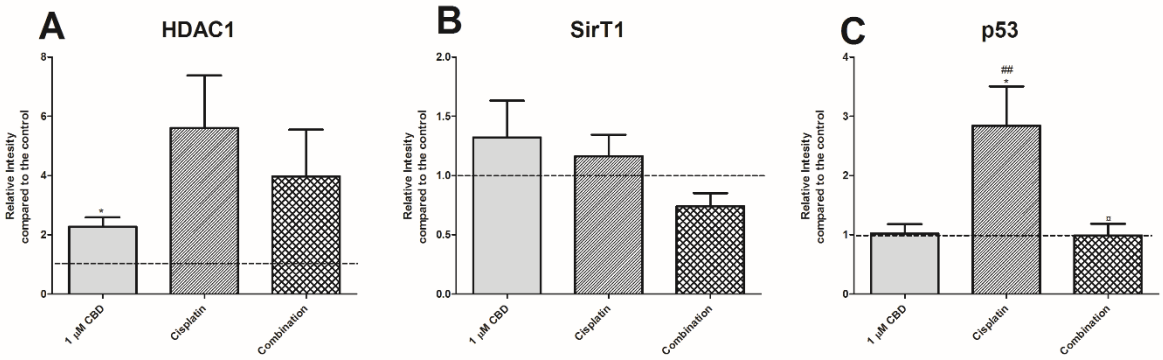

Figure 4.25. The effect of CBD, cisplatin and combination treatment on the gene expression of control cell's p53 pathway.
Control cells were treated with 1 μM CBD, 100 μM cisplatin or combination treatment for 24 hours prior to mRNA extraction. **(A)** HDAC1, **(B)** SirT1 and **(C)** p53 expression was examined. The dashed line represents the untreated expression levels. Data shown is repeated 3-5 times. Data is represented as mean ± SEM. * $p<0.05$ *vs* Untreated; ## $p<0.01$ *vs* CBD.

4.10.2.2. Cisplatin treatment and combination treatment's effect on the gene expression of the Nrf2 pathway in control cells

The expression of MAPk was significantly reduced following treatment with 1 μM CBD (0.43 ± 0.105 R.I.) ($p<0.05$) (Figure 4.26.A.). A trend to increase in MAPk gene expression was observed following cisplatin treatment was not significant to untreated levels. However, treatment with cisplatin was significantly increased compared to CBD alone ($p<0.01$) and combination treatment ($p<0.01$). No change in MAPk expression was observed following combination treatment.

Nrf2 expression was unchanged following treatment with 1 μM CBD (Figure 4.26.B.). A significant increase in the gene expression of Nrf2 was observed following cisplatin treatment (7.35 ± 1.818 R.I.) ($p<0.05$). Furthermore, the increase observed with cisplatin was also significant to CBD ($p<0.01$) treatment. There was no change in Nrf2 expression with combination treatment.

No change in IL-6 expression was observed following CBD and combination treatment (Figure 4.26.C.). However, when the cells were treated with cisplatin there was a significant increase in IL-6 expression (2.06 ± 0.198 R.I.) ($p<0.05$).

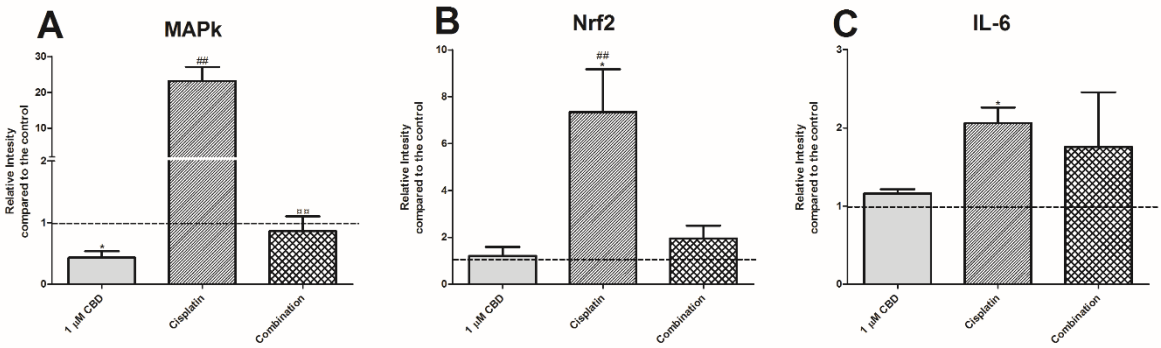

Figure 4.26. The effect of CBD, cisplatin and combination treatment on the gene expression of the Nrf2 pathway in control cells.
Control cells were treated with 1 μM CBD, 100 μM cisplatin or combination treatment for 24 hours prior to mRNA extraction. **(A)** MAPk, **(B)** Nrf2 and **(C)** IL-6 expression was examined. The dashed line represents the untreated expression levels. Data shown is repeated 3-5 times. Data is represented as mean ± SEM. * $p<0.05$ *vs* Untreated; ## $p<0.01$ *vs* CBD; ¤¤ $p<0.01$ *vs* Cisplatin.

4.10.2.3. The effect of cisplatin treatment and combination treatment on the gene expression of the AKT Pathway in control cells

AKT's gene expression was unchanged following treatment with 1 μM CBD and combination (Figure 4.27.A.). There was however a significant increase of AKT gene expression following treatment with cisplatin (2.91 ± 0.402 R.I.) ($p<0.05$).

No change in mTOR gene expression was observed following 1 μM CBD treatment (Figure 4.27.B.). An increase in mTOR gene expression was observed following cisplatin treatment (3.36 ± 0.0.483 R.I.) compared to untreated cells ($p<0.05$), CBD ($p<0.05$) and combination treatment ($p<0.01$). No change in mTOR gene expression was observed following combination treatment.

NFkB expression was significantly increased following treatment with 1 μM CBD (1.64 ± 0.241 R.I.) ($p<0.05$) (Figure 4.27.A.). No change was observed following cisplatin and combination treatments compared to untreated levels.

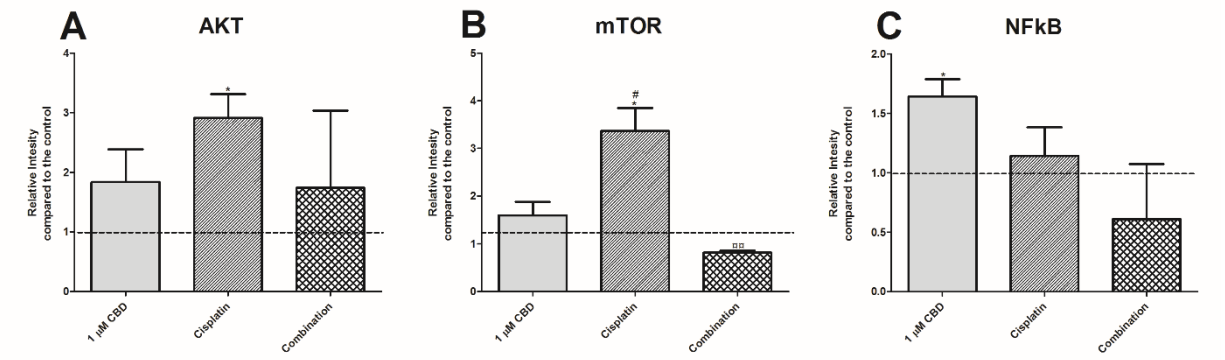

Figure 4.27. The effect of CBD, cisplatin and combination on the gene expression of MCF10A cell's AKT pathway.
Control cells were treated with 1 μM CBD, 100 μM cisplatin or combination treatment for 24 hours prior to mRNA extraction. **(A)** AKT, **(B)** mTOR and **(C)** NFkB expression was examined. The dashed line represents the untreated expression levels. Data shown is repeated 3-5 times. Data is represented as mean ± SEM. * $p<0.05$ *vs* Untreated; # $p<0.05$ *vs* CBD; ¤¤ $p<0.01$ *vs* Cisplatin.

4.10.2.4. Cisplatin treatment and combination treatment's effect on the gene expression of FOXO1 in control cells

FOXO1 gene expression was increased following 1 μM CBD treatment (2.07 ± 0.294 R.I.) ($p<0.05$) (Figure 4.28.). No change was observed following cisplatin treatment. However, combination treatment led to a significant reduction in FOXO1 gene expression (0.15 ± 0.042 R.I.) compared to untreated levels ($p<0.01$) and CBD alone ($p<0.01$).

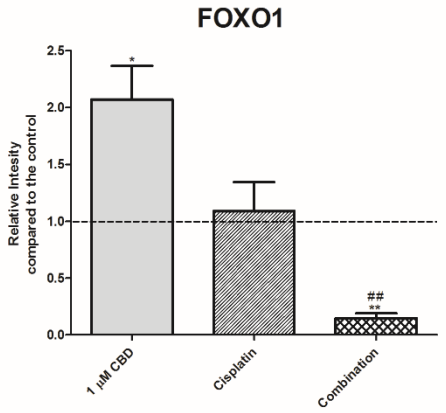

Figure 4.28. The effect of FOXO1 gene expression following CBD, cisplatin and combination treatment in control cells.
Control cells were treated with 1 μM CBD, 100 μM cisplatin and combination treatment for 24 hours prior to mRNA extraction for FOXO1 analysis. The dashed line represents the untreated expression levels. Data shown is repeated 3-5 times. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated; ## $p<0.01$ *vs* CBD.

4.11. The effect of cisplatin treatment and combination treatment on the protein levels in the cell

The cells protein alteration was assessed to determine if treating with cisplatin alone or with combination could have an augmented effect on the cancer cells. Protein levels were analysed based on the same pathways as with CBD (Section 3.12.) and were carried out using western blotting (Section 2.13.).

4.11.1. Cisplatin treatment and combination treatments effect on the protein level's in cancer cells

The cancer cells were examined for any alteration in protein expression following cisplatin and combination treatments. The pathways selected were the pro-apoptotic p53 pathway which included HDAC1, SirT1 and p53 (Section 3.11.1.1.). A cell stress response pathway, Nrf2, was also analysed which included HDAC6 and Nrf2 (Section 3.11.1.2.). Finally, the AKT pathway which controls cell growth was analysed which included AKT, mTOR and NFκB (Section 3.11.1.3.).

4.11.1.1. The effect of cisplatin treatment and combination treatment on the protein levels of the p53 pathway in cancer cells

HDAC1 expression levels were increased following treatment with 1 μM CBD (1.89 ± 0.081 R.I.) ($p<0.01$) and showed a trend to increase with cisplatin treatment (5.66 ± 1.925 R.I.) ($p=0.06$) (Figure 4.29.A.). However, when cells were treated with the combination therapy there was a significant reduction in HDAC1 expression compared to untreated cells (0.40 ± 0.069 R.I.) ($p<0.001$) and cisplatin ($p<0.05$).

There was no alteration in SirT1 protein expression levels following CBD and combination treatment (Figure 4.29.B.). However, there was a significant increase with

cisplatin treatment compared to untreated cells (3.01 ± 0.747 R.I.) ($p<0.05$), CBD ($p<0.05$) and combination ($p<0.05$).

When p53 protein levels were examined an increase was observed following 1 μM CBD (2.27 ± 0.310 R.I.) ($p<0.05$) and cisplatin treatment (59.76 ± 6.531 R.I.) ($p<0.001$) compared to untreated cells (Figure 4.29.C.). Cisplatin was also significantly increased compared to CBD ($p<0.001$) and combination treatment ($p<0.001$). Combination treatment showed a significant reduction in p53 levels (0.39 ± 0.095 R.I.) ($p<0.001$).

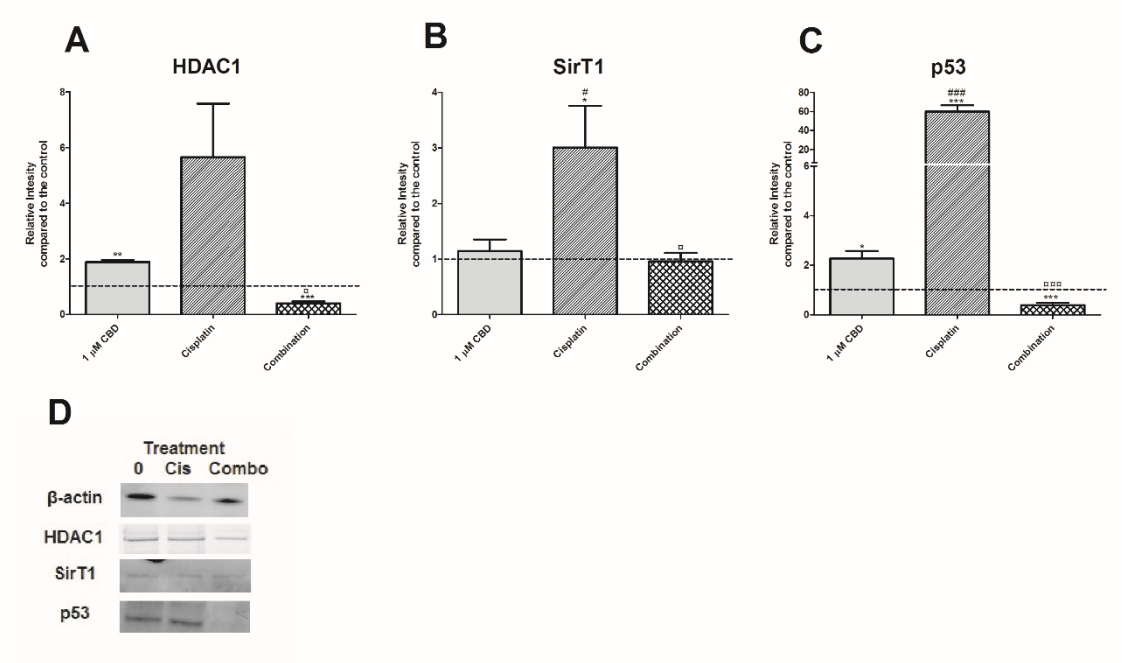

Figure 4.29. The effect of CBD, cisplatin and combination treatment on the protein levels in the p53 pathway of MDA-MB231 cells.
Following MDA-MB231 treatment with 1 μM CBD, 100 μM cisplatin and combination their protein levels were analysed for alterations in the p53 pathway. **(A)** HDAC1, **(B)** SirT1 and **(C)** p53 levels were all examined. The dashed line represents the untreated cell levels. **(D)** Western blot of relevant graphs with untreated (0) and the housekeeper β-actin as control protein. Data shown is repeated 3-5 times. 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.

4.11.1.2. The effect of cisplatin treatment and combination treatment on the protein levels in the Nrf2 pathway in cancer cells

HDAC6 protein levels were unchanged following treatment with both 1 μM CBD and the combination treatment (Figure 4.30.A.). There was a significant increase in

HDAC6 protein levels when the cells were treated with cisplatin (2.68 ± 0.769 R.I.) ($p<0.05$).

An increase in Nrf2 protein levels was observed following treatment with 1 μM CBD (2.43 ± 0.291 R.I.) ($p<0.01$) and cisplatin (2.09 ± 0.299 R.I.) ($p<0.05$) (Figure 4.30.B.). However, no change was observed of Nrf2 protein levels when cancer cells were treated with combination treatment compared to untreated cells.

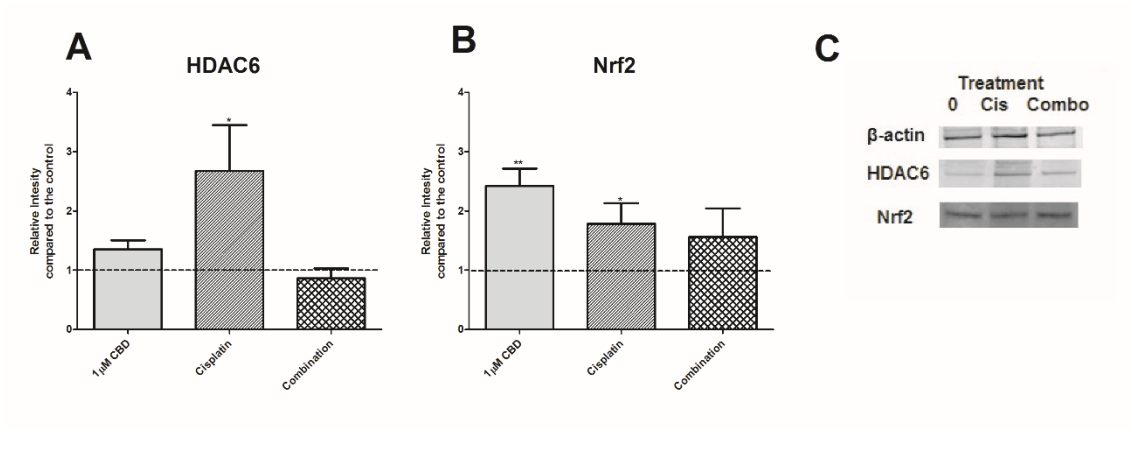

Figure 4.30. Following treatment with CBD, cisplatin and combination, the protein levels of the Nrf2 pathway in MDA-MB231 cells.
Following MDA-MB231 treatment with 1 μM CBD, 100 μM cisplatin and combination their protein expression levels were analysed for alterations to the Nrf2 pathway. **(A)** HDAC6 and **(B)** Nrf2 levels were all examined. The dashed line represents the untreated cell level. Data shown is repeated 3-5 times. **(C)** Western blot of relevant graphs with untreated (0) and the housekeeper β-actin as control protein. Data is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated.

4.11.1.3. The AKT pathway's protein levels following cisplatin treatment and combination treatment in cancer cells

AKT protein levels were unchanged following treatment with 1 μM CBD and the combination treatment (Figure 4.31.A.). There was a trend to increase AKT protein levels following cisplatin treatment.

Following 1 μM CBD treatment a significant increase in mTOR protein levels was observed (1.49 ± 0.171 R.I.) ($p<0.05$) (Figure 4.31.B.). This was not seen following treatment with cisplatin. However, combination treatment did see a significant increase in mTOR levels (1.52 ± 0.042 R.I.) ($p<0.01$).

There was also no change in NFkB protein levels following treatment with 1 μM CBD and cisplatin (Figure 4.31.C). However, there was a trend to increase in NFkB protein levels following combination treatment.

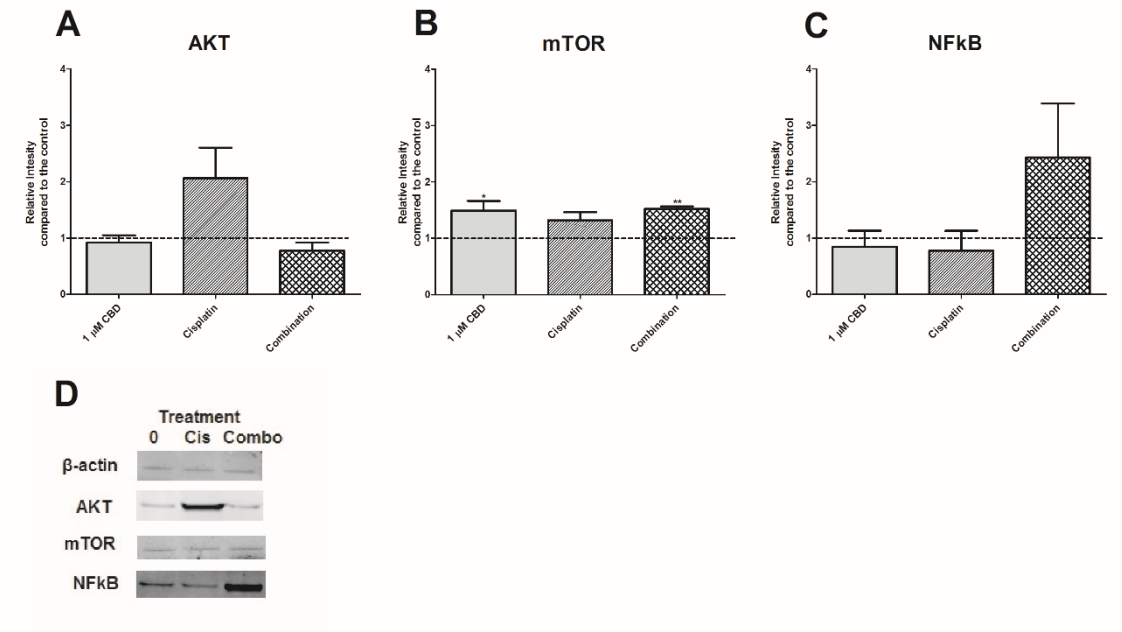

Figure 4.31. The effect of CBD, cisplatin and combination treatment on the protein expression levels of MDA-MB231 cells.
Following treatment with 1 μM CBD, 100 μM cisplatin and combination MDA-MB231 cells protein expression levels were analysed for alterations in the AKT pathway. **(A)** AKT, **(B)** mTOR and **(C)** NFkB levels were all examined. The dashed line represents the untreated cell levels. Data shown is repeated 3-5 times. **(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.

4.11.2. The effect of cisplatin treatment and combination treatment on the protein levels of control cells

Control cells were also analysed as above in section 4.11.1..

4.11.2.1. Cisplatin treatment and combination treatment's effect on the protein levels in the p53 pathway of control cells

No change was observed following 1 μM CBD treatment (Figure 4.32.A.). However, when treated with cisplatin (0.54 ± 0.104 R.I.) and combination (0.44 ± 0.046 R.I.) there was a significant reduction in HDAC1 protein levels ($p<0.01$). This reduction

observed with cisplatin and combination was also significant compared to CBD treatment alone (p<0.01).

SirT1 levels were significant increased following treatment with 1 μM CBD (1.62 ± 0.141 R.I.) (p<0.01) (Figure 4.32.B.). Interestingly, there was no change in SirT1 levels following cisplatin or combination treatment compared to untreated levels but there was a significant difference compared to CBD alone for both treatments (p<0.05).

No change in p53 levels was observed following 1 μM CBD treatment (Figure 4.32.C.). However, a significant reduction in protein levels was observed following treatment with cisplatin (0.32 ± 0.041 R.I.) (p<0.001) and the combination treatment (0.49 ± 0.022 R.I.) (p<0.001).

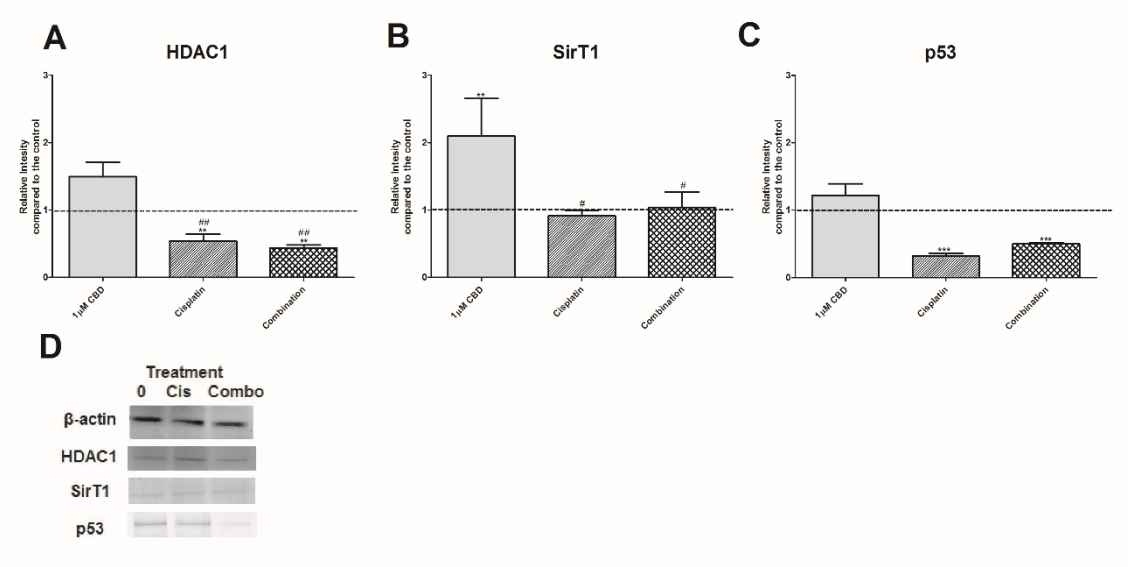

Figure 4.32. The control cell's p53 pathway protein levels following CBD, cisplatin and combination treatment.
Control cells were analysed for alterations to the p53 pathway following 1 μM CBD, 100 μM cisplatin or combination treatments. **(A)** HDAC1, **(B)** SirT1 and **(C)** p53 levels were all examined. The dashed line represents the untreated cell levels. Data shown is repeated 3-5 times. **(D)** Western blot of relevant graphs with untreated (0) and the housekeeper β-actin as control protein. Data is represented as mean ± SEM. ** p<0.01, *** p<0.001 *vs* Untreated; ## p<0.01 *vs* CBD.

4.11.2.2. The effect of cisplatin treatment and combination treatment on the protein levels of the Nrf2 pathway in control cells

HDAC6 protein levels were unchanged following 1 μM CBD and combination treatment (Figure 4.33.A.). However, a significant decrease was observed following cisplatin treatment (0.66 ± 0.085 R.I.) compared to untreated cells ($p<0.001$).

There was no change in Nrf2 expression levels following CBD, cisplatin and combination treatments compared to untreated cell levels (Figure 4.33.B.).

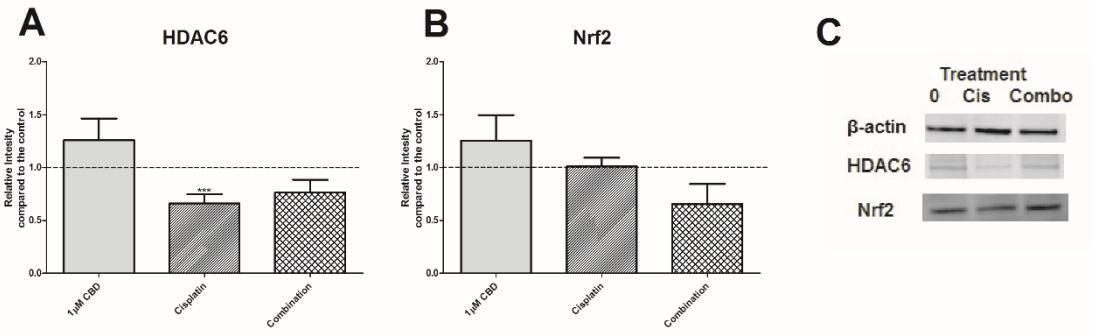

Figure 4.33. The effect of CBD, cisplatin and combination treatment on control cell's Nrf2 pathway protein levels.
Control cells were analysed for alterations to the Nrf2 pathway following 1 μM CBD, 100 μM cisplatin or combination treatment. **(A)** HDAC6 and **(B)** Nrf2 levels were all examined. The dashed line represents the untreated cell levels. Data shown is repeated 3-5 times. **(C)** Western blot of relevant graphs with untreated (0) and the housekeeper β-actin as control protein. Data is represented as mean ± SEM. *** $p<0.001$ *vs* Untreated.

4.11.2.3. Cisplatin treatment and combination treatment's effect on the protein levels of the AKT pathway in control cells

A trend to decrease of AKT protein levels was observed following CBD treatment (Figure 4.34.A.). However, there was no change following CBD, cisplatin and combination treatment.

mTOR levels were significantly increased following 1 μM CBD treatment (1.82 ± 0.257 R.I.) ($p<0.05$) (Figure 4.34.B.). However, levels were reduced following cisplatin (0.55 ± 0.115 R.I.) ($p<0.05$) and combination treatment (0.46 ± 0.074 R.I.) ($p<0.001$). Furthermore, cisplatin and combination treatment were significantly reduced compared to CBD treatment ($p<0.001$).

Control cell's NFkB protein levels were increased following CBD treatment (1.57 ± 0.113 R.I.) ($p<0.05$) (Figure 4.34.C.). No change was observed following cisplatin and combination treatment. However, the increase observed with CBD was also significantly increased compared to cisplatin treatment ($p<0.05$).

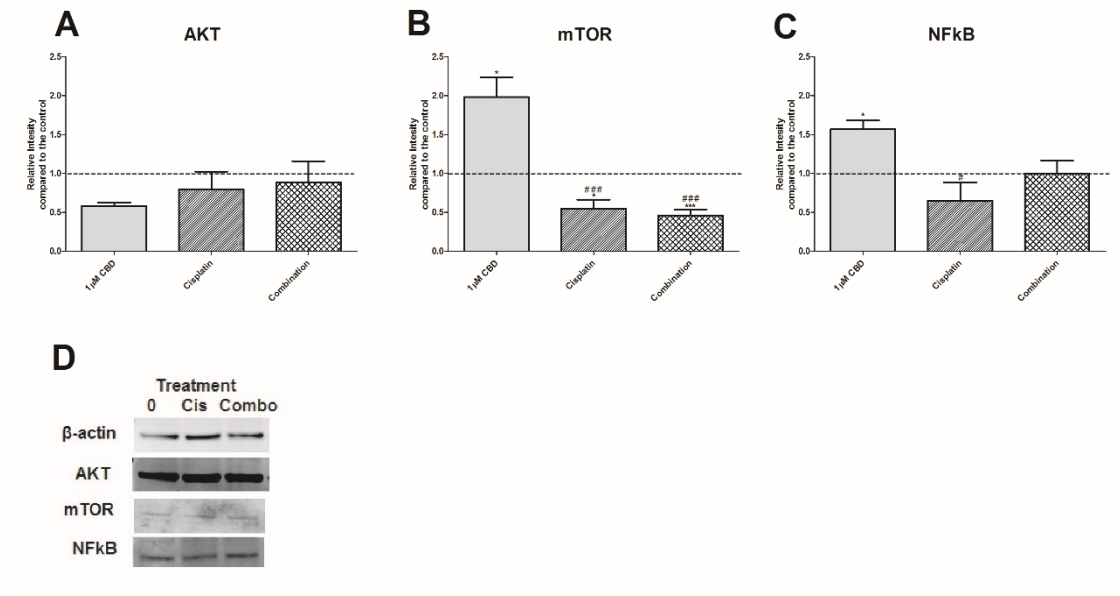

Figure 4.34. The effect of CBD, cisplatin and combination treatment on control cell's protein levels in the AKT pathway.
Control cells were analysed for alterations to the AKT pathway following 1 μM CBD, 100 μM cisplatin and combination treatment. **(A)** AKT, **(B)** mTOR and **(C)** NFkB levels were all examined. The dashed line represents the untreated cell levels. Data shown is repeated 3-5 times. **(D)** Western blot of relevant graphs with untreated (0) and the housekeeper β-actin as control protein. Data is represented as mean ± SEM. * $p<0.05$, *** $p<0.001$ *vs* Untreated; # $p<0.05$, ### $p<0.001$ *vs* CBD.

4.12. Cisplatin treatment and combination treatment's effect on the global DNA methylation of the cell

4.12.1. Cancer cell's global DNA methylation state following cisplatin treatment and combination treatment

The ratio of 5-methylCytosine to Guanine was investigated as in section 3.13. (Section 2.14.). The overall DNA methylation state of cancer cells showed no change following 1 μM CBD treatment, however, there was a significant decrease in 5methylcytosine/dGuanine levels following treatment with cisplatin (2.22 ± 0.003 %) ($p<0.01$) and combination (2.07. ± 0.019 %) ($p<0.05$) (Control, 2.45 ± 0.128 %) (Figure 4.35.).

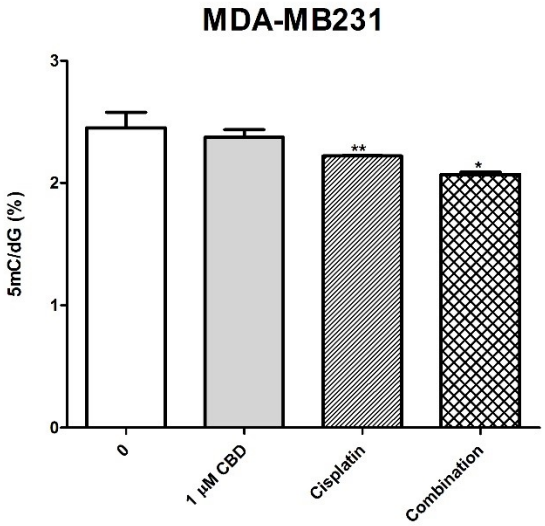

Figure 4.35. The effect of CBD, cisplatin and combination on the global methylation state of MDA-MB231 cells.
MDA-MB231 cells were treated for 24 hours with 1 μM CBD, 100 μM cisplatin or combination prior to extraction for methylation examination. Data shown is repeated 3-5 times and is represented as mean ± SEM. * $p<0.05$, ** $p<0.01$ *vs* Untreated.

4.12.2. Global methylation state in control cells following cisplatin treatment and combination treatment

Control cells showed a significant reduction in methylation state following 1 μM CBD treatment (2.65 ± 0.169 %) ($p<0.05$) (Untreated, 2.87 ± 0.119 %) (Figure 4.36.). There was also a decreasing trend with cisplatin ($p=0.09$) and combination ($p=0.08$) though this did not reach significance.

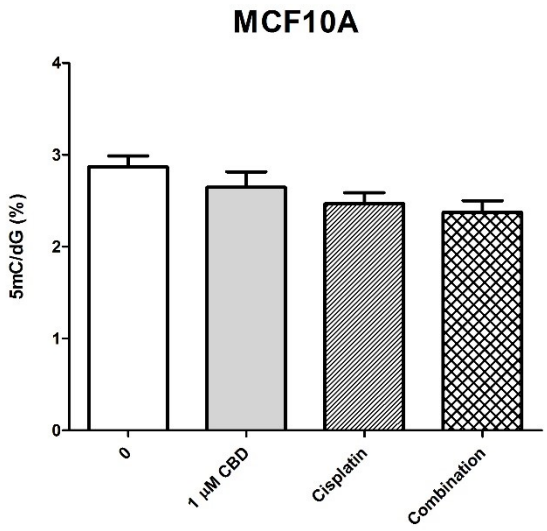

Figure 4.36. MCF10A cells methylation state following CBD, cisplatin and combination treatment.
Control cells whole methylation was examined following 1 μM CBD, 100 μM cisplatin and combination treatment. Data shown is repeated 3-5 times and is represented as mean ± SEM. * $p<0.05$ *vs* Untreated.

4.13. Summary of the effects of cisplatin treatment and combination treatment on both cancer and control cells

- **The mode of action of cisplatin in cancer and control cells**

Cisplatin was shown to reduce the viability of both cancer and control cells ($p<0.001$) (Section 4.3.). Previous work has shown that cisplatin can increase cell death through increased ROS and DNA damage (Bragado *et al.* 2007, Martins *et al.* 2008, Florea and Busselberg 2011, Galluzzi *et al.* 2012).

Here I have established that the molecular mechanism of cisplatin in cancer cells is through increased ROS and Ca^{2+} concentrations to unsustainable levels that modulate the mitochondria's response to induce cell death (Section 4.5.1., 4.6.1. and 4.7.1.). In parallel there was a trend to increased VDAC1 activity which has been linked to increased apoptosis (Section 4.9.1.). Moreover, p53 levels were increased to high levels following cisplatin treatment ($p<0.001$) along with increased Nrf2 levels (Section 4.10.1. and 4.11.1.). AKT levels were unchanged and FOXO1 was significantly reduced (Section 4.10.1. and 4.11.1.). As observed following CBD treatment (Section 3.14) increased dual activation of p53 and Nrf2 can lead to a mild-stress response which increases cell death furthermore (Lau *et al*. 2008, Chen *et al*. 2012).

Interestingly the molecular mechanism of cisplatin was not similar in control cells. An increase in ROS and Ca^{2+} concentrations to unsustainable levels was observed (Section 4.5.2. and 4.6.2.). However, a reduction in basal mitochondrial respiration along with a significant reduction in proton leak ($p<0.001$) was observed. Along with this the cell's maximal respiration was reduced following cisplatin treatment ($p<0.05$) (Section 4.7.2.). Moreover VDAC1 activity was reduced following cisplatin treatment in the control cells ($p<0.001$) (Section 4.9.2.). Interestingly a reduction in p53 protein levels and Nrf2 levels were unchanged compared to untreated levels (Section 4.11.2.). Finally, there

was no change to global methylation state which was reduced in cancer cells (Section 4.12.).

Cancer cell's mode of action following cisplatin treatment is similar to CBD treatment. However, it is interesting to note that even with the reduced cell viability in control cells following cisplatin treatment the molecular mechanism is different. This shows the importance of cisplatin as a chemotherapeutic agent.

- **The potential of CBD and cisplatin in enhancing cell death when combined**

Both CBD and cisplatin reduced cell viability over the 24 hour treatment period (Section 3.4.1. and 4.3.1.). However, a "protective" effect from the increased cell viability of cisplatin was observed in cancer cells (Section 4.3.3.). Following combination treatment an increase in ROS concentrations was observed though it was not as significant as cisplatin treatment alone (Section 4.5.). Ca^{2+} concentrations were reduced with cancer cells but increased with control cells ($p<0.01$) (Section 4.6.). Interestingly, a reduction in VDAC1 activity was observed in cancer ($p<0.001$) and control ($p<0.01$) cells following combination treatment (section 4.9.). As previously mentioned VDAC1 is an important regulator in mitochondrial apoptosis and cytochrome *c* release (Section 1.4.4. and 3.14.) (Madesh and Hajnóczky 2001, Shoshan-Barmatz *et al.* 2010, Shoshan-Barmatz *et al.* 2014, Tewari *et al.* 2015). Moreover a reduction in p53 ($p<0.001$) was observed in both cancer and control cells (Section 4.11.1.1. and 4.11.2.1.). No change in Nrf2 levels or AKT levels was observed following combination treatment (Section 4.11.1.2., 4.11.1.3., 4.11.2.2. and 4.11.2.3.).

Interestingly, combination treatment did not increase the efficiency of cisplatin treatment. The molecular mechanism of combination was similar for both cancer and control cells as mentioned above. Here I have shown that the "protective" effect

observed following combination treatment is through decreased p53 expression and unchanged Nrf2 activity while in parallel VDAC1 activity was reduced. This suggests that the mitochondria are protecting their function from the stress of combination treatment.