Chapter 6

Discussion

6. Discussion

In this body of work I have shown that CBD has a beneficial effect in reducing cancer cell growth through the novel mechanism of “priming”. I have identified and characterised the mode of action by which “priming” with CBD leads to enhanced cell death, mainly through the activation of the high stress p53/Nrf2 pathway which increased mitochondrial damage and cell stress and inhibited the chemotherapy resistant Nrf2 pathway. Furthermore, an increased VDAC1 activity also reduced cell viability by increasing cytochrome *c* release and apoptosis. This mechanism was specific to cancer cells with no effect in control cells compared to cisplatin treatment. Furthermore, preliminary works suggest that this effect may be translatable to an *in vivo* model.

6.1. Optimising CBD treatment for “priming” experiment

Cannabinoids have been shown to be potent anti-tumour agents in colon, prostate, liver, breast and lung cancer (Guzmán 2003, Sarfaraz *et al.* 2005, Ligresti *et al.* 2006, Izzo *et al.* 2009, Lim *et al.* 2011, McAllister *et al.* 2011, Shrivastava *et al.* 2011, Aviello *et al.* 2012, De Petrocellis *et al.* 2012b, Ramer *et al.* 2012, Ramer *et al.* 2013, Macpherson *et al.* 2014, Romano *et al.* 2014). However, there is no consistent dose range for the cannabinoids nor treatment time period. Furthermore, recent research showed a reduction in viability from as little as 2 hours following treatment (Ramer *et al.* 2013). This is important to note when interpreting data as a longer time frame may show a higher cell viability with lower doses and vice versa, a shorter time point with a higher dose may show the same response. Optimising the dose range of CBD was vital prior to examining CBD’s potential in “priming”.

An acute treatment with CBD was carried out on both cancer and control cells to observe if there was any alteration to the cellular regulation with a 1 hour, acute, time

point. Breast cancer cells showed no change in cell viability following acute treatment with CBD. An increase in ROS levels and mitochondrial respiration was observed in the cancer cells. Is has been shown that increased ROS levels, increase cancer cell proliferation (Gupta *et al.* 2012, Sena and Chandel 2012). Recent research has also shown that liver cells remained in an arrested state following 1 hour of H_2O_2 exposure (Koczor *et al.* 2013). This suggests that cancer cells showed no response to CBD treatment due to sudden increased ROS levels in the cell. No change was also observed following acute treatment in control cells of cell viability or ROS levels. Interestingly, lowered stress levels from the EnR following CBD treatment has been shown to have a protective role in keeping ROS reduced in oligodendrocyte progenitor cells, preventing cell death in liver steatosis (Mecha *et al.* 2012, Yang *et al.* 2014). As mentioned previously in section 1.1.3.4. ROS can aid in maintaining homeostasis within the cell, protecting the cell from damage and increased ROS production (Figure 1.3.) (Sena and Chandel 2012, Yang *et al.* 2014).

A chronic, 24 hour treatment was pursued as no change in cell viability was observed following acute treatment. Most studies on CBD have been carried out following 24 hours of treatment from dose ranges between 0.01 μM to 10 μM (Sarfaraz *et al.* 2005, Shrivastava *et al.* 2011, Aviello *et al.* 2012, Romano *et al.* 2014). In this study, MDA-MB231 cancer cells saw a reduction in cell viability following chronic treatment with 0.5 μM CBD. This is consistent with previous work carried out in MDA-MB231 cells with doses of 2.5 μM to 10 μM CBD for 24 hours (McAllister *et al.* 2011, Shrivastava *et al.* 2011). Recent work carried out by Shrivastava and colleagues on several different breast cancer and control cell lines showed a reduction in control cell (MCF10A) viability as from 5 μM to 10 μM CBD (Shrivastava *et al.* 2011). A reduction in control cell's viability was observed following CBD treatment in this study also confirming the previous reports. CBD has been shown to preferentially kill cancer cells due to the increase of CB_2 receptor on tumour cell's membrane (Athanasiou *et al.* 2007). Increased

cancer cell death from CBD is due to the increased entry of cannabinoids into cancer cells compared to control cells. From this a treatment of 1 μM CBD was chosen for combination and "priming" treatment due to its effect on reducing cancer cell viability and having no effect on control cells.

6.2. CBD's mode of action and its potential as a "priming" agent

Cancer cell metabolism, as previously mentioned in section 1.1.3.1., is directed primarily towards glycolysis even in the presence of oxygen in a process called the "Warburg Effect" (Samudio *et al.* 2009, Vander Heiden *et al.* 2009, Wallace 2012). This mechanism allows the cancer cells to grow more rapidly due to the continual supply of glucose available from circulating blood as is mimicked in cell culture (Vander Heiden *et al.* 2009). In acute treatment with CBD no change in cell viability was reported. Interestingly, the "Warburg Effect" was not observed in cancer cells following acute CBD treatment. Rather a reverse of the cell back to an OXPHOS state, with an increase in basal mitochondrial respiration. This flexibility of the mitochondria has been documented due to the "Crabtree Effect" which can reverse the glycolytic drive (Dranka *et al.* 2010, Diaz-Ruiz *et al.* 2011). Recent research has shown that another plant compound, curcumin can reverse the "Warburg Effect" towards the "Crabtree Effect" in breast cancer cells even in the presence of the pro-oncogenic tumour necrosis factor α (TNFα) (Vaughan *et al.* 2013). In comparison, control cells showed no effect to mitochondrial stress levels and function following acute CBD treatment.

Interestingly, mitochondria have been described as having a pivotal role in the mechanism behind "priming" (Chonghaile *et al.* 2011, Reed 2011, Sarosiek *et al.* 2013). A recent study has shown that administration of CBD can increase the functionality of mitochondria through the complexes by increasing Ca^{2+} within the mitochondria itself and thus increasing NADH accessibility (Valvassori *et al.* 2013). The alteration in

mitochondrial function with CBD treatment is interesting as in my study there was an increase in mitochondrial respiration and ATP production suggesting a reverse of the "Warburg Effect" (Terada 1990, Rapizzi *et al.* 2002, Benard *et al.* 2007, Diers *et al.* 2012). Furthermore, increased ROS has been shown to increase cell death when in excess (Gupta *et al.* 2012, Sena and Chandel 2012, Singer *et al.* 2015). Previous studies have shown that increased ROS levels along with increased Ca^{2+} led to further increased apoptosis (Brookes *et al.* 2004, De Petrocellis *et al.* 2012b). Furthermore, a recent study has confirmed that CBD inhibits chemotherapy resistant glioblastoma cell viability in a ROS-dependant manner (Singer *et al.* 2015). The control of ROS and Ca^{2+} is interlinked whereby, increased Ca^{2+} leads to increased ROS generation (Brookes *et al.* 2004). However, increased Ca^{2+} levels can increase ATP production but ROS depletes ATP levels (Brookes *et al.* 2004, Wang *et al.* 2013). In this study, an increase in ATP levels was observed suggesting that cancer cells are trying to overcome the ROS-mediated depletion of ATP through reversing the "Warburg Effect" which is more efficient at ATP production (Tiwari *et al.* 2002, Vander Heiden *et al.* 2009, Kroon and Taanman 2014). However, control cells showed an increase in Ca^{2+} levels but no increase in mitochondrial respiration, ATP production or ROS levels. Progenitor cells have shown that CBD can regulate oxidative stress by decreasing ROS production and increasing anti-apoptotic Bcl-2 (Mecha *et al.* 2012). This suggests that the high concentration of CBD required to reduce cell viability in control cells may be due to the levels of ROS not being increased. However, an increase in Ca^{2+} was observed and along with increased ROS, increased Ca^{2+} triggers apoptosis when it is taken up into the mitochondria (Rizzuto *et al.* 2003). A recent study showed that in grape seed extract an increase in apoptosis was due to increased ROS and a subsequent loss of MOMP (Shrotriya *et al.* 2014). Furthermore, elevation in oxidative stress can not only damage the mitochondrial membrane but increase the release of cytochrome *c* into the cell upregulating apoptosis (Tiwari *et al.* 2002, Shrotriya *et al.* 2014). The primary role of Ca^{2+} is in stimulating OXPHOS however, an overload of Ca^{2+} can trigger increased ROS

and increase the MOMP which is regulated by key components such as VDAC1 (Brookes *et al.* 2004, Szabadkai *et al.* 2006). Overexpression of Ca^{2+} leads to stimulation of the TCA cycle enhancing ROS production through complex I and making the mitochondria work faster (Brookes *et al.* 2004). In this study an increase in the mitochondria's overall maximal respiration potential in cancer cells was observed due to increased Ca^{2+}, directing the mitochondria to function at full capacity. Proton leak is unchanged or reduced when a faster functioning metabolism is observed (Tiwari *et al.* 2002, Rizzuto *et al.* 2003, Brookes *et al.* 2004). CBD treatment increased Ca^{2+} levels in cancer cells, increasing mitochondrial OXPHOS, reversing the "Warburg Effect" and increased ROS further though the mitochondria. Moreover, it is important to note that a trending decrease in AMPK activity was observed which has been linked to an increase in OXPHOS and an increase in mTOR which can lead to mitochondrial dysfunction (Wu and Wei 2012). In control cells this same effect was not observed however, both cell types showed an increase in Ca^{2+} levels and control cells did have a reduction in cell viability at high doses. The proton leak of the control cell's mitochondria was significantly decreased suggesting that the mitochondria were increasing their stability (Brookes *et al.* 2004, Galluzzi *et al.* 2011). This suggests that the difference in CBD treatment for cancer and control cells could be through their mitochondria's response to the agent. Whereby control cell's mitochondria are not altering their function with CBD treatment but are instead reducing mitochondria permeability and thus inhibiting the direct influence of metabolic regulators from the cytoplasm. Ca^{2+} homeostasis is tightly controlled and any elevation can lead to increased apoptosis (Szabadkai *et al.* 2006, Kondratskyi *et al.* 2014). Moreover, increasing VDAC1 receptor, increases cell death through apoptosis from direct interaction with CBD and the mitochondria (Tiwari *et al.* 2002, Tewari *et al.* 2015). The plant polyphenol, curcumin, has been shown to reduce cancer cell viability by binding to the VDAC1 receptor (Tewari *et al.* 2015) CBD has previously been shown to interact with VDAC1 receptors and increase apoptosis in cancer cells (Rimmerman *et al.* 2013). MDA-MB231 cells showed an increase in VDAC1

expression however, control cell's expression was significantly reduced. This further strengthens the difference between the two cells in their response to CBD through the mitochondria and also confirms the potential of CBD as a favourable "priming" agent.

Known as the master regulator of a diverse range of cellular networks, p53 is capable of controlling both cell death and cell survival (Ito *et al.* 2002, Bansal *et al.* 2011, Hanahan and Weinberg 2011). In unstressed conditions p53 is maintained at low levels by HDAC1 suppression (Ito *et al.* 2002, Witt *et al.* 2009). This allows the cells to proliferate at normal levels (Ito *et al.* 2002). This regulation of p53 was observed in control cells and their viability. However, p53 has also been shown to act as a pro-apoptotic controller in high stress conditions whereby it is activated by the tumour suppressor SirT1 (Ventura *et al.* 2007, Yi and Luo 2010, De Amicis *et al.* 2011, Wade *et al.* 2013). SirT1 is enhanced by increased levels of ROS in the cell which blocks transcription-mediated apoptosis but translocates p53 to the mitochondria to allow release of cytochrome *c* and consequently apoptosis (Figure 6.1) (Yi and Luo 2010, De Amicis *et al.* 2011). This interactive regulation of apoptosis has been seen with resveratrol whereby an increase in p53 due to HDAC1 activation led to a down regulation of EnR in breast cancer cells which have been shown to drive cell proliferation (De Amicis *et al.* 2011). However, CBD treatment in breast cancer cells led to a decrease in HDAC1 gene expression with a corresponding increase in SirT1 expression and p53 expression which led to an increase in cell death through p53 translocation to the mitochondria. Resveratrol has been documented to control mitochondrial function and protect it from damage by increasing OXPHOS levels in the cell through SirT1 (Lagouge *et al.* 2006, Desquiret-Dumas *et al.* 2013). Activation of SirT1 can increase NADH levels within the cell and activate complex I in the mitochondria inducing respiratory chain and OXPHOS activity as was described earlier. It is also important to note that SirT1 has been shown to have anti-tumourogenic properties though this is dependent on tumour types (Tan *et al.* 2014). This also translated to protein levels though there was a slight

increase in HDAC1 protein levels. Recent work has shown that high HDAC1 levels in breast tissue correlated with reduced tumour sizes and a better prognosis which could suggest that at these high levels HDAC1 is pro-apoptotic (Zhang *et al.* 2005).

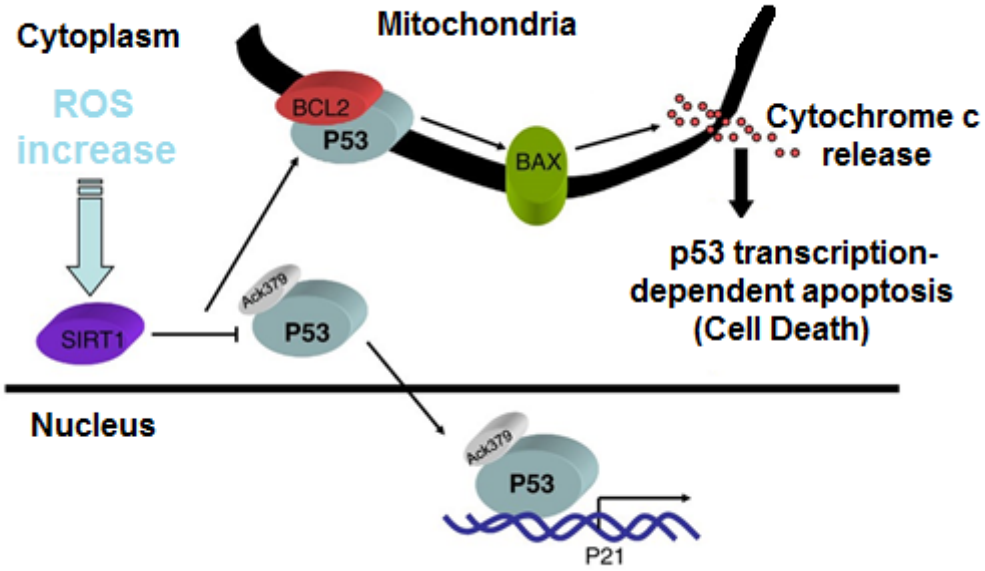

Figure 6.1. In the presence of increased ROS, SirT1 promotes translocation of p53 to the mitochondria for transcription-dependent apoptosis.
In the presence of increased ROS, SirT1 is activation to bind to and block p53 nuclear translocation. The deacetylated p53 is translocated to the mitochondrial outer membrane interacting with BCL-xL and Bcl-2. This allows the release of BAX which releases cytochrome *c* from the mitochondria to induce apoptotic cell death. Image modified from Yi et al., 2010.

Low levels of p53 activation have been shown to upregulate the cell stress regulator Nrf2 (Chen *et al.* 2012). Nrf2 is a key regulator in controlling cell stress as mentioned in section 1.1.3.4.1. whereby its activation can reduce ROS levels. Nrf2 is bound tightly to KEAP1 in the cytoplasm until an increase in ROS is detected (Kensler *et al.* 2007, Pi *et al.* 2007, Ohtsuji *et al.* 2008, Tufekci *et al.* 2011). Increased Nrf2, as observed in cancer cells here following CBD treatment, increases the AREs which promote antioxidant and anti-inflammatory pathways as has been shown previously with Broccoli seed treatment (McWalter *et al.* 2004, Tufekci *et al.* 2011, Namani *et al.* 2014). The AREs can increase expression of NAD(P)H:quinone oxidoreductase 1 (NQO1), and

down regulate IL6 and NFκB inflammatory markers (Hodge *et al.* 2005, Kensler *et al.* 2007, Li *et al.* 2008, Reuter *et al.* 2010). In cancer cells, an increase in Nrf2 gene expression and protein levels was observed along with an increase in ROS and p53 levels. However, an increase in MAPk gene expression was also observed which upregulates the genetic expression of the inflammatory marker IL6 (Son *et al.* 2008). Interestingly though this gene expression increase was not translated to an increase in protein levels for either MAPk or IL6 suggesting that Nrf2 was able to initiate an inhibition of their protein translation. Increased Nrf2 levels can lead to decreased oxidative stress levels which would allow the cell to regulate the damage incurred from increased ROS concentrations and protect the cell mechanisms (Li *et al.* 2008). Furthermore, in control cells there was no change in Nrf2 levels observed and no increased ROS production. A decrease in Nrf2 levels has been suggested to lead to cell death however, as Nrf2 control levels remained similar to untreated cells there was no evidence for an increased cell death through Nrf2 (Morito *et al.* 2003). Nrf2 has also been linked to increased ATP and mitochondrial function protection (Tufekci *et al.* 2011, Holmström *et al.* 2013). Increased p53 and Nrf2 levels in cancer cells suggest that CBD treatment activated the two-phase p53/Nrf2 mechanism whereby the cell is reacting to increased oxidative stress through increasing mitochondrial function, increasing ROS production and activating a controlled cell death (Figure 6.2.) (Chen *et al.* 2012, Holmström *et al.* 2013). Important to note is that control cell's mitochondrial function was not altered following CBD treatment confirming that CBD does not affect control cells the same as cancer cells (Athanasiou *et al.* 2007, Fukuda *et al.* 2014). It is also important to note that Nrf2 has been linked to activating AKT in inducing cell growth and proliferation (Wang *et al.* 2008). However, in cancer cells CBD does not increase AKT gene expression or protein levels which in turn does not increase cell growth nor increase glycolysis (Wang *et al.* 2008, Scotland *et al.* 2013, Yadav and Chandra 2014). Furthermore, AKT regulates FOXO activation which can regulate many cell fate pathways including apoptosis (Fu and Tindall 2008, Nunn *et al.* 2010). Activation of FOXO1 can increase apoptosis and

decrease cellular growth (Fu and Tindall 2008, Jiang *et al.* 2013, Prasad *et al.* 2014). An upregulation of FOXO3 results in cell cycle arrest and a reduction in a cancerous state (Bullock *et al.* 2013, Prasad *et al.* 2014). Interestingly, as mentioned previously control cells saw no change to Nrf2 levels following CBD treatment but did see an increase in AKT activation. This cell survival pathway was activated following CBD treatment in control cells (Wang *et al.* 2008). Moreover, AKT has been shown to inhibit VDAC1 expression directly to inhibit apoptosis (Yadav and Chandra 2014). Again, in cancer cells there was no change with AKT activity and an increase in both VDAC1 expression and cell death. Furthermore, VDAC1 was controlled by AKT activation in control cells. Moreover, mTOR which is linked to AKT activity, has been shown to increase mitochondrial activity and ATP production (Mu *et al.* 2014, Yadav and Chandra 2014). This further amplifies that CBD treatment in cancer cells increases cell death through the p53/Nrf2 two-phase pathway, increasing mitochondrial VDAC1 receptor's activation of cell death. In control cells this action of CBD was not observed.

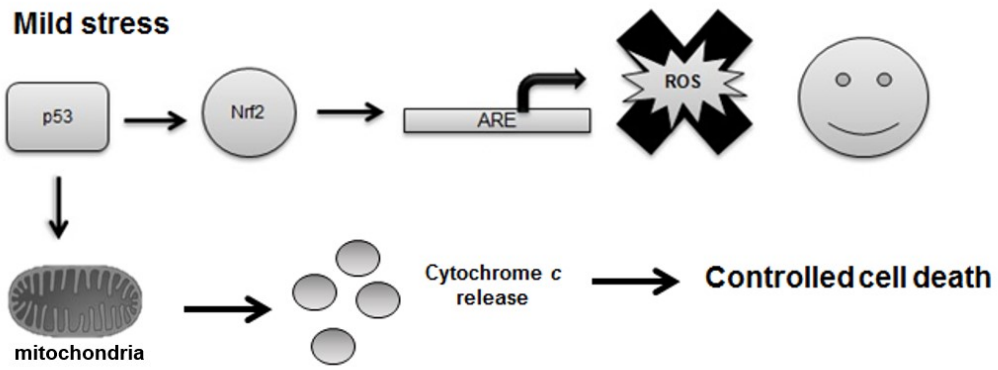

Figure 6.2. Mild stress such as CBD treatment, activates p53 to increase Nrf2 activity to reduce excess ROS and interact with the mitochondria to release apoptotic cytochrome *c*.
In a mild stress condition where ROS is increased, such as CBD treatment in cancer cells, p53 is activated. Increased p53 due to high ROS levels can lead to a direct activation of Nrf2 to inhibit further ROS production through the AREs. Increased p53 is also translocated to the mitochondria releasing cytochrome *c* and inducing apoptosis.

6.2.1. Conclusion

This study has shown that the mode of action of CBD is not the same in cancer cells as it is in normal cells. There is an ulterior mode of action in control cells that does not lead to increased oxidative stress nor increased mitochondrial function which is dysfunction in the cancer cells. Cancer cells when treated with CBD have been shown to induce cell death through activation of the p53/Nrf2 pathway and VDAC1 expression due to an increase in Ca^{2+} and ROS levels. This in turn reverses the "Warburg Effect" to increase the functionality and maximal respiration of the mitochondria in a dysfunctional manner, leading the cell to a controlled mitochondrial cell death. CBD's mode of action in control cells does not lead to increased oxidative stress nor increased mitochondrial function which became dysfunctional in the cancer cells following CBD treatment. The control cells reduced any potential for further oxidative stress by not increasing OXPHOS by keeping mitochondrial respiration and functions the same as untreated cells (Sena and Chandel 2012). Instead an increase in the expression of regulators that can aid in cell growth and repair, the AKT pathway, was observed. The proposed mode of action in control cells highlights the advantageous capacity of CBD as a tumour specific repressor. The difference observed in the molecular mechanism between cancer cells and control cells following CBD treatment highlights the importance of CBD in cancer therapy. Not only do cancer cells have a higher abundancy of cannabinoid receptors (CB) receptors and VDAC1 receptors as previously reported (Simamura *et al.* 2008). Here, I have shown that in cancer cells there is an increase in cell death, increased ROS concentration and increased VDAC1 activity. The role that CBD has to play in cancer therapy is clearly discussed here and I have shown its potential in enhancing chemotherapeutic treatment. Furthermore the role of CBD as a "priming" agent which could enhance chemotherapeutic cell death is even more promising.

6.3. Cisplatin as a suitable chemotherapy agent in MDA-MB231 cells

Cisplatin is one of the most potent anti-tumour agent used in the treatment of solid cancers and has been known to activate this toxic mechanism through both DNA damage and mitochondrial apoptosis (Jordan and Carmo-Fonseca 2000, Yang *et al.* 2006, Galluzzi *et al.* 2012, Santin *et al.* 2013, Al-Taweel *et al.* 2014). Based on previous research carried out with MDA-MB231 cells, a dose range was examined in this study to verify the positive effect cisplatin had on killing cancer cells (Hong *et al.* 2008, Arisan *et al.* 2010). A significant reduction in both cancer and control cells was observed with 100 μM cisplatin which was chosen as the effective dose range to observe any molecular alterations in the cell.

Cisplatin has been shown to induce apoptosis through different pathways including increased DNA adduct damage and mitochondrial apoptosis (Jordan and Carmo-Fonseca 2000, Yang *et al.* 2006, Arisan *et al.* 2010, Galluzzi *et al.* 2012). Cisplatin increases ROS production to increase cell toxicity and cell death (Bragado *et al.* 2007, Martins *et al.* 2008). Increased ROS levels in the cell can also induce an increase in Ca^{2+} level concentration which are normally at a consistent homeostatic level (Kawai *et al.* 2006, Muscella *et al.* 2011). In both cancer and control cells there was a significantly high level of ROS concentration compared to CBD treatment. Furthermore, an increase in Ca^{2+} levels was also observed in both cell types. These increases in oxidative stress can drive a cell towards apoptosis either through mitochondrial or through DNA damage (Brookes *et al.* 2004, Gupta *et al.* 2012, Sena and Chandel 2012). As previously mentioned, Ca^{2+} activity can drive OXPHOS however, with cisplatin treatment there was no change in mitochondrial respiration of cancer cells or maximal respiration (Rizzuto *et al.* 2003, Brookes *et al.* 2004, Parkash and Asotra 2010, Kuznetsov *et al.* 2011b). In the control cells there was actually a significant decrease in mitochondrial respiration, proton leak and in maximal respiration. This could suggest that again like with CBD there is a

shutdown of mitochondrial function when a toxic agent is introduced to the control cells. When looking at cisplatin's known interaction with VDAC1 channels in the mitochondria it has been shown already that cisplatin binds to VDAC1 increasing the interaction with cytochrome *c* release for an apoptotic cell death (Yang *et al.* 2006, Tajeddine *et al.* 2008, Galluzzi *et al.* 2012). Cancer cells showed no change in VDAC1 expression compared to untreated cells however, in control cells there is a significantly reduced expression of VDAC1 suggesting a resistance to cell death (Tajeddine *et al.* 2008, Galluzzi *et al.* 2012). A reduction in VDAC1 expression has been shown to be linked to a resistance in cell death (Galluzzi *et al.* 2012). The control cells are responding to the chemotherapeutic agent by increasing cell death though perhaps the cells are controlling this increase in Ca^{2+} levels and ROS by closing the VDAC1 channel to potentially resist these cells to further cell death.

Furthermore, much research has shown cisplatin's interaction with cell fate regulators such as p53, AKT and MAPk (Siddik 2003, Bragado *et al.* 2007, Hong *et al.* 2008, Galluzzi *et al.* 2012, Santin *et al.* 2013). Cisplatin death has been shown to be activated by initiating p53 expression with increased ROS, this high toxic stress activates the second-phase of p53 whereby ROS levels are further increased to toxic levels (Bragado *et al.* 2007, Chen *et al.* 2012). Furthermore, MAPk is also a cisplatin target as it can also activate p53 and increase ROS levels in the cell (Siddik 2003, Bragado *et al.* 2007). With the MDA-MB231 cells there was an increase in protein levels of p53 and of MAPk which could explain the high levels of both ROS and Ca^{2+} levels in the cell (Figure 6.3.A.) (Brookes *et al.* 2004, Bragado *et al.* 2007, Florea and Busselberg 2011). However, in the control cells a significant reduction of p53 activity and MAPk expression was observed following cisplatin treatment suggesting that there is a protection of the cell (Figure 6.3.B) (Wang *et al.* 1998, Bragado *et al.* 2007, Koczor *et al.* 2013). The cell survival target, Nrf2 had no change to its gene expression levels in cancer cells and no change to protein levels in control cells (Lau *et al.* 2008, Ren *et al.* 2011, Vinod *et al.*

2013). Nrf2 inhibition encourages cell death and many plant compounds upregulate its activity to decrease ROS (Kensler *et al.* 2007, Lau *et al.* 2008, Vinod *et al.* 2013). Furthermore, the cell growth pathway of AKT and NFκB were not activated with cisplatin treatment further cementing the fact that cisplatin inhibits cell growth (Vivanco and Sawyers 2002, Elstrom *et al.* 2004, Scotland *et al.* 2013). Finally, the DNA methylation state of cancer cells was significantly reduced with cisplatin treatment. Global hypomethylation has been shown to increase chromosomal instability and can be linked to activating tumour activating proteins (Chen *et al.* 1998, Szyf *et al.* 2004). This was not observed in control cells.

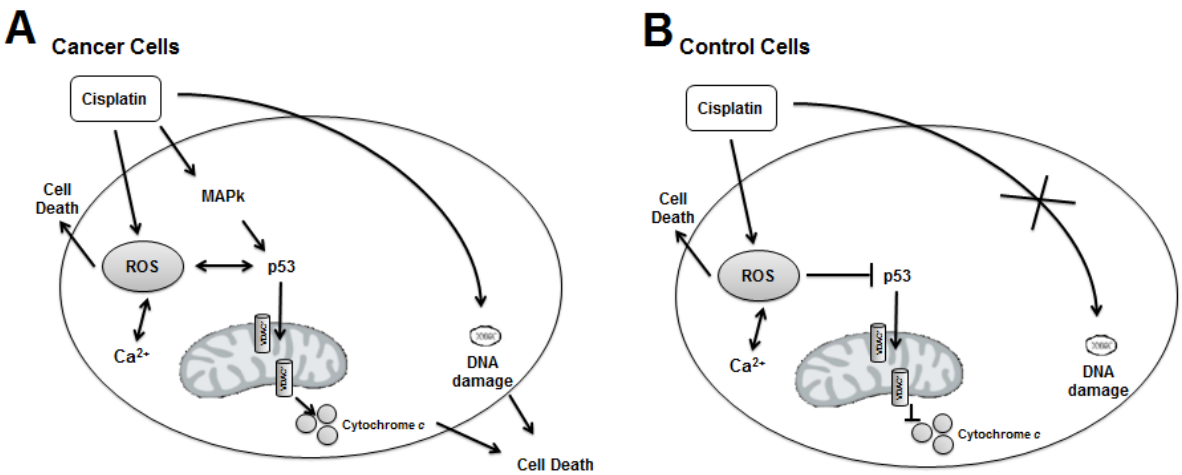

Figure 6.3. The mode of action of cisplatin in cancer and control cells.
The mode of action of cisplatin in **(A)** cancer cells leads to cell death through several different parameters. These include an increase in ROS and Ca^{2+} levels which encourage cell death. The molecular mechanism of p53 induces cell death through mitochondrial cytochrome *c* release and finally there was an observed DNA damage in the cell following treatment. Whereas in **(B)** control cells there was an increase in ROS levels as was seen in cancer cells which increased cell death. Though there was an inhibition of p53 which did not increase VDAC1 and mitochondrial apoptosis and unlike cancer cells there was no enhanced DNA damage.

6.3.1. Conclusion

The role of cisplatin in cancer cells has been shown to increase ROS concentration to a level that is toxic to the cell and to induce DNA damage through

crosslinking damage (Siddik 2003, Kawai *et al.* 2006, Bragado *et al.* 2007, Martins *et al.* 2008, Galluzzi *et al.* 2012). Following cisplatin treatment, I showed that cancer cells do have increased ROS concentration, Ca^{2+} levels and increased p53 leading to further increases of ROS and finally a hypomethylated state whereby DNA damage is being induced. Conversely I showed that in control cells, cell death was observed however this seems to be solely through ROS and Ca^{2+} level increase. There is no further activation of the p53 toxic route instead there is an inhibition of p53 and MAPk. This suggests that the control cells protective mechanism has been activated to regulate cell survival of the population that remains following treatment through closing the apoptotic VDAC1 channel in the mitochondria and controlling the rising ROS and Ca^{2+} levels. This work I have carried out has highlighted cisplatin's differing molecular mechanism between cancer and control cells. Herein I have shown that cisplatin increases cancer cell death through several regulatory pathways which include DNA damage, oxidative stress and mitochondrial damage. However, in control cells the same mechanism is not activated. This impaction in cancer therapy encourages the further use of cisplatin as a chemotherapeutic agent due to the differing molecular mechanism between cancer and control cells.

6.4. Could combining CBD with cisplatin have an enhanced effect?

Combining chemotherapeutic agents has been shown to be highly beneficial since the 1960s as mentioned previously (section 1.2.1.) (Frei *et al.* 1965, Moxley *et al.* 1967, Chabner and Roberts 2005, Caley and Jones 2012). However, much care is needed when combining chemotherapeutic agents to ensure that they are beneficial (Caley and Jones 2012). One main imperative is that both the agents are successful in reducing cell growth when used separately (Caley and Jones 2012, Galmarini *et al.* 2012). Here I have shown that both CBD and cisplatin treatment increased cell death on

their own (section 6.2. and 6.3.). Increased cell death by these separate agents on their own suggests that combining them could enhance these effects of cell death (Chabner and Roberts 2005, Kunnumakkara *et al.* 2007, Caley and Jones 2012). Phytochemicals have been shown to act as chemosensitizers and prevent cancer growth (Shankar *et al.* 2012, Vinod *et al.* 2013). As stated earlier, the dose for the combination study was 1 μM CBD as it showed a significant reduction in cell viability with cancer cells but not in control cells and 100 μM cisplatin as it was more potent a dose in both cell lines. Combinations of cannabinoids (80 nM-100 μM) have been previously used successfully with gemcitabine and bortezomib (Donadelli *et al.* 2011, Morelli *et al.* 2014). However, in my study, when CBD and cisplatin were combined there was no added effect on cancer cell death. Interestingly, there was a resistant effect to cell death from the combination treatment compared to cisplatin treatment alone. The same effect was observed with control cells. Combining positive cancer killing agents is not always successful as they may alter target proteins or compete for receptor targets (Chabner and Roberts 2005, Caley and Jones 2012). Interestingly though, when a combination of cisplatin and resveratrol was used in proximal tubular cells this same response was observed, whereby resveratrol and cisplatin alone showed a reduction in cell viability but when combined there was a significant protective effect (Kim *et al.* 2011). This protective effect was suggested to be through SirT1 activation which reduced cisplatin's chemotherapeutic effect (Yi and Luo 2010, Kim *et al.* 2011).

The mechanism of CBD in cancer cell death was through the activation of the p53/Nrf2 pathway and VDAC1 expression increase due to an increase in ROS and Ca^{2+} levels. The effectiveness of the combination of gemcitabine and the synthetic cannabinoid, GW, was through an increase in ROS level and in EnR stress increases (Donadelli *et al.* 2011). Furthermore, the decrease in cell viability of CBD in combination with bortezomib was through increased ROS levels and decreased AKT activity (Morelli *et al.* 2014). In cancer cell's following CBD and cisplatin combination treatment an

increase in ROS levels was observed though it was not as increased as cisplatin treatment alone. Furthermore, there was no added difference to mitochondrial respiration or maximal respiration from the combination treatment compared to untreated cells and cisplatin treatment. A reduction in glycolytic energy production and reserves remained also which suggests that the cells are not competing for cell growth as could be suggested (Scotland *et al.* 2013, Yadav and Chandra 2014, Yang *et al.* 2014). In control cells the same response was observed with ROS levels not being increased compared to cisplatin treatment alone. ROS signalling is an important factor in chemoresistance (Ren *et al.* 2011, Galluzzi *et al.* 2012, Vinod *et al.* 2013). The control of ROS levels by Nrf2 is a well-known mechanism for homeostatic cell control (Kensler *et al.* 2007, Lau *et al.* 2008, Martin-Montalvo *et al.* 2011, Ren *et al.* 2011, Chen *et al.* 2012, Sena and Chandel 2012, Vinod *et al.* 2013). Nrf2 activity has also been shown to inhibit ROS levels in a chemoresistant way whereby, cell growth is not inhibited but rather the cell is protected from the chemotherapeutic agent (Lau *et al.* 2008, Ren *et al.* 2011, Vinod *et al.* 2013). Many plant compounds have been shown to activate Nrf2 to control ROS and stress levels in the cell, such as curcumin, EGC and resveratrol (Kode *et al.* 2008, Lau *et al.* 2008, Na and Surh 2008, Asensi *et al.* 2011). When combined Nrf2 levels were unchanged compared to control levels in both cancer and control cells. Furthermore, there was a significant decrease in p53 levels which is similar to the response of resveratrol and cisplatin combination (Kim *et al.* 2011).

Furthermore to the chemoresistance observed with the combination treatment, recent work has suggested a role for cisplatin in controlling VDAC1 and in turn this controls ROS levels in the cell reducing cell death (Siddik 2003, Bragado *et al.* 2007, Martins *et al.* 2008, Tajeddine *et al.* 2008, Galluzzi *et al.* 2012). Overexpression of VDAC1 has been linked to increased sensitivity to apoptosis (Rapizzi *et al.* 2002). Both CBD and cisplatin treatment alone showed an increase in VDAC1 expression. Both agents have been shown to have an affinity to bind and activate VDAC1 (Yang *et al.*

2006, Galluzzi *et al.* 2012, Rimmerman *et al.* 2013). Interestingly though there was a significant reduction in VDAC1 expression with “combination” treatment in both cell types. Decreased VDAC1 expression has been linked to increased cell resistance (Tajeddine *et al.* 2008, Galluzzi *et al.* 2012). Furthermore, decreased p53 activity along with MAPk inhibition has been linked to cisplatin resistance in cells (Galluzzi *et al.* 2012). There is no activation of mitochondrial cell death due to this closure of VDAC1 and reduction in p53 expression (Mañes *et al.* 2003, Koczor *et al.* 2013).The fact that both of these tumour killing agents target VDAC1 on the mitochondria to increase apoptotic signals could be why there is no increased cell death. Moreover, the decrease in p53 activity and inhibition of MAPk confirms that there is no activation of mitochondrial apoptosis or increase of ROS by combination treatment (Figure 6.4.). The silencing of p53 has been linked to increased cell growth and decreased ROS and mitochondrial apoptosis (Mañes *et al.* 2003, Yi and Luo 2010, Kim *et al.* 2011, Galluzzi *et al.* 2012, Koczor *et al.* 2013).

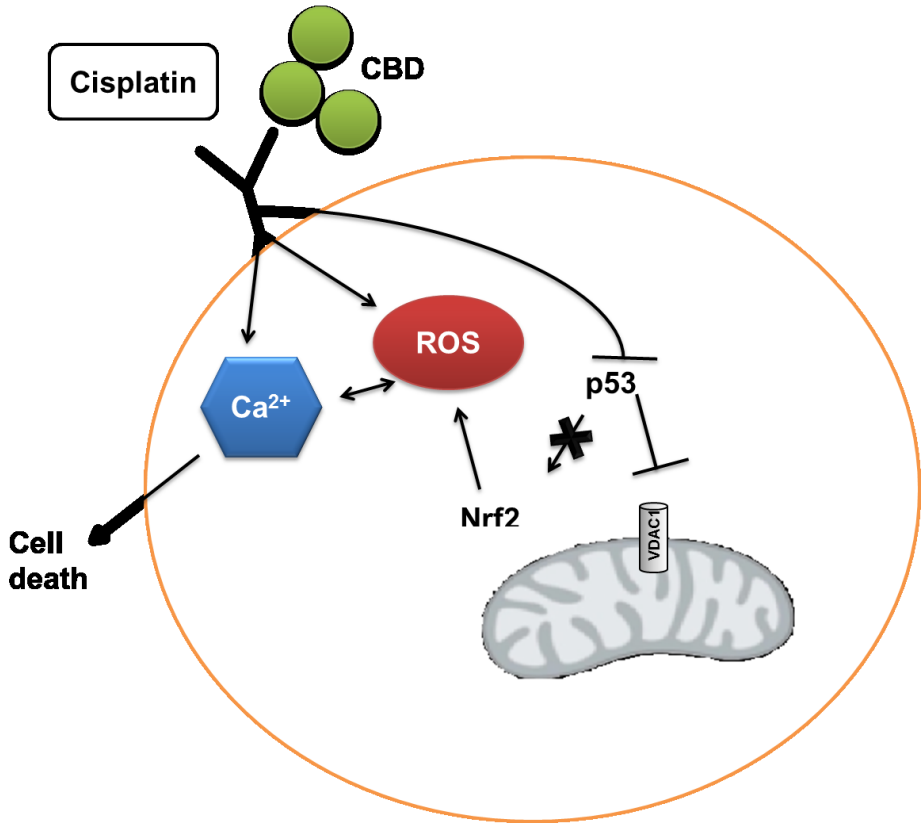

Figure 6.4. The mode of action of combining of CBD and cisplatin treatment.
The mode of action for the combination and cisplatin for both cancer and control cells was similar. An increase in Ca^{2+} levels was observed for both cell lines which led to cell death. No change in MAPk meant a down regulation of p53 and no change in Nrf2 activation. A significant reduction in VDAC1 proposed no release of cytochrome *c* to induce apoptosis.

6.4.1. Conclusion

The effect of combining CBD and cisplatin treatment together did not enhance cell death. There was a similar mechanism by which both agents were working which inhibited an enhanced effect. Chemotherapy resistance was observed due to increased Nrf2 activity promoting cell survival through controlling altered ROS levels from the agent's treatment (Lau *et al.* 2008, Kuznetsov *et al.* 2011a, Ren *et al.* 2011, Vinod *et al.* 2013). Furthermore, the competition for the VDAC1 receptor by both agents decreased cytochrome *c* release and apoptosis (Chabner and Roberts 2005, Yang *et al.* 2006, Rimmerman *et al.* 2011, Caley and Jones 2012). Furthermore, a decrease in p53 following combination treatment increased cell viability. Combinational treatments are

not always successful though interestingly, both cell types, cancer and control, responded in the same way to the “combination” effect. The mode of action of this “combination” effect had no specific difference to cancer or control cells. Therefore I would not suggest any further investigation into the “combination” of CBD and cisplatin for future cancer therapy.

6.5. “Priming”, its effect on cancer cell viability

The killing of cancer cells with chemotherapeutic agents has been shown to be a very effective cancer treatment (Siddik 2003, Chabner and Roberts 2005, Arisan *et al.* 2010, Florea and Busselberg 2011, Caley and Jones 2012). However, chemotherapy resistance which is observed with some chemotherapy treatments shows that novel, up to date mechanisms are desired (Chabner and Roberts 2005, Hong *et al.* 2008, Caley and Jones 2012, Galluzzi *et al.* 2012). One such method is pre-treating (“priming”) cancer cells with an agent that sensitizes cells to chemotherapy and hence enhances cell death (Sharma *et al.* 2005, Kuhar *et al.* 2007, Chonghaile *et al.* 2011, Reed 2011, Kumar and Singh 2012, Mu *et al.* 2014). Many natural compounds have been targeted as chemosensitizers and can reduce chemotherapy resistance (Sharma *et al.* 2005, Kuhar *et al.* 2007, Reed 2011, Vinod *et al.* 2013). In my study <u>CBD was shown to enhance cancer cell death through “priming”</u> prior to cisplatin treatment. Moreover, as the dose of CBD increased so too did the “priming” effect.

In control cells there was no effect following “priming” treatment. The effect observed with control cells was similar to cisplatin treatment viability. This lack of “priming” in control cells is advantageous if we are to move towards a novel chemotherapeutic strategy (Caley and Jones 2012). As I previously described, there was a different mode of action for both cancer and control cells following CBD and cisplatin treatment. I investigated if there also was a separate mode of action for control cells following “priming”.

6.6. The mode of action of "priming"

"Priming" has been suggested to work through mitochondrial sensitisation (Sharma *et al.* 2005, Chonghaile *et al.* 2011, Reed 2011). Alteration in cellular metabolism is a key hallmark of all tumour cells and it is this drive towards "the Warburg effect" that can make the cancer cell's mitochondria more sensitive to drug targets (Hanahan and Weinberg 2000, Brookes *et al.* 2004, Vander Heiden *et al.* 2009, Hanahan and Weinberg 2011). Mitochondrial dysfunction which includes reduced mitochondrial respiration, inhibition of the MOMP and increased VDAC1 activation can all lead to an increase in the cells susceptibility to death and thus making the mitochondria a key target for cancer therapy (Shoshan-Barmatz and Ben-Hail 2012, Huang *et al.* 2013, Zhang *et al.* 2014). The role of mitochondria in both cell survival and cell death is a tightly controlled mechanism and can be easily manipulated in stressed conditions (Galluzzi *et al.* 2011, Ashrafi and Schwarz 2013, Zhang *et al.* 2014). It is this sensitivity of the mitochondria that is key in the mode of action of "priming" (Chonghaile *et al.* 2011, Reed 2011, Wallace 2012). The mitochondria are both generators and targets for oxidative stress such as ROS (Donadelli *et al.* 2011, Lee *et al.* 2012). As described previously increases in ROS levels can lead to cell death and have been linked to damaged mitochondria (Kuhar *et al.* 2006, Reuter *et al.* 2010, Donadelli *et al.* 2011, Galluzzi *et al.* 2011, Gupta *et al.* 2012, Lee *et al.* 2012, Sena and Chandel 2012, Singer *et al.* 2015). Cancer cell's response to "priming" led to an increase in ROS and increased Ca^{2+} levels which were significantly higher than cisplatin treatment alone. Ca^{2+} as mentioned earlier can control ROS levels in the cell and are important in cancer cell regulation (Pinton *et al.* 2001, Rapizzi *et al.* 2002, Brookes *et al.* 2004, Saidak *et al.* 2009, Parkash and Asotra 2010). These high levels of ROS and Ca^{2+} observed in cancer cells was not mimicked in control cells. Increased ROS and Ca^{2+} levels led to permeabilization of the MOMP and increase cell death which was not observed in control cells (Rapizzi *et al.* 2002, Rizzuto *et al.* 2003, Parkash and Asotra 2010, Kuznetsov *et*

al. 2011a, Kuznetsov *et al.* 2011b, Lee *et al.* 2012). Interestingly, when cancer cells were "primed" with quercetin, an increase in ROS led to a 71% increase of cytochrome *c* release and consequently an increase in cell death compared to either treatments alone (Kuhar *et al.* 2006). However, even though the mitochondria itself was never examined in Kuhar's study, it points out that the mechanism of "priming" may be through ROS increase resulting in mitochondrial damage (Kuhar *et al.* 2006, Donadelli *et al.* 2011, Lee *et al.* 2012, Mei *et al.* 2014).

ROS, Ca^{2+} and ATP are tightly linked and any alteration in their balance can lead to an increase in cell death (Brookes *et al.* 2004). As mentioned previously, increased Ca^{2+} levels increase mitochondrial swelling and sensitisation leading to apoptosis (Pinton *et al.* 2001, Mathiasen *et al.* 2002, Rapizzi *et al.* 2002, Rizzuto *et al.* 2003). Altered calcium homeostasis leads to increased MOMP, VDAC1 activation and ATP depletion (Figure 6.5.) (Tiwari *et al.* 2002, Mayer and Oberbauer 2003, Brookes *et al.* 2004, Jackson and Schoenwaelder 2010). In cancer cells, "priming" with CBD resulted in decreased mitochondrial respiration, ATP production levels and a stable maximal respiration which have all been linked to increased apoptosis (Jackson and Schoenwaelder 2010, Hahm *et al.* 2011). Decreased ATP production levels have been linked to increased oxidative stress and increased apoptosis and programmed necrosis (Tiwari *et al.* 2002, Jackson and Schoenwaelder 2010, Ouyang *et al.* 2012, Sena and Chandel 2012). Moreover, no increase in AMPK was observed to counteract decreased ATP levels or increased Ca^{2+} levels observed (Ojuka 2004, Hardie 2008). Improved metabolic control and a reduction in cell death is linked to AMPK therefore a reduction in AMPK activity suggests that "priming" is independent of AMPK (Zhang *et al.* 2009, Jeon *et al.* 2012). In control cells, there was no change to ATP levels or maximal respiration. Ca^{2+} increases along with increased ROS leads to increased MOMP, increased ATP production due to the reduction of mitochondria permeability along with increased sensitivity to apoptosis (Pinton *et al.* 2001, Rapizzi *et al.* 2002, Rizzuto *et al.*

2003, Lisanti *et al.* 2010, Parkash and Asotra 2010, Kuznetsov *et al.* 2011b, Lee *et al.* 2012). In control cells there was no increase in ROS, rather a significant decrease was observed leading to no change in ATP levels significantly reduced proton leak and no enhanced cell death. The effect of "priming" in control cells is not the same as cancer cells from this observation. The mitochondria are not sensitized and are instead reducing the release of ATP and apoptotic bodies through decreased proton leak protecting the control cells from any "priming" damage (Tiwari *et al.* 2002, Chonghaile *et al.* 2011, Reed 2011, Wallace 2012, Sarosiek *et al.* 2013, Valvassori *et al.* 2013).

Increased Ca^{2+} can lead to increased cytochrome *c* release due to increased VDAC1 activity (Rapizzi *et al.* 2002, Brookes *et al.* 2004, Kawai *et al.* 2006, Shoshan-Barmatz *et al.* 2014). In the presence of increased ROS, VDAC1 has been shown to activate the release of cytochrome *c* and thus induce apoptosis (Madesh and Hajnóczky 2001). Furthermore, both CBD and cisplatin's affinity to VDAC1 can increase its sensitivity to increase cytochrome *c* through the "priming" mechanism (Figure 6.5.) (Rapizzi *et al.* 2002, Yang *et al.* 2006, Shoshan-Barmatz *et al.* 2010, Rimmerman *et al.* 2013, Shoshan-Barmatz *et al.* 2014). Moreover, Madesh and colleagues showed that ATP was required by the cell to preserve the MOMP which was reduced following "priming" treatment (Madesh and Hajnóczky 2001, Chonghaile *et al.* 2011, Sarosiek *et al.* 2013). The sensitivity of the mitochondria is a key mechanism of "priming" and this sensitivity seems to be reached by increased VDAC1 activity, which has not been shown previously with "priming" (Figure 6.5.) (Chonghaile *et al.* 2011, Reed 2011, Rimmerman *et al.* 2013, Sarosiek *et al.* 2013, Shoshan-Barmatz *et al.* 2014). There is a higher abundance of VDAC1 receptors in cancer cells compared to control cells (Simamura *et al.* 2008). The reduction of VDAC1 receptors in control cells has been suggested to be due to the "Warburg Effect" whereby the cancer cells require more energy to drive metabolism and cell growth and therefore require a heavier flux of transportation of molecules requiring more VDAC1 receptors (Simamura *et al.* 2008, Vander Heiden *et*

al. 2009, Israelsen and Vander Heiden 2010, Hanahan and Weinberg 2011). "Priming" in control cells saw an increase in VDAC1 activity though no change in cell viability compared to cisplatin treatment.

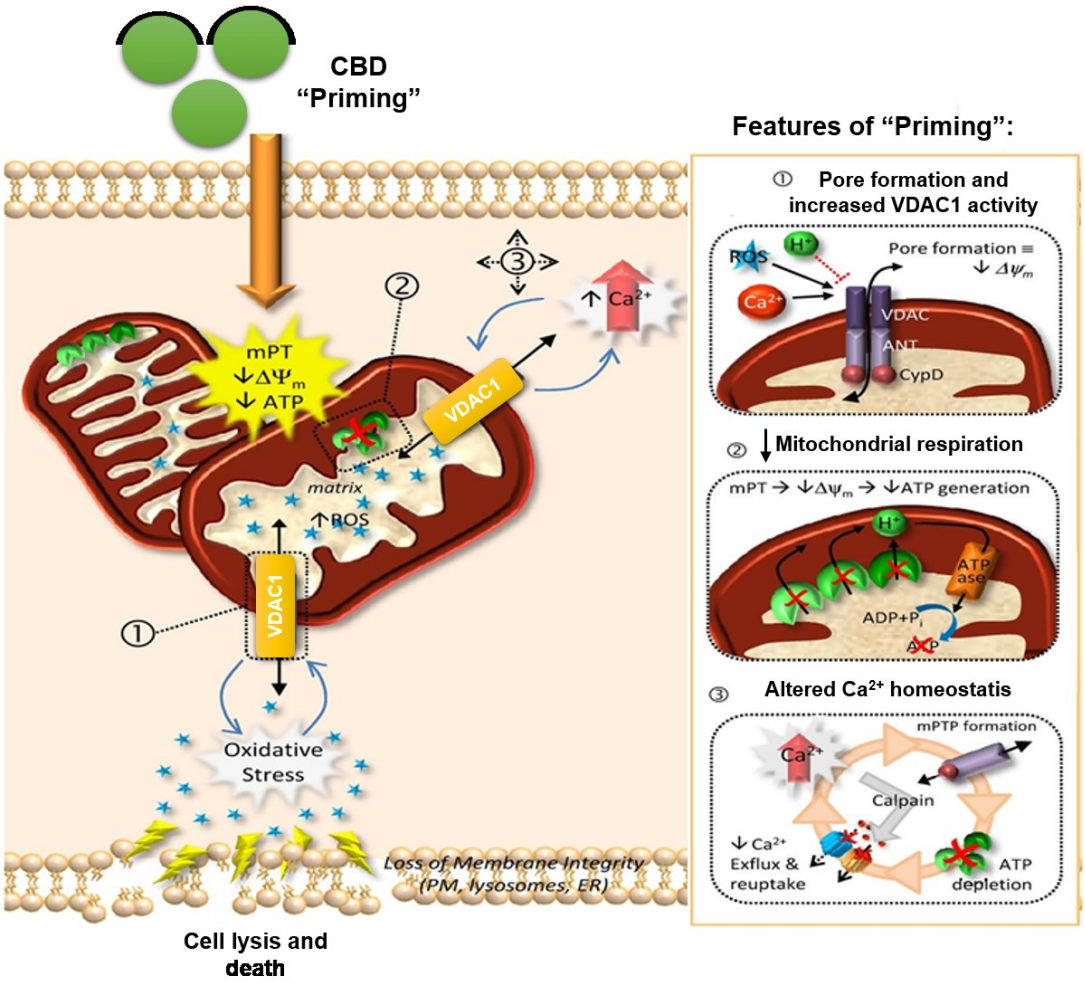

Figure 6.5. The effect of mitochondrial sensitisation following "priming" in cancer cells
"Priming" promotes mitochondrial sensitisation through increased VDAC1 activity, decreased ATP production and altered Ca^{2+}activity. These are all related to the programmed cell death known as necrosis, thus enhancing "priming" cell death through mitochondrial sensitzation. Adapted from Jackson and Schoenwaelder, 2010).

At a molecular level, further to the increased MOMP in the mitochondria through VDAC1 activity increase and consequent ROS and Ca^{2+} level increase, p53 has been shown to induce MOMP and recruit pro-apoptotic proteins in high stress conditions (Madesh and Hajnóczky 2001, Pinton *et al.* 2001, Rapizzi *et al.* 2002, Pastorino *et al.* 2005, Chen *et al.* 2012, Koczor *et al.* 2013). As mentioned previously p53 is a master

regulator of apoptosis and there is a fine balance of when p53 can control cell death through activation of cell controlling pathways e.g. Nrf2, and when it can lead to an uncontrolled cell death through increased ROS and Ca^{2+} levels (Yi and Luo 2010, Bansal *et al.* 2011, Chen *et al.* 2012). "Priming" has been shown to increase p53 to levels whereby it increases cellular ROS and decreases the controllers of cell growth, AKT and Nrf2 (Bragado *et al.* 2007, Chen *et al.* 2012, Galluzzi *et al.* 2012, Kumar and Singh 2012, Mu *et al.* 2014). In some "priming" cases mutated or abnormal p53 have also been activated to levels that lead to apoptosis (Putral *et al.* 2005, Ventura *et al.* 2007, Kumar and Singh 2012, Koczor *et al.* 2013, Mu *et al.* 2014). "Priming" with CBD in cancer cells showed an increase in protein levels of p53 and furthermore an increase in SirT1 protein and gene expression suggesting that SirT1 is trafficking p53 to the mitochondria to increase cell death through cytochrome *c* release (Figure 6.1.) (Ventura *et al.* 2007, Yi and Luo 2010, Wade *et al.* 2013). VDAC1 activity has also been shown to be induced by p53 activation (Siddik 2003, Yang *et al.* 2006, Galluzzi *et al.* 2012). This interaction between SirT1 and p53 is driven through the increase in ROS already described (Yi and Luo 2010). Furthermore, there was no change in HDAC1 expression illustrating that p53 is not being directed to cell proliferation in cancer cells (De Amicis *et al.* 2011).

Nrf2 is an important chemoresistant agent whereby it protects against oxidative stress by initiating the AREs which reduces and controls ROS levels in the cell, increases cell growth through AKT interaction thus decreasing the amount of cell death (Morito *et al.* 2003, Pi *et al.* 2007, Li *et al.* 2008, Wang *et al.* 2008, Bitar and Al-Mulla 2011, Tufekci *et al.* 2011, Chen *et al.* 2012, Vinod *et al.* 2013, Namani *et al.* 2014). Loss of Nrf2 can lead to mitochondrial depolarization, increased MOMP, ATP reduction and in increase in ROS (Morito *et al.* 2003, Lau *et al.* 2008, Ohtsuji *et al.* 2008, Bitar and Al-Mulla 2011, Holmström *et al.* 2013). Interestingly, there was a significant reduction in Nrf2 gene expression and protein levels following "priming" in cancer cells but control cells showed no change. AKT controls cell growth and proliferation along with mTOR

which is a controller of mitochondrial activity (Liu *et al.* 2013, Mannaa *et al.* 2013). No change in AKT activation and a reduction in the controller of mitochondrial activity, mTOR was observed following “priming” in cancer cells (Scotland *et al.* 2013). Interestingly, control cells did not show the same response but rather had an increase in Nrf2 and AKT activity following “priming”. Increased AKT increase the resistance the cell has to chemotherapy treatment (Hong *et al.* 2008). Furthermore, Nrf2 activity has been linked to chemoprevention also by reducing ROS resulting in chemoresistance (Lau *et al.* 2008, Ren *et al.* 2011, Vinod *et al.* 2013). Increased AKT recruits NFκB and FOXOs to activate pro survivial genes and inhibit apoptosis from the mitochondria (Nakanishi and Toi 2005, Yadav and Chandra 2014). Furthermore, AKT has also been shown to interact with and down regulate VDAC1 activity (Yadav and Chandra 2014). Mitochondrial survival can be controlled by p53 activation through weakening the cancer cell (Shimizu *et al.* 1999, Matoba *et al.* 2006, Price *et al.* 2012). As shown in section 6.2., following CBD treatment in cancer cells, a mild stress response through p53 and Nrf2 activity which control ROS levels and induced controlled cell death was activated (Figure 6.2.) (Chen *et al.* 2012). This same mechanism was activated when control cells were “primed” with CBD resulting in a chemoresistant mechanism to “priming” (Ren *et al.* 2011, Chen *et al.* 2012, Vinod *et al.* 2013). However, “priming” in cancer cells activates the second high cell stress response whereby p53 was significantly increased and transported to the mitochondria through SirT1 while Nrf2 was significantly down regulated (Figure 6.6.) (Yi and Luo 2010, Chen *et al.* 2012). The high stress within the cell from increased ROS and Ca^{2+} levels drive p53 to pro-apoptotic mediation, reducing the Nrf2-mediated survival mechanism and increasing ROS levels in a feedback loop mechanism from the mitochondria (Bragado *et al.* 2007, Chen *et al.* 2012). It is this activation in cancer cells of the second phase of the p53/Nrf2 pathway that can enhance cell death through “priming”.

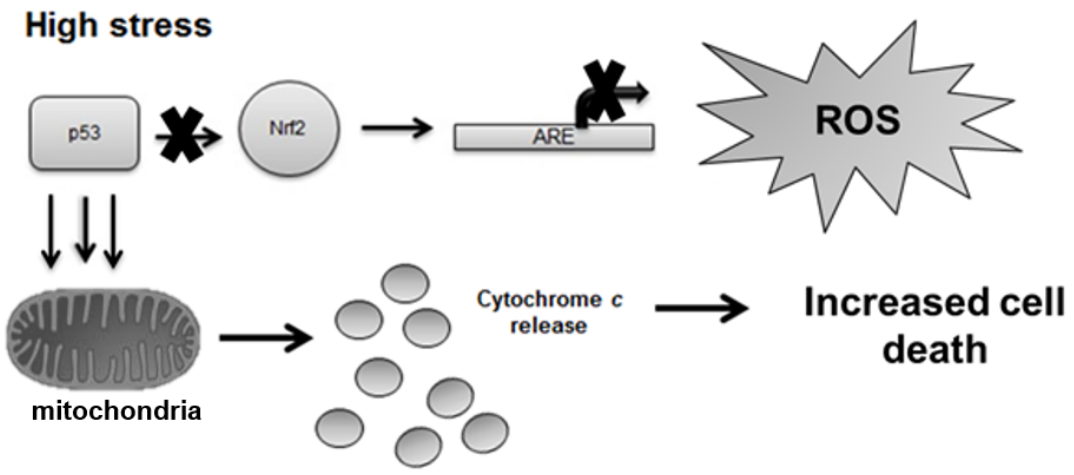

Figure 6.6. "Priming" in cancer cells induces a high stress response by activating p53 to toxic levels.
Due to high stress levels, such as "priming, p53 can act as a pro-apoptotic activator through inhibition of Nrf2 activation which increases cellular ROS levels. Increased ROS levels lead to uncontrolled cell death. Furthermore, p53 increased the release of cytochrome *c* from the mitochondria to induce further apoptotic cell death.

At a genetic level, cancer cells have been shown to be globally hypomethylated and cisplatin has been shown here and in other research to reduce cell viability through nuclear crosslinking damage (Jordan and Carmo-Fonseca 2000, Das and Singal 2004, Szyf *et al.* 2004, Florea and Busselberg 2011, Galluzzi *et al.* 2012). Interestingly, drug targets for hypomethylation have been shown to cause short-term therapeutic treatment for cancer reduction and this is what was observed with cisplatin alone (Feinberg and Tycko 2004). Hypomethylation has been suggested to initiate chromosomal instability (Szyf *et al.* 2004). DNA hypermethylation has been shown to reduce cell viability and ultimately lead to multidrug resistance in cancer cells and is a prime target in chemotherapy (Feinberg and Tycko 2004). Cisplatin has recently been shown to hypermethylate cancer cells both *in vitro* and *in vivo* (Shen *et al.* 2012). "Priming" resulted in a global hypermethylation of cancer cells which proposes an idea that the DNA is deregulated and that genes are being silenced (Feinberg and Tycko 2004,

Roossink *et al.* 2012). This loss of gene function by hypermethylation can lead to increased mutations and hence cell death and could be pivotal in advancing chemotherapeutics for a long-term anticancer treatment (Ehrlich 2002, Herman and Baylin 2003).

6.6.1. Conclusion

In summary, “priming” in cancer cells was shown to enhance cell death through sensitising the mitochondria following increased ROS levels, Ca^{2+} levels and VDAC1 channel to increase cytochrome *c* release and thus increase cell death (Figure 6.5. and 6.7.A.) (Madesh and Hajnóczky 2001, Pinton *et al.* 2001, Rapizzi *et al.* 2002, Brookes *et al.* 2004, Donadelli *et al.* 2011, Lee *et al.* 2012, Shoshan-Barmatz *et al.* 2014). Furthermore, “priming” in cancer cells also initiates the high stress p53/Nrf2 two-phase pathway to further cell death in the cancer cell by encouraging cell death and inhibiting chemotherapy resistance (Lau *et al.* 2008, Ren *et al.* 2011, Chen *et al.* 2012, Vinod *et al.* 2013). The VDAC1 receptor is activated through ROS, Ca^{2+} and p53 initiation to reduce the MOMP, leading the mitochondrial sensitisation and cell death (Figure 6.7.A.) (Madesh and Hajnóczky 2001, Simamura *et al.* 2008, Shoshan-Barmatz and Ben-Hail 2012, Huang *et al.* 2013, Tewari *et al.* 2015).

Interestingly, in control cells the same mechanism for “priming” as not observed. In fact, the cell death observed following “priming” in control cells is similar to cisplatin cell death whereby there is no increase in mitochondrial function and no DNA damage (Figure 6.3.B. and 6.7.B.). There was an activation of the mild p53/Nrf2 pathways reducing cell death but controlling ROS levels in the cell resulting in a chemo resistant phenotype to “priming” (Lau *et al.* 2008, Ren *et al.* 2011, Chen *et al.* 2012, Sena and Chandel 2012, Vinod *et al.* 2013).

As mentioned previously in section 1.2.2.3., a major problem with cancer therapy is the rise of chemoresistance to the treatment. There has been much advancement and progression in novel cancer agents however the same issues arise (Siddik 2003, Galluzzi *et al.* 2006, Santin *et al.* 2013). The importance of "priming" as a novel mechanism is that due to its staged treatment would lead to lower treatment usage and hence lowered chance of resistance. The future of cancer therapy relies on the combating of chemoresistance and "priming" will overcome this. I have shown here that "priming" with CBD enhances chemotherapeutic treatment by increasing mitochondrial sensitivity. Increased mitochondrial sensitivity reduces the amount of required chemotherapy agent resulting in fewer complications and resistance.

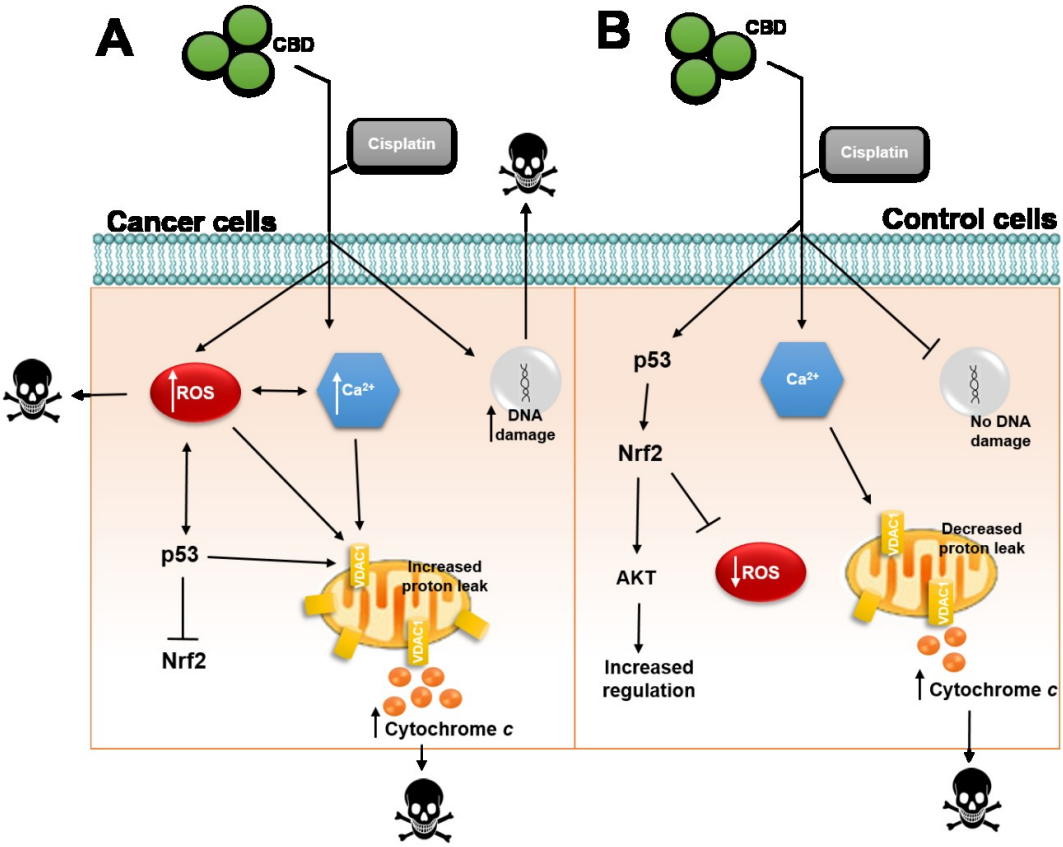

Figure 6.7. "Priming" mode of action in cancer and control cells.
The mode of action of "priming" in **(A)** cancer cells is through the over activation of ROS levels, p53 activation and DNA damage. This leads to an increase in apoptosis due to increased MOMP and increased in cytochrome *c* release. **(B)** Control cells cell death was unaltered by "priming" and follows similar path to that observed with cisplatin alone of an increase in VDAC1 expression. No increase in ROS was observed due to the activation of the mild stress p53.Nrf2 pathway.

6.7. Additional cancer cells lines examined for their potential of "priming" with CBD

As mentioned previously, CBD's use in inhibiting tumour growth has been extensively documented in lung, glioma, breast, colon and prostate cancer to name a few (McAllister *et al.* 2011, Shrivastava *et al.* 2011, Aviello *et al.* 2012, Caffarel *et al.* 2012, De Petrocellis *et al.* 2012b, Ramer *et al.* 2012, Massi *et al.* 2013, Macpherson *et al.* 2014, Sanchez *et al.* 2014). In this study additional cancer cells were examined for a potential "priming" effect with CBD. A recent study using primary hepatocytes showed that after 8 hours of CBD treatment a reduction in cell viability was observed with 5 μM -20 μM CBD (Lim *et al.* 2011). Liver cancer cells HepG2 showed a reduction of cell viability following 0.1 μM CBD treatment in this study and when treated with cisplatin also showed a significant reduction. Since there was an effect from both agents on cell viability when treated separately their effect at "priming" would be positive (Caley and Jones 2012). Following "priming" a significant reduction compared to cisplatin was observed from 5 μM. Colon cancer cells, Caco-2, have been recorded to reduce viability with CBD following 24 hours and 72 hours (Aviello *et al.* 2012, Macpherson *et al.* 2014). Interestingly, treating Caco-2 cells with CBD from 0.0.1 μM to 10 μM CBD for 24 hours showed no change in cell viability but did reduce proliferation rates at 10 μM treatment (Aviello *et al.* 2012). Whereas treatment for 72 hours showed a reduction in proliferation of Caco-2 cells from 1 μM CBD (Macpherson *et al.* 2014). Some of the difference observed between this study and other research, could be due to the different type of colon cells used. Caco-2 a polarized monolayer that mimics epithelial cells of the small intestine whereas the HT29 cells are heterogeneous in culture in that they contain a small proportion of mucus-secreting cells and columnar absorptive cells (Gagnon *et al.* 2013, Macpherson *et al.* 2014). These small changes could allow CBD to act differently in different cell types. Colon cancer treated with CBD has been shown to reduce AKT/mTOR activity and increase ROS levels (Aviello *et al.* 2012, Velasco *et al.* 2012, Massi *et al.* 2013, Romano *et al.* 2014). These reported mechanisms for CBD in colon

cancer are shared with what has been observed in MDA-MB231 cells here following "priming" suggesting a positive role for colon cancer in "priming". In this study, colon cancer cells showed a reduction with CBD treatment from a low dose of 0.5 μM CBD. While, colon cancer cells showed a reduction in viability following 1 μM CBD "priming" treatment.

MCF7 cells have been shown along with MDA-MB231 cells to exhibit reduced cell viability following CBD treatment (McAllister *et al.* 2011, Shrivastava *et al.* 2011, Caffarel *et al.* 2012, Massi *et al.* 2013). A reduction in MCF7 viability was observed following CBD treatment which was comparable to a previous study with similar doses (Shrivastava *et al.* 2011). Interestingly, there was no effect on cell viability following cisplatin treatment which suggested that "priming" would not work as both agents need to reduce viability on their own (Reed 2011, Caley and Jones 2012). This was further confirmed through examining cell viability following "priming" which showed an increased cell viability from 0.1 μM CBD treatment. A more pronounced effect of "negative" "priming" was observed in A549 cells. A549 have shown to be reduced by 51% with 3 μM CBD treatment after 48 hours (Ramer *et al.* 2012). Again, comparable time points and treatment dose may show the same result but for this study 24 hours was the treatment period selected. Furthermore, there was no effect on A549 cells following cisplatin treatment. Neither CBD nor cisplatin reduced cell viability following 24 hours of treatment which meant "priming" would not be successful (Reed 2011, Caley and Jones 2012, Sarosiek *et al.* 2013).

Since CBD has been shown to increase ROS, decrease the MOMP and interact with the mitochondrial receptor VDAC1 it was important to evaluate the mitochondria itself in controlling cell viability (Shrivastava *et al.* 2011, Massi *et al.* 2013, Rimmerman *et al.* 2013, Iannotti *et al.* 2014, Singer *et al.* 2015). Interestingly, cells that have mutated complex I and IV and a lack of mitochondrial DNA (Rho0) were shown to have an increase in cell viability following 2.5 μM CBD treatment (Annunen-Rasila *et al.* 2007,

Rimmerman *et al.* 2011, Mecha *et al.* 2012). Interestingly, it seems that using a cell type that have dysfunctional mitochondria led to an increase in cell viability which suggest that the mitochondria play a greater role in CBD's tumourogenic effects than previously thought (Bénard *et al.* 2012). Potentially CBD is not accessing the mitochondria and therefore not inhibiting cell growth through apoptosis. Interestingly, there was a reduction of cell viability following cisplatin treatment and furthermore, showed a significant reduction in viability following "priming" with CBD. CBD treatment has been shown to increase the activity of mitochondrial complexes in rat brain (Valvassori *et al.* 2013). This impairment of complexes I and IV, inhibited the effect of CBD in killing cancer cells which suggests that CBD's anti-tumour effect is mitochondrial based and linked to OXPHOS. Inhibiting complex IV can result in dysfunctional OXPHOS however with MDA-MB231 cells there was an increase in mitochondrial respiration suggesting that CBD can compensate for the impaired complex IV (Nelson and Cox 2006, Valvassori *et al.* 2013). Interestingly, following "priming" treatment there was an increase in cell death compared to cisplatin treatment alone and seemed to be more potent than any other cancer cell tested. This could be through increased cytochrome *c* build up due to the lack of a functioning complex IV (Nelson and Cox 2006). However, as will be mentioned later caution with these cells should be noted as any impairment to vital regulators could affect factors not targeted with the proposed mechanisms.

6.7.1. "Priming" with other plant compounds

"Priming" with natural polyphenols has been previously shown with quercetin in laryngeal cancer cells showing a reduction of ~48% following "priming" with 40 μM quercetin (Sharma *et al.* 2005, Kuhar *et al.* 2007). Curcumin was also shown to reduce cell viability in laryngeal cancer cells by ~40% and in T-cell lymphomas "priming" with aspirin had a 10% further reduction in viability (Kuhar *et al.* 2007, Kumar and Singh

2012). Here, quercetin was repeated using the doses described but there was no effect on the cancer or control cells. Furthermore, no effect following “priming” with quercetin was observed in either cell line. This further confirms that the anti-tumour plant compound should be effective at killing the cancer cells when used as a single treatment (Chabner and Roberts 2005, Caley and Jones 2012).

6.7.2. “Priming” with acetate

The short chain fatty acid (SCFA), acetate has been shown to reduce cancer cell growth from about 10 mM in colon cancer (Gamet *et al.* 1992, Hague *et al.* 1995). However, other reports have shown it has no effect on cancer cells from lower concentrations (Leschelle *et al.* 2000). Using 1 mM acetate, I did not observe any effect on cancer or control cell viability. However, when cancer cells were “primed” prior to 50 μM cisplatin there was a significant reduction in cell viability compared to untreated cells and cisplatin. No such effect was observed with control cells. Recent work has shown that SCFAs can increase ROS and ablate the mitochondria’s function (Leschelle *et al.* 2000, Lan *et al.* 2007). This effect could be behind the effect observed with the cancer cells “primed” with acetate. Furthermore, in control cells there was a significant increase in cell viability with “priming”. This was significantly different to untreated cells and cisplatin.

Interestingly, when the cisplatin dose was increased to 100 μM there was no enhanced effect of cell death in the cancer cells and the increase in cell growth in control cells was not as effective. This is interesting as the higher dose of cisplatin with CBD enhances cell death further as would be expected. The role of acetate in cancer cells is not well elucidated as high physiological doses are required as a single treatment to get any effect on cell death (Gamet *et al.* 1992, Hague *et al.* 1995, Leschelle *et al.* 2000). “Priming” could alleviate this problem in SCFA cancer treatment however, further work

into the mechanism of both cancer cell death and control cell growth through “priming” is required.

6.7.3. The translation of “priming” to an *in vivo* model

Much research has shown the potential of CBD as an anti-tumour agent both *in vitro* and *in vivo* (Ligresti *et al.* 2006, McAllister *et al.* 2011, Shrivastava *et al.* 2011, Aviello *et al.* 2012, De Petrocellis *et al.* 2012a, Ramer *et al.* 2013, McAllister and Desprez 2014, Romano *et al.* 2014). This translation from cell work through to murine model shows the potential CBD has as a chemotherapeutic agent (McAllister and Desprez 2014, Sanchez *et al.* 2014, Velasco Diez *et al.* 2014). Many different xenograft models have been used for this type of work, including breast, prostate, lung and colon (Ligresti *et al.* 2006, McAllister *et al.* 2011, De Petrocellis *et al.* 2012a, Ramer *et al.* 2013, Romano *et al.* 2014). Confirming previous results, CBD treatment led to a significant decrease in tumour volume in the MDA-MB231 xenograft when compared to control. Like with the cell work there are many different ways of producing a similar result of reduced tumour volume such as using CBD-BDS (CBD-Botanical drug substance) straight from the plant at 100 mg/kg daily in a prostate xenograft (De Petrocellis *et al.* 2012a). However, most CBD studies have used 5 mg/kg treatments, carried out either daily or every 72 hours (Ligresti *et al.* 2006, McAllister *et al.* 2011, Ramer *et al.* 2013, Romano *et al.* 2014). Daily injections could skew the results of the *in vivo* model due to increased stress therefore in this study treatments were carried out every other day (1 μM CBD) with intra-tumour injections. Interestingly, following “priming” treatment there was a significant reduction in tumour volume compared to cisplatin treatment alone. “Priming” has so far been concentrated on *in vitro* studies and no work has yet been brought forward to show that “priming” can enhance chemotherapy *in vivo* as was done here (Sharma *et al.* 2005, Kuhar *et al.* 2007, Chonghaile *et al.* 2011, Reed 2011, Kumar and Singh 2012).

6.8. Final discussion

The research completed here has shown a novel mechanism for CBD “priming” treatment in cancer cells along with a complete story behind CBD and cisplatin’s mode of action in both cancer cells and control cells. More importantly however, “priming” has been shown to be a novel technique specific to cancer cells in enhancing cell death compared to chemotherapy treatment alone. Furthermore, the full mode of action for “priming” has been completed showing a different mechanism between cancer cells and control cells which highlight its advantages towards tumour specific cell death. Finally, “priming” has also been shown not only *in vitro* to reduce cell death compared to chemotherapy treatment but *in vivo* also. In this body of work, I have shown that “priming” with CBD enhances chemotherapeutic treatment through mitochondrial sensitisation.

6.9. Limitations to study

The present work has several limitations that should be considered and are presented below.

6.9.1. MTT Assay

The MTT assay is a well-established method for observing cell death in a cell population. The ease, sensitivity and rapidity of the assay have made the MTT assay one of the most widely used however, there are several disadvantages to this metabolic assay along with others of its kind (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide (XTT), WST-1) (Sumantran 2011, van Meerloo *et al.* 2011). There have been reports that show these assays underestimate cell death as they measure the late stages of apoptosis, when cell activity is severely reduced (Wang *et al.* 2010, Sumantran 2011). The MTT assay works on the principle that mitochondrial activity is constant when cells are viable (van Meerloo *et al.* 2011).

To circumvent these problems several additional techniques were used. A FACS analysis using PI was utilised to observe if there were any difference to cell viability (Section 2.7.1.) along with a lactate dehydrogenase assay that examined cell damage and toxicity (Section 2.7.3.). Furthermore, a trypan blue exclusion assay and morphology examination (not shown here) was also performed initially to confirm the original MTT results before continuing with the study. This was completed by staining the cells with trypan Blue in a 1:1 ratio following treatment. The live cells possess an intact cell membrane which excludes the trypan blue while dead cells do not. This allows quantitative analysis of the live and dead cell population following treatment. Another set of experiments could have been carried out which include caspase detection through an enzyme-linked immunosorbent assay (ELISA) assay for early apoptosis detection and DNA fragmentation for late apoptosis detection (Sumantran 2011). The limitations

observed with the MTT assay do not impair the viability tests completed here as the effects were confirmed in several different ways.

6.9.2. Mitochondria-altered Rho0 cells

The novel mitochondria-altered Rho0 cells were interesting to use in this study given the proposed mechanism of “priming”. These cells have deleted complex I and IV which means that the conversion from NADH to Ubiquinone which is a cytochrome *c* precursor is inhibited as well as the conversion of cytochrome *c* to O_2 which is the electron releasing step (Figure 6.8) (Nelson and Cox 2006). This deletion would suggest reduced ATP production and a build of cytochrome *c* from complex III meaning a more sensitive aerobic flux and mitochondrial apoptosis. These cells showed increased viability with CBD treatment and significantly reduced cell viability with “priming”. The reduction of cell growth with CBD was suggested to be through mitochondrial sensitivity however CBD had no effect on Rho0 cells. “Priming” relies on mitochondrial sensitisation which was seen to be enhanced with Rho0 cells which is what could be expected with complex I and IV deletion. These cells are poorly understood and the data from it should be taken with caution as these cells do not act like normal cells. They are a good example of impaired mitochondria in cells though should be used cautiously for further investigations.

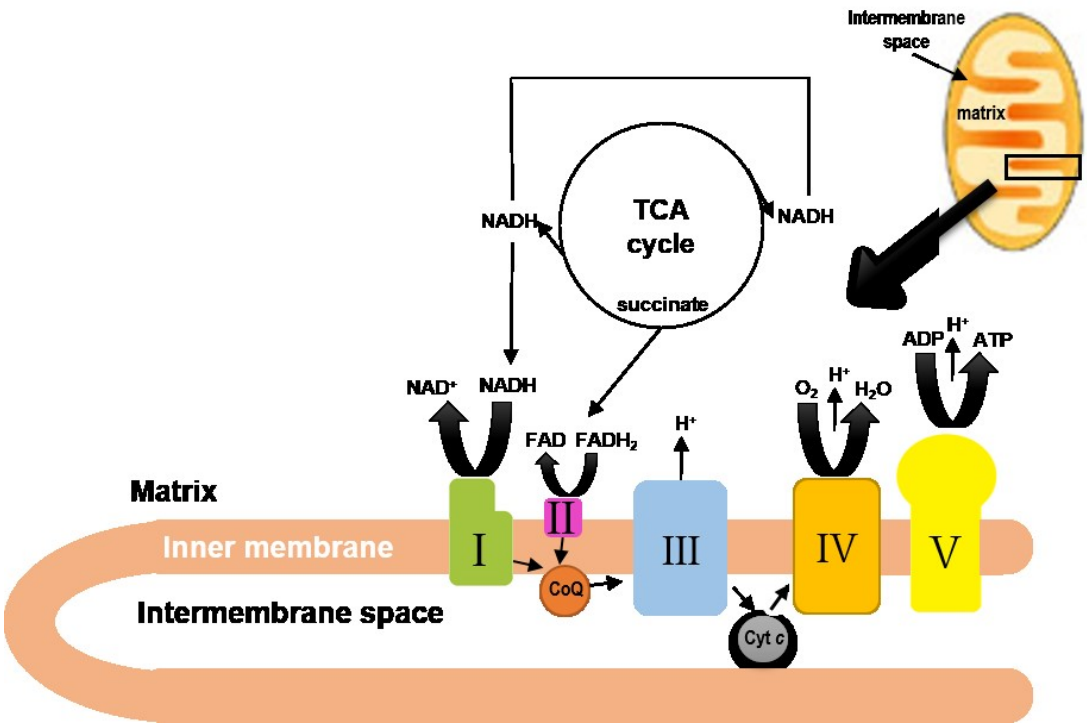

Figure 6.8. Mitochondrial complexes.
The mitochondrial complexes compose of 4 units and an ATP synthase complex (V). Complex I and complex II (which is part of the TCA cycle) activate coenzyme Q (CoQ). This is then utilised by complex III to increase the proton pump (H^+) potential and activate cytochrome *c* (Cyt *c*). This is then is used by complex IV where molecular oxygen (O_2) is reduced to water (H_2O). The ATP synthase, complex V, mediates the conversion of ADP to ATP with the help of the proton pump potential.

6.9.3. AMPK activity

AMPK can be activated in stressed conditions, when the AMP/ATP ratio is low and is required for maintaining glucose homeostasis (Ojuka 2004, Hardie 2008, Zhang *et al.* 2009, Vara *et al.* 2011, Hawley *et al.* 2012, Dando *et al.* 2013). There are 2 key pathways that activate AMPK activity and they are through a Ca^{2+}-dependent mechanism and through activation of LKB1 (Hardie 2008, Shackelford and Shaw 2009, Zhang *et al.* 2009, Vara *et al.* 2011). Cannabinoids have been previously reported to induce ROS levels and AMPK activity through decreasing the AMP/ATP ration through LKB1 initiation (Dando *et al.* 2013). However, MDA-MB231 cells lack functioning LKB1 (Shen *et al.* 2002). This lack of LKB1 did not seem to effect the cancer cells with CBD treatment as an increase in ATP was observed with a trending decrease of AMPK.

However, increased Ca^{2+} results in increased AMPK which again is the opposite of what was observed here (Ojuka 2004, Zhang *et al.* 2009). Again, “priming” did not show the regular activation and control observed with AMPK following Ca^{2+}-dependant pathways or the AMP/ATP ration. This could be due to lack of LKB1 or another mechanism for AMPK.

Furthermore, isolation of AMPK can be quite varied due to the nature of the harvesting, the speed between isolation and activity reading and finally freeze-thaw complications. Due to the nature of the collaboration many of these limitations were regulated to ensure there was no false activation of AMPK however, an increase in sample number could aid in verifying the abnormal results.

6.9.4. Glycolysis comparisons

To measure glycolysis in the cell following treatments, the Seahorse bioanalyser was used to give a real-time quantification of ECAR rates which could be analysed further to provide evidence of different anaerobic conditions. Lactate dehydrogenase was also analysed to observe alterations to the anaerobic pathway following treatments. However, the results from these separate experiments did not compare well most of the time. This could be due to the different mechanisms in quantifying the amount of activity. Seahorse bioanalyser’s glycolytic assay is based on ECAR of the cells and the manipulation of the ECAR through different reagents. The lactate dehydrogenase assay is based on a colorimetric measurement of NAD conversion to NADH which interacts with a probe to produce a quantifiable colour. However, it is not specific to a certain lactate dehydrogenase molecule and certain substances can impede results. The difference in results for glycolysis function should be noted and potentially not be used as a direct comparison of anaerobic glycolysis since the specificity of lactate dehydrogenase is not indicated.

6.9.5. Global methylation sample sizes

Global DNA methylation was measured using mass spectrometry which is a well detailed and reproducible mechanism of measurement (Amouroux *et al.* 2014). In this study the number of samples tested varied due to several issues with isolation and collaboration timing. DNA isolation from cells requires a large sample volume and precision when completing the isolation. The quality of the content must be above a certain threshold which was adhered to in this study. However, due to this and a collaboration timing problem the sample numbers for cisplatin and combination treatment for cancer and control cells was low (n=3) which is not ideal. This resulted in poor statistical output even with a trending result. To overcome this and fulfil the power of this experiment, further samples should be tested to confirm the trend observed.

6.9.6. *In vivo* efficiency

The *in vivo* work that was carried out in this study showed promising results towards "priming" treatment. One suggestive limitation was that the effectiveness of cisplatin was not enough for the animal model. CBD showed a reduction in tumour volume which has been previously described (Ligresti *et al.* 2006, McAllister *et al.* 2011, De Petrocellis *et al.* 2012a, Ramer *et al.* 2013, Romano *et al.* 2014). This suggests that the concentration of CBD was sufficient for intra-tumour injections. However, cisplatin alone had a reduction in tumour growth though it was not sufficiently significant compared to untreated tumours. The dose and treatment using cisplatin may not have been optimal for this study. Recent work has shown that doses of 1 mg/kg–5 mg/kg have been successful in administration (Dhar *et al.* 2011, Rattan *et al.* 2011). Potentially increasing the dose of cisplatin to mimic these levels could be more ideal and prove more efficient in an animal model. This however is to enhance the effect further since a positive effect was observed on tumour volume.

6.10. Future Work

There are many different routes that can be pursued with this study which include further intrinsic cell line analysis using similar methods, increasing the efficiency of the *in vivo* model and investigating other potential plant compounds that could be ideal in "priming".

6.10.1. CBD transportation into the cell

One avenue that would be interesting to pursue would be the mode of action of CBD's transportation into the cell. There has been much interest in receptor bound mechanisms of CBD's transport mechanism however the lipophilic nature of CBD could suggest a more lipid based mechanism. Lipid rafts have been shown to interact with endocannabinoids, AEA and 2-AG (Bari *et al.* 2006). Lipid rafts are areas of increased cholesterol and glycosphingolipids found in grape-like clusters on the plasma membrane (Pike 2003, Sarnataro *et al.* 2005, Sarnataro *et al.* 2006). One such lipid raft is caveolin-1 which has been shown to be reduced in cancer tissues due to its role in supressing tumour growth (Zajchowski and Robbins 2002, Hino *et al.* 2003, Pike 2003). Caveolin-1 is found on the plasma membrane of MDA-MB231 cells but has been shown in stressed conditions to translocate into the cytoplasm (Sun *et al.* 2002, Sarnataro *et al.* 2005). The affiliation between endocannabinoids and caveolin-1 suggest that lipophilic phytocannabinoids could also work through lipid rafts.

Preliminary work showed that in cancer cells 12 hours of CBD treatment with 1 μM CBD showed no change in caveolin-1 location (Figure 6.9.A.) (Methods in section 7.1.1.). However, following 24 hours of CBD treatment there was an increase in untranslocated caveolin in the cytoplasm and cell membrane (Figure 6.9.B, 6.10., 6.11.). This may show that caveolin-1 does have the potential to increase CBD translocation into the cytoplasm though not through actual stressed translocation of the caveolin-1

itself. Increased caveolin-1 has been shown to decrease cell growth which is what was also observed with CBD treatment (Hino *et al.* 2003).

The potential role of the lipid raft caveolin-1 should be further pursued to confirm a new mechanism by which CBD locates into the cell. CBD has been shown to reduce cell death in a non-receptor mechanism (Shrivastava *et al.* 2011). Therefore, the potential of this hypothesis should be followed.

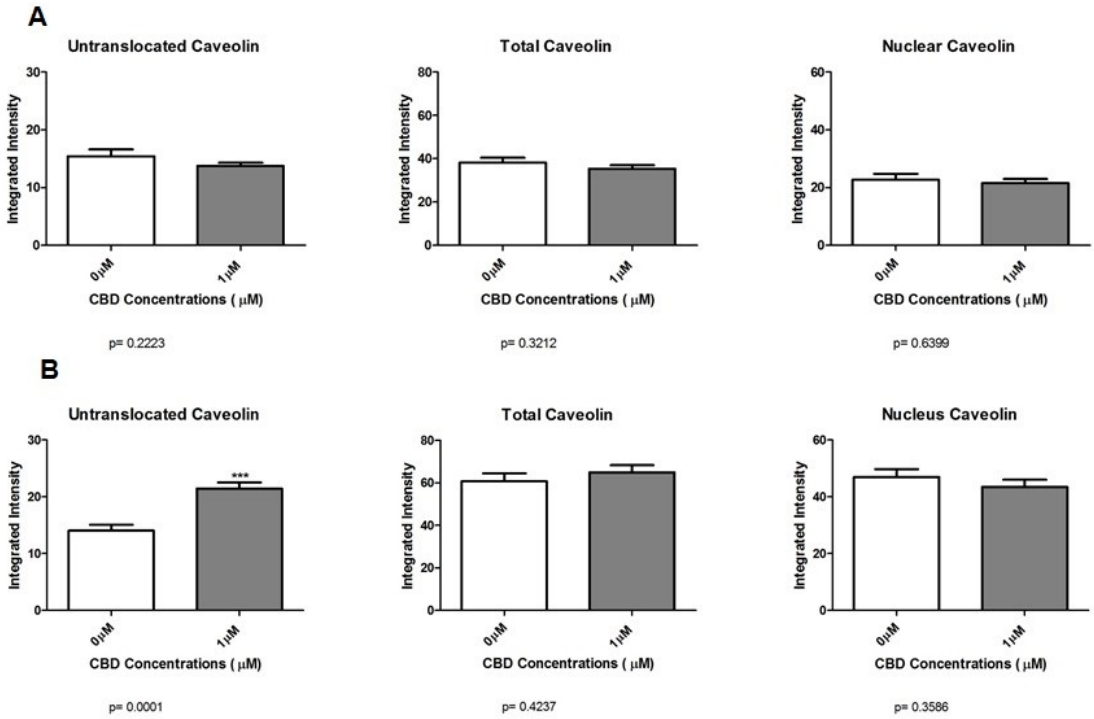

Figure 6.9. Cavoelin-1 concentration in cancer cells
Measurement of translocation of caveolin dye within MB231 cells. **(A)** 12 hours of control treatment versus 1 μM CBD treatment. **(B)** 24 hours treatment of control treatment versus 1 μM CBD treatment. Data shown is a repeated for 20 images with an average of 86 cells/image. Data is represented as mean ± SEM. ***p<0.001 *vs* Untreated.

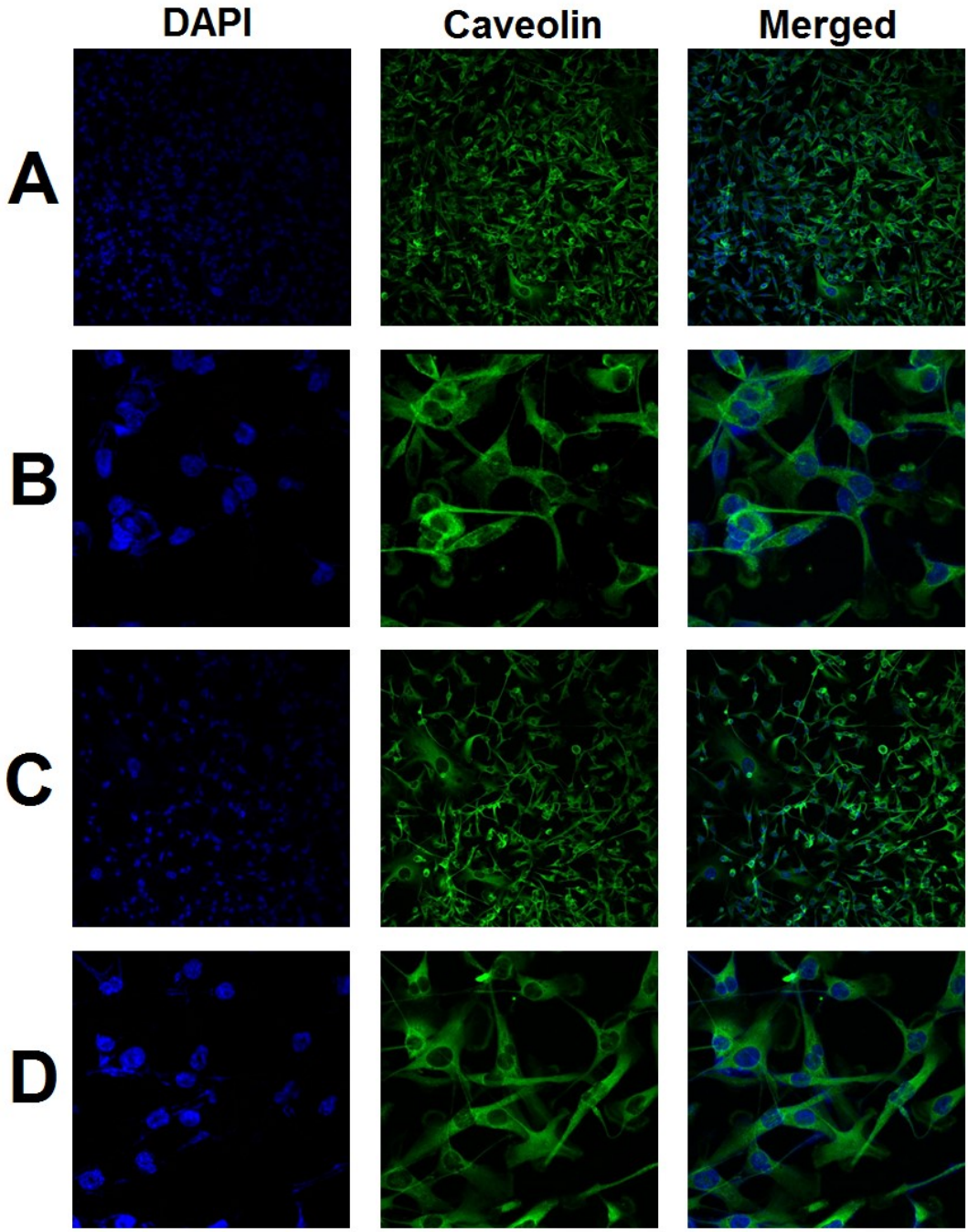

Figure 6.10. Caveolin-1 confocal images used for quantification following 24 hours treatment in cancer cells.
MDA-MB231 cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue) and anti-caveolin (green) after treatment. Anti-caveolin stained for caveolin and DAPI for the nucleus. **(A)** Cells were treated with control treatment for 24 hours and visualised for DAPI and caveolin at 20x optical zoom. **(B)** The same image was zoomed in 5.1x and re-examined. **(C)** MDA-MB231 cells were treated with 1 μM CBD and imaged with confocal at 20x optical zoom. **(D)** The same section was observed with 5.1x zoom.

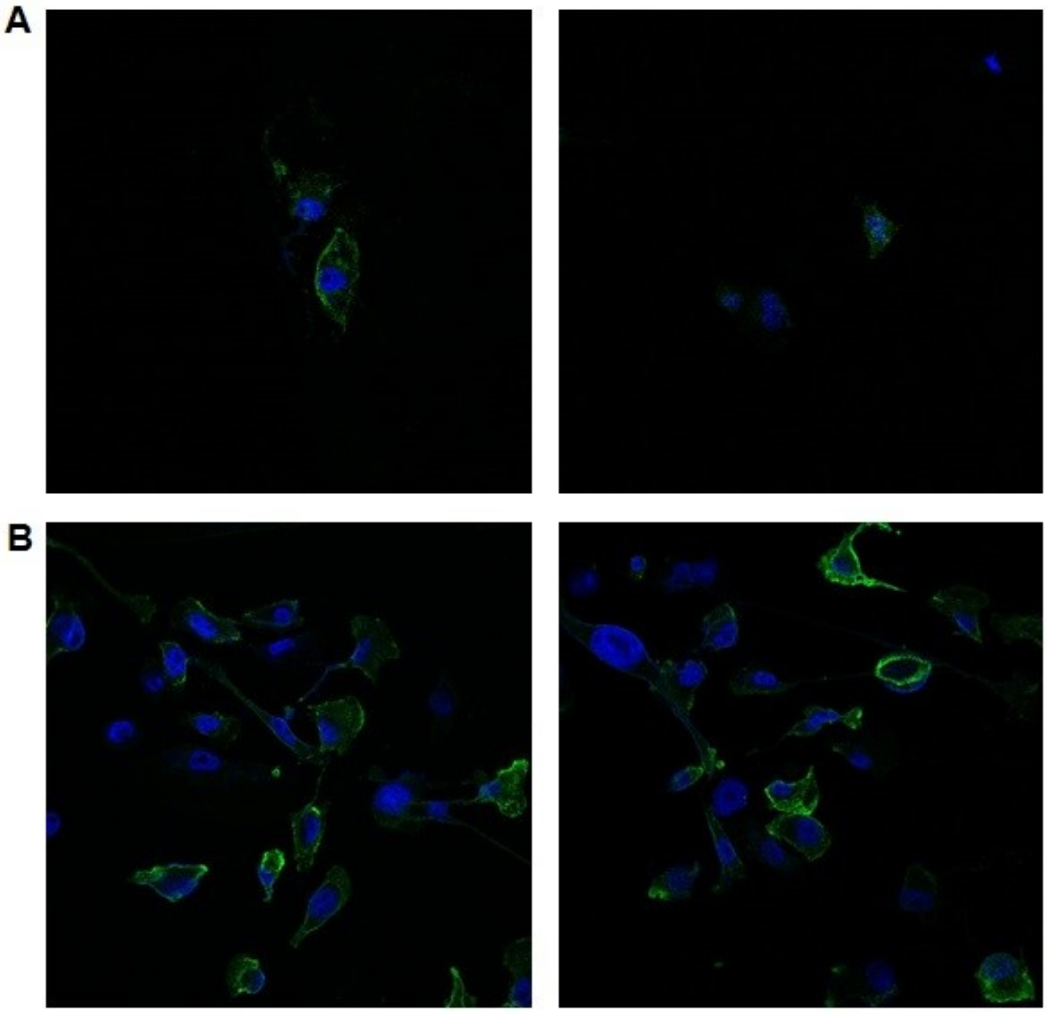

Figure 6.11. Lipid raft specific dye following 24 hours CBD treatment.
MDA-MB231 cells were imaged using confocal using a lipid raft specific dye. The lipid specific dye (green) and nucleus specific DAPI (blue) were used. **(A)** Cells were untreated for 24 hours and then imaged for changes. **(B)** Cells were treated with 1 μM CBD for 24 hours prior to staining.

6.10.2. Mitochondrial permeability assessment

The permeability of the mitochondria has been suggested to be the mechanism for “priming” and has been investigated in several research reports (Kuhar *et al.* 2007, Chonghaile *et al.* 2011, Wallace 2012, Sarosiek *et al.* 2013). It is important then to follow this basis and examine the MOMP following CBD, cisplatin and “priming” treatment. Initial experiments that were carried out using FACS with a mitochondrial sensitive dye,

DiOC showed that the permeability of the mitochondria was reduced following 24 hours of CBD treatment as predicted (Figure 6.12.).

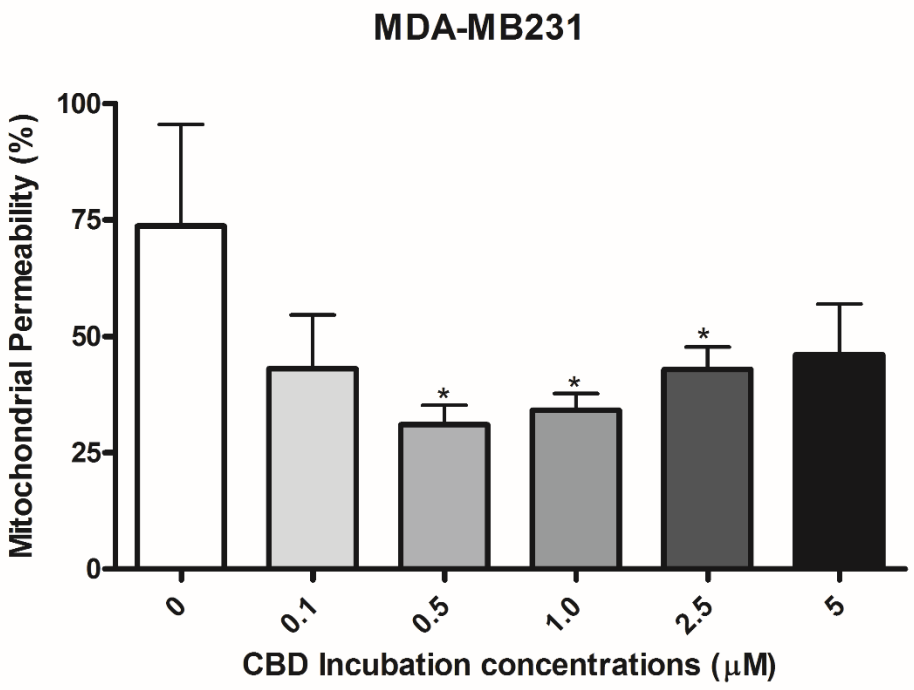

Figure 6.12. Mitochondrial permeability analysis following 24 hours of CBD treatment in cancer cells.
Cancer cells were treated with CBD for 24 hours prior to DiOC treatment. Data shown is repeated 3 times and is represented as mean ± SEM. *p<0.05 *vs* Untreated.

6.10.3. Cytochrome *c* levels

Following "priming" treatment an increase in ROS, Ca^{2+} levels and p53 suggest that the increased cell death is due to a decrease in the MOMP and consequently increased cytochrome *c* release (Madesh and Hajnóczky 2001, Rapizzi *et al.* 2002, Brookes *et al.* 2004, Kawai *et al.* 2006, Bragado *et al.* 2007, Yi and Luo 2010, Galluzzi *et al.* 2011). It would be important to carrying out a cytochrome *c* release assay to confirm both apoptotic death and that "priming" could be activating an increased release. There are some indications that "priming" could be activating necrosis due to the decreased ATP, and increased VDAC1 and Ca^{2+} levels (Jackson and Schoenwaelder

2010, Ouyang *et al.* 2012). However, this confirmation could be completed simply with a fluorescence microscopy study in either live or fixed cells (Sumantran 2011).

6.10.4. Nucleotide analysis

Following the AMPK activity measurements carried out in this study, looking at the nucleotides themselves separately would be an ideal future experiment. This could be carried out using high-performance liquid chromatography (HPLC) after harvesting following the specific treatments (Schweinsberg and Loo 1980). Thereby AMP, ADP and ATP could be measured separately to investigate whether the ATP production levels from the Seahorse bioanalyser were correct and if the mode of action of CBD is AMPK-independent. Preliminary work was carried out measuring AMP levels following all treatments in this study (Figure 6.13.) (Methods in section 7.1.2.). The levels of AMPK trended to increase following CBD treatment with cancer cells and no change in control cells (Figure 6.13.A and B). A significant reduction in combination treatment AMP levels were observed with the opposite trend with cancer cells following “priming” (Figure 6.13.C.). Finally, there was a significant reduction of AMP levels with cisplatin and combination but no change following “priming” (Figure 6.13.D.). This reduction in AMP with combination for both cancer and control cells could potentially aid in answering the difference between “priming” and combination’s mode of action. Increased AMPK leads to protection of the cell though it also increases Ca^{2+} levels which can increase cell death (Ojuka 2004, Zhang *et al.* 2009). Increased ROS increased the AMP/ATP ration which could explain the preliminary work here (Dando *et al.* 2013). Thus, the activation of AMP could be beneficial to either pathway. However, further work needs to be carried out with this study as the sample numbers are low (n=3) and ADP and ATP were not measured.

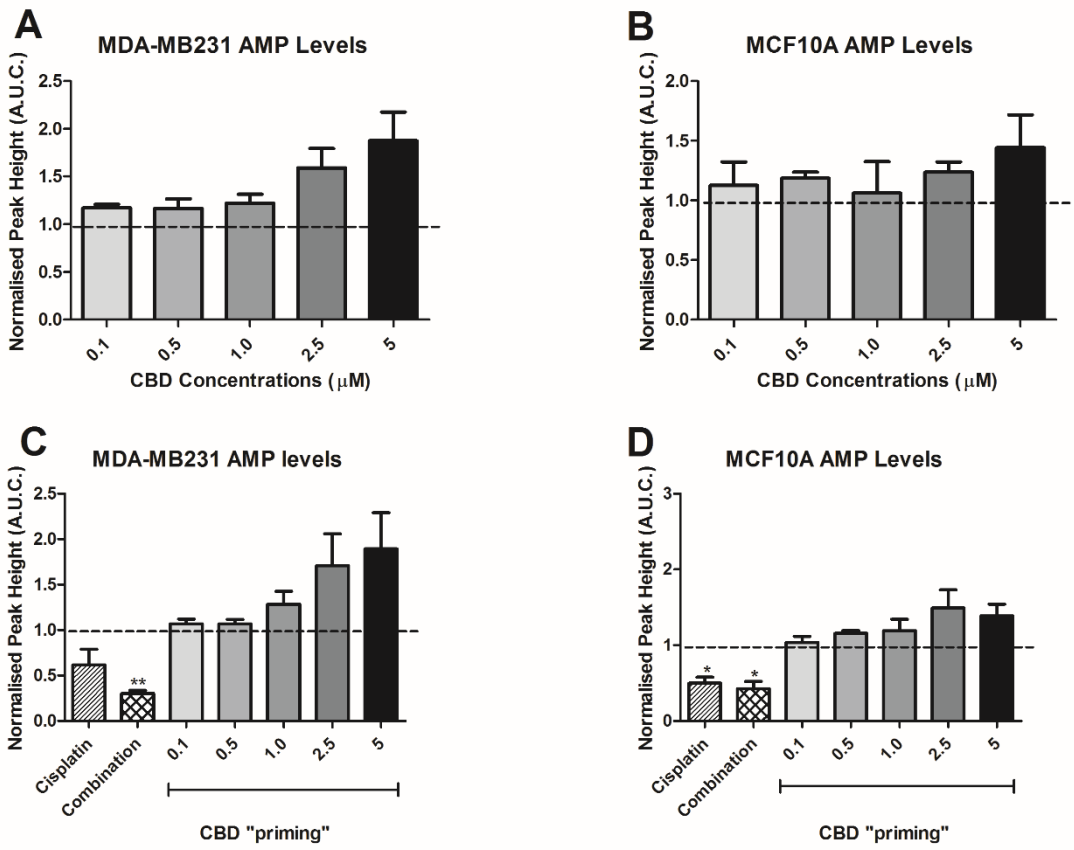

Figure 6.13. AMP activity in cancer and control cells
Following (A) CBD treatment MDA-MB231 cell's AMP activity was measured as were (B) control cells. (C) Cisplatin, combination and "priming" treatments were also analysed and examined for AMP activity alteration in cancer cells and (D) control cells. Data shown is repeated 3 times and is represented as mean ± SEM. *p<0.05, ** p<0.01 *vs* Untreated.

7. Appendix

7.1. Presentations and awards

Poster presentations

"Cannabidiol (CBD) priming enhances cisplatin killing of cancer cells"

A.B. Henley, A.V. Nunn, G.S. Frost, J.D. Bell

The British Pharmacological Society 6TH European Cannabinoid Workshop, Dublin, April 2013

Awards

MRC CSC Image of the Year 2012

A.B. Henley

MRC CSC, December 2012