2016

Novel Therapeutic Approaches to Induce Autophagy in Brain Cancer

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Novel Therapeutic Approaches to Induce Autophagy in Brain Cancer

Submitted by
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PhD

Food Science and Environmental Health, FOCAS Research Institute, Dublin Institute of Technology

Supervisors:
Dr James F. Curtin, Dr Orla Howe

2016
Abstract

Glioblastoma Multiforme (GBM) is classified as a malignant grade IV astrocytoma, and is considered to be the most biologically aggressive brain tumour with a 5-year survival rate of ~4%. The most frequent issue arising with GBM tumours is the high level of resistance observed to conventional therapeutic methods i.e. surgery, chemotherapy and radiation, thus signifying the urgency for the development of novel therapeutic methods. This study aims to investigate and develop both a complimentary and novel method to overcome the current treatment barriers. We have investigated the use of novel technologies such as cold atmospheric plasma (CAP) as an alternative to radiotherapy. Employing methods such as cytotoxicity assays, molecular inhibitors, confocal microscopy and flow cytometry, for the first time, we report that CAP induces a ROS, JNK and Caspase independent mechanism of cell death in the GBM cells that can be greatly enhanced when used in combination with low doses of TMZ. We have also further elucidated that CAP results in the activation of autophagy prior to cell death.

Current chemotherapeutics used for the treatment of GBM have offered little progression in the reduction of years of life lost. We have investigated the efficacy of naturally available bioactive compound Ursolic acid (UA) as a potential alternative to current chemotherapeutics. Using migratory assays, cytotoxicity assay, confocal microscopy and flow cytometry we have identified that UA activates autophagy prior to JNK dependent mechanism of cell death. A significant issue with GBM is its ability to spread and infiltrate throughout normal brain tissue. We demonstrated that UA successfully inhibits GBM cell migration through a JNK independent mechanism, more effectively than the current chemotherapeutics. It is evident that there are inconsistencies in the literature regarding the mechanism of cell death which occurs in GBM cells, through critical analysis of the literature and multiple independent treatment
modalities, and following the guidelines set out by the national cell death committee, we have demonstrated that U373MG GBM cells result in activation of the autophagy following treatment prior to the cells demise
Declaration

I certify that this thesis which I now submit for examination for the award of PhD, is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work. This thesis was prepared according to the regulations for graduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for another award in any other third level institution. The work reported on in this thesis conforms to the principles and requirements of the DIT's guidelines for ethics in research.

Signature__________________Date__________

(Candidate)
Acknowledgments

Words cannot express how extremely grateful and very fortunate to have been able to study for my PhD under the supervision of Dr. James Curtin. I can honestly say that the last 4 years have been the most transformative years of my life, both scientifically and on a personal level. It is strange to look back over this period and think how much I have evolved as a person and watched my research grow from an idea on a page into a successful body of work. It is difficult to believe that its almost 5 years since I first got in touch with James and asked if he would have an interest in supporting me in my application for an IRC PhD fellowship. I was so thrilled that you accepted the challenge. It has been the most wonderful, exciting and stressful (in a good way) period of my life. I have taken on challenges that I once never thought possible. I am now a published author, have travelled to the other side of the world to present my data. I overcame my fears of public speaking, secured academic and teaching experience, and more importantly I learned to trust myself and be confident in my work. Everything I have gained academically and scientifically would not have been possible without the help of a few very important people;

Dr. James Curtin, it has been an absolute pleasure to have you watch over me for the last 4 years. You have guided me through the successful grant applications process which was anything but easy, we being at opposite ends of the world! You have provided me with endless assistance and continued support throughout this whole process. You pushed me out of my comfort zone, helped mould me into the scientific scholar I am today. Today I am so eager and excited at the thought of the many new challenges that lie ahead which is in no small part due to you and for this I am eternally grateful. Thank you for believing in me and for your trust in giving me scientific license and freedom to reach my goals and thanks you for taking a chance on me when I was
just fresh out of college and not sure what I wanted to do or where I wanted to go. You are a role model to follow in every sense of the word, your work ethic is beyond commendable and you always had time for my endless questions. I am so lucky and immensely grateful to have had the chance to be a student under your tutorage.

To my second supervisor Dr. Orla Howe. Your advice and knowledge throughout my PhD has been unequivocal. It was a pleasure to have you on board this project. You always gave me your undivided attention even in the depths of very busy schedule and undergraduate exam period! Your gregarious and infectious personality coupled with such a positive attitude shone like a ray of sunshine when I needed it most. I may have feared you’re very thorough correction process, but as a result I am much better for it, not only in scientific writing, but also as a scientist. Your contribution to my PhD has enabled me to develop skills that I will take with me to wherever life should take me.

I am indeed privileged to have had the chance to work with two such brilliant academic minds. The guidance that I have received from each one of you has had an immense impact on my development as a scientist. I hope this will not be the last time we will collaborate together in future studies.

Thanks to all my past and present peers and staff at DIT FOCAS for making me at home. In fact, there were nights when it literally felt did become home! I will truly miss you all; our endless coffee trips, our in-depth scientific meetings that took place in two highly regarded centers of academic excellence on Camden St: ‘Ryan’s’ and ‘The Palace’, the comradery and memories I will cherish forever. I cannot express in words how much I have appreciated the help and support you have afforded me over the past four years. I am not sure I would have made it to the finish line without three people in particular, Lisa White, Adrian Maguire and Dáire O Donnell. The friends I have made
during my time at FOCAS have made this experience one of the most enjoyable times in my life.

I would sincerely like to thank the entire BioPlasma research group and all its members, faculty and students, particularly Daniella Boehm and Catie Heslin, for whom I will miss greatly. In particular, a special thanks to Dr. Vladimir Milosavljevic and Dr. PJ Cullen for all their endless support, assistance and for educating me on all there is to know about plasma science. To all the members of the RESC research groups, for allowing me the use of their laboratory when undertaking my research. The experience and knowledge I have gained while being a part of these groups has been immeasurable. I would also like to thank all those who have collaborated and contributed to the project culminating in the successful publication of my first paper and two others currently under review by international peer review journals, your participation has been greatly appreciated.

Last but by no means least, the two most influential and important people in my life; my Mother and Father, whom had they not believed in me from the beginning I would not be where I am today. They have provided endless support to me, guiding me all the way to the finish line. Finally, after all those years of study, I can hang up my role as a student and stand on my own two feet. You have been there for me through all the highs and lows, and not just during my PhD. You always told me ‘nothing is impossible’ and for that I can’t thank you enough and I will be forever grateful to you both, for you believed in me when there were times that I didn’t believe in myself. It is because of the love you both have shown me over the years that I have accomplished what I have today.

A big thank you must go to my sisters Elaine and Louise. You have helped through tough times and were always there for me. You both have turned out to be strong and independent
young women and I am so proud and privileged to have you as my sisters. Mom and Dad did good! I am immensely proud of what you have both achieved thus far in life and I have no doubt you will go on to do great things in your chosen fields. I Love you both.

I must also pay a very special tribute to my late grandmother, who was one of my biggest drivers. A woman I admired beyond belief and whom I will forever miss. She was the reason I embarked on my PhD journey. She was truly one of the greatest intellects I have ever had the privilege of knowing. At the ripe old age of 94 years she proof read every document or paper that I wrote and listened to every presentation I had to give, immaterial of whether she understood the content or not. She instilled in me the belief that I could do anything I put my mind to, and it is fact had it not been for her I wouldn’t be here getting a PhD. In fact, I don’t know where I would be or what I would be doing right now as she always guided my every decision. Although she is not here to see the final result, I know she is with me in spirit and for the last six months it was her who pushed me all the way to finish line. ‘Always in our heart and never forgotten’ Thank you Gran.

Thank you all so much.

“Truly great friends are hard to find, difficult to leave and impossible to forget”

G. Randolf
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>GBM</td>
<td>Glioblastoma multiforme</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health organisation</td>
</tr>
<tr>
<td>CBRTUS</td>
<td>Brain Tumor Registry of United States</td>
</tr>
<tr>
<td>NCRI</td>
<td>National Cancer Registry of Ireland</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor Tyrosine Kinase</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet Derived Growth Factor Receptor</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and Tensin Homolog</td>
</tr>
<tr>
<td>MDM2</td>
<td>Mouse double minute 2 homolog</td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilisation</td>
</tr>
<tr>
<td>MOPP</td>
<td>Mustargen, Oncovin, Procarbazine, Predinsone</td>
</tr>
<tr>
<td>TMZ</td>
<td>Temozolomide</td>
</tr>
<tr>
<td>MGMT</td>
<td>O6-methylguanin-DNA-methyltransferase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
</tr>
<tr>
<td>BCNU</td>
<td>bis-chloroethylnitrosourea/ carmustine</td>
</tr>
</tbody>
</table>
PFS  Progression Free Survival
PCV  Pro-carbazine, lomustine (CCNU) and Vincristine
CCNU  Lomustine
mAb  Monoclonal antibodies
RTKIs  Receptor tyrosine kinase inhibitors
HER  Human Epidermal Growth Factor Receptor
NSCLC  Non-Small Cell Lung Cancer
ATP  Adenosine Triphosphate
CAP  Cold Atmospheric Plasma
ROS  Reactive Oxygen Species
RNS  Reactive Nitrogen Species
LTE  Local Temperature Equilibrium
DBD  Dielectric Barrier Discharge
APP  Atmospheric Pressure Plasma
FE-DBD  Floating Electrode Dielectric Barrier Discharge
SOD  Superoxide Dismutase
GSH  Glutathione
UA  Ursolic Acid
PCD  Programmed Cell Death
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BID</td>
<td>BH3 Interacting Domain Death Agonist</td>
</tr>
<tr>
<td>BCL-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>DISC</td>
<td>Death Inducing Signalling Complex</td>
</tr>
<tr>
<td>TNF-R1</td>
<td>Tumour Necrosis Factor Receptor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related Apoptosis-inducing Ligand Receptor</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic Target of Rapamycin’</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>AO</td>
<td>Acridine orange</td>
</tr>
<tr>
<td>3-MA</td>
<td>3-methyladeneine</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography- Mass Spectroscopy</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared spectroscopy</td>
</tr>
</tbody>
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1 Introduction
1.1 Glial Tumours

The term glioma was first used to describe a tumour that arises from the glial cells within the brain by Rudolf Virchow in 1863 (Van de Kelft, 1997). Astrocytes, oligodendrocytes and ependymal cells all fall under the classification of a glial cell. They are differentiated based on location and structure within the central nervous system (CNS) and therefore produce independent and distinct tumours as seen in figure 1.1 below; astrocytoma, oligodendroglioma, ependymomas, and mixed glioma, each of which can produce independent and distinct glial tumours.

![Figure 1.1 The neuroglial cell lineage](Huse & Holland, 2010a)

It is thought that self-renewing, progenitors produce committed neuronal and glial progenitors that eventually differentiate into mature neurons, astrocytes and
oligodendrocytes. The precise cells of origin for glioma variants and medulloblastoma remain largely unknown, candidates for each are indicated in figure 1.1 above by dashed arrows. These tumours can then be further sub-divided into categories according to the World Health Organisation (WHO) depending on the perceived level of malignancy (grade I-IV). Glial tumours are the most commonly occurring neoplasm of the central nervous system (CNS), accounting for 50% of CNS tumours in adults (Bower & Waxman, 2011). The overall prognosis for patients diagnosed with high grade glioma is very bleak, with survival correlated to the age at which the patient is diagnosed and the histological features of the tumour. Brain tumours are usually attributed to change in the genetic composition of the cells; this can be inherited or can be caused by external factors in the environment. It has been postulated that only 5% of brain tumours can be linked to a genetic predisposition, although there are rare genetic conditions such as Li-Fraumeni syndrome, retinoblastoma, Turcots syndrome, and neurofibromatosis that are seen to be predisposing factors in the development of certain tumours (Schwartzbaum et al. 2006; Hardwidge & Hettige 2012). Environmental factor such as exposure to high dose radiation and chemicals such as formaldehyde have increase risks of developing a malignant glioma (Schwartzbaum et al, 2006).

1.1.1 Astrocytomas

Aastrocytoma’s develop from astrocytes, star-shaped glial cells, which are part of the supportive tissue of the brain. The primary function of astrocytes is to provide support and nourishment to surrounding neurons, regulation of energy metabolism, transport of blood-borne material to the neuron, and reaction to injury (Kimelberg & Nedergaard, 2010). Astrocytoma represents the majority of brain tumours found in adults making up approximately 81% of all brain and CNS tumours (National cancer Registry Ireland, 2010). According to the WHO astrocytomas can be classified into four types (as
described in the table 1.1 below) and are graded on a scale from I to IV based on frequency of mitosis, necrosis and degree of differentiation. (Louis et al, 2007).

Table 1.1: WHO classification of astrocytic tumours

<table>
<thead>
<tr>
<th>WHO grade</th>
<th>WHO description</th>
</tr>
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<tbody>
<tr>
<td>Grade I</td>
<td>Pilocytic Astrocytoma</td>
</tr>
<tr>
<td>Grade II</td>
<td>Low-Grade Astrocytoma</td>
</tr>
<tr>
<td>Grade III</td>
<td>Anaplastic Astrocytoma</td>
</tr>
<tr>
<td>Grade IV</td>
<td>Glioblastoma</td>
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</tbody>
</table>

Pilocytic astrocytomas (Grade I) are the most common brain tumour found in children. The pilocytic tumour is benign and tends not to spread from the area in which they grow and are most commonly treated by surgical resection (Reifenberger et al, 2010). Low-grade astrocytomas, (also known as diffuse astrocytomas) are regarded as malignant tumours. They generally present with vast penetration of the surrounding brain tissue making surgical resection more difficult. These tumours, if left untreated, are likely to develop into a grade IV astrocytomas (Pouratian & Schiff, 2010). Anaplastic astrocytomas are faster growing tumours then the previous tumours mentioned. They are more difficult to remove; treatment usually involves radiation therapy or chemotherapy and sometimes both. These grade III tumours most commonly occur in males between the ages of 30-50 years (DeVita & Rosenberg, 2008; Pouratian & Schiff, 2010). Glioblastoma multiforme (GBM) is a highly malignant grade IV astrocytoma, and is considered the most biologically aggressive brain tumour. These tumours are associated with poor prognosis and a low 5 year survival rate of 5% post diagnosis (Ostrom et al, 2014, 2015). According to the 2014 Central Brain Tumor Registry of United States (CBRTUS) report, GBM makes up approximately 45.6% of all primary
malignant brain tumours in USA (Ostrom et al, 2014). These tumours show high level of resistance to conventional therapies i.e. surgery, chemotherapy and radiation, indicating urgency for the development of new therapeutic strategies.

1.2 Glioblastoma multiforme

According to the National Cancer Registry of Ireland (NCRI), glioblastomas account for 56% of astrocytic tumours (National cancer Registry Ireland, 2010). There is a 1.6 times higher incidence rate of GBM found in males compared to females (Dolecek et al, 2012). The median age for diagnosis is approximately 64 years of age but it can occur at any age (Preusser et al, 2011). Epidemiological studies have shown that there is a higher incidence of GBM cases in developed countries, for example in Western society there is approximately 4.5/100,000 persons affected/year compared with that of under-developed countries where there is approximately 2.4/100,000 persons affected/year. This is thought to be due to under-detection, different diagnostic practices, and limited access to healthcare, and moreover this variance could also be due to ethnic, lifestyle or environmental differences in susceptibility to developing GBM’s (Butowski & Chang, 2007; Ohgaki, 2009).

1.2.1 Primary and Secondary GBM’S

The majority of GBM’s develop in the cerebral hemisphere. Histologically, Glioblastoma multiforme, as the name implies, displays attributes of various morphologies, and is a heterogeneous mixture of cells that demonstrate varying degrees of cellular and nuclear polymorphism. GBM is a non-metastasising tumour and is characterized by regions of necrosis, mitotic activity, pleomorphic nuclei, and micro-vascular proliferation, all of which is essential for its histopathological diagnosis (Reifenberger et al, 2010). GBM can be divided into primary and secondary subtypes
according to the astrocytic lineage and on the basis of clinical presentation and genetic make-up. The first distinction between primary and secondary GBM was first defined by the German neuropathologist Hans-Joachim Scherer in 1940. He wrote,

“From a biological and clinical point of view, the secondary glioblastomas developing in astrocytomas must be distinguished from ‘primary’ glioblastomas. They are probably responsible for most of the ‘glioblastomas of long clinical duration’.”(Scherer, 1940)

Primary glioblastomas, also known as ‘de novo’ glioblastomas, develop rapidly, with no clinical history and with no evidence of a previous clinical precursor lesion or malignancy (Ohgaki & Kleihues, 2007; Reifenberger et al, 2010). Secondary glioblastomas develop much slower, by progression from a pre-existing lower grade (WHO grade II) astrocytomas (Ohgaki & Kleihues, 2007). Primary GBM is usually diagnosed following a description of an acute clinical presentation coupled with biopsy showing features of IV glioblastoma, whereas diagnosing a secondary glioblastoma requires clinical or histological confirmation of progression from a previously identified less malignant lower grade astrocytoma.

Although the distinction has been made between primary and secondary glioblastomas, these terms have yet to be used diagnostically, mostly due to the fact that primary and secondary glioblastomas cannot be distinguished morphologically (Kleihues & Ohgaki, 2000; Reifenberger et al, 2010). Despite this, clinical evidence shows a difference between these two subtypes of glioblastomas in terms of prevalence. Primary glioblastomas are much more prevalent in older patients (>60 years) whereas secondary glioblastoma occur in younger patients (<45 years) (Kleihues & Ohgaki, 2000). There are also certain genetic alterations that have been associated with each subtype. The
EGFR/PTEN/Akt/mTOR pathway is a principle signalling pathway in the development of primary glioblastoma as illustrated in figure 1.2 below. Epidermal Growth Factor Receptor (EGFR) over expression or amplification occurs in approximately 40 and 50% respectively of primary glioblastomas but rarely occurs secondary glioblastomas (Ekstrand et al, 1992). On the other hand the TP53 pathway is associated with the development of secondary glioblastomas with approximately 60% of cases demonstrating TP53 mutations (Riemenschneider & Reifenberger, 2009). The pathways involved in the development of GBM will be discussed in further detail under Section 1.4 Gliomagenesis.

![Figure 1.2 Genetic pathways involved in primary and secondary glioblastoma development (Endersby & Baker, 2008).](image)

**1.3 Incidence of Central Nervous System Tumors.**

The CNS is composed of the brain and the spinal cord. It is the processing centre for the entire nervous system. In 2008 the NCRI reported that from 1994-2008 there was a 2.3% increase in the number of cases of brain & other CNS tumours being diagnosed,
most likely attributed to the increase in the population (National cancer Registry Ireland, 2010). In the 2013 report of Cancer in Ireland it stated that there were 344 new cases of brain and CNS tumours diagnosed from 2008-2010, making up approximately 2% of all invasive cancers diagnosed (National Cancer Registry, 2013). According to the International Agency for Research on Cancer (IARC) it is estimated that approximately 238,000 malignant brain & CNS tumours were diagnosed worldwide in 2008 (Ferlay et al, 2010). The number of cases diagnosed are significantly lower compared to those of other cancers such as, prostate, breast and colorectal which are among the five most frequently diagnosed cancers worldwide, but these cancers have a considerably higher five year survival rate i.e. prostate: 81%, breast: 85% and colorectal: 55% compared with brain & CNS tumours which is a bleak 15% (Office for National Statistics, 2011).

1.4 Gliomagenesis

Gliomagenesis describes the development of glial tumours. With advances in technology over the past two decades our knowledge of genetic and molecular processes in which GBM develops has increased dramatically but there is still much to be discovered. There are numerous genetic abnormalities that have been linked to gliomagenesis, many of which affect processes such as proliferation, cell cycle control, signal transduction, apoptosis, and differentiation (Zhang & Fine, 2006). It is important to develop a better understanding of the events that result in gliomagenesis in order to allow for the development of target specific therapies. Demonstrated in figure 1.3 below, are some of the most commonly deregulated genes/major signalling pathways found in GBM, the RTK/PI3K/Akt pathway, p14ARF/MDM2/TP53 and the RB1-p16INK4a pathway.
As seen in figure 1.3 above (a) depicts the RTK/PI3K/Akt signal transduction pathway that promotes survival and growth in response to extracellular signals, and has been shown to be mutated in 88% of GBM. (b) demonstrates the p14ARF/MDM2/TP53, this pathway responds to diverse cellular stresses to regulate target genes that induce cell cycle arrest, cell death, cell differentiation, senescence, DNA repair. Inactivation of this pathway is cause by mutations that occur in the p53 gene, which occur in 87% of GBM. (c) The retinoblastoma protein (RB1) is a tumour suppressor protein that is
dysfunctional in 78% of GBM. RB1 functions to prevent excessive cell growth by inhibiting cell cycle progression until a cell is ready to divide.

**I. RTK/PI3k/Akt/ Pathway**

The RTK/PI3K/Akt refers to a cellular proliferation pathway. This first involves the binding of extracellular growth factors to the receptor tyrosine kinase (RTK) proteins on the cell membrane which results in autophosphorylation of the RTK where PI3 kinase binds to a phosphorylated domain on the RTK, which then signals the phosphorylation of serine/threonine kinase known as Akt to regulate cellular proliferation, apoptosis and glucose metabolism (Manning & Cantley, 2007; Nakada *et al.*, 2011a).

The receptor tyrosine kinase (RTK) family are a group of transmembrane glycoprotein’s that activate growth factor mediated signal transduction. They play an important role in cellular proliferation and differentiation. Aberrant signalling of the RTK family has been associated with a variety of cancers such as breast and brain cancer. Genetic mutations of the RTKs result in gene amplification or over expression thus leading to constitutive activity of that protein. One of the main mechanisms of deregulated RTK function is the activation of the autocrine growth factor/receptor loops (Zhang & Fine, 2006), which result in the promotion of tumour cell proliferation. The Epidermal Growth Factor Receptor (EGFR) and Platelet Derived Growth Factor Receptor (PDGFR) have been repeatedly demonstrated to play a role in gliomagenesis (Holland, 2001; Nakada *et al.*, 2011b). EGFR is the most frequent of all RTK mutations, with approximately 45% of all primary GBM exhibiting gene amplification (Ohgaki & Kleihues, 2009; Tanaka *et al.*, 2012). EGFR amplification is commonly associated with gene amplifications and activated mutations in the EGFR locus, the most documented being the EGFR variant III (EGFRvIII) deletion which is observed in 20-30% of all
primary GBM with a further 50-60% of those also displaying EGFR amplification (Ohgaki & Kleihues, 2009; Huse & Holland, 2010b; Nakada et al, 2011a).

PI3K are a family of lipid kinases that are involved in an array of diverse cellular signalling pathways including cellular proliferation, differentiation, motility, and survival (Nakada et al, 2011a). The PI3K complex is composed of a catalytically active protein p110α encoded by \textit{PIK3CA}) and a regulatory protein p85α (encoded by \textit{PIK3RI}) (Ohgaki & Kleihues, 2009). PIK3CA mutations have been reported in a variety of cancers including brain tumours. In a study by Gallia, G. L. et al. 2006, it was observed that in 73 GBM samples, 15% possessed \textit{PIK3CA} mutations. In addition to this, a study by Kita, D. et al. 2007 noted that in a sample of 107 primary GBM, PIK3CA missense mutations and amplifications were observed in 5% and 13% respectively. It can be said that PIK3CA mutations are quite prevalent in GBM unlike the regulatory protein PIK3R1 which has been rarely reported. The Cancer Genome Atlas Research Network carried out a project using GBM samples (n=206) that determines 10% of their cohort displayed PIK3R1 mutations (The Cancer Genome Atlas Research Network, 2008).

Phosphatase and tensin homolog (PTEN) gene is a tumour suppressor gene that is mutated in a large number of cancers at high frequency. It negatively regulates intracellular levels of phosphatidylinositol-3,4,5-trisphosphate in cells and also negatively regulates Akt pathway (NCBI), by antagonizing PI3-kinase-mediated signalling cascades. The PTEN gene has been shown to be mutated in approximately 40% of primary GBM (Ohgaki & Kleihues, 2009), resulting in constitutive activation of the RTK/PI3K/Akt pathway. Akt is serine/threonine kinase that regulates cellular proliferation, apoptosis and glucose metabolism. To date there have been no oncogenic Akt mutations detected (Nakada et al, 2011a).
2. p14ARF/MDM2/TP53 pathway

p14ARF/MDM2/TP53 pathway refers to activation of the alternate reading frame protein p14ARF by oncogenic signalling, which binds Mouse double minute 2 homolog (MDM2), inhibiting its E3 ubiquitin ligase function and results in the release of P53 and therefore reinstating its transcriptional activity (Shaoman & Van Meir, 2009).

The TP53 gene encodes for a 43.7kDa tumour suppressor protein p53. This tumour suppressor protein is located in the nucleus of cells where it binds directly to DNA. While distinct mutations are predominant in each subtype of GBM, alterations of tumour suppressor p53 are observed in 28 % of primary GBM and 65 % of secondary GBM (England et al, 2013; Nakada et al, 2014), making it the most common genetic alteration observed in GBM. The p53 protein plays a crucial part in many cellular processes including the cell cycle, cellular DNA damage response, cell death and differentiation (Ohgaki & Kleihues, 2009). Subsequent to DNA damage, TP53 is activated and induces the transcription of other genes such as p21Waf1/Cip1 which acts as a regulator of cell cycle progression (Ohgaki & Kleihues, 2009; Nakada et al, 2011a). TP53 plays a crucial role in the development of GBM, since the majority of GBM tumours demonstrate TP53 mutations. The majority of p53 mutations in secondary GBM have been located at two key codons 248 and 273, whereas they have been demonstrated to be more widely distributed in primary GBMs (Mao et al, 2012). This therefore proposes that p53 mutations in primary GBM occur as a secondary incident due to general genomic instability of the GBM tumour environment. The MDM2 protein is involved in promoting tumour formation by targeting tumour suppressor proteins such as p53 for proteosomal degradation when over expressed (Nakada et al, 2011a), and this has been shown in 10% of all malignant GBM’s (Mao et al, 2012). MDM2 binds to both mutant and wild type p53 preventing the ability of TP53 to
activate transcription. Contrarily, transcription of the MDM2 gene is induced by wild-type TP53, acting as an auto-regulatory feedback loop regulating the expression of MDM2 and p53 activity (Ohgaki & Kleihues, 2009; Nakada et al, 2011a). p14ARF also known as ARF tumour suppressor, is an alternate reading frame protein, which forms stable complexes with MDM2. It acts as a tumour suppressor initiating P53 dependant cell cycle arrest. ARF and p16\textsuperscript{INK4a}, are crucial regulators of the key growth control pathways p53 and pRB, respectively. One of the most frequent mutations found in GBMs is the homozygous deletion of the p16\textsuperscript{INK4a}/p14\textsuperscript{ARF}/p15\textsuperscript{INK4b} locus (Solomon et al, 2008). P53 plays an important role in cell cycle. The G1/S checkpoint prevents initiation of DNA replication in cells that have damaged DNA. Expression of p53 following DNA damage, arrests cells at the G1/S transition. Similarly, entry into mitosis is blocked by the G2 checkpoint mechanism when DNA is damaged. DNA damage signals cause activation of p53, which induces the expression of p21\textsuperscript{WAF1/CIP1/Sdi1}, an inhibitor of the cyclin dependent kinases (CDKs)2, 3, 4 and 6 (Taylor & Stark, 2001). It has been demonstrated that astrocytic gliomas with altered G1/S transition control genes also have abnormalities of the p14\textsuperscript{ARF}/MDM2/p53 pathway genes (Ichimura et al, 2000), and therefore indicating that tumours with G1/S transition control gene abnormalities exhibit disruption of the p53 pathway.

3. RB1-p16\textsuperscript{INK4a} pathway

The retinoblastoma tumour suppressor protein (pRB) plays a critical role in inhibiting cell cycle progression in the G1/S phase by binding and inhibiting transcription factors of the E2 factor (E2f) family. The 107-kDa RB1 protein encoded by \textit{RB1} (at 13q14) controls progression through G1 into the S-phase of the cell cycle and is regulated by the complex of cyclin-dependent kinases (CDKs)(Mao \textit{et al}, 2012). The loss of pRB expression has been detected in GBM and the loss of 13q was also associated with the
transition from low- to intermediate-grade gliomas (Henson et al, 1994; Bahuau et al, 1998). Genetic loss of RB1 (40%), deletion of CDKN2A (40%) and/or CDK4 amplification (15%) have been demonstrated in the majority of GBM’s (Schmidt et al, 1994; Ueki et al, 1996).

1.5 History of cancer therapy

A German chemist Paul Ehrlich in the early 1900’s, who is considered the father of modern immunology and chemotherapy, first coined the term chemotherapy. He defined chemotherapy as the use of chemicals to treat disease (DeVita & Chu, 2008). The 1960s was an exciting decade for advancements in the chemotherapeutics field. The Eli Lilly Company unearthed the bio-activity of over 30 plant alkaloids from the plant, *Vinca rosea*, one of which is vincristine (Johnson et al, 1963) used for the treatment of a variety of cancers such as acute leukaemia. Another was the discovery of the activity of ibenzmethyzin (now known as pro-carbazine) for the treatment of Hodgkin’s disease by Brunner and Young (Brunner, 1965). Both of these drugs are also commonly used in the treatment of GBM today. Devita and his colleagues developed the mitochondrial outer membrane permeabilisation (MOMP) programme, the first combinational chemotherapy regime, consisting of nitrogen mustard with vincristine, methotrexate, and prednisone. This has since become known as the MOPP (Mustargen, Oncovin, Procarbazine, and Predinsone) programme, which has substituted methotrexate with Procarbazine. This treatment led to Hodgkin’s disease being deemed curable by the 1970s, with approximately 80% of cases resulting in complete remissions (Devita et al, 1970). Following the success of combinational chemotherapy came the era of adjuvant and neo-adjuvant chemotherapy. Adjuvant chemotherapy is when chemotherapy is administered to patients in addition to the primary or initial treatment to reduce the risk
of recurrence. Neo adjuvant chemotherapy is when chemotherapy is given pre surgery to shrink the tumour, to aid in resection. Since the 1940s, the successful advancements of chemotherapy to treat cancer has led to the age of specific targeted therapies, the most successful being the BCR-Abl tyrosine kinase inhibitor ‘Imatinib’ for the treatment of chronic myelocytic leukaemia (DeVita & Chu, 2008). Although chemotherapy cannot be deemed to cure all forms of cancers, it has provided significant improvements to patients, prolonging life expectancy, increasing the quality of life.

Radiotherapy first began in 1895, with the discovery of x-rays, and in 1896 only six months later the first patients were treated with radiotherapy (Thariat et al, 2013a). It was discovered that daily doses of radiation over several weeks greatly improved the chances of a patient’s recovery. This breakthrough was closely followed by the isolation of radium by Curie in 1898 (Thariat et al, 2013b). It was not until the early 20th century that it was identified that radiation exposure also induced cancer formation, and therefore, steps were taken to optimise dosage and exposure, forming a basis for safe and effective therapies. Therefore, when radiation therapy is used to treat cancerous cells in the body, it is important to measure the dose correctly to avoid unnecessary damage to normal cells as radiation is not selective to tumour cells and can target any cells that are in the process of replication. It has been previously demonstrated that exposure to radiation damages the genetic material of a cell, therefore altering their ability to divide and proliferate (Baskar et al, 2012). The Gray (Gy) is the unit used to measure the total amount of radiation the patient is exposed to. This can also be recorded as centigray (cGy), which is 0.01 of a single gray unit. The total radiation dose is usually divided into several fractions. For most patients that require radiation therapy, the total dose is broken up into daily doses for a total period of weeks. The current standard of care for GBM patients receiving radiotherapy, is as follows; concurrent use
of TMZ with radiotherapy to 6,000 cGy over 30 days followed by adjuvant TMZ treatment for 6 months (Stupp et al, 2010). Studies have demonstrated increases in GBM patient survival with postoperative radiation therapy (RT) to doses of 5,000-6,000 cGy but efforts have reported attempts beyond 6,000 cGy that resulted in increased toxicity and no additional survival benefit (Barani & Larson, 2015). Over the years there have been major advances in radiotherapy, with the development of techniques such as conformal radiation therapy, stereotactic radiation therapy, intraoperative radiation therapy, and the development of radiosensitisers, all of which are used today for the treatment of many malignancies including GBM (Baskar et al, 2012).

1.6 Treatment and prognosis of GBM.

The current prognosis for those diagnosed with glioblastoma is unfavourable. Patients who receive maximal treatment have a survival rate of approximately 12-18 months, where as those who do not obtain treatment have a survival rate of <1 year (Merlo, 2003). It is clear from these statistics that despite maximal treatment for patients with glioblastoma the outlook is grave, when compared to successful treatments of others cancers such as breast and prostate cancer with a survival rate of >80% (National Cancer Registry, 2013). It highlights just how aggressive and fatal GBM is, and how challenging it is to treat, even with the advancement in treatment and therapeutics over past decades.

As illustrated below in figure 1.4, the current standard of care for those who have been newly diagnosed with a glioblastoma is as follows, surgical resection to the extent that is safely feasible. Following histopathology, which identifies the stage of the tumour according to WHO classification, the patient is either treated with radiotherapy, chemotherapy or both. For GBM tumours following surgical resection the patient is
treated with a 6 week course of radiotherapy (typical dose is around 60 Gy) with concomitant systemic therapy using alkylating agent TMZ (75 mg/m² daily), followed by the minimum of 6 months of adjuvant TMZ (150-200 mg/m² for 5 days every 28 days) (Stupp et al, 2005a). Patients who have recurrent GBM tumours or are less likely to respond to treatment are also given the option to take part in clinical trials.

Figure 1.4 Illustrates a typical algorithm used by neurologists to identify the best course of treatment following identification of a glioma (Wick et al, 2014)

A key factor as to why there is such a poor prognosis for those with GBM is due to the high tendency for tumour recurrence. It has been suggested that the majority of patients will develop recurring tumours after a median survival time of 32-36 weeks (Hou et al, 2006). Treating recurrent GBM requires individualisation and is patient dependent. There are numerous factors that must be taken into account before deciding on the most suitable therapy for that patient, i.e. age, histology, O6-methylguaninE-DNA-
methyltransferase (MGMT) status, extent of initial resection, initial therapy received and their response, whether recurrence is local or diffuse (Weller et al, 2013).

1.7 Commonly used chemotherapeutics employed for treatment of GBM

Treatment of GBM has remained poor. Despite the advancements in chemotherapy in recent years, the disease still remains incurable. The current standard of care is maximal resection of the tumour followed by administration of TMZ, an alkylating cytotoxic agent that is administered orally on a daily basis at a dose of 75 mg/m² throughout external beam radiotherapy. Four weeks later, magnetic resonance imaging (MRI) is repeated, and TMZ is then given at a dose of 150-200 mg/m² daily for 5 days every 28 days for maintenance. MRIs are performed after every 2-3 cycles of TMZ treatment to ensure continuous stability or response of the tumor to treatment (Hottinger et al, 2014). The following are some of the most common chemotherapeutics used for the treatment of GBM.

1.7.1 Alkylating agents

An alkylating agent attaches an alkyl group to the guanine base of DNA, thus resulting in DNA damage. In 2005 the US Food and Drug Administration (FDA) approved the use of TMZ chemotherapeutic agent for the treatment of GBM’s. It is a cytotoxic alkylating agent that can effectively cross the blood brain barrier (BBB). The blood brain barrier is a diffusion barrier that excludes blood borne substances entering the brain and protects the central nervous system. Tight junctions exist between the vascular endothelial cells AND the BBB, and therefore can restrict many chemotherapeutic agents from crossing into the invasive GBM cells. TMZ was first synthesized by Stevens and his colleagues in 1984 (Stevens et al, 1984), they also demonstrated that
TMZ has anti-tumour activity (Stevens et al, 1987). TMZ is a small lipophilic molecule with a molecular weight of 194 Daltons and therefore, is able to cross the blood-brain barrier. When TMZ comes in contact with the slightly basic pH of the blood and tissues, it undergoes hydrolysis, which rapidly breaks down to form the reactive methyldiazonium ion. The methyldiazonium ion formed by the breakdown of (3-methyl-(triazen-1-yl)imidazole-4-carboxamide) MTIC primarily methylate’s guanine residues in the DNA molecule, resulting in the formation of $O^6$- and $N^7$-methylguanine, damaging DNA. When DNA mismatch repair enzymes attempt to remove $O^6$-methylguanine, they generate single- and double-strand breaks in the DNA, leading to an activation of apoptotic pathways (Agarwala & Kirkwood, 2000).

Resistance to alkylating agents such as TMZ is a large cause of treatment failure in patients. TMZ resistance is mediated by enzyme (methylguanine methyltransferase) MGMT. TMZ cytotoxicity is mediated through $O6$-methylguanine ($O6$-MeG), a mutagenic, toxic lesion. $O6$-MeG is removed by MGMT in tumours expressing this protein thus counteracting the toxicity induced by TMZ. After DNA alkylation, MGMT attaches to damaged substrate DNA, the target base is then flipped out of the helix and bound to MGMT, altering the conformation of the DNA binding domain allowing alkylated MGMT to be detached from DNA and degraded through the ubiquitin/proteasomal system (Zhang et al, 2012) Therefore increased MGMT expression levels results in resistance to alkylating agents. As demonstrated by Hegi et al, 55% of all newly diagnosed GBM cases do not benefit from the addition of TMZ to their treatment, in contrast to this the 45% that did benefit also saw an increase of 38% in the two year survival (Hegi et al, 2008). Another study providing similar statistics, was a large randomized phase III trial that found TMZ treatment along with
radiotherapy resulted in an improved median overall survival from 12.1 to 14.6 months and an increase in the 2-year survival rate from 10% to 27% (Mirimanoff et al, 2007).

Carmustine, also known as BCNU (1, 3- bis (2-chloroethyl)-1-nitrosurea), is a dialkylating nitrosourea based compound that has been FDA approved for the treatment of cancers such as GBM (recurrent and newly diagnosed), Hodgkin’s and non-Hodgkin’s lymphoma. It is a cell cycle independent lipophilic compound and can cross the blood brain barrier. In 2004 a phase II study of BCNU in combination with TMZ was carried out on patients with either progressive or recurrent GBM. Results showed that progression free survival at 6 months (PFS-6) was 21% and survival at one year was 26%, previous data for PFS-6 after TMZ treatment alone was 21%, and therefore it was concluded that combined treatment was no more effective than TMZ treatment alone (Prados et al, 2004). BCNU has also been developed into a biodegradable polymer that has been impregnated with BCNU, after surgical resection the wafer is implanted into the cavity enhancing effective local drug delivery and minimising side effects. The wafer slowly dissolves releasing the drug into the surrounding tissue, treating any remaining malignant cells. It is the only form of treatment for brain tumours that does not cross the blood brain barrier. Westphal et al conducted a phase III trial in patients diagnosed with recurrent GBM, two groups, one who received either BCNU wafer or a placebo wafer at the time of surgery followed by external beam radiation postoperatively (Westphal et al, 2003). The results indicated that patients that received the BCNU wafer had a 28% reduction in the risk of mortality, with a median survival time of 13.9 months.

PCV (Pro-carbazine, lomustine (CCNU) and Vincristine) is combinational chemotherapy therapy, involving treatment with Procarbazine, Lomustine, and
Vincristine. Procarbazine gained FDA approval in 1969 for the use in treating Hodgkin’s lymphoma. It is a pro-drug that undergoes metabolic transformation into its active form where it targets and inhibits DNA, RNA and protein synthesis (Armand et al, 2007). The development of safe combinational therapies resulted in a more popular use for pro-carbazine, It was a key component of the combinational therapy MOPP in the 1970 (Devita et al, 1970) as described earlier, for the treatment of Hodgkin’s lymphoma. A phase II study carried out by (Yung et al, 2000) which compared TMZ with single agent pro-carbazine in 225 GBM patients with first relapse. Patients who received TMZ had a PFS-6 of 21% and for those who received pro-carbazine, had a PFS-6 of 8%, and the 6-month overall survival was 60% versus 44% with TMZ and pro-carbazine respectively, concluding the safety and efficacy in TMZ for treatment of recurrent GBM as opposed to pro-carbazine.

Lomustine is FDA approved for treatment in patients with brain tumour who have had surgery or radiation therapy, it is also used for the treatment of Hodgkin’s lymphoma. Lomustine also known as CCNU is an alkylating agent that forms cross links with DNA inhibiting DNA and RNA synthesis. There are no recent studies indicating a benefit of increased survival for treatment with Lomustine alone compared to that of other available therapies. In 2009, a study evaluating the long-term survival of GBM patients after treatment with radiotherapy and TMZ in combination with lomustine, reported a significantly high 2-year survival rate of 47.4% with 18.5% survived 4 years. Concluding that the combination of TMZ and Lomustine display promising efficacy for the treatment of newly diagnosed GBM (Glas et al, 2009).

Vincristine is classified as a plant alkaloid. Vincristine is an anti-microtubule agent and therefore inhibits the microtubule structures within the cell. Microtubules are part of
the cell's apparatus for dividing and replicating itself. Inhibition of these structures results in cell death. Vincristine has been extensively studied as a single agent for the treatment of GBM since the 1960’s (Lassman et al, 1965; Smart et al, 1968), but it is more commonly used in combinational therapeutic approaches.

The PCV regimen is made up of multiple 6 week long cycles, with a full course consisting of 6 cycles lasting up 9 months. PCV was first studied as treatment for malignant brain tumours in 1975 (Gutin et al, 1975). To date it is more commonly used for the treatment of oligodenrogliomas and also anaplastic astrocytomas, which are similar to GBM but lack the vascular proliferation of GBM tumours. A study carried out in 2001 demonstrated that after treatment with PCV 25% of patients out of a total of 63 patients, resulted stabilisation of disease, and observed responses were observed in 11%, although further randomised phase 3 trials were required to determine the efficacy of PCV as a chemotherapeutic for the treatment of recurrent GBM (Stupp et al, 2006).

1.7.2 Monoclonal Antibodies

Monoclonal antibodies (mAb) were first used for the treatment of cancer in the 1980s, when it was determined that a major problem with the current therapies was the lack of specificity for the cancer cells. The first patient treated with this monoclonal antibody therapy was a patient suffering from Hodgkin’s lymphoma. The antibody was a murine mAb known as AB 89 (Oldham & Dillman, 2008). The major limitation with using murine mAb was that immunogenicity of the protein and its short half-life. Over the last two decades there have been considerable advances made in the development of monoclonal antibodies, table 1.2 below describes 10 current FDA approved mAb used for the treatment of various cancers.
Table 1.2 10 current FDA approved monoclonal antibodies for the treatment of various malignancies

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Target</th>
<th>Malignancy</th>
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<tbody>
<tr>
<td>Trastuzumab (Herceptin)</td>
<td>ERBB2</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>Bevacizumab (Avastin)</td>
<td>VEGF</td>
<td>Colon, ovarian cancer, GBM</td>
</tr>
<tr>
<td>Cetuximab (Erbitux)</td>
<td>EGFR</td>
<td>Colorectal, Small cell lung cancer, head and neck cancer</td>
</tr>
<tr>
<td>Panitumumab (Vectibix)</td>
<td>EGFR</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>Ipilimumab (Yervoy)</td>
<td>CTLA4</td>
<td>Melanoma</td>
</tr>
<tr>
<td>Rituximab (Mabthera)</td>
<td>CD20</td>
<td>Lymphomas, Leukemias,</td>
</tr>
<tr>
<td>Alemtuzumab (Campath)</td>
<td>CD52</td>
<td>Chronic lymphocytic leukemia, T-cell Lymphoma</td>
</tr>
<tr>
<td>Ofatumumab (Arzerra)</td>
<td>CD20</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>Brentuximab vedotin (Adcetris)</td>
<td>CD30</td>
<td>Hodgkin’s Lymphoma,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anaplastic large cell lymphoma</td>
</tr>
</tbody>
</table>
Bevacizumab was approved by the FDA in 2009 as a single agent, second line treatment for progressive GBM following prior therapy. This is currently under review by the FDA. Two randomized, placebo-controlled, double-blind, phase 3 trials (AVAGLIO and RTOG 08-25) were recently carried out, both of which suggest there was a minor increase in progression free survival from 6.2 to 10.6 months (AVAGLIO), 7.3 to 10.7 months (RTOG 08-25) but no significant increase in the overall survival, when bevacizumab was added to the standard of care (TMZ and radiation therapy) (Weller & Yung, 2013; Gilbert et al, 2014; Taphoorn et al, 2015).

1.7.3 Receptor Tyrosine Kinase Inhibitors

Receptor tyrosine kinase inhibitors (RTKIs) are a class of chemotherapeutics that inhibit, or block, the enzyme tyrosine kinase, allowing for targeted specific treatment for different cancers. In 1962, Stanly Cohen successfully isolated the nerve growth factor from mouse salivary gland and snake venom alongside Levi Montalcini, Cohen then isolated and characterised the epidermal growth factor from mouse salivary gland extract. It was found to stimulate the proliferation of epithelial cells (Gschwind et al, 2004). In 1983 EGFR was targeted with mouse monoclonal antibody to block proliferation of human cancer cells in vitro. Throughout the following decade it was discovered that many cancers such as breast and cervical cancer displayed amplified EGFR or EGFR related gene (human epidermal growth factor receptor 2 (HER2) (Gschwind et al, 2004). This discovery provided prospects for the development of target specific therapies. In 1990 the Genentech group characterise the murine monoclonal antibody 4D5 that binds to HER2/neu on the surface of cancer cells, and this led to the development of the first targeted anti-kinase therapeutic agent based on genomic research, known as Trastuzumab (Herceptin) (Shawver et al, 2002; Gschwind et al,
2004). Following the success of Herceptin in the treatment of breast cancer, it propagated the development of small molecule inhibitors for cancer therapy. In 2002 the first EGFR tyrosine kinase inhibitor became available in Japan, as a novel therapeutic approach for the treatment of non-resectable non-small cell lung cancer (NSCLC). Gefitinib is a low–molecular weight molecule and a member of the anilinoquinazoline family that reversibly inhibits the tyrosine kinase activity associated with EGFR (Rich et al, 2004). After treatment it was noted that there were a small population of patients who responded well to the treatment but unfortunately for approximately 70-80% of patients it had no effect (Araki et al, 2012). Since then many different techniques have been used to try and predict whether a patient will respond to Gefitinib treatment.

Genotyping work identified patients with EGFR mutations in the area of the gene coding for adenosine triphosphate (ATP) binding pocket of tyrosine kinase domain were those that respond to Gefitinib (Araki et al, 2012). Another issue with the use of Gefitinib is that the majority of patients develop resistance to the treatment after a couple of years.

Approximately 50% of malignant gliomas overexpress EGFR, resulting from aberrant signalling of the RTK/PI3K/Akt pathway (and as described in the gliomagenesis in section 1.4,) therefore making them an ideal target for EGFR tyrosine kinase therapy (Halatsch et al, 2006). They also have the ability to penetrate the blood brain barrier (Rich et al, 2004). In 2002 a report was published on a phase II clinical trial to determine the tolerability of Gefitinib for the treatment of GBM at first recurrence. They demonstrated tolerability with moderate activity of Gefitinib in patients. It was observed that a population of the patients experienced beneficial effects of the therapy, with over 50% of the patients showing progressive disease or toxicity. They observed a 6-month progression free survival rate of 13.2% which is worse than observed in patients with
first relapse GBM that were treated with TMZ (21%) but better than Procarbazine (8%) (Rich et al, 2004). While the results from this study were poorer than expected, Gefitinib demonstrated low toxicity and moderate efficacy therefore suggesting that future studies should investigate alternative dosages (Rich et al, 2004). Also due to the low toxicity of the drug, there might be a future for Gefitinib in the treatment of GBM as a combinational therapy.

1.7.4 Plant Alkaloids

Plant alkaloids are chemotherapeutics derived from certain types of plants. Plant alkaloids can be classified into 4 specific categories shown in table 1.3 below. Camptothecin is a natural product, derived from traditional Chinese medication. It is a DNA topoisomerase I (topo I) and induces DNA single-strand breaks during transcription and DNA replication (Efferth et al, 2007). Vinca alkaloids such as vincristine bind to β-tubulin and inhibit microtubule assembly. Vindesine is a novel vinca alkaloid derivative that exhibits enhanced clinical features for tumour therapy (Efferth et al, 2007), as stated earlier vincristine is used as part of the PCV combinational therapy for the treatment of GBM.
Table 1.3 Different categories of plant alkaloids

<table>
<thead>
<tr>
<th>Plant alkaloids</th>
<th>Plant source</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vinca alkaloids</td>
<td>Periwinkle plant ((catharanthus rosea))</td>
<td>Vincrisitne</td>
</tr>
<tr>
<td>Taxanes</td>
<td>Pacific Yew tree (taxus)</td>
<td>Paclitaxel (Taxol)</td>
</tr>
<tr>
<td>Podophyllotoxins</td>
<td>May apple plant</td>
<td>Etoposide</td>
</tr>
<tr>
<td>Camptothecin analogues</td>
<td>Asian &quot;Happy Tree&quot; ((Camptotheca acuminate))</td>
<td>Topotecan</td>
</tr>
</tbody>
</table>

Medicinal herbs have played an important role in the development of western medicine. Since the advancement of the pharmaceutical industry the role of rational for Chinese/herbal medicines have had less of an impact on the treatment of disease. In more recent years there has been a noticeable revival of interest in the use of bioactive natural compounds to be used as therapeutic targets for the treatment of disease (Efferth \textit{et al}, 2007). A large proportion of natural products are currently under development as potential anti-cancer therapies.

1.8 Nutraceuticals

According to the scientists around 400BC the Hippocrates were the first to identify the association of food and its medicinal properties stating \textit{“Let the food be thy medicine and medicine be thy food.”} –Hippocrates (Gupta \textit{et al}, 2010), although it is only in the past 20 years, that there has been a big emphasis on the link between nutrition and pharmaceuticals. The rapidly growing interest in the area of bioactive natural
compounds and their health enhancing properties, led to the development of the term ‘nutraceutical’, a term coined by Stephen DeFelice, the founder and chairman of the Foundation for Inno-vation in Medicine (FIM). The term nutraceutical can be defined as ‘A functional food that aids in the prevention and/or treatment of disease or disorder (except anaemia) therefore allowing for the distinction between functional food, dietary supplements and a nutraceutical (Kalra, 2003).

1.8.1 Ursolic Acid as a novel treatment for GBM.

Ursolic acid (3β-hydroxy-urs-12-en-28-oic-acid), a bioactive active pentacyclic triterpene acid which has been successfully isolated from various types of medicinal plants for example, holy basil (Ocinum sanctum L.) (Vetal et.al, 2010), Salvia officinalis (sage) (Kassi et al, 2007) and fruits such as apples, pears, cranberries (Vaccinium macrocarpon Air) or more significantly in their peel (Kondo et al, 2011; Shanmugam et al, 2013a). Ursolic acid (UA) as seen in figure 1.5 below, is a sterol-like chemical with 5 fused rings. It has a chemical formula of C_{30}H_{48}O_{3}, has a melting point is 283-285°C and the molecular weight of UA is 456.7g/mol. UA belongs to a family of plant-indigenous phytosterols. These are a group of chemical compounds that are similar to the structure and properties of cholesterol.
Figure 1.5: Chemical structure ursolic acid

Ursolic acid has been the major component of many herbal medicines worldwide that are used to treat anti-inflammatory conditions (Ikeda et al., 2008). Current research suggests that UA has anti-proliferative and anti-tumour properties when tested on various malignant cell lines in vitro (Shanmugam et al., 2013a; Weng et al., 2014). Mechanisms for UA activity have been identified in some in vitro cancer models, for example, UA down-regulates Mcl-1 and activates caspase gene expression in leukaemia cells (Gao et al., 2012). In an ovarian cancer cell line, UA was shown to induce caspase activation while suppressing the transcription of survival genes such as c-Myc and Bcl-xL and interrupting phosphorylation of extracellular regulated kinases (ERK) (Song et al., 2012). In a colon cancer cell line, UA was found to induce anti-proliferating effects by suppressing the phosphorylation of EGFR, ERK, JNK, and P-38 (Shan et al., 2009). It was recently demonstrated by (Yeh et al., 2010) that pretreatment of cells with UA chemosensitises ASTC-a-1, lung adenocarcinoma cells, but not normal cells, therefore significantly reducing the required chemotherapeutic drug dose without sacrificing treatment outcome. Yeh et al. also identified that UA is dependent on the inhibition of
NF-κB, leading to amplified activation of the intrinsic apoptotic pathway via augmentation of BID cleavage and activation of Fas/FasL-caspase-8 extrinsic apoptotic pathway. (Shanmugam et al, 2011) demonstrated both in vitro and in vivo, that UA exerts anti-metastatic effects through the suppression of CXCR4 expression in prostate cancer. Recently in 2014, a small Phase I trial evaluating the multiple-dose safety and antitumor activity of UA liposomes in subjects with advanced solid tumors was carried out and demonstrated that UA liposomes were tolerable, had controllable toxicity, and could potentially improve patient remission rates. A larger study is required to confirm the findings observed by (Qian et al, 2014). Little research at present has been published focusing on GBM, despite clear evidence of positive efficacy and safety data in other tumour models and the lack of therapeutic alternatives for treating GBM. Antitumour activity by UA was determined in previous studies (Wang et al, 2012b; Shen et al, 2014) where either apoptosis or autophagy in U251MG or U87MG GBM cells respectively was induced by UA. More recently it was also demonstrated that UA triggers non-programmed cells death (necrosis) in the GBM TMZ resistant cell line DBTRG-05MG (Lu et al, 2014). UA is a promising anti-proliferative compound and has great potential as a natural chemo-preventative target.

1.9 Novel experimental treatment for GBM

The following sections describe the novel experimental therapies that are employed in this report for the treatment of GBM cells. Current treatment for GBM involves using a combination of treatments including surgical resection with treatment of radiotherapy combined with chemotherapy. With survival rates remaining low over the past decade despite the multitude of treatments available, indicts a necessity for the development of novel therapies for the treatment of GBM. Cold Atmospheric Plasma (CAP) is a novel non-ionizing form of radiation that has shown great promise as a combinational therapy
for the treatment of GBM. An advantage of CAP over treatments such as radiotherapy is the localisation of the treatment to the site of the tumour, with reduced side effects to the surrounding healthy tissue. There is great potential for CAP to be utilised as a combination therapy with both existing and novel chemotherapeutics.

1.10 Plasma

During the 19th century the term ‘plasma’ was first coined by the Czech physiologist Jan Evangelista Purkinje. It symbolised the transluicid liquid that remains after completely removing the corpuscles from the blood (Bellan, 2006). Following this in 1927 an American chemist and physicist Irving Langmuir became the first scientist to describe the term ‘plasma’ as ionized gas. Langmuir developed the theory of ‘plasma sheaths’, the boundary layers that form between ionized plasmas and solid surfaces, he also discovered Langmuir waves, nowadays known as plasma oscillations, which are electrostatic waves that propagate in a plasma, because of variations in the plasma's electron density. Gradually overtime plasma research expanded in different directions depending on its application for example space plasma physics, thermonuclear fusion, and plasma medicine.

Over the past decade plasma science has emerged as a novel tool for applications such as food sterilization, medical devices, polymer science, biomedicine, microelectronics (von Woedtke et al, 2013; Ziuzina et al, 2014). This continuously evolving field has led to the recent discovery of the biological effects of plasma, now known as plasma medicine. This emerging field combines plasma physics, life sciences and clinical medicine in the hope of developing therapeutic applications. Despite all of the recent advances in medical research over the past century there are still many diseases that are deemed incurable as there is no available therapy, or the disease has evolved and is now
resistant to such therapies. Recent advances have highlighted the potential of technological solutions to treat a variety of medical conditions. Over the past decade there have been many studies investigating the biological effects of CAP in mammalian cells both *in vitro* and *in vivo*, these studies established that CAP applied at sub-lethal doses can induce a cytotoxic effect resulting in apoptosis, cell death, cell detachment and cycle arrest in a variety of mammalian cells tumour cells of which have been extensively reviewed by (Ratovitski *et al.*, 2014). In contrast to this CAP has also been shown to promote cellular proliferation, providing painless disinfection and reducing the healing time of venous ulcers, and other lesions as a result of skin disorders, proving a potential application for wound healing (Isbary *et al.*, 2012; Arndt *et al.*, 2013; Haertel *et al.*, 2014). The mechanisms that affect mammalian cells and prokaryotes are as a result of a variety of biologically active agents generated by CAP.

Plasma is often referred to as the fourth state of matter as illustrated in figure 1.6 below. Plasmas are defined as fully or partially ionized gas consisting of ions and electrons (Piel, 2010). It is the combination of free electrical charges, ions and electrons that makes plasma electrically conductive and responsive to an electromagnetic field (Fridman & Friedman, 2012). Plasmas possess a variety of unique properties such as ability to radiate, react chemically and conduct electricity, these properties result in plasmas having different temperatures. Plasmas are can be distinguished as either thermal or non-thermal.
Thermal plasmas are obtained at high pressure ($>10^5$ Pa) and require substantial power (~50MW) to be observed (Moreau et al, 2008). They are characterized by high electron densities and high temperatures between 5000 -50000K, and thus are known to be in local temperature equilibrium (LTE), meaning they can be characterised by a single temperature value (Heberlein, 1992). Thermal plasmas are naturally occurring e.g. the sun, they are also found in plasma torches and circuit breakers (Heberlein, 1992; Fridman & Friedman, 2012). Non thermal plasmas are non-equilibrium plasmas and are often referred to as ‘cold’ plasmas. They are obtained at low or atmospheric pressure, have low power requirements and also have the ability generate chemically active species within gasses while remaining at a low temperature. The temperature of the electrons can reach between 10,000 -100,000 K while the gas temperature can remain as low as room temperature. An example of naturally occurring non thermal plasma is the effect known as the aurora borealis as seen in figure 1.7 below. Throughout the 21st century non thermal plasmas have been established by many industries for various
applications; sterilization of foods, dentistry, bacterial decontamination, wound healing, improvement of packaging materials, waste and air pollution management, polymer processing, low energy light bulbs (Kalghatgi et al., 2011; von Woedtke et al., 2013).

![Image of Aurora Borealis](image)

**Figure 1.7:** The Aurora borealis is an example of non-thermal plasmas occurring in nature as opposed to the sun which is an example of thermal plasma found in nature. **Aurora Borealis, Co. Donegal** (Diver, 2013)

Plasmas are generated by applying energy i.e. electrical, thermal, or electromagnetic radiations, to a gas which primarily produces energized electrons and ions (Fridman et al. 2005; Tendero et al. 2006). The majority of the energy applied results in the generation of energized ‘hot’ electrons while the gas stream remains at a low energetic state i.e. low plasma temperature (von Woedtke et al., 2013). Throughout the past two decades there have been a variety of plasma sources developed and commercialised for the biomedical field, the majority of which are based on using atmospheric pressure plasmas as opposed to low-pressure plasmas which involve the use of a vacuum chamber (von Woedtke et al., 2013). The most significant advantage being that it allows for localized treatment of a specific area.
1.10.1 Cold Atmospheric Plasmas

Cold atmospheric plasma occurs at atmospheric pressure in air or other gases when a high voltage is applied between two electrodes. Energy is required in order to generate plasma; this can be achieved using electrical energy, electromagnetic waves and gravity. Plasma can be created using noble gases, ambient air, or aqueous vapours (Ogata et al., 2000). There are a number of requirements that must be taken into account for the design of CAP for any given application for example; temperatures, chemical composition, air flow, frequency of applied voltage, pressure and sustainment of the electrical field (Conrads & Schmidt, 2000; Fridman et al., 2008). Thus each design is created specifically for its application. The method most commonly used for creating plasma involves the electrical breakdown of a neutral gas in the presence of an electric field (Conrads & Schmidt, 2000). The electrical energy that is discharged firstly goes to the electrons. This results in the temperature of the electrons to rise quickly to approximately 10,000K and the electrons become excited, but gradually over time the heat is transferred to the ions and neutral species (Kumar et al., 2008; Kalghatgi, 2010). This is known as the ‘electron avalanche’ until the electrons becomes balanced and steady-state plasma is formed.

Cold atmospheric plasmas can be generated by a diversity of electrical discharges, and the following are examples of those which have been developed for the biomedical industry; corona discharge, micro hollow cathode discharge, atmospheric pressure plasma jet, dielectric barrier discharge (DBD), floating-electrode dielectric barrier discharge (FE-DBD) plasma guns and plasma needle (von Woedtke et al., 2013). These can generally be broken down technically into barrier discharges, plasma jets and corona discharges. Barrier discharges are characterised by the presence of at least one insulating layer in the discharge gap (von Woedtke et al., 2013). Atmospheric pressure
plasma jets (APP jets) consists of one or two electrodes through which gas flows, when the gas discharge is ignited the plasma flows through the nozzle (Schutze et al, 1998). Corona discharge was the first scheme used to generate cold atmospheric plasma. It can be defined as non-uniform discharges that develop in the high field region near the sharp electrode spreading out towards the planar electrode (Kumar et al, 2008).

1.10.1.1 Dielectric Barrier Discharge

The dielectric barrier discharge also known as ‘silent discharge’ is a well-established plasma source. It was first developed in 1857 by Werner von Siemens who focused on the generation of ozone (Kogelschatz, 2003). A DBD is an alternating current (AC) discharge, which provides a strong thermodynamic, non-equilibrium plasma at atmospheric pressure and at a low gas temperature (Kumar et al, 2008). DBD’s are composed of one or more dielectric layers placed in the current path between metal electrodes, the presence of an insulating layer between two electrodes as illustrated in figure 1.8 below, it is the easiest approach to creating a non-equilibrium atmospheric discharge. The dielectric barrier can be made from glass quartz ceramics and other materials of low dielectric cost and high breakdown strength (Fridman et al, 2005). Most DBD’s operate with an amplitude of about 10-120 kV and frequency of 10-100 kHz (Bibinov et al, 2011). The DBD structure can be operated in a variety of configurations for example volume discharge which can be planar electrode arrangements, or cylindrical electrode arrangements, surface discharge and coplanar discharge.
Figure 1.8: Common DBD configuration.

Illustrating the two electrodes, separated by a dielectric barrier. In this schematic the sample is placed between the two electrodes, where the reactive species are generated in the plasma field.

To ensure stable plasma operations, the gap between the electrodes is limited to a few centimetres; while this set-up is advantageous for the purpose of sterilization and has the ability to cover large surface areas there are limitations in certain biomedical applications, as the surface must be smooth otherwise treatment can become patchy (Kong et al., 2009). Stable plasmas can also be generated between electrodes with a larger gap by applying higher voltages, as demonstrated in the system used in this report. DBD’s that are used in medical applications use only one electrode which is covered with a dielectric layer, allowing close contact with the skin, the human body acts as the second electrode (Bibinov et al., 2011), this is known as the floating electrode dielectric barrier discharge (FE-DBD). Fridman et al developed the FE-DBD in order to enhance the safety of directly applying high voltage non thermal plasma discharges to the skin. DBD’s are operated at atmospheric pressure i.e. air is characterised by the formation filament structure known as ‘microdischarges’. Microdischarges have a high
electron density and thus dissociation of oxygen and nitrogen molecules during electron impact takes place abundantly (Fridman et al., 2005; Bibinov et al., 2011).

1.10.2 Biologically active components generated by CAP

The most significant biologically active agents generated by CAP are, Reactive Oxygen/Nitrogen Species (ROS/RNS) and electrical current, which if present at high concentrations can result in inhibition or stimulation of cellular function (von Woedtke et al., 2013)

Most CAP’s generate an electrical current, primarily the dielectric barrier discharge system. The electrical energy produced from plasma generation is used to produce energetic electrons, this is an alternative to heating the entire gas stream (Fridman et al., 2005). The International Commission on Non-Ionizing Radiation Protection has determined the touch perception limit of current flowing through the human body to 25 mA at both 1 and 100 kHz (ICNIRP, 1998). There are many therapies that rely on electrical current to take effect, for example the fentanyl HCl iontophoretic transdermal system (fentanyl ITS), it uses an electrical current (170mA) to drive ionized drug molecules across the skin and into blood circulation. This system is used throughout Europe and the USA for post-operative pain management, allowing for self-administration by patients (Power, 2007). Therefore, provided the electrical current of the plasma device is kept to a minimum, it can be deemed safe.

Once the plasma source is turned on there is a series of cascading events that occur during plasma generation. Plasma is only generated once the power supply has been switched on, once it is switched off the plasma rapidly dissipates. As described previously CAP is generated under atmospheric conditions i.e. air. Samples can be in liquid form (water or cell culture media), as dry samples (food products such as fruit) or
in the case of adherent mammalian cell cultures there is a thin film of media that will always remain in the wells covering the cells. The interface between CAP and liquid is important. It can be present in the sample as described above or present as humidity in the air. Without water, CAP will not generate long lived reactive species. CAP induces longer lived species that accumulate in liquid (i.e. water or media) such as the production of the ROS / RNS as described below. Water, or media, allows the longer lived species to remain in the vicinity of the treatment, and of course facilitates the production of long lived reactive species. Studies have shown that media that has been treated with CAP (plasma activated media, PAM) is able to retain its cytotoxic and genotoxic activity upon storage at +4 °C or -80 °C, as well as H2O2 concentration in PAM remains stable during at least 7 days of storage at +4 °C and −80 °C (Judée et al, 2016). In the case of CAP and direct treatment on cellular material such as cancer cells, there are a number of physical and chemical changes on biological surfaces that occur. When compared to normal cells, cancer cells display elevated levels of ROS which enhances their ability to proliferate and promote their malignant progression (Marengo et al, 2016). This results in redox adaptation in response to the increase in oxidative stress, leading to an upregulation of antioxidant molecules such as glutathione (GSH). As a result, this renders the cancer cells highly dependent on these antioxidant systems, therefore making them an ideal target to treatment modalities that abrogate these antioxidant molecules (Trachootham et al, 2009). This provides biochemical bases for selective attack of cancer cells by CAP, mediated by the influx of extracellular RONS, resulting in oxidative damage overcoming the antioxidant capacity of the cell, resulting in cell death. It has also recently been demonstrated that CAP can modify the expression of nearly 3,000 genes encoding structural proteins and inflammatory mediators, such as growth factors and cytokines (Gay-Mimbrera et al, 2016; Schmidt et al, 2016). The
mechanism of how CAP exactly interacts with tissues is not completely understood, but unlike penetrating ionisation radiation, CAP is a surface or near-surface treatment modality. It is unknown whether the intracellular RNS following CAP treatment is being created by the cell in response to CAP treatment or as a results of the generated ROS penetrated through the cell membrane (Graves, 2014).

![Cell-cell communication: 'bystander effect' leading to tumor apoptosis? Immune cell involvement? Plasma-induced increased blood flow/O2?](Image)

**Figure 1.9 CAP and cell interaction** (Graves, 2014)

As demonstrated above in figure 1.9, CAP generates RONS that can either enter a cell surface-covering liquid layer or enter the cells directly. The effects of the solvated RONS and their products are in the surface layer of cells that are exposed to them, the effects on deeper layers of tissue therefore, involve some cell–cell communication. It has been postulated that these mechanisms are similar to radiation-induced “bystander effects,” such as the involvement of the immune system.

**Reactive Oxygen/Nitrogen Species**

CAP attained at low or atmospheric pressure, generate chemically active species within gases such as reactive oxygen species (ROS) (O2•, O, O3, OH•, and H2O2) and reactive nitrogen species (RNS). One of the major components generated as a result of CAP is
Ozone (O₃). The US Environmental Protection Agency in 2008 revised the level of the 8-hour national ambient air quality standards to 0.075 parts per million (ppm) (von Woedtke et al, 2013). Ozone has been used in the medical field for over 80 years, and still remains a topic of controversy. A recent review by (Graves, 2012) describes published articles that are both for and against the use of ozone therapy. The American Cancer Society’s journal CA: A Cancer Journal for Clinicians published an article that strongly against recommending the use of ozone and other hyper oxygenation therapies, although they conclude with the following statement:

‘This recommendation, however, is not intended to preclude the use of oxygen-rich compounds or hyperbaric oxygen therapy in situations for which efficacy has been demonstrated. Nor is it meant to discourage responsible study of these methods by the scientific community.’

In opposition to this is (Bocci, 2006, 2007) has published articles showing that although the effects of ozone exposure can be toxic, the effects can be controlled when administered correctly and can be used in the treatment on many diseases (Graves, 2012).

1.10.3 Reactive Oxygen/Nitrogen Species

The term ROS (reactive oxygen species), has been coined to define an emerging class of endogenous, highly reactive, oxygen (and nitrogen) -bearing molecules, derived from molecular oxygen. They mediate many biological functions within the cell such as phagocytosis of bacteria and cellular redox signalling (Simon et al, 2000; Ahn et al, 2011). Molecular oxygen (O₂) is a free radical containing two unpaired electrons with the same spin in its outer shell. O₂ is capable of accepting electrons to its anti-bonding orbital’s and thus becomes reduced (Thannickal & Fanburg, 2000). The mitochondrion
is the main source for ROS in mammalian cells (Murphy, 2009). The reduction of oxygen by one electron results in the formation of superoxide anion \( \text{O}_2^{-}\bullet \), which is a precursor of many ROS. Rapid dismutation of \( \text{O}_2^{-}\bullet \) by superoxide dismutase (MnSOD) results in formation of hydrogen peroxide \( \text{H}_2\text{O}_2 \) which can then be further reduced to hydroxyl radical (OH\footnote{\textbullet{\text{•}}}) which is considered one of the strongest oxidants in nature (Turrens, 2003), this process is known as the Fenton reaction:

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}\bullet + \text{OH}^{-}
\]

Hydroxyl molecules can also be generated through the Haber-Weiss reaction:

\[
\text{O}_2^{-}\bullet + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{OH}\bullet + \text{OH}^{-}
\]

While the dismutation of \( \text{O}_2^{-}\bullet \) accounts for the majority of \( \text{H}_2\text{O}_2 \) produced in the cell, \( \text{H}_2\text{O}_2 \) can also be generated by two electron reductions of \( \text{O}_2 \). Hydrogen peroxide is more stable than \( \text{O}_2^{-}\bullet \) and also has the ability to diffuse across the mitochondrial membrane unlike \( \text{O}_2^{-}\bullet \) (Thannickal & Fanburg, 2000). In table 1.4 is a list of the most common ROS/RNS species found in normal cells.
**Table 1.4 : List of the most common ROS and RNS species found in normal cells**
(del Río *et al*, 2006; Fransen *et al*, 2012).

<table>
<thead>
<tr>
<th>Reactive oxygen Species (Radicals)</th>
<th>Reactive Oxygen Species (Non-Radicals)</th>
<th>Reactive Nitrogen Species (RNS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxyl</td>
<td>OH•</td>
<td>Nitrous Oxide</td>
</tr>
<tr>
<td>Superoxide</td>
<td>O$_2$•</td>
<td>Nitrous Oxide</td>
</tr>
<tr>
<td>Nitric Oxide</td>
<td>NO•</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>Peroxyl</td>
<td>RO$_2$•</td>
<td>Nitrogen dioxide</td>
</tr>
<tr>
<td>Lipid Peroxyl</td>
<td>LOO•</td>
<td>Nitrous acid</td>
</tr>
<tr>
<td></td>
<td>Singlet Oxygen</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$^1$O$_2$</td>
<td></td>
</tr>
</tbody>
</table>

Reactive nitrogen species are recognised as a derivative of nitric oxide (NO). NO is biosynthesised endogenously from the oxidation of L-arginine to L-citrulline by the family of enzymes, nitric oxide synthases (NOS) (*Curtin et al*, 2002). NO plays a pivotal role in many biological processes, for example is a mediator for smooth muscle relaxation, regulator of skeletal muscle contractility (*Bredt*, 1999), activates GTP binding protein on p21Ras pathway, and regulation of Ras/NFκB pathway (*Patel et al.*, 1999). Free radicals such as superoxide (O$_2$•) react with NO to form peroxynitrite (ONOO•) a short lived powerful oxidant. RNS formed in the cytosol by nNOS, eNOS, and iNOS and also in the mitochondrion by mtNOS (*Curtin et al.*, 2002).
1.1.1.1 Antioxidants

It is important for the cells to maintain a steady state concentration of $O_2^{-\bullet}$ in order to prevent oxidative stress, which is described as the deleterious process resulting from an imbalance between the over production of ROS/RNS and inadequate antioxidant defences (Turrens, 2003). Cellular damage and apoptosis due to the high reactivity of ROS/RNS is avoided by various antioxidant enzymes present in the cell such as glutathione peroxidise, superoxide dismutase (SOD), and catalase which act as scavengers, detoxifying ROS (King & Robins, 2006). There are three types of SOD that have been identified (intra-mitochondrial manganese MnSOD, cytosolic Cu,Zn-SOD, and extracellular SOD) (Raha & Robinson, 2000), all of which catalyses the dismutation of $O_2^{-\bullet}$ to $H_2O_2$ and $O_2$. Since $O_2^{-\bullet}$ has the ability to reduce both transition metals which results in $OH\cdot$ formation or spontaneously react with NO resulting in $ONOO\bar{\cdot}$ formation, it is vital that the concentration of $O_2^{-\bullet}$ is maintained at a low steady state. One of the primary defences against hydrogen peroxide is the catalase enzyme which is found in peroxisomes, it catalyses the decomposition of $H_2O_2$ to form water and molecular oxygen (Curtin et al, 2002):

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

There have been numerous studies that have demonstrated the cytotoxic effects of endogenous and exogenous $H_2O_2$ (Datta et al, 2002a; Ahn et al, 2014), which highlight the importance of the catalase and a cells antioxidant defence system. Glutathione (GSH) is a tripeptide consisting of glutamate, cysteine and glycine ($L-\gamma$-glutamyl-$L$-cysteinyl-glycine), is low molecular weight thiol molecule which is a very important antioxidant in a cell defence system in its protection against ROS. GSH has two main functions; it acts as a ROS scavenger and also regulates the intracellular redox state.
GSH is synthesised by all cells and approximately 15% of cytosolic GSH is transported to the mitochondria (Stańczyk et al., 2005). The GSH system is composed of GSH, glutathione peroxidase and glutathione reductase. GSH is involved in the regulation of apoptosis, reduced levels of GSH have been shown to induce oxidative stress and therefore induce apoptosis (Franco & Cidlowski, 2009). Glutathione peroxidase catalyses the reduction of H$_2$O$_2$ into water, and the conversion of GSH into glutathione disulphide (GSSG). The regulation of the redox cycle is based on the GSH: GSSG ratio, and not the concentration of GSH present.

1.11 History of cell death

Cell death has been recognised and understood since the 19th century, but there was no experimental examination until the mid-20th century. The first to recognise ‘cell death’ was Carl Vogt in 1842 (Clarke & Clarke, 1996) although at that time it was not identified as cell death. It was till much later in 1885 that Walter Flemming was the first to describe what we now identify today as apoptotic morphologies, such as shrinkage of cells, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies; he termed this process chromatolysis (Curtin & Cotter, 2003). Research on cell death drifted for much of the 20th century until the 1960’s, when Glücksmann described the characteristics of karyorrhexis and karyopyknosis, that we now know to be events associated with apoptosis (Glücksmann, 1951; Curtin & Cotter, 2003). As a consequence of Glücksmann’s findings, this led to a revived interest in the investigation of cell death. In 1965, Australian pathologist John F. Kerr, noted an unusual form of cell death in liver tissue from rats. He described the unique morphological changes as shrinkage necrosis, which was subsequently identified through electron microscopy as condensation of the cytoplasm and the nuclear chromatin (Curtin & Cotter, 2003).
wasn’t until 1972 when Kerr published one of the most highly regarded papers to date in relation to cell death, where he first coins the term apoptosis, which replaced his original terminology of necrosis shrinkage (Kerr et al, 1972). At a similar time, in 1964 Lockshin & Williams from their studies on the demise of intersegmental muscles in developing silk moths, coined the term ‘programmed cell death’ (Lockshin & Williams, 1964), where they described the death of any cell is mediated by an intracellular programme. Today, programmed cell death (PCD) can be divided into three main categories which ultimately decide a cell’s fate; PCD type 1 also known as apoptosis, PCD type 2 known as autophagy and more recently described type 3 programmed necrosis/necroptosis (SEN, 1992).

In recent years, our knowledge on the mechanisms of cell death have developed thus resulting in the identification of alternative cell death pathways such as caspase independent cell death. This has led to different mechanisms of cell death that have been defined by morphological criteria, without a clear reference to precise biochemical mechanisms. The development underscores the importance of clearly defining what is referred to as 'cell death' as well as the multiple processes leading to it. As a result, members of the scientific community with expertise in the area of cell death have come together and established a committee known as the ‘The Nomenclature Committee on Cell Death’ (NCCD). The aim of this committee is to unified criteria for the definition of cell death and of different cell death morphologies, while formulating several warnings against the misuse of words and concepts that slow down progress in the area of cell death research (Kroemer et al, 2005). They recommend the use of functional descriptive terms for example, expressions such as percentage cell death or percentage apoptosis, percentage cell survival must now be accompanied with more descriptive terms such as percentage cell death with condensed chromatin, or percent of dead cells.
with a low mitochondrial transmembrane potential'. Encouraging the quantification of cell death with more than one assay (Kroemer et al, 2005). The NCCD have published four articles since 2005 critically evaluating how cell death is represented in the literature and the most appropriate ways to do so in terms of how to identify different mechanisms of cell death and the correct expressions and terms in which to do so.

1.11.1 Caspases

Caspases are a family of endoproteases that are important for maintaining homeostasis through regulating cell death and apoptosis. In humans, there are 11 known caspases, and have been classified based on the function, caspase 2, 3, 6, 7, 8, 9, 10 and 14 are involved in apoptosis, 1, 4, 5, are involved in inflammation. Caspase 12 is present in the murine genome but is not expressed in humans. Caspase 11 is a pro-inflammatory caspase found in mice, and its human homolog is caspase 4. Caspase 13 was originally reported to be a member of the human caspases but following studies could not identify caspase 13 expression in any human tissue. Studies have shown caspase 13 is not of human origin but represents a bovine gene (Humke, 1998; Koenig et al, 2001). The apoptotic caspases can be sub classified based on their role in the apoptotic process, they are divided into initiator (8, 9) and executioner (also known as ‘effector’) caspases (3, 6, and 7). Caspase 2 plays a central role in the execution phase of apoptosis, it is involved in proteolytic cleavage of other proteins. It is a highly conserved protein but function remains poorly defined. It belongs to a family of cysteine proteases that cleave proteins only at an amino acid following an aspartic acid residue. Caspase-2 has been shown to be involved in the regulation of cell death that is induced by metabolic imbalance, DNA damage, endoplasmic reticulum (ER) stress, mitotic catastrophe (Fava et al, 2012) and more recently it has been shown to exist in the mitochondria and that it is essential for mitochondrial oxidative stress-induced apoptosis (Lopez-Cruzan et al,
Caspase 10 is highly homologous to caspase-8, it is involved in the activation cascade of caspases and is responsible for the execution of apoptosis. However, the full role of caspase 10 in extrinsic apoptosis remains unclear. A recent article by McIlwain et al reviews the conflicting reports suggesting a role for Caspase-10 in CD95-mediated apoptosis in the absence of Caspase-8 (McIlwain et al, 2013a), while others have demonstrated that Caspase-10 acts in an atypical CD95-induced cell death pathway (Sprick et al, 2002; Lafont et al, 2010). It has also been suggested that Caspase 10 is recruited to both Fas- and TNFR-1 receptors in a FADD dependent manner as well as cleaves and activates Caspase-3, -4, -6, -7, -8, and -9 (Wang et al, 2001; McIlwain et al, 2013a). The initiator caspases exist as pro-caspases which when become dimerised are activated. The activated initiator caspases then activate the executioner caspases. Caspases and the apoptotic signalling pathway are tightly regulated, it would be logical to consider that any form of altered expression of these proteins would result in dysregulation and therefore aid in disease and tumorigenesis. Although mutations in caspases are rare, other elements of the apoptotic signalling pathway such as P53 are commonly mutated, especially in GBM (as noted in section 1.4) and therefore as a result effect their expression downstream. There have been some reports noting mutations in caspase 8 in a variety of cancers (McIlwain et al, 2013b). In a study of 180 cases of human colorectal carcinoma 5% were shown to express mutated caspase 8 (Kim et al, 2003).

The initiator caspases (8, 9) exist as pro-caspases that retain low levels of intrinsic activity and cleave in trans when they become dimerised, resulting in their activation. The activated initiator caspases then activate the executioner caspases (3, 6, 7), which do not possess any intrinsic activity in the pro-caspase form. Caspase 8 plays a pivotal role in the extrinsic apoptotic signalling pathway via death receptors, it has been
demonstrated (Kruidering & Evan, 2000) that ligand binding of death receptors results in the recruitment of the receptor-specific adapter protein Fas-associated death domain (FADD), which in turn recruits caspase-8. Activated caspase-8 can transmit apoptotic signals by directly cleaving and activating downstream caspases or by cleaving the BH3 containing Bcl2-interacting protein, which in turn leads to the release of cytochrome c, resulting in the activation of caspase 9 by binding Apaf-1 and dATP (Kruidering & Evan, 2000; Kuida, 2000). Activated Caspase 9 cleaves downstream caspases such as Caspase 3, 6 and 7 initiating the caspase cascade (Kuida, 2000).

1.11.2 Apoptosis

Apoptosis occurs normally during development and aging but also as a homeostatic mechanism for tissue development (Elmore, 2007), on the other hand apoptosis also occurs as a defense mechanism in response to stress for example DNA damage. Apoptosis is the most studied mechanism of cell death, and is now accepted as a distinctive and important mode of “programmed” cell death, which involves the genetically determined elimination of cells. As first described by Kerr et al, apoptosis is characterised morphologically by the following attributes: condensation of both nucleus and cytoplasm, nuclear fragmentation, pyknosis and separation of protrusions that form on the cell surface (karyorrhexis) (Kerr et al, 1972; Elmore, 2007). Cells can respond differently to the same apoptotic stimuli, for example drugs that induce DNA damage can result in activation of apoptosis in some cells while others remain unaffected. Also it should be noted that low doses of external stimuli such as radiation can induce and apoptotic response whereas at higher doses induce necrosis (Elmore, 2007). Apoptosis is a highly regulated process, and once Caspase 3 has been cleaved and activated it will unavoidably lead to cell death as beyond this point it is an irreversible process, below will describe in details the two mechanisms by which apoptosis is initiated.
Apoptosis can be initiated by one of two pathways; intrinsic or extrinsic pathway. The intrinsic pathway also known as the mitochondrial pathway as it is under control of the mitochondria. The pathway becomes activated by extracellular and intracellular stressors such as cytotoxic drugs and oxidative stress. This causes a conformational change in the pro-apoptotic B-cell lymphoma 2 (Bcl-2) members Bax and Bak and which results in permeability of the mitochondrial membrane (by producing MOMP) and then releasing cytochrome c along with mitochondrial proteins (apoptotic inhibitors Smac and Diablo) into the cytosol. As demonstrated in figure 1.10 below, Cytochrome c that is released into the cytosol binds to Apaf-1 which initiates the formation of the apoptosome and catalyses the activation of initiator Caspase 9 which in turn activates executioner Caspase 3 that carries out the known physiological steps of apoptosis (membrane blebbing, pyknosis, karryohexis etc.)
The extrinsic apoptotic pathway is also known as the death receptor mediated pathway and is the other major pathway of apoptosis. Death receptors exist on the plasma membrane and the most studied are Fas-R, tumour necrosis factor receptor (TNF-R1) and TNF-related apoptosis-inducing ligand receptor (TRAIL). Extracellular ligands, FasL, TNF-α, and TRAIL bind to these receptors and initiate apoptotic pathway (Lawen, 2003; McIlwain et al, 2013b). Binding of the death receptor ligand to the death receptors results in recruitment of initiator Caspase 8 and 10 to produce the death inducing signalling complex (DISC) (Lawen, 2003) and this then activates the executioner
Caspase 3 which results in apoptosis. As demonstrated in figure 1.10 above, there is cross talk between these two pathways, whether apoptosis has become activated by the extrinsic pathway or the intrinsic pathway, the mitochondrial membrane becomes permeable and Cytochrome c is released which triggers Caspase 9/3 signalling cascade and ultimately resulting in apoptosis (Li & Yuan, 2008).

1.11.2.2 Apoptosis resistance in GBM cells

The poor survival statistics of GBM is fundamentally due to their highly deregulated tumour genome, expressing deletions of tumour suppressor genes, amplification and/or mutations resulting in hyper activation of tyrosine kinase receptors (Krakstad & Chekenya, 2010). It is these genetic alternations that results in the deregulation of both survival and apoptotic signalling pathway and therefore development of tumour resistance to therapies. GBM’s are associated with apoptotic resistance, a recent study carried out demonstrated heterogeneity of apoptosis associated protein expression in GBM (Murphy et al, 2013). They identify low basal levels of Procasparse-9 both in vitro and in vivo, which has been shown previously by others to be expressed at high levels (Bodey et al). Whereas, on the contrary they demonstrate differential levels of Procasparse-3 in both GBM patient samples and GBM cell lines. Moreover, they noted differential expression of procaspase-3 in the GBM patient samples and in the in vitro studies, but interestingly was found to correlate with TMZ sensitivity in the cell lines and also progression free survival times in GBM patients (Murphy et al, 2013). A key player in the intrinsic apoptotic pathway, Bcl-2 family of proteins which consists of both a proapoptotic form such as Bax and Bak, which induce mitochondrial permeability progression of the intrinsic apoptotic pathway, but also exist in anti-apoptotic form such as Bcl-2 and Bcl-XL which bind and inhibit Bax and Bak therefore acts as a cell survival mechanism. One study has demonstrated an upregulation of pro-
survival proteins Bcl-2 and Bcl-XL and a down regulation of pro- apoptotic protein Bax in recurrent GBM patients (Strik et al, 1999). It has also been shown that anti-apoptotic protein Bcl2-like protein 12 (Bcl-2L12) is over expressed in nearly all GBM’s which also leads to dysregulation of the apoptotic signalling network (Stegh et al, 2007).

The expression levels of the Bcl-2 family are controlled by P53. Dysregulation of this tightly controlled system signifies an important feature in GBM. As discussed earlier some of the most common mutations found in GBM are TP53, PTEN, and EGFR. P53 and Bcl-2 have a close relationship with P53 promoting transcription of BCL-2 family member Bax (Krakstad & Chekenya, 2010). Therefore, with altered expression of both of these proteins commonly occurring in GBM, it therefore increases the resistance to the apoptotic mechanism of cell death, enhancing cell survival. This presents a possible rational as to why autophagy has been a well-documented mechanism of cell death in GBM, for example treatment of TMZ and radiotherapy are known to induce autophagy and not apoptosis (Ito et al, 2005a).

1.11.3 Caspase Independent Cell Death (CICD)

In recent years, there has been accumulating evidence in the literature that supports the existence of caspase independent cell death pathways. Similar to classical apoptosis the mitochondrion is the main organelle orchestrating the series of events which are required for the induction of CICD, in addition, the pro-apoptotic proteins Bax and Bid also remain to be key participants. CICD can be induced by the addition of chemical caspase inhibitors such as zVAD-fmk, but studies have also demonstrated CICD in cells expressing genetically encoded caspase inhibitors such as XIAP, or p35 (Okuno et al, 1998; Wilkinson et al, 2004; Tait & Green, 2008a). Two ways in which cell can die
independent of caspase which has been extensively reviewed by (Tait & Green, 2008a) can be induced through death receptors, which has been described as death receptor activated necrosis, and Mitochondrial outer membrane permeabilisation (MOMP). Following induction of MOMP in response to a stimulus, various mitochondrial proteins are released triggering loss of mitochondrial function and/or proactively contributing to cell death. The reasons for this have not fully been elucidated, but as demonstrated in figure 1.11 below, studies have suggested that loss in ATP generation following disruption of the mitochondria, AIF and Endonuclease G (EndoG), Omi/HtrA2 (mammalian serine protease) are implicated in MOMP-CICD (Lorenzo & Susin, 2004; Tait & Green, 2008a).
1.11.4 Autophagy

Autophagy has been the subject of much attention with regards to cell death in recent years, with some scientists suggesting that autophagy can act in both pro-survival mechanism or as a mechanism of cell death depending on the stimulus and surrounding environment (Codogno & Meijer, 2005). Autophagy is a critical process for all cells whereby damaged or long-lived proteins and organelles are removed. Autophagy involves engulfment of portions of cytoplasm and proteins by autophagosomes, bound for lysosome fusion and degradation. The degraded autophagic contents serve as
building blocks for protein synthesis and energy production. Until recently, autophagy was considered to be a non-selective process for degradation of cytoplasmic components during nutrient deprivation, it has since been discovered that this is not the case, the process of selective autophagy relies on cargo-specific autophagy receptors that facilitate sequestration of cytoplasmic material into autophagosomes (Glick et al, 2010a; Boya et al, 2013). There are three types of autophagy that have been described in the literature; (A) macro-autophagy (most commonly referred to as autophagy) which delivers cytoplasmic material to the lysosome through a double membrane-bound vesicle, referred to as an autophagosome, that fuses with the lysosome to form an autolysosome (Kroemer et al, 2009). (B) micro-autophagy which is when cytosolic material is directly taken up by the lysosome through invagination of the lysosomal membrane. Both macro-and micro-autophagy are able to engulf large structures through both selective and non-selective mechanisms (Glick et al, 2010a; Boya et al, 2013). (C) chaperone-mediated autophagy (CMA), targeted proteins are translocated across the lysosomal membrane in a complex with chaperone proteins (i.e. Hsc-70) these are recognized by the lysosomal membrane receptor and triggers their unfolding and degradation (Glick et al, 2010a). For the purpose of this thesis we will refer to macro-autophagy as autophagy unless stated otherwise.

Autophagy can be distinguished morphologically from apoptosis by the extensive double membrane vacuolisation of the cytoplasm, which will be described in more detail below. Autophagy is classified as class II PCD, an alternative mechanism to the conventional type I programmed cell death ‘apoptosis’. It is a highly regulated process that all eukaryotic cells can carry out. It is a self-degradative process that’s acts by sequestering and degrading the bulk of cytoplasmic contents, abnormal protein aggregates, and excess of damaged or defective organelles within a double membrane
bound vesicle called an autophagosome, which then fuses with a lysosome to form an autolysosome and in turn degrades or recycles the aggregated material and organelles (Kondo et al., 2005; Levine & Kroemer, 2008). As noted previously, some studies have shown autophagy to exhibit a dual function in the regulation of both cell survival and cell death. When under stressful conditions such as nutrient deprivation a cell will promote autophagy by removing degraded organelles and proteins before they have a negative impact on the cell and therefore sustaining cellular integrity, and maintaining cellular homeostasis (Levine & Kroemer, 2008; Mizushima et al., 2008). Depending on the stimulus and surrounding environment, autophagy can also promote cell death and it is now appreciated that defective autophagy contributes to cellular pathology in certain cases as described below by the NCCD. In 2012 the NCCD published an article stating that the term ‘autophagic cell death' has widely been used to indicate instances of cell death that are accompanied by a massive cytoplasmic vacuolization, which often (but not always) indicates increased autophagic flux with the implication that the autophagic process is actually executing the cells demise. They did note that this can occur in some cases, more specially some cancer cells that lack essential apoptotic modulators such as BAX and BAK or caspases, that respond to certain chemotherapeutic agents. (Shimizu et al., 2004; Fazi et al., 2008; Galluzzi et al., 2012). However, the autophagic process most commonly acts in a cytoprotective manner and that the term autophagic cell death must be used with caution. As our knowledge on the autophagic processes develop, the NCCD in the their most recent article published in 2015 have further expanded on their recommendations for identifying autophagic cell death. Stating that this term is only for ‘regulated cell death instances that can be influenced by the pharmacologic or genetic interventions targeting at least two distinct components of the molecular machinery for autophagy’ (Galluzzi et al., 2015). Interestingly they also make note of increasing
evidence that inhibition of the processes that are essential for cell death often does not result in inhibition of the cells demise but more often result in altering the biochemical pathway results in cell death via a different mechanism. Therefore, indicating that if a cell is destined for death employing inhibitors as a therapeutic intervention, this might prevent cell death from occurring via that pathway but ultimately the cell will succumb to cell death via an alternative pathway. This has been demonstrated in a number of studies (Prabhakaran et al, 2004; Dunai et al, 2012; Steinhart et al, 2013).

1.11.5 Cross talk between autophagy and apoptosis.

Both apoptosis and autophagy are highly conserved processes where their main function is in the maintenance of the homeostasis. Apoptosis is a tightly regulated process that is implicated in the removal of damaged cells, whereas autophagy is involved in lysosomal degradation and recycling of damaged proteins and organelles, and is considered an important survival/protective mechanism for the maintenance of homeostasis within an organism but also in cancer cells as their molecular mechanisms can be counteracted in by protective mechanisms of cancer cells. Although the mechanisms of autophagy and apoptosis are different, some proteins modulate both autophagy and the apoptosis pathway. Recent evidence suggests that interactions among the crucial proteins of autophagy and apoptosis are involved in the crosstalk between the two processes (Mariño et al, 2014). The B-cell lymphoma 2 (Bcl-2) proteins plays a significant role in both autophagy and apoptosis (Mariño et al, 2014; Li et al, 2016a). These proteins direct the mitochondria outer membrane permeabilisation (MOMP) thus releasing Cytochrome c into the cytosol. As described previously these proteins exist as either pro or anti apoptotic form and play an important role in the apoptotic process. Beclin-1 is a component of the class III PI3K/Vps34 complex and as described previously in section 1.14 and is necessary for the formation of the autophagosome. In
autophagy, Bcl-2 binds to Beclin-1 and prevents the binding of Beclin-1 to class III PI3K, leading to an inhibition autophagosome formation, preventing autophagy. In contrast, mutations of the BH3 domain of either Beclin-1 or the Bcl-2 will alter the Bcl-2-Beclin-1 complex, resulting in the activation of autophagy (Mariño et al, 2014; Li et al, 2016a). The interplay between Bcl-2 and Beclin-1 is an essential component of the regulation involved in the crosstalk between autophagy and apoptosis. Under stressful conditions such as nutrient deprivation, autophagy is activated to promote cell survival. C-Jun N-terminal protein kinase 1 (JNK1) is activated which results in the destruction of the Bcl-2-Beclin-1 complex, therefore inducing autophagy. During this process phosphorylated Bcl-2 combines with Bax inhibiting apoptosis, allowing the autophagic process to occur and act in a pro survival mechanism (El-Khattouti et al, 2013; Mariño et al, 2014; Li et al, 2016a). On the other hand, if the autophagic process cannot alleviate the stress induced by nutrient starvation, JNK promotes the Bcl-2 hyper phosphorylation, resulting in the in the separation of Bcl-2 from Bax (El-Khattouti et al, 2013; Mariño et al, 2014; Li et al, 2016a), thus inducing apoptosis via caspase-3-dependent pathway and initiating cell death.

**Autophagosome formation**

There are five key stages in autolysosome formation as depicted in figure 1.12 below, 1. **Phagophore formation:** The autophagic process begins with the isolation membrane known as the phagophore. It is thought that this is derived from the lipid bilayer contributed by the endoplasmic reticulum (ER), but this process is not fully understood. 2. **Atg5–Atg12 conjugation:** The phagophore elongates to form a autophagosome which is controlled by Atg genes through Atg12-Atg5 and LC3 complexes (Xie & Klionsky, 2007; Glick et al, 2010b). Atg12-Atg5 also interacts with Atg16 to form larger complexes (Xie & Klionsky, 2007). Modification of Atg5 by Atg12 is essential for the
elongation of the initial membrane. 3. **Capture of targets for degradation**: The phagophore expands and begins to sequester and engulf protein aggregates, degraded organelles, and ribosomes. 4. **LC3 processing and extension of phagophore membrane**: LC3 is cleaved by ATG4 protease to generate cytosolic LC3. Cleavage of LC3 is necessary for the terminal fusion of the phagophore in order to become a autophagosome (Liou *et al.*, 1997). 5. **Fusion of the autophagosome with the lysosome**: The loaded autophagosome becomes a mature autolysosome through fusion with a lysosome, which functions in providing lysosomal acid proteases and aids in the degradation of the internal constituents.

![Autophagosome Formation and Lysosomal Degradation](image)

**Figure 1.12 Autophagosome formation and lysosomal degradation** (Ciechanover & Kwon, 2015).

### 1.11.5.1 Regulation of autophagy

All cells undergo a basal level of autophagy, therefore cells need a well-organized mechanism to effectively up-regulate and down-regulate during times of stress and to maintain the order of homeostasis. One of the key players in the autophagy signalling
network is the mechanistic target of rapamycin (mTOR) complex. This protein kinase is a critical regulator of autophagy induction with activated mTOR (Akt and MAPK signalling) suppressing autophagy, and negative regulation of mTOR (AMPK and p53 signalling) promoting it. mTOR is a serine/threonine kinase that is involved in the regulation of many cellular signalling networks such cell growth, proliferation, transcription, cell survival and also autophagy. When mTOR is activated by Akt and MAPK signalling, autophagy is suppressed, but mTOR is also negatively regulated by AMPK and p53 signalling therefore promoting autophagy. mTOR binds several proteins to form two distinct protein complexes mTORC1 (mTOR complex 1) (rapamycin sensitive) and mTORC2 (mTOR complex 2) which contains rapamycin insensitive companion of mTOR (rictor) (Jung et al, 2010).

Figure 1.13 mTOR signalling pathway (Fleming et al, 2010)
Autophagy is induced through reduced growth factor signalling as well as nutrient starvation. The growth factor signalling as observed above in figure 1.13 that regulates mTORC1 mainly involves the insulin/insulin-like growth factor (IGF-1)-PI3K (phosphoinositide 3-kinase class I)-Akt pathway, which negatively regulates autophagy induction. The insulin/IGF-1 pathway involves PDK1 and Rheb, the positive regulators of mTORC1 signalling, and PTEN and tuberous sclerosis complex 2 (TSC2), the negative regulators of mTORC1 signalling (Jung et al., 2010). It has been previously demonstrated that there is a close link between the mitochondria and mTORC-1 (Desai et al., 2002), it has been postulated that cellular stressors such as reactive oxygen species and their obstructive effects on mitochondrial function could therefore trigger mitochondrial autophagy (Jung et al., 2010). This also provides evidence that CAP, as a result of the significant production of ROS could induce mitochondrial autophagy. mTORc-1 is also inhibited by rapamycin or nutrient starvation which results in de-phosphorylation of Unc-51 Like Autophagy Activating Kinase 1 (ULK1) and initiation of autophagy.

Another regulator of autophagy is the PI3K which is upstream of mTOR, the survival PI3K/AKT pathway modulates mTOR activity. The PI3K signalling network plays critical roles in the regulation of cell growth, proliferation, differentiation, motility, and cell survival and when deregulated, it is a major driver of oncogenesis. There are three major classes of PI3K, class I, class II, and class III. PI3K class I responsible for the production of phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol (3,4)-bisphosphate (PIP2), and phosphatidylinositol (3,4,5)-trisphosphate (PIP3) (Li et al., 2016b). PI3K class II and III are differentiated from class I by their structure. Class II catalyses the production of PIP3 through phosphorylation of PI and PIP2 from PIP.
whereas class III only produces PIP3 from PI. Phosphorylation to PIP3 results in downstream activation of AKT which has also been shown to function as a nutrient-regulated lipid kinase mediating signalling through mTOR indicating a potential role in regulating cell growth (Liu et al, 2009; Li et al, 2016b). As previously described, members of the PI3K/Akt signalling cascade are among the most frequently altered in GBM, resulting in hyperactivation. The PI3K/Akt/mTOR survival signalling cascade has been identified as a promising therapeutic target, particularly as PTEN, its negative regulator, is among the most frequent mutated proteins in GBM. The PI3k/AKT/mTOR pathway when activated negatively regulates autophagy. The various dysregulations of the PI3K pathway can lead to increased downstream activation of mTOR and inhibition of autophagy-mediated cytoprotection. It is the over expression of the PI3K signalling pathway that results in resistance to chemotherapeutics such as TMZ (Stupp et al, 2005a), therefore regulating PI3K expression through targeted therapy could sensitise GBM cells to TMZ therapy. Studies have shown that targeting PI3K signalling pathway with small molecule inhibitors may prove to be an effective therapeutic target. Initial studies employing pan-PI3K inhibitors such as wortmannin and LY294002 demonstrated excellent anti-cancer effect both in vivo and in vitro (Vlahos et al, 1994; Guerreiro et al, 2008). Unfortunately, both drugs were halted at preclinical studies due to the toxicity, and poor pharmacodynamics. More recently a new group of PI3K inhibitors are being investigated in a number of tumour models. BKM120 has demonstrated lower toxicity with better drug properties and has passed phase I clinical trials and is currently undergoing phase II trials among patients with recurrent glioblastoma and activated PI3K pathway (Bendell et al, 2012). In addition, BKM120 is also undergoing several clinical trials as a potential combinational therapy with radiation and Bevacizumab (Huang et al, 2009). While in theory targeting mTOR has
many advantages, it is important to note that the main function of autophagy is to act as a prosurvival mechanism for the cell, some studies have demonstrated the downstream effects of activating autophagy resulting in a prosurvival mechanisms for tumour cells by recycling damaged cellular contents (Vignot et al., 2005a; Xie et al., 2013). A suggested alternative to overcome this problem is to combined mTOR inhibitor with its antitumor properties concurrently with an autophagy inhibitor to inhibit the recycling process of the degradation tumour cells (Xie et al., 2013).

1.11.6 Necrosis

Necrosis is deemed to be a non-physiological process because it occurs in response to external stimuli such as injury or infection. For many years’ necrosis has been noted as an accidental uncontrolled form of cell death, but with accumulating evidence in recent literature the international cell death committee has revised this statement and is noted that necrosis can result in a controlled and regulated manner (Galluzzi et al., 2012), regulated necrosis has also been described as necroptosis. Regulated necrosis has been characterized as non-apoptotic accidental cell death resulting from environmental distresses with release of inflammatory cellular contents, in contrast to apoptosis and autophagy which are regulated and controlled processes. Necrosis does not follow a signaling cascade as observed in apoptosis but results in loss of membrane integrity, releasing cellular contents into the cytosol (Proskuryakov et al., 2003), which in turn initiates an immune response and results in damaged cell being phagocytosed. The causative elements that result in necrosis remain to be fully elucidated, and therefore necrotic cell death is still largely identified in negative terms by the absence of apoptotic or autophagic markers (Kroemer et al., 2009; Galluzzi et al., 2012). It has been widely accepted that the loss of structural integrity of the plasma membrane is a hallmark of necrosis and represents the common final endpoint which leads to an inflammatory
responses by immune cells (Ouyang et al, 2012), and most frequently exhibits changes in the nuclear morphology but not the organized chromatin condensation as observed in apoptotic cell death. Biochemically the release of cytosolic enzymes such as lactate dehydrogenase and uptake of membrane-impermeant dyes such as trypan blue or propidium iodide are also indicators of necrosis but cannot be considered confirmatory assays as loss of membrane integrity is also associated with apoptosis (Fink & Cookson, 2005).

Necrosis can be initiated by the same cell death signals that initiate apoptosis and depending on the context of the cell can undergo necrosis rather than apoptosis, i.e. if caspases were blocked, or ATP was low. In the case of inactivated or blocked caspases, death receptor ligation to TNFR1 results in assembly of a complex involving caspase-8, FADD, receptor-interacting serine-threonine kinase 1 (RIP1) and RIP3. Leading to increased ROS formation, mitochondrial membrane permeabilisation (MMP) and subsequent programmed necrosis (Ouyang et al, 2012) as demonstrated below in figure 1.14.
In GBM necrosis is not only studied as a mechanism of cell death in response to treatment but also a classical feature of GBM is that the tumour maintenance of a central hypoxic necrotic core (Martínez-González et al, 2012) and this form of necrosis within GBM has been demonstrated to be a powerful predictor of poor patient prognosis (Noch & Khalili, 2009). Here we are going to discuss necrosis as a mechanism of cell death in response to treatment. Over the years, there have been many advances in our understanding of the molecular mechanisms underlying glioblastoma formation, but it is widely accepted that GBM is defective in the apoptotic process, on the other hand both pathological and radiological observations almost always reveal obvious necrosis foci within GBM (Jensen, 2009). Necrosis seems to be related with GBM proliferation, angiogenesis and invasion, and therefore necrosis as a mechanism of cell death offers
great potential therapeutic value for the treatment of GBM. It has been demonstrated that Mouse embryo fibroblasts die by necrosis in response to DNA alkylating agents such as staurosporine and nitrogen mustard. Also novel anticancer therapy photodynamic treatment (PDT) results in necrotic tumour cell death as a result of loss of plasma membrane integrity by ROS generation in GBM cells (Fabris et al, 2001; Miki et al, 2013). Shikonin is a quinone-containing natural product commonly used in Chinese herbal medicine, has been shown to induce regulated necrosis mediated by RIP-1 in C6 and U87 GBM cells and ROS (Huang et al, 2013) therefore providing efficacy for necrosis as an alternative signalling target to apoptosis to initiate cell death.

1.11.7 Cysteine proteases

There are three distinct subtypes of cysteine proteases: caspases, calpains and cathepsins. While the importance of caspases in apoptosis, and necrosis have been clearly established, studies have indicated that several other types of non-caspase proteases may also play a role in the execution of cell death, for example calpains. Calpains are a family of Ca\(^{2+}\)-activated neutral cysteine, nonlysosomal endoproteases found in all mammalian cells that can be divided into tissue specific calpains (calpain 3 and 9) or universally expressed (µ-calpain (I) and m-calpain (II))(Harwood et al, 2005). They are distinguished based on the different Ca\(^{2+}\) requirements. There is a lot of cross talk between apoptotic caspases and calpains, as calpains have been demonstrated to be able to cleave pro-caspase 3,7,8 and 9 into inactive fragments, and equally, calpain is known to be an activator of caspase 3 and 12 (Nakagawa et al, 2000; Blomgren et al, 2001). Calpains have been noted to play a role in a number of diseases such Alzheimer’s, cataracts, strokes, Parkinson’s disease (Momeni, 2011), and have also been implicated in apoptotic glial cell death (Ray et al, 2002). More recently it has been demonstrated that radiation induced necrosis though a caspase 3 independent pathway mediated by
apoptosis-inducing factor (AIF), calpains, and cathepsins B and D in GBM (Das et al., 2016).

Cathepsins are a group of protease enzymes predominantly found within lysosomes, as for optimal activity they require a more acidic environment, such as that found in lysosomes. There are 15 human cathepsins, of which 11 are cysteine based, B, C, F, H, K, L, O, S, V, X and W. Their function was originally understood to be intra-lysosomal protein degradation but it has since established they have a role in necrosis, autophagy and to a lesser degree apoptosis. As described early, under certain conditions and stimuli autophagy can be detrimental to a cell and result in autophagic mediated cell death. During the autophagic process, lysosomes fuse with the autophagosome into an autolysosome, of which cathepsins D, B and L all play a role in this process as their primary function is to degrade proteins and are therefore important regulators of autophagy (Turk & Turk, 2009). Dysregulation of autophagy leads to increases in oxidative stress, it has been demonstrated that inhibition of cathepsin D increases the formation of reactive species (Yamasaki et al., 2007). It has also been recently demonstrated that inhibition of cathepsin S induces both autophagy and apoptosis in GBM cells through ROS mediated PI3K/AKT/mTOR/p70S6K and JNK signalling pathways (Zhang et al., 2014) and moreover that silencing the EGFR led to an improved autophagic response to irradiation and suppressed migration in the T98G (Palumbo et al., 2014a). A similar study carried out by Plaumbo et al. identified that response to TMZ and radiation both alone or combined induced an autophagic response with both cell lines U87MG (radiosensitive) and U373MG (radioresistant) both inducing autophagy but not apoptosis. Interestingly when U373MG cell were pre-treated with mTOR inhibitor rapamycin and autophagy inducer, it sensitised the U373MG radioresistant cells to radiation (Palumbo et al., 2012b). These studies provide evidence that the
autophagic cell death pathway is more dominant then the apoptotic pathway in irradiated GBM cells, and indicates a possible adjuvant therapeutic strategy to enhance the conventional GBM treatment, as well as rational for the identification of novel targeted autophagy-interfering agents either with radiation and TMZ alone or in combination as a new potential strategy for the treatment of GBM.

**Aims & Objectives**

The aim of this project is to develop and investigate the application of novel technological and medicinal therapies for the treatment of brain cancer. GBM is considered to be the most biologically aggressive brain tumour which accounts for approximately 16% of all brain tumours (Dolecek et al, 2012). The prognosis for patients diagnosed with GBM is bleak, with low 5 year survival rate of <10% (Ostrom et al, 2014), thus highlighting the need for novel and more effective methods for treating these tumours. Even with the many chemotherapeutic agents available for the treatment, GBM’s survival rates have remained stagnant over the past three decades. This is due to the high number of tumours being resistant to the currently available therapies and also the inability of most chemotherapeutics to cross the blood brain barrier. The molecular signalling pathway of GBM is complex and remains poorly understood.

In this PhD thesis, the first study (Chapter 2) of this PhD thesis investigates the anti-tumour properties including cytotoxicity and molecular mechanisms of commonly employed medicinal and chemotherapeutic drugs in our GBM model. The novel nutraceutical UA is also investigated. This is a compound commonly found in fruits and plants. Employing validated procedures for extraction of UA from cranberries and High
performance liquid chromatography (HPLC) to purify and quantify the crude extract. The cytotoxicity of the extracted compound was analysed by cell viability and compared to that of a known ursolic acid standard. Migration studies were also performed using UA, and several chemotherapeutic compounds. A distinct feature of GBM tumours is how rapidly the tumour cells infiltrate normal surrounding tissue. UA demonstrated promising anti-proliferative capabilities with potential as a natural chemo-preventative target over existing chemotherapies. It was demonstrated that UA activates autophagy prior to cell death in GBM cells, which to date has not been shown in this cell line. Part of this chapter is being prepared for submission to a relevant scientific journal for peer review.

The second study outlined in Chapter 3 of which in part was published in peer reviewed journal the ‘British Journal of Cancer’, investigates and characterises the anti-tumour effects of a potential new therapy for treatment known as CAP using a novel DBD system in vitro on U373MG glioblastoma and HeLa cervical cancer cell lines. Using ROS detection/inhibition assays an understanding into the underlying molecular mechanism that induces cell death within these cell lines began to unfold.

Chapter 4 of this PhD thesis identifies an autophagic mechanism of cell death in U373MG cells in response to cold atmospheric plasma treatment. We have successfully proven a mechanism of cell death that is independent of caspase and JNK through both biochemical and imaging techniques in our GBM model. Following the appropriate guidelines for the detection of autophagy we have identified the formation of autophagosomes through electron microscopy, AVO’s through fluorescence microscopy in U373MG cells. We are the first to identify autophagic mediated cell death following treatment cold atmospheric plasma.
This study has therefore greatly enhanced the knowledge of the molecular signaling process of GBM, and the induction of autophagy following both UA and CAP treatment.
Optimisation of GBM cell models and characterisation of cell death in novel nutraceutical Ursolic acid for the treatment of brain cancer.

Part of this chapter has been submitted for publication and is currently under review.
2.1 Introduction

Glioblastoma multiform (GBM) remains one of the most difficult tumours to treat; this is due to a variety of factors. Localisation of the tumour within the brain tissue of the CNS can make it difficult for surgical resection. GBM tumours are highly invasive, infiltrating surrounding brain parenchyma and are very heterogeneous. The majority of patients have numerous genetic mutations which increase a patient’s resistance to therapies i.e. p53, EGF, MGMT. Patients expressing high levels of MGMT are more resistant to TMZ (Köritzer et al, 2013). The tumour suppressor gene p53 is mutated in 30–50% of GBM. Mutation in the TP53 gene leads to tumorigenesis, because of the loss of the transcription function of wild-type TP53 (Wang et al, 2014). Mutations or loss of function of PTEN has also been implicated in the malignant progression of astrocytic gliomas and linked with resistance to GBM chemotherapeutic erlotinib. Chemotherapeutic drugs that are transported across the membrane in ATP-independent manners transported across concentration gradients. The ATP-dependent group consists of a protein which includes P-glycoprotein (P-gp) which is often overexpressed in GBM and is associated with resistance to GBM chemotherapy erlotinib, vincristine and doxorubicin (Haar et al, 2012). Poor prognosis of patients with GBM is also contributed to the lack of successful drug delivery across the blood brain barrier (BBB). The BBB prevents efficient passage of cancer therapeutics, including small molecules and antibodies. Therefore, many drugs can still be blocked from reaching the GBM tumour cells. The onset of symptoms, usually only occur when tumour has reached stage 3 and 4, this is especially evident in primary GBM’s, therefore reducing the chances of successful treatment. In addition, GBM tumours contain self-renewing, tumorigenic cancer stem cells (CSCs) that contribute to tumour initiation and therapeutic resistance. This diverse grade IV malignant astrocytoma along with many other cancers have
developed over time and become genetically unstable resulting in developed resistance to chemotherapeutic treatments, therefore as a consequence survival statistics have remained low. GBM accounts for 50% of the tumours of the CNS (Bower & Waxman, 2011).

The BBB is a diffusion barrier that excludes blood borne substances entering the brain and protects the central nervous system. There are tight junctions between the vascular endothelial cells which restricts many chemotherapeutics agents from crossing thorough into the invasive GBM cells. This is an important feature to take into account when investigating the use of novel therapies for the potential treatment of GBM. The chemotherapeutic TMZ which dose cross the BBB, is converted to an alkylating methylidiazonium cation which then methylate’s the guanine residues in the DNA molecule, resulting in the formation of O6- and N7-methylguanine, which the inhibits DNA repair mechanisms therefore results in DNA damage such as DNA double strand breaks (Agarwala, 2000). Although TMZ is the current standard of care chemotherapeutic, over 55% of newly diagnosed GBM tumours are resistant to TMZ and therefore supplements no value when added to the patients treatment regime (Hegi et al, 2008). This has been attributed to the overexpression of (O6-methylguanine-DNA methyltransferase) MGMT in GBM cells (Köritzer et al, 2013). Other commonly used chemotherapeutics that have been approved by the FDA for use in the treatment of GBM include BCNU (carmustine), an alkylating nitrosourea that forms inter-strand crosslinks in DNA and interferes with DNA replication. Due to the high rate of RTK mutations in GBM, TKI inhibitors have emerged as an attractive therapeutic approach, and while demonstrating success in other cancers, unfortunately results from clinical trials have been poor in GBM (Joshi et al, 2012). Gefitinib, a receptor tyrosine kinase that inhibits, or blocks, the EGF receptor, allowing for targeted specific treatment has
been explored as a possible treatment for GBM and has been approved by the FDA for treatment (Rich et al., 2004), with somewhat favorable results due to the low toxicity of the compound, overall survival rates remain unchanged. Other compounds include plant alkaloids which are employed in the combinational treatment regime, PCA (procarbazine, CCNU, vincristine), and monoclonal antibodies which are utilized as a second line treatment for progressive GBM following prior therapy, for example Bevacizumab. In recent years, investigations into novel approaches for the treatment of brain cancer have been at the forefront of the research industry, with some therapeutic targets demonstrating promising results. As TMZ is considered the standard of care for the treatment of GBM, a lot of the current research is investigating ways to overcome TMZ resistance. One current strategy is direct enzyme inhibition using O6-benzylguanine, which has been studied in combination with TMZ. Phase two clinical trials have demonstrated promising results in restoring TMZ sensitivity in anaplastic glioma but unfortunately results in GBM have remained poor (Quinn et al., 2009). As we learn more about the biology of GBM and its aberrant signalling pathways, it has paved the way for novel therapeutics, targeting and inhibiting aspects of these pathways. The majority of these agents are small-molecule inhibitors or monoclonal antibodies. Antibodies to EGFR such as cetuximab are currently under evaluation in early-phase clinical trials (Combs et al., 2006). Temsirolimus is an inhibitor of the mammalian target of rapamycin, the best-studied of the targets in the phosphoinositide-3 kinase pathway. During clinical trials it was noted to be well tolerated by patients, demonstrating radiographic improvement in 36% of patients, however in a recent trial it was observed that when combined with radiotherapy there was no increase in overall survival compared to current that of radiotherapy plus TMZ (Wick et al., 2016), in addition there was no increase in survival when combined with bevacizumab (Lassen et
Even with the multitude of therapeutic approaches available, survival rates have remained stagnant for the past decade and therefore indicate a need for the development of novel technologies and new strategies towards the treatment of GBM in order to overcome the high reoccurrence rate and ever increasing resistance of these tumours to the current therapeutic approaches.

Bioactive compounds isolated from plants and other living sources offer a source of new compounds with therapeutic potential that may afford fewer side effects. There have been numerous studies investigating the efficacy of phytochemicals in the treatment of cancer (Neto, 2007; Sasidharan et al, 2011; Wang et al, 2012a; Øverby et al, 2014; Koldaş et al, 2015). In fact, it has been postulated that drug discovery from plant sources has been the single most successful avenue for the development of anti-cancer therapies (Harvey, 2000). This is not a new concept, for example, as previously shown in table 3 (section 1.7.4) Camptothecin, isolated and extracted from the bark of the *Camptotheca acuminata* tree. Two camptothecin analogues; topotecan and irinotecan have been approved and are used in cancer chemotherapy today (Haustedt & Siems, 2015). Vincristine which is a vinca alkaloids, extracted from the Periwinkle plant (*catharanthus rosea*) and is also used in the treatment of GBM. It has been hypothesised, through an international case controlled study that phytochemicals found in yellow/orange and leafy green vegetables have an inverse effect on the incidence of glioma (Terry et al, 2009). A similar observation was also observed by Das et al who demonstrated that flavonoids apigenin, (-) epigallocatechin, EGCG, and genistein, which are commonly found in fruit and vegetables, induce apoptosis via caspase activation in GBM cells (Das et al, 2010). These studies underscore the long observed association between nutrition and the incidence of cancer, highlighting the potential
therapeutic targets of bioactive phytochemicals for the prevention or treatment of brain cancer.

Ursolic acid (UA) is an ursane-type pentacyclic triterpenoid acid and primary component of the thin waxy coating in popular western foods including cranberries, apples and olives. Current research has shown that UA demonstrates potent anti-proliferative properties when tested on various malignant cell lines in vitro for example DU145 prostate cancer cells, MCF-7 breast cancer cells and A549 lung cancer cells (Shanmugam et al., 2013a) Ursolic acid has demonstrated anti-cancer properties in a number of glioma models (Wang et al., 2012b; Lu et al., 2014). The effects of in vivo administration are currently under investigation in a number of pre-clinical trials (Shanmugam et al., 2013b). Mechanisms for UA activity have been intensively studied in in vitro cancer models, and have been extensively reviewed by (Woźniak et al., 2015).

In colon cancer cells, UA was found to induce anti-proliferating effects by suppressing the phosphorylation of EGFR, ERK, JNK, and P38 (Shan et al., 2009). More recently it has been demonstrated that UA not only exhibits anti-cancer properties by suppressing Vaccinia-related kinase 1 (VRK1) mediated DNA damage repair but also that a synergy exists between UA and other DNA damaging drugs in lung cancer cells (Kim et al., 2015). There have been few studies published focusing on GBM, despite clear evidence of its positive efficacy, safety data in other tumour models and the lack of therapeutic alternatives for treating GBM. It has been shown that UA demonstrated anti-tumour activity and induced either apoptosis or autophagy in U251MG or U87MG GBM cells respectively (Khan et al., 2012; Shen et al., 2014). In contrast it has recently been reported that ursolic acid triggers a typical non-programmed cell death (necrosis) in the GBM TMZ resistant cell line DBTRG-05MG through mitochondrial permeability transition (MPT) pores (Lu et al., 2014). These studies provide evidence of the efficacy
of UA as a potential anti-cancer drug but further studies are required to elucidate the molecular mechanism involved in GBM cell models. Recently, there has been data published on the effects of UA on cell migration and invasion, an important feature when being considered as a potential target for the treatment for cancer, for example, UA suppresses migration and invasion in gastric cancer cells, breast and lung cancer cells (Yeh et al, 2010; Huang et al, 2011; Kim & Moon, 2015). However, another compound known as oleanolic acid, also a pentacyclic triterpenoid, which is structurally similar to UA differing only by the position of one methyl group on the ring E (Ovesná et al, 2006) has demonstrated anti migratory and invasion in two GBM cell lines, U251MG and U87MG, by inactivating the MAPK/ERK signalling pathway (Guo et al, 2013). It is postulated that due to the similarity in their structures, UA might also display anti-migratory properties. Therefore, in conjunction with published data, stating that UA is a promising anti-cancer compound that has high bioavailability enhances its efficacy as a potential natural chemo-preventative target.

The aim of this study was to establish a working GBM culture model and establish working protocols for cell death assay, and inhibitor studies. We have investigated the mechanism of cell death in a panel of medicinal compounds including chemotherapeutics used for the treatment of GBM and further novel nutraceutical ursolic acid. We also investigate the effect of both current chemotherapeutics and UA on the rate of cell migration.
2.2 Materials & Methods

Routine cell culture, maintenance and sub culture

The human glioblastoma cell line (U373MG-CD14) was kindly gifted from Dr Michael Carty (Trinity College Dublin) and was the main cell line used in this study, also employed for parts of this study were the human cervical cancer (HeLa, ATCC® CCL-2™) cells were purchased from American Type Culture Collection (LGC Standards, Middlesex, UK)

U373MG cells, were cultured in DMEM (Dulbecco’s Modified Eagles Medium high glucose) (Sigma) supplemented with 10% FBS (Sigma) (known as full media for remainder of the study). HeLa cells, were cultured in RPMI-1640 (Roswell Park Memorial Institute) (Sigma) supplemented with 10% FBS. HeLa cells were used only in chapter two of this thesis. Both are adherent cell lines. No antibiotics were used in the regular culturing of these cells. Cell lines were maintained in a humidified incubator containing 5% CO₂ at 37°C. Media was changed every 2-3 days until 70-80% confluency was reached to ensure logarithmic phase of growth.

Cell were routinely sub-cultured as follows: Cells were removed from the bottom of the stock flask by using a using a 1:1 ratio EDTA: Trypsin, (EDTA: 0.1 g of EDTA in 500 ml PBS: Trypsin 2.5% 10X). Cells were spun down at 1200 revolution per minute (RPM) using a bench top centrifuge at 4°C for 5 minutes. The supernatant was then removed as waste and the afforded pellet was re-suspended in full media. Cells were then counted using a haemocytometer (Abcam).
Optimisation of cell lines

Cell growth curves were established for both cell lines in order to identify the appropriate seeding density for further cytotoxicity analysis and various time-point assays.

Cells were counted using a haemocytometer and then seeded at different cell densities (1 x 10^1 to 5 x 10^4) in a 96-well plate (Corning Costar) at 100 µl per well. The plates were then incubated at 37°C until ready for analyses. Cell viability was at four different time points (24, 48, 72, 96 hours).

2.2.1 Panel of medicinal and nutraceutical compounds

Dose response curves for commonly employed chemotherapeutic drugs used for the treatment of GBM: TMZ (Sigma-Aldrich, Arklow, Ireland), Carmustine (BCNU) (Sigma-Aldrich, Arklow, Ireland) and Gefitinib (Insight Biotechnology ltd, Wembley, UK) Cisplatin (Sigma-Aldrich, Arklow, Ireland) and Ursolic acid (Sigma-Aldrich, Arklow, Ireland) were established. To make stock solutions UA standard, TMZ, and Gefitinib were all dissolved in Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Arklow, Ireland), BCNU was dissolved in sterile H_2O and stored in -20°C and cisplatin was dissolved in PBS and stored at 4°C for a maximum of 2 weeks. It should be noted that cisplatin should not be dissolved in DMSO, as previously carried out by the majority of published articles. It has been recently demonstrated that DMSO reacts with the cisplatin complexes, resulting in the replacement of ligands on the platinum complexes, which inhibited its cytotoxicity and its ability to initiate cell death (Hall et al., 2014). This was noted for all platinum based compounds i.e. oxaliplatin. Therefore, we have followed the guidelines suggested by (Hall et al., 2014), where they suggest a saline or PBS based solution, making to a 2mM stock solution that is stable at 4 °C for a
maximum of two weeks. Stocks were subsequently used to make the working standard solutions in media. The highest concentration of DMSO was 0.5%. U373MG cells were seeded at a density of 1x10⁴ (24 and 48hr exposure time points) in 96 well plates (Sigma-Aldrich, Arklow, Ireland) with 100µl media per well. In order to establish accurate IC₅₀ values for the chemotherapeutic compounds, a lower seeding density of 2.5x10³ and a 6-day exposure period was required. Plates were left overnight in the incubator at 37°C with 5% CO₂ to allow the cells to adhere. Existing media was removed from each well and cells were treated with either a medicinal compound, ursolic acid, or solvent control (0.5% DMSO) and incubated for the appropriate time point.

2.2.2 Cytotoxicity assays

Alamar Blue

Alamar blue (Invitrogen) assay was used to investigate cell viability when performing the cytotoxicity and proliferation studies in both cell lines. After the appropriate incubation time was been reached, all media was removed from the wells. Each well was washed once with sterile phosphate buffered saline (PBS) (Sigma Aldrich). PBS was pipetted out of each well and 100µL of fresh Alamar blue solution (10%) in DMEM (minus FBS) was added to each well. Blank control wells contained medium and Alamar blue dye only without cells. Alamar blue reagent is sensitive to light, thus plates were wrapped in tin foil and incubated at 37°C for 2.5 hours. Absorbance was read immediately after incubation period on a Victor 3V 1420 (Perkin Elmer) multi-plate reader. Fluorescence was quantified using excitation and emission wavelengths of 540 nm and 595 nm, respectively. The data (in fluorescence units from the microplate
reader) for the test wells were normalised to the assay control (DMEM or RPMI medium only) and cell growth was calculated as a change of viability over time.

2.2.3 Inhibitor studies

zVAD-fmk

Commonly employed general caspase inhibitor zVAD-fmk (BD Bioscience, Oxford, England), was used in order to determine whether caspases play a role in cell death in U373MG GBM cells. zVAD-fmk was reconstituted in 100% DMSO and frozen at -20°C at a stock concentration of 10mM. GBM cells were pre-treated for 1 hour with zVAD-fmk (BD Bioscience, Oxford, England). zVAD-fmk was then removed from each well and replaced with full media. Cells were then analysed after 48 hours with Alamar blue cell viability assay. Multiple concentrations were tested to determine the most appropriate. Cells were also treated with a 0.5% DMSO vehicle control.

N-acetyl-cysteine

N-acetyl-cysteine (NAC) (Sigma Aldrich, Arklow, Ireland) a ROS inhibitor was used to identify whether ROS was involved in cell death following treatment with various medicinal/nutraceutical drugs in U373MG cells. NAC powder was reconstituted in full cell culture media and made up fresh each time. Cells were pre-treated for 1 hour with N-Acetyl Cysteine (NAC) (Sigma Aldrich, Arklow, Ireland). NAC was then removed from each well and replaced with medicinal/nutraceutical compound made up in full media. Cell viability was assessed 48 hours later using Alamar blue cell viability assay. Dose response curves were established using a range of concentrations to determine the most effective concentrations of NAC.
**SP600125**

SP600125 is an inhibitor of JNK which is commonly associated with apoptosis. SP600125 was reconstituted in DMSO at a stock concentration of 25mM and stored at -20°C. U373MG cells were pre-treated for 1 hour with SP600125 (Sigma Aldrich, Arklow, Ireland), SP600125 was then removed by pipetting and fresh drug was added to each well at a concentration around the IC₅₀. Cell viability was assessed 48 hours later using Alamar blue cell viability assay. Dose response curves were established for SP600125 to determine the most effective concentration in U373MG cells. Cells were also treated with a vehicle control of 0.5% DMSO.

**3-methyladenine (3-MA)**

3-Methyladenine (3-MA) (Sigma Aldrich, Arklow, Ireland) is an inhibitor of phosphatidylinositol 3-kinases (PI3K). PI3K plays an important role in many biological processes, including controlling the activation of mTOR, a key regulator of autophagy. 3-MA was prepared as a 100mM stock solution in sterile PBS, and gently heated in 37°C to aid solubility. Stock solution was aliquoted out and stored in the -20°C freezer for future use. Working stock solution were made up from the 100mM stock in full media fresh for each experiment.

### 2.2.4 Cell Migration Assay

U373MG cells seeded at 0.9 x 10⁶ cells in individual 35mm dishes and incubated for 24 hours. A scratch was performed in each dish prior to treatment using a 200 µl sterile pipette tip. Utilising the data observed from the dose response curve a sub-toxic concentration of TMZ (100µM), BCNU (50µM), gefitinib (25µM) and UA (12.5µM) was chosen. Cells were treated with media alone, DMSO (0.1%), or drug. Each scratch was examined under a light microscope and images were taken using the (Nikon Eclipse
700). Cells were also treated in the presence of UA (12.5µM) and JNK inhibitor SP600125 (12.5µM) and analysed over a period of 7 hours. Multiple images were taken for each time point and the average size of scratch for that time point was obtained. Image analysis was performed using image processing and analysis software, Image J (Rasband).

2.2.5 Flow cytometry

*JC-I mitochondrial membrane potential assay*

Stock solutions of JC-1 dye (Biosciences, Dublin, Ireland) were prepared at 1 mg/mL in dimethylsulfoxide (DMSO), aliquoted out and stored in freezer (–20°C) until required for use. Cells were then harvested by trypsinisation and pelleted at 1200rpm for 5 minutes. A working solution of 10µg/ml JC-1 was made up in full media and kept away from light. The cell pellet was resuspended and stained with 10µg/ml JC-1 dye and kept at room temperature for 10 minutes. Cells were then washed twice with sterile PBS and analysed by flow cytometry (BD Accuri C6). Fluorescence was measured using the FL1 (530 nm) and FL2 (585 nm) channels with emission.

*Acridine orange*

Stock solutions of acridine orange (Sigma Aldrich, Arklow Ireland) were prepared at 1 mg/mL in sterile PBS and stored in the fridge (4°C) away from light until required for use. Cells were harvested by trypsinisation and pelleted at 1200rpm for 5 minutes. A working solution of 1mg/ml AO was made up in full media and kept away from light. Cell pellet was resuspended and stained with 1µg/ml acridine orange and incubated at 37°C for 20 minutes. The cells were then washed twice with sterile PBS and analysed by flow cytometry (BD Accuri C6) with an excitation of 475nm and emission 590nm.
2.2.6 Data analysis

Spectrophotometer data

Data retrieved from the Perkin Elmer microplate reader measures the fluorescence in each well of the microplate and generates readings as arbitrary fluorescent unit values. These fluorescent unit values were extrapolated for viability calculations using statistical analyses software Prism 5, GraphPad software, Inc. (USA). All experiments were performed at least three independent times with a minimum of five replicates per experiment. Cell viability was calculated, by first subtracting any background fluorescence from the Alamar blue blank reading. The mean (average) fluorescence value from each test per plate was calculated, n=5 per individual experiment of which was repeated independently three times.

Image J Software

The total cell fluorescence was calculated using ImageJ (v1.49, NIH) software. An outline was drawn around each individual cell, as previously described by (McCloy et al, 2014). The total corrected fluorescence was then calculated as follows; Total corrected cell fluorescence (TCCF) = Integrated density – (area of selected cell x mean fluorescence of background readings) in excel. Calculated data was then transferred to prism where is was normalised to untreated control and statistical analysis carried out using regression analysis and statistical significance.

2.2.7 Statistical analysis

Statistical analysis was performed on raw fluorescent values (after first removing the background fluorescence) retrieved from the spectrophotometer or mean fluorescence values retrieved from cytometry BD Accuri C6 software as indicated in each result
section. Using the Prism GraphPad statistical software the mean values were normalised to the untreated assay controls, by subtracting the mean values from the untreated internal assay controls to give a final result. Percentage viability was calculated relative to the assay control, which was set to 100%. Percentage viability of each bioassay is graphically displayed as bar charts or regression plots with. Presented as mean ± SEM (n= total number of replicates) unless stated otherwise. Statistical analysis was carried out using one sample tests, student t tests, One-way ANOVA, Two-way ANOVA with Bonferroni post-test. Curve fitting on dose response curves were carried out using both linear and nonlinear regression analysis allowing for the calculation and comparison of IC$_{50}$ values. Unless otherwise indicated differences were considered significant with a $p$ value <0.05.
2.3 Results

2.3.1 Optimisation of mammalian cancer cells.

The results for the initial optimisation of both cells lines are graphically displayed below in figures 2.1 (A-B). Growth curves allowed for the determination of the appropriate seeding densities for both short and long term experiments i.e. 48 hours up to 6 days. It is evident from figure 2.1 that both cell lines exhibit significantly different growth characteristics and therefore require different seeding densities when carrying out experiments.

A.  

B.

![Growth curves](image)

Figure 2.1 Growth curves.

Cell viability of (A) U373MG and (B) HeLa cells over time. Both cell lines were seeded at different cell densities ranging from 100 to 50K cells/per well. Cell viability was then analysed using Alamar blue assay at different time points. Experiments were completed a minimum of three time and data was transformed to log form and plotted as fluorescence units.
2.3.2 Cytotoxicity curves for a panel of conventional therapeutic drugs in GBM cells.

Cytotoxicity curves were established for each compound utilised in this thesis. Cells were treated with a vehicle control the highest concentration present in drug, either 0.2% DMSO or 0.6% H2O (Appendix II). No significant reduction in cell viability was observed in the vehicle controls, calculated using student t-test. As demonstrated in figure 2.2 (A-D) below, after 48 hours there was little reduction in cell viability. With a reduction of only 35%, 28%, 29% in BCNU, TMZ and Gefitinib respectively, accurate IC50 could not be calculated. A longer exposure time was carried out, after a 6-day time point, IC50 values were sufficiently calculated as observed in table 2.1 below. For Cisplatin, a 48-hour time-point sufficed in order to observe a significant cytotoxic response compared to that of the other the chemotherapeutic compounds. Cisplatin with an IC50 (9.17µM) (table 2.2) demonstrates the greatest level of toxicity compared to that of other therapeutics such as clozapine (appendix VI) with IC50 values of 48.32µM at 48 hours. While cisplatin demonstrates a greater level of cytotoxicity, it also has a high molecular weight of 300.01g/mol, is a charged compound, is hydrophilic and therefore would be unable to cross the blood brain barrier.
Figure 2.2 Dose response curves for therapeutic compounds.

*U373MG* cells were treated with increasing concentrations (0 ≤ 500 µM) of either (A) BCNU, (B) TMZ, (C) Gefitinib, (D) cisplatin. Cells were also treated with a vehicle control of 0.2% DMSO or 0.6% H₂O. After 48 hours or 6 days’ cells were analysed using Alamar blue cell viability assay (see appendix II). All experiments were repeated minimum in triplicate. Data shown was normalised to the untreated control and are shown as the % mean ± S.E.M. Non-linear regression statistical analysis was carried out using Prism5 statistical software.
Table 2.1 Statistical analysis of GBM specific chemotherapeutic compounds over a 48 hour and 6 day period

<table>
<thead>
<tr>
<th>Log(Inhibitor) vs. Normalized response Variable slope</th>
<th>BCNU - 48 Hours</th>
<th>BCNU - 6 Days</th>
<th>TMZ - 48 hours</th>
<th>TMZ - 6 days</th>
<th>Gefitinib - 48 hours</th>
<th>Gefitinib - 6 Days</th>
</tr>
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<tbody>
<tr>
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<td>1.631</td>
<td>2.822</td>
<td>1.345</td>
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<td>-2.619</td>
<td>-0.9583</td>
<td>-1.011</td>
<td>-3.178</td>
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</tr>
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<td>0.1934</td>
<td>0.1144</td>
<td>0.161</td>
<td>0.7887</td>
<td>0.6382</td>
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<tr>
<td><strong>95% Confidence Intervals</strong></td>
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</tr>
<tr>
<td>LOGIC50</td>
<td>2.747 to 2.859</td>
<td>1.928 to 1.986</td>
<td>3.013 to 3.354</td>
<td>1.482 to 1.781</td>
<td>2.748 to 2.896</td>
<td>1.240 to 1.450</td>
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<td>-1.186 to -0.7310</td>
<td>-1.335 to -0.6867</td>
<td>-4.746 to -1.611</td>
<td>-3.772 to -1.204</td>
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<tr>
<td>IC50</td>
<td>558.0 to 722.6</td>
<td>84.76 to 96.78</td>
<td>1031 to 2259</td>
<td>30.32 to 60.40</td>
<td>559.8 to 786.8</td>
<td>17.37 to 28.18</td>
</tr>
<tr>
<td><strong>Goodness of Fit</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Degrees of Freedom</td>
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<td>98</td>
<td>48</td>
<td>97</td>
<td>48</td>
</tr>
<tr>
<td>R²</td>
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<td>0.7541</td>
<td>0.5897</td>
<td>0.8449</td>
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<tr>
<td>Absolute Sum of Squares</td>
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<td>2976</td>
<td>16937</td>
<td>4973</td>
<td>20412</td>
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<td>Sy.x</td>
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<td>5.511</td>
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Table 2.2 Statistical analyses of other medicinal compounds over a 48hour period.

<table>
<thead>
<tr>
<th>Log(inhibitor) vs. Normalized response Variable slope</th>
<th>Cisplatin - 48 Hours</th>
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<tbody>
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<td><strong>Best-fit values</strong></td>
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<td>LOGIC50</td>
<td>0.9627</td>
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<td>HILLSLOPE</td>
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<td>IC50</td>
<td>9.176</td>
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<td>Std. Error</td>
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<td>LOGIC50</td>
<td>0.01704</td>
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<tr>
<td>HILLSLOPE</td>
<td>0.02336</td>
</tr>
<tr>
<td><strong>95% Confidence Intervals</strong></td>
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</tr>
<tr>
<td>LOGIC50</td>
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<td>HILLSLOPE</td>
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<td>IC50</td>
<td>8.486 to 9.922</td>
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<td><strong>Goodness of Fit</strong></td>
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<tr>
<td>Degrees of Freedom</td>
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<td>R²</td>
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<tr>
<td>Absolute Sum of Squares</td>
<td>2067</td>
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<tr>
<td>Sx x</td>
<td>4.99</td>
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<tr>
<td>Outliers (nct excluded, Q=1.0%)</td>
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</tr>
</tbody>
</table>
2.3.3 Extraction and purification of Ursolic Acid from Cranberries by HPLC

Figure 2.3 (A) demonstrates the potential of UA as anti-tumour compound and possible chemoprevention agent using both commercially available chemically synthesised UA and UA enriched fraction isolated from cranberries. Indigenous UA was sufficiently extracted from fresh whole lyophilized cranberry fruits using a solvent extraction method with soxhlet apparatus and ethyl acetate (EtAOc) solvent. In order to quantify the amount of UA present in an extracted sample of lyophilized cranberry fruits, Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) was performed on both UA standard and cranberry extract, the method was based on a similar method as described by (Zacchigna et al., 2009). The IC$_{50}$ value for the enhanced UA extract was determined to be 144µM. The enhanced UA extract, results in cell death at concentrations greater than 100µM. Interestingly, the synthetic ursolic acid has a lower IC$_{50}$ value 21.72 µM. It is thought that this is due either to the impurities that might be still present in the enhanced UA extract or due to functional modifications of UA derived from Cranberries. In order to minimise variability between samples, the synthetic UA standard was used for the remainder of this thesis to investigate the biological effects in GBM cells. As demonstrated in figure 2.3a, there was no significant difference in the IC$_{50}$ value for UA when treated for 12hr, 24hr or 6 days (P <0.05). The enhanced extracted was identified as UA by means of Liquid chromatography-mass Spectrometry (LC-MS) (figure 2.3 (D)). Similar peaks were observed from both the UA standard and enhanced extract by LC-MS as seen in figure 2.3d, the ions observed on the mass spectra at m/z 455.3 for both the UA standard and the enhanced extract represents the deprotonated mass of the intact molecule [M-H]$^-$. The ion at m/z 419 can be attributed to the loss of two water molecules from the intact molecule. The chromatograms also suggest the presence of cis and trans isomers.
Therefore, we have identified the enhanced extract as UA. Note that the UA extraction from cranberries was carried out by a post-doc Jafar Alqudah in DIT, who is an expert in bioactive extraction.
Figure 2.3 Purified Enriched UA from cranberries induces dose dependent cytotoxicity of GBM.

U373MG cells were treated with increasing concentrations (0-200µM) of (A) chemically synthesised UA over a period from 24 hours, 48 hours and 6 days and (B) Enriched UA fraction over 48 hours and analysed by Alamar blue analyses. Cells were
incubated for 48 hours with enriched UA fraction and analysed by Alamar blue cell viability assay. All experiments were repeated minimum in triplicate. Data shown was normalised to the untreated control and are shown as the % mean ± S.E.M. Statistical analysis was performed using non-linear regression analysis. (C) Demonstrates the difference in the cytotoxicity between chemically synthesised UA and enriched UA fraction over a 48 hours’ period. Non-linear regression statistical analysis was carried out using Prism5 statistical software. (D) Shows the LC-MS analysis of purified UA fraction from cranberries (bottom panel) compared to UA standard (top panel)
Table 2.3 Statistical analyses for UA over a 24, 48 hour and 6 day period and UA enriched fraction isolated from cranberries.

<table>
<thead>
<tr>
<th>Log(inhibitor) vs. Normalized response Variable slope</th>
<th>UA - 24 Hours</th>
<th>UA - 48 Hours</th>
<th>UA - 6 days</th>
<th>UA enriched fraction</th>
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<tr>
<td><strong>Best-fit values</strong></td>
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</tr>
<tr>
<td>LOGIC50</td>
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<td>~ 1.356</td>
<td>1.246</td>
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<td>IC50</td>
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<td>~ 22.71</td>
<td>17.6</td>
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<tr>
<td><strong>Std. Error</strong></td>
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</tr>
<tr>
<td>LOGIC50</td>
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<td>~ 120.5</td>
<td>846500000</td>
<td>0.02535</td>
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<td>HILLSLOPE</td>
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<td>~ 69549</td>
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<tr>
<td><strong>95% Confidence Intervals</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOGIC50</td>
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<td>(Very wide)</td>
<td>-1.685e+009 to 1.685e+009</td>
<td>2.106 to 2.211</td>
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<td>(Very wide)</td>
<td>-12.36 to -5.903</td>
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</tr>
<tr>
<td>IC50</td>
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<td>(Very wide)</td>
<td>127.7 to 162.5</td>
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<tr>
<td><strong>Goodness of Fit</strong></td>
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<td></td>
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<tr>
<td>Degrees of Freedom</td>
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<td>88</td>
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<tr>
<td>R²</td>
<td>0.9825</td>
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<td>Absolute Sum of Squares</td>
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<td>Sy.x</td>
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<td>Outliers (not excluded, Q=1.0%)</td>
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<td>0</td>
</tr>
</tbody>
</table>
2.3.4 Ursolic Acids demonstrates greater cytotoxicity over conventional chemotherapeutics

We have confirmed that chemically synthesized UA can sufficiently inhibit cell viability in U373MG GBM cells as demonstrated above in (Figure 2.3a). Following this we evaluated the efficacy of UA compared to the current standard chemotherapeutic drugs used for the treatment of GBM. There was little reduction in cell viability following treatment with the chemotherapeutics compound at 48 hours, thus IC$_{50}$ could not accurately be calculated (Figure 2.4a). In contrast, UA demonstrated a significant reduction in cell viability following treatment after 48 hours, with an IC$_{50}$ value of 22 µM. IC$_{50}$ values for the standard chemotherapeutics were calculated after a 6 days exposed time period (TMZ 28 µM, BCNU 79 µM, and Gefitinib 16 µM) (Figure 2.4 b).

Cells were also treated with a vehicle control the highest concentration present in each drug, either 0.2% DMSO or 0.6% H$_2$O. No significant reduction in cell viability was observed in the vehicle controls, calculated using student t-test.

Figure 2.4 Ursolic acid exhibits increased cytotoxicity over conventional chemotherapeutics.

(A, B) U373MG cells were treated with increasing concentrations of UA (0-200µM), TMZ, BCNU, or Gefitinib (0-500µM) for 48 hours (A) or 6 days (B), and analysed using
Alamar blue cell viability assay. Cells were also treated with a vehicle control of 0.2% DMSO or 0.6% H2O. No deleterious effects were observed. Experiments were repeated in triplicate. All experiments were normalised to untreated control. Statistical analysis was carried out using non-linear regression analysis and Two-way ANOVA with Bonferroni post-tests. (*P<0.001).

2.3.5 Optimisation of a panel of apoptotic, JNK inhibitors and autophagy inhibitors

U373MG cells were exposed to a number of commonly employed inhibitors; caspase inhibitor zVAD-fmk, JNK inhibitor SP600125 and ROS inhibitor NAC, and PI3K inhibitor 3-methyladenine (3-MA) in order to determine the molecular mechanism involved surrounding GBM cells death in response to various compounds. Dose response curves were carried out for each inhibitor to determine an appropriate nontoxic concentration to be used in future experiments. As demonstrated in figure 2.5 (A) no significant cytotoxicity was observed even at higher concentrations of NAC. Based on this data, for all future experiments 4mM was employed. It was evident in figure 2.5 (B) that concentrations of SP600125 above 25µM were significantly toxic (P<0.05) reducing cell viability by 33% at 25µM and 50% at 50µM. For all future experiments, a concentration of 12.5µM was used. As observed in figure 2.5 (C), there was no significant cytotoxicity induced from general caspase inhibitor zVAD-fmk, with less a 10% reduction on cell viability. Based on our data and that of the literature using zVAD-fmk with U373MG cells (Kanzawa et al, 2003), for all future experiments 50µM zVAD-fmk was employed. Both the JNK and caspase inhibitor were dissolved in DMSO (sigma Aldrich) and therefore to ensure any cytotoxic effects observed as a result of the inhibitor and not the vehicle control. Therefore, the cells were treated with the high concentration of DMSO present (0.5% DMSO). There were no deleterious
effects observed from the control in either JNK or ZVAD-fmk. In figure 2.5 (D) below cells were exposed to increasing concentrations of 3-MA, a well-known autophagy inhibitor. 3-MA inhibits autophagy by blocking autophagosome formation via the inhibition of class I and class III PI3K. As per the manufactures instructions, the most commonly used concentration used is 5mM, as demonstrated below we observe no evidence of cytotoxicity following treatment with 5mM 3-MA. There appears to be a slight increase in cell viability at 7.5mM but this was not deemed significant and is variability within the pooled data. For all future experiments employing 3-MA, 5mM was be used.

Figure 2.5 Optimisation of ROS, caspase, JNK and PI3K class III Inhibitors.
(A -D) Each inhibitor was made up in full media. U373MG cells were treated with increasing concentrations of either NAC (ROS), SP600125 (JNK), zVAD-fmk (caspase) or 3-MA (autophagy). Following incubation of 48 hours’ cell viability was analysed by Alamar blue analyses. Experiments were repeated in triplicate. Cells were also treated with a vehicle control of 0.2% DMSO or 0.6% H₂O. No deleterious effects were observed. All experiments were normalised to untreated control and expressed as a % of the SEM. Statistical analysis was carried out using One-Way ANOVA with Bonferroni post-test (p<0.05).

Positive controls

Using the statistical data generated for the compounds used in this study, concentrations around the observed IC₅₀ values for each compound were chosen to be exposed to each inhibitor. Cells were pre-treated with each inhibitor for 1 hour, after which were co-treated with drug and inhibitor for 48 hours. As demonstrated below figure 2.6 (A) H₂O₂ was used as positive control for NAC-sensitive cell death, (B) TMZ was used as a positive control for zVAD-fmk-sensitive cell death and (C) Ursolic acid was used as a positive control for SP600125-sensitive cell death. There was no significant loss in cell viability following treatment with vehicle control (0.5%) DMSO. Cold atmospheric plasma (CAP) was used as a positive control for autophagy inhibitor 3-MA. It is evident form figure 2.6 (D) the 3-MA significantly protects against cytotoxicity induced by CAP (P<0.001). It is evident from figure 2.6 (A-D) that each inhibitor has significantly reduced the level of cytotoxicity induced by each compound (P<0.05), similarly the compounds are shown to be nontoxic when added to U373MG cells alone.
Figure 2.6 Optimisation of biochemical inhibitors employed to investigate mechanisms of cell death.

U373MG cells were pre-treated with each inhibitor (A-D) for 1 hour prior to addition of the positive control. Following incubation of 48 hours’ cell viability was analysed by Alamar blue analyses. Experiments were repeated in triplicate. All experiments were normalised to untreated control and expressed as a % of the SEM. Statistical analysis was carried out using One-Way ANOVA with Bonferroni post-test (p<0.05).
2.3.6 Characterisation of cell death induced by a panel of compounds.

A selection of compounds from the panel were chosen for further characterisation of the molecular pathways that are involved in cell death. Recent studies have demonstrated efficacy for the use of a modified form of cisplatin called platin-M (Pt(iv)), a prodrug, which has been shown to successfully cross the BBB by a lipophilic polymeric nanoparticle in a canine glioma model (Feldhaeusser et al., 2015). In additional current therapeutic strategies such as the Gliadel wafer as described previously, this could potentially be loaded with cisplatin and placed in the cavity of the brain post-surgery therefore overcoming the need to cross the BBB. Therefore, we choose to investigate non-conventional GBM therapeutics and assess their efficacy in a GBM model. Using the results obtained from figure 2.5 for each inhibitor and the IC\textsubscript{50} data established in Tables 2.1 and 2.2 TMZ, cisplatin, was exposed to each inhibitor for either 48 hours (cisplatin) or 6 days (TMZ). As demonstrated in figure 2.7 (A) Both zVAD and SP successfully attenuated the effects of TMZ induced cytotoxicity by 17% and 26% respectively (P<0.05). Our data suggests that TMZ follows a caspase dependent, JNK dependent mechanism of cell death, interestingly with cisplatin (B) neither of the two inhibitors were able to attenuate any cytotoxicity induced. There was no significant reduction in cell viability in the inhibitors alone, as observed in the control data in figure (2.5). A significant reduction in cell viability was observed following treatment with cisplatin (48%) alone, therefore the compounds were effective killing the cells. The vehicle control did not produce any deleterious effects. Our data suggests that cisplatin induces cell death by a caspase and JNK independent mechanism.
Figure 2.7 TMZ induces cell death through a caspase and JNK dependent mechanisms.

(A-B) Cells were pre-treated with either zVAD-fmk (50µM) or SP600125 (12.5µM) for 1 hours prior to addition of the either TMZ (26.5µM) for 6 days or cisplatin (10µM) for 48 hours. Cell viability was analysed by Alamar blue analyses. Experiments were repeated in triplicate. DMSO and negative control were carried out as described in 2.3.5. All experiments were normalised to untreated control and expressed as a % of the SEM. Statistical analysis was carried out using One-Way ANOVA with Bonferroni post-test (p<0.05).
2.3.7 Ursolic acids stimulates a reduction in the mitochondrial membrane potential in GBM cells

To investigate whether UA induces apoptosis in U373MG GBM, cells were stained with JC-1 dye and analysed using flow cytometry. Mitochondrial membrane potential (ΔΨm) is an important factor of mitochondrial function and can be an indicator of early intrinsic apoptosis. Collapse of the ΔΨm results in the release of cytochrome C into the cytosol and thus leading to cell death (Salido et al, 2007). Figure 2.8 demonstrates the loss in Mitochondrial Membrane Potential (ΔΨm) following treatment with UA. The voltage sensitive fluorescent probe JC-1 that is sensitive to changes in ΔΨm was used to determine whether UA could induce mitochondrial membrane depolarisation in GBM cells. U373MG cells show a significant (P < 0.05) loss in mitochondrial function in a dose dependent manner indicating a reduction in cell viability after UA treatment when compared to untreated control. This result correlates with the Alamar blue results and indicate that depolarisation of mitochondria is a feature of cell death induced by UA. The rapid execution of the cellular death programme observed by UA possibly indicates an alternative mechanism of activation of cell death compared with the other chemotherapeutic agents tested.
Figure 2.8 UA induces mitochondrial membrane depolarisation in GBM.

U373MG cells were exposed to increasing concentrations of UA (standard). After a 48hr incubation period cells were loaded with 10µg/ml JC-1 dye and analysed by flow cytometry. Data shown depicts apoptosis measured by quantitative shifts in the ΔΨm (red to green) fluorescence intensity ratio with increasing concentrations of UA. Cells were also treated with a vehicle control of 0.2% DMSO. No deleterious effects were observed. All experiments were repeated minimum in triplicate. Data shown was normalised to the untreated control and are shown as the % mean ± S.E.M. Statistical analysis was carried out using non-linear regression.
2.3.8 Ursolic acid induces JNK dependent cell death

As previously described, UA was successfully isolated from cranberries, and was demonstrated to selectively induce a cytotoxic response in GBM cells. In order to minimise variability between samples, the chemically synthesised UA standard was used for the remainder of the study to investigate the biological effects in GBM cells. The hill slope for UA is high and small changes in concentration around the IC50 value can lead to large changes in viability. Therefore, three concentrations around the established IC_{50} value were used. There was no evidence of any inhibitory effects by the broad spectrum caspase inhibitor zVAD-fmk as seen in figure 2.9 (A), indicating that caspase enzymatic activity may not be playing a role. It was speculated that stress kinase signalling through JNK may be involved. Figure 2.8 (B) demonstrates that the JNK inhibitor SP600125 alleviates the cytotoxic effects induced by UA in U373MG cells. A dose response curve was established for SP600125 and it was determined that concentrations above 12.5μM were cytotoxic, as previously described above. As seen in (B), co-treatment with the JNK-specific inhibitor SP600125 significantly inhibited (P<0.001) cytotoxicity induced by 20μM UA, and the IC_{50} value was also significantly increased (p<0.05). Our results suggest that UA induces caspase independent, JNK dependent cytotoxicity. It is important to note that while we identify JNK dependent cytotoxicity following the use of JNK inhibitor SP600125, there are limitations to the conclusion that can be drawn from this experimental approach due to lack of specificity of this kinase inhibitor (Bubici & Papa, 2014a). In addition, cytotoxicity of the JNK inhibitor must be taken into account also, as previously demonstrated in figure 2.5b, for concentrations greater than 12.5μM, a significant loss in cell viability was observed using the JNK inhibitor alone. In order to fully elucidate and confirm JNK dependent cytotoxicity further analysis would be required in order to identify JNK activation. The
next step would to carry out Western blot which can used to detect expression of specific proteins for example phosphorylated JNK, and total JNK levels, which would confirm its involvement in UA induced cell death.

Figure 2.9 Ursolic acid induces JNK dependent cytotoxicity in GBM cells.

U373MG cells were pre treated with 50µM zVAD-fmk (A) for 1 hr. prior to UA treatment. Cells were then incubated for 48 hrs and analysed by Alamar blue. (B) Cells were pre loaded 12.5 µM SP600125 JNK inhibitor for 1 hour, increasing concentrations of UA were added in the presence of 12.5 µM SP600125 and incubated for 48 hrs. Cells were also treated with a vehicle control of 0.2% DMSO. No deleterious
effects were observed. Cells were then analysed using Alamar blue cell viability assay. Data shown was normalised to the untreated control and are shown as the % mean ± S.E.M. Statistical analysis was carried out using One-Way ANOVA with Bonferroni post test (*P<0.001).

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2.3.9 UA and TMZ Combinational approach

If used in a clinical setting, UA therapy would most likely be used in combination with existing chemotherapeutic regimens such as the current gold standard TMZ. We hypothesised that a combinational approach with existing chemotherapeutic agents could enhance the overall cytotoxicity of TMZ. TMZ is currently used in combination with radiotherapy, which induces unfavourable off target side effects to the patient, with no significant increase in survival. As UA is bioactive naturally available compound, and is consumed regularly through plants and fruits, and therefore, has a high safety profile. As individual agents we have observed that UA demonstrated greater cytotoxicity over shorter period of time then TMZ, more over at significantly lower concentrations then that used for TMZ. To date there has been only one published article that has combined ursolic acid with TMZ, and have demonstrated the combination treatment of TMZ and UA synergistically enhanced cytotoxicity and senescence in TMZ-resistant GBM cells and moreover they observed a reduction in tumour volume by reducing MGMT in vivo, demonstrating that UA down regulates MGMT status in GBM cells. (Zhu et al, 2016). As demonstrated below in figure 2.10 we observed no additive or synergistic effect between low doses of TMZ and UA. We assume the reason for this is that U373MG are TMZ sensitive and therefore UA is not having an effect on MGMT.
Figure 2.10 Combination of UA and low doses of TMZ

U373MG cells were treated with 0-20 µM UA after which 15µM TMZ was added to the wells. Cells were then incubated for 6 days and analysed by Alamar blue. Cells were also treated with a vehicle control of 0.2% DMSO. No deleterious effects were observed. Data shown was normalised to the untreated control and are shown as the % mean ± S.E.M. Statistical analysis was carried out using Two-Way ANOVA with Bonferroni post test (*P<0.001).
2.3.10 UA inhibits GBM cell migration

One of the most important hallmarks of GBM is an invasive behaviour and ability to migrate into surrounding healthy brain tissue. Despite surgical resection, the majority of the time the tumour recurs within 1cm of the surgical site (Demuth & Berens, 2004). An important feature of a potential novel therapeutic for the treatment of GBM is its ability to inhibit migration. A recent report has demonstrated that UA successfully inhibits chemotaxis in polymorphonuclear leukocyte (PMN) using human whole blood (Mawa et al, 2016). The scratch assay allows for the preliminary examination of the effects of TMZ, Gefitinib, BCNU, and UA on the migration of U373MG cells. Cells were treated with each compound, at a sub toxic concentration; so that it would not initiate a cytotoxic response but potentially inhibit a migratory response. Figure 2.11 (A) shows the transformation process when using ImageJ to calculate the size of the scratch. It can be seen from the images in figure 2.11 (B) over the course of 7 hours, untreated cells have migrated into the area of the scratch, therefore significantly reducing the size of the scratch (P<0.05). TMZ demonstrates the greatest capacity to inhibit cell migration of the chemotherapeutic compounds, reducing closure by 34% (P<0.05) compared to untreated control. In comparison to this, there is a significant difference in the size of the scratch in UA treated cells compared to the untreated control (P<0.05) as seen in figure 2.11 (C). Using linear regression analysis, we have identified the slopes for all compounds as being significantly different (P<0.0001). Thus, indicating that the rate of migration has significantly slowed down as a result of exposure to sub toxic concentrations of both the chemotherapeutic compounds and UA, therefore preventing the scratch from closing. It would also appear that UA has a stronger capability to inhibit migration over the current standard chemotherapeutics.
Figure 2.11 UA inhibits GBM migration.
Demonstrates the transformation process using image J software to analyse % size of the scratch over time. (A) U373MG cell were either left untreated, or treated with TMZ (100µM), Gefitinib (25µM), or BCNU (50µM), (DMSO 0.1%). Time dependent closure of the scratch when was then analysed over a 7 hour period. All experiments were performed in triplicate. Image analysis was performed using image J Software. Statistical analysis was carried out using linear regression analysis and two-way ANOVA with Bonferroni post-tests. (*P<0.001). (B) Time dependent closure of the scratch, U373MG cells were left untreated, or treated with 12µM UA for 7 hours. All experiments were performed in triplicate. Image analysis was performed using image J software. Statistical analysis was carried out using linear regression analysis and two-way ANOVA with Bonferroni post-tests. (*P<0.001).
2.3.11 JNK independent inhibition of migration by UA.

As stated previously, there has been much speculation as to the molecular mechanism that induces cell death following UA treatment in vitro. Our results have previously demonstrated that JNK plays a role in the cell death induced by ursolic acid, in addition we have also demonstrated that UA is effective in inhibiting cellular migration in GBM cells. To determine if JNK also played a role in the migration of GBM, cells were treated with UA, SP600125, or both UA and SP600125 (12.5 µM), along with DMSO control at a sub toxic concentration; so that it would not initiate a cytotoxic response but potentially inhibit a migratory response. It is evident from our data (figure 2.12) that treatment with UA has significantly slowed down the rate of migration when compared to the untreated control (P < 0.05). No cytotoxicity or significant difference was observed between DMSO control and untreated cells. Interestingly, we observed a partial inhibition in migration following the use of a JNK inhibitor alone, demonstrating that JNK pathway play a role in GBM cell migration, which correlates this that observed by (Zhou et al, 2012). However, when we combine UA with JNK inhibitor SP600125, it does not enhance the inhibitory effects observed by UA alone. Our results suggest that JNK does not play a role in the migration of U373MG cells in response to UA treatment, but plays a role in alleviation of cell death.
Figure 2.12 Ursolic acid induces JNK independent inhibition of cell migration in GBM cells.

The effects of JNK inhibition using SP600125 on scratch closure when treated with UA. Cells were treated with either, DMSO 0.1%, 12.5µM UA, SP600125 12.5µM, alone and UA/SP combined. Images were taken at the time of the scratch and 7 hours later. There is no significant difference observed between UA and UA combined with SP600125. Image analysis was performed using ImageJ software. Statistical analysis was carried out using One-Way ANOVA with Bonferroni post test. (*P<0.05).
2.3.12 Ursolic acid triggers formation of acidic vesicle formation in GBM cells.

Having ruled out a role for caspases, we explored the hypothesis that UA resulted in activation of the autophagic pathway Phosphatidylinositol 3-kinase PI3K/AKT/mTOR signalling is involved in both the tumorigenesis and invasion of tumour cells. Cells were exposed to autophagy inhibitor 3-methyladenine, and then treated with ursolic acid. As observed below in figure 2.13 (A), 3-MA did not attenuate cytotoxic effects induced by UA. In order to investigate the autophagy pathway was being activated by UA, U373MG were also examined by flow cytometry using Acridine Orange following treatment with UA, which is commonly employed for the detection of acidic vesicular organelles (AVO’s). AVO’s are an important morphological characteristic in the autophagic process. As seen in figure 2.13 (B) there is an increase in the formation of acidic vesicular organelles (AVO’s) as demonstrated by an increase in the red fluorescence signal following UA treatment with AO compared with the untreated cells stained with AO. This data was quantified by the mean fluorescence value and a significant difference was observed between treated and untreated cells (P<0.005, figure 2.13 (C)). Acridine orange fluorescence in cells validates the formation of AVO’S, which is a significant characteristic of the autophagic process suggesting that the autophagy pathway may be activated during UA induced cell death.
Figure 2.13 UA results in the formation of Acidic vesicle organelles.

(A) U373MG cells were pre treated with 5mM 3-MA for 1 hr prior to addition of UA. Cells were then incubated for 48 hrs and analysed by Alamar blue. Data shown was normalised to the untreated control and are shown as the % mean ± S.E.M. Statistical
analysis was carried out using One-Way ANOVA with Bonferroni post test (*P<0.001). (B) U373MG cells were exposed to 25µM UA. After a 48hr incubation period cells were loaded with 1µg/ml AO fluorescent probe and analysed by flow cytometry. Data shown depicts the formation of acidic vesicular organelles measured by quantitative shifts in the FL-2 channel (red fluorescence) intensity ratio following treatment with UA. Data shown was normalised to the untreated control and represented as arbitrary unit’s ± S.E.M. (C) Overlay of U373MG untreated cells with AO (black) with UA (25µM) with AO (red). Quantification of mean fluorescence index and statistical analysis using non parametric t-tests (P<0.05).
2.4 Discussion

Current commonly used chemotherapies used for the treatment of GBM include a mixture of alkylating agents, receptor tyrosine kinase inhibitors, antibiotics, alkaloid agents and combinational therapies. The current standard of care for the treatment of GBM, as previously described, requires a combination of therapies including surgery, radiation therapy and chemotherapy with TMZ. Unfortunately, even with the multitude of therapies available, survival rates have remain stagnant over the past 30 years and for those who receive maximal treatment the 5 year survival rate is <10% (Ostrom et al, 2014). Poor survival statistics and resistance to current treatment modalities, demonstrates a need for the devolvement of novel approaches for the treatment of GBM. Our data has demonstrated that commonly used chemotherapeutics induce a significant cytotoxic insult over a longer 6-day period, in contrast to that of other medicinal compounds, cisplatin and clozapine (see appendix VI), where cytotoxicity was identified after 48 hours. A key recurring issue with using compounds such as cisplatin for the treatment of GBM is the high molecular weight of the compound. Compounds that maintain a high molecular weight are unable to successfully cross the highly selective blood brain barrier unlike that of Clozapine which has a low molecular weight and therefore has no difficulty crossing the blood brain barrier. Although it has been postulated that with a combined therapeutic approach, using radiotherapy which disrupts that BBB therefore allowing passage of larger molecules to the site of the tumour (Agarwal et al, 2013). There have been numerous studies investigating novel delivery strategies to enhance the permeability of the BBB in a non-invasive manner, to deliver therapeutics across the BBB to reach the brain, which have been extensively reviewed by Zhang et al (Zhang et al, 2015), demonstrating efficacy for novel combinational approaches for the treatment of GBM.
There has been considerable interest in the nutraceutical industry over the past 20 years, since the development of the link between nutrition and pharmaceuticals to aid in the prevention or curing of disease. There are numerous phytochemicals derived from food sources that have been used for their potential health benefits, for example folic acid, found in dark green vegetables which are said to prevent neural tube defects and catechins that are present in green tea, are said to be cancer preventive (Oleszek, 2002). Recent studies suggest that cranberries contain health enhancing bioactive ingredients can induce a cytotoxic response in cancer cells (Shanmugam et al, 2013a; Weng et al, 2014) while remaining nontoxic to normal cells, this has also been demonstrated in vivo (Wang et al, 2011). One of those indigenous bioactive ingredients is ursolic acid (UA) demonstrating efficacy as a potential therapy or as a chemo-preventative. To date there have been few reports on the effects UA in GBM cells, It was reported that UA inhibits the endogenous reverse transcriptase (RT) activity GBM cells which was previously shown to be involved in the control of proliferation (Bonaccorsi et al, 2008). More recently a study has presented the anti-tumours activities of UA in GBM cells demonstrating that apoptosis is induced by UA-triggered TGF-b1/miR-21/PDCD4 pathway (Khan et al, 2012). Using validated methods for extraction, purification and identification, our extract sample was confirmed to be the pentacyclic triterpene UA by means of LC-MS. This is thought be due to the isomeric formations of the compounds. Similarly, to others as mentioned previously, our data demonstrates that UA exhibits anti-tumour activity in the U373MG GBM cell line. We have shown that UA has increased cytotoxic activity compared to that of the commonly used chemotherapeutics used for the treatment of GBM. No synergy was observed between UA and TMZ combined. Currently TMZ is administered orally on a daily basis at a dose of 75 mg/m2 throughout external beam radiotherapy. Post radiotherapy, TMZ is then administered at a dose of 150-200 mg/m2 daily
for 5 days every 28 days for maintenance (Hottinger et al, 2014). This level of treatment is both invasive and prolonged. This further validates the efficacy of UA and its potential as a therapeutic. It induces a greater cytotoxic response over a shorter period of time, making it an ideal candidate from a therapeutic point of view. In addition, it has great potential as a chemo preventive as it is readily bioavailable, making it inexpensive and easily accessible.

One of the most important hall marks of GBM is its invasive behaviour and ability to migrate into surrounding healthy brain tissue. Despite surgical resection, tumour recurrence commonly occurs within 1cm of the surgical site (Demuth & Berens, 2004). The distinct characteristic of migration is therefore an important feature of any potential new chemotherapeutic for treatment of GBM is the ability to inhibit migration of any remaining cells post-surgery. In contrast to results observed by (Choi et al, 2014), our data demonstrated significant reduction in cellular migration following treatment with TMZ, this could due to differences in cell lines used and the sensitivity to TMZ. Moreover, we have observed a significant reduction in migration after only 4 hours following treatment with a low dose UA when compared to untreated cells. This finding is novel and echoes a recent report that UA successfully inhibits chemotaxis in polymorphonuclear leukocyte (PMN) using human whole blood (Mawa et al, 2016). We have also identified the role of JNK in promoting GBM cell migration. We observed a significant partial inhibition in GBM migration following the addition of a JNK inhibitor SP600125. Interestingly, when UA was co-treated with JNK inhibitor SP600125 we observed no enhanced inhibition in migration. However, we demonstrate a significant increased inhibition of cell migration following the combination of UA with JNK inhibitor when compared to the JNK inhibitor alone. This demonstrates the efficacy of UA as a potential anti-migratory therapeutic for the treatment of GBM and also confirming the role of JNK in GBM migration.
To date there has been many studies published with aim to developing an understanding of the molecular mechanisms involved in the induction of cell death following treatment with UA *in vitro*. With reports confirming that UA induces a cytotoxic effect on a variety of cancer cells, there have been inconsistencies as to the mechanism in which cells are dying. Various studies have suggested, apoptosis, autophagy and necrosis as the underlying mechanisms, therefore it would appear to be cell line dependent. The more conventional mechanism of cell death, apoptosis was demonstrated in a variety of cancer cells as a result UA treatment, for example apoptosis in gallbladder cancer was observed both *in vitro* and *in vivo* and is thought to be through the intrinsic mitochondrial-mediated apoptosis pathway (Weng *et al*, 2014). UA-mediated caspase activation and apoptosis in human leukaemia cells both *in vivo* and *in vitro* and has been demonstrated to be linked with PKB inactivation and JNK activation (Gao *et al*, 2012). In colon cancer cells UA induced apoptosis by suppressing the EGFR/MAPK pathway (Shan *et al*, 2009).

There are few reports of UA induced cell death in GBM models. One report has demonstrated apoptosis in a different GBM model through increased caspase-3 expression and suppression of the TGF-b1/ miR-21/PDCD4 Pathway (Wang *et al*, 2012b) miR-21 is a protein involved in the regulation of apoptosis and has been shown to be up-regulated in GBM, it is thought to exert anti-apoptotic effects through regulation of target-programmed cell death 4 (PDCD4) (Chan *et al*, 2005; Wang *et al*, 2012b) whereas in contrast more recently necrosis has been demonstrated in DBTRG-05MG GBM cells through rapid mitochondrial permeability transition (MPT) and opening of the MTP pores (Lu *et al*, 2014). Recently a third mechanism of cell death has also been demonstrated in U87MG GBM cells, UA induces autophagy following Ca\(^{2+}\) release from the ER lumen and activation of the AMPK-mTOR kinase signalling cascade (Shen *et al*, 2014).
As observed from figure 2.9b, our data suggests that UA induces caspase independent, JNK dependent cell death. Our data demonstrates a significant inhibition of cell death at a concentration of 20µM but no inhibition was observed a 25 µM. As demonstrated previously in section 2.3.3, the dose response curve for UA demonstrated a very steep hill slope, identifying a narrow concentration range in which the IC₅₀ (~21.5µM) and that anything above this was very cytotoxic. It is postulated that the cytotoxicity induced at the higher concentration of UA was too toxic for the inhibitor to overcome. It was initially hypothesised that where significance was observed at 20 µM was due to an anomaly in the data or an outlier, however this experiment was repeated in triplicate and the same outcome was observed each time indicating that observations were true. This correlates with data observed by (Zhang et al, 2010), who demonstrated that UA induces cell death via activation of JNK in prostate cancer cells. Increasing evidence has shown that UA activates autophagy cancer cells (Leng et al, 2013; Shen et al, 2014) which will be discussed further in chapter three. Similarly, as shown by Xavier et al also demonstrated a caspase independent, JNK dependent cell death by UA in colorectal cancer cells. Interestingly, they also note that apoptosis corresponds to only a small percentage of the total cell death induced by UA (Xavier et al, 2013). As mentioned previously it is important to note the limitations of using a single inhibitor assay to identify the role of JNK in UA induced cell death. SP600125 has been previously shown to can bind to a broad range of protein kinases and inhibit some of them with similar or greater potency than JNK, thus it is important to note that additional kinases may be targets of SP600125, for example, SP600125 displays action against all three JNK proteins (JNK1, JNK2 and JNK3), and at higher concentrations, can also inhibit the closely related ERKs and p38 MAPKs (Bennett et al, 2001; Tanemura et al, 2010; Bubici & Papa, 2014b). To further confirm the role of JNK in UA induced cytotoxicity it would be important
to demonstrate biochemical evidence of JNK activation. Similar to the experimental approach carried out by Xavier et al, such as employing the use of Western blots, as this offers higher specificity and sensitivity over the use of a single inhibitor. It was previously reported that UA inhibits migration and invasion in vitro in human breast cancer cell line, and was observed that the anti-invasive effects of UA were speculated to be through the inhibition of Jun N-terminal kinase, AKT and a reduction of the level of NFκB transcriptional regulator protein therefore leading to down-regulation of metalloproteinase-2 (MMP-2) and u-PA expression (Yeh et al, 2010). It was also observed in C6 glioma cells that UA inhibits MMP-9 resulting in the inhibition of tumour invasion (Huang et al, 2011). Our findings provide evidence that UA is inhibiting/altering the migratory pathways of U373MG cells independent of the JNK pathway. Further analysis is required to determine whether JNK is playing a role in the regulation of MMP proteins in U373MG cells as a result of UA.

Following identification of a caspase independent mechanism, it was postulated that autophagy maybe play a role in U373MG cell death following UA treatment. For the studies using 3-MA we used CAP as a positive control. A studied carried out by GE et al in HeLa cells that LC-3 protein could not be detected during starvation when cells were exposed to 3-MA (Ge et al, 2014). Therefore, another control that could be added to this study is the use of a serum or nutrient starvation as an alternative positive control for 3 MA, as autophagy is naturally induced following nutrient starvation, this would be a good physiological representation of the induction of autophagy. Our data demonstrates that 3-MA did not alleviate the cytotoxic effects of UA. However, this correlates with others who also demonstrated that 3-MA did not inhibit cell death, although it has been demonstrated that other autophagy inhibitors such as Wortmannin and LY294002 augmented the cytotoxic insult induced by UA. Therefore, suggesting autophagy as a mechanism of cell death. It has
been noted previously that 3-MA acts as both an inhibitor of Class I PI3K activity (inhibits autophagy) and Class III PI3K activity (necessary for autophagy), and therefore the effect of 3-MA may not be observable in all cases (Wu et al, 2010). We speculate that this is the case with our data as further studies employing the use of acridine orange by flow cytometry, verified the formation AVOs following UA treatment, which are a morphological characteristic of autophagy. Following UA treatment, we have identified the formation of acidic vesicle organelles using flow cytometry which as described previously are a morphological indicator of autophagic vesicles. It is important to note that this assay cannot identify autophagic vesicles but is used as an indicator and further analysis is required to categorically identify the autophagic pathway. Acridine orange crosses into acidic compartments such as AVO’s and lysosomes and becomes protonated. Therefore, it cannot be ruled out that the shift in fluorescence observed is from the uptake of acridine orange by lysosomes and not acidic vesicle organelles that are associated with autophagy. However, it is also noted that acridine orange stains late autophagic vacuoles, therefore, fluorescence observed could indicate the presence of both lysosomes as well as autolysosomes. Two methods that are commonly employed for the detection of autophagic processes are TEM and the presence of autophagic processes is identification of microtubule-associated protein 1A/1B-light chain 3 LC3, both of which should be employed when trying to identify autophagy. During autophagy, a cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine to form LC3-phosphatidylethanolamine conjugate (LC3-II). LC3-II degraded in the autolysosome. Thus, lysosomal turnover of the autophagosomal marker LC3-II indicates starvation-induced autophagic activity (Tanida et al, 2004), and detecting LC3 by immunoblotting or immunofluorescence has become a reliable method for monitoring autophagy. Another commonly employed reliable approach is the use of TEM.
TEM characterizes autophagy qualitatively, as early stage autophagosomes containing morphologically intact cytosol or organelles or as late stage autolysosomes containing partially degraded cytoplasmic as well as organelle material. However, it is important to note that when classifying autophagy as a mechanism of cell death the NCCD have provided very specific guidelines on how to do so. To identify cell death that is mediated by autophagy, is that it can be suppressed by the inhibition of the autophagic pathway by chemicals (e.g. VPS34) and/or genetic means (e.g., gene knockout/mutation) (Galluzzi et al, 2015). They also express that cases of cell death that exhibit markers of autophagy such as the LC3/Atg8 or an increased degradation of autophagic substrates like sequestosome 1 (SQSTM1), but cannot be blocked by autophagy inhibition should not be classified as autophagic cell death (Galluzzi et al, 2015). Therefore, it is important to specify whether you are just identifying the autophagic process which is most likely acting as a pro-survival mechanism or whether you are identifying autophagy as a mechanism of cell death.

The data observed form this chapter has offered great insight into how GBM cells respond to various compounds and provides significant basis for further experimental work with ursolic acid. A limitation that has been noted in this chapter and perhaps throughout the thesis is not having employed the use of normal non-malignant cell line to use as a comparator against the U373MG GBM cells. When testing compounds and drugs that are potential therapeutic compounds it is essential to identify the therapeutic window. The therapeutic window is a range of doses that induce a therapeutic response without causing any significant adverse effect in patients. Generally, the therapeutic window is a ratio between the minimum effective concentrations (MEC) to the minimum toxic concentration (MTC). By employing the use of a non-malignant cell line combined with the U373MG it would allow for the identification of the maximum effective concentrations and minimum toxic concentration for ursolic acid.
In conclusion we have identified a novel nutraceutical compound that has a greater cytotoxic capacity against GBM cells than the currently used chemotherapeutics for the treatment of brain cancer. We have identified a ROS-dependent, JNK-dependent and caspase dependent mechanism of cell death in the current gold standard chemotherapeutic TMZ that is in line with published data. Interestingly, in contrast to published data we demonstrated that low doses of TMZ inhibits cell migrations in GBM cells. We show that Ursolic acid induces JNK dependent cell death and JNK independent suppression of migration in human Glioblastoma multiforme cells. It is widely accepted that TMZ induces an autophagic mechanism of cell death in GBM cells (Kanzawa et al, 2004a; Natsumeda et al, 2011). Caspase independent cell death is often linked with autophagy (Kroemer & Martin, 2005b; Rieckher & Tavernarakis, 2010). We demonstrated no inhibition of cell death using 3-MA, we have demonstrated evidence of the formation of AVO’s which could indicate the presence of the autophagic process. Further experiments are required in order to fully elucidate the mechanism of cell death that is induced by UA, and whether autophagy is activated, which if it is the case could be acting in a pro survival mechanism preceding cell death or cell death with autophagy.
3 Cold Atmospheric Plasma induces ROS-independent cell death in U373MG Glioma cells & demonstrates both synergistic and inhibitory effects when combined with medicinal compounds.

Part of this chapter has been published (see Conway GE et al., Br J Cancer, 2016)
3.1 Introduction

Over the past decade plasma science has emerged as a novel approach for applications in areas such as food sterilization, medical devices, polymer science and biomedicine (von Woedtke et al, 2013; Ziuzina et al, 2014). Physical plasmas are fully or partially ionized gases which contain various concentrations of free electrical charges, atoms, ions and electrons which are generated by an energy supply to a neutral gas (von Woedtke et al, 2013). Plasmas can be categorised as either thermal or non-thermal. Non-thermal plasma (NTP) gains its reactivity from the high energy electrons while the ions and neutral species remain at a low temperature (von Woedtke et al, 2013). NTP’s attained at low or atmospheric pressure, have low power requirements and are also able to generate chemically active species within gases such as reactive oxygen species (ROS) (O₂⁺, O, and O₃, OH⁺, H₂O₂) and reactive nitrogen species (RNS) (NO₃, N⁺₂, NO). The operating principle of the plasma discharge system used is Dielectric Barrier Discharge (DBD), as seen in figure 3.1 below.

![Dielectric barrier discharge (DBD) system.](image)

Figure 3.1 Dielectric barrier discharge (DBD) system.
Schematic of the Cold Atmospheric Plasma Dielectric Barrier (CAP-DBD) system utilised in this study. The sample is placed in between two electrodes, where both reactive oxygen and reactive nitrogen species are generated.

The DBD generates high voltage pulsed cold plasma between two electrodes, one of which is insulated. The insulator prevents the build-up of current between the electrodes and any significant gas heating (Fridman et al., 2006). CAP can effectively control the concentrations of intracellular ROS/RNS. CAP generated NO species results in the elevation of intracellular NO concentration which therefore leads to increase of intracellular ROS (Vandamme et al., 2012; Ishaq et al., 2014a).

ROS are naturally present in the body and at low concentrations mediate many biological functions including cellular redox signalling and high concentrations of ROS are utilised as a cytotoxic mechanism by innate immune cells (Ahn et al., 2011). In order to prevent cellular damage and apoptosis due to the high reactivity of ROS, the cell contains antioxidant enzymes such as glutathione peroxidise which act as scavengers to detoxify ROS (King & Robins, 2006). Over the years it has become increasingly apparent that the generation of ROS can be utilised therapeutically for the treatment of cancer (Curtin et al., 2002). A recurring issue with the current chemotherapeutics used in the treatment for GBM is the adverse side effects induced due to the high toxicity of the drugs, and developed resistance to the therapeutic over time.

Recent studies have shown that high concentrations of ROS produced from CAP exposure can generate a cytotoxic effect and induce apoptosis of cancer cells via disruption of the mitochondrial membrane (Fridman et al., 2008; Ahn et al., 2011; Vandamme et al., 2012; Köritzer et al., 2013). An important feature that has been demonstrated in previous studies, is that CAP not only has the ability to induce cell death in GBM cells but also has a much
lower cytotoxic effect on normal astrocytes (Recek et al, 2015; Siu et al, 2015). While there is vast potential for the use of CAP as both a single agent and as a combinational therapy in the treatment of cancers, experimental evidence supporting this application in GBM remains to be carried out.

The aim of this chapter is to generate and optimise the CAP DBD for treatment with mammalian cells. The anti-tumour effects of CAP using our DBD system in vitro on U373MG GBM cells and the cervical cancer cell line HeLa will be evaluated. The molecular mechanism involved that result in cell death following CAP treatment will be investigated, and the combinational effect between CAP and a panel of medicinal and nutraceutical compounds will be determined.

3.2 Materials & methods

3.2.1 Routine cell culture, maintenance and sub culture

The human glioblastoma cell line (U373MG-CD14) was kindly gifted from Dr Michael Carty (Trinity College Dublin) and was the main cell line used in this study. Also employed for part of this study was the human cervical cancer (HeLa, ATCC® CCL-2™) cell line which was purchased from American Type Culture Collection (LGC Standards, Middlesex, UK) and cultured as previously described in chapter two (section 2.1.1).

3.2.2 Non-thermal atmospheric plasma Dielectric barrier discharge (CAP-DBD) set-up

The CAP-DBD device used is a novel prototype atmospheric low temperature plasma generator (Ziuzina et al, 2013). The system consists of a variable high voltage transformer
with an input voltage of 230 V at 50 Hz and a maximum high voltage output of 120 kV. The two 15-cm-diameter aluminium disc electrodes were separated by a polypropylene sheet which served both as a sample holder and as a dielectric barrier with a thickness of 1-2 mm. The distance between the two electrodes was 14.2 mm - 26.6 mm depending on the size of culture plate used. Voltage was monitored using an InfiniVision 2000 X-Series Oscillo-scope (Agilent Technologies Inc., Santa Clara, CA, USA). The atmospheric air condition at the time of treatment was 45% relative humidity (RH) and 22 °C. The samples were treated at 75 kV for 0 – 300 seconds. Optical emission spectroscopy (OES) readings were taken between the range of 200-850 nm using the Low Straylight Smart CCD Exemplar LS spectrophotometer (BRC115U), with a 3,000 m/s integration time. Data was analysed using BWSpec4 and OriginPro 8.

*Optimisation of CAP-DBD experimentation with mammalian cells.*

U373mg cells were seeded at a density of 1x10^4 and HeLa cells were seeded at 0.8x10^4 into 96 well plates (Sigma-Aldrich, Arklow, Ireland) with 100µl media per well. Plates were left overnight in the incubator at 37°C with 5% CO₂ to allow the cells to adhere. Dose response curves were established with both cells lines, with exposure time being the variable. Cell were also exposed to CAP both in the presence and absence of cell culture media, to investigate the most appropriate treatment conditions. Media was removed from the wells prior to CAP treatment and fresh media was replaced immediately after treatment and incubated at 37°C as indicated. No deleterious effects were observed in the vehicle control samples. The 96 well plates were also placed both in and out of the CAP field. In field studies required the 96 well plates being placed directly between the 2 electrodes. Out of field (indirect) studies were carried out by placing the 96 well plates inside a plastic sample...
container which allowed for the samples to be placed out of the plasma field, these are denoted as direct and indirect treatment of CAP.

### 3.2.3 Cytotoxicity assays

**Alamar blue Assay**

Alamar blue (Invitrogen) assay was used to investigate cell viability when performing the cytotoxicity and proliferation studies in both cell lines as described previously in chapter 2 (section 2.3.3). The data (in fluorescence units from the microplate reader) for the test wells were normalised to the assay control (DMEM or RPMI medium only) and cell growth was calculated as a change of viability over time.

**Trypan Blue assay**

The Trypan Blue (Biosciences, Dun Laoghaire, Ireland) cell dye exclusion assay was also performed 48 hrs post treatment in HeLa cells and 96 hrs post treatment in U373MG cells. Both floating and trypsinised cells were collected and a 1x10^6 cells/ml cell suspension was prepared. A 1:1 mixture of cell suspension and 0.4% trypan blue solution was loaded onto a haemocytometer for counting.

### 3.2.4 JC-1 apoptosis Assay

After CAP treatment, cells were harvested and stained with 10 µg/ml JC-1 dye (Biosciences, Dublin, Ireland) (Galluzzi et al, 2007), at room temperature for 10 minutes and analysed by flow cytometry (BD Accuri C6). JC-1 was excited using the argon laser at a wavelength of 488 nm. Fluorescence was measured using the FL1 (530 nm) and FL2 (585 nm) channels with emission spectral overlap compensation (7.5% FL1/FL2 and 15% FL2/ FL1).
3.2.5 Spectrophotometric identification of reactive oxygen species (ROS).

ROS generation was determined using the cell permeable non-fluorescent probe H$_2$DCFDA (Biosciences, Dublin, Ireland) (Wu & Yotnda, 2011). A stock solution of 100µM H$_2$DCFDA was made up in DMSO, aliquot out and stored in -20°C freezer. Working solutions of 50µM were made up fresh for each experiment in full media. Cells were plated in black flat bottomed 96 well plates (Fischer Scientific, Ballycoolin, Ireland) overnight. Cells were then preloaded with 10 µM H$_2$DCFDA and placed in the incubator for 1 hr in the dark. Following the incubation period cells were washed once with PBS. The wells of the 96 well plates were emptied using a pipetting following PBS wash and exposed to CAP for 180 seconds at 75kV or treated with H$_2$O$_2$ where indicated. After CAP treatment media was added to the wells and fluorescence intensity was measured 1 hour later on a micro-plate reader (Victor 3V) at 492–495/517–527 nm.

3.2.6 Confocal microscopy

Identification of ROS by confocal microscopy.

Both U373MG and HeLa cells were plated in 35 mm glass bottom dishes (MatTek Corporation, USA) at a density of 5x10$^4$ and 8x10$^4$ cells per dish respectively. Cells were left to adhere for 24 hrs in a 37°C incubator. Cells were then preloaded with 10µM H$_2$DCFDA and treated as stated above, cells were then analysed 1-hour post treatment using confocal microscopy (ZEISS LSM 510 Meta). Images were captured using an excitation wavelength 488nm and emission wavelength of 515nm with a 40x objective.
Mitochondrial ROS production by confocal microscopy

Stock solutions of MitoSOX (Biosciences, Dublin, Ireland) were made up as per manufactures instruction. The contents of one vial (50µg) was dissolved in 13µL of DMSO to make a 5mM stock solution which was aliquoted and stored at -20°C. A 2µM working solution were made fresh for each experiment in full media. Both U373MG and HeLa cells were plated in 35 mm glass bottom dishes (MatTek Corporation, USA) at a density of 5x10^4 and 8x10^4 cells per dish respectively. Cells were left to adhere for 24 hrs in a 37°C incubator. Cells were loaded with 2µM MitoSOX 1-hour post treatment. Cells were incubated for 10 minutes at 37°C and analysed by confocal microscopy (ZEISS LSM 510 Meta). Images were captured using an excitation wavelength 510nm and emission wavelength of 580nm with a 40x objective.

3.2.7 Identification of ROS by confocal microscopy.

ROS was also quantified by flow cytometry using H$_2$DCFDA probe. Cells were stained with 0.1µM H$_2$DCFDA for 30 minutes prior to CAP treatment. 1-hour post treatment cells were harvested by means of trypsinisation, washed twice in PBS and analysed by flow cytometry. Fluorescence was measured using the FL1 channel (530 nm).

3.2.8 Inhibitor studies

Commonly employed general caspase inhibitor zVAD-fmk was used in order to determine whether caspases play a role in cell death as a result of CAP treatment. A working solution of zVAD-fmk was prepared in full media from previously prepared stored stock solutions as described in chapter 2 (sec 2.3.4.1). Cells were pre-treated for 1 hour with zVAD-fmk. Wells were then emptied and exposed CAP. Cell viability was assessed 48 hours later using Alamar Blue cell viability assay.
N-acetyl-cysteine (NAC) a ROS inhibitor was used to identify whether ROS was involved in cell death following CAP treatment. As described in chapter 2 (sec 2.3.4.2) 4mM NAC was made up fresh for each experiment in full media. Cells were preloaded with NAC for 1 hour. Wells were then emptied and exposed to CAP and NAC, fresh NAC was added immediately after treatment. Cell viability was assessed 48 hours later using Alamar Blue cell viability assay.

SP600125 is inhibitor of JNK which is commonly associated with apoptosis. Cells were pre-treated with SP600125 (Sigma Aldrich, Arklow, Ireland) and also re-added immediately after CAP treatment. Cell viability was assessed 48 hours later using Alamar Blue cell viability assay.

3.2.9 Combinational Studies

*Combinational studies employing short durations of CAP.*

Cells were seeded into 96 well plates as previously described in chapter one, and left to adhere overnight. Media was then removed from each well and exposed to between 10-60 secs CAP. Following CAP treatment cells were treated with a low medium and high dose of either TMZ (0, 5, 10, 20µM), clozapine (6.25, 12.5, 50 µM), cisplatin (1.5, 3.1, 6.25, 25 µM), or UA (6.25, 12.5, 25 µM). Cells treated with TMZ were analysed after 6 days, cells treated with cisplatin, clozapine or UA were analysed by Alamar blue analysis after 48 hours. No deleterious effects were observed from vehicle DMSO control of 0.1%.

*Direct exposure of drug to CAP.*

Cells were seeded into 96 well plates as previously described in chapter one, and left to adhere overnight. Stock concentrations of TMZ (50mM, DMSO), clozapine (50mM, DMSO),
UA (40mM, DMSO), Cisplatin (2mM, PBS), were directly exposed to CAP at 75kV for 15 minutes. Immediately after CAP treatment, working stocks of each compound were made up in full media and added to untreated cells. As a negative control, cells were also treated with the same concentrations of each compound that was not exposed to CAP. No deleterious effects were observed from vehicle DMSO control of 0.25%

3.2.10 Statistical Analysis

All experiments were performed at least three independent times with a minimum of five replicates per experiment. Data shown is pooled and presented as mean ± SEM (n= total number of replicates) unless stated otherwise. Statistical analysis and curve fitting was performed using Prism 5, GraphPad Software, Inc. (USA), as previously described in chapter 2. Unless otherwise indicated differences were considered significant with a p value < 0.05. Correlation graphics were attained using R statistical software (Lucent Technologies) (See appendix (III-V)).

3.3 Results

3.3.1 CAP induces cell death in cancer cells.

As demonstrated in figure 3.2 both (A) U373MG cells and (B) HeLa cells were exposed to CAP in the presence of culture media and analysed after 48 and 72 hours, to determine the cytotoxic effect over time. As observed U373MG cells demonstrated increased capacity to withstand CAP treatment compared to that observed by HeLa cells. H$_2$O$_2$ was used as a positive control for both cells lines which induced an effective 100% cell death. Interestingly in the U373MG cells we observed a significant (P<0.05) difference in cell viability between
48 and 72 hours, this could be due to variability in cell amongst plates, or the cells might be undergoing the repair process and therefore a small increase in viability was observed. All future experiments carried out were over a 24 or 48-hour period. Figure 3.2 (C and D) shows a dose response curve of CAP treatment in both in the presence and absence of cell culture media. Cells were either treated in the presence of media or media was removed prior to CAP treatment. Media was also removed on the control plate for matched observations, no deleterious effects were detected. There is a significant (p<0.05) reduction in cell viability in response to CAP treatment in the U373MG (C) when the media is removed prior to treatment compared to when media was present. It is evident that the Hela cells (D) are highly susceptible to CAP treatment, showing 100% reduction in cell viability after 10 sec of treatment when media was removed prior to treatment. No significance was observed between media and no media, following statistical analysis by means of Two-way AONVA with Bonferroni post-test.
Figure 3.2 U373MG cells demonstrated increased resistance to CAP over time.

(A) U373MG cells and (B) HeLa cells were exposed to CAP for 120 seconds in the presence of cell culture media. Cell viability was then analysed using Alamar assay at different time points. Experiments were completed a minimum of three time. Statistical analysis was carried out using Two-Way ANOVA with Bonferroni comparison post-test. (*P <0.01). (C)U373MG and (D) HeLa were exposed to CAP for up to 180 seconds at 75kV in 96 well plates in the presence and absence of 100 µl cell culture media. 48 hours post treatment cell viability was analysed by Alamar blue analyses. All experiments were repeated in triplicate with n=5 per experiment. Statistical analysis was carried out using Two-Way ANOVA with Bonferroni comparison post-test. (*P <0.05).
3.3.2 Antioxidants only afford partial protection against cold atmospheric plasma (CAP) cytotoxicity.

Upon investigation it was noted that the cell culture media used for the U373MG cells, DMEM high glucose contained pyruvate, a well-known reactive oxygen species scavenger. As seen in figure 3.3 (A), there is a correlation between increasing concentrations of pyruvate with increases in cell viability. There is a significant difference (P<0.001) in cell viability between CAP treatment and no CAP treatment at each concentration pyruvate. As demonstrated (B), following CAP treatment, pyruvate is absent from the media. There is only 8% cell viability compared to when there is 100% pyruvate present, were we demonstrate a significant (P<0.001) increase in cell viability by 35%. Therefore it is evident that pyruvate appears to have some capacity to protect against the effects of CAP induced cell death. Interestingly when the cells were also pre-treated with NAC ROS scavenger in media containing 100% pyruvate (normal full media), there was no additional protection against cell death. Therefore, indicating that CAP induced cytotoxicity in GBM cells is only partially ROS dependent and that other signalling pathways may be playing an important role in cell death.

It was concluded that for all further studies the experimental conditions for CAP treatment would be as following unless otherwise stated; media would be removed prior to CAP treatment and fresh media (with pyruvate) added immediately after treatment in order to simulate similar conditions observed during surgery. All CAP treatments are carried out at 75kV with exposure time and post treatment time as the variables.
Figure 3.3 CAP treatment in the presence and absence of pyruvate in cell culture media.

(A) U373MG cells were exposed to CAP for 180 seconds at 75kV both in the presence and absence of cell culture media containing increasing concentrations of pyruvate. 48 hours post treatment cell viability was analysed by Alamar blue analyses. All experiments were repeated in triplicate with n=5 per experiment. Statistical analysis was carried out using Two-Way ANOVA with Bonferroni comparison post-test comparing CAP treated and no CAP treated samples (*P<0.001) (B) CAP treated U373MG cells with increasing concentrations of pyruvate were compared to CAP treated U373MG cells in the presence of pyruvate and 4mM NAC. Statistical analysis was carried out on CAP treated samples with increasing concentrations of pyruvate and NAC by means of One-way ANOVA with Bonferroni post-test (*P<0.001).

3.3.3 GBM cells demonstrated increased resistance to CAP treatment

Previous studies have suggested that CAP has an antitumor effect in vitro in cell lines such as cervical, colorectal, lung cancer and glioma (Ahn et al, 2011; Köritzer et al, 2013; Cheng et
Despite the wide range of different CAP generating devices being used worldwide, evidence is emerging that cell-type specific resistance can be observed in tumour cells and GBM cells (and other brain-derived cells) appear to be relatively resistant to CAP. We compared the sensitivity of U373MG GBM cells to a commonly used human cervical carcinoma cell line (HeLa) (Ahn et al, 2011, 2014; Kim et al, 2014). Figure 3.4 depicts representative kinetic cytotoxicity results obtained when GBM cells are exposed to CAP for 180 seconds at 75kV. Inhibition of normal metabolic processes in GBM cells by CAP treatment was almost immediate, it was observed that only 4 hours post CAP treatment there was a significant reduction in cell viability compared to untreated controls, and after 24 hours almost 80% loss in cell viability was observed. Dose response curves for both U373MG and HeLa were also established in a dose dependent manner by first exposing cells to CAP for between 3 and 300 seconds at 75kV, as seen in (B). Cell viability was quantified 48 hours post treatment. We confirmed findings by Ahn et al, 2014 that HeLa cells are sensitive to CAP using our system, the IC\textsubscript{50} value was determined to be 4.8 seconds (95% confidence range of 4.2 - 5.6 seconds). As observed U373MG GBM cells, exhibited a significant increase in resistance to CAP with an IC\textsubscript{50} of 74.26 seconds (95% confidence range of 47.24 - 116.8 seconds). A comparison of fit demonstrated a significant difference in the IC\textsubscript{50} values (P < 0.05) between both the U373MG and HeLa as a result of CAP treatment and significant differences of the dataset were confirmed using a correlation analysis (see appendix III). The cytotoxic response was confirmed using the trypan blue cell membrane integrity assay and U373MG cells demonstrate a significantly (P<0.001) higher resistance to the CAP treatment than that observed by the HeLa cells after 180sec treatment as demonstrated in (C).
We have previously demonstrated the increased resistance observed by U373MG GBM cells following CAP treatment both the presence and absence of antioxidant pyruvate and also with extended exposures to CAP (5 minutes). This correlates with the current literature on the increase resistance observed in GBM tumour that they resist most treatment modalities and have high reoccurrence rates. If used in a clinical setting, CAP or any variant of this technological therapy would most likely be used multiple times and in combination with existing chemotherapeutic regimens. We hypothesised that multiple treatments and/or combinational therapy with existing chemotherapeutic agents, would be sufficient to overcome the relative resistance of U373MG cells to CAP, for example TMZ combined with low doses of CAP (which is demonstrated later in this chapter). As demonstrated in (D), triplicate CAP exposures of 75kV for 180 seconds results in a significant reduction in U373MG cell viability compared to a single treatment (5.669 - 43.16 %, P < 0.05).
Figure 3.4 CAP induces a dose dependent cytotoxic effect in both U373MG cells and HeLa cells

(A) U373MG cells were exposed to CAP at 75kV for 180 seconds and analysed by Alamar blue cell viability assay at 1, 4, 8, 21.5, 33.5, 45.5 hours post CAP treatment. All experiments were repeated a minimum of three times. Each time point was normalised to an untreated control that was left under the same conditions as the CAP treated plate. (B) Cells were exposed to CAP at 75kV for up to 5 minutes. 48 hours later the cells were analysed using the Alamar Blue cell viability assay. (C) Both U373MG cells and HeLa cells were exposed to CAP at 75kV for up to 180 seconds. HeLa cells were trypsinised and counted using a haemocytometer 48 hours post treatment. U373MG cells were analysed 96 hours post treatment. (D) The effects of multiple CAP exposures were determined in both HeLa and U373MG. Cells were exposed 3 times within a 10 hr. period with a minimum 4 hours between...
treatments. 48 hours later cells were then analysed using the Alamar blue assay. Results are shown as the mean ± S.E.M. (n= minimum 24). Statistical analysis was carried out using One-Way ANOVA with Tukey’s multiple comparison post-test, (*P <0.05 against untreated control).

3.3.4 Loss in Mitochondrial membrane potential following CAP treatment.

Mitochondrial membrane potential (ΔΨm) is an important indicator of mitochondrial function and can also be an indicator of early intrinsic apoptosis. A distinctive feature of the early stages of programmed cell death is the disruption of active mitochondria. This mitochondrial disruption includes changes in the membrane potential and alterations to the oxidation-reduction potential of the mitochondria. Collapse of the ΔΨm results in the release of cytochrome C into the cytosol and thus leading to cell death (Salido et al, 2007). In healthy cells with high ΔΨm, JC-1 forms aggregates with intense red fluorescence. However, when the ΔΨm has been disrupted the JC-1 remains in its monomeric form and fluoresces green. Figure 3.5 (A) demonstrates the loss in Mitochondrial Membrane Potential (ΔΨm) due to NTAP treatment. Both cell lines show a significant (P < 0.05) loss in mitochondrial function indicating a reduction in cell viability after CAP treatment when compared to untreated controls (B). This result correlates with both the Alamar blue and trypan blue results indicating that NTAP induces differential cytotoxicity in HeLa and U373MG cells.
Figure 3.5 CAP induces mitochondrial membrane depolarisation in HeLa and U373MG cells.

Both cell lines were exposed to CAP at 75kV for 3mins. (A) After a 48hr incubation period cells were loaded with 1µg/ml JC-1 dye and analysed by flow cytometry. (B) Data shown depicts apoptosis measured by quantitative shifts in the ΔΨm (red to green) fluorescence intensity ratio before and after H₂O₂ (1Mm – U373MG, 200µM- HeLa) and CAP exposure (60 sec). All experiments were repeated triplicate. Statistical analysis was carried out using One-Way ANOVA with Tukey’s multiple comparison post-test. (*P <0.05).
3.3.5 CAP induces mitochondrial ROS formation in cancer cells *in situ*

Recent literature using HeLa and GBM cell lines has stated that CAP generated ROS causes DNA damage resulting in apoptosis (Vandamme *et al.*, 2012; Cheng *et al.*, 2014). It is also well known that mitochondrial depolarisation can be a result of oxidative stress produced by ROS (Ahn *et al.*, 2011). In order to determine that the cytotoxicity observed was from ROS generated by CAP, both cell lines were preloaded with the cell permeable ROS-sensitive fluorescence dye, H$_2$DCFDA. ROS generation was first visualised using confocal microscopy and we confirmed that CAP induced ROS-dependent H$_2$DCFDA fluorescence in both cell lines. The level of fluorescence in U373MG cells appeared to be lower than that observed in HeLa cells (figure 3.6 (A)) and this was then confirmed using both spectrophotometry and flow cytometry. As seen in figure 3.6 B, significantly higher levels of ROS fluorescence were observed following CAP treatment in HeLa cells (P < 0.05), compared to untreated controls and to CAP treated U373MG cells (p<0.05).

A similar result was also observed by flow cytometry as demonstrated in (C) CAP treatment demonstrated a significant increase in ROS generation (P<0.001) compared to the untreated control in HeLa cells. A slight dextral shift was observed in U373MG cells but the measured mean fluorescence index was not significantly altered. Following the identification of ROS formation by H$_2$DCFDA, cells were treated with mitochondrial superoxide indicator, MitoSOX red. Mitochondrial ROS production as a result of CAP treatment has been previously demonstrated in HeLa cell by (Ahn *et al.*, 2014). Figure 3.6 (D) demonstrates the increase in intracellular fluorescence of mitochondrial ROS formation by confocal microscopy in both HeLa and U373MG cells compared to the untreated control. The level of fluorescence was quantified using ImageJ (v1.49, NIH) software in both treated and untreated cells.
(McCloy et al, 2014). There is a significant increase in mitochondrial ROS after CAP treatment observed in both U373MG (P<0.05) and HeLa cells (P<0.001) compared to the untreated controls.
Figure 3.6 *In situ* verification of ROS production in both U373MG cells and HeLa cells following CAP treatment.
(A) In situ verification of ROS production in both U373MG cells and HeLa cells was measured 1 hr after CAP exposure (75kV for 180sec) by confocal microscopy using 10µM H$_2$DCFDA. (B) ROS fluorescence intensity was quantified spectrophotometrically, 1hr after CAP exposure (75kV for 180sec). All experiments were repeated in triplicate. Statistical analysis was carried out using One-Way ANOVA with Tukey's multiple comparison post test (*P<0.05). (C) ROS production in both U373MG and HeLa was also measured by flow cytometry using 0.1µM H$_2$DCFDA. Fluorescence was quantified using the mean H$_2$DCFDA fluorescence and compared to the untreated control (P<0.001). (D) In situ verification of mitochondrial ROS production in both U373MG cells and HeLa cells was measured 1 hr after CAP exposure (75kV for 180sec) by confocal microscopy using 2µM MitoSOX red. The level of fluorescence was quantified using image J software and compared to the untreated control. Statistical analysis was carried out using t-test with Mann Whitney test post test with (P<0.05) U373MG and (P<0.001) HeLa cells.

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3.3.6 GBM cells demonstrate higher anti-oxidant activity against H$_2$O$_2$

The production of a variety of ROS by CAP, including H$_2$O$_2$ has been shown previously to induce cytotoxicity (Ahn et al, 2014; Hirst et al, 2015; Xu et al, 2015). These free radicals, particularly the CAP-generated NO species, are believed to result in an increase in intracellular ROS, which in turn signals a cascade of events leading to apoptosis (Ishaq et al, 2014a). As observed from the optical emission spectroscopy (OES) data, figure 3.7 (A), we have identified the presence of OH in our OES measurements, OH is generated as a result of O$_2$ and H$_2$O$_2$ interactions in the plasma field, which as a result induces cell death (Xu et al, 2015). To investigate GBM cells response to specific extracellular reactive oxygen species, we treated U373MG cells with increasing concentrations of H$_2$O$_2$. Interestingly similarly to that observed with CAP treatment, U373MG demonstrated increased resistance to H$_2$O$_2$ compared to HeLa cells. We hypothesised that the increased resistance displayed by U373MG cells to CAP could be replicated using H$_2$O$_2$ as an oxidative stressor. (B) Demonstrates that U373MG cells indeed have an increased tolerance for H$_2$O$_2$ compared to that of HeLa cells. The IC$_{50}$ value for H$_2$O$_2$ cytotoxicity is significantly higher (p<0.0001) for U373MG cells (544.8 µM) compared to HeLa cells (27.91 µM) and significant differences of the dataset were confirmed using a correlation analysis (see appendix IV). The generation of H$_2$O$_2$ was significantly lower in U373MG cells (EC$_{50}$ 458.1µM) compared with HeLa cells (EC$_{50}$ 55.36µM) (C) and significant differences of the dataset were confirmed using a correlation analysis (see appendix IV). The presence of intracellular and mitochondrial ROS was confirmed using MitoSOX red and H$_2$DCFDA for both cell lines using confocal microscopy as demonstrated below in figure 3.7 (D).
Figure 3.7 GBM cells demonstrate a higher anti-oxidant activity against $\text{H}_2\text{O}_2$. 
(A) Using optical emission spectroscopy, chemical species produced by the CAP DBD system were identified. OES measurements were taken between the range of 200-850nm (Exemplar LS). This spectrum demonstrates distinct atomic lines at the wavelengths 280-400nm in the ultraviolet region, these denote strong emissions from various nitrogen ions. Species within the wavelength 300-390 nm are part of the N$_2$ second positive system. Associated nitrogen molecules from the nitrogen second positive system and ionised nitrogen molecules found within the range 390-480 nm are part of the N$_2^+$ first negative system. (B) Both U373MG cells and HeLa cells were treated with increasing concentrations of H$_2$O$_2$ (0-2mM). After 48 hours cell were analysed using the Alamar Blue assay. Statistical analysis was carried out using non linear regression analyses. (C) Both U373MG cells and HeLa cells were preloaded for 1hr with 10µM H$_2$DCFDA. H$_2$DCFDA was removed and a dose-response analysis was performed using H$_2$O$_2$. The relative increase in fluorescence was determined one hour after addition of H$_2$O$_2$. Statistical analyses was carried our using nonlinear regression analyses. (D) Shows In-situ verification of ROS production in both U373MG cells and HeLa cells which was measured 1 hr after the addition of H$_2$O$_2$ by confocal microscopy using both 10µM H$_2$DCFDA and 2µM MitoSOX red. All experiments were repeated minimum in triplicate.
3.3.7 CAP can induce ROS, JNK and Caspase independent cell death in U373MG cells

Previously it has been reported that CAP induces ROS, JNK and caspase activation in cancer cells (Vandamme et al, 2011; Hou et al, 2015; Siu et al, 2015). To confirm the role of intracellular ROS in CAP and H₂O₂ induced apoptosis, HeLa (A) and U373MG cells (B) were loaded with the well-known thiol antioxidant NAC for 48 hours after CAP treatment (figure 3.8). As expected, the addition of NAC to H₂O₂ treated HeLa (A) cells and U373MG cells (B) improved cell viability (p<0.05) indicating that NAC was protecting against ROS induced cell death in both cell lines. While NAC improved cell viability in HeLa cells following treatment of both cells and media with CAP (P < 0.05), there was no protective effect noted for U373MG cells treated with CAP. Therefore, the data strongly suggests that unlike HeLa cells, U373MG cells undergo ROS independent cell death in response to CAP. To further elucidate the molecular mechanism underlying U373MG cell death following CAP treatment, the protective effect of increasing concentrations of the caspase inhibitor, zVAD-fmk and the JNK inhibitor, SP600125 were determined. No significant difference in cell viability was observed with increasing concentrations of either zVAD-fmk (C) or SP600125 (D). Together, our results indicate that CAP induces apoptosis by a ROS-, JNK- and caspase-independent manner in GBM cells.
Figure 3.8 CAP induces ROS, JNK and caspase independant cytotoxicity in GBM cells.

Both HeLa (A) and U373MG (B) cells were preloaded for 1hr with 4mM NAC. Cells were then treated with H₂O₂ or exposed to NTAP. After 48 hours, cells were analysed...
using the Alamar blue assay. Data shown was normalised to the untreated control and are shown as the % mean ± S.E.M. (n= minimum 20). All experiments were repeated at least three times. Statistical analysis was carried out using One-Way ANOVA with Tukeys multiple comparison post test. (*P<0.05). (C) Following CAP treatment cells were loaded with increasing concentrations of SP600125 (0-50μM) inhibitor and incubated for 48 hrs. Black bars represent increasing concentrations of inhibitor alone with no CAP treatment. Dark and light grey bar represents the combination of inhibitor and CAP treatment. The first three bars depict CAP treatment only with no 0μM inhibitor. Cells were then analysed using Alamar blue cell viability assay. (D) U373MG cells were pretreated with increasing concentrations of zVAD-fmk for 1 hr prior to CAP treatment. Cells were then incubated for 48 hrs and analysed by Alamar blue. Black bars represent increasing concentrations of inhibitor alone with no CAP treatment. Dark and light grey bar represents the combination of inhibitor and CAP treatment. The first three bars depict CAP treatment only with no 0μM inhibitor. Data shown was normalised to the untreated control and are shown as the % mean ± S.E.M. (n= minimum 20). All experiments were repeated at least three times.
3.3.8 **A low dose of CAP demonstrates an additive capacity when combined with therapeutic compounds overcoming the relative resistance of U373MG cells.**

If used in a clinical setting, CAP or any variant of this technological therapy would most likely be used multiple times and in combination with existing chemotherapeutic regimens. We hypothesised that a combinational therapy with existing chemotherapeutic agents or novel compounds, would be sufficient to overcome the relative resistance to CAP. We also employed the use of an additional medicinal compound, clozapine, in this study. Clozapine is an antipsychotic drug that has been previously shown to cause increased oxidative stress in cells and tissues and also has been associated with increased production of ROS and antioxidants (Polydoro *et al.*, 2004; Pillai *et al.*, 2007; Miljevic *et al.*, 2010) Therefore our rationale for employing clozapine was as a possible prodrug that could be activated by ROS generated from CAP and enhance the overall cytotoxic effect with low doses of CAP (see appendix VI for optimisation of clozapine). As seen in figure 3.9 (A), low doses (10 µM) of TMZ greatly augmented the cytotoxicity observed due to CAP with toxicity levels approaching 80-100%. Two-way ANOVA confirmed that there is variance in the data due to the combination of TMZ and CAP which cannot be explained by TMZ and CAP alone. This indicates the presence of an additive effect as observed on the graph (A) at 30sec CAP and 10µM TMZ with $P<0.0001$. As observed for clozapine (B), no synergistic or additive effect was observed between low doses of the medicinal compound and CAP. Interestingly the most dramatic effect was observed with cisplatin (C) at 60 sec CAP, with an additive effect observed amongst all concentrations examined, with an average 30% further reduction in cell viability following the combination of these two methods ($P<0.05$). There was no additive or synergy observed between UA and CAP (D).
Figure 3.9 Additive effect observed between TMZ and CAP.

Following CAP treatment cells were treated with low concentrations of either (A) TMZ, (B) clozapine, (C) cisplatin or (D) UA. Cells treated with TMZ were incubated for 6 days’ post and analysed by Alamar blue assay all other compounds were analysed after 48 hours. Data shown was normalised to the untreated control and are shown as the % mean ± S.E.M. Statistical analysis was carried out using both One-Way ANOVA (*P<0.05) and Two-Way ANOVA with Bonferroni post test. (*P<0.001). All experiments were repeated at least three times.
3.3.9 Chemotherapeutic compounds generally highly resistant to degradation by CAP.

A risk when employing new technology as a potential combinational treatment with other therapeutic compounds is that despite evidence of additive effects with some compounds, that the chemical transformation of chemotherapeutic agents by CAP will reduce its cytotoxicity. Therefore, using stock concentrations of TMZ (50mM, DMSO), clozapine (50mM, DMSO), UA (40mM, DMSO), Cisplatin (2mM, PBS), the compounds were exposed CAP at 75kV for 15 minutes, in advance of adding to GBM cells. As demonstrated in figure 3.10 (A) below there is no significant difference between TMZ that has been directly treated with CAP and untreated drug. Using non-linear regression analyses there was also no significant difference demonstrated in the IC\textsubscript{50} value of the compound post treatment. This demonstrates the stability of the compound if were to be used in a combinational therapeutic approach. However more interestingly an inhibitory effect was observed with Clozapine. It is evident from figure 3.10 B, there is a difference between clozapine that has been exposed to CAP for an extended period of time compared to that which was not exposed to CAP there was a change in the IC\textsubscript{50} value of clozapine from 62µM to 73µM following CAP exposure, although this was calculated to not be significant using nonlinear regression. However, Two-way ANOVA confirmed that there is variance (P<0.001) in the data due to the combination of untreated clozapine and CAP treated clozapine. A significance difference was observed at several concentrations of clozapine as demonstrated below (B) (*P<0.005 - P<0.001). Our data suggests that there is a chemical interaction between CAP and clozapine, as there is a reduction in the cytotoxicity of the drug when directly exposed to the CAP. There was no difference observed in the IC\textsubscript{50} values between CAP treated cisplatin and cisplatin alone (C), although there was a significance
observed at a concentration of 31.5µM (*P<0.05), which was above the calculated IC_{50} of cisplatin (12µM). Similarly, with UA (D) there was little difference observed between the two treatment conditions. At 15.5µM, significance was observed following direct treatment of the compound (*P<0.001). As there was no significant difference between the IC_{50} values for UA and cisplatin following direct treatment, in combination with only one significant value being observed using Two-way ANOVA it is postulated that these values may be anomalies in the data, which could be explained by the combination of triplicate experiment, intra experiment variation.
Figure 3.10 Long term exposure to CAP does not reduce the cytotoxicity of compounds.

Stock concentration of compounds (A) TMZ and (B) clozapine (C) cisplatin and (D) UA were exposed directly to CAP at 75kV for 15 minutes. Compounds were then diluted down in full media and added to the cells. Cells treated with TMZ were incubated for 6 days’ post and analysed by Alamar blue assay clozapine, cisplatin and UA were analysed after 48 hours. Data shown was normalised to the untreated control and are shown as the % mean ± S.E.M. Statistical analysis was carried out using both Two-Way ANOVA, with Bonferroni post-test (*P<0.05- P<0.001) All experiments were repeated at least three times.
3.4 Discussion

GBM’s are very aggressive and are resistant to most chemotherapies and radiation therapy (Curtin et al, 2005). Therefore, it is necessary to develop a novel therapy that can induce cell death in GBM cancer cells. For this report, the effect of CAP was investigated on two in vitro cells lines; HeLa cervical carcinoma cells, which are a well characterised CAP model (Ahn et al, 2011; Panngom et al, 2013; Ma et al, 2014) and the U373MG GBM cells which have yet to be characterised using CAP-DBD. The evolving technology industry has led to the possibility of replacing or complementing existing chemotherapies using technology based approaches. In this regard, there has been great development in the area of plasma medicine over the past decade, with a huge focus on the area of non-thermal atmospheric plasma as a potential therapy for the treatment of cancer. Advances in wearable medical devices have led to the possibility of replacing or complementing existing chemotherapies using a technology-based approach. In accordance with previous studies, the anti-proliferative effect of CAP treatment was confirmed in vitro (Ahn et al, 2011; Vandamme et al, 2012) in both cells lines. Our data demonstrated a significant difference in the survival of U373MG when treated in both the presence and absence of media. Following the removal of media there is a small film of media that covers the cells; this prevents the cells from completely drying out during the course of the treatment and having a harmful effect on the cell viability. Upon further research, it was determined that the antioxidant pyruvate was present in the U373MG cell culture media. Pyruvate is a well characterised ROS scavenger, which we have shown to play a significant role in the survival of U373MG cells following CAP treatment. Similar findings were also observed by others who employed the use of similar media formulations i.e. media that included pyruvate. (Adachi et al, 2015; Boehm et al, 2016). Even with the addition of pyruvate to the
media, our data still showed a significant 50 percent reduction in cell viability following CAP treatment over a 48-hour period, it was then noted that this cytotoxic insult could be further enhanced following multiple treatments, resulting in an overall reduction in cell viability of 68%, therefore overcoming the relative resistance of U373MG cells. When looking from a clinical aspect, it would be envisaged that first application of CAP could be applied following surgical resection and therefore to mimic these conditions i.e. a moist environment where scavengers are plentiful, it was decided that treatment of CAP for U373MG cells would be in the absence of media with media (with pyruvate) added immediately after treatment.

The resistance to H₂O₂ induced apoptosis has been reported previously in GBM cells (Datta et al, 2002a), but to the best of our knowledge, ROS-independent CAP cytotoxicity has yet to be reported and characterised. We demonstrate here that U373MG GBM cells display a significant resistance to cytotoxicity and ROS generation (P<0.0001) when compared with a commonly studied CAP-sensitive cancer cell line HeLa. It has been documented that reactive oxygen species generated by CAP treatment can induce apoptosis in glioma cells as well as many other cancer cells (Vandamme et al, 2012; Ahn et al, 2014; Siu et al, 2015). Our OES measurements confirmed notably larger emissions for nitrogen species and very little emissions at the atomic oxygen region of 777 nm and 845 nm, in agreement with those reported by others using unmodified atmosphere, it is as a result of quenching with N₂ and O₂ (Walsh et al, 2010; Misra et al, 2014). The presence of atomic oxygen is not required for the generation of ROS. CAP generated NO results in intensification of intracellular NO concentration that initiates a cascade of events, subsequently increases intracellular ROS (Vandamme et al, 2012; Ishaq et al, 2014a).
The current standard of care for the treatment of glioma involves repeated exposure to chemotherapy/radiation over a period of weeks/months in order to ensure eradication of the entire tumour. A similar effect was demonstrated with multiple exposures to CAP, a triple exposure over a 10 hr period greatly enhanced cytotoxicity of the U373MG cells, indicating that CAP can be both effective and cumulative against GBM. In the current clinical setting, radiation therapy is used in combination with surgery and chemotherapy to treat GBM. CAP is also based on the formation of intracellular chemically active species. As the technology develops, the primary advantage of CAP over radiotherapy is the ability to generate these species in situ, at the site of the tumour and reduce the resulting systemic side effects (Ishaq et al, 2014a). Moreover, the species generated by CAP are short lived in comparison to the relatively long lived radioactive isotopes introduced during radiation therapy (Stoffels et al, 2008). We were able to confirm the generation of ROS by CAP treatment in HeLa cells as previously reported by (Ahn et al, 2011). ROS generation was also observed in CAP treated U373MG cells (figure 2) but, interestingly, at a significant lower level compared with CAP treated HeLa cells. These results echoed H2O2 induced ROS levels, indicating that U373MG cells have a 5 to 10-fold greater capacity to neutralise ROS compared with HeLa cells.

ROS, JNK and caspase activation have all been reported in cancer cells following CAP treatment (Vandamme et al, 2011; Hou et al, 2015; Siu et al, 2015). We hypothesised that by activating multiple cell death pathways together, specific inhibition of these pathways would not be preventative of cell death after longer treatments of CAP in U373MG cells. To test this hypothesis, we titrated common inhibitors of ROS, JNK and caspases. In line with previous findings (Köritzer et al, 2013), we found that caspase inhibitors do not protect against the effects of CAP treatment. Furthermore, we were unable to protect against cell death using ROS scavengers or JNK inhibitors in
GBM cells. It has been hypothesised that CAP offers great potential as a possible therapy for the treatment of GBM due to the wide variety of biological processes affected and our own data is supporting this hypothesis. Our data suggests that several pathways have been activated simultaneously by the CAP treatment for example autophagy and blocking individual pathways is insufficient to inhibit cell death. To the best of our knowledge, this is the first evidence of a cell line where CAP treatment can induce cell death even in the presence of JNK, caspases and ROS inhibitors. Our data has suggested a caspase independent mechanism based on the use of general caspase inhibitor Z-VAD. However, as stated previously, it has been recently noted by the NCCD that ‘a growing body of evidence indicates that the pharmacologic or genetic inhibition of the processes that are commonly considered as essential for cell death execution often does not avoid the demise of mammalian cells, but rather alters its kinetics and biochemical (and morphologic) manifestations.’ and therefore inhibitors such as Z-VAD administered as therapeutic interventions fail to significantly limit primary regulated cell death, in spite of the fact that they efficiently limit caspase activation (Galluzzi et al, 2015). Therefore, based on our data alone without the use of genetic knock down models or demonstration of caspase activation we cannot rule an apoptotic mechanism of cell death. In contrast to the results observed for HeLa cells, cytotoxicity in U373MG cells following CAP treatment is not alleviated by the presence of NAC. NAC does protect against high concentrations (2mM) of H₂O₂ in U373MG cells. Therefore, CAP is primarily inducing cell death through a H₂O₂ and ROS-independent mechanism in U373MG cells.

As observed our data demonstrates significant mitochondrial membrane depolarisation as a result of cap treatment in both HeLa cells and U373MG cells, also in addition we demonstrated an increase in mitochondrial ROS following CAP treatment, both of
which are classic modifications observed during intrinsic apoptosis. During the early stages of apoptosis, mitochondrial ROS production is stimulated, this results in the release of cytochrome c from the mitochondria, this activates the executioner caspases 9 which activates caspase 3, resulting in the cells demise. As we have demonstrated significant depolarisation of the mitochondria, another step to investigate apoptosis would be to test for cytochrome c translocation from mitochondria into cytosol to further identify apoptosis.

However, we demonstrate that NAC, a ROS inhibitor was unable to alleviate the cytotoxicity induced by CAP, therefore one must also consider the process of mitophagy. Mitophagy is a form of macroautophagy that selectively degrades damaged mitochondria. Double-membraned autophagosomes enclose whole mitochondria, or selectively target the damaged areas. Mitophagy is considered a homeostatic program that maintains a healthy mitochondrial population and plays largely cytoprotective roles in the context of disease pathogenesis (Ding & Yin, 2012), in contrast to this is has also been postulated that mitophagy could act as an effector of cell death programs. For example a recent study has demonstrated ceramide induced cell death required mitophagy, involving LC3-ceramide interactions at the mitochondria (Sentelle et al, 2012). One way to determine if mitophagy is playing a role is to analysis mitochondrial function via PTEN-induced putative kinase 1 (PINK1), which is involved with mitochondrial quality control by identifying damaged mitochondria and targeting specific mitochondria for degradation. PINK1 activity causes the parkin protein to bind to depolarized mitochondria to induce mitophagy (Ding & Yin, 2012). As we observed significant depolarisation of the mitochondria following CAP treatment, it could be that the ROS damage that has been induced by CAP is so significant that inhibitors such as
NAC would be unable to protect against cell death and therefore, mitophagy offers a plausible mechanism of action.

We observed that U373MG cells were relatively resistant to CAP treatment, and so we investigated methods to overcome this. We found that both multiple exposures to CAP, and use of low concentrations of TMZ were sufficient to overcome the relative resistance of U373MG cells to CAP. Both these approaches let to an improved cytotoxicity of between 80% and 95%. Harsh side effects are associated with existing standard of care for patients diagnosed with GBM and despite this; very limited gains in long-term survival are evident. Therefore, lower doses of TMZ when used in combination with CAP may lead to a more effective treatment whilst reducing the harmful side effects. We have also demonstrated that longer direct treatment of different therapeutic compounds with CAP (i.e. 15 minutes) can demonstrate only a marginal inhibitory effect on the cytotoxicity of the compound. We believe that this is a very important finding, especially since CAP is likely to be used in the clinic in combination with existing chemotherapeutic agents. Further analysis of the interactions between CAP and compounds will need to be carried out, using techniques such as LC-MS and NMR, to determine the major chemical changes and rate of degradation of the compound structure following direct exposure.

In conclusion, this chapter demonstrates that CAP induces a significant cytotoxic insult that induces cell death in both U373MG GBM cells and HeLa cervical cancer cells. U373MG cells demonstrate increased resistance to CAP when compared to HeLa cells, which can be overcome when applied with cumulative exposures to CAP. We can conclude that CAP induces a ROS, JNK and Caspase independent mechanism of cell death in the U373MG GBM cells, where as we have shown HeLa cells die by ROS
dependant mechanism. However, based on this data alone we cannot rule out any specific pathway based on the use of inhibitors studies alone. We have demonstrated that inhibition of caspase activity does not alleviate cell death, it could be that Z-VAD does not have the capacity to inhibit all of the apoptotic caspases, also in contrast to this its widely accepted that caspases are commonly dysregulated in cancer cells, resulting in reduced caspase function (Devarajan et al, 2002; Fong et al, 2006; Shen et al, 2010), However in order to rule out apoptosis as a mechanism of cell death further studies are required, such as investigation of caspase cleavage and activation, cytochrome c release as recommended by the NCCD. It is evident that the way in which cells respond to CAP is cell specific and therefore the molecular mechanisms surrounding cell death following CAP treatment need to be evaluated for each cell type being treated. This study highlights the potential of in situ CAP generation as a future brain cancer therapy. Further refinement of the technology will be necessary and the generation of micro-scale plasma fields promises to facilitate localised activation of cytotoxicity against GBM cells when used in combination with new and existing chemotherapeutic regimens.
CAP results in activation of autophagy preceding cell death in resistant glioblastoma cells

Part of this chapter has been submitted for publication and is currently under review.
4.1 Abstract

Non thermal atmospheric plasma (CAP) has shown promising efficacy for the treatment of cancer but the exact mechanisms of action remain unclear. Both apoptosis and necrosis have been implicated as the mode of cell death in various cancer cells. We have previously demonstrated a caspase-independent mechanism of cell death in p53-mutated glioblastoma multiforme (GBM) cells. The purpose of this study was to elucidate the molecular mechanisms involved in caspase-independent cell death induced by CAP treatment. We confirmed that CAP induces rapid cell death in GBM cells, independent of caspases. While depolarised mitochondria and permeable plasma membranes were observed, we hypothesise the involvement of multiple signalling pathways as a results of CAP treatment. We have identified a non-classical mechanism of apoptotic cell death on the basis of morphological assessment and the use of pharmacological inhibitors of apoptosis. We speculate that autophagy is being activated as a pro survival strategy that precedes cell death, as we have demonstrated the rapid formation of Acidic vesicle organelles (AVO’s) in response to CAP treatment. Inhibition of Phosphoinositide 3-kinase (PI3K) class III protected against cell death in CAP treated GBM cells, which further endorses our hypothesis that autophagy is activated following CAP treatment. We determined that the resistance of U373MG GBM cells to the mTOR inhibitor rapamycin could be overcome using low doses of CAP in combination with rapamycin. In conclusion, we report here for the first time that CAP induces autophagy in cancer cells prior to cell death and overcomes rapamycin resistance in GBM cells.
4.2 Introduction

Autophagy is a highly regulated process that all eukaryotic cells can carry out by sequestering damaged or defective organelles within a double membrane bound vesicle called an autophagosome, which then fuses with a lysosome to form an autolysosome and in turn degrades or recycles the aggregated material and organelles (Kondo et al., 2005; Levine & Kroemer, 2008). Autophagy has been shown to exhibit a dual function in the regulation of both cell survival and cell death. When under stressful conditions such as nutrient deprivation a cell will promote autophagy by removing degraded organelles and proteins before they have a negative impact on the cell and therefore sustaining cellular integrity, and maintaining cellular homeostasis (Levine & Kroemer, 2008; Mizushima et al., 2008). Depending on the stimulus and surrounding environment, autophagy has been shown to promote cell death and it has been identified in a small number of cases that defective autophagy contributes to cellular pathology (Denton et al., 2012). Autophagy has been classified as class II programmed cell death (PCD) an alternative to the conventional type I programmed cell death ‘apoptosis’ (Bursch et al., 2000). However, as stated previously there is an increasing body of evidence of a complex crosstalk between the apoptotic and autophagic machinery, where autophagy is required for PCD to occur but is not necessarily responsible for the cells demise. It could be that autophagy degrades cellular components that then results in activation of the apoptosis machinery but this doesn’t mean autophagy is directly responsible for cell death; the effect could be secondary (Gump & Thorburn, 2011).

Apoptosis as described by Kerr et al is characterised morphologically by the following attributes: condensation of both nucleus and cytoplasm, nuclear fragmentation, and
separation of protrusions that form on the cell surface (Kerr et al., 1972). Molecular mechanisms have since been extensively studied both in vivo and in vitro and it is now accepted that caspases play a central role in both the intrinsic and extrinsic apoptotic pathway, but it is also noted that caspase independent apoptosis (CICD) has also been demonstrated, and can manifest with morphological signs of apoptosis, autophagy or necrosis (Kroemer et al., 2005, 2009; Galluzzi et al., 2012). Intrinsic apoptosis is characterised by permeabilisation of the mitochondria outer membrane and release of cytochrome c into the cytoplasm which initiates activation of a caspase cascade though caspase 9 and 3, whereas the extrinsic pathway involves, binding of ligands (FasL/TNFα) to cell surface death receptors which leads to activation of caspase 8 and 3, which through the activation of BID also involves mitochondrial outer membrane permeabilisation (MOMP) (Galluzzi et al., 2015). There are significant morphological and molecular differences between PCD I (apoptosis) and PCD II (autophagy), with autophagy having been associated with caspase independent cell death (Kroemer & Martin, 2005b; Rieckher & Tavernarakis, 2010), along with other morphological features for example the presence of double membrane autophagosomes, or ‘vesicular redistribution of LC3 which have been reviewed previously (Kroemer et al., 2005, 2009). GBM a grade IV malignant astrocytomas, are commonly associated with PTEN mutations, amplification of Receptor Tyrosine Kinases and hyper-activation of Phosphoinositide 3-kinase PI3K (Hartmann et al; Akhavan et al, 2010), all of which are involved in the regulation of the autophagic pathway.

Recent studies have suggested that treatment of GBM induces an autophagy prior to cell death. More specifically it has been demonstrated that the current gold standard chemotherapeutic for the treatment of GBM, TMZ, induces autophagy (Galluzzi et al., 2012), and not apoptosis as demonstrated by LC3 accumulation on the membranes of
autophagosomes (Kanzawa et al, 2004b). Although activation of autophagy through acute stimulus of TMZ can act in a cytoprotective manner, leading to transient activation of autophagy and therefore contributing to TMZ resistant (Filippi-Chiela et al, 2015). On the other hand it has also been demonstrated that inhibition of TMZ induced autophagy by blocking end stage autophagosome and lysosome fusion, enhances apoptotic cell death therefore enhancing a therapeutic outcome of TMZ (Koukourakis et al, 2016a), similar findings has been observed and have been recently reviewed by Yan et all (Yan et al, 2016). Through a number of genetic mutations that inactivate pathways, GBM’s are considered resistant to apoptosis (Lefranc & Kiss, 2006). Inhibiting targets that keep autophagy downregulated or silenced such as mTOR, could subsequently enhance TMZ induced autophagy. Similarly, it has been demonstrated that radiotherapy also induces autophagy and not apoptosis in GBM (Ito et al, 2005a; Lomonaco et al, 2009), mechanisms of which have been recently reviewed by (Koukourakis et al, 2016a).

GBM’s are generally treated using a combination of physical, chemical and biological treatments. Surgical interventions and radiation therapy in combination with either pharmaceuticals or biopharmaceuticals have been the mainstay of cancer treatment for decades (Fine, 2005; Stupp et al, 2005b; Begg et al, 2011). While successful in improving patient prognosis in some cancers, many others such as GBM remain refractory to treatment. In recent years, advances in materials and technologies have led to the possibility of physical interventions for the treatment of cancer either alone (drug free) or in combination with existing therapeutic modalities (Keidar et al, 2013; Thakor & Gambhir, 2013).
This emerging scientific field, plasma medicine, aims to use physical plasmas for therapeutic applications and has been subject to intensive study over the last decade. Of particular interest is the capability of cold atmospheric plasma (CAP) to induce cell death preferentially in tumour cells, although the precise mechanisms by which cell death is induced remain to be determined. CAP is also known as cold plasma and non-thermal atmospheric plasma. There have been a number of cell death mechanisms reported such as cell cycle arrest (Köritzer et al., 2013; Siu et al., 2015), apoptosis (Keidar et al., 2011; Ishaq et al., 2014b), necrosis (Hirst et al., 2015) in various cancer models. An extensive review carried out on the therapeutic potential of autophagy in brain cancer noted a number of studies investigating both novel and existing compounds that activate autophagy in brain cancer models, for example, arsenic trioxide, EMC6 (endoplasmic reticulum membrane protein complex subunit 6), and cannabinoids (Salazar et al., 2009; Kaza et al., 2012). Both novel and established approaches such as those listed above promote an autophagic response in brain cancer and demonstrate the potential to improve combinational therapeutic strategies.

Apoptosis has been the most studied mechanism of cell death, and is the more conventional method of cancer cell death in response to treatment. Therefore, it is logical that it be the first point of investigation when analysing a mechanism of cell death. Following a thorough review of literature, previous studies using CAP in GBM cells and studies carried out by others using different methods of treatment such as UA, TMZ and radiation in resistant GBM cells it is apparent that autophagy appears to play a significant role prior to cell death. This most likely relates to the genetic alterations that are most commonly observed in GBM such as P53 mutations, PTEN deletions and EGFR amplification. We have previously shown that CAP can induce cell death independent of ROS, JNK and Caspases in P53 mutated GBM cells (Conway et al.,...
The aim of this study is to investigate whether CAP induces an autophagic response in apoptosis resistant GBM cells. In addition to this investigate whether a combination of CAP with mTOR inhibitors can provoke a synergistic effect in the rapamycin resistant GBM cells.

4.3 Materials & Methods

4.3.1 Cell culture

Human glioblastoma (U373MG-CD14) cells were obtained from Dr Michael Carty (Trinity College Dublin). U373MG cells, were cultured in DMEM high glucose (Sigma-Aldrich, Arklow, Ireland) supplemented with 10% FBS (Sigma-Aldrich, Arklow, Ireland) which were maintained in a humidified incubator containing 5% CO2 at 37°C. Media was changed every 2-3 days until 80% confluency was reached. Cells were routinely sub-cultured using a final 1:1 ratio of 0.25% trypsin (Sigma-Aldrich, Arklow, Ireland) and 0.1% EDTA (Sigma-Aldrich, Arklow, Ireland).

4.3.2 CAP device

The operating principle of the plasma discharge system used in this study is a Dielectric Barrier Discharge (DBD) which generates high voltage pulsed non thermal plasma between two electrodes, one of which is insulated, as previously described in chapter 3 and also by Ziuzina et al and Conway et al (Ziuzina et al, 2015; Conway et al, 2016). The samples were treated at 75 kV for between 0-180 seconds depending on the experiment.
4.3.3 Alamar blue cell viability assay

U373MG cells were seeded at a density of $1 \times 10^4$ into 96 well plates (Sigma-Aldrich, Arklow, Ireland) and allowed to adhere and grow overnight. Media was removed for the duration of CAP treatment and fresh media was replaced immediately after treatment and incubated at 37 °C as indicated. No deleterious effects were observed in the vehicle control samples. In cytotoxicity studies using rapamycin (Sigma-Aldrich, Arklow, Ireland), cells were seeded as described above, exposed to CAP (for combinational studies) and were exposed to rapamycin (250 nM) and incubated for up to 48 hours. Cell viability was analysed using Alamar blue. Cells were washed once with sterile PBS, incubated for 2.5 hours at 37 °C with a 10% Alamar blue solution. Fluorescence was measured using an excitation wavelength of 530 nm and emission wavelength of 595 nm on a Victor 3V 1420 (Perkin Elmer) multi-plate reader.

4.3.4 Haematoxylin & Eosin staining

Cells were plated in a T25 flask and incubated overnight as above, cells were treated with CAP as described above or left untreated. Cell were harvested, the pellet was fixed using 70% ethanol (Sigma Aldrich, Arklow, Ireland). A monolayer of both treated and untreated of cells was generated using a cytopin. Cells were then stained with Harris Haematoxylin for two minutes and 1% Eosin for 3 minutes. Cells were dehydrated in alcohol and xylene and analysed by light microscopy (Olympus Bx41).

4.3.5 Confocal Microscopy

U373MG cells were plated in 35 mm glass bottom dishes at $1 \times 10^4$ cells per dish and incubated for 24 hrs. Media was removed and cells were exposed to CAP for 60sec at 75kV after which fresh media was added. 4 hours after treatment cells were loaded with propidium iodide (10µg/ml for 10minutes at 37°C), AO (1µg/ml for 15 minutes 37°C)
or JC-1 mitochondrial membrane potential indicator (2.5µg/ml for 30 minutes 37°C) and the images were captured on a Zeiss 510 LZSM confocal inverted microscope. Propidium iodide was excited with the aid of an external argon ion 488 nm laser and detected by its emission above 585nm long pass filter. JC1 was detected by a multi-track dual excitation and emission scan, namely 488nm excitation and JC1 emission between 505 - 530nm with a band pass filter and secondly 543nm excitation and JC1 emission above 560nm with a 560nm long pass filter. Finally, AO was also detected by a multi-track dual excitation and emission scan, namely 477nm excitation and AO emission above 585nm with the aid of a long pass 585nm filter and secondly by 488nm excitation and the AO emission between 505 - 530nm with a band pass filter. All images were recorded live through PBS. The total cell fluorescence was calculated using ImageJ (v1.49, NIH) software. An outline was drawn around each individual cell, as previously described by the total corrected fluorescence was then calculated as follows; Total corrected cell fluorescence (TCCF) = Integrated density – (area of selected cell x mean fluorescence of background readings) (McCloy et al, 2014).

4.3.6 Inhibitor studies

U373MG cells were pre-treated with 3-methyladenine (3-MA) (5mM) for 1 hour prior (as described previously in chapter 2) to CAP treatment, and was added again immediately after CAP treatment. Cells were treated for 180sec at 75kV. Cell viability was assessed 48 hours later using Alamar blue.

4.3.7 Flow Cytometry

Cells were seeded in 6 well plates; cells were then treated with CAP as described above for 60 secs, and or 3-MA (5mM) and further incubated for 48 hours. Cells were harvested both 5 hours and 48 hours post treatment and stained with 1µg/ml AO
incubated at 37°C for 20 minutes, washed twice with sterile PBS and analysed by flow cytometry excited at 488 nm.

4.3.8 Statistical Analysis

All experiments were performed at least three independent times with a minimum of five replicates per experiment. Data shown is pooled and presented as mean ± SEM (n= total number of replicates) unless stated otherwise. Statistical analysis, and curve fitting was performed using Prism 5, GraphPad Software, Inc. (USA). Using Shapiro-Wilk normality test to calculate normality, our data was then normalised to the untreated control. Unless otherwise indicated differences were considered significant with a *P value < 0.05.

4.4 Results

4.4.1 CAP induces non apoptotic cell death in GBM cells.

We have previously demonstrated dose dependent cytotoxicity of CAP in U373MG GBM cells (Conway et al, 2016) in chapter 3. Inhibition of normal metabolic processes in GBM cells by CAP treatment was almost immediate, it was observed that only 4 hours post CAP treatment there was a significant reduction in cell viability compared to untreated controls (figure 3.5 chapter 3). We have further demonstrated that gross morphological changes were detected in cells 4 hours after CAP treatment, including the apparent presence of internal vesicles (Figure 4.1 (A)). Morphological assessment of cell death was carried out using H&E staining 24 hours after treatment (B). We did not find evidence of any of the morphological hallmarks of apoptosis, specifically, there was no evidence of any increase in membrane apoptotic blebbing, nuclear condensation
fragmentation or apoptotic body formation in the CAP treated cells. Similarly, we did not find any evidence of necrosis. However, based on morphological analysis alone we cannot rule out necrosis. In order to determine is necrosis is occurring, use of necrosis inhibitor necrostatin (Nec-1) could be employed. Multiple internal vesicles were observed in the CAP treated cells that were not present in untreated controls. To further mechanism of cell death, we employed nuclear dye propidium iodide (PI) and the ΔΨm probe JC1, both of which are used in the analysis of cell death. PI was added to the cells 4 hours post CAP treatment. (C) Demonstrates localisation of PI, which has bound to the nucleic acids within the nucleus of CAP-treated GBM cells. In contrast, no fluorescent signal was observed in the untreated control cells, indicating the presence of a healthy plasma membrane. The increase in fluorescence was quantified, as demonstrated (C), and a significant increase in fluorescence following CAP treatment was recorded. The mitochondria play a central role in both intrinsic apoptosis and autophagy along with the removal of defective mitochondria therefore acting as a control mechanism (Saito & Sadoshima, 2015). It is widely accepted that oxidative stress, such as reactive oxygen species generated from CAP, target the mitochondria which can result in mitochondrial dysfunction and therefore cell death, but similarly up regulation of the autophagy process can result in mitochondrial dysfunction and cell death (Karna et al, 2010; Lee et al, 2012). Previous studies have demonstrated that CAP treatment results in loss of the mitochondrial membrane potential (ΔΨm) (Ahn et al, 2011; Conway et al, 2016). We examined the ΔΨm using the molecular fluorescent probe JC-1 by confocal microscopy. As observed (D), in the untreated control JC-1 aggregates in healthy mitochondria exhibiting punctated red fluorescence, whereas in comparison there is a significant reduction in JC-1 aggregates (red fluorescence) where the membrane potential has decreased as a result of CAP treatment. The fluorescence
intensity was quantified and we observed a significant decrease (p<0.05) in the red fluorescent signal between the CAP treated cells and the untreated health controls. ROS-dependent activation of caspases and apoptosis are believed to be the primary routes for cell death in cancer cells exposed to CAP’s. U373MG cells loaded with the well-known thiol antioxidant N-acetyl cysteine or caspase inhibitor zVAD-fmk interestingly offer no protective effect when treated with CAP, which in agreement with our previous data, (Conway et al, 2016) as observed in chapter three. Together, our results confirm a ROS-dependent activation of apoptosis is not necessary for cell death when U373MG GBM cells are exposed to CAP as demonstrated below (E).
Figure 4.1 CAP induces ROS and caspase independent cells death in GBM cells.

(A) U373MG cells were treated with CAP for 60 sec. Images were taken using confocal microscopy 4 hours’ post treated of both treated and untreated samples to determine morphological differences. (B) Cells were also subjected to CAP for 180 seconds, and
fixed and stained with H&E 24 hours post treatment (bottom two panels). Cells were examined under light microscopy and images were captured. All experiments were repeated a minimum of three times. (C) In situ verification of nuclear membrane degradation U373MG was measured 4 hr after CAP exposure (75kV for 60sec) by confocal microscopy using prodium iodide 10µg/ml. The level of fluorescence was quantified using image J software and compared to the untreated control. Statistical analysis was carried out using a one sample t-test (*P<0.05). (D) In-situ verification of ΔΨm was measured 4 hr after CAP exposure (75kV for 60sec) by confocal microscopy using JC-1 2.5µg/ml. The level of fluorescence was quantified using image J software and compared to the untreated control. Statistical analysis was carried out using a unpaired t-test (*P<0.05). (E) U373MG cells were preloaded for 1hr with 4mM NAC, 50µM zVAD-fmk, or a combination of both prior to CAP treatment. After 48 hours, cells were analysed using the Alamar blue assay. Data shown was normalised to the untreated control and are shown as the % mean ± S.E.M. (n= minimum 20). All experiments were repeated at least three times. Statistical analysis was carried out using One-Way ANOVA with Bonferroni’s post test. (*P<0.05).
4.4.2 Identification of Acidic vesicle organelles (AVO’s) determined by confocal microscopy

It was then postulated as to whether autophagy is being up-regulated following CAP treatment and thus resulting in cell death. The observation of intracellular vesicles by confocal microscopy in figure 4.2 below supported this hypothesis. As stated earlier in chapter 2, it is important to note that this assay can only be employed as an indicator of acidic vesicle organelles and further analysis is required to categorically identify the autophagic pathway. Cells were loaded with AO, a cell-permeable, nucleic acid-selective fluorescent cationic dye that aggregates and fluoresces in acidic vesicular organelles, which are a known morphological characteristic in the autophagic process.

The presence of AVO’s was observed in CAP treated cells, but not in untreated cells, this was quantified and a significant increase in formation of AVOS’s was determined (A). We also confirmed and quantified AVO formation using flow cytometry 48 hours post treatment with CAP (B). An increase in the formation of AVO’s was evident by the increase in FL2 red fluorescence signal compared with the untreated cells and a significant difference in the mean fluorescence index was observed between treated and untreated cells (P<0.05). The presence of red fluorescence from acridine orange staining validates the formation of AVO’S, which is a significant characteristic of the autophagic process. There is a significant difference between the formation of AVO’s as determined by flow cytometry which correlates with that observed from the confocal images above (C).
Figure 4.2 CAP induces the formation of acidic vesicles organelles (AVO’s) by confocal microscopy.
(A) In situ verification of AVO formation was measured 4 hr after CAP exposure (75kV for 60 sec) by confocal microscopy using Acridine orange 1µg/ml. The level of fluorescence was quantified using image J software and compared to the untreated control. Statistical analysis was carried out using unpaired t-test (*P<0.05). (B) U373MG cells were exposed to CAP at 75kV for 60 seconds. After a 48hr incubation period cells were loaded with 1µg/ml acridine orange dye and analysed by flow cytometry. (C) The overlay shown between untreated and CAP treated GBM cells depicts the formation of AVO’s by quantitative shifts the FL2 channel, red fluorescence intensity ratio in both treated and untreated samples. Data was quantified using the mean fluorescence intensity and normalised to untreated control. All experiments were repeated in triplicate. Statistical analysis was carried out using unpaired t-test (*P <0.05).
4.4.3 3-MA inhibits cell death in response to CAP but not the formation of AVO’S

Having identified the formation of AVO’s by flow cytometry and confocal microscopy, with the hypothesis that CAP results in activation of autophagy, the inhibitor 3-methyladenenine (3-MA) was employed. The use of 3-MA significantly alleviated the cytotoxic effect of CAP over a 48-hour period using spectrophotometric analysis (Figure 4.3 (A)). Interestingly, there was no evidence of a reduction in the formation of AVO’s 48 hours following CAP treatment when co-treated with 3-MA. This was confirmed using flow cytometry (B), the data was then quantified using the mean fluorescence values and statistically analysed using One-way AONVA. We observed statistical significance, as expected between untreated samples and CAP treated samples but no significance was observed between cells treated with and with 3-MA (C), indicating that 3-MA does not inhibit the formation of AVO’s after 48 hours CAP treatment. We had previously observed the formation of AVO’s by confocal microscopy as little as 4 hours post treatment, therefore, we postulated that 48 was two late detect any changes, because 3-MA has been previously described to induce a cytotoxic effect over long exposure periods (Wu et al, 2010). Therefore, the same experiment was also carried out 4 hours post treatment (D) and as observed below there was no alleviation on the formation of the AVO’s. The mean fluorescence value was quantified and statistical analysis confirmed that 3-MA does not yield any significant difference in the formation of AVO’s following incubation of GBM cells with CAP in either 48hr post treatment or 4 hours post treatment.
Figure 4.3 CAP induces PI3K independent AVO formation in GBM cells.
(A) U373MG cells were preloaded for 1hr with 5mM 3-MA. Cells were exposed to CAP for 180sec at 75kV. After 48 hours’ cell were analysed using the Alamar blue assay. Data shown was normalised to the untreated control and are shown as the % mean ± S.E.M. (n= minimum 20). All experiments were repeated at least three times. Statistical analysis was carried out using one-way AONVA (*P<0.05). (B) U373MG cells were pre-treated with or without 5mM 3-MA for 1 hour then exposed to CAP at 75kV for 60 seconds. After a 48hr incubation period cells were loaded with 1µg/ml Acridine orange dye and analysed by flow cytometry. (1. 3-MA (red), 2. Untreated (black), 3. CAP (green), 4. CAP & 3-MA (blue)). (C) Data shown (flow cytometry overlay) depicts the formation of AVO’s by quantitative shifts the FL2 channel, red fluorescence intensity ratio in both treated and untreated samples in the presence of absence of the 3-MA. (D) U373MG cells were pre-treated with or without 5mM 3-MA for 1 hour then exposed to ACP at 75kV for 60 seconds. After a 5hr incubation period cells were loaded with 1µg/ml Acridine orange dye and analysed by flow cytometry. (1.Untreated (black), 2. 3-MA (blue), 3. CAP (red), 4. CAP & 3-MA(green)) Data was quantified using the mean fluorescence intensity and normalised to untreated control. All experiments were repeated in triplicate. Statistical analysis was carried out using one-way ANOVA with Bonferroni post-test (*p<0.05) (C-D).
4.4.4 GBM cells demonstrate resistant to well-known autophagy inducers, rapamycin and metformin.

It has been previously demonstrated that U373MG cells are resistant to autophagy inducers (Takeuchi et al., 2005). Rapamycin is an inhibitor of the serine/threonine protein kinase mTOR and acts as an inducer of autophagy in cells by negatively regulating the P13K-Akt-mTOR pathway. Therefore, we postulated that by combining CAP with Rapamycin we could enhance the cytotoxic effect of CAP, and overcome rapamycin resistance. We investigate the combinational effect with another well-known autophagy inducer metformin, which has previously been shown to activate adenosine monophosphate-activated protein kinase (AMPK), an enzyme that coordinates control of cell growth (Zhou et al., 2001). A variety of GBM cells have previously been shown to be sensitive to the cytotoxic effect of 10mM metformin, in addition it has also been demonstrated that metformin enhances the cytotoxicity of TMZ and radiation (Sesen et al., 2015). We confirmed that U373MG are rapamycin resistant, the recommended working concentrations for rapamycin, according to manufactures instructions for the induction of autophagy is between 10-500nM. We noted that even when exposed to high concentrations (>1.25 µM) there was no significant reduction in cell death observed over a 48-hour period (figure 4.4 (A)). Interestingly we noted that U373MG cells also demonstrate resistance to metformin (B). As there was no significant reduction in cell death for rapamycin and metformin, the IC₅₀ could not be calculated.
Figure 4.4 GBM cells demonstrated increased resistance to autophagy inducers.

(A-B) U373MG cells were exposed to increasing concentrations of rapamycin (0-1250nM), and Metformin and analysed by Alamar blue cell viability assay after 48 hours. All experiments were repeated a minimum of three times. Cells were normalised to an untreated control. No deleterious effects were observed from DMSO vehicle control. Statistical analysis was carried out using non-linear regression analyses.

4.4.5 CAP demonstrates synergistic effect when combined with rapamycin in GBM cells.

From the dose response curves established above we were able to identify a nontoxic concentration for each compound for the combination with CAP. As demonstrated in figure 4.5, there is a significant synergistic effect observed with all compounds when combined with low doses of CAP (60 secs, 75kV) compared to cells treated with each compound alone (P<0.001) (A). Rapamycin demonstrated a 58% difference in cell viability following the combination of CAP with Rapamycin when compared to rapamycin alone therefore overcoming rapamycin resistance. More interestingly there is a significant synergy between CAP alone and CAP with rapamycin, demonstrating a
further 30% reduction in cell viability than CAP alone (B). There was no significant additional loss in cell viability following the combination of CAP with metformin. Therefore, the combination of low doses of CAP (60sec) with non-toxic concentrations of rapamycin induces a synergistic effect, significantly enhancing the cytotoxicity induced by either treatment alone and overcoming GBM resistance.
Figure 4.5 CAP induces an synergistic effect in rapamycin resistance GBM cells.

(A-B) Cells were pre-treated with 250nM rapamycin or 5mM metformin for 1 hour, cells were then exposed to CAP (60sec at 75kV), and fresh rapamycin was added immediately after. Cells were analysed 48 hours later by Alamar Blue analyses. All experiments were repeated in triplicate. (A) Statistical analysis was carried out using Two-way ANOVA with Bonferroni post-test (*P<0.001). (B) Statistical analysis of CAP treated samples with inducers compared to CAP treated only cells were carried out using One-way ANOVA with Bonferroni post-test (*P<0.05).
4.5 Discussion

Recent advances in technology, engineering and materials science have led to a wide range of biological applications for non-thermal atmospheric plasma. There has been substantial development in the area of plasma medicine over the past decade, including the use of CAP as a potential therapy for the treatment of cancer. The major advantage of CAP over conventional therapies as an anti-tumor agent is the broad range of biological responses that can be initiated, reducing the likelihood for resistance to develop. The generation of short and long lived reactive species, generation of photons, heat, pressure gradients, charged particles, and electrostatic and electromagnetic fields have all been shown to induce biological effects (Stoffels et al., 2003; Kong et al., 2011; Babington et al., 2015). CAP is a partially ionized gas that contains various concentrations of free electrical charges, atoms, ions and electrons which are generated by an energy supply to a neutral gas (von Woedtke et al., 2013), which generates a unique physical and chemical environment when exposed to biological tissues including activating short and long lived reactive oxygen species (ROS) (Stoffels et al., 2003; Kong et al., 2011; Babington et al., 2015), many of which are known to induce biological effects. Many studies have shown that CAP can induce a cytotoxic response in vitro in a variety of cell lines, for example glioblastoma, cervical, breast, colorectal, and lung (Kalghatgi et al., 2011; Ma et al., 2014; Hou et al., 2015; Conway et al., 2016), of which the cell death mechanisms have been reported as apoptosis (Keidar et al., 2011; Ishaq et al., 2014b), cell cycle arrest (Köritzer et al., 2013; Siu et al., 2015), and necrosis (Hirst et al., 2015) depending on the tumour model studied and the CAP system used in these studies. Many studies to date have demonstrated an important role for reactive oxygen species generated by CAP treatment, including, H$_2$O$_2$, that induces apoptosis in glioma cells as well as many other cancer cells (Vandamme et al., 2012; Ahn et al., 2014; Siu et
al., 2015; Conway et al., 2016). We previously identified a ROS-independent mechanism of cell death in GBM cells which are known to have a very high tolerance to ROS such as H₂O₂ owing in part to expressing mutated P53 (Datta et al., 2002b) This mechanism of cell death was dose dependent and was independent of both JNK, and caspase activation. To date there has been little to no studies identifying autophagy as a mechanism of cell death in tumour cells following CAP treatment in GBM cells, although it should be noted that Hirst and colleagues have observed both necrotic and autophagic responses in primary prostate epithelial cells following CAP treatment, in contrast with prostate cancer cell lines where apoptosis was the primary mechanism of cell death (Hirst et al., 2015). We hypothesise that autophagy that is being activated, and is acting in a cytoprotective manner prior to cells death when GBM cells are treated with CAP in a caspase independent manner, similar to Koritzer et al who have also demonstrated a caspase independent mechanism of cell death in CAP treated GBM cells (Köritzer et al., 2013). Apoptosis was ruled out which was noted by lack of caspase activation, however, they did identify cell senescence of the G2/M phase (Köritzer et al., 2013).

We determined here that the cytotoxic insult induced by 75kV CAP for 180 seconds occurs rapidly, with 50% cell death observed after only 5 hours. Our findings that ROS and caspase inhibitors did not affect cell death was confirmed by morphological assessment of cells using H&E, where we observed no evidence of morphological indicators of either apoptosis or necrosis (Elmore et al., 2016). These results correlate with recently published data using CAP in U373MG cells, where the authors note a significant increase in cytotoxicity after 24 hours using a different indicator of cell death (MTT) (Siu et al., 2015). In the manuscript, the authors suggest that cell death of U373MG in response to helium jet CAP is a result of apoptosis or necrosis depending
on treatment time (Siu et al, 2015). However, the authors observed a loss in cell viability within 24 hours, whereas a small increase in caspase 3 / 7 activity was only observed at 48 hours and not at earlier time-points, indicating that caspases may not play an important role. Recent recommendations on detection of apoptosis suggest a combinational approach; the use of inhibitor studies, combined with biochemical criteria and corroboration using morphological data (Vanden Berghe et al, 2013). We have combined inhibitors with biochemical assays and morphological assessment to determine that while mitochondrial dysfunction and loss of plasma membrane integrity are features of cell death induced by CAP, caspase inhibition and hallmark morphological features of apoptosis are not apparent. Therefore, it is likely that apoptosis does not play an important role in our model.

Caspase independent cell death has often been associated with autophagy and macroautophagosome formation (Tait & Green, 2008b). There is a very close relationship between the autophagic process and apoptosis (Kroemer et al, 2009; Galluzzi et al, 2012), with some studies suggesting that autophagy may coordinate with apoptosis to induce cell death (Booth et al, 2014). Similarly it has previously been suggested that the mitochondria maybe an organelle that integrates and regulates both processes (Elmore et al, 2001). We previously confirmed depolarisation of the mitochondrial membrane potential by flow cytometry 48 hours post CAP treatment (Conway et al, 2016). Here, we confirm that CAP in fact results in a rapid depolarisation of mitochondrial membrane potential, as quickly as 4 hours post CAP treatment. The kinetics of mitochondrial depolarisation is indicative of a response that does not require de novo gene expression. A distinct morphological feature of autophagy is the formation of acidic vesicle organelles (AVO’s) (Paglin et al, 2001b). We have identified the formation of AVO’s by flow cytometry and confocal microscopy.
as early as 4 hours post CAP treatment. To confirm the autophagic process we employed the use of well known autophagy inhibitor 3-MA (Petiot et al, 2000), which successfully protected against the cytotoxic effects of CAP. To the best of our knowledge there has been no published data indicating autophagy mediating CAP induced toxicity. 3-MA is a selective inhibitor of Class III PI3K activity which is necessary for autophagy, however, long term exposure to 3-MA can induce autophagy (Wu et al, 2010) by inhibiting class I PI3K activity (Ito et al, 2007; Leng et al, 2013). Interestingly, when combined with flow cytometry, we did not observe any reduction in the formation of AVO’s after 4 or 48 hours. It has been noted that in many cases, autophagosomes accumulate despite a block in macroautophagy and despite a decrease in flux through the entire autophagic process (Klionsky et al, 2016). It has been recently shown in LNCaP prostate cells treated with docetaxel that 3-MA was toxic by itself and also increased autophagic vesicle formation and apoptosis. These paradoxical effects of 3-methyladenine were largely independent of reactive oxygen species production. Similar to our results, 3-MA treatment did not attenuate AV formation, which is suggestive of increased autophagic activity. This phenomenon of increased autophagy following 3-MA treatment is thought to be due to differing temporal effects on class I and class III PI3K under nutrient rich conditions as described above by wu et al. Our results suggest that while 3-MA protects GBM cells against CAP cytotoxicity, accumulation and acidification of intracellular vesicles and formation of AVO’s continues to take place in the presence of 3-MA. Therefore, it could be that 3-MA is driving autophagy, Wu et al noted that in autophagy is induced due to prolonged treatment with 3-MA in nutrient-rich condition is due to suppression of class I PI3K, while sparing the class III PI3K (Wu et al, 2010). The effects of PI3K inhibitors may vary depending on the activation of the two classes. The protective effect of 3-MA
following CAP treatment could be that of autophagy acting in a cytoprotective role. 3-Methyladenine also inhibits phosphorylation of Akt, JNK and p38 MAPK (Xue et al, 1999), thus demonstrating the possible off target effects of 3MA, and therefore as stated by the NCCD, it is not sufficient to use inhibitors alone when identifying autophagy. It is important to also consider as GBM cells are known to have mutated PI3k activity that autophagy could be being induced independent of either PI3k class I or III. Recent studies have provided evidence that loss of mitochondrial membrane induces autophagy through a mechanism independent of the class III phosphatidylinositol 3 kinase (PI3K/Vps34)-Beclin 1 pathway (Zhu et al, 2007). With still a lot to discover surrounding the mechanisms of autophagy, and the debate as to whether autophagy, is just feature of cell death, or is providing a cytoprotective role resulting in delayed cell death by apoptosis, therefore in accordance with the Nomenclature Committee on Cell Death (NCCD) (Galluzzi et al, 2012) based on our data, we suggest the CAP induces autophagy prior to cell death occurring. In order to elucidate the precise role of following CAP treatment further experimentation is required as advised by the NCCD such as siRNA knock down of the autophagy genes.

Downstream of the PI3K/Akt/mTOR signalling pathway is the effector mammalian target of rapamycin (mTOR), which has become a convincing therapeutic target. mTOR is associated with growth factor signalling through PI3K, protein translation, autophagy, and tumour cell metabolism. Induction of autophagic cell death as a therapeutic strategy can be achieved by modulation of autophagy using regulators such as rapamycin (mTOR) and metformin (protein kinase AMPK) (Kim & Guan, 2015). While rapamycin is currently being used for the treatment of limgageolimyomatosis (LAM) lung disease and prevention of transplant rejection, recent studies have shown its effectiveness in the treatment of various malignancies such as GBM. It has been demonstrated that
inactivation of mTORC1 by rapamycin selectively induced autophagy and not apoptosis in rapamycin sensitive GBM cells, it was also been noted that rapamycin has no cytotoxic capacity on rapamycin resistant GBM cells, but interestingly they observed that PI3K inhibitor, LY294002, and AKT inhibitor, UCN-01, synergistically sensitized rapamycin resistant cells to rapamycin by stimulating the induction of autophagy (Takeuchi et al, 2005). Interestingly we demonstrate that no effect of autophagy inducer rapamycin. It has been previously shown that Ulk regulation by mTORC1 in response to nutrients is commonly observed in eukaryotes. In addition, treatment of S. cerevisiae with rapamycin has the capacity to induce autophagy in the presence of nutrients (Kamada et al, 2000). Inhibition of mTORC1 by nutrient starvation or rapamycin treatment activates autophagy, through the regulation of the ATG1/ULK kinase complex by TORC1 and AMPK (Russell et al, 2014). In order to confirm whether U373MG cells are rapamycin resistant, this experiment should be carried out in the absence of nutrients, i.e. serum starvation to determine if autophagy could be induced.

A combination of mTOR inhibitor rapamycin with radiotherapy has also demonstrated a greater therapeutic capacity by autophagy and cellular senescence in cancer cells (Nam et al, 2013). It has also been previously demonstrated that rapamycin induces autophagy in rapamycin sensitive GBM cell lines but many GBM cells are resistant to rapamycin through the expression of the promyelocytic leukemia (PML) gene (Iwanami et al, 2013). We both pre- and co-treated rapamycin with 3-methyladenine but saw no sensitisation to rapamycin (data not shown). Metformin, another autophagy inducer is the most commonly used oral normoglycemic agent for type 2 diabetes but has also been shown to exhibit anti-tumoral effects (Li, 2011; Sesen et al, 2015). Metformin has been shown to inhibit mitochondrial activity through metformin induced AMPK activation. Similarly to rapamycin, metformin inhibits mTOR signaling. Interestingly,
P53 which in our GBM model is mutated, plays a central role in the extrinsic and intrinsic apoptotic pathway but also functions to inhibit mTOR activity and regulate its downstream targets ie. autophagy. We have demonstrated that U373MG GBM cells are both rapapymcin and metformin resistant, this provides further evidence that the resistance displayed by U373MG cells is as a result of its P53 status and therefore cells are more likely to induce autophagy prior to cell death occuring.

Recently it has been demonstrated that rapamycin can enhance the cytotoxic capacity of chemotherapeutics by apoptosis in rapamycin sensitive breast cancer cells, but the group did not observe any additive or synergistic effect when combined with rapamycin resistance breast cancer cell (Mondesire et al, 2004). We demonstrated a synergistic effect when combining rapamycin with low doses of CAP in rapamycin resistant GBM cells. Bcl-2 is a molecular marker associated with rapamycin resistance which is also associated with P53, transcriptionally activates the BAX promoter or activates apoptosis and could be a potential therapeutic target (Gomez-Manzano et al, 1997; Vignot et al, 2005b). More interestingly it was observed that inhibition of Akt expression resulted in sensitisation of rapamycin to rapamycin resistant p53 mutated U373MG GBM cells which therefore enhanced autophagic cell death (Takeuchi et al, 2005). We found no synergy between metformin and CAP. Further studies are required to fully comprehend the pathways involved in GBM induced autophagy, and to elucidate the interactions taking place between CAP and rapamycin, also the mechanisms that promote the additive effect, but demonstrates a promising potential therapeutic target for resistant tumour cells. This study and that of others indicates that there is the potential to overcome rapamycin resistance in GBM cells, and therefore potentially augment the response observed between CAP and rapamycin.
5 General Discussion
Glioblastoma is considered to be the most biologically aggressive brain tumour due to the localisation within the brain tissue, its highly invasive nature and has developed increased resistance to most therapies for example TMZ chemotherapy and radiation. It is evident that there is a need for the development and investigation of novel therapeutic approaches for the treatment of brain cancer. It is essential that these targets have the ability to overcome GBM resistance, be able to cross the blood brain barrier, however not reduce the patient’s quality of life due to off target side effects. This thesis has investigated two novel approaches to overcome these obstacles, whilst providing a better understanding as to the molecular mechanism surrounding GBM cell death in response to therapies.

A significant issue we observed was that U373MG cells do not follow the conventional method of cell death; apoptosis but demonstrated possible activation of autophagy that is preceding cell death. This is in contrast with the majority of papers that have been published with regards to GBM, employing either CAP or UA, an apoptotic mechanism of cell death was reported. An attention-grabbing article recently published, has marked significant impact on GBM research for those who have employed in vitro studies using the U87MG GBM model and possibly explains the findings reported in the literature. The U87MG cells used in over 2000 published articles does not genetically resemble the original tumour tissue used to seed the original U87MG cell line (Allen et al, 2016; Dolgin, 2016). Gene expression analysis has identified that this cell line might possess some resemblance to brain cancer but this needs to be further confirmed. This report obviously puts into question the majority of the research undertaken over the past 50 years with regards to U87MG cells brain cancer research. Therefore, it is impossible to compare and contrast data observed with other published work who claim to be using GBM models, by means of the U87MG cells. A similar issue had arisen in the past
between U373MG cells, where U251MG were formerly distributed as U373 MG, However, this has since been rectified and a new deposit of U373MG known as U-373 MG (Uppsala) is now available, of which we use in this study.

It is widely accepted that autophagy has dual roles in cancer, acting as both a tumour suppressor, preventing the accumulation of damaged proteins and organelles and also as a mechanism of cell survival that can promote the growth of established tumors. It not only acts as in a cytoprotective role but it is now evident that autophagy in a small; number of cases has been established as a mechanism of cell death in response to certain stimuli or cytotoxic agents. It is postulated that the prolonged stress incapacitates the cytoprotective nature of autophagy and results in the induction of cell death (Yang et al, 2011). The unique nature of GBM cells appear to make them resistant to apoptotic stimuli, (Yao et al, 2003; Kanzawa et al, 2004b) and as a result an alternative caspase independent mechanism of cell death is being implicated. It has been demonstrated that certain cytotoxic agents used for the treatment of GBM induce autophagy associated cell death for example, TMZ, radiation therapy and arsenic trioxide (Paglin et al, 2001a; Kanzawa et al, 2003; Kondo et al, 2005; Natsumeda et al, 2011). Whether a cell undergoes autophagic mediated cell death or autophagy in a cytoprotective manner prior to cell death, most likely depends on the cellular framework. It has been demonstrated that treatment with TMZ induced the inhibition of Akt-mTOR signalling pathway which produced a temporary induction of autophagy, leading to cell resistance of the therapy after which resulted in apoptotic cell death (Filippi-Chiela et al, 2015). Similarly, another study demonstrated that autophagy, induced a cell survival mechanism was activated following addition of therapeutic concentrations of TMZ in GBM cells over a 3-day period. They also demonstrate that inhibition of autophagy with inhibitor bafilomycin A1 may enhance the antitumor effect of TMZ by inducing apoptosis
(Kanzawa et al, 2004b). The same group observed autophagic cell death in GBM cells treated with clinical concentrations of arsenic trioxide, therefore autophagy was functioning in self-destructive manner (Kanzawa et al, 2003). In addition a study monitoring patients both before and after TMZ treatment through immunohistochemical analysis noted that induction of autophagy was increased in surgical samples after treatment with TMZ (Natsumeda et al, 2011) These studies indicate that stressors such as TMZ, that accelerate autophagy may result in autophagic cell death by disrupting the balance between survival and cell death, Or in contrast this could demonstrate the cells capacity to overcome the cytotoxic stress that is being induced as a results of treatment or as noted by the NCCD in a small number of cases be mediating cell death. Similarly, the literature has demonstrated that radiation therapy, induces autophagic cell death which can be enhanced when combined with other therapies. There have been numerous reports demonstrating that GBM cells exposed to radiation therapy that result in apoptosis resistance (Koukourakis et al, 2016b), and as a result activates autophagy (Ito et al, 2005b; Fujiwara et al, 2007; Benzina et al, 2008; Mehta et al, 2015). More recently, it has been demonstrated that Akt inhibitors induce autophagic death, and not apoptotic death in both radioresistant and radiosensitive U87 cell lines and enhance overall sensitivity to radiation (Benzina et al, 2008; Mehta et al, 2015), moreover that silencing the EGFR led to an improved autophagic response to irradiation and suppressed migration in the T98G cell line (Palumbo et al, 2014a). A similar study carried out by Plaumbo et al identified that the response to TMZ and radiation both alone or combined induced an autophagic response, moreover U87MG (radiosensitive) and U373MG (radioresistant) both induced autophagy but not apoptosis (Palumbo et al, 2014b). Interestingly, when both cell lines were pre-treated with mTOR inhibitor rapamycin and an autophagy inducer, it sensitised the U373MG radioresistant cells to
radiation (Palumbo et al, 2012b). These studies provide evidence that the autophagy-associated cell death pathway is the dominant mode of cell death rather than apoptosis in irradiated GBM cells, and indicates a possible adjuvant therapeutic strategy to enhance the conventional GBM treatment. It follows logically that the identification of novel targeted autophagy-interfering agents either with radiation and TMZ alone or in combination may represent a new strategy for the treatment of GBM. It is evident form the literature that term the use of the term ‘autophagic cell death’ has been a matter of much debate. As noted by the NCCD there are only a small number of cases in which autophagic mediated cell death have been demonstrated (Grandér et al, 2009; Laane et al, 2009; Liu et al, 2013). Grander et al showed that inhibition of autophagy through siRNA-mediated repression of Beclin 1 expression inhibited apoptosis demonstrating a significant role of autophagy in dexamethasone-induced cell death, using techniques that are recommended by the NCCD it is satisfying that autophagy is playing a role in cell death in this case. But in most cases that are being demonstrated as autophagic cell death it is more likely that the autophagic process that is being identified is the cell undergoing autophagy in a cytoprotective manner that is preceding cell death. The cell tries to overcome the cytotoxic insult but in most cases the cell becomes overwhelmed and therefore sends signals to activate a mechanism of cell death. Therefore, it is hypothesised that this is where the confusion lies in the literature. As described earlier there is a lot of cross talk between autophagy and apoptosis and that they can activate each other when required. Therefore, it is necessary that the correcting terminology is used when describing autophagy in relation to cell death. Similarly, data should not be perceived as demonstrating autophagic cell death without following the guidelines of the NCCD. It is important that in coming years that there is a standardised approach to
identify autophagy and its involvement in cell death in conjunction with appropriate use of terminology in publications to prevent misrepresentation of data.

To date and to the best of our knowledge we are the first to suggest activation of autophagy in response to CAP treatment in cancer cells. While we have preliminary evidence to support that apoptosis is not involved in our GBM model as described in chapter three and chapter 4, further experimentation is required in order to fully rule out an apoptotic mechanism of cell death. Our hypothesis is based on the lack of evidence of any morphological or biochemical apoptotic indicators. While we did not directly investigate necrosis as a potential mechanism, based on the H&E staining and confocal imaging there was no morphological evidence of necrosis. However, experimentation such as necrostatin stating to rule out necrosis and further demonstration of caspase activation is required. We successfully identified the presence of acidic vesicle organelles and using biochemical inhibitor 3-MA, we propose that autophagy is being activated. We were able to overcome this resistance with a multiple dose treatment demonstrating that CAP has the ability to overcome GBM resistance to therapies.

Equally, to that observed with CAP, there has been conflicting reports to date in GBM as to the mechanism of cell death reported by UA. It has been demonstrated that UA induces autophagy, in a ROS dependent manner (Shen et al, 2014), cell death by apoptosis was also reported, demonstrating that UA induced inhibition of miR-21, an anti-apoptotic oncogenic found in glioma therefore inducing apoptosis (Wang et al, 2012b). In addition, necrosis has also been identified as a possible mechanism through demonstration of LDH leakage and HMGB-1 release following UA treatment. Similarly, to results observed in our studies, this group also determined caspase independent cell death using general caspase inhibitor zVAD-fmk, but interestingly in the supplementary data provided they also investigated the possibility of autophagy.
They did not observe any formation of acidic vesicle organelle by flow cytometry by acridine orange staining. It should be noted that each of these studies were carried out using different GBM cell lines, and therefore contain different genetic backgrounds. For example, the DTGB-05MG cell line expresses wild type P53 (Kruse et al, 1992) whereas our GBM model expresses a mutant form of P53. Interestingly, Wang et al reported, the identification of apoptosis based on the use of acridine orange staining along with TEM and Caspase 3 cleavage (Wang et al, 2012b). While caspase dependent cell death is commonly associated with apoptosis, acridine orange and TEM are more commonly employed in the identification of autophagy (Klionsky et al, 2016). To date there has been no data published on the role of JNK following treatment with UA in GBM. We have demonstrated that JNK plays a role in UA induced cell death in GBM cells. This correlates with a study carried out by Xavier et al, who demonstrated that, in apoptotic resistant colorectal cells, UA activates the autophagic pathway prior to cell death through activation of the JNK pathway (Xavier et al, 2013). Moreover, initially their study suggested an apoptotic mechanism of cell death, but their results which indicated apoptosis, only accounted for 4% of the total number of dead cells, therefore indicating activation of another cell death signalling pathway, which was then identified to be autophagy. This study demonstrates the importance of critical analyses of the data. Xavier and her colleagues had observed a significant positive result for apoptosis and therefore could easily have been interrupted solely as an apoptotic mechanism of cell death, and therefore misreporting the involvement of the autophagic signalling pathway. We hypothesise that this misinterpretation of cell death is occurring in the case of CAP induced cell death as described later in this discussion.

The degree of the stressor also plays a role on whether a cell will undergo autophagy as a survival response, or mediate cell death. More notably in cancer cells there are a
number of genetic mutations which make it more likely that a cell will undergo autophagic cell death rather than apoptosis, for example P53, a mutation commonly found in GBM cells. P53 is responsible for triggering apoptosis and activation of caspases 3 and 8 (Haupt et al., 2003), therefore it stands to reason that cells that are mutated in P53 are resistant to apoptotic cell death, this assumption correlates with data observed in chapter two and three, where we have demonstrated a ROS and caspase independent mechanism of cell death following CAP treatment. When we investigated the effects of pyruvate (ROS scavenger) present in out DMEM media, we noted a partial alleviation in the cytotoxicity induced by CAP by Pyruvate. Pyruvate is a scavenger for H_{2}O_{2}, a species that is generated in abundance by CAP. More interestingly when we examined the effects NAC with and without CAP there was no significant difference observed, this is assumed to be due to the presence of pyruvate in the media but more interestingly there was still only partial inhibition of cell death even in the presence of both scavengers. Together, the data suggests a ROS-dependent and a ROS-independent mechanism of cell death induced by CAP. U373MG cells are known to express mutated P53 and in all cytotoxic treatments examine including CAP, chemotherapeutics and UA, bar TMZ, we have failed to demonstrate caspase activity through the use of general caspase inhibitor. Previously it has been shown that TMZ, the current gold standard for the treatment of GBM, induces an autophagy. Interestingly they demonstrated low levels of caspase activation (0.3 fold increase) using caspase inhibitor in P53 mutated U373MG cells with noted in caspase 3/7 activity (Lee et al., 2015). Similarly, to our results obtained in chapter one, we observed that cells treated with TMZ, combined with caspase inhibitor, resulted in a small but significant (17%) increase in cell viability. This data again relates with that observed by Xavier et al, it would be easy to assume that a significant increase in cell viability following the
addition of a caspase inhibitor would indicate apoptotic mechanism of cell death, but in this case it has been well established that TMZ induces autophagy, based on the NCCD’s recommendations whether it is in a cytoprotective role or mediating cell death remains to be elucidated. We postulated that this represents a basal level of activity of caspases, but is not strong enough to initiate an apoptotic response. Autophagy has also been previously associated with caspase independent cell death (Rieckher & Tavernarakis, 2010). In contrast to this, UA and CAP, demonstrate caspase independent mechanism of cell death. There was no significant alleviation of cell death following the addition of zVAD-fmk. As mentioned earlier we speculate that detection of small amounts of caspase activity is being misrepresented for apoptosis. (Siu et al, 2015) recently reported apoptotic effects in U373MG cells following CAP treatment. While their cytotoxicity and viability studies (MTT assay) correlates with our data, they have identified apoptosis as the mechanism of cell death, using only the Caspase-Glo® 3/7 assay, which measures caspase-3 and -7 activities in purified enzyme preparations. It is evident from their data that the significance observed is mild, with less than 1-fold increase in DVED cleavage, which is only noted at 48 hours after 120sec of treatment. We postulate that although this was noted to be significant that it might be basal level caspase activity and that cell death is being induced by alternative mechanisms, similar to that observed by (Xavier et al, 2013) In the case of CAP and UA one might argue that CICD is not the only mechanism cell death that could in playing a role here i.e. necrosis, or programmed cell death 1 (apoptosis) (Kroemer & Martin, 2005b). Using morphological techniques such as microscopic analysis of unstained and stained cells by following both UA and CAP treatment, we have concluded there was no evidence of cell swelling or rupturing, disintegrated cellular organelles, or loss of intracellular contents, all of which are morphological characteristics of necrosis (Baskar et al, 2012).
Similarly, we saw no evidence of apoptosis, chromatin condensation, plasma membrane blebbing, nuclear fragmentation. However, it is evident that this data is limited and can only be considered preliminary and further experimentation is required before these can be ruled out. We provided evidence suggesting autophagy that is being activated proceeding cell death by identifying the presence acidic vesicles organelles, microscopic analysis and biochemical inhibitors. Autophagy has been defined morphologically by Yu et al as a type of cell death that occurs in the absence of chromatin condensation but is accompanied by autophagic vacuolization of the cytoplasm (Yu et al, 2004a, 2004b) this is in accordance with the recently set guidelines for the detection of autophagy (Klionsky et al, 2016) to which we have observed in our data. However, in order to fully elucidate the role of autophagy following CAP and UA treatment further experimentation is required that is in line with the NCCD.

Treatment of cancers rarely employs a single standalone drug or one form of treatment. Most therapies comprise of a regime that involves multiple different modalities, for example as described previously for GBM tumours, generally speaking, following surgical resection the patient is treated with a 6 week course of radiotherapy (typical dose is around 60 Gy) with concomitant systemic therapy using alkylating agent (Stupp et al, 2005a). The main issues arising with radiotherapy is radioresistance in GBM tumours, and the invasive treatment regime of external beam radiation therapy, which includes six weeks of localized radiation therapy five times per week (Carlsson et al, 2014). While radiotherapy remains a part of the gold standard regime, in many cases it is only palliative because of radioresistance. GBM tumours that are radiosensitive, similarly to that observed with TMZ, have been shown to undergo autophagy as a mechanism of cell death (Ramirez et al, 2013). It has be demonstrated that knockdown of proteins in the autophagic signalling pathway, Beclin-1 and Atg-5, reduce sensitivity
to radiotherapy both alone or when combined with TMZ in radiosensitive GBM cells (Palumbo et al., 2012a). The mechanisms surrounding radioresistance still remains to be fully elucidated, and therefore demonstrates a need for the development of novel technologies that overcome GBM resistance.

Developments in molecular and cell biology have led to better understanding of GBM tumour development, leading to novel treatment strategies and novel approaches for drug delivery. In an approach to overcome the issues associated with current therapeutic strategies such as radiotherapy and chemotherapy, we have established methodology and investigated a novel approach for treating our resistant GBM model using CAP. Targeted drug delivery strategies to avoid the blood-brain barrier have shown efficiency in clinical trials. The Gliadel wafer is a novel approach to treat GBM, which involves after surgical resection, the wafer is placed in the tumour resected cavity and provides controlled release delivery of carmustine (BCNU) from biodegradable polymer wafers. However widespread use has been limited due to uncertainty in the toxicity of the wafer along with adverse side effects observed in clinical trials. The most common adverse side effects noted convulsions, confusion, brain oedema, infection, hemiparesis (weakness of one side of the body), aphasia (inability to comprehend and formulate language) visual field defects and surgical sites infections. A recent study analysing the survival outcome and safety of the Gliadel wafer for those treated with high grade glioma. They noted that there was an added 3-month increase in the median overall survival compared to patients who were treated with other modalities, and a significant increase in survival with combination of the Gliadel wafer with combined treatment with TMZ (Chowdhary et al., 2015). Other forms of targeted therapies include small molecule inhibitors which are organic compounds that are able to cross cell membranes and target specific intracellular constituents, for example; tyrosine kinase inhibitors
(TKIs). Monoclonal antibodies, on the other hand are used to target cell surface proteins and other extracellular peptides, as they are big to cross the cell membrane. Both of these methods have been explored as an immunotherapy with vaccination delivery. A review carried out by Thomas et al noted that of 7 published clinical trials using dendritic cell vaccines there was only an average overall survival of 24 months (Thomas et al, 2012).

An attractive feature of CAP is its potential to act as a targeted therapy due to its high selectivity for cancer cells while having little to no effect on normal cells, this phenomenon has been observed in a variety of cell types including GBM (Wang et al, 2013; Guerrero-Preston et al, 2014; Kim & Chung, 2016), unlike that observed with radiation and chemotherapy therapy. Similarly, another advantage of CAP is the redundancy of the need to cross the blood brain barrier. While there are numerous studies identifying the ability of CAP to induce a cytotoxic response in cancer cells, the molecular mechanism involved still remains poorly understood. The majority of the published work has suggested ROS mediated cell death, therefore indicating apoptosis, there have also been studies reporting necrosis (Hirst et al, 2015). There is substantial potential in the use of CAP as a combinational treatment, as observed we demonstrated a synergy between CAP and low doses of TMZ, with a significant reduction in cell viability than either treatment alone. A study was carried out by Köritzer et al which demonstrated that CAP treatment was able to restore sensitivity in chemo resistant GBM cells when combined with TMZ by inducing cell cycle arrest (Köritzer et al, 2013). The results outlined above demonstrate a potential mechanism for overcoming chemo resistance in GBM cells by demonstrating a potential combinational targeted therapeutic approach for the treatment of GBM, enhancing the overall cytotoxic potential of either modality while reducing the concentrations of TMZ required while
employing low doses of CAP. When developing a new therapy, more importantly a combinational therapy, it is necessary to investigate the pharmokinetic behaviour between the two components and also the inhibitory or enhanced cytotoxicity when combined, for example it has been shown that cisplatin based chemotherapies act as radiosensititiers, therefore limiting the dose of radiation required (Boeckman et al, 2005; Candelaria et al, 2006). Interestingly, as the dose of CAP was increased the synergistic or additive effect between CAP and TMZ was lost. On the other hand, we detected a marginal but significant inhibitory action on the cytotoxicity of certain drugs when directly treated with CAP, indicating that there is an interaction occurring between the species generated by CAP and the compound itself. Further studies including techniques such as LS-MS or MNR and FTIR would be able to elucidate any alteration in the compound after CAP treatment. Similarly, a recent study demonstrated the synergistic effect between UA and radiation treatment, sub toxic concentrations of ursolic acid sensitised adenocarcinoma gastric cancer cells to radiation therapy, through enhanced G2/M arrest, increased ROS resulting in cell death by apoptosis (Yang et al, 2015). Interestingly we demonstrated no synergy between UA and CAP as demonstrated in chapter 2, and as described in chapter 4, on the other hand we have provided preliminary evidence to suggest that autophagy is being active and is preceding cell death. In addition, our studies have demonstrated that U373MG cells are resistant to ROS generation following CAP treatment, and therefore might play a role in the lack of synergy observed in GBM cells. From a clinical point of view this study gives evidence of the safety CAP when used as a combinational approach. There is only negligible loss in the bioactivity of the majority of the compounds when exposed to CAP for extended periods of time. Therefore, demonstrating efficacy, it would be unlikely that CAP would interfere with other treatment modalities that a patient might also be ongoing.
One of the most important hallmarks of malignant gliomas is their invasive behaviour. Gliomas can be observed as comprising of two subpopulations of cells, the proliferative cells at the tumour core, and cells invading the surrounding brain tissue. To date, the majority of approved therapies are targeted towards inhibiting tumour cell growth by targeting cell proliferation. EGFR targeted therapy employing TKIs (Gefitinib), monoclonal antibodies (Cetuximab). TMZ and BCNU are both alkylating agents targeting DNA and prevent proper DNA replication and thus inhibiting cell proliferation. Studies have demonstrated that RTK are an appropriate target for inhibiting cell migration and invasion as activation of RTK’s have been shown to demonstrate anti-migratory activity in vitro and in vivo (Iwamoto et al, 2010; Zhu et al, 2011; Balaña et al, 2014; Chinchar et al, 2014; Zhao et al, 2015), however these promising results have not translated during clinical trials (Iwamoto et al, 2010; Balaña et al, 2014). Another target that has shown promising for inhibiting cell migration is the 26S proteasome. The 26S proteasome is an intracellular protease which degrades proteins via the ubiquitin pathway (Areeb et al, 2016). Proteasome inhibitor Bortezomib has been approved for the use in treating multiple myeloma, and once again demonstrated promising results in vitro but this failed to translate in clinical trials (Odia et al, 2015; Vlachostergios & Papandreou, 2015; Areeb et al, 2016). Interest has revived recently in the investigation of medicinal plants to identify novel active phytochemicals that might potentially lead to novel drug development. Anticancer drugs derived from plants have been utilised in cancer therapy for years for example plant alkaloids such as vincristine, the activities of flavonoids and the synergistic action shown by them with other drugs make them ideal candidate for anticancer therapies. We have demonstrated the ability of Ursolic acid, a naturally available bioactive compound has the ability to suppress migration in GBM cells, which has also been successfully demonstrated by
other in a variety of other cancer cells. It has been previously demonstrated that radiotherapy induces cell migration and invasion in glioma cells, the same group have also has demonstrated that the MAPK pathway plays a significant role, with inhibition of JNK reducing the rate of migration in glioma initiating and glioma stem cells (Alapati et al, 2014). This also correlates with our data, were we observed a partial inhibition in migration following the use of a JNK inhibitor. More interestingly we demonstrate significantly increased inhibition of cell migration following the combination of UA with JNK inhibitor then JNK alone, therefore demonstrating the efficacy of UA as a potential anti-migratory therapeutic for the treatment of GBM but also confirming the role of JNK in GBM migration.

In conclusion, Chapter two of this thesis has presented cytotoxicity profiles of a panel of medicinal and phytochemical compounds, and also provides preliminary evidence of autophagy following UA treatment. We have presented a strong rational for the potential use of naturally available phytochemical ursolic acid as a potential therapeutic target for the treatment of GBM. Not only does it exhibit a greater cytotoxic profile to currently used chemotherapeutics over a shorter period of time, we have provided sufficient evidence to support our hypothesis of an autophagic mechanism of cell death and of a concomitant, JNK-independent inhibition of migration.

In chapter three we optimised and investigated the efficacy of CAP as a potential therapeutic target to induce cytotoxicity and overcome GBM resistance. We have successfully established a working model using GBM and HeLa cells using our CAP-DBD system. Through repeated CAP exposures we have successfully overcome the natural resistance of GBM to CAP. We demonstrated a synergistic effect between low doses of TMZ and CAP. We have also demonstrated a mild inhibition of bioactivity
following direct long term on some compounds. Part of this chapter was accepted and published in the British Journal of Cancer in February 2016.

In chapter four we investigated autophagic process that precedes cell death following CAP treatment. As per the guidelines for detecting both autophagy and apoptosis we have provided preliminary evidence to rule out apoptosis as a mechanism of cell death in our model and for the first time we demonstrate autophagy that is preceding a caspase independent mechanism of cell death following CAP treatment in GBM cells. This thesis provides evidence to suggest that GBM follows the non-conventional mechanism of cell death in response to a variety of treatment modalities and therefore, we hypothesise the mechanism of cell death in which in vitro cell lines follow is based on the genetic mutations that are present in that cell line and further studies are required to fully elucidate what pathways are involved.
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Appendix I

Cell counting using a Haemocytometer.

The haemocytometer was washed with 70% ethanol and a glass cover slip was placed on top. 10µl of cell suspension to be counted was added to the haemocytometer. Through capillary action the sample was drawn under the coverslip. The haemocytometer was placed under a light microscope and focused using the x10 objective. Cells were counted in the four outer set of squares (each containing 16 smaller squares), as shown below (Maria Fuentes, 2013).
Appendix II

Vehicle controls for (A) BCNU, (B) TMZ, (C) Gefitinib, (D) Cisplatin, (E) Clozapine and (F) UA. Cells were treated with the highest concentration present in drug, either 0.2% DMSO or 0.6% H₂O. No significant reduction in cell viability was observed in the vehicle controls, calculated using student t-test.

A.

![Graph A](image)

B.

![Graph B](image)
C.

![Graph showing Relative Cell Viability (%)](image)

D.

![Graph showing Relative Cell Viability (%)](image)
Appendix III

Correlation analysis was carried out by a member of the DIT school of computing, Dr. Yupeng Liu (Appendix III-V)

Comparison of dose response curves between U373MG and HeLa cells in response to NTAP treatment (data plotted in chapter 3, figure 3.4). Using the statistical software R, correlation analysis was carried out on both cell lines (U373MG and HeLa) in response to NTAP treatment. The statistical procedure of calculating correlation is one of the most frequently used procedures in biomedicine. Correlation is the agreement of values from two data sets, and it expresses the degree of association between the investigated phenomena. The following relationship base on correlation coefficient.

Exactly –1. A perfect downhill (negative) relationship (deep red colour)

–0.70. A strong downhill (negative) relationship

–0.50. A moderate downhill (negative) relationship

–0.30. A weak downhill (negative) relationship

0. No relationship (white colour)

+0.30. A weak uphill (positive) relationship

+0.50. A moderate uphill (positive) relationship

+0.70. A strong uphill (positive) relationship

Exactly +1. A perfect uphill (positive) relationship, (Blue colour)

It is evident that there is no correlation between the two cell lines, which coincides with the results observed in the dose response experiment.
Appendix IV

Comparison of dose response curves between U373MG and HeLa cells following treatment with H$_2$O$_2$ (data plotted in chapter 3, figure 3.14). Using the statistical software R, correlation analysis was carried out on both cell lines in response to H$_2$O$_2$. According to the colour scale the correlation coefficient appears to lie near zero, indicating that U373MG and HeLa cells do not correlate. It is evident that there is no correlation between the two cell lines, which coincides with the results observed in the dose response experiment.
Appendix V

Comparison of dose response curves between U373MG and HeLa cells following treatment with H$_2$O$_2$ (data plotted in chapter 3, figure 3.15). According to the colour scale the correlation coefficient appears to lie near zero, indicating that U373MG and HeLa cells do not correlate.
Appendix VI

Optimisation of clozapine.

Dose response curve was carried out as described in chapter for all other compounds. Cells were analysed by Alamar blue after 48-hour period (A). Using statistical analysis and nonlinear regression, the IC$_{50}$ was calculated to 49.13µM interestingly neither of the three inhibitors were able to attenuate any cytotoxicity induced. There was no significant reduction in cell viability in the inhibitors alone, as observed in the control data in figure (B). A significant reduction in cell viability was observed following treatment with cisplatin (48%) and clozapine (41%) alone, therefore the compounds were effective killing the cells. The vehicle control did not produce any deleterious effects, therefore is not responsible for the significant additive effect seen between clozapine and SP600125, resulting in a further 10% reduction in cell viability. Our data suggests that cisplatin and clozapine induces cell death via a ROS, caspase and JNK independent mechanism.

A
(A) U373MG cells were treated with increasing concentrations (0 ≤ 500) of clozapine. Cells were also treated with a vehicle control of 0.2% DMSO (see appendix II). After 48 hours were analysed using Alamar blue cell viability assay. All experiments were repeated minimum in triplicate. Data shown was normalised to the untreated control and are shown as the % mean ± S.E.M. Non-linear regression statistical analysis was carried out using Prism5 statistical software. (B) Each inhibitor was made up in full media. U373MG cells were pre-treated for 1 hour with increasing concentrations of either NAC (ROS), SP600125 (JNK), zVAD-fmk (caspase), after which 50µM clozapine (IC₅₀) was added to each well. Following incubation of 48 hours’ cell viability was analysed by Alamar blue analyses. Experiments were repeated in triplicate. All experiments were normalised to untreated control and expressed as a % of the SEM. Statistical analysis was carried out using One-Way ANOVA with Bonferroni post-test (p<0.05).
Appendix VII

Haematoxylin &Eosin staining protocol

Cells are trypsinised and pelleted by centrifugation at 1200 RPM for 5 minutes. The pellet in the resuspended in ThinPrep PreservcCyte which is a methanol based buffered preservative solution. 1.5ml of cell suspension is then added to the cytospin cassette (containing a glass slide) and spun at 800rpm for 5 minutes to form a monolayer of cells on the glass slide. Cells are the fixed in industrial methylated spirits.

H+E staining

1) Bring cells back to water.
   • Dip slides into 70% alcohol for 3 minutes
   • Dip slides into 50% alcohol for 3 minutes
   • Dip slides into distilled water for 3 minutes
2) Stain with Harris haematoxylin (Sigma Aldrich, Arklow) for 2 minutes
3) Rinse well with tap water
4) Stain with 1% eosin (Sigma Aldrich, Arklow) for 3 minutes
5) Rinse well with tap water
6) Dehydrate cells
   • Dip slides into 50% alcohol for 3 minutes
   • Dip slides into 70% alcohol for 3 minutes
   • Dip slides into 100% alcohol for 3 minutes
   • Dip slides into xylene for 3 minutes, mount in DPX mounting medium (Sigma Aldrich, Arklow) and coverslip and view under microscope.