Preclinical evaluation of apoptosis resensitisation with Inhibitor-of-apoptosis-protein (IAP) antagonist in glioblastoma cell lines and in an orthotopic mouse GBM model

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Mr Donncha O’Brien
Candidate Thesis Declaration

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a higher degree of Doctor of Philosophy, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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<th>Definition</th>
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<tr>
<td>5-ALA</td>
<td>Amino levulinic acid</td>
</tr>
<tr>
<td>5-AZA</td>
<td>Azacitidine</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>AA</td>
<td>Anaplastic astrocytoma</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis-inducing factor</td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine transaminase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>One-way analysis of variance</td>
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<tr>
<td>AST</td>
<td>Aspartate aminotransferase</td>
</tr>
<tr>
<td>AVPI</td>
<td>Ala-Val-Pro-Ile</td>
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<tr>
<td>Apaf-1</td>
<td>Apoptotic protease-activating factor 1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Bid</td>
<td>BH3 interacting domain death agonist</td>
</tr>
<tr>
<td>B-BB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BCNU</td>
<td>Bischloroethylnitrosourea</td>
</tr>
<tr>
<td>BIR</td>
<td>Baculoviral IAP repeat</td>
</tr>
<tr>
<td>BLI</td>
<td>Bioluminescence imaging</td>
</tr>
<tr>
<td>BRUCE</td>
<td>BIR–containing ubiquitin-conjugating enzyme</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cFLIP</td>
<td>cellular FLICE-inhibitory protein</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase recruitment domain</td>
</tr>
<tr>
<td>CCD</td>
<td>Charged coupled device</td>
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<tr>
<td>CDK4</td>
<td>Cyclin-dependent kinase 4</td>
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<tr>
<td>CCL2</td>
<td>Chemokine ligand 2</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-inducing signaling complex</td>
</tr>
<tr>
<td>DD</td>
<td>Death domain</td>
</tr>
<tr>
<td>DGGR</td>
<td>1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6′-methylresorufin)</td>
</tr>
<tr>
<td>DLT</td>
<td>Dose limiting toxicity</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DR</td>
<td>Death receptor</td>
</tr>
<tr>
<td>DTI</td>
<td>Defined Trypsin Inhibitor</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>EORTC</td>
<td>European Organization for Research and Treatment of Cancer</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-Associated protein with death domain</td>
</tr>
<tr>
<td>FBC</td>
<td>Full blood count</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>FDG</td>
<td>Fluorine-2-Deoxy-D-Glucose</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin-embedded</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FLI</td>
<td>Fluorescence imaging</td>
</tr>
<tr>
<td>FOV</td>
<td>Field of view</td>
</tr>
<tr>
<td>GSC</td>
<td>GBM stem cell</td>
</tr>
<tr>
<td>GDH</td>
<td>Glutamate dehydrogenase</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma multiforme or Glioblastoma</td>
</tr>
<tr>
<td>GEM</td>
<td>Genetically engineered mice</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GTR</td>
<td>Gross total resection</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
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<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
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<td>Human epidermal growth factor receptor 2</td>
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<td>HPV</td>
<td>Human papilloma virus</td>
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<td>i.p.</td>
<td>Intraperitoneal</td>
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<td>IAPs</td>
<td>Inhibitor of apoptosis proteins</td>
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<tr>
<td>IDH</td>
<td>Isocitrate dehydrogenase</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>ILP-2</td>
<td>IAP-like protein 2</td>
</tr>
<tr>
<td>IMPACT</td>
<td>Microbe PCR Amplification Test</td>
</tr>
<tr>
<td>IVC</td>
<td>Individually ventilated cages</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte chemoattractant protein</td>
</tr>
<tr>
<td>MGMT</td>
<td>O6-methylguanine methyltransferase</td>
</tr>
<tr>
<td>ML-IAP</td>
<td>Melanoma inhibitor of apoptosis proteins</td>
</tr>
<tr>
<td>MMR</td>
<td>Mismatch repair</td>
</tr>
<tr>
<td>MOMP</td>
<td>Mitochondrial outer membrane permeabilisation</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum tolerated dose</td>
</tr>
<tr>
<td>MTIC</td>
<td>5-(3-methyltriazen-1-yl) imidazole-4-carboximide</td>
</tr>
<tr>
<td>MZMs</td>
<td>Marginal zone macrophages</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NAIP</td>
<td>Neuronal apoptosis inhibitory protein</td>
</tr>
<tr>
<td>NCIC</td>
<td>National Cancer Institute of Canada</td>
</tr>
<tr>
<td>NF</td>
<td>Neurofibromatosis</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NIK</td>
<td>Nuclear factor kappa B inducing kinase</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding and oligomerization domain</td>
</tr>
<tr>
<td>NTR</td>
<td>Near total resection</td>
</tr>
<tr>
<td>OS</td>
<td>Overall survival</td>
</tr>
<tr>
<td>p75NTR</td>
<td>p75 neutrotrophin receptor</td>
</tr>
<tr>
<td>PARP</td>
<td>poly(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PD</td>
<td>Pharmacodynamics</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression free survival</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetic</td>
</tr>
<tr>
<td>PET</td>
<td>Positron-emission tomography</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulation and activated normal T cell-expressed and - secreted</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene</td>
</tr>
<tr>
<td>RIP1</td>
<td>Receptor interacting kinase 1</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>Smac</td>
<td>Second mitochondria-derived activator of caspases</td>
</tr>
<tr>
<td>SPECT</td>
<td>Single-photon emission CT</td>
</tr>
<tr>
<td>Spec fPL</td>
<td>Spec pancreatic lipase immunoreactivity</td>
</tr>
<tr>
<td>SRS</td>
<td>Stereotactic radiosurgery</td>
</tr>
<tr>
<td>STR</td>
<td>Subtotal resection</td>
</tr>
<tr>
<td>TACE</td>
<td>TNF-converting enzyme</td>
</tr>
<tr>
<td>tBid</td>
<td>Truncated Bid</td>
</tr>
<tr>
<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming growth factor-β1</td>
</tr>
<tr>
<td>TMZ</td>
<td>Temozolomide</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumour necrosis factor receptor</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumour protein 53</td>
</tr>
<tr>
<td>TRAFs</td>
<td>TNF receptor-associated factors</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNF receptor 1-associated death domain protein</td>
</tr>
<tr>
<td>TWEAK</td>
<td>TNF-related weak inducer of apoptosis</td>
</tr>
<tr>
<td>UBA</td>
<td>Ubiquitin-associated domain</td>
</tr>
<tr>
<td>UBC</td>
<td>Ubiquitin-conjugate-like</td>
</tr>
<tr>
<td>US</td>
<td>Ultrasonography</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VPA</td>
<td>Valproic acid</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cells</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organisation</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis proteins</td>
</tr>
<tr>
<td>zVAD</td>
<td>Benzylxoycarbonyl-Val-Ala-Asp-fluoromethylketone</td>
</tr>
</tbody>
</table>
Summary

Resistance to temozolomide (TMZ) greatly limits chemotherapeutic effectiveness in glioblastoma (GBM). Within the currently presented thesis, we analysed the ability of the Inhibitor-of-apoptosis-protein (IAP) antagonist Birinapant to enhance treatment responses to TMZ in a panel of commercially available and patient-derived GBM cell lines. Responses to TMZ and Birinapant were analysed using colorimetric viability assays, flow cytometry, morphological analysis and protein expression profiling of anti- and pro-apoptotic proteins involve in intrinsic and extrinsic pathways. Cell death analysis identified three principal response patterns: Type A cells that readily activated caspase-8 and cell death in response to TMZ with minimal sensitisation following addition of Birinapant; Type B cells that readily activated caspase-8 and cell death in response to Birinapant but did not show further sensitization with TMZ; and Type C cells that showed no significant cell death or moderately enhanced cell death in the combined treatment paradigm.

Next, using the system biology model of effector caspase activation (APOPTO-CELL), we implemented the concentrations of five key apoptotic proteins (XIAP, pro-caspase-3, pro-caspase-9, Smac and Apaf-1), as analysed from western blot, into the model. The model’s output is identified as substrate cleavage of caspase-3 over time. Findings indicate APOPTO-CELL is a predictive tool of TMZ response in vitro, but no correlation were found between TMZ plus Birinapant treatment in vitro and APOPTO-CELL output following addition of IAP antagonist, suggesting that other pathway might be activated independent of MOMP-dependent caspase-3 activation. When comparing primary and recurrent patient tumour samples, significantly increased levels of pro-caspase-3 were found in primary tumour samples. The APOPTO-CELL model was able to predict a shorter time to substrate cleavage in these tumour cells, which was associated with a higher rate of apoptotic activity, suggesting these patients were more likely to respond to adjuvant treatment.

In vivo toxicity study was performed to achieve the maximum tolerated dose for the combination of TMZ and Birinapant in rats, followed by haematological and
biochemistry profiles of the treatment regimes. Responses *in vivo* were analysed in an orthotopic xenograft GBM model. The toxicity study, using the same treatment schedule, showed a higher degree of tolerance of combination treatments in immunocompromised than immunocompetent rats. The acute inflammatory response, as confirmed from significant level of neutrophils as well as high level of glutamate dehydrogenase and lipase, suggesting liver and pancreatic failure in immunocompetent rats. In an orthotopic mouse GBM model, application of patient derived GBM cells, which are aggressive and poorly differentiated, revealed resection of the tumour did not confer survival advantage when compared to the non-resection group. Furthermore, a Type C patient-derived cell line that was TMZ-insensitive *in vitro* and showed a strong sensitivity to TMZ and TMZ plus Birinapant treatments. Our results demonstrate remarkable differences in responses of patient-derived GBM cells to Birinapant single and combination treatments and suggest that therapeutic responses *in vivo* may be greatly affected by the tumor microenvironment. Thus, in preclinical testing of new therapeutics, a careful experimental design to include both *in vitro* and *in vivo* study are warranted for a well-balanced experimental interpretation.
CHAPTER 1:
General Introduction
1.1 Cancer and the Central Nervous System (CNS)

A neoplasm is an abnormal new growth of cells, whereby cells proliferate without control and can invade nearby tissues or metastasise to distant organs. These abnormal cells become a cancerous/malignant tumour. Somatic alterations in oncogenes and/or tumour suppressor genes in normal tissues/cells are thought to initiate neoplastic formation, followed by a stepwise accumulation of molecular and cellular changes that ultimately result in development of a malignancy (Vogelstein and Kinzler, 2004). More than one million people in the United States get cancer each year, while in Ireland an average of 30,000 new cases are diagnosed each year (www.cancer.ie). Treatment options vary, depending on the origin of cancer cells, the stage of diagnosis and treatment availability. Cancers are divided into central nervous system (CNS; brain and spinal cords) and non-CNS cancers, which can be primary or metastatic. The brain and spinal cord consist of different types of tissues and cells, which can develop into different types of tumours.

The normal brain is made up of two major types of cells: nerve cells (neurons) and glia. The most common types of glia are astrocytes, oligodendrocytes, microglia and ependymal cells, each with different functions and phenotypes. Astrocytes perform a variety of functions including the provision of biochemical support to neurons and other cell types (Haydon and Carmignoto, 2006), a major role in the repair and scarring process of the brain and spinal cord following injuries, supporting endothelial cells (cells involved in the formation of the blood-brain barrier) and also act in a "guidance" capacity for growing neurons or axons during the development of the brain (Cohen, 2014). CNS cancer is the fifteenth most common cancer in Ireland (www.ncri.ie), with approximately 300 people diagnosed with brain tumours each year. 80% of adult brain tumours are gliomas, which is also the most fast growing brain tumour (Dunbar and Yachnis, 2010). In recent years, there have been several breakthrough developments in brain tumour treatments; these include advance imaging and surgical techniques, newer types of radiation therapy, testing of new chemotherapy drugs, and other alternative treatments such as tumour vaccines and growth factor inhibitors (Taylor et al., 2012, Xu et al., 2014,
http://www.eortc.org/clinical-trials/). The continuous developments have shown a promise in preventing tumour progression and improving prognosis of glioma patients. However, *glioblastoma multiforme* (GBM), the most common primary malignant brain tumour in adults (Brodbelt et al., 2015), remains incurable, with less than 5% of patients still alive 2 years after diagnosis (Kumar et al., 2013).

### 1.2 Glioblastoma and standard of care

GBM accounts for 50% - 60% of all intracranial gliomas and carries one of the worst prognoses of all cancers (Filippini et al., 2008). Primary intracranial tumours are graded from I - IV. Astrocytomas, which are tumours derived from astrocytes, include WHO grade II (known as diffuse), WHO grade III (known as anaplastic) and WHO grade IV (GBM). The grading system and types of glioma are associated with patient’s prognosis (Ohgaki and Kleihues, 2005). GBM are histologically similar to anaplastic astrocytoma but the distinguishing features are characterized by the presence of microvascular proliferation, endothelial hypertrophy and hyperplasia, as well as glomeruloid vessels and/or necrosis. GBM are significantly more proliferative, invasive, and angiogenic compared with grade II and III astrocytomas (Agnihotri et al., 2013, Kleihues and Sobin, 2000).

**Figure 1.1** shows the differences between diffuse astrocytoma and GBM from magnetic resonance imaging (MRI), brain specimen and histology samples.

The incidence rate of GBM in the United States is 2.96 new cases per 100,000 population per year (Ohgaki and Kleihues, 2007, Ohgaki and Kleihues, 2005). Accumulation of genetic alterations is involved in the initiation and progression of GBM, leading to great genetic heterogeneity of these tumours (Popova et al., 2014). In 2008, The Cancer Genome Atlas (TCGA) Research Network described the interim results of its analyses of GBM, the first type of cancer to be studied in the TCGA pilot (Verhaak et al., 2010). Integrated genomic analysis described a gene expression-based molecular classification of GBM into classical, mesenchymal, proneural and neural subtypes. They found four important aberrations and gene expression profiles that defined these molecular
subtypes: neurofibromatosis 1 (NF1), epidermal growth factor receptor (EGFR), isocitrate dehydrogenase (IDH) and platelet-derived growth factor receptor alpha (PDGFRA). Table 1.1 highlights the characteristics associated with each of the GBM subtypes.
Magnetic resonance imaging of the human brain with contrast in a diffuse astrocytoma demonstrates no contrast material enhancement, minimal brain oedema and no visible necrosis (A), while GBM demonstrates typically poor tumour margins and diffusely infiltrating necrotic masses localised to the cerebral hemispheres. Infiltration beyond the visible tumour margin is always present (B). From the brain specimen, diffuse astrocytoma has a poor demarcation with difficult tumour margins between tumour ends and normal tissue. It is depicted by an enlargement of the involved portion of the brain and blurring of anatomical landmarks (C). GBM, also known as butterfly glioma, crosses the midline via the corpus callosum. It is very vascular with prominent areas of necrosis and haemorrhage (D). Histologically, diffuse astrocytoma shows mild cellularity with subtle nuclear anaplasia (E), while GBM has marked cellularity, with notable hyperchromatism and pleomorphism. Note the prominent vascularity (arrow), as well as the area of necrosis at the left (dash arrow) with neoplastic cells (F). Adapted from (Gonçalves et al., 2011, Abul-Kasim et al., 2013, http://library.med.utah.edu/WebPath/, www.eurorad.org, 2015).
Table 1.1 Molecular subtype of glioblastoma

<table>
<thead>
<tr>
<th>Subtypes</th>
<th>Mutations correlated</th>
<th>Neural cell types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Classical</td>
<td>100% chromosome 7 amplification with loss of chromosome 10</td>
<td>Highly astrocytic signature</td>
</tr>
<tr>
<td></td>
<td>97% amplification of EGFR</td>
<td></td>
</tr>
<tr>
<td>Mesenchymal</td>
<td>53% NF1 abnormality</td>
<td>Strongly associated with the cultured astroglial signature</td>
</tr>
<tr>
<td></td>
<td>Genes in the tumour necrosis factor super family pathway and NFκB pathway activation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>and highly expressed in this subtype, potentially as a consequence of higher overall</td>
<td></td>
</tr>
<tr>
<td></td>
<td>necrosis and associated inflammatory infiltrates in the mesenchymal class</td>
<td></td>
</tr>
<tr>
<td>Proneural</td>
<td>Amplification of PDFRA gene expression and mutation of IDH1/2</td>
<td>Highly enriched with the oligodendrocytic signature but not the astrocytic signature</td>
</tr>
<tr>
<td></td>
<td></td>
<td>* Occur in younger age</td>
</tr>
<tr>
<td>Neural</td>
<td>EGFR overexpression</td>
<td>Association with oligodendrocytic and astrocytic differentiation but additionally</td>
</tr>
<tr>
<td></td>
<td>HER2 overexpression</td>
<td>had a strong enrichment for genes differentially expressed by neurons</td>
</tr>
<tr>
<td></td>
<td>Expression of neural markers such as NEFL, GABRA1</td>
<td></td>
</tr>
</tbody>
</table>

EGFR: Epidermal growth factor receptor; IDH: Isocitrate dehydrogenase; NF1: neurofibromatosis 1; NFκB: Nuclear factor kappaB; HER2: human epidermal growth factor receptor 2; NEFL: Neurofilament light polypeptide; PDGFRA: Platelet-Derived Growth Factor Receptor Alpha; GABRA1: gamma-aminobutyric acid A receptor, alpha 1. Adapted from (Murat et al., 2008, http://cancergenome.nih.gov/cancersselected/glioblastomamultiforme, Verhaak et al., 2010)
On May this year, the 2016 WHO Classifications of Tumours of the CNS was published, replacing the old classification from 2007 (Louis et al., 2016, Louis et al., 2007). The 2016 WHO classification divided GBM based on isocitrate dehydrogenase (IDH) status, namely: 1) IDH-wildtype GBM; 2) IDH-mutant GBM; and 3) not otherwise specified (NOS)-GBM (Louis et al., 2016). A new variant, epithelioid GBM has now been added under the umbrella of IDH-wildtype GBM (Figure 1.2). The function of normal IDH is the decarboxylation of isocitrate to generate α-ketoglutarate (αKG) (Zadeh and Aldape, 2016). αKG regulates a number of cellular functions, including glutamate production, glucose sensing, and in the cellular protection and response to oxidative and energetic stress (Turkalp et al., 2014, Waitkus et al., 2016). Mutations in IDH occurs early in gliomagenesis, which change the function of the enzymes (Cohen et al., 2013). The enzymatic activity of mutant IDH is significantly reduced and results in an 100-fold increase in the amount of oncometabolite D-2-hydroxyglutarate (D2HG) (Xu et al., 2011). D2HG accumulation leads to epigenetic alterations in glioma (Flavahan et al., 2016). IDH mutations results in reduced cellular protection and survival following exposure to oxidative stress (Reitman and Yan, 2010), such as from both gamma and ultraviolet radiations, resulting in DNA damage (Lee et al., 2002). DNA damage leads to DNA double strand breaks and cell death (Kaina, 2003).

As displayed in Table 1.2, identification of IDH status defines key characteristics of GBM. IDH-wildtype GBM is also synonym for primary GBM, which develops de novo. It accounts for approximately 94% of all GBM (Morokoff et al., 2015). The tumour development is rapidly aggressive, with the majority (68%) of patients presenting a mean clinical history of approximately 4 months (Ohgaki and Kleihues, 2007). IDH-mutant GBM is synonym for secondary GBM. It develops slowly from less malignant diffuse astrocytoma (WHO grade II) or anaplastic astrocytoma (WHO grade III), with patients experiencing a mean clinical history of approximately 15 months.

Primary GBM is more common in older patients (mean age: 62 years), whereas secondary GBM generally develops in younger patients (mean age: 44 years) (Ohgaki et al., 2004, Ohgaki and Kleihues, 2005). Primary GBM also
demonstrates a slight preponderance to men (male-to-female ratio, 1.42:1) compared to secondary GBM (male-to-female ratio, 1.05:1). DNA alterations such as TP53 mutation and EGFR overexpression are mutually exclusive between primary and secondary GBM (Li et al., 2015). However, loss of PTEN is typical observed only in primary GBM, whereas secondary GBM often contains loss of chromosome 19q along with TP53 (Korshunov et al., 2006, Morokoff et al., 2015). As for the median survival rate, secondary GBM shows a better prognosis of 31 months following maximal therapy, while prognosis for primary GBM is only 15 months (Ohgaki and Kleihues, 2013).
Figure 1.2 A simplified algorithm to differentiate between 2007 and 2016 WHO classification of tumours of the central nervous system
Table 1.2 Key characteristics of isocitrate dehydrogenase (IDH)-wildtype and IDH-mutant glioblastoma

<table>
<thead>
<tr>
<th></th>
<th>IDH-wildtype</th>
<th>IDH-mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Synonym</strong></td>
<td>Primary GBM</td>
<td>Secondary GBM</td>
</tr>
<tr>
<td></td>
<td>IDH-wildtype</td>
<td>IDH-mutant</td>
</tr>
<tr>
<td><strong>Precursor lesion</strong></td>
<td>Not identifiable; Develops <em>de novo</em></td>
<td>Diffuse astrocytoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anaplastic astrocytoma</td>
</tr>
<tr>
<td><strong>Proportion of GBM</strong></td>
<td>~90%</td>
<td>~10%</td>
</tr>
<tr>
<td><strong>Median age at diagnosis</strong></td>
<td>~62 years</td>
<td>~44 years</td>
</tr>
<tr>
<td><strong>Male-female ratio</strong></td>
<td>1.42:1</td>
<td>1.05:1</td>
</tr>
<tr>
<td><strong>Mean length of clinical history</strong></td>
<td>4 months</td>
<td>15 months</td>
</tr>
<tr>
<td><strong>Location</strong></td>
<td>Supratentorial</td>
<td>Prefentially frontal</td>
</tr>
<tr>
<td><strong>Necrosis</strong></td>
<td>Extensive</td>
<td>Limited</td>
</tr>
<tr>
<td><strong>TERT promoter mutations</strong></td>
<td>72%</td>
<td>26%</td>
</tr>
<tr>
<td><strong>TP53 mutations</strong></td>
<td>27%</td>
<td>81%</td>
</tr>
<tr>
<td><strong>ATRX mutations</strong></td>
<td>Exceptional</td>
<td>71%</td>
</tr>
<tr>
<td><strong>EGFR amplification</strong></td>
<td>35%</td>
<td>Exceptional</td>
</tr>
<tr>
<td><strong>PTEN mutations</strong></td>
<td>24%</td>
<td>Exceptional</td>
</tr>
</tbody>
</table>

IDH: Isocitrate dehydrogenase; TERT: Telomerase reverse transcriptase; ATRX: alpha-thalassemia/mental retardation syndrome X-linked; EGFR: Epidermal growth factor receptor; PTEN: Phosphatase and tensin homolog. Adapted from (Louis et al., 2016)
As with most other forms of brain tumours, attempt for curative therapy by maximal neurosurgical resection remains the initial treatment for patients with GBM. Ideally, the goal of surgery is to achieve a gross-total resection (GTR), which means all visible tumour is removed by the surgeon and no apparent tumour is observed post-surgery using MRI with contrast (Gadolinium). Use of neuronavigation systems or intraoperative MRI during the operation procedure has been related to higher resection rates (Kawano et al., 2014). A GTR greater than 90% is associated with prolonged 1-year survival (Orringer et al., 2012) and a delay in tumour progression and malignant degeneration (McGirt et al., 2008).

Following surgery, adjuvant treatment is advocated to the remaining cancer cells. The Stupp protocol is the most widely implemented treatment regimen, as suggested by a phase III study conducted by the European Organization for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC) in 2005 (Stupp et al., 2005b). According to the protocol, patients should receive concomitant radiotherapy (RT) and temozolomide (TMZ) followed by adjuvant TMZ. Radiotherapy consists of fractionated focal irradiation at a dose of 2 Gy per fraction given once daily, five days per week over a period of six weeks, for a total dose of 60 Gy. It is delivered to the gross tumour volume with a 2 to 3 cm margin for the clinical target volume. Concomitant chemotherapy consists of TMZ at a dose of 75 mg per square metre (mg/m²) per day, given 7 days per week during radiotherapy. Upon completion of chemo- and radiotherapy, there should be a 4 week break. After that, patients should receive up to six cycles of adjuvant TMZ, given for 5 days every 28 days, with a starting dose of 150 mg/m² for the first cycle. This dose is subsequently increased to 200 mg/m² at the beginning of the second cycle, as long as there are no haematologic toxic effects.
1.3 Temozolomide as a standard first line anti-cancer agent

TMZ is an oral chemotherapeutic agent, used in the treatment of GBM patients undergoing the Stupp protocol. TMZ represents a new class of second generation imidazotetrazine prodrugs that undergo spontaneous conversion under physiological conditions to the active alkylating agent 5-(3-methyltriazen-1-yl) imidazole-4-carboximide (MTIC), which further degrades to 5(4)-aminoimidazole-4(5)-carboxamide and a highly reactive methyl-diazonium cation (Figure 1.3). This means that it is a controlled reaction with little or no enzymatic component and therefore does not require hepatic metabolism for activation (Colvin, 2003).

Pharmacokinetic (PK) analysis has consistently shown that the PK of TMZ is linear, with 100% bioavailability after oral administration (Friedman et al., 2000). Maximum brain concentrations of TMZ were observed within one hour post-dose for both orally and intravenous route, suggesting rapid penetration of circulating TMZ into brain tissue (Reyderman et al., 2004). Time to maximum plasma concentration ($t_{\text{max}}$) is approximately 1 hour and the elimination half-life ($t_{\text{1/2}}$) is 1.6 – 1.8 hours. PK parameters do not vary with repeated doses over five days, which indicates that TMZ does not accumulate after multiple doses. The primary pathway of clearance is via the kidneys, with 36% of the dose excreted as the intact drug (Gerson and Friedman, 2011).
Figure 1.3 Molecular structure of TMZ

Temozolomide undergoes spontaneous conversion under physiological conditions to the active alkylating agent. It is a controlled reaction with little or no enzymatic component and therefore, does not require hepatic metabolism for activation.
TMZ, and related drugs, cause potentially cytotoxic deoxyribonucleic acid (DNA) lesions from methylation of O$_6$ position of guanine (O6-MG) forming O6-methylguanin (O6-meG) (Liu and Gerson, 2006, Kohsaka and Tanaka, 2013). Importantly in GBM, O6-MG incorrectly pairs with thymine hence interfering with DNA replications. The mismatch that occurs is recognized by a mismatch repair (MMR) system and once activated, it induces double strand breaks in the genome that result in cell cycle arrest, triggering of chromosomal damage and ultimately cell death; cell death is executed mainly by apoptosis (Fulda and Debatin, 2006). However, a deficiency in the MMR pathway can result in a failure to recognize and repair the O6-MG adducts produced by TMZ and other methylating agents (Wyatt and Pittman, 2006), and in such instances, DNA replication continues without cell cycle arrest and apoptosis.

The reported benefit of starting TMZ together with radiotherapy is due to their synergistic effect on cell growth inhibition and cell cycle arrest in the G2/M phase in GBM cells; this is the most radiosensitive phase of the cell cycle (Hirose et al., 2001). Furthermore, TMZ has been shown to increase radiation-induced DNA double-strand breaks and cell death in a GBM model, an effect that was enhanced when the drug was given concomitantly with radiotherapy and not sequentially (Chakravarti et al., 2006). The spontaneous conversion of TMZ into the active metabolite, its low molecular mass, lipophilic properties, and its ability to cross the B-BB also favours this regimen (Ostermann et al., 2004, Patel et al., 2003).

However, the administration of TMZ comes with some drawbacks, as MGMT and other DNA repair mechanisms have the capacity to deal with the DNA damage induced by this drug. MGMT is ubiquitously expressed in normal human tissues but is overexpressed in all types of human tumours (Gerson, 2004). Tumour cells can show a 4- to 10-fold expression increase in MGMT, thereby rendering these cells less sensitive to the alkylating agent (Christmann and Kaina, 2016). The \textit{MGMT} gene is located on chromosome 10q26 and encodes a DNA-repair protein that rapidly reverses alkylation (including methylation) at the O$_6$ position of guanine, thereby neutralizing the cytotoxic
effects of TMZ (Thon et al., 2013, Hegi et al., 2005). The expression level of *MGMT* is determined in large by the methylation status of the gene’s promoter. This “epigenetic silencing” of *MGMT* methylation occurs in approximately 40% of cases (Dunn et al., 2009, Rivera et al., 2010, Wick et al., 2014), and can be assessed by its promoter methylation status on polymerase chain reaction (PCR)-based tests of genomic DNA (Brat, 2012). **Figure 1.4** shows that the MGMT-mediated repair is unique compared with other DNA repair pathways in that it acts alone without relying on any other proteins or cofactors. It is also called a suicidal protein as it can inactivate itself after receiving the alkyl-group from guanine (Gerson, 2004, Pegg, 2000).
Figure 1.4 The DNA repair process mediated by O\textsuperscript{6}-alkylguanine DNA alkyltransferase (MGMT)

The MGMT enzyme transfers the methyl group from the O6-methylguanine DNA adduct to a cysteine residue in the enzyme and becomes irreversibly inactivated. Adapted from (Hegi et al., 2008).
TMZ has been shown to be capable of depleting MGMT levels over prolonged exposure to the drug. A continuous daily dosing of TMZ for seven weeks permits a 2.1 fold increase in drug exposure (Garcia et al., 2009). GBM cells highly expressing a methylated MGMT promoter, and therefore with reduced MGMT expression, are more susceptible to alkylating agents and have a better treatment response to TMZ therapy (Hegi et al., 2005, Stupp et al., 2007, Stupp et al., 2009). In 2005, Hegi et al. reported that there was a significant difference in overall survival between GBM patients whose tumours had promoter methylation and those who did not, irrespective of treatment assignment (radiotherapy alone versus radiotherapy and TMZ) (Hegi et al., 2005). In a 5-year analysis of the phase III EORTC-NCIC trial, the median overall survival among “methylated” patients who received radiotherapy and TMZ was 23.4 months compared with 15.3 months among those who received radiotherapy alone. As for “unmethylated” patients, the median survival of patients who received radiotherapy and TMZ was 12.6 months compared with 11.8 months in those who received radiotherapy alone (Stupp et al., 2009).

1.4 Mechanisms of treatment resistance in glioblastoma

Despite the above-mentioned standard therapy, patient prognosis remains dismal. Various factors contribute to this poor survival, such as patient’s condition, tumour location as well as heterogeneous instability within GBM cells. Furthermore, older age of poor functional status may lead to a decreased ability for a patient to withstand neurological insults caused by the tumour, surgery, and/or adjuvant therapy (Krex et al., 2007, Lamborn et al., 2004). The following pre-operative factors are independently associated with shorter survival: older age (> 60 years), poor functional status categorised by a Karnofsky performance status score value of ≤ 80, patients presenting with motor or language deficits, and patients with tumours immediately adjacent to the lateral ventricles (Chaichana et al., 2010). Rather than tumour debulking, these patients would typically undergo stereotactic biopsy of their tumour for histological diagnosis prior to palliative adjuvant treatment.
The anatomical location of the tumour quite often makes GBM very difficult to treat. Even in a fit patient, it may not be possible to completely remove the tumour during surgery, particularly deep-seated tumours or those located in eloquent regions of the brain. Intra-operatively, it can also be difficult to differentiate between normal brain tissue and tumour. Furthermore, microscopic tumour deposits are frequently found centimetres from the tumour origin, which were not previously visible on MRI or at the time of surgery (Minniti et al., 2010). Despite a GTR of the visible tumour, the tumour deposit continue to growth, needing further surgery and treatment plan.

Another potential mechanism of treatment failure is the blood-brain barrier (B-BB), which consists of specialised endothelial cells with tight junctions that serves to restrict brain uptake of drugs (Upadhyay, 2014). Even though the integrity of the B-BB in GBM is heterogeneous and more permeable when compared with normal brain tissue (Pokorny et al., 2015), this only represents the central necrotic core, while the B-BB in the periphery remains essentially intact (Zhang et al., 2015). The infiltrative tumour cells from the periphery that are invading healthy tissue are shielded by the intact B-BB, which gives an obstacle for any drug penetration and radiation therapy into that area (Sengupta et al., 2012, Zhang et al., 2015, Agarwal et al., 2013). TMZ has the advantages of being lipophilic and can efficiently cross the B-BB (Agarwala and Kirkwood, 2000). Overcoming the B-BB is something that should be considered when developing new drugs.

Importantly, GBM cells have been shown to proliferate and recur within 2 cm from the primary tumour’s margin in 90 - 95% of cases (De Bonis et al., 2013). This may be linked to the fact that these tumours contain stem cells, which are believed to be the real driving force behind tumour growth (Altaner, 2008); this subset of cells can both self-renew and give rise to differentiated progenies (Ailles and Weissman, 2007). The mechanisms of cancer stem cells that contribute to therapeutic resistance include increase DNA repair capacity (Bao et al., 2006), possession of a high level of CD133⁺ (Lathia et al., 2015), high expression of multiple drug resistance membrane transporters (e.g., ABC transporters) (Garrido et al., 2014), a higher expression of O⁶-methylguanine-
methyltransferase (MGMT) as well as anti-apoptosis proteins and inhibitors (Liu et al., 2006, Johannessen et al., 2008).

The immunosuppressive microenvironment and location close to perivascular niches promotes tumour growth and neo-angiogenesis (Calabrese et al., 2007, Lathia et al., 2015). GBM has a high degree of neo-angiogenesis, reflected by the fact that malignant gliomas are the most vascularised tumours found in humans (Eberhard et al., 2000). The rich capillary network increases tumour blood perfusion and VEGF is thought to be the most important pro-angiogenic factor involved in normal and pathogenic angiogenesis (Prager et al., 2012). This protein increases endothelial cell proliferation, vascular permeability and recruitment of endothelial progenitor cells, playing a central role in the pathological process of tumour growth (Kleihues et al., 2002). GBM cells express VEGF and the VEGF receptor, providing a rationale for the use of antiangiogenic agents such as bevacizumab (Avastin®) (Zhang et al., 2012a) in the treatment of GBM; however, a concern about the VEGF receptor treatment is the effect of anti-angiogenic agents on tumour biology. For example, a preliminary study found that GBM upregulates other pro-angiogenic factors and invades normal brain tissue, thereby shifting GBM to a more infiltrative phenotype when exposed to anti-angiogenic therapy (Easaw et al., 2011); such therapies may also directly interfere with gadolinium uptake in tumours, making it difficult to ascertain tumour margins and to evaluate clinical response, thereby masking the treatment effect (Lucio-Eterovic et al., 2009, Tuettenberg et al., 2009). Recent experiments have demonstrated that GBM stem cells have an angiogenesis-independent and highly invasive phenotype, which suggests that blocking angiogenesis may not be sufficient to control GBM (Sakariassen et al., 2006).

TMZ, as well as most other anti-cancer therapies, exerts its cytotoxic effect by triggering apoptosis in cancer cells (Fulda and Debatin, 2006), thus it follows that defective apoptosis provides another mechanisms by which these treatments could fail. Therefore, maximizing the apoptotic activity within GBM cells may help to reduce resistance of GBM to cell death (Fulda, 2014).
1.5 Apoptosis - the extrinsic and intrinsic apoptotic pathways

Apoptosis is a mode of programmed cell death that is distinct from necrosis and autophagic cell death. Programmed cell death is beneficial for the prevention of tumour growth and the spread of infectious diseases, as it enables elimination of damaged or infected cells, as well as cells that harbour too many mutations (de Almagro and Vucic, 2012). Apoptosis execution requires activation of caspases. The name caspase is an abbreviation of cysteine-dependent, aspartate-specific peptidase, because caspases have a dominant specificity for protein substrates that contain an aspartate (McLuskey and Mottram, 2015). In humans, 11 caspases have been identified (caspase-1 to -10 and caspase-14); these are produced in cells as catalytically inactive zymogens that need to be proteolytically processed to become active. Caspases can be categorised into two groups based on their functional role in inflammation (caspase-1, -4 and -5) or apoptotic processes.

Caspase activation is a complex and tightly regulated process. The apoptotic pathways engage two types of caspases: initiator caspases (caspase-2, -8 and -9) and executioner caspases (caspase-3, -6 and -7) (Riedl and Shi, 2004, McIlwain et al., 2015). Apoptosis is initiated when cells are damaged by anticancer drugs, gamma and ultraviolet irradiation, deprivation of survival factors, and various other cytokines that activate “death receptors”. The extrinsic (death receptor-ligand) pathway is initiated by extracellular stimuli that are recognized by the binding of death ligands such as Fas/Apo-1, tumour necrosis factor α (TNFα), and Apo2L/TNF-related apoptosis-inducing ligand (TRAIL) to their cognate death receptors (CD95/FasR, TNFR1 and DR4/DR5) at the cell-surface.

Upon binding of ligands to death receptors, the adaptor protein, Fas-associated death domain containing protein (FADD), binds to the intracellular death domains of the receptor, promoting the recruitment and activation of initiator procaspase-8 within the death-inducing signalling complex (DISC) (Boldin et al., 1995, Kischkel et al., 1995). Individual procaspase-8 molecules initially associate with each other through their death effector domain or pro-domain to
form dimers. Activation of procaspase-8 involves two sequential cleavage events: the separation of the large and small subunits, followed by the separation of the large subunit from the pro-domain (Chang et al., 2003). Active caspase-8 initiates two coordinated pathways involved in apoptosis: the type 1 (extrinsic) pathway that does not involve the mitochondria and the type 2 (intrinsic) pathway that does initiates mitochondrial dysfunction (Ashkenazi and Dixit, 1998).

In the type 1 pathway, caspase-8 is activated within the DISC (Hao and Mak, 2010) and can go on to directly process procaspase-3 and -7 to generate active caspase-3 and -7. In the type 2 pathway, caspase-8 cleaves the pro-apoptotic Bcl-2 family protein, Bid into its active form, truncated Bid (tBid) (Li et al., 1998). tBid then translocates to the mitochondria where it induces mitochondrial outer membrane permeabilisation (MOMP) that is dependent on the pro-apoptotic proteins Bax and/or Bak (Luo et al., 1998, Gross et al., 1999, Billen et al., 2008). tBid recruits and activates Bax and/or Bak by inducing conformational changes and association of these proteins at specific sites of the mitochondrial outer membrane. The subsequent event produces membrane opening that allow apoptotic protein release from mitochondrial cells (Kuwana et al., 2002). Bax and/or Bak can also be directly activated by certain conditions, such as acidic pH (Cartron et al., 2004), heat (Pagliari et al., 2005), and phosphorylation (Kim et al., 2006).

The intrinsic or mitochondrial pathway of apoptosis is mediated by the release of apoptotic proteins, including cytochrome c and second mitochondria-derived activator of caspases (Smac) from the mitochondrial intermembrane space into the cytosol (Cande et al., 2002, Saelens et al., 2004) where it binds and activates Apaf-1, forming the heptameric backbone of the apoptosome complex to recruit caspase-9 in the presence of dATP (Li et al., 1997b). Once activated, this initiator caspase can then activate effector caspases such as caspase-3, leading to caspase-dependent apoptosis. This activation of effector caspases ultimately leads to the downstream cleavage of various cytoplasmic and nuclear substrates, such as poly(ADP-ribose) polymerase (PARP) (Elmore, 2007).
PARP is an enzymatic protein that plays an important role in transcription, cardiac remodelling, vasoconstriction, regulation of astrocytes and microglial function, long term memory, and aging (Chaitanya et al., 2010). PARP has also been shown to act as a survival factor involved in the detection and repair of DNA damage (Kim et al., 2005), thereby attenuating the effect of chemotherapeutic agents at basal levels. Active caspases are able to block this process by rapidly cleaving PARP (Los et al., 2002). Cleaved PARP is incapable of responding to DNA damage (Yang et al., 2004).

1.6 Crosstalk between apoptosis, necro(pto)sis and autophagy

The tight regulation of cell survival and cell death plays an important role in a variety of biological processes. It is a well-controlled process; in which under- or overstimulation ratify the cells into a state of pathological events, such as neurodegeneration, musculoskeletal disorder, autoimmune disease, and tumour formation. To compensate, should apoptosis fail to occur, a crosstalk with other non-apoptotic forms of cell death is necessary; these include necro(pto)sis and autophagy. Necrosis is strictly non-programmed or unregulated cell death. Necrosis, is a non-caspase-dependent event involving the sudden loss of membrane integrity (Kam and Ferch, 2000), followed by organelle breakdown and plasma membrane rupture (Elmore, 2007). It is a non-controlled process, which leads to activation of the immune system and extensive inflammation (Kroemer and Martin, 2005). Apoptosis consumes energy that comes from intracellular ATP. Therefore, in an event whereby ATP is depleted, the cells switch between apoptotic and necrotic modes of cell death (Los et al., 2002) (Figure 1.5).

Necroptosis, however, is a programmed form of active cell death. It is morphologically characterised by the increase in cell volume, swelling of organelles, plasma membrane permeabilisation, cellular collapse, and release of cellular contents (Belizario et al., 2015). Cells undergo necroptosis after activation of canonical and non-canonical pathways triggered by TNF (Tait et al., 2014), interferons, viral products (Polykratis et al., 2014), and diverse
pathophysiological signals (Vanden Berghe et al., 2014). There are several candidate mediators of necroptosis. The proteins, receptor interacting protein kinase (RIPK)1, RIPK3, mixed lineage kinase domain-like protein (MLKL) and phosphoglycerate mutase family member 5 (PGAM5), have been shown to convey the necrototic signal by inducing mitochondrial fragmentation (Mocarski et al., 2012, Silke et al., 2015). In the physiological condition, the FADD/caspase-8 platform plays a role as an inhibitor of excessive RIPK1 and RIPK3 activation (Green et al., 2011, Martin et al., 2012). When caspase-8 is deleted, depleted or inhibited, ligation of TNFR1 regulates TNF-induced necroptosis (Liu et al., 2016). Caspase-8 inhibition prevents the cleavage of receptor interacting kinase (RIP)1 and RIP3 allowing RIP1 to phosphorylate RIP3, which results in the assembly of (RIP)1/RIP3 necrosome complex (Li et al., 2012). Activation of RIP1-RIPK3 necrosome initiates the cascade of phosphorylation of several downstream target proteins including the proteases calpains and cathepsins, MLKL, and PGAM5 (Degterev et al., 2008, Mocarski et al., 2012, Wang et al., 2012). MLKL, when phosphorylated, forms an oligomer that moves from the cytosol to plasma and intracellular membranes, thereby disrupting plasma membrane integrity (Wang et al., 2014, Liu et al., 2016).

Autophagy is an important homeostatic process that enables the turnover and elimination of superfluous or damaged cellular constituents in an ‘eating of self’ process (Gozuacik and Kimchi, 2004). (Glick et al., 2010). It also forms an adaptive response induced by damage of DNA and deprivation of nutrients, hormones and/or energy (He and Klionsky, 2009). Autophagy is active at a basal level in most cells in the body (Codogno and Meijer, 2005). The mechanism involves a selective intracellular degradation system that delivers cytoplasmic constituents to the lysosome following autophagosome formation (Mizushima, 2007, Glick et al., 2010). Autophagosomes fuse with lysosomes to form autolysosomes (Nikoletopoulou et al., 2013), after which the cytoplasmic constituents are degraded by specific acidic hydrolases (Levine and Kroemer, 2008). The process is mainly regulated by autophagy-related (ATG) proteins and Beclin-1.
Autophagy and apoptosis share common stimuli and signalling pathways (Kang et al., 2011, Gordy and He, 2012). Beclin 1 is a novel Bcl-2-homology (BH)-3 domain only protein (Oberstein et al., 2007). It is localised primarily within cytoplasmic structures, such as endoplasmic reticulum and mitochondria (Kang et al., 2011). Beclin 1 acts as an essential activator of autophagy, however, in a nutrient rich environment, the anti-apoptotic Bcl-2 binds to Beclin 1 and inhibits its autophagic function (Levine et al., 2008). During starvation, the stress-activated signalling molecule, c-Jun N-terminal kinase 1 (JNK1) phosphorylates Bcl-2 and disrupts its association with Beclin 1, leading to autophagy activation (Wei et al., 2008b).

Although autophagy is a cytoprotective mechanism and presents a beneficial pro-survival effect, the process may also induce cell death (Yonekawa and Thorburn, 2013). An example where autophagy may cause cell death is in situations where the apoptosis mechanism is defective (Yonekawa and Thorburn, 2013). In a study of cells from Bax and Bak double knockout mice, upon stimulation with various apoptotic stimuli, a significant number of cells appeared to have undergone cell death that was dependent on autophagic regulators ATG5 and Beclin-1. This was associated with generation of autophagosomes/autolysosomes, suggesting that in situations where the cells have no ability to induce apoptosis, DNA damage activates autophagy that kills the cells (Shimizu et al., 2004). In other situation where a certain level of intracellular damage is reached and constitutive activation of autophagy failed to maintain the cell survival, autophagy may serve to remove the damaged cells by launching cell death (Kroemer et al., 2007). In cervical cancer cell line HeLa and breast cancer cell line MDA-MB-231, high level of oxidative stress induces apoptosis through ATG5-triggered mitochondrial responses by releasing Smac and activating caspase-3 (Sun et al., 2015). The findings show that autophagy able to inhibit tumour cell proliferation, which provides a rational approach to manipulate autophagy for clinical benefit (Thorburn et al., 2014).
Figure 1.5 Morphological features of apoptosis, autophagy and necrosis

Apoptotic cells are characterised by cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies. Autophagy is characterised by the accumulation of autophagosomes and autophagolysosomes for cytoplasm, organelle, and protein degradation. Necrosis is characterised by the swelling of the cell and its organelles as well as early membrane damage. Adapted from (Tan et al., 2014).
1.7 Inhibitor-of-apoptosis-proteins

Inhibitor-of-apoptosis-proteins (IAPs) was originally discovered in baculoviruses two decades ago because of their ability to suppress the host-cell death response during viral infection (Dubrez et al., 2013). IAP proteins are comprised of a family of anti-apoptotic proteins that promote pro-survival signalling pathways and prevent the activation of the effector phase of apoptosis by interfering with the activation of caspases (Fulda and Vucic, 2012). All IAPs contain one to three of the signature baculoviral IAP repeat (BIR) domains, a 70–80 amino acid segment that mediates protein-protein interactions between IAP proteins and caspases (Fulda, 2014, Vince et al., 2007). To date, there are eight human IAP gene family members, including cellular IAP1 (cIAP1), cIAP2, X-linked inhibitor of apoptosis protein (XIAP), neuronal apoptosis inhibitory protein (NAIP), survivin, BIR–containing ubiquitin-conjugating enzyme (BRUCE/Apollon), melanoma IAP (ML-IAP) (also called Livin) and IAP-like protein 2 (ILP-2) (Fulda, 2014, Dubrez et al., 2013) (Figure 1.6). As suggested by the name, IAPs in normal tissues have several potential physiological roles and display additional non-apoptotic functions in the regulation of cell proliferation, cell division, cell differentiation, cell motility, and in pro-inflammatory and immune responses (Chen et al., 2013). Of all the members, cIAP1, cIAP2 and XIAP are known to play a direct role in the blockade of apoptosis (Bai et al., 2014).

XIAP contains three BIR domains (BIR1–BIR3), a UBA and a RING domain. XIAP is the most potent member of the IAP family of proteins due to its capability to directly inhibit enzymatic activity of caspases (Silke and Meier, 2013). The XIAP-BIR2 is a potent inhibitor for caspase-3 and -7, whereas XIAP-BIR3 selectively targets caspase-9 by blocking its dimerization and activation (Scott et al., 2005) (Figure 1.7). Overexpression of XIAP has been shown to inhibit the onset of cytochrome c and Smac release from the mitochondria (Rehm et al., 2006, Flanagan et al., 2010). Many human tumours such as high-grade glioma express high levels of IAPs and aberrant expression has been linked to defects in apoptosis, therapy resistance and poor prognosis (LaCasse et al., 2008, Gillissen et al., 2013, Kashkar et al., 2003).
Members of the mammalian IAP gene family are characterised by the presence of at least one canonical BIR domain. The BIR domains are involved in protein–protein interactions with factors such as tumour necrosis factor receptor-1-associated factors (TRAFs), NODs, caspases and Smac. Some IAPs contain a really interesting new gene (RING) E3 ubiquitin ligase domain that ubiquitinates various substrates, a ubiquitin-associated domain (UBA) domain that binds to ubiquitin and polyubiquitinated chains, and/or caspase recruitment domain (CARD) that suppresses the E3 ligase activity under steady-state conditions. Adapted from (Beug et al., 2012).
The IAPs have multiple and distinct domains that are responsible for different functions of the protein. IAP proteins such as XIAP, cIAP1, cIAP2, and ML-IAP contain the RING domain that exhibits E3 ubiquitin ligase activity (Vucic et al., 2011). E3 ligase acts to “tag” proteins for degradation (Lopez et al., 2011). Two IAP E3 domains must interact with each other to be functional. Thus, when two IAPs come together to form a homodimer, allowing two E3 ligase to interact, it will permit auto-ubiquitination and their self-degradation. However, unique to cIAP1 and cIAP2 is the CARD domain, which functions to prevent RING-mediated dimerization by inhibiting the E3 domain, thereby preventing the two IAPs from coming together and interacting with each other (Oberoi-Khanuja et al., 2013).

cIAPs stimulate the activation of canonical NFκB signalling to increase cell survival (Figure 1.7). Furthermore, it has previously been shown that cIAP1 can block apoptosome formation, which in turn inhibits the processing and activation of procaspase-3 (Burke et al., 2010). Despite the efficacy of TMZ to induce cytotoxic DNA damage (Dunn et al., 2009), high levels of IAPs could have the potential to block the apoptotic signal evoked by TMZ, leading to treatment resistance.
Figure 1.7 Mechanism of action of XIAP and cIAPs

XIAP is able to bind to caspase-3 and -7 via its BIR2 domain, and caspase-9 via BIR3 domain. cIAPs stimulate the activation of canonical NFκB signalling to increase cell survival. Adapted from (http://tetralogicpharma.com/presentations-publications/).
1.8 Inhibitor-of-apoptosis-protein antagonist (Smac)

Smac, the best studied antagonist of IAPs, once released from mitochondria, is able to promote apoptosis through its ability to antagonize IAP-mediated caspase inhibition (Du et al., 2000). Smac is able to do so by having N-terminal four residues, Ala-Val-Pro-Ile (AVPI), that recognise a surface groove on BIR protein. Smac targets the BIR2 and BIR3 domains of XIAP and competitively and sterically displaces caspase-3, -7 and -9 from their XIAP interaction sites (Scott et al., 2005), thereby lowering the threshold for apoptosis induction (Fox and MacFarlane, 2016, Wei et al., 2008a).

Smac also binds to the BIR3 domain of cIAP1 and cIAP2 via its AVPI binding motif. Smac causes a conformational change that opens the cIAP1 structure and allows cIAP RING domain dimerization (Figure 1.8). RING mediated dimerization activates cIAP1/2 E3 ligase activity, leading to auto-ubiquitination and subsequent proteasomal degradation (Dueber et al., 2011).
IAP antagonists such as Smac and Smac mimetic trigger a conformational change in cIAP1 prompting RING dimerization and activation of E3 ligase activity. Adapted from (de Almagro and Vucic, 2012).
1.8.1 Inhibitor-of-apoptosis-protein antagonist and canonical NFκB signalling pathway

NFκB activation is associated with the ability of tumour cells to progress, mainly from stimulation of transcription of genes involved in blocking the induction of apoptosis, promoting cell growth, cell proliferation, angiogenesis, inflammation, and metastasis (Park and Hong, 2016). It has been rationalised that blocking NFκB can cause tumour cells to stop proliferating, to die, or enhance sensitisation to certain types of chemotherapy (Escarcega et al., 2007). cIAPs, through their ability to act as E3 ligases, regulate canonical and noncanonical NFκB signalling pathways in opposite directions (Tenev et al., 2011). cIAPs are essential positive regulators of the canonical pathway and are essential to suppress activation of the extrinsic cell death pathway (Darding and Meier, 2012). cIAPs are also required to suppress constitutive activation of noncanonical NFκB signalling by associating with NFκB inducing kinase (NIK), TRAF2 and TRAF3, resulting in proteasomal degradation of NIK (Tenev et al., 2011, Gyrd-Hansen and Meier, 2010).

There are two known TNFR; TNFR-1 and -2. TNFR1 is expressed in all cells and has a death domain that is absent in TNFR2. This pathway can be upregulated or downregulated depending on the activity of cIAP1 and cIAP2 (Aggarwal, 2003, van Horssen et al., 2006). The canonical NFκB pathway is stimulated upon binding of TNFα to TNFR1. This will trigger the recruitment of the proximal receptor-associated complex consisting of TNFR1-associated death domain protein (TRADD), TNF Receptor-Associated Factor 2 (TRAF2), cIAP1, cIAP2 and RIP1. The BIR1 domain of cIAPs binds to TNFRs through association with TRAF2. Ligand-stimulated aggregation of receptor complexes leads to cIAP1 or cIAP2 dimerization, activation of cIAP E3 ligase activity and consequent ubiquitination of RIP1 (Riedl et al., 2001, Varfolomeev et al., 2012). Polyubiquitinated RIP1 suppresses the activation of caspase-8, thereby preventing apoptosis (Nikoletopoulou et al., 2013). At the same time, it also activates the TAK1 (transforming growth factor-β-activated kinase-1) kinase complex. This complex mediates the phosphorylation of IkB kinase (IKK) (a trimeric complex composed of IKKα, IKKβ and NEMO). Ubiquitination of IKBs
ultimately results in their degradation by the proteasome (Park and Hong, 2016), and frees the NFκB transcription factor p65/RelA; this enables the p65/RelA to bind to p50, forming an active transcription factor complex. The complex translocates from the cytoplasm to the nucleus, where it can ‘turn on’ the expression of several genes that regulate cell survival (de Almagro and Vucic, 2012).

However, in the event where cIAPs are downregulated by IAP antagonist or when cIAPs’ E3 activity is defective, the unubiquinated RIP1 can dissociate from the TNFR1 complex, where it is able to recruit FADD and caspase-8 (Darding and Meier, 2012). The assembly of the cytosolic complex RIP1/FADD/caspase-8 drives caspase-8 activation (Cristofanon et al., 2015), leading to apoptotic cell death. Caspase-8 also cleaves and inactivates RIP1, abolishing its pro-survival NFκB-inducing ability (Savva et al., 2016). A crosstalk between the intrinsic and extrinsic pathway can also occur following caspase-8 activation (Tait and Green, 2010).

1.8.2 Inhibitor-of-apoptosis-protein antagonist and the non-canonical NFκB pathway

The non-canonical NFκB pathway involves the binding of TNF family members to their receptor, such as CD40. Under resting condition, cIAP1 and cIAP2 are able to shut down the non-canonical pathway by inducing proteasomal degradation of NFκB-Inducing Kinase (NIK) protein, an essential kinase of this pathway (Silke et al., 2005). Depletion of cIAPs results in stabilisation of NIK, which is subsequently able to phosphorylate inhibitor of κB Kinase alpha (IKKα). Phosphorylated IKKα induces phosphorylation of NFκB precursor, p100, which forms a complex with RelB in the cytoplasm (Darding and Meier, 2012). Further partial degradation by the proteasome is needed to form p52-RelB dimers (Park and Hong, 2016). The dimers then translocate to the nucleus to induce transcription of target genes, such as TNFα. In the absence of cIAPs, TNFα can bind and stimulate the TNFR pathway and mediate the activation of the RIP1/FADD/caspase-8 complex for apoptosis (Fulda, 2014).
1.9 Smac mimetic

1.9.1 Monovalent and bivalent Smac mimetics

It has been rationalized that removal of IAPs helps diseased cells to enter apoptosis while sparing those that are not primed for programmed cell death (Straub, 2011). The growing repertoire of molecular-based chemotherapeutics has brought forth the discovery of Smac mimetics as IAP antagonists, which were developed as monovalent and bivalent molecules. In the last decade, a large number of new IAP antagonist-based drug discoveries have entered the market (Fulda and Vucic, 2012). To date, four monovalent and two bivalent Smac mimetics have been tested in clinical trials for their safety, maximum tolerated dose (MTD), pharmacokinetics (PK), pharmacodynamics (PD), biomarker identification, and initial efficacy in patients with advanced solid tumours and haematological malignancies (Bai and Wang, 2014). Table 1.3 summarises Smac mimetics that are currently being tested in clinical trials.
Table 1.3 List of Smac mimetics that are currently being tested in clinical trial

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Clinical trial</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM-406 (DEBIO1143)</td>
<td>Monovalent</td>
<td>Phase I/II</td>
<td>Ovarian cancer, Breast cancer, Advanced solid tumours and lymphoma (Hurwitz et al., 2015) Locally advanced squamous cell carcinoma of the head and neck (Le Tourneau et al., 2014)</td>
</tr>
<tr>
<td>GDC-0917</td>
<td>Monovalent</td>
<td>Phase I</td>
<td>Advanced solid tumours and lymphoma (Tolcher et al., 2013)</td>
</tr>
<tr>
<td>LCL161</td>
<td>Monovalent</td>
<td>Phase I/II</td>
<td>Advanced solid tumours, pancreatic cancer (Infante et al., 2014) Breast cancer, Myelofibrosis, Multiple myeloma (Lluch-Hernández et al., 2013)</td>
</tr>
<tr>
<td>TL32711 (Birinapant)</td>
<td>Bivalent</td>
<td>Phase I/II</td>
<td>Refractory solid tumours or lymphoma (Amaravadi et al., 2015) Advanced ovarian, fallopian tube and peritoneal cancers (Senzer et al., 2013)</td>
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</tbody>
</table>
Monovalent Smac mimetics contain one AVPI motif (Figure 1.9A), while bivalent Smac mimetics comprise two AVPI motifs connected by a chemical linker (Figure 1.9B) (Elsayed et al., 2015). Examples of monovalent mimetics include Compound 1, SM-406 (compound 2/AT-406/DEBIO1143), LBW242, and LCL161 (Bai et al., 2014). SM-406 was initially designed to mimic the interaction of the Smac AVPI peptide with XIAP BIR3 protein. The therapeutic effect was evident in ovarian cancer cells, whereby significant single agent activity was observed in 60% of cancer cell lines investigated; this was further enhanced when used in combination with carboplatin agent (Brunckhorst et al., 2012). However, in subsequent in vitro and in vivo experiments using breast cancer cell lines, it was shown not only to inhibit the activity of XIAP but also to induce the degradation of cIAP1/2 (Cai et al., 2011). This compound was renamed AT-406 and later DEBIO1143 and brought to phase I clinical trial in patients with metastatic solid tumours or lymphoma. In general, tolerability and safety of this class of drugs was found to be acceptable. Furthermore, they achieved the target of inducing degradation of cIAP1 (Hurwitz et al., 2015). Currently, this molecule is under evaluation in phase I/II clinical trials in patients with locally advanced squamous cell carcinoma of the head and neck (http://www.chuv.ch/onc-zoom-debio-1143-201.pdf).

Examples of bivalent mimetics include SM-164 and Birinapant. Bivalent mimetics provide advantages in terms of higher binding affinities and potency to promote caspase activation, as well as ubiquitination and proteasomal degradation of IAPs (Varfolomeev et al., 2007, Vince et al., 2007, Krepler et al., 2013, Fulda, 2014). Based on this, the Smac mimetic SM-164 was investigated in different cancer cell lines, such as breast, prostate, hepatocellular and pancreatic cancer cell lines (Lu et al., 2011, Zhang et al., 2012b, Zhou et al., 2013, Bai et al., 2014). In in vitro breast cancer cells (MDA-MB-231), SM-164 activity was increased upon addition of TNFα, indicating the requirement for the TNFR pathway for cell death to occur. In addition, SM-164 is a pan-IAP molecule in its ability to downregulate cIAP1/2 with relatively weak functional antagonism against XIAP. When tested in vivo, SM-164 induces robust apoptosis and achieves tumour regression in MDA-MB-231 xenograft tumour tissue in mice (Lu et al., 2008).
Figure 1.9 Molecular structure of monovalent and bivalent Smac mimetic

SM-406 is a monovalent Smac mimetic (A), while Birinapant is a bivalent Smac mimetic (B). IAP antagonist is designed to inhibit IAPs and by doing so is able to increase the apoptotic activity within the cancer cells. Adapted from (Fulda and Vucic, 2012).
1.9.2 Smac mimetic Birinapant

Birinapant is the most advanced bivalent Smac mimetic and is currently in phase II clinical assessment for solid tumours and haematological malignancies. A recent finding indicates good responses in patients with lymphoma (Yang et al., 2016). Since this is one of the main drugs used in this project, we looked in further detail at its molecular pathway and therapeutic effect in vitro. Previous studies using knockout mice have demonstrated variable development when IAPs were deleted (Moulin et al., 2012b). For example, single mutant cIAP1, cIAP2 or XIAP mice are reported to be viable and with no reproductive or developmental defects; however the absence of both cIAP1 and cIAP2 or XIAP results in lethality at or before birth. Together these results show that cIAP1 is sufficient for development in the absence of cIAP2 and XIAP but that the presence of both cIAP2 and XIAP is needed if cIAP1 is absent (Moulin et al., 2012a). Based on the fact that critical IAP functions can be fulfilled by just cIAP1, or by XIAP and cIAP2 acting together, Birinapant was developed with a well-defined therapeutic index on cIAP1 (Condon et al., 2014).

Owing to the structural differences between bivalent and monovalent Smac mimetics, the biochemical activity of Birinapant was compared to and contrasted with several monovalent IAP antagonists, including a monovalent version of Birinapant, MV711 (Mitsuuchi et al., 2014). In EVSA-T breast cancer cell lines, bivalent Birinapant was superior to the corresponding monovalent Smac mimetic, MV711, for the degradation of cIAP1, reduction of cell survival, and activation of the RIP1:caspase-8 complex. Compound-induced formation of the RIP1:caspase-8 complex was associated with cytotoxicity induced cell death in sensitive ovarian (SKOV3) and breast cancer cell lines (EVSA-T), suggesting that early activation of caspase-8 is critical for the overall cytotoxicity for this class of drugs.

The potency of Birinapant was examined in many cancer cell lines. In the MDA-MB-231 cancer cell line, Birinapant at 1 nmol/L resulted in >50% cIAP1 loss, without any observed effect on total cIAP2 levels, together with activation of
caspases and induction of cell death. This agent specificity to cIAP1 was also confirmed in melanoma cells (Krepler et al., 2013). While the activity of Birinapant has never been tested in combination with TMZ, this agent has been tested in combination with TNF and TRAIL in a panel of 111 cell lines representing multiple tumour types. Eighteen of the 111 cell lines were sensitive to Birinapant as a single agent, with IC\textsubscript{50} values <1 \textmu mol/L. The addition of TNF or TRAIL resulted in the sensitization to Birinapant of approximately 45% of the single agent Birinapant-resistant cell lines (Benetatos et al., 2014). This result has led to the supposition that the combination of Birinapant with TNF or TRAIL could potentially sensitize certain resistant cancer cells to cell death.

To date, animal studies have demonstrated good tolerability to Birinapant (Benetatos et al., 2014, Krepler et al., 2013). Nevertheless, studies of Birinapant treatment on animals with GBM have not yet been performed. TMZ has shown good tolerability in animal models of GBM (Fourniols et al., 2015, Viel et al., 2013); however, studies using the combination of both drugs have not yet been reported.

1.10 Pre-clinical glioblastoma animal models

Animal models play an important role in pre-clinical neuro-oncology research. The study of tumourigenesis and the evaluation of new therapies for GBM require accurate and reproducible brain tumour animal models, which ideally should recapitulate key features of the human disease, be reproducible and resemble anti-tumour immune responses of spontaneous GBM (Maher et al., 2001, Candolfi et al., 2007). This feature could be deficient \textit{in vitro}.

Whilst many of the biological pathways involved in cancer have been studied extensively \textit{in vitro}, the complex biology of the \textit{in vivo} environment is not entirely approachable \textit{in vitro} (Stylli et al., 2015). Therefore, there are innate limitations for modelling invasion, angiogenesis and metastasis in a cell culture system. Rodent glioma models have been used in preclinical glioma research
for over 30 years and successful development of GBM mouse models helps to identify relevant therapeutic targets pre-clinically and translate them into clinical settings (Candolfi et al., 2007). There are many models available for neuro-oncology research, these include: genetically engineered, congenic strain, and chemically-induced and immunodeficient xenograft models (Stylli et al., 2015). Due to the variability of these models, it is essential to identify the strengths and weaknesses of different available models in order to select the most appropriate GBM model depending upon the nature of the study to be conducted (Candolfi et al., 2007, Yi et al., 2011).

Molecular progression of gliomas, like many tumours, involves the accumulation of genetic and epigenetic alterations that result in the loss of tumour suppressor gene function (phosphatase and tensin homolog (PTEN), tumour protein 53 (TP53)) or activation of oncogenic pathways (p21-Ras, EGFR, cyclin-dependent kinase 4 (CDK4)) (Agnihotri et al., 2013). The identification of the genes involved led to development of ’mouse models of GBM modifier genes’, ‘transgenic mice’ or ‘genetically engineered mice (GEM)’. These mice harbour overexpression or downregulation of relevant oncogenic receptors or downstream signalling pathways that are involved in gliomagenesis. An example is glial fibrillary acidic protein (GFAP) induced p21-Ras (an intermediate filament protein found exclusively in astrocytes) mouse or wild-type epidermal growth factor receptor (EGFR)/mutant EGFRvIII mouse, as these genes are the common gain of function alterations in malignant human astrocytomas (Gan et al., 2009, Agnihotri et al., 2013). These mouse models are necessary not only to investigate the potential of novel pre-clinical reagent but also to increase our understanding of the in vivo interactions of these molecular alterations in the pathogenesis of gliomas (Agnihotri et al., 2013). However, the GEM model has its own weakness, such as relatively poor prediction of drug therapeutic response, high cost, time consuming and slow tumour development (Chen et al., 2013). Also, the genetic properties of mouse GBM is different to human GBM, which can complicate data interpretation.

There are two types of xenograft models; heterotypic (tumour cells implanted under the skin in the subcutaneous flank) or orthotopic (tumour cells implanted...
intracranially). The subcutaneously grown tumour xenograft is frequently applied for therapy development and preclinical testing (Kerbel, 2003). Nevertheless, the subcutaneous microenvironment is not sufficient to mimic the unique circumstances of the growth within the brain, such as the lack of a B-BB, which is known to influence drug delivery and clearance kinetics (Shankavaram et al., 2012). Also, the non-existence of an immune-privileged site such as the brain does not allow for potential neurotoxic effects of therapeutic agents to be ascertained (Agarwal et al., 2013). The orthotopic grown tumour xenograft can be generated in two ways. Firstly, human GBM tumours are transferred to tissue culture flasks to generate cell lines, which are then inoculated intracerebrally into immunodeficient animals. Secondly, human GBM solid tissues (patient surgical specimens) are retransplanted and maintained as subcutaneous xenografts through serial passaging in the flank of immunedeficient mice, which is then passaged in vitro for further studies (Chen et al., 2013, Yi et al., 2011, Styli et al., 2015, Antunes et al., 2000). Still, the established GBM cell lines form discrete, noninvasive tumours with well-circumscribed borders that push aside rather than invade adjacent normal tissue. In addition, these xenograft gliomas in immunocompromised mice grow in the absence of a natural tumour-harbouring environment (Chen et al., 2013). The result can be conflicting as others found that the rodent models of GBM exhibit histopathological features compatible with tumour invasion into the non-neoplastic brain parenchyma (Candolfi et al., 2007). Despite the disparity between the GEM and xenograft model, this model still holds significant clinical value in initial drug screening, and is the model which is used for this current project.
1.11 Imaging techniques for small animal models

Long-standing concerns about the predictive value of conventional subcutaneous xenograft models has spurred the increased use of orthotopic and genetically engineered mouse models (GEMMs) for cancer (Kung, 2007). With the growing need for an adjunct to the experimental armoury in neuro-oncology, techniques that could offer the possibility for frequent intravital measurements and quantification of tumour growth are well appreciated. Beginning with the discovery of X-rays in 1895, advances in imaging technologies have improved the ability to visualise normal tissues and pathological lesions from the gross centimetre scale at the turn of the last century to micrometre resolution in the current era (Wang, 2015). This includes the use of small-animal imaging instruments that are miniaturised derivatives of preclinical and clinical imaging modalities, including computed tomography (CT), magnetic resonance imaging (MRI), positron-emission tomography (PET), single-photon emission CT (SPECT), fluorescence imaging (FLI), ultrasonography (US), and bioluminescence imaging (BLI) (Figure 1.10). These imaging techniques come with specific strengths and limitations (Table 1.4).
Bioluminescence imaging of a DBT-FG intracranial mouse GBM (Jost et al., 2009), fluorescence imaging of a U87-Luc intracranial mouse GBM (Huang et al., 2014), SPECT/CT imaging of CXCR4 expression in subcutaneous U87 xenografts (Nimmagadda et al., 2009), coronal view CT brain with contrast imaging of a U87MG intracranial mouse GBM (Kirschner et al., 2015), coronal view T1-weighted MRI brain with contrast imaging of DBT-FG intracranial mouse GBM (Jost et al., 2009), PET imaging of a OH1 h-SCLC tumour bearing nude mice (Demmer et al., 2011).
Table 1.4 Comparison between different imaging modality for *in vivo* model

<table>
<thead>
<tr>
<th>Imaging modality</th>
<th>Strengths</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>X-ray</strong></td>
<td>Gross alterations of bone&lt;br&gt;Less radiation dose compared to CT</td>
<td>Poor soft-tissue contrast&lt;br&gt;Poor sensitivity in detecting small tumours in animals</td>
</tr>
<tr>
<td><strong>CT</strong></td>
<td>Can achieve high resolution in areas of high contrast (lung, bone)&lt;br&gt;Widely available reagents</td>
<td>Limited intrinsic soft tissue contrast&lt;br&gt;Serial high-resolution imaging requires significant imaging time, resulting in a considerable radiation dose</td>
</tr>
<tr>
<td><strong>SPECT-PET</strong></td>
<td>High sensitivity in detecting small tumour&lt;br&gt;Whole-body imaging can be performed&lt;br&gt;Best for molecular imaging probe for tumour metabolism rather than tumour growth</td>
<td>Poor spatial resolution therefore less normal anatomy visualised&lt;br&gt;Radiation to animals</td>
</tr>
<tr>
<td><strong>MRI</strong></td>
<td>Ability to provide <em>in vivo</em> information with high resolution and good soft tissue contrast&lt;br&gt;Non-invasively report on cell localisation and migration with detailed anatomical background information&lt;br&gt;Better features of tumour growth with great consistency over time compared to BLI</td>
<td>Long imaging time putting small animals at further risk of general anaesthetic complications especially when serial imaging is needed&lt;br&gt;High instrument costs and space requirements&lt;br&gt;Requires high technical expertise in imaging techniques and data interpretations&lt;br&gt;Less sensitive than BLI in the case of the small tumours</td>
</tr>
<tr>
<td>Imaging modality</td>
<td>Strengths</td>
<td>Limitations</td>
</tr>
<tr>
<td>------------------</td>
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</tr>
</tbody>
</table>
| US               | Best suited for anatomical imaging of soft tissues  
Serial imaging can be performed without risk of radiation  
Able to assess blood flow and angiogenesis using Doppler mode imaging | Limited field of view  
Cannot be used for whole animal imaging |
| Fluorescence imaging | Widely available reagents  
No radiation  
Cellular and molecular imaging applications | Prone to attenuation with increased tissue depth |
| BLI              | Commonly used to monitor tumour regrowth following treatment and survival benefit  
Able to demonstrate cell proliferation at earlier time point than MRI  
Serial imaging can be performed without risk of radiation  
Many animals can be imaged at the same time  
Short image acquisition time than MRI | False bioluminescent signal can be generated by the presence of intraparenchymal haemorrhages  
Different depth of tumour location can affect the bioluminescent signal |

The summary and adaptation from (O'Farrell et al., 2013, Wang et al., 2015, Jarzabek, 2012, Koo et al., 2006).
1.11.1 Bioluminescence imaging

BLI was initially developed for modelling infectious diseases, allowing bioluminescent bacteria to be imaged in live animals (Baban et al., 2012). BLI necessitates that cells or animals be genetically engineered to express a variety of luciferase species, most commonly firefly luciferase (Wang, 2015). BLI is based on the oxidation of luciferin \((D-(\ce{\neg})-2-(\ce{60-hydroxy-20-benzothiazolyl})-\text{thiazone-4-carboxylic acid})\) in the presence of molecular oxygen and adenosine triphosphate. This reaction is catalysed by the enzyme luciferase, which converts chemical energy into photon energy, resulting in the emission of light in cells or organisms that have been genetically modified to express luciferase (Dinca et al., 2007). Visible yellow-green to yellow-orange light is emitted following the relaxation of excited oxy-luciferin to its ground state (Nakatsu et al., 2006). BLI has been identified for high sensitivity, comparatively reduced equipment costs, high throughput, short image acquisition times, relative ease of use, and minimal image post-processing requirements (Doyle et al., 2004, Dinca et al., 2007, Close et al., 2011).

The application of BLI has been numerous and include: monitoring tumour growth and regression in response to therapy, demonstrating cell migration, monitoring the locations as well as the timing of promoter-dependent luciferase expression (Shah et al., 2005, Dinca et al., 2007, Contag and Bachmann, 2002, Sarraf-Yazdi et al., 2004, Doyle et al., 2004). In an intracranial xenograft model, Dinca et al. (2007) used BLI to monitor tumour regrowth following administration of TMZ, and they found that the BLI monitoring could be used as a surrogate for predicting survival benefit from TMZ treatment (Dinca et al., 2007). Moreover, luciferase and its substrate, luciferin, have not been shown to be toxic to mammalian cells, and no functional differences have been observed, thus far, between cells expressing luciferase compared with parental cell lines (Fleming and Brekken, 2003). When comparing between the BLI and MRI, both techniques permitted longitudinal assessment in a mouse tumour model. However, due to a high sensitivity of BLI in detecting the luciferase-expressing cells, \textit{in vivo} BLI was able to demonstrate cell proliferation at an earlier time point than MRI (Inoue et al., 2007). In addition, D-luciferin (the substrate) readily
crosses cell membranes and was shown to penetrate the B-BB after intraperitoneal or intravenous injection in mice (Ozawa and James, 2010). In brain cancer research, serial imaging can be performed without radiation effect (as compare to CT imaging) to allow comparison of tumour growth between animal groups. As the whole animal is imaged, this gives further advantages to other cancer cells owing to rapid identification of brain metastasis or any unpredictable sites of metastasis (Honigman et al., 2001, Adiseshaiah et al., 2014).

The application of BLI comes with limitations. Upon bioluminescence emission from the tumour, the biological tissue such as cell membranes, nuclei, collagen or other cellular microstructures can cause scattering and diffraction of light through tissue (Rice et al., 2001, Contero et al., 2009). This can mask the size of tumour growth. Therefore, a standard field of view and minimizing charge-couple device (CCD) saturation can minimize this effect (Connolly and Hensley, 2009). Another limitation is that the sensitivity of detecting internal light sources is dependent on several factors, including the oxygen concentration within the tumour or brain tissue (de Almeida et al., 2011), the level of luciferase expression, the depth of labelled cells within the body (the distance that the photons must travel through tissue), and the sensitivity of the detection system.

1.12 Project aims

The overall aim of this project was to investigate the therapeutic effect of TMZ and Birinapant as monotherapies and as a combination therapy for GBM in vitro and in a clinically relevant in vivo model of the disease.

Specifically, the aims of each chapter are as follows:

Chapter 3:
- To characterize the efficacy of Birinapant as a monotherapy and in combination with TMZ in a panel of GBM cell lines
• To investigate the mechanism of cell death in GBM cell lines using differential quantification methods
• To investigate the specificity of Birinapant as an inhibitor of apoptosis in GBM cell lines

Chapter 4:
• To quantify protein expression profiles of GBM for the APOPTO-CELL model
• To validate the APOPTO-CELL model for GBM cell lines and patient tumour samples and identify patterns of protein expressions between the groups
• To identify characteristics of treatment sensitisation of GBM cells with the addition of a XIAP antagonist using APOPTO-CELL model

Chapter 5
• To determine the maximum tolerated dose of Birinapant TMZ combination treatment in rats in vivo
• To investigate haematological and biochemistry profiles of Birinapant and/or TMZ in rats

Chapter 6:
• To develop and validate the intracranial surgical resection model using U87RGFP-Luc2 cells in Athymic nude rats
• To develop and validate an intracranial surgical resection model using RN11uc cells in NOD/SCID mice
• To validate the efficacy of Birinapant monotherapy and combination therapy with TMZ in an in vivo intracranial mouse model of GBM
CHAPTER 2:
Materials and Methods
2.1 *In vitro* GBM cell cultures

2.1.1 Establishment of GBM cell lines

GBM cell lines U87, U251, U343, U373 and A172 are commercially available cell lines (Asai et al., 1994, Badie et al., 1994). The characterization of the cell lines is available from the American Type Culture Collection. All MZ-derived GBM cell lines were established and kindly donated by Professor Donat Kögel, Center for Neurology and Neurosurgery, Johann Wolfgang Goethe University Hospital, Frankfurt, Germany. Tumour samples were obtained during craniotomies from the operating theatre of Neurosurgery, University Hospital Mainz, under sterile conditions from patients with intracranial tumour. Pathological and immunohistochemical analyses of the tumour specimens were performed in the Institute of Neuropathology in cryo sections and in formalin-fixed and paraffin-embedded tissues. Confirmations of the diagnosis were according to the WHO classification (Louis et al., 2007).

GBM cell lines MZ18, MZ294 and MZ327 were derived from primary grade IV GBM; while MZ-256 and MZ-304 were derived from recurrent grade IV GBM (Hetschko et al., 2008). These cell lines have previously been characterised by immunostaining (Mittelbronn et al., 2011). To isolate the GBM cells, the tumour specimens were minced to a homogeneous compound with scalpels, suspended in 1 x PBS and centrifuged at 400 x g. Pellets were resuspended in 10 ml Dulbecco’s Modified Eagle’s Medium (DMEM) (Lonza, Basel, Switzerland) containing 3.7mg/ml NaHCO₃, 10% heat inactivated foetal bovine serum (FBS), 1% L-glutamine (2 mM), 100 U/ml penicillin and 100 mg/ml streptomycin, and cultivated at 37°C in a humidified incubator containing 5% CO₂.

U87RGFP cell line was a generous gift from Professor Marc Symons, The Feinstein Institute for Medical Research, Manhasset, NY, USA. U87RGFP cell line is the invasive cells isolated from U87GFP cell following serial implantation within the mouse brain (Johnston et al., 2007). Lentiviral transduction of U87RGFP cell line was previously performed to generate U87RGFP-luc2 stably...
expressing luciferase (Jarzabek, 2012). U87RGFP-luc2 cell line was provided by Dr Monika Jarzabek, Physiology Department, Royal College of Surgeons in Ireland, Dublin.

RN1luc, JK2luc, WK1luc and SJH1luc are patient-derived GBM cell lines stably expressing luciferase. These cell lines were established and kindly donated by Queensland Institute of Medical Research (QIMR) Berghofer Medical Research Institute, Brisbane, Australia (Day et al., 2013, Tivnan et al., 2014, Abuhusain et al., 2013, Puttick et al., 2015). Clinical data was provided by Dr Brett Stringer, QIMR Berghofer Medical Research Institute. Isolation of patient tumours for the generation of GBM cell lines were approved by the human ethics committee of the QIMR and Royal Brisbane and Women’s Hospital (RBWH). Table 2.1 summarises the P53 and MGMT methylation status based on previously described results found in the literature.
Table 2.1 GBM cell lines used in this study

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>WHO grade</th>
<th>P53 status</th>
<th>MGMT promoter status</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MZ18</td>
<td>Primary GBM</td>
<td>Unknown</td>
<td>Methylated</td>
<td>Hetschko et al., 2007, Murphy et al., 2013</td>
</tr>
<tr>
<td>MZ51</td>
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<td>Unknown</td>
<td>Methylated</td>
<td>Hetschko et al., 2007, Murphy et al., 2013</td>
</tr>
<tr>
<td>MZ256</td>
<td>Recurrent GBM</td>
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<td>Unmethylated</td>
<td>Hetschko et al., 2007, Murphy et al., 2013</td>
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<tr>
<td>MZ294</td>
<td>Primary GBM</td>
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<td>Hetschko et al., 2007, Murphy et al., 2013</td>
</tr>
<tr>
<td>MZ304</td>
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<td>Unmethylated</td>
<td>Hetschko et al., 2007, Murphy et al., 2013</td>
</tr>
<tr>
<td>MZ327</td>
<td>Primary GBM</td>
<td>Unknown</td>
<td>Unmethylated</td>
<td>Hetschko et al., 2007, Murphy et al., 2013</td>
</tr>
<tr>
<td>U87</td>
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<td>Wildtype</td>
<td>Methylated</td>
<td>Asai et al., 1994; Mittelbronn et al., 2011</td>
</tr>
<tr>
<td>U87RGFP</td>
<td>GBM</td>
<td>Wildtype</td>
<td>Methylated</td>
<td>Johnston et al., 2007</td>
</tr>
<tr>
<td>U343</td>
<td>GBM</td>
<td>Mutant</td>
<td>Methylated</td>
<td>Murphy et al., 2013</td>
</tr>
<tr>
<td>U373</td>
<td>GBM</td>
<td>Mutant</td>
<td>Methylated</td>
<td>Mittelbronn et al., 2011; Murphy et al., 2013</td>
</tr>
<tr>
<td>U251</td>
<td>GBM</td>
<td>Mutant</td>
<td>Methylated</td>
<td>Asai et al., 1994; Mittelbronn et al., 2011</td>
</tr>
<tr>
<td>A172</td>
<td>GBM</td>
<td>Wildtype</td>
<td>Methylated</td>
<td>Badie et al., 1994; Mittelbronn et al., 2011</td>
</tr>
<tr>
<td>Cell lines</td>
<td>WHO grade</td>
<td>P53 status</td>
<td>MGMT promoter status</td>
<td>Reference</td>
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<tr>
<td>---------------</td>
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<td>----------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>U87RGFP-luc2</td>
<td>Primary GBM</td>
<td>Wildtype</td>
<td>Methylated</td>
<td>Johnston <em>et al.</em>, 2007; Jarzabek, 2012</td>
</tr>
<tr>
<td>RN1luc</td>
<td>Primary GBM</td>
<td>Unknown</td>
<td>Unmethylated</td>
<td>Tivnan <em>et al.</em>, 2014</td>
</tr>
<tr>
<td>WK1luc</td>
<td>Primary GBM</td>
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<td>Unmethylated</td>
<td>Day <em>et al.</em>, 2013; Puttick <em>et al.</em>, 2015</td>
</tr>
<tr>
<td>JK2luc</td>
<td>Primary GBM</td>
<td>Unknown</td>
<td>Unmethylated</td>
<td>Data provided by Dr Brett Stringer</td>
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<tr>
<td>SJH1luc</td>
<td>Primary GBM</td>
<td>Unknown</td>
<td>Unmethylated</td>
<td>Data provided by Dr Brett Stringer</td>
</tr>
</tbody>
</table>
2.1.2 GBM cell lines culture conditions

MZ18, MZ294, MZ327, MZ256, MZ304, U87, U251, U343, U373 and A172 cell lines were cultured in high-glucose (4500 mg/l) DMEM supplemented with 10% heat inactivated FBS, 1% L-glutamine (2 mM), 100 U/ml penicillin and 100 mg/ml streptomycin, all from Sigma-Aldrich (St. Louis, USA). U87RGFP and U87RGFP-luc2 cell lines were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco, Invitrogen, Carlsbad, CA, USA) containing all the supplements listed above.

RN1luc, JK2luc, WK1luc and SJH1luc cell lines stably expressing luciferase were cultured in neural stem cell serum free medium-Knockout DMEM/F12 (Gibco, USA) supplemented with 10 ml StemPro Neural Supplement, 1% L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. This basal medium were filtered and aliquot into 50 ml falcon tubes and stored at -20°C. Each time 50 ml of basal medium was pre-warmed, 20 ng/ml of Epidermal growth factor (EGF) (Gibco, USA) and 10 ng/ml of basic Fibroblast growth factor (β-FGF) (Gibco, USA) were added. The cells were grown on 1% matrigel-coated flasks or plates. All GBM cell lines listed above were cultivated at 37°C in a humidified incubator containing 5% CO₂.

2.1.3 Preparation of matrigel coated tissue culture flasks and plates

BD Matrigel basement membrane matrix (BD Biosciences, UK) was used to coat the tissue culture flasks/plates to cover the polylysine coating of the flasks/plates. It is a solubilized basement membrane preparation and is effective for the cell attachment. Prior to the procedure, the flasks/plates, pipettes and Phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, USA) were pre-cooled and kept at 4°C. The matrigel was initially stored at -20°C and later thawed at 4°C. All materials were transferred and kept on ice. Using cooled pipettes, the matrigel was diluted at 1:100 using sterile cold PBS. Even coating of the surface of the flasks/plates were checked by even tilting, and later were kept in incubator for a minimum of 2 hours and not longer than
overnight. After that, the unbound solutions from the flasks/plates were rinsed gently with the medium prior to use. The diluted matrigel was added to the flasks/plates and coated as below:

### Table 2.2 Amount of diluted matrigel added for coating each flask or plates

<table>
<thead>
<tr>
<th>Flask/Plate</th>
<th>Coating</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-25 flask</td>
<td>2 - 3 ml</td>
</tr>
<tr>
<td>T-75 flask</td>
<td>7 - 8 ml</td>
</tr>
<tr>
<td>T-175 flask</td>
<td>12 - 13 ml</td>
</tr>
<tr>
<td>96 well plate</td>
<td>50 µl</td>
</tr>
<tr>
<td>24 well plate</td>
<td>150 µl</td>
</tr>
<tr>
<td>6 well plate</td>
<td>1 ml</td>
</tr>
</tbody>
</table>
2.1.4 Defrosting of GBM cell lines

GBM cell lines were stored in -80°C liquid nitrogen in cryo tube vials as replicated of the original samples. Cells were carefully defrosted by adding the pre-warmed culture medium, transferred to a sterile centrifuge tube and spun at 1000 rpm for 3 min. The supernatant was removed and the resulting pellet was re-suspended again in culture media. The process was repeated once to make sure that the DMSO was fully removed to avoid toxic effect on cells. Finally, the cells were seeded at the required density in an appropriate flasks and maintained in incubator at 37°C in humidified air with 5% CO₂.

2.1.5 Sub-culturing of GBM cell lines

GBM cell lines (except RN1luc, JK2luc, WK1luc, SJH1luc) were cultured in T-75 flask and kept in a humidified incubator until reached approximately 70 -80% confluences. The waste media was removed and the cells were washed with sterile Hanks Balanced Salt Solution (Sigma-Aldrich, St. Louis, USA). The cells were than added with 2 ml of a 0.25% Trypsin-EDTA solution (Sigma-Aldrich, St. Louis, USA) to detach the cells from the surface of the tissue culture flask. The flasks were returned back into incubator for 3 min to allow total cell detachment. Another 8 ml of complete growth medium was added into same flask and later was pipetted into sterile centrifuge tube and spun at 1000 rpm for 3 min. After that, the waste media was removed leaving the pellet at the bottom. 10 ml of culture medium was added into new culture T-75 flask. Another 1 ml of culture medium was added into the centrifuge tube and mixed well with the pellet. The cells were mixed into culture flask and for each experiment; cells were seeded at a different density. Cells were returned to the incubator and maintained at 37°C in humidified incubator with 5% CO₂.

RN1luc, JK2luc, WK1luc, SJH1luc cell lines were all cultured in a matrigel coated flask and kept in a humidified incubator until reached approximately 70 -80% confluence. The waste media was removed and the cells were washed with PBS. The cells were added with 2 ml pre-warmed StemPro Accutase.
(Gibco, USA) to ensure complete coverage of cell monolayer. The flasks were returned back into incubator for 3 min to allow total cell detachment. After incubation, the flask was checked with an inverted microscope to see if the cells have detached. Then, the flask was added with 4 ml Defined Trypsin Inhibitor (DTI) (Gibco, USA) at 1:2 ratio to neutralize the effects of Accutase after the release of cells from a cell culture surface. Another 3 - 4 ml of complete growth medium was added into same flask and later was pipetted into sterile centrifuge tube and spun at 1000 rpm for 3 min. After that, the waste media was removed leaving the pellet at the bottom. The coating solution from newly matrigel coated T-75 flask was removed and 10 ml of complete growth medium was added. Another 1 ml of same medium was added into the centrifuge tube and mixed well with the pellet. The cells were mixed into flask and for each experiment; cells were seeded at different density. All GBM cells lines were passaged at least once weekly. Cultures were discarded before they reached passage 20 and new frozen samples were cultivated as replacements. Cells were analysed every 2 months for the presence of mycoplasma.

2.1.6 Viable cell counting

The harvested cells were counted using a haemocytometer (Hawksley, Sussex, UK). For this method, adherent cell lines were enzymatically detached from the flask using the appropriate solutions. The cell suspension was then pelleted by centrifugation at 1000 rpm for 3 minutes and re-suspended in 5 or 10 ml of complete growth medium depending on pellet size. 100 µl of the cell suspension was placed in an eppendorf tube and well mixed with 100 µl of trypan blue (Sigma-Aldrich, St. Louis, MO, USA). 10 µl of the cell suspension containing trypan blue was then transferred onto the haemocytometer and the number of live cells (unstained by trypan blue) was counted in 4 sets of 16 corner squares under a phase contrast microscope. To obtain the number of cell per ml, the total count from 4 sets of 16 corners squares was divided by 4, multiplied by 2 to adjust for the 1:2 dilution in trypan blue and multiplied by $10^4$, which accounts for the volume of the haemocytometer. Using this method, a
representative value of the viable cell number per ml in the original cell suspension was obtained.

2.1.7 HeLa cells

The human cervical carcinoma cell line HeLa D98 was kindly provided by Markus Rehm, Physiology Department, Royal College of Surgeons in Ireland, Dublin. This cell line was established in 1951 from a tumour sample of the patient Henrietta Lacks (Silberman, 2010). The cells grow adherent with an epithelial morphology. HeLa cells have a modal number of 82 chromosomes and are 100% aneuploid. This cell line has very low p53 expression level due to the insertion of the human papilloma virus 18 (HPV 18) in the genome. HPV 18 encodes for the E6 oncoprotein, which promotes the rapid degradation of p53 (Scheffner et al., 1990). HeLa cell line were cultivated in Roswell Park Memorial Institute (RPMI) (Lonza, Basel, Switzerland) medium supplemented with 10% heat inactivated FBS, 1% L-glutamine (2 mM), 100 U/ml penicillin and 100 mg/ml streptomycin. Cell line handling was carried out as described for GBM cell lines.

2.1.8 Patient tumour samples

All human GBM patient samples were kindly provided by Dr Donat Koegel, Experimental Neurosurgery, Johann Wolfgang Goethe University Hospital, Frankfurt, Germany. Their use was approved by the local ethics committee of the Frankfurt University Hospital (Murphy et al., 2013). The samples were transported and stored at -80°C prior to experiment. Human tumour samples were obtained as described for GBM cell lines. The pellet resulting from tumour specimen homogenisation was directly mixed with SDS-lysis buffer (62.5 mmol/l Tris-HCl (pH 6.8), 10% Glycerin, 2% SDS, 10 μM protease inhibitor cocktail) and samples were stored at -80°C.
2.2 Calculation of agents used prior to *in vitro* experiment

2.2.1 Treatment of cells with standard chemotherapy TMZ

Temozolomide (TMZ) is a DNA-alkylating agent that indices the intrinsic apoptotic pathway (Roos et al., 2007). TMZ (Sigma-Aldrich, Ireland) has a molecular weight of 194.15 Daltons and comes in 100 mg powder per bottle. A stock solution of 0.1 M was made by adding DMSO (1:10), aliquoted in eppendorf tubes and stored at -20°C. All GBM cell lines were incubated with TMZ at 150 µM, a concentration that has been proven to be pharmacodynamically active in cancer cell lines (Murphy et al., 2013).

2.2.2 Treatment of cells with IAP antagonist Birinapant

TL32711 or Birinapant is a small molecule IAP antagonist currently in phase II clinical trial with proven efficacy in inducing apoptosis in certain non-CNS malignancy such as melanoma, leukaemia, breast and colorectal cancer where the IAPs are frequently overexpressed (Allensworth et al., 2013, Krepler et al., 2013, Benetatos et al., 2014, https://www.clinicaltrials.gov). At 1 µM, Birinapant treatment has been shown to prime cancer cells for death (Allensworth et al., 2013), and to restore the sensitivity to apoptotic stimuli, such as TNFα and TRAIL, in both *in vitro* and *in vivo* experiments (Krepler et al., 2013, Allensworth et al., 2013). In this study, a single and combination treatment with TMZ was investigated for potential of Birinapant to induced cell death in GBM cells. TL32711 (Activebiochem, USA) has a molecular weight of 806.91 Daltons and comes in a powder. A stock concentration of 100 µM was made by adding DMSO and sterile H2O (DMSO volume is 1:10 of H2O), aliquoted in eppendorf tubes and stored at -20°C. For the combination treatment, the drugs were added simultaneously.
2.2.3 Treatment of cells with Caspase-inhibition with zVAD

In order to analyse whether the evident of cell death was caspase dependent, the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (ZVAD) was added as an additional control to the experiments (Li et al., 1997b). ZVAD (Bachem, Germany) binds to the active site of cleaved caspases, thereby inhibiting them. ZVAD has a molecular weight of 467 Daltons and comes in a 1 mg powder. A stock solution of 10 mM was made by adding DMSO (1:10), aliquoted in eppendorf tubes and stored at -20°C. During the experiment, all GBM cell lines were pretreated with ZVAD at 50 µM, one hour prior to treatment.

2.3 In vitro experimental designs

2.3.1 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay

Cellular viability was assessed using a MTT (Sigma-Aldrich, St. Louis, USA). Briefly, the assay was based on the reduction of the soluble yellow tetrazolium MTT salt, via the mitochondria of metabolically active cells, leading to the precipitation of purple water-insoluble formazan crystals (Riss et al., 2013). Relative numbers of viable cells were determined spectrophotometrically following solubilisation of the formazan crystals in DMSO. One the day prior to drug(s) treatment, GBM cell lines were enzymatically detached from the flask using 0.25% of Trypsin-EDTA or Accutase and seeded at a density of 2000 cells in 150 µl of complete growth medium per well in a 96-well plate using multi-channel pipette. Following overnight culture, 50 µl of complete growth medium containing DMSO (control wells) or drug(s) were added in appropriate concentration (treated wells). For the combination treatment, the drugs were added simultaneously. The plates were incubated in 37°C in humidified air with 5% CO₂ for indicated time point (24 hour, 48 hour, 72 hour, 96 hour, 120 hour). On the day of analysis, 50 µl of a fresh sterile filtered solution of MTT/DPBS (5 mg/ml) was added to each well using a multichannel pipette. MTT was
dissolved in sterile PBS, vortexed until completely dissolved and filter sterilised using a 0.2 µm filter (Millipore, Billerica, MA, USA). The plates were then returned to the incubator for 4 hour. The media containing MTT solution was carefully removed from each well and the crystals were dissolved following the addition of 150 µl of DMSO. Finally, the absorbance of the resulting solution was measured at wavelength of 560 nm using micro-plate reader (Tecan Group Ltd, Männedorf, Switzerland). Cell viability was determined by calculating relative changes of absorbance compared to the DMSO control, which was set to 100% viability.

### 2.3.2 Hoechst staining and phase contrast microscopy

Hoechst staining was performed on GBM cells treated with TMZ (150 µM) and/or Birinapant (1 µM) or DMSO for 96 hour. For the combination treatment, the drugs were added simultaneously. Cells were stained with Hoechst 33258 (1 µg/ml; Sigma Aldrich), incubated for 10 min at 37°C. Nuclear morphology was visualized using an Eclipse TE 300 inverted microscope (Nikon, Dusseldorf, Germany) using a 20 x objective. Minimums of 300 cells were counted in three subfields of each culture. Those cells with condensed/fragmented nuclei were deemed apoptotic, counted (using Image J software, National Institute of Health, Bethesda, MD, USA) and expressed as a percentage of total cell number.

### 2.3.3 Flow cytometric analysis

Flow cytometry is a technique for counting cells or other subcellular organelle. It uses the principle of light scattering to generate data from various samples. The samples are passed through a laser path, and as they pass through they intercept the light source. This scatters the light and fluorochromes are excited to a higher energy state. This energy is released as a photon of light, which can be recorded. Flow cytometry was performed on Partec Cyflow ML 16 flow cytometer (Partec, Münster, Germany).
In apoptotic cells, the membrane phospholipid, phosphatidylserine, is translocated from the inner surface of the plasma membrane to the outer surface where it is exposed. Annexin V is a Ca\textsuperscript{2+} dependent phospholipid-binding protein that has a high affinity for phosphatidylserine, and so the Annexin V binds to the phosphatidylserine that is exposed on apoptotic cells (Martin et al., 1995). Annexin V may be conjugated to fluorochromes, such as FITC so that its level can then be detected. Staining with AnnexinV-FITC is usually used in conjunction with PI to distinguish between early apoptotic, late apoptotic and necrotic cell death.

Using two-parameter dot-plots, cells in the early stages of apoptosis will be located on the bottom right of each panel as single positive Annexin V-binding cells, since at this stage cell membrane are still intact and PI cannot enter cells. Healthy cells should be PI negative and Annexin V negative. As the apoptotic process progresses, cell membrane integrity is lost and PI can enter cells leading to a double positive population (top-right hand corner of each panel). Whereas the membranes of dead cells are permeable to PI, hence, necrotic cells would be PI positive and Annexin V negative (Henry et al., 2013).

GBM cells were cultivated in 24-well plates for flow cytometry analysis at 10,000 cells/ well. The cells were pretreated with zVAD (50 µM) for 60 min and then treated with TMZ (150 µM) and/or Birinapant (1 µM) or DMSO. For the combination treatment, the drugs were added simultaneously. Following 96 hour treatment, monolayer cells were harvested with 0.25% of Trypsin-EDTA or Accutase and washed with Hanks or PBS solution. For cell death analysis, cells were incubated in 200 µl binding buffer (10 mM HEPES, 135 mM NaCl, 5 mM CaCl\textsubscript{2}) containing AnnexinV-FITC conjugate (1 µl/ml; Bio Vision, Mountain View, CA, USA) and PI (1 µg/ml; Bio Vision, Mountain View, CA, USA) for 15 min on ice. Following incubation, additional 800 µl ice-cold binding buffer was added before measurement. Cells were subsequently analysed on a flow cytometer. Annexin V-FITC was excited with the 488 nm laser, and fluorescence emission was collected in the FL1 channel through a 520 nm band pass filter. PI was excited with the 488 nm laser, and fluorescence emission was collected in the FL2 or FL3 channel through 575 - 605 nm or 630 nm band
pass filter, respectively. Compensation for crosstalk was performed as required. 1 x 10^6 gated cells were required for each sample.

2.3.4 Western blotting of GBM cell lines

2.3.4.1 Preparation of whole cell extract for western blotting

To examine protein levels and protein expression changes, extracts from whole cells were examined. Cells were seeded at 80,000 cells per well in 6-well plates and were allowed to adhere for 24 hour in the incubator. After that, the medium was replaced with fresh medium containing the treatment drugs and the plates were returned to incubator. Following 96 hour treatment, monolayer cells were harvested with 0.25% of Trypsin-EDTA or Accutase and washed with Hanks or PBS solution, spun at 1000 rpm for 3 min and washed with PBS. Whole cell extracts were homogenized in lysis buffer containing 0.5 mmol/l Tris-HCL (pH 6.8), 10% (w/v) glycerine, 2% (w/v) SDS, and protease and phosphatase inhibitor cocktails (Sigma Aldrich, Ireland) and heated at 95°C for 10 min. The total protein concentration of each whole cell extract was measured using the Micro BCA protein assay (Pierce, Rockford, IL, USA). For calibration, a standard curve ranging from 0 - 12 µg was set up using a bovine serum albumin (BSA) standard. 2 µl of each sample was added to 150 µl 0.9% NaCl and 150 µl of the ABC mixture (ABC mixture consists of reagents A, B and C ratios at 25:25:1). Samples and standards were performed in duplicate. As a control 2 µl of SDS lysis buffer was also measured. Samples and standards were incubated for 45 min and the absorbance was measured at 560 nm. The average absorbances were calculated for each sample and standard. The slope of the BSA standard curve was used to calculate the protein concentration of each sample.
2.3.4.2 Protein separation using SDS-PAGE

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis was carried out as previously described (Sambrook et al., 1989). 10 - 15% resolving gels were casted, the stacking gel was prepared afterwards on top and 10- or 15-well combs were inserted as space holders for sample loading. Percentage of the resolving gel was decided depending on the size of the protein of interest, the smaller the protein the higher the gel percentage used. Protein samples were prepared with SDS loading buffer and denatured at 95°C for 10 min. An equal amount of 20 µg protein was loaded onto each lane of the SDS-polyacrylamide gels. An electric field was applied across the gel, causing the negatively charged proteins to migrate through the gel towards the positive electrode. Gels were run at a voltage of 80 V until the proteins had migrated through the stacking gel and then at 120 V to drive the proteins through the resolving gel. Proteins migrate through the gel depending on their size: small proteins move more easily than larger proteins through the gel, so the smaller the protein the further it migrates through the gel. Along with the protein a molecular weight ladder was also run as a means of determining sample size (Fermentas Page Ruler Plus). The running buffer was composed of 25 mM Tris-Cl (pH 8.3), 250 mM glycine and 0.1% SDS. Once the proteins had migrated through the gel the current was stopped and gels were transferred to nitrocellulose membranes.
Table 2.3 Composition of resolving gel

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<thead>
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<th>Per 10 ml</th>
<th>10%</th>
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</tr>
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Table 2.4 Composition of stacking gel

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2.3.4.3 Protein transfer

Protein transfer was performed either using the iBlot® gel transfer device and the iBlot kit (Invitrogen, Bioscience, Ireland) or manually assembled and transferred using the semi-dry transfer gels. For manual protein transfer, gels were blotted to nitrocellulose membrane (Protean BA 83; Schleicher & Schuell, Germany) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol (v/v), and 0.1% SDS). Membranes were placed on two pieces of Whatmann paper, pre-soaked in transfer buffer and the gel was placed over the gel. Transfer cassettes were assembled and gels were transferred at 18 V for 90 min. The electric charge drives negatively charged proteins from the gel onto the positively charged nitrocellulose membrane. The proteins will form a pattern on the nitrocellulose that is identical to the pattern they created in the gel.

2.3.4.4 Western blot and protein detection

After western blotting, the nitrocellulose membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween (TBS-T) (15 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 0.1% Tween 20) at room temperature for 1 hour. During this period, fresh solution was replaced every 20 min. The blocking step helps to prevent non-specific binding of antibodies to the membranes as the proteins in the blocking solution saturate the non-specific binding sites in the membrane. Membranes were incubated with the primary antibodies overnight at 4°C on a falcon tube rotator.

Primary antibodies are raised against a specific target in animals, most commonly rabbits, mice, rats and goats. The animal is immunized with the antigen of interest and produces antibodies directed against this protein. These specific antibodies can then be extracted from the blood of the animal. A wide range of such antibodies is commercially available. When the membranes are incubated with a specific antibody, the antibody will bind to the target protein on the membrane. Secondary antibodies are directed against the primary antibody and have a probe or marker by which they can be detected, for example biotin.
labelling or horseradish peroxidase labelling. They recognise the primary antibody and attach to them specifically.

After an overnight incubation with the primary antibody, blots were washed with TBS-T three times for 15 min and incubated with anti-mouse, anti-goat or anti-rabbit peroxidase-conjugated secondary antibodies. The blots were incubated at room temperature for one hour and were then again washed with TBS-T three times for 15 min. The blots were developed using the enhanced chemiluminescence detection reagent (Milipore, Ireland). Images were captured using a LAS-3000 Imager equipped with a cooled 12 bit digital CCD camera (FUJIFILM UK Ltd Systems, Sheffield, UK). The chemiluminescence reagent recognises the peroxidase label on the secondary antibody and can thus be detected. To verify an even amount of protein loading, the membranes were probed against β-actin, the ‘housekeeping genes’ that are expressed at high levels constitutively in the sample. All western blots shown are representative of at least two independent experiments with similar results.
<table>
<thead>
<tr>
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M= monoclonal; P= polyclonal
2.3.4.5 Quantitative western blotting and densitometry analysis

For quantitative western blotting, standard curves from HeLa cell extracts (5 – 20 mg) were run concurrently with the GBM cell line and patient lysates (20 mg) to ensure linearity of the signal detection range. The protein concentrations of the antigen of interest within each GBM cell lines and individual GBM patient samples were determined by comparison to signals from HeLa cell extracts, which served as controls. Digital densitometry was used to evaluate the protein levels from the images generated of the western blots. The intensity of each band was calculated using the open source Image J software (http://rsbweb.nih.gov/ij) and Microsoft Excel. Special care was taken not to overexpose any pixels to guarantee accurate quantifications. The background in each lane was subtracted from the intensity of the protein bands. The intensity of the loading control (actin) is then deducted from the intensity of the band of interest to eliminate any differences in protein levels owing to uneven loading, so that each sample is normalized and can be compared. Protein levels are expressed as percentage of HeLa protein expression levels (set to 100%).

To represent calculation as formula:

\[
A = \frac{\text{Intensity of protein band} - \text{background intensity}}{\text{Intensity of actin} - \text{background intensity}}
\]

\[
B = \frac{\text{Intensity of HeLa} - \text{background intensity}}{\text{Intensity of actin} - \text{background intensity}}
\]

Protein expression levels = \((A / B) \times 100\)
2.4 Systems modelling and APOPTO-CELL

APOPTO-CELL is a computational model designed to simulate the late steps of the intrinsic apoptotic pathway, the apoptosome-dependent effector caspase activation of caspase-3 and -7 and the subsequent substrate cleavage (Huber et al., 2007, Rehm et al., 2006). This model is based on a set of ordinary differential equations representing a reaction network of 53 reactions, 19 reaction partners and 75 reaction parameters, previously implemented in MATLAB (Rehm et al., 2006). APOPTO-CELL was developed in HeLa cells and validated in cellulo and by adapting the model to cancer cell lines deficient in apoptosis signalling proteins (Rehm et al., 2006, O'Connor et al., 2008). The reaction network is initiated by cytochrome c-dependent apoptosome formation and mitochondrial Smac release while substrate cleavage by effector caspases is the model output (Huber et al., 2007).

To generate substrate cleavage profiles for the GBM cells, protein expression values for procaspase-3 and -9, XIAP, Apaf-1 and Smac were calculated for each individual patient samples and cell lines in relation to concentration as measured in HeLa cells (Table 2.6). These absolute protein values served as input for APOPTO-CELL. The output displays substrate cleavage by caspase-3 after the release of cytochrome c. Varied substrate cleavage profiles were predicted at 60 min after the release of cytochrome c, ranging from <1% to 100%. Apoptosis susceptibility in GBM tumour resections and GBM cell lines was defined as the ability to efficiently generate >80% substrate cleavage within this 60 min timeframe. A >80% substrate cleavage was chosen as it has previously been determined that this % enables apoptosis to proceed efficiently (Rehm et al., 2006). Drug sensitivity can be interpreted as the ability to perform substrate cleavage by caspase-3 after the release of cytochrome c.
Table 2.6 HeLa protein concentrations for APOPTO-CELL modelling

<table>
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<th>Protein</th>
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<td>Procaspase-3</td>
<td>0.12</td>
<td>Rehm <em>et al.</em>, 2006; supplementary information 2</td>
</tr>
<tr>
<td>XIAP</td>
<td>0.063</td>
<td>Rehm <em>et al.</em>, 2006; supplementary information 2</td>
</tr>
<tr>
<td>Smac</td>
<td>0.126</td>
<td>Estimated to be twice the XIAP concentration (Rehm <em>et al.</em>, 2006; supplementary information 2)</td>
</tr>
</tbody>
</table>
2.5 *In vitro* assessment of luciferase expression was performed using *in vivo* imaging system

*In vitro* assessment of luciferase expression was performed using *in vivo* imaging system (IVIS) Spectrum (Caliper Life Science, Perkin Elmer Company, Hopkinton, MA, USA), which includes a cooled charged coupled device (CCD) mounted on a light-tight imaging chamber, camera power supply, thermoelectric cooling unit, fluorescence light source, and a Windows®-based computer system for data acquisition and analysis. Using Living Image Software version 3.2, *in vitro* bioluminescent images were acquired and the signals measured respectively.

Briefly, cell lines were seeded in triplicate wells of a black 96-well plate (U87RGFP-luc2) or a black 24-well plate (RN1luc) with a clear bottom at varying densities at a volume of 100 µl of complete growth medium. 200 X stock solution of D-luciferin (30 mg/ml) was prepared in sterile water and stored at -20ºC. Prior to imaging, a 1:100 dilution of stock solution (2 x D-luciferin) was made in pre-warmed complete medium to obtain 300 µg/ml solution of D-luciferin. 100 µl of 2 x D-luciferin was added to each well containing 100 µl of complete medium just prior to imaging. This resulted in the final concentration of D-luciferin of 150 µg/ml. The plates were returned to incubator for 20 min prior to imaging. Parameters used to assess *in vitro* bioluminescence were as follows: (i) exposure time: 1 - 60 sec, (ii) binning: medium, (iii) f-stop: 1 and (iv) field of view (FOV): C (12.5 cm). Parameters of *in vitro* bioluminescence and was adjusted to obtain the signal of interest above the noise level (> 600 counts) and below CCD saturation (< 60 000 counts). A region of interest (ROI) was traced for each well containing cells and corresponding total flux photons/sec values were obtained.

2.6 *In vivo* GBM studies

All animal experiments were licensed by the Department of Health and Children, Dublin, Ireland. Protocols were reviewed by the Royal College of
Surgeons in Ireland (RCSI) Animal Research Ethics Committee (AREC). Application of the three Rs (Replacement, Reduction and Refinement) were followed and animal studies were conducted in an ethical manner (Wolfensohn and Lloyd, 2013). 5 - 6 week-old female Foxn1\textsuperscript{mu} T-cell deficient, athymic nude rats (n = 15 animals) were purchased from Charles River Laboratories (Germany). These animals were selected for dose escalation study and surgical resection rat GBM model, and weighed approximately 150 - 180 g at the commencement of treatment. Six-week-old female NOD/SCID (NOD.CB17-Prkdc\textsuperscript{scid}/J) mice (n = 60 animals) were purchased from Charles River Laboratories (UK). These animals were used for intracranial implantation of mouse GBM model and weighed approximately 18 - 25 g at the commencement of treatment. As those animals were immunosuppressed, special care were taken whereby these animals are maintained in Individually Ventilated Cages (IVC)-system and kept in specific SPF-grade scantainer (Scanbur technology, Karlslunde, Denmark). 5 - 6 week-old female Sprague Dawley rats (n = 20 animals) were purchased from Charles River Laboratories, UK. These animals were used for toxicity study and weighed approximately 150 - 200g at the commencement of treatment. We selected to run our studies in female mice and rats due to practical reasons, such as female mice are less aggressive and easier to handle (Sandnabba et al., 1994). Previous studies from our labs also use female animals as a choice for intracranial GBM model (Jarzabek, 2012, Sweeney et al., 2014, O'Halloran et al., 2016). Particularly for mice, they are smaller so require less weight-administered drugs and generally less expensive. The animals were maintained in IVC-system. All animals are kept within the RCSI Biomedical Research Facility (BRF) and allowed to acclimatise for at least 1 week following delivery and prior to commencement of procedures.
2.7 *In vivo* preparation of TMZ and Birinapant

For *in vivo* experiments, Foxn1<sup>nu</sup> T-cell-deficient athymic nude rats, Sprague Dawley rats and NOD/SCID mice bearing intracranial RN1luc tumour were treated with TMZ and/or Birinapant or vehicle. TMZ was prepared in a vehicle solution of 10% DMSO and kept as stock solution. On the day of treatment, the drug was diluted in distilled H<sub>2</sub>O to give a final concentration of 25 mg/kg. Birinapant was kept in its original product (powder). On the day of treatment; the drug was prepared in 20% captisol (Ligand Pharmaceuticals, California) in distilled H<sub>2</sub>O and adjusted to pH 4.0 using concentrated hydrochloric acid (HCl) to give a final concentration of 20 mg/kg.

2.7.1 Oral gavage (p.o.) administration of TMZ or vehicle

The animals were firmly restricted by pulling up the loose skin on the flanks to immobilize the head and straight on the animal's body. A 22 gauge x 25 mm curved oral dosing needle (mice) (Vet Tech Solutions Ltd., Cheshire, UK) or 15 gauge x 78 mm x 2.6 mm tip teflon tube (rat) (AgnTho's AB, Sweden) was inserted down the oesophagus and freshly formulated TMZ or vehicle alone was slowly injected. The needle was carefully withdrawn and immediately following drug administration the animals were returned to their cages and monitored for any adverse effects.

2.7.2 Intraperitoneal (i.p.) administration of Birinapant or vehicle

The animals were firmly immobilised by pulling up the loose skin on the flanks. The head and body were tilted downward. The right lower abdominal wall was disinfected with 70% isopropyl alcohol swabs (Mid Meds Limited, Essex, UK). Freshly formulated Birinapant or vehicle alone was quickly injected into the peritoneal cavity using a 26 gauge 3/8" (10 mm) brown needle (BD Biosciences, Franklin Lakes, NJ, USA). Immediately following drug administration, the animals were returned to their cages and monitored for any adverse effects.
2.8 *In vivo* dose escalation study for establishment of maximum tolerated dose

As Birinapant has never been administered with TMZ, it was important to determine whether the animals would tolerate the drugs. Thus, initially a dose escalation study to determine the maximum tolerated dose using Athymic rats (n = 5) was performed. On each animal, the TMZ dose was maintained at 25 mg/kg p.o for 7 consecutive days, while the Birinapant doses was for i.p every 3 days and 9 doses in total. The experiment was conducted by administering the starting dose of Birinapant at 10 mg/kg i.p. on one animal. After 7 doses of TMZ and three doses of Birinapant, if the animal tolerated the dose then the next animal was started with TMZ at standard dose of 25 mg/kg for 7 consecutive days, while the Birinapant was increased to 15 mg/kg. The experiment was repeated on next animal whereby the dose of Birinapant was escalated by 5 mg/kg until the animals could not tolerate the dose. The highest dose achieved on this experiment was considered as the maximum tolerated dose.

2.9 Blood profile experiment for toxicity study in immunocompetent rats (Pilot study)

Sprague dawley rats were randomly assigned to four groups (n = 5 animals per group). Animals in control group were administered with vehicle agents, p.o. DMSO/H₂O and i.p. Captisol/H₂O. Animals in TMZ group were administered with TMZ p.o formulated at 25 mg/kg for 7 consecutive days. Animals in Birinapant group were administered with Birinapant i.p. formulated at 20 mg/kg every 3 days for 9 doses in total. Finally, animals in the combination group were administered with TMZ and Birinapant.

The health of each rat was checked daily including weight during the study. Handling and blood sample collection was performed by the same experimenter. A week prior to the experiment, each rat was handled daily by the experimenter, including placing the rat in the restrainer device to minimize the stress. Puncture of the lateral vein was performed in the morning while the rat in
the restraining device. No anaesthesia was used for this technique. The base of the tail placed between the index and middle fingers. Pressure was applied to distend the vein, and a 25 gauge x 16 mm needle was inserted into the vein in a cranial direction. The collected blood was transferred straight into 1.2 ml ethylen-diamino-tetracetic-acid tube (S-Monovettes EDTA K₃, Sartstedt, Ireland) for haematology and 1.2 ml tube (S-Monovettes serum clotting activator, Sartstedt, Ireland) for biochemistry profile. The needle was removed and digital pressure applied to the puncture site for 10 sec prior returning the rat to its cage. In this study, four sets of blood sample were collected; 1) Pre-treatment- the bloods were collected one week before any treatment commenced, 2) Week 1- the bloods were collected at the end of first week of treatment, 3) Week 2- the bloods were collected at the end of second week of treatment and 4) Recovery- the bloods were collected one week after the completion of all treatments. Haematology samples were analysed within 12 hours of collection using Sysmex XT-2000iV (Sysmex, Kobe, Japan). All samples for biochemistry profiles were labelled and stored at -20°C until end of study. Later on, using F360 clinical biochemistry analyser (Menarini Diagnostics, Florence, Italy), the biochemical tests from each group/treatment were pooled together and tested as an average. At the end of experiment, the animals were euthanized by carbon dioxide (CO₂) inhalation and cervical dislocation. Data interpretations for both haematology and biochemistry profiles were performed by a veterinary toxicologist Professor Peter O'Brien, School of Veterinary Medicine, University College Dublin, Ireland.

2.10 Microbe PCR Amplification Test (IMPACT) 1

U87RGFP-luc2 and RN1luc cell lines were selected for the animal study. Prior to experiment, these cells were tested for Microbe PCR Amplification Test (IMPACT) 1 and PCR mycoplasma tests (Harlan Laboratory, UK). Briefly, GBM cells were enzymatically detached from the flask using 0.25% of Trypsin-EDTA or StemPro Accutase, counted to 1 x 10⁶ cells and were transferred to Cryo Tube vial. This cells were shipped in -80°C container to Harlan Laboratory (UK) for IMPACT I test (Table 2.7). All cell lines were tested negative. For animals
work, all GBM cell lines were kept in dedicated incubator and specific tissue culture hood. The cells were maintained for maximum 6 - 8 weeks prior to intracranial implantation.
Table 2.7 Lists for agents tested for IMPACT 1

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Test method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectromelia</td>
<td>PCR</td>
</tr>
<tr>
<td>Hantaan</td>
<td>PCR</td>
</tr>
<tr>
<td>Lactate dehydrogenase elevating virus</td>
<td>PCR</td>
</tr>
<tr>
<td>Lymphocytic choriomeningitis virus</td>
<td>PCR</td>
</tr>
<tr>
<td>Minute virus of mice</td>
<td>PCR</td>
</tr>
<tr>
<td>Mouse Adenovirus</td>
<td>PCR</td>
</tr>
<tr>
<td>Mouse cytomegalovirus</td>
<td>PCR</td>
</tr>
<tr>
<td>Mouse hepatitis virus</td>
<td>PCR</td>
</tr>
<tr>
<td>Mouse K virus</td>
<td>PCR</td>
</tr>
<tr>
<td>Mouse norovirus</td>
<td>PCR</td>
</tr>
<tr>
<td>Mouse Parvovirus</td>
<td>PCR</td>
</tr>
<tr>
<td>Mouse polyoma virus</td>
<td>PCR</td>
</tr>
<tr>
<td>Mouse Rotavirus (EDIM)</td>
<td>PCR</td>
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<tr>
<td>Mouse Thymic virus</td>
<td>PCR</td>
</tr>
<tr>
<td>Pneumonia virus of mice</td>
<td>PCR</td>
</tr>
<tr>
<td>Reovirus type 3</td>
<td>PCR</td>
</tr>
<tr>
<td>Sendai virus</td>
<td>PCR</td>
</tr>
<tr>
<td>Theiler’s murine encephalomyelitis virus</td>
<td>PCR</td>
</tr>
<tr>
<td>Bacteria, Mycoplasma</td>
<td></td>
</tr>
<tr>
<td>Mycoplasma spp</td>
<td>PCR</td>
</tr>
</tbody>
</table>
2.11 Bioluminescence imaging (BLI) for assessment of tumour volume

2.11.1 In vivo optical imaging

In vivo BLI was performed using the IVIS Spectrum (Caliper Life Science, PerkinElmer Company, Hopkinton, MA, USA). Before in vivo BLI, the heads of each mouse were shaved to decrease attenuation and scattering of transmitted light by hair. Approximately 5 min following an i.p. injection of 150 mg/kg of D-luciferin (Caliper Life Science, PerkinElmer Company, Hopkinton, MA, USA), animals were anaesthetized with isoflurane/ O₂ mixture and BLI began 10 min after D-luciferin administration. Isoflurane/ O₂ anaesthesia was maintained during imaging by nose cone delivery. In vivo BLI of luciferase expression were attained using a 1-120 sec exposure time with medium binning, f-stop of 1 and field of view D (22.5 cm, when 5 mice imaged at the same time) or C (12.5 cm, when 3 mice imaged at the same time). Parameters were adjusted to obtain the in vivo bioluminescent signal above the noise level (recommended > 600 counts) and blow CCD saturation (< 60 000 counts). A region of interest (ROI) was traced around the tumour area manually and corresponding total flux photons/sec values were obtained for each image.

2.11.2 Ex vivo optical imaging

Shortly following necropsy, brains were washed in D-PBS and placed on sterile petri dishes inside IVIS Spectrum. To assess ex vivo bioluminescent signal, 200 µl of D-luciferin (15 mg/ml) was added topically right before ex vivo imaging. Ex vivo BLI images were attained using a 1-60 sec exposure time with medium binning, f-stop of 1 and field of view C (12.5 cm).
2.12 Intracranial implantation of tumour cells and surgical resection of tumour in GBM model

2.12.1 Intracranial implantation of tumour cells in rat GBM model

In this study, 10 female Athymic rats were selected for intracranial implantation of GBM cells. The same model and number of animals was previously been used by our group for the establishment of surgical resection model using U87MG cell lines (Sweeney et al., 2014). However, U87RGFP-luc2 is an invasive variant of U87MG cell lines, which is more clinically relevant (Jarzabek, 2012). This experiment will enable us to validate an exponential growth phase in rats using bioluminescent signal and comparing between these GBM cell lines. Directly before tumour cells implantation, the animals will be weighed and anaesthetized via i.p. delivery of anaesthetic ketamine (80 mg/kg) (Narketan 10%, Vétoquinol, Lure cedex, France)/Xylazine (10 mg/kg) (Xylapan®, Vétoquinol, Lure cedex, France). Respiration was closely monitored along with the general condition of the animal. Skin was prepared by removing hair with a depilatory cream. The rats were then fixed in a stereotactic frame and the skin was disinfected with alcohol. A small right parasagittal skin incision was made followed by a craniectomy with a high speed dental drill at the level of the bregma 3 mm right of the midline. After the dura was punctured, 2 µl of cell suspension containing $1 \times 10^6$ U87RGFP-luc2 cells were aspirated to a Hamilton syringe. The syringe was loaded into the stereotactic arm and cell suspension slowly injected at a depth of 2.5 mm. The syringe was left in place for 5 min prior to withdrawal. Any residual cell suspension was removed. The skin was closed in a single layer with monocryl 4/0 interrupted simple sutures (Ethicon Inc., Novartis Animal Health Inc., Basel, Switzerland). After surgery, the animals were placed in a recovery chamber before being returned to their cages. Close observation of the animals was maintained until recovery from anaesthesia and resumption of normal movement around the cage. Following tumour cells implantation, animals were health checked daily with animal weights. Any changes in general condition/behavior were noted in score sheets. These animals were then subjected to bioluminescence imaging and
randomized into two groups for surgical resection models (further details will be described).

### 2.12.2 Intracranial implantation of tumour cells in mouse GBM model

Animals were weighed and anesthetised by i.p. administration of Ketamine (100 mg/kg) (Narketan 10%, Vétoquinol, Lure cedex, France)/ Xylazine (5 mg/ kg) (Xylapan®, Vétoquinol, Lure cedex, France). The head of the recipient mouse was secured in a stereotactic frame (David Kopf, Tujunga, CA, USA). Local analgesia/ anaesthesia (7 mg/kg of Lidocaine) was applied 3 min before a short longitudinal incision was made. A burr-hole was prepared 0.5 mm posterior to the bregma and 2.5 mm to the right of the sagittal suture using a micro-drill. 5 x 10^5 RN1luc cells were implanted into the cerebral cortex of mice. The GBM cells were slowly injected, using an appropriate 27 G Hamilton needle for cell line implantation (Hamilton, Bonaduz, GR, Switzerland) at 2 mm depth below the brain surface. The syringe was left in place for 5 min prior to withdrawal. The skin was closed with a monocryl 4/0 interrupted simple sutures. After surgery, the animals were placed in a recovery chamber before being returned to their cages. Close observation of the animals was maintained until recovery from anaesthesia and resumption of normal movement around the cage. Following tumour cells implantation, animal’s health were checked daily with animal weights. Any changes in general condition/ behavior were noted in score sheets. These animals were then subjected to bioluminescence imaging and randomized for the efficacy study and surgical resection models (further details will be described).

### 2.12.3 Validation of surgical resection model (Pilot study)

In this study, 10 female Athymic rats and NOD/SCID mice were inoculated with U87RGFP-luc2 and RN1luc cells, respectively (as described above). The number of animals used in the surgical resection model was similar to previously published by our group (Sweeney et al., 2014), which has also
identified minimum number required to reach statistical significance. When tumours were in exponential growth phase as determined by bioluminescence signal, animals were randomized into 2 groups: non-resection (control) and resection group. Bioluminescence imaging (BLI) was performed a day prior to surgery. Rats and mice were weighed and anaesthetised using similar concentration of anaesthetic drugs (as described above). No antibiotics were administered for the experiment. Animal respiration and general condition were closely monitored. The skin was prepared by removing hair with a depilatory cream. The animals were then fixed in a stereotactic frame and the skin was prepared in standard surgical fashion. The previous skin incision was extended in a curvilinear fashion and tissues reflected back. The temporalis muscle was sharply dissected from the cranium and reflected. The coordinate of previous intracranial implantation and the BLI prior to tumour resections were used as a landmark. Using an operating microscope and high-speed dental drill, a 1.5 mm craniectomy was made centred on the landmark. A durotomy was made and surface of the brain identified. Under an operating microscope, a macroscopic surgical plane was developed and a micro dissector was used to remove the tumour. Homeostasis was achieved by applying a sterile swab on the area for a few min. The surgical cavity was irrigated and filled with sterile saline. The skin was closed with monocryl 4/0 interrupted simple sutures and cleaned with alcohol. Animals underwent immediate post-operative BLI and were returned to their cage when fully recovered. Animals were weighed daily and assessed for wound infection and general condition.

2.12.4 Efficacy studies of TMZ and/or Birinapant in NOD/SCID mice

In this study, 50 NOD/SCID female mice were inoculated with RN1luc cells, as described above. The sample study calculations were extrapolated from the established intracranial GBM model performed by our group (Jarzabek, 2012, Jarzabek et al., 2013). Randomisation was then performed in order to reduce selection bias, generate comparable results particularly between treated and untreated animals, which is needed for testing the treatment efficacy. The experiment involves comparing the tumour volumes following treatment(s), and
animals within the same cage must have the same treatment(s), hence the experimental unit would be the cage of animals and the metric used in the statistical analysis would be cage means of tumour volumes (Festing et al., 2002). A completely randomised design was selected, in which experimental units are allocated to a treatment at random, with n = 10 animals per group.

Animals were randomised into five groups:

Group 1: Control (untreated) (n = 10)
Group 2: Vehicle (n = 10)
Group 3: TMZ (25 mg/kg) p.o. daily for seven consecutive days (n = 10)
Group 4: Birinapant (20 mg/kg) i.p. every three days for nine doses (n = 10)
Group 5: TMZ + Birinapant (n = 10)

Kaplan-Meier survival analysis was performed and median survival was calculated.

2.13 Histology

2.13.1 Tissue processing for haematoxylin and eosin (H&E) staining

Excised brains and other organs were placed in 10 ml of 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, USA). A standard tissue processing and haematoxylin and eosin (H&E) staining were kindly performed by the histopathology technician in School of Veterinary Medicine, University College Dublin, Ireland. Further analysis and data interpretation was performed by a veterinary pathologist Professor Sean Callanan, School of Veterinary Medicine, University College Dublin, Ireland.
2.13.2 Immunohistochemistry for luciferase antibody

Formalin-fixed paraffin-embedded (FFPE) tissue sections were placed on a heating plate at 65°C for 10 min and melted paraffin was wiped off. Following de-paraffinization, performed in xylene, the tissue sections were re-hydrated in descending gradient alcohols (2 x 3 min in 100% ethanol and 2 x 3 min in 96% ethanol/dH₂O) and dH₂O (1 x 2 min). Heat-mediated antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6.0) in microwave (5 min at high power, 10 min at low power). The sections were then cooled on bench for 20 min and washed in TBS-T (2 x 5 min). All slides were blocked with 200 ul per slide of 5% serum/TBS-T (serum from the same species as the secondary antibody) for 1 hour at room temperature, followed by incubation with goat anti-luciferase (1:50 dilution) (Promega, Madison, WI, USA) primary antibody for 90 min. Following incubation, all unbound antibody was removed by washing the slides in TBS-T (3 x 5 min). The endogenous peroxidases were blocked by incubating the sections with peroxidase blocking reagent (Dako, Glostrup, Denmark) for 10 min following washing in TBS-T. The appropriate biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) was diluted (1:200) and applied to each slide for 1 hour at room temperature. Again, all unbound antibody was removed by washing with TBS-T (2 x 5 min). A drop of ABC complex (VECTASTAIN® ABC system, Vector Laboratories, Burlingame, CA, USA) was applied to the slides for 30 min at room temperature to allow the Avidin-Biotin Complex to form. All unbound Avidin complex was removed by washing with TBS-T (2 x 5 min) after which the diaminobenzidine (Liquid DAB+, Dako, Glostrup, Denmark) was applied for 1 - 10 min. This highly sensitive substrate chromagen system is used in the peroxidase-based immunohistochemical staining methods resulting in a very stable, brown stain located wherever the primary antibody is bound. The reaction was stopped by washing slides in running tap water before haematoxylin staining was performed for 20 sec. Excess haematoxylin was rinsed in running tap water until the water was no longer stained blue. Finally, the slides were dehydrated in ascending gradient alcohols (2 x 3 min in 96% ethanol/dH₂O and 2 x 3 min in 100% ethanol). Following, clearing with xylene, the DPX mounting medium
(Sigma-Aldrich, St. Louis, MO, USA) was used to mount cover slips on the sections.

### 2.14 Statistical methods

The statistical analysis was performed using SAS Version 9.2, Prism v6.0c (Graphpad), MATLAB (MATLAB and Statistics Toolbox Release 2014b, The MathWorks) and R (version 3.2.0). A one-way ANOVA followed by Tukey-Kramer post-hoc test was used to analyse the response of the TMZ and/or Birinapant treatments versus control for MTT assay. For flow cytometry and Hoechst staining, a two-way ANOVA followed by Tukey-Kramer post-hoc test was used to analyse the response to 8 different combinations of TMZ, Birinapant and zVAD and the experiment-to-experiment variation in these cell lines. A nominal 5% level of significance was used. Data are shown as boxplot with overlaid data points for flow cytometry and Hoechst experiments. In each boxplot, the upper and lower edges represent the 25th and 75th percentile; the ticker central line indicated the median and the whiskers cover 1.5 times the interquartile range. For the in vivo experiment, linear mixed-effects models were used to analyze changes in bioluminescence over time and Kaplan-Meier curves plus log-rank tests were used to compare survival times between treatment groups. The mixed-effects analysis included a random effect for each mouse plus group fixed effects, linear time effects and group*time interaction. Likelihood-ratio tests were used to determine overall differences in trends between groups. A nominal 5% level of significance was used. To account for multiple testing, a Bonferroni-adjusted significance level of 0.5% (p-value < 0.005) was implemented.
CHAPTER 3:
Combination effect of Temozolomide and Inhibitor of apoptosis protein (IAP) antagonist, Birinapant (TL32711), in in vitro glioblastoma cell lines
3.1 Introduction

3.1.1 Apoptosis and cell death are regulated by various pro- and anti-apoptotic proteins

Many human tumours such as high-grade glioma express high levels of IAPs; this has been linked to defective apoptosis, therapy resistance and poor prognosis (LaCasse et al., 2008, Gillissen et al., 2013). Irradiation and many chemotherapy agents trigger tumour cell apoptosis via the intrinsic pathway as an indirect consequence of causing cellular damage (Ashkenazi, 2002); however, in many cases, crosstalk between the intrinsic and extrinsic pathways are still needed for cells to die.

Many apoptotic inhibitors are known to prevent proper apoptosis induction. Cellular FLICE-inhibitory protein (cFLIP) is able to modulate activation of procaspase-8 (Kataoka, 2005). cFLIP is highly expressed in GBM (Grund et al., 2008, Bellail et al., 2010) and interacts with FADD and caspase-8 to block caspase-8 binding to the DISC (Irmler et al., 1997). Bcl-2 molecules can bind to tBid and Bax and thereby inhibit formation of active Bax dimers (Raychaudhuri and Das, 2013). High levels of human IAPs has been associated with dysregulation the apoptotic balance (Mesri et al., 2001). The binding of survivin to XIAP increases the stability of latter, and reinforces the antiapoptotic activity of this IAP-IAP complex (Dohi et al., 2004). Survivin and XIAP are direct inhibitors of caspases (Deveraux and Reed, 1999), and the presence of survivin correlated with reduced apoptotic index in vivo (Mesri et al., 2001). cIAPs modulate the NFκB pathways (Gyrd-Hansen and Meier, 2010, Tenev et al., 2011). cIAP1 and cIAP2 are also negative regulators of RIP1; high levels of these inhibitors prevent the association of RIP1 with FADD-caspase-8 (Tenev et al., 2011, Varfolomeev et al., 2007).

The discovery of RIP1 kinase has been of interest. Previous analysis of glioma patient samples showed that RIP1 is overexpressed in ~30% of GBM but not in low-grade gliomas (Park et al., 2009). Under normal conditions, RIP1 is constitutively ubiquitinated by cIAP proteins and the ubiquitinated RIP1 serves
as a signaling platform for the activation of NFκB pathways (Bai et al., 2014). Inhibitions of cIAPs, such as by Smac or Smac mimetics, switch the function of RIP1 and canonical NFκB pathway from pro-survival to pro-apoptotic.

3.1.2 Birinapant targets inhibitor-of-apoptosis-proteins

As shown in Figure 3.1, Birinapant targets IAP proteins. Downregulation of cIAPs result in the activation of non-canonical NFκB signalling (Lau and Pratt, 2012), which subsequently upregulates NFκB target genes, such as TNFα (Fulda, 2014). Autocrine produced TNF stimulates TNR receptor 1 (TNFR1) and, in the absence of cIAP proteins, ubiquitination of RIP1 does not occur promoting the assembly of the RIP1/FADD/caspase-8 complex (Bai and Wang, 2014, Witt et al., 2015). Caspase-8 cleaves RIP1 and prevents it from delivering TNF signals, subsequently abolishing the induction of anti-apoptotic factors (Lin et al., 1999). As displayed in Table 3.1, Birinapant has been trialled in different cancer cell lines, but not yet in GBM cell lines.
Figure 3.1 Schematic presentation of Birinapant (TL32711) in inducing death of tumour cells

Smac mimetics are IAP antagonists that can exert their effect on both intrinsic and extrinsic pathways. Inhibition of cIAP can switch the canonical NF-κB signalling pathways from pro-survival to pro-apoptotic. Activation of the non-canonical NF-κB pathway increases autocrine TNF production, which further increases ligand binding to the death receptors. Activation of the intrinsic pathway can occur either from direct action by caspase-8 or from inhibition of IAPs, which increases the availability of caspase-3 and -7 to induce apoptosis of cancer cells. From (http://tetralogicpharma.com/birinapant/).
Table 3.1 List of previous studies using Birinapant as a single agent or in combination with other treatment

<table>
<thead>
<tr>
<th>Authors</th>
<th>Conditions</th>
<th>Single and/or combination treatment</th>
</tr>
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<tbody>
<tr>
<td>(Lalaoui et al., 2016,</td>
<td>Acute myeloid leukemia</td>
<td>LY2228820</td>
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<tr>
<td>Carter et al., 2014,</td>
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<td>Mak et al., 2014)</td>
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<td></td>
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<td>Decitabine</td>
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<td>(Amaravadi et al.,</td>
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<td>Carboplatin</td>
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<td>Janzen et al., 2015)</td>
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<td>(Ebert et al., 2015)</td>
<td>Chronic hepatitis B infection</td>
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<td>Benetatos et al., 2014)</td>
<td></td>
<td>Doxetaxel</td>
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<td>(Allensworth et al., 2013)</td>
<td>Melanoma</td>
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<td>(Ma et al., 2012, Zhu et</td>
<td>Pancreatic adenocarcinoma</td>
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While Birinapant has proven efficacy in inducing apoptosis in certain non-CNS malignancies where IAPs are frequently overexpressed (Allensworth et al., 2013, Krepler et al., 2013, Benetatos et al., 2014), little however is known about the efficacy of Birinapant in CNS malignancies such as GBM, particularly when treatment is combined with the standard chemotherapy agent, TMZ. In this present chapter, we explore the potential of Birinapant as a therapeutic agent in GBM. Here, we performed a comprehensive study to explore the sensitivity of a large panel of GBM cell lines to Birinapant as a single agent and in combination treatment with TMZ. We looked at the response pattern of GBM cell lines and at the mechanism of cell death induced by Birinapant alone and in combination with TMZ.

### 3.1.2 Aims

In an effort to improve the performance of GBM therapy, our first aim is to investigate the effectiveness of the bivalent Smac mimetic, Birinapant, in a panel of 16 GBM cell lines and to analyse the advantage of combining Birinapant therapy with TMZ. Our second aim is to evaluate IAP proteins levels in GBM cell lines treated with TMZ and Birinapant alone and in combination. Our third and last aim is to identify the response pattern to cell death, particularly by analysing the effect of zVAD, nuclear condensation, and extrinsic and intrinsic pathway activation following the same treatment paradigm.
3.2 Results

3.2.1 Glioblastoma cell lines show a heterogeneous sensitivity to TMZ and Birinapant monotherapy with enhanced sensitisation achieved with combined treatment

To reflect the heterogeneity of GBM cells, we used a panel of sixteen GBM cell lines, which consisted of six commercially available GBM cell lines (U87, U87RGFP, U251, A172, U343 and U373), a cohort of cell lines derived from patient GBMs including four primary (MZ294, MZ327, MZ51 and MZ18) and two recurrent (MZ304 and MZ256) (Hetschko et al., 2008), and four patient-derived GBM cell lines that were stably transfected with luciferase gene (JK2luc, WK1luc, RN1luc and SJH1luc) (Tivnan et al., 2014, Day et al., 2013). The cell lines were incubated with TMZ at 150 µM, and/or with Birinapant at 1 µM. For the combination treatment, the drugs were added simultaneously. Analysis was performed using MTT cell viability assay. For the purpose of statistical calculation, a one-way ANOVA followed by Tukey-Kramer post-hoc test was used to analyse the response of the TMZ and/or Birinapant treatments versus control.

Following incubation at five time points, three distinct response patterns were observed: TMZ-sensitive cells, Birinapant-sensitive cells, as well as cells that were insensitive to either treatment were observed. During the first 48 hour of incubation, TMZ and Birinapant monotherapies were not effective at inducing cell death (Figure 3.2 and 3.3). The cells responded to TMZ treatment earlier than Birinapant treatment, with two of the commercially available cell lines (A172 and U251) showing TMZ-sensitivity at 72 hour post treatment onset (p < 0.05). All commercially available GBM cell lines showed persistent reduction in cell survival at 96 hour of treatment (p < 0.01) (Figure 3.2 A). On the other hand, the TMZ-resistant group were the cell lines that did not show statistically significant reduction of cell survival despite the prolonged incubation period. Those cell lines comprised of all patient-derived GBM cell lines. (Figure 3.2 B, C). In contrast, the Birinapant-sensitive cells, comprised of a mixture of the commercial and patient-derived GBM cell lines (A172, U251, U87, U87RGFP
and JK2luc), showed a moderate reduction in cell survival, which reached significant value at 96 hour treatment \((p < 0.05)\). Eight of the sixteen cell lines investigated were sensitised at 120 hour (Figure 3.3 A). The rest of the cells did not reach significant value (Figure 3.3 B, C).

We next explored whether the combination of TMZ plus Birinapant induced a further reduction in cell survival in cell lines resistant and sensitive to monotherapy. We found that the combination treatment further categorised the cell lines into four distinct response patterns. During the first 48 hour of the incubation, two cell lines (A172 and U251), which were sensitive to both TMZ and Birinapant single treatments, were below 80% survival in the combined regimen (Figure 3.4 A), with further reductions observed at 72 hour of treatment \((p < 0.05)\). A similar effect was observed in the TMZ-sensitive U373 cell line, where a significant reduction in cell survival was evident at 72 hour of treatment, and in two Birinapant-sensitive cell lines (RN1luc and JK2luc) at 96 hour \((p < 0.05)\) (Figure 3.4 B). Two cell lines (WK1luc and MZ18) that were resistant to both single treatments reach statistical value \((p < 0.05)\) after 96 hour of combination treatment, with a further (MZ327 cell line) at 120 hour \((p < 0.05)\) (Figure 3.4 C). Nevertheless, statistical analysis showed no significant reduction on cell survival in four GBM cell lines (MZ294, SJH1luc, MZ51 and MZ256) even at 120 hour (Figure 3.4 D).

Together this set of experiments demonstrates that GBM cell cells show different sensitivity towards TMZ and Birinapant single treatment, with a subset of cell lines overcoming resistance following combination treatment. For subsequent experiments, we wanted to look at the effect of single and combination treatment on IAP proteins. Therefore, we randomly selected three commercially available GBM cell lines (U87, U87RGFP and U251), two primary and recurrent MZ-derived patient GBM cell lines (MZ294 and MZ304), and three patient-derived GBM cell lines that were stably transfected with luciferase gene (RN1luc, WK1luc and JK2luc).
Figure 3.2 Effect of TMZ treatment on cell survival in a panel of GBM cell lines

All GBM cell lines were treated with TMZ for the indicated time points. The characteristics of TMZ-sensitive cells were observed at the beginning of 72 hour treatment (A). All patient derived GBM cell lines were resistant to the treatment (B, C). Data are expressed as mean ± S.E.M from at least three independent experiments (one-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001).
Figure 3.3 Effect of Birinapant treatment on cell survival in a panel of GBM cell lines

All GBM cell lines were treated with Birinapant for the indicated time points. The first 72 hour of the treatment did not show significant activity on cell death. At 120 hour, eight GBM cell lines showed the characteristics of Birinapant-sensitive (A). The rest of the cell lines were resistant to the treatment (B, C). Data are expressed as mean ± S.E.M from at least three independent experiments (one-way ANOVA, *p < 0.05).
Figure 3.4 Effect of combination treatments on cell survival in a panel of GBM cell lines

All GBM cell lines were treated with TMZ and Birinapant for the indicated time points. All cell lines sensitive to TMZ and/or Birinapant monotherapies show further reductions in cells survival following combination treatments (A, B). Three of the cell lines that were initially resistant to both TMZ and Birinapant monotherapies benefited from combined treatment (C). Only four of the sixteen cell lines did not show statistical reduction following the combination treatments (D). Data are expressed as mean ± S.E.M from at least three independent experiments (one-way ANOVA, *p < 0.05, **p < 0.01, ***p < 0.001).
3.2.2 Evaluation of IAP proteins levels in glioblastoma cell lines

To validate that Birinapant targeted IAP proteins, we randomly selected several GBM cell lines and conducted a western blot analysis of cIAP1, cIAP2 and XIAP protein expression levels. The results of three commercially available GBM cell lines (U87, U251 and U87RGFP) are shown in Figure 3.5 A; two MZ-derived patient GBM cell lines (MZ294 and MZ304) are shown in Figure 3.5 B; and three patient-derived GBM cell lines that were stably transfected with luciferase gene (RN1luc, WK1luc and JK2luc) are shown in Figure 3.5 C. The U87RGFP cell lines represent a highly invasive U87 cells, which were previously established through serial in vivo selection of tumour cells that have invaded out of the tumour mass (Johnston et al., 2007, Jarzabek, 2012).

Following 96 hour treatment, Birinapant as a single agent causes the degradation of cIAP1, as the protein is autoubiquitinated and degraded via the proteasome in response to IAP antagonist treatment (Fulda, 2014). We observed degradation of cIAP1 in all selected GBM cell lines, with U251 cells showing complete degradation only in response to TMZ and TMZ plus Birinapant treatment. In all other cell lines, the cIAP1 degradation was not further enhanced by the addition of TMZ. There was little evidence for changes in cIAP2 and XIAP protein levels in line with the fact that autoubiquitination and proteasomal degradation is a feature of cIAP1 but not cIAP2 and XIAP (Vince et al., 2007, Condon et al., 2014). An exception to this were U251 cell line, where the exposure to TMZ alone and in combination with Birinapant induced degradation of cIAP2 and XIAP, and MZ304 cell line where the exposure to Birinapant alone or in combination with TMZ induced degradation of XIAP.

Together, this experiment demonstrated high specificity to cIAP1 following Birinapant treatment with little evidence for significant changes in cIAP2 and XIAP protein levels. The cIAP1 degradation was not enhanced when the treatment was combined. Following this finding, using three different experimental techniques, we wanted to look at further details of the apoptotic cell death pathways activation.
Figure 3.5 Effects of single and combination treatments on IAPs expression profiling

Birinapant as a single agent causes the degradation of cIAP1 in all selected GBM cell lines, with U251 cells showing complete degradation only in response to the TMZ plus Birinapant or TMZ single treatment. There was no variability on cIAP2 and XIAP, except for U251, JK2luc and MZ304 cell lines. Exposure to TMZ alone and in combination with Birinapant induced degradation of cIAP2 (U251) and XIAP (U251 and JK2luc). Exposure to Birinapant alone or in combination with TMZ induced degradation of XIAP in MZ304 cell line. Western blots shown are representative of at least two independent experiments with similar results, with β-actin used as a loading control.
3.2.3 Cell death analysis after TMZ and Birinapant single and combined treatments identifies Type A response pattern

In order to further elucidate this apparent heterogeneity in treatment responses, we next performed a more detailed analysis of the cell death pathways activated in response to TMZ and Birinapant. Flow cytometry analysis of propidium iodide (PI) uptake and Annexin V-FITC staining was conducted following 96 hour of treatment with single or combination therapies, both in the presence and absence of the caspase inhibitor, zVAD. Using two-parameter dot-plots for Annexin V-FITC (FL1 channel) and PI (FL2 channels), cells positive for Annexin V-binding (as a marker for apoptosis activation) but with an intact cell membrane will be located in the bottom right of each panel; Annexin V-FITC- and PI-positive cells in later stages of apoptosis as double positive cells at the top-right hand corner of each panel; and cells undergoing necrotic cell death in the absence of Annexin V-FITC staining in the top left of each panel (Henry et al., 2013). To further characterize morphology of GBM cell lines, the condensation and fragmentation of nuclei was imaged using Hoechst staining.

Cell death analysis in response to single and combined treatments identified three major principal response patterns. The first response pattern (‘Type A’ cells) was seen in the TMZ-sensitive U251 cell line. Flow cytometry analysis showed significant apoptotic and secondary necrotic (i.e. Annexin-V and PI-positive) cell death in response to TMZ but not Birinapant, which was only partially sensitive to zVAD treatment (Figure 3.6 A-D). From the morphological analysis of nuclear condensation, TMZ plus Birinapant treatment showed a small increase of apoptotic cell death (Figure 3.7 A, B). Western blot analysis after 96 hour of treatment demonstrated processing of pro caspase-8 into p41 and p18 subunits in TMZ and TMZ plus Birinapant treatment, indicative of caspase-8 activation. This was accompanied by an increase in FADD and a decrease in RIP1 and cFLIP proteins (Figure 3.7 C). A similar event was observed in caspase-3 activation, which demonstrated detectable p19/17 subunits on western blot. This was accompanied by PARP cleavage (Figure 3.7 D).
Following 96 hour treatment, Type A response pattern was identified in U251 cell line. A significant apoptotic and secondary necrotic (i.e. Annexin-V and PI-positive) cell death in response to TMZ but not Birinapant was observed, with partial sensitivity to zVAD treatment (A-D). Data is shown as boxplot with overlaid data points from at least three independent experiments with technical replicates (two-way ANOVA, *p < 0.05, ***p < 0.001 versus control cells).

Figure 3.6 Type A response pattern in TMZ-sensitive U251 cell line (flow cytometric analysis)
Following 96 hour treatment, a morphological analysis of nuclear condensation showed a significant apoptotic cell death in response to TMZ but not Birinapant (A, B). Data is shown as boxplot with overlaid data points from at least three independent experiments with technical replicates (two-way ANOVA, ***p < 0.001 versus control cells). Scale bar = 50 µm. Protein expressions showed that the TMZ alone and in combination accelerate caspase-8 cleavage and induced reduction of RIP1 and cFLIP, and further increased of FADD expression (C). A similar activation of caspase-3 and PARP were also observed (D). Western blots shown are representative of at least two independent experiments with similar results, with β-actin used as a loading control.
3.2.4 Cell death analysis after TMZ and Birinapant single and combined treatments identifies Type B response pattern

A second principal response pattern ('Type B' cells) was seen in the Birinapant-sensitive WK1luc and JK2luc cell lines. Following 96 hour treatment, flow cytometry analysis of WK1luc cell line revealed significant apoptotic and secondary necrotic cell death in response to Birinapant or Birinapant plus TMZ, but not to TMZ alone (Figure 3.8 A-D). Cell death was completely inhibited by zVAD. These findings were confirmed by quantitative analysis of cell death using Hoechst staining (Figure 3.9 A, B). Western blot analysis indicated that Birinapant treatment readily activated caspase-8, and led to an increase in FADD and reduction in RIP1 protein levels. There is no variation of cFLIP protein expression (Figure 3.9 C). A similar activation of caspase-3 and PARP were also observed when treated with Birinapant and Birinapant plus TMZ (Figure 3.9 D).

For JK2luc cell line, western blot analysis also confirmed activation of caspase-8 and caspase-3 when cells were treated with Birinapant and Birinapant plus TMZ (Figure 3.10 A, B). However, the activation of both caspases was also evident in the TMZ treatment group, though the expression of these proteins was weaker when compared to other treatments. In addition, there was no variation in RIP1 and cFLIP expressions, as well as no activation of PARP.
Following 96 hour treatment, Type B response pattern was identified in WK1luc cell line. Using flow cytometry technique, Birinapant and Birinapant plus TMZ revealed significant apoptotic and secondary necrotic cell death. Cell death was completely inhibited by zVAD (A-D). Data is shown as a boxplot with overlaid data points from at least three independent experiments with technical replicates (two-way ANOVA, ***p < 0.001 versus control cells, +++p < 0.001 versus treated cells).
Figure 3.9 Type B response pattern in Birinapant-sensitive WK1luc cell line (Hoechst staining and western blot analysis)

Following 96 hour treatment, a morphological analysis of nuclear condensation showed that Birinapant and Birinapant plus TMZ revealed significant apoptotic cell death (A, B). Data is shown as a boxplot with overlaid data points from at least three independent experiments with technical replicates (two-way ANOVA, ***p < 0.001 versus control cells). Scale bar = 50 µm. Western blot analysis indicated that Birinapant treatment readily activated caspase-8, and led to an increase in FADD and reduction in RIP1 protein levels (C). A similar activation of caspase-3 and PARP were also observed when treated with Birinapant and Birinapant plus TMZ (D). Western blots shown are representative of at least two independent experiments with similar results, with β-actin used as a loading control.
Figure 3.10 Type B response pattern in Birinapant-sensitive JK2luc cell line

Following 96 hour treatment, Type B response pattern was identified in JK2luc cell line. Western blot analysis demonstrated that Birinapant and Birinapant plus TMZ treatment induced activation of caspase-8. There were no variations of RIP1 and cFLIP proteins on any treatment plan, while strong expression of FADD proteins was observed in Birinapant and Birinapant plus TMZ treatment (A). A similar activation of caspase-3 was observed but this event did not lead to PARP cleavage (B). Western blots shown are representative of at least two independent experiments with similar results, with β-actin used as a loading control.
3.2.5 Cell death analysis after TMZ and Birinapant single and combined treatments identifies Type C response pattern

A third response pattern (‘Type C’ cells) was seen in RN1luc, MZ294, MZ304, U87 and U87RGFP cell lines. Following 96 hour treatment, flow cytometry analysis of RN1luc cell line showed moderate apoptotic cell death with the combined treatment, which reached the level of statistical significance in the flow cytometry (p < 0.05) and Hoechst staining (p < 0.01). This sensitization was sensitive to zVAD treatment (Figure 3.11 and 3.12 A-B). Western blot analysis demonstrated a loss of RIP1 expression following Birinapant treatment but addition of TMZ reversed the effect of Birinapant. Neither single nor combination treatment induced any loss to FADD and cFLIP expressions nor cleavage of caspase-8 (Figure 3.12 C). This finding was similar for caspase-3 and PARP, where active cleavage was not detected (Figure 3.12 D).
Figure 3.11 Type C response pattern in RN1luc cell line (flow cytometric analysis)

Following 96 hour treatment, Type C response pattern were identified in RN1luc cell line. Flow cytometry analysis showed moderate sensitization with the combined treatment and sublethal caspase activation following treatment with zVAD (A-D). Data are shown as boxplot with overlaid data points from at least three independent experiments with technical replicates (two-way ANOVA, *p < 0.05 versus control cells, +p < 0.05 versus treated cells).
Figure 3.12 Type C response pattern in RN1luc cell line (Hoechst staining and western blot analysis)

Following 96 hour treatment, a morphological analysis of nuclear condensation showed moderate sensitization with the combined treatment (A, B). Data are shown as boxplot with overlaid data points from at least three independent experiments with technical replicates (two-way ANOVA, **p < 0.01 versus control cells). Scale bar = 50 µm. Western blot analysis did not reveal any loss of FADD and cFLIP expressions or cleavage of caspase-8 (C). Activation of caspase-3 and PARP were also not detected (D). Western blots shown are representative of at least two independent experiments with similar results, with β-actin used as a loading control.
MZ294, MZ304 and U87 cell lines showed no effects to the combined treatments with regards to PI uptake (Figure 3.13 A-B, 3.15 A-B and 3.17 A-B). MZ294 and MZ304 cell lines however, showed moderate Annexin V-FITC staining and nuclear condensation in response to TMZ plus Birinapant, which reached the level of statistical significance in the flow cytometry and Hoechst staining (Figure 3.13 C-D, 3.14 A-B, 3.15 C-D and 3.16 A-B). For U87 cell line, moderate secondary necrosis (PI-positive and Annexin V-negative) and further apoptotic (Annexin V-positive) cell death was observed in response to TMZ plus Birinapant treatment, which reached the level of statistical significance in the flow cytometry and Hoechst staining (Figure 3.17 and 3.18 A-B). This was sensitive to zVAD, suggesting that these cells exhibited sublethal caspase activation. For U87RGFP cell line, Hoechst staining showed moderate cell death in response to the combined treatment (Figure 3.19 A, B). Flow cytometry analysis was initially conducted on U87RGFP cell line, however, the green fluorescent protein (GFP) from the cells react with Annexin V-FITC staining, which affect data interpretation.

Western blot analysis demonstrated variable effects of TMZ and Birinapant single and combination treatments on RIP1, FADD and cFLIP expressions with no activation of caspase-8 or -3 in this group (Figure 3.12 C-D, 3.14 C-D, 3.16 C-D, 3.18 C-D and 3.19 C-D). MZ294 cell line had no detectable PARP cleavage, while activation of PARP was observed in TMZ and Birinapant single and combined treatments (MZ304), and TMZ and combined treatment (U87RGFP).
Following 96 hour treatment, Type C response pattern was identified in MZ294 cell line. Flow cytometry analysis showed no effects of the combined treatments on cell death as measured by PI uptake (A, B). A moderate Annexin V-FITC staining was found in response to TMZ and TMZ plus Birinapant, while addition of zVAD induced sublethal caspase activation (C, D). Data are shown as boxplot with overlaid data points from at least three independent experiments (two-way ANOVA, *p < 0.05 versus control cells, *p < 0.05 and **p < 0.01 versus treated cells).
Following 96 hour treatment, a morphological analysis of nuclear condensation showed moderate sensitization with TMZ and TMZ plus Birinapant treatment (A, B). Data are shown as boxplot with overlaid data points from at least three independent experiments (two-way ANOVA, *p < 0.05, **p < 0.01 versus control cells). Scale bar = 50 µm. Western blot analysis demonstrated a downregulation of RIP1 expression, while Birinapant and Birinapant plus TMZ decreased FADD expression. There was no detectable caspase-8 (C), caspase-3 or PARP activation in this cell line (D). Western blots shown are representative of at least two independent experiments with similar results, with β-actin used as a loading control.
Following 96 hour treatment, Type C response pattern was identified in MZ304 cell line. Flow cytometry analysis showed no effect of the combined treatments on cell death as measured by PI uptake (A, B). A moderate Annexin V-FITC staining was found in response to combined treatments, while addition of zVAD induced sublethal caspase activation (C, D). Data are shown as boxplot with overlaid data points from at least three independent experiments (two-way ANOVA, *p < 0.05, **p < 0.01 versus control cells, † p < 0.05 versus treated cells).
Figure 3.16 Type C response pattern in MZ304 cell line (Hoechst staining and western blot analysis)

Following 96 hour treatment, a morphological analysis of nuclear condensation showed moderate sensitization with the combined treatment, which reached the level of statistical significance (A, B). Data are shown as boxplot with overlaid data points from at least three independent experiments (two-way ANOVA, **p < 0.01 versus control cells). Scale bar = 50 µm. Western blot analysis demonstrated a decreased in RIP1 and cFLIP expressions in TMZ and Birinapant single and combined treatment. There were no detectable caspase-8 (C) or caspase-3 activation (D), however TMZ and Birinapant single and combined treatment induced cleavage of PARP. Western blots shown are representative of at least two independent experiments with similar results, with β-actin used as a loading control.
Following 96 hour treatment, Type C response pattern was identified in U87 cell line. Flow cytometry analysis showed no effects of the combined treatments on cell death as measured by PI uptake. A moderate secondary necrosis (PI-positive and Annexin V-negative) and further apoptotic (Annexin V-positive) cell death was observed in response to TMZ and TMZ plus Birinapant treatment (A-D). Data are shown as boxplot with overlaid data points from at least three independent experiments (two-way ANOVA, *p < 0.05, **p < 0.01 and ***p < 0.001 versus control cells, +++p < 0.001 versus treated cells).
Following 96 hour treatment, a morphological analysis of nuclear condensation showed an increased cell death with TMZ and TMZ plus Birinapant treatment, which reached the level of statistical significance (A, B). Data are shown as boxplot with overlaid data points from at least three independent experiments (two-way ANOVA, \( p < 0.05 \), and \( ***p < 0.001 \) versus control cells). Scale bar = 50 µm. Western blot analysis demonstrated no variation of RIP1 but a decrease in FADD and cFLIP following treatment with TMZ and TMZ plus Birinapant. There was no detectable caspase-8 (C) and caspase-3 activation (D), however, Birinapant and Birinapant plus TMZ induced cleavage of PARP. Western blots shown are representative of at least two independent experiments with similar results, with β-actin used as a loading control.
Figure 3.19 Type C response pattern in U87RGFP cell line (Hoechst staining and western blot analysis)

Following 96 hour treatment, Type C response pattern was identified in U87RGFP cell line. Hoechst staining showed moderate cell death in response to TMZ plus Birinapant treatment ((A, B); data are shown as boxplot with overlaid data points from at least three independent experiments (two-way ANOVA). Scale bar = 50 µm. Western blot analysis demonstrated no variation in RIP1 and cFLIP expression. FADD expression was increased with Birinapant treatment and reverse effect was observed when treatment was combined. There were no detectable caspase-8 (C) and caspase-3 activation (D), however, TMZ and TMZ plus Birinapant induced PARP cleavage. Western blots shown are representative of at least two independent experiments with similar results, with β-actin used as a loading control.
3.3 Discussion

The development of IAP antagonists as potential anticancer agents has gained significant interest following the finding that inhibition of IAP proteins sensitizes various cancer cells to pro-apoptotic agents (Fulda, 2014, Varfolomeev et al., 2007). Our results demonstrate a surprisingly heterogeneous response in commercial and patient-derived GBM cell lines treated with Birinapant in vitro, either as a single agent or when combined with TMZ. Furthermore, we showed variable expression in GBM cells of proteins involved in the extrinsic and intrinsic pathways, with some downregulated following treatment and others not affected at all. Heterogeneous responses have been reported in non-small-cell lung cancer cells and in A172 GBM cell line (Petersen et al., 2010, Wagner et al., 2013), while other reports have described more homogeneous effects in acute myeloid leukaemia and breast cancer cell lines (Benetatos et al., 2014, Carter et al., 2014).

Heterogeneous responses in our panel of GBM cell lines were evident from the outset of our study when we explored the activity of TMZ and Birinapant in vitro. When looking at the cell survival at five time points, we noted that all commercially available GBM cell lines were sensitive to TMZ. However, for patient-derived GBM cell lines, analysis using a one-way ANOVA to compare the treatment(s) versus the control did not show a statistical reduction in cell survival even after 120 hour incubation. In contrast, ‘responders’ to Birinapant were observed in both groups, albeit the magnitude of responses to Birinapant was significantly smaller in both commercial and patient-derived GBM cell lines. Silencing of the gene encoding the DNA repair protein MGMT that occurs relatively frequently in culture (Harris et al., 1996, Taylor et al., 2000), may result in commercially available cell lines being more susceptible to TMZ therapy. In the combination treatment, we also observed a subset of cell lines (WK1luc, MZ327 and MZ18) that were previously resistant to single treatment, but reach significant value (p < 0.05) with a prolonged incubation period, although the magnitude of responses were also smaller than cell lines that were previously sensitive to single treatment. Collectively, our cell viability studies highlight the different sensitivities between commercially and patient-derived
GBM cell lines. Therefore, we believe that the inclusion of patient-derived GBM cell lines is important in pre-clinical *in vitro* studies.

In the present study, we identified three major response patterns. Of all the cell lines that were selected for analysis, Type A (U251) cells were sensitive to TMZ treatment (**Figure 3.6 and 3.7**). The mode of cell death was mainly via apoptosis and secondary necrosis, as identified by Annexin V and PI uptake, while zVAD only partially blocked the caspase activity. The Hoechst staining technique did show ~15% increase in apoptotic cell counts when the treatments were combined. In this cell, western blot analysis showed that TMZ alone and TMZ plus Birinapant increased the upregulation of FADD protein, which was then recruited to the intracellular death domains of the receptors, in turn promoting the activation caspase-8 within the DISC complex. Furthermore, caspase-3 activation was evident in response to TMZ, with further increased expression when the treatment was combined, suggesting the presence of downstream intrinsic cell death activation (Hellwig and Rehm, 2012).

The absence or low expression of cleaved caspase-8 and overexpression of RIP1 has previously been identified in tumours and serves as an independent negative prognostic indicator in GBM. Particularly, overexpression of RIP1 prevents the activation of caspase-8 (Park et al., 2009, Saggioro et al., 2014). In Type A cell, Birinapant alone treatment did not have any effect on cIAP1 and XIAP proteins, but TMZ treatment showed degradation of these proteins, which likely contributed to an increase in caspase-8 activity. Caspase-3 activation followed by cleavage of PARP was also evident in this cell line, suggesting cellular and DNA cytotoxicity from TMZ treatment activates apoptotic cell death (Roos and Kaina, 2013).

Type B (WK1luc and JK2luc) cells were sensitive to single agent treatment with Birinapant; however, no further sensitisation was observed when used in combination with TMZ (**Figure 3.8 - 3.10**). The mode of cell death was different than that observed in the U251 cell line, whereby Birinapant or Birinapant plus TMZ treatment induced secondary necrotic cell death (annexin V and PI-positive cells). This cell death was however zVAD-sensitive, which significantly
blocked caspase activity within the cells. We also observed annexin V-positive and PI-negative cells. This cell death was, however, also zVAD-sensitive, and Hoechst staining indicated strong nuclear condensation, indicating that these cells did not undergo a primary necrotic cell death. The appearance of such double positive cells is likely a consequence of ruptured membranes during late apoptosis that sheared off apoptotic nuclei during flow cytometric sample reading.

Complete degradation of cIAP1 protein, but not cIAP2 and XIAP, demonstrated a high specificity of Birinapant treatment on WK1luc cell. At 96 hour treatment, we also noted high upregulation of FADD proteins in this cell line, which increases the assembly of RIP1/FADD/caspase-8 upon treatment with Birinapant. Previous western blot analysis of GBM cells with Smac mimetic also resulted in the same event, even as early as 12 hour treatment (Roesler et al., 2016). Other study has found that high expression of FADD ubiquitinates IκKβ and stabilizes IκBα to canonically obstruct cytosolic to nuclear translocation of p65. Thus, high expression of this protein suggested an important contribution of FADD in suppressing NFκB activation and induction of apoptosis (Ranjan and Pathak, 2016). Birinapant treatment alone resulted in caspase-8 activation, which then downregulated RIP1 protein (Bai et al., 2014); this action was more evident when the treatments were combined. Similar to the U251 cell line, caspase-3 and PARP cleavage was detected in the WK1luc cell line, which contributed to cell death.

In the WK1luc cell line, we also observed discrepancies between MTT and flow cytometry as well as Hoechst staining. The MTT result showed a sensitisation with combined treatment but not with TMZ and Birinapant single agent treatment, while flow cytometry and Hoechst staining showed single agent sensitisation with Birinapant. The discrepancy observed could result from different experimental techniques, where MTT reports the results of cell proliferation, while Hoechst staining assess the nuclear morphology.

Type C cells, however, were mainly capable of activating moderate apoptotic cell death when the treatments were combined (Figure 3.11 - 3.19).
Importantly, Western-blot analysis did not demonstrate evidence of caspase-8 or -3 activation in these cell lines. In the absence of cIAP1 following Birinipant and Birinapant plus TMZ treatment, it causes stabilisation of NIK, which results in translocation of p52-RelB dimers into the nucleus to induce transcription of TNFα (Chapter 1.9.3) (Darding and Meier, 2012, Park and Hong, 2016). In other cancer cell line, TNFα helps to increase TNFR1 activation and mediated Smac mimetic-induced cell death (Roesler et al., 2016). However, in GBM cell line, other studies have found that Smac mimetic stimulated RIP1/FADD/caspase-8 complex occur in TNFα-independent manner (Wagner et al., 2013).

Variation of FADD expression was observed, with an increased expression following Birinapant alone treatment in U87 cell lines and Birinapant plus TMZ treatment in MZ304 cell line. Particularly, there was a reduced FLIP expression in MZ304 cell line. The signal however, was not fundamental to the activation of caspase-8 or caspase-3. We stipulated that since no observed activation of caspase-8 on western-blot, an interaction of RIP1/FADD/caspase-8 complex did not occur. In this case, crosstalk could occur, whereby RIP1 interacts with RIP3 and undergo RIP1-dependent necroptosis. While our study mainly looked at the apoptotic cell death, Smac mimetic-stimulated IAP depletion can engage caspase-independent non-apoptotic mechanism when caspase activity is impaired (Christofferson et al., 2014).

The presence of cleaved PARP on Western-blot has previously been used as an indicator of apoptosis in many cells (Duriez and Shah, 1997, Bressenot et al., 2009). However, we found variation in PARP activity within GBM cell lines. In JK2luc cell line, despite the activation of caspase-8 and caspase-3, PARP is not cleaved in response to Birinapant, but seems to become upregulated. We stipulated that other inhibitory factors contribute to this resistance. The PARP cleavage is not only mediated by caspasas activity, but also by many related proteases including calpains, cathepsins, and grandzymes. These proteases could directly or indirectly mediates PARP activity within cells (Chaitanya et al., 2010). Although we could not find any literature to support the activity of PARP following Smac mimetic treatment in GBM cells, in other cancer cells, FLIP-
mediated inhibition of Smac activity was found to attenuate PARP cleavage (Crawford et al., 2013).

Furthermore, in Type C cells (MZ304, U87 and U87RGFP), PARP cleavage was detected without caspase-8 or -3 activation. This is not uncommon, as others have found similar events. Masdehors et al. (2000) found that radiation treatment in human lymphocytes cells induces PARP cleavage and seems to be caspase independent, as no activation of caspase-3 or -7 was observed. In addition, the use of a caspase inhibitor did not modify PARP cleavage (Masdehors et al., 2000). In another study of apoptosis, AML-12 cells treated with transforming growth factor-β1 (TGF-β1), Yang et al. (2004) also found that PARP cleavage during apoptosis did not involve caspase-3 and suggested that PARP cleavage may occur as an independent event that can be dissociated with cell apoptosis (Yang et al., 2004). Another stipulation was that PARP activation could be induced by other caspases, such as caspase-2, which is activated when cells undergo apoptosis (Li et al., 1997a). Caspase-2 promotes cytochrome c release from mitochondria (Bonzon et al., 2006, Guo et al., 2002). While activation of caspase-2 occurs earlier than caspase-3 (Penna et al., 2012), PARP cleavage could occur in the presence of active caspase-2 and not caspase-3 (Stefanis et al., 1998). From moderate cell death activity in Type C cells, we believe that the cleavage of cellular proteins such as PARP may be just one unrelated event during apoptosis (Nicholson and Thornberry, 1997, Herceg and Wang, 1999), or activated by other caspases within the cells.

Taken together, our data demonstrates that GBM cell lines show heterogeneous treatment responses in vitro to Birinapant alone or in combination with TMZ. Birinapant treatment is highly specific in inducing degradation of cIAP1 protein in the majority of GBM cell lines, without affecting other IAPs. TMZ and Birinapant alone and in combination principally subgrouped GBM cell lines into three different responses patterns. As evidenced in Type A and Type B cells, effective cell death requires activation of initiator and executioner caspases. There was no activation of caspases in Type C cells, which resulted in only moderate cell death. Following the in vitro results, we next moved to the mathematical model of the caspase execution
network previously developed by our group (‘APOPTO-CELL’) and undertook further analysis of the proteins involved in the execution of caspase activation in the mitochondrial apoptosis pathway.
CHAPTER 4:
Application of systems biology model of effector caspase activation (APOPTO-CELL) for glioblastoma
4.1 Introduction

4.1.1 Proteins involved in the execution of intrinsic (mitochondrial) pathways are dysregulated in cancer cells

The apoptotic cascade can be triggered by various stimuli such as DNA- or organelle damage and proteasomal stress as well as growth factor withdrawal and other extracellular signals (Nowsheen and Yang, 2012, Hellwig and Rehm, 2012). In GBM patients, following surgical resection of all tumour cells, TMZ therapy and radiotherapy are the adjuvant treatment regime (Stupp et al., 2005a), resulting in the triggeration of apoptotic cell death (Shi et al., 2012, Murphy et al., 2013). Caspase-dependent mitochondrial apoptosis occurs following mitochondrial outer membrane permeabilization (MOMP) and release of cytochrome c and Smac into the cytosol (Figure 4.1) (Fulda et al., 2010). Subsequent formation of the apoptosome, a multiprotein complex comprising of Apaf-1, procaspase-9, dATP/ATP and cytochrome c results in auto-activation of procaspase-9, which in turn activates procaspase-3 (Zhou et al., 2015). Activation of caspase-3 is the final step in apoptosis activation (Schmid et al., 2012), as it induces DNA fragmentation and cleavage of cytoskeletal proteins, DNA repair enzymes and signaling proteins leading to the demise of the cell.

Evasion of apoptosis is a typical hallmark of cancer and is often conferred by an upregulation of anti-apoptotic or a downregulation or deletion of pro-apoptotic proteins and genes (Plati et al., 2011, Pistritto et al., 2016). For this reason, it has been suggested that the basal expression of apoptotic proteins could be used as a marker to predict therapy response to apoptosis-inducing agents and to establish a prognosis for cancer patients (Hector et al., 2012a, Hector et al., 2012b, Zlobec et al., 2006). For example, a significant decline in Apaf-1 expression in melanoma was observed with the progression of disease and thickness of the tumour (Mustika et al., 2005). In patients with rectal cancer, a positive Apaf-1 expression in pre-treatment tumour biopsy is a predictive marker of response to preoperative radiotherapy (Zlobec et al., 2006). The Apaf-1/Caspase-9 was also correlated with 5-fluorouracil (5-FU) treatment, whereby evaluation of this treatment in patients showed that the 5-FU induces apoptosis
via this pathway (Shang et al., 2014). In GBM, no association between Apaf-1 and clinical outcome has been identified, yet Apaf-1 gene expression has been shown to be downregulated (Wybranska et al., 2013, Watanabe et al., 2003), a process that may contribute to apoptosis resistance following TMZ treatment (Shi et al., 2012).

The endogenous Smac, once released into cytosol, targets the BIR2/BIR3 domains of XIAP and competitively displaces caspases from their interaction sites (Obexer and Ausserlechner, 2014). This process increases the availability of free caspases within cytosol, promoting apoptosome and apoptosis execution (Obexer and Ausserlechner, 2014). Smac levels are shown to be lower in lung cancer tissue than in normal tissue and the prognosis of patients with tumours exhibiting low Smac expression was worse than those with a high Smac expression (Sekimura et al., 2004). A low serum level of Smac may also be a predictor of early recurrence in patients with bladder cancer (Mizutani et al., 2012). In GBM, previous analysis by our group has displayed a variable expression level of Smac in patient samples (Murphy et al., 2013). Of note, small molecule Smac mimetics are currently developed to re-sensitise resistant cancer cells to apoptosis (Wagner et al., 2013).

However, the presence of XIAP is one culprit involved in the resistance to extrinsic apoptotic signalling (Dai et al., 2009). As shown in Figure 4.2, XIAP contains three BIR domains (BIR1–3). Whereas BIR1 interacts with proteins that modulate NFκB signalling, BIR2 and BIR3 are critical for interactions with caspase-3 and -7 (BIR2), and caspase-9 (BIR3). The N-terminal tetra-peptide of the processed caspase-9 that binds into the BIR3 pocket shares significant homology with the N-terminus of mitochondrial Smac/Diablo, suggesting that these two binding motives compete for XIAP–BIR3 interaction (Srinivasula et al., 2001). In clear-cell renal cell carcinoma (RCC), XIAP expression levels were reported to significantly increase with advancing tumour stage, tumour dedifferentiation and aggressive tumour growth (Ramp et al., 2004, Mizutani et al., 2007). High expression of XIAP has also been suggested as an independent prognostic marker for the early recurrence of non-muscle-invasive bladder cancer (Chen et al., 2013), and is associated with significantly shorter
survival in patients with cholangiocarcinoma and oesophageal squamous cell carcinoma (Zhou et al., 2013). Analysis of 78 oncogenes within GBM patients sample yielded 3-fold amplification of XIAP gene (Beckner et al., 2016), which is associated with increased protein levels of XIAP and other IAP proteins (Yang et al., 2014). Concordantly, inhibition of IAP proteins can sensitize cancer cells to pro-apoptotic agents (Fulda and Vucic, 2012).
Figure 4.1 Intrinsic apoptotic pathway following mitochondrial outer membrane permeabilization (MOMP)

Following a response to cellular stress, mitochondrial outer membrane permeabilization (MOMP) is activated, followed by the release of cytochrome c and Smac (red dots) into the cytosol. Formation of apoptosome complex and activation of effector caspases, particularly caspase-3, can be inhibited by XIAP, whose action is antagonised through its interaction with Smac. Adapted from (Hector et al., 2012b).
Figure 4.2 Structural basis of the interaction between Smac and XIAP-BIR domains

Schematic diagram shows that Smac can bind to both BIR2 (interact with caspase-3 and -7) and BIR3 (interact with caspase-9). This, in turn, increases the amount of free caspases needed to activate cell death.
4.1.2 APOPTO-CELL model for glioblastoma

While the expression of certain apoptotic proteins may deliver useful single markers of therapy responses, they appear to have limited or no prognostic significance (Sehn, 2006). The decision to activate apoptosis in tumour cells depends on the interaction of multiple key apoptotic proteins and their feedback loops (Rehm et al., 2006). Therefore, examination of these interactions as a whole rather than assessment of individual components provides us with more insight into a cell's capability to activate caspases following apoptotic stimuli.

One approach is the APOPTO-CELL, which is a computational model of downstream apoptosis execution firstly calibrated in HeLa cells and further validated in other cancer cell lines and patient samples (Schmid et al., 2012, Rehm et al., 2006, Murphy et al., 2013). This model is composed of a set of 53 ordinary differential equations representing the dynamic behavior of 19 species and governed by 75 kinetic parameters (Rehm et al., 2006). From the expression of procaspase-3, procaspase-9, Smac, XIAP and Apaf-1, the APOPTO-CELL predicts the ability of the cells to undergo apoptosome-dependent effector caspase activation following MOMP and cytochrome c release.

Varied substrate cleavage profiles were predicted after the release of cytochrome c, ranging from <1% to 100%. To validate this model in the clinical setting, the first study was performed by our group using colorectal tumours and normal tissue (Hector et al., 2012b). Systems analysis indicated that apoptosis is impaired with advancing disease stage, and able to predict and identify those patients who were unlikely to respond to 5-FU based chemotherapy due to defect in their efficiency to execute apoptosis. Murphy et al. (2013) further examined this model in GBM cell lines and patient tumour samples. Apoptosis susceptibility in GBM cell lines and patient tumour samples was defined as the ability to efficiently generate >80% substrate cleavage within this 60 min time frame. From the positive outcome of the APOPTO-CELL model, we continue to analyse the usability of a systems analysis-based approach in a larger cohort of GBM cell lines and patient tumour samples.
4.1.3 Aims

The aim of this study was to investigate the molecular expression profiles of five key apoptotic proteins (procaspase-3, procaspase-9, Smac, Apaf-1 and XIAP) using quantitative western blotting. Following this, our first objective was to compare the protein expression profiles of sixteen GBM cell lines between commercial versus patient derived GBM cell lines. We also analysed forty-eight GBM patient tumour samples and compared between primary versus recurrent samples. Our second objectives was to investigate if the expression and interaction of these apoptotic proteins could be utilized to determine GBM cell line or patient sample response to apoptosis-inducing therapy using the APOPTO-CELL model. The exact time to achieve 80% substrate cleavage for each cell line was calculated and again compared. Our third objective was to include the effects of XIAP antagonist (0 - 1 µM) to evaluate the therapeutic benefit of targeted treatment in high risk patients (Hector et al., 2012b).
4.2 Result

4.2.1 Application of the system model, APOPTO-CELL, in GBM cell lines

4.2.1.1 Analysis of basal protein expression profiles in GBM cell lines

Chemotherapeutic responses are tightly mediated by apoptosis signaling (Plati et al., 2011). To analyse whether the apoptotic machinery downstream of the mitochondria is impaired in GBM, we first sought to establish the basal expression levels of these five proteins in our panel of sixteen GBM cell lines using western blots and densitometric analysis (Figure 4.3). Protein expression levels were normalised to the actin loading control and expressed relative to HeLa protein levels set at 100% (Figure 4.4 and 4.5). We observed generally variable protein expression levels when compared to HeLa control, with procaspase-9 showing the lowest protein concentration within GBM cells.

For the purpose of statistical calculation, we employed the Mann-Whitney test, a non-parametric test used to compare two independent groups of sampled data, to determine differences in the protein expression levels in commercial (black) and patient derived GBM cell lines (grey). There were no statistically significant differences in the protein expression measured for procaspase-9 ($p = 0.6832$), procaspase-3 ($p = 0.5584$) and Apaf-1 ($p = 0.2118$). Tendencies towards higher Smac expression levels in commercially available GBM cell lines were not significant ($p = 0.0727$). However, patient derived GBM cell lines showed a trend in increased XIAP, which reached significant value ($p = 0.0415$).

Together, the results so far have shown a differential basal expression of single proteins analysis of the commercial versus GBM cell lines. Having found this result, we next sought to examine the expression and interaction of these apoptotic proteins using the experimentally validated, mathematical model, APOPTO-CELL.
Figure 4.3 Differential protein expressions of apoptotic proteins in GBM cell lines

Representative western blots showing Apaf-1, procaspase-3, procaspase-9, Smac and XIAP expression in GBM whole cell lysates (A, B). The western blot in (A) was previously performed by our group (Murphy et al., 2013). β-actin was used as a loading control.
Figure 4.4 Densitometry analysis of the expression of procaspase-3, Smac and Apaf-1

The HeLa cell line is in white, while the commercial and patient derived GBM cell lines are in black and dark grey, respectively. There were no statistically significant differences in the protein expression measured for procaspase-9, procaspase-3 and Apaf-1. Mann-Whitney test; $p < 0.05$. 
The HeLa cell line is in white, while the commercial and patient derived GBM cell lines are in black and dark grey, respectively. All GBM cell lines displayed low basal expression of procaspase-9 when compared to the HeLa cell line. The patient derived GBM cell lines showed a trend in increased XIAP, which reached significant value. Mann-Whitney test; p < 0.05.
4.2.1.2 The APOPTO-CELL model predicts the ability of GBM cell lines to undergo apoptosome-dependent effector caspase activation

To investigate if the abundance and the interaction of these apoptotic proteins could be utilized to calculate GBM cell line response to apoptosis-inducing therapy, we employed the APOPTO-CELL model. To calculate protein concentrations of all five proteins (procaspase-3, procaspase-9, XIAP, Smac and Apaf-1), we utilised the previously described protein expression profiles and expressed them relative to published protein concentrations for HeLa (Table 4.1) (Rehm et al., 2006, supplementary material). The determined protein concentrations served as an input, while the output is the prediction of cellular susceptibility to undergo caspase cleavage and activation within a 60 min time frame.
Table 4.1 Protein concentrations of procaspase-3, procaspase-9, XIAP, Smac and Apaf-1 and within GBM cell lines

<table>
<thead>
<tr>
<th>GBM cell lines</th>
<th>Procaspase-3 [µM]</th>
<th>Procaspase-9 [µM]</th>
<th>XIAP [µM]</th>
<th>Smac [µM]</th>
<th>Apaf-1 [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>0.120</td>
<td>0.030</td>
<td>0.063</td>
<td>0.126</td>
<td>0.372</td>
</tr>
<tr>
<td>U87</td>
<td>0.144</td>
<td>0.005</td>
<td>0.066</td>
<td>0.273</td>
<td>0.072</td>
</tr>
<tr>
<td>U87RGFP</td>
<td>0.082</td>
<td>0.002</td>
<td>0.07</td>
<td>0.266</td>
<td>0.069</td>
</tr>
<tr>
<td>U251</td>
<td>0.168</td>
<td>0.002</td>
<td>0.04</td>
<td>0.284</td>
<td>0.033</td>
</tr>
<tr>
<td>U343</td>
<td>0.115</td>
<td>0.002</td>
<td>0.057</td>
<td>0.245</td>
<td>0.067</td>
</tr>
<tr>
<td>U373</td>
<td>0.109</td>
<td>0.003</td>
<td>0.053</td>
<td>0.159</td>
<td>0.092</td>
</tr>
<tr>
<td>A172</td>
<td>0.136</td>
<td>0.001</td>
<td>0.023</td>
<td>0.132</td>
<td>0.022</td>
</tr>
<tr>
<td>MZ18</td>
<td>0.138</td>
<td>0.002</td>
<td>0.061</td>
<td>0.107</td>
<td>0.234</td>
</tr>
<tr>
<td>MZ51</td>
<td>0.067</td>
<td>0.001</td>
<td>0.051</td>
<td>0.114</td>
<td>0.052</td>
</tr>
<tr>
<td>MZ256</td>
<td>0.08</td>
<td>0.002</td>
<td>0.083</td>
<td>0.213</td>
<td>0.036</td>
</tr>
<tr>
<td>MZ294</td>
<td>0.097</td>
<td>0.001</td>
<td>0.067</td>
<td>0.232</td>
<td>0.026</td>
</tr>
<tr>
<td>MZ304</td>
<td>0.074</td>
<td>0.003</td>
<td>0.113</td>
<td>0.158</td>
<td>0.086</td>
</tr>
<tr>
<td>MZ327</td>
<td>0.061</td>
<td>0.002</td>
<td>0.054</td>
<td>0.199</td>
<td>0.040</td>
</tr>
<tr>
<td>JK2luc</td>
<td>0.150</td>
<td>0.004</td>
<td>0.082</td>
<td>0.205</td>
<td>0.333</td>
</tr>
<tr>
<td>WK1luc</td>
<td>0.183</td>
<td>0.003</td>
<td>0.082</td>
<td>0.143</td>
<td>0.386</td>
</tr>
<tr>
<td>SJH1luc</td>
<td>0.147</td>
<td>0.006</td>
<td>0.083</td>
<td>0.144</td>
<td>0.291</td>
</tr>
<tr>
<td>RN1luc</td>
<td>0.123</td>
<td>0.004</td>
<td>0.067</td>
<td>0.151</td>
<td>0.269</td>
</tr>
</tbody>
</table>
In HeLa cell line, 80% of the substrate was cleaved by 12.6 min after release of cytochrome c (Figure 4.6 A). The HeLa cell line is thus considered highly likely to undergo apoptosis following activation of the mitochondrial pathway. Previously (Figure 3.2), we have demonstrated that all commercially available GBM cell lines were sensitive to TMZ treatment while all patient derived GBM cell lines were resistant. The APOPTO-CELL output accurately predicted the GBM cell line sensitivities in 13 of the 16 cell lines, which corresponds to a predictive rate of 81.3%. All commercially available GBM cell lines were correctly predicted to achieve an early substrate cleavage within the 60 min simulation time frame. Of the patient derived GBM cell lines, which failed to respond to TMZ in vitro, 70% also failed to reach sufficient substrate cleavage within the 60 min simulation time frame (Figure 4.6 B). Only three of these cell lines (JK2luc at 44 min, SJH1luc at 48.5 min, and RN1luc at 51 min) achieved the targeted optimal substrate cleavage but at a higher time point when compared to the commercial cell lines.

Together, the results demonstrate that based on basal proteins expression levels of each GBM cell line, the APOPTO-CELL approach is able to predict the cellular susceptibility to undergo caspase cleavage following MOMP and cytochrome c release, as explained by the differing sensitivities to TMZ treatment in vitro. Next, by in silico administration of XIAP antagonist at varying concentration (0 - 1 µM), we wanted to investigate if the delay in the caspase-3 substrate cleavage was due to the inhibitory action of anti-apoptotic protein XIAP.
Figure 4.6 The APOPTO-CELL outputs for GBM cell lines

The time (min) taken to achieve optimal 80% caspase-3 substrate cleavage after cytochrome c release was calculated. Standard HeLa cells were shown to have optimal value at 12.6 min (A). The GBM cells could be divided into two groups; the commercially available GBM cell lines that were sensitive to TMZ treatment in vitro were predicted to have an early substrate cleavage while the patient derived GBM cell lines that were resistant in vitro were predicted to have a delayed substrate cleavage (B). Data input as absolute protein concentrations [µM] based on established protein expression profiles (Table 4.1).
4.2.1.3 Application of XIAP antagonist in APOPTO-CELL accelerates the likelihood of the cells to undergo caspase activation

Previously (Figure 3.4), *in vitro* experiments with TMZ plus Birinapant demonstrated that all commercially available GBM cell lines displayed further reduction in cell survival, while only four patient derived GBM cell lines (MZ294, MZ51, MZ256 and SJH1luc) showed no statistically significant reduction in cell survival, underlying no benefit from the treatment regime. Previous analysis of basal protein expression levels showed a trend in increased XIAP in patient derived GBM cell lines (Figure 4.5), and analysis of effector caspase activation using APOPTO-CELL has produced an output that predicted an increase in times needed to achieved 80% caspase-3 substrate cleavage in this group.

Similar to endogeneous Smac, synthetically designed Smac mimetics have been shown to compete with caspses for the binding of XIAP (Li et al., 2004). Having identified the role of XIAP, we next wondered whether the presence of Smac could enhance caspase-dependent apoptosis. Using XIAP antagonists at different concentrations (0 - 1 µM) and mimicking the behaviour of Smac, we therefore experimentally examined the consequence of XIAP inhibition on the likelihood of the cells to undergo caspase activation.

Following addition of XIAP antagonist at varying concentrations, APOPTO-CELL output of GBM cell lines showed variability in response to this agent. At 1 µM, a concentration that correlates to the current *in vitro* treatment, the result shifted to the left, confirming a shorter time was needed to achieve optimal substrate cleavage (Figure 4.7). All the commercially available GBM cell lines were found to respond to the addition of XIAP antagonist, with shorter predicted time needed to achieve 80% substrate cleavage within a 60 min time frame (Table 4.2). However, for the patient derived GBM cell lines, we also found that four of the cell lines that were previously shown to be resistant to the combined treatment were predicted to have a sufficient caspase-3 substrate cleavage (Table 4.3).
Figure 4.7 Application of XIAP antagonist accelerates substrate cleavage in GBM cell lines

The calculated protein expression levels of procaspase-9, procaspase-3, Apaf-1, Smac and XIAP were used as an input for the APOPTO-CELL model. Further addition of XIAP antagonist at different concentration (0-1 µM) was performed. Within 60 min simulation time, the outputs were calculated for the time (min) needed for optimal 80% caspase-3 substrate cleavage after cytochrome c release. All GBM cell lines were predicted to have a sufficient caspase-3 substrate cleavage.
Table 4.2 *In vitro* responses and APOPTO-CELL prediction of the commercially available GBM cell lines

<table>
<thead>
<tr>
<th>Commercially available GBM cell lines</th>
<th>TMZ response</th>
<th>APOPTO-CELL prediction of sufficient substrate cleavage</th>
<th>TMZ + Birinapant response</th>
<th>APOPTO-CELL prediction with addition of XIAP antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>U87</td>
<td>Sensitive</td>
<td>Predicted</td>
<td>Sensitive</td>
<td>Predicted</td>
</tr>
<tr>
<td>U87RGFP</td>
<td>Sensitive</td>
<td>Predicted</td>
<td>Sensitive</td>
<td>Predicted</td>
</tr>
<tr>
<td>U251</td>
<td>Sensitive</td>
<td>Predicted</td>
<td>Sensitive</td>
<td>Predicted</td>
</tr>
<tr>
<td>U343</td>
<td>Sensitive</td>
<td>Predicted</td>
<td>Sensitive</td>
<td>Predicted</td>
</tr>
<tr>
<td>U373</td>
<td>Sensitive</td>
<td>Predicted</td>
<td>Sensitive</td>
<td>Predicted</td>
</tr>
<tr>
<td>U251</td>
<td>Sensitive</td>
<td>Predicted</td>
<td>Sensitive</td>
<td>Predicted</td>
</tr>
</tbody>
</table>

Table 4.3 *In vitro* responses and APOPTO-CELL prediction of the patient derived GBM cell lines

<table>
<thead>
<tr>
<th>Patient derived GBM cell lines</th>
<th>TMZ response</th>
<th>APOPTO-CELL prediction of sufficient substrate cleavage</th>
<th>TMZ + Birinapant response</th>
<th>APOPTO-CELL prediction with addition of XIAP antagonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>MZ304</td>
<td>Resistant</td>
<td>Failed</td>
<td>Sensitive</td>
<td>Predicted</td>
</tr>
<tr>
<td>MZ327</td>
<td>Resistant</td>
<td>Failed</td>
<td>Sensitive</td>
<td>Predicted</td>
</tr>
<tr>
<td>MZ18</td>
<td>Resistant</td>
<td>Failed</td>
<td>Sensitive</td>
<td>Predicted</td>
</tr>
<tr>
<td>MZ294</td>
<td>Resistant</td>
<td>Failed</td>
<td>Resistant</td>
<td>Predicted</td>
</tr>
<tr>
<td>MZ51</td>
<td>Resistant</td>
<td>Failed</td>
<td>Resistant</td>
<td>Predicted</td>
</tr>
<tr>
<td>MZ256</td>
<td>Resistant</td>
<td>Failed</td>
<td>Resistant</td>
<td>Predicted</td>
</tr>
<tr>
<td>SJH1luc</td>
<td>Resistant</td>
<td>Predicted</td>
<td>Resistant</td>
<td>Predicted</td>
</tr>
<tr>
<td>RN1luc</td>
<td>Resistant</td>
<td>Predicted</td>
<td>Sensitive</td>
<td>Predicted</td>
</tr>
<tr>
<td>WK1luc</td>
<td>Resistant</td>
<td>Failed</td>
<td>Sensitive</td>
<td>Predicted</td>
</tr>
<tr>
<td>JK2luc</td>
<td>Resistant</td>
<td>Predicted</td>
<td>Sensitive</td>
<td>Predicted</td>
</tr>
</tbody>
</table>
Kaplan-Meier curves showed that, within the 60 min time frame, the predicted times to 80% caspase-3 substrate cleavage vary significantly between commercially available and patient derived GBM cell lines (Log-rank, p = 0.0001; Figure 4.8). There was a significant difference between commercially available GBM cell lines following addition of XIAP antagonist at 1 µM than without any addition of XIAP antagonist (Log-rank, p = 0.0005). Similarly, there was a significant difference between patient derived GBM cell lines following addition of XIAP antagonist at 1 µM than without any addition of XIAP antagonist (Log-rank, p < 0.0001). The median time needed to achieve 80% substrate cleavage for the commercially available GBM cell lines with and without the addition of XIAP antagonist were 10.75 and 29.5 min, respectively. The median time needed to achieve 80% substrate cleavage for the patients derived GBM cell line with and without addition of XIAP antagonist were 14 and 60 min, respectively.

In summary, we have shown that based on western blotting technique, GBM cell lines have different basal proteins expression levels. Rather than analysis of individual proteins, we applied the APOPTO-CELL approach to look at the network of interactions of these proteins. We found that the ability of GBM cell lines to activate sufficient 80% caspase-3 substrate cleavage within the 60 simulation time correlated with the sensitivity of the GBM cell lines to TMZ treatment in vitro, with a prediction rate of 81.3%. However, upon application of XIAP antagonist at 1 µM, the APOPTO-CELL is not a suitable tool to predict the targeted therapeutic and did not correlate with in vitro TMZ plus Birinapant treatment response.
The calculated protein expression levels of procaspase-9, procaspase-3, Apaf-1, Smac and XIAP in sixteen GBM cell lines were used as an input for the model. The Kaplan-Meier curve showed that, within the 60 min time frame, the predicted times to 80% caspase-3 substrate cleavage vary significantly between commercially available and patient derived GBM cell lines ($p = 0.0001$), and between cell lines with and without addition of XIAP antagonist (commercially available GBM cell lines with and without addition of XIAP antagonist ($p = 0.0005$); patient derived GBM cell lines with and without addition of XIAP antagonist ($p = 0.0001$)). Log-rank test; $p < 0.05$.
4.2.2 Application of system model, APOPTO-CELL, in a clinical setting in patients with GBM

4.2.2.1 Clinical characteristics of glioblastoma patients

The standard treatment for patients with GBM is maximal surgical resection that is safely feasible, followed by the Stupp protocol, which is the most widely implemented treatment regimen (Stupp et al., 2005a). The tumour samples taken from the operating theatre are analysed to determine the histological tumour grade, tumour subtype and MGMT status. Having identified the role of the APOPTO-CELL model in GBM cell lines, we next examined this model in a clinical setting and evaluated whether the identified pattern of apoptosis resistance in GBM cell lines is transferrable for a clinical setting situation. Therefore, we pooled the entire primary and recurrent patient tumour samples available in our facility: 17 primary and 4 recurrent tumour samples were previously analysed by our group (indicated as P1, P2, P3, etc.) (Murphy et al., 2013), while 16 primary and 11 recurrent tumour samples were available for further analysis. In total, there were 48 tumour samples, with 33 primary and 15 recurrent GBM samples.

Primary GBM samples were obtained from patients who underwent primary therapeutics tumour resection, while recurrent GBM samples were obtained from patients who underwent second tumour resection due to recurrences. The clinical characteristics of GBM patients were shown in Table 4.4. The median age of patients with primary GBMs was 57 years, which is lower than the current median age at diagnosis (64 years) (Thakkar et al., 2014, Ostrom et al., 2013); however, this is still high when compared to other types of glioma (Schwartzbaum et al., 2006). The majority of GBMs were located in the supratentorial region, with 60% was on the right side. As per standard protocol, all primary tumour samples did not receive neoadjuvant treatment, while 60% of patients who underwent recurrent tumour resection received at least TMZ therapy and/or radiotherapy.
Table 4.4 Clinical characteristics of the glioblastoma patient cohort

<table>
<thead>
<tr>
<th></th>
<th>Primary</th>
<th>Recurrent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patients [n, %]</strong></td>
<td>33 (68.8)</td>
<td>15 (31.2)</td>
</tr>
<tr>
<td><strong>Median age, years (range)</strong></td>
<td>57 (16 - 75)</td>
<td>53 (12 - 74)</td>
</tr>
<tr>
<td><strong>Gender [n, %]</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>15 (45.5)</td>
<td>10 (66.7)</td>
</tr>
<tr>
<td>female</td>
<td>18 (54.5)</td>
<td>5 (33.3)</td>
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<tr>
<td><strong>Treatment strategy before resection [n, %]</strong></td>
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<td></td>
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<tr>
<td>none</td>
<td>33 (100)</td>
<td>6 (40.0)</td>
</tr>
<tr>
<td>TMZ therapy + radiotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMZ + cilengitide therapy + radiotherapy</td>
<td>7 (46.7)</td>
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<tr>
<td>TMZ + avastin + irinotecan therapy + radiotherapy</td>
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<tr>
<td><strong>Tumour location [n, %]</strong></td>
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<tr>
<td>right</td>
<td>19 (57.6)</td>
<td>10 (66.7)</td>
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<tr>
<td>left</td>
<td>11 (33.3)</td>
<td>3 (20.0)</td>
</tr>
<tr>
<td>other/unknown</td>
<td>3 (9.1)</td>
<td>2 (13.3)</td>
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<tr>
<td><strong>MGMT promoter methylation [n, %]</strong></td>
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<tr>
<td>methylated</td>
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<td>6 (40.0)</td>
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<tr>
<td>unmethylated</td>
<td>15 (45.5)</td>
<td>8 (53.3)</td>
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<tr>
<td>unknown</td>
<td>6 (18.2)</td>
<td>1 (6.7)</td>
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<tr>
<td><strong>Median progression-free-survival, months (95% CI)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11.1</td>
<td>5.8</td>
</tr>
<tr>
<td></td>
<td>(8.9 – 17.2)</td>
<td>(2.3 – 7.6)</td>
</tr>
</tbody>
</table>

TMZ: temozolomide; MGMT: O\(^b\)-methylguanine-DNA methyltransferase
We also examined the prognostic value of the clinical variables and reported their Kaplan-Meier estimate. Progression-free-survival (PFS) is defined as the period of time between surgical removal of the tumour and subsequent tumour regrowth. When comparing patients who underwent primary and recurrent tumour resection, Kaplan-Meier survival plot demonstrated a significant difference in median PFS, with 11.1 months (95% CI 8.9 - 17.2) following primary tumour resection and 5.8 months (95% CI 2.3 - 7.6) following recurrent tumour resection (p = 0.009) (Figure 4.9 A).

Statistically significant differences were observed across the PFS curves when patients were categorised as under 50, 51 - 60 and over 60 years old (p = 0.019, log-rank test) (Figure 4.9 B). Analysis using Cox proportional hazards regression models also shows that ageing was associated with worse prognosis (HR 1.03, 95% CI 1.00 - 1.06, p = 0.023). In addition, females tend to have a lower risk of tumour recurrence (HR 0.90 95% CI 0.48 - 1.68), while patients from whom tumour samples had unmethylated MGMT promoter tend to progress quicker (HR 1.48, 95% CI 0.73 - 3.01). Nevertheless, the results were not significant (p > 0.05, log-rank test) (Figure 4.10 A and B).
Figure 4.9 Prognostic value of the clinical variables in glioblastoma patients

Kaplan-Meier analysis shows a significant difference in median PFS between primary and recurrent GBM (A), while ageing was associated with worse prognosis (B). Log-rank test; $p < 0.05$. 
Figure 4.10 Prognostic value of the clinical variables in glioblastoma patients

Kaplan-Meier analysis shows that gender (A), and *MGMT* promoter methylation status (B) are not a prognostic marker in this cohort (Log-rank test; \( p < 0.05 \)).
4.2.2.2 Analysis of basal protein expression profiles in primary and recurrent tumour samples from GBM patients

Western blot and densitometry analysis of the new tumour samples was performed and protein expression levels of procaspase-9, procaspase-3, Apaf-1, Smac and XIAP were analysed (Figure 4.11). However, of the new tumour samples, actin level from 6 of 32 samples disappeared (arrows) and could not be quantified, which we believed was due to degradation of the extracted lysates. This could be contributed to sample collection procedures (De Cecco et al., 2009), since tissue degradation could occur in surgical pathology specimens immediately after tissue resection (Li et al., 2013). Therefore, in total, there were 48 tumour samples, with 33 primary and 15 recurrent GBM samples.
Figure 4.11 Primary and recurrent patient tumour samples differ in their basal protein expression levels of apoptotic proteins

Representative western blots showing Apaf-1, procaspase-3, procaspase-9, Smac and XIAP expression in patient tumour samples. Arrows indicated the disappeared actin band and these samples were excluded from study.
Protein expression levels were normalised to the actin loading control and expressed relative to HeLa protein levels set at 100% (Figure 4.12 and 4.13). When looking at the protein expression of patient tumour samples, the expression of procaspase-9, Apaf-1 and XIAP were lower than the HeLa control. When comparing between the primary and recurrent patient tumour samples, statistical analysis indicated a significantly higher expression of procaspase-3 in primary tumour samples ($p = 0.0311$). Of note, however, we found a lower expression level of procaspase-3 in primary tumour samples compared to what was previously found by our group (Murphy et al., 2013). Therefore, together with new tumour samples (238 and 259), some of the samples (P1, P2, P3, P7 and P9) were repeated on the same western blot and compared with those previously performed (Figure 4.14). We found that the expression level of procaspase-3 was lower in the repeated samples compared to the original samples. Since these tumour samples were four years old and had repeated thaw-freeze cycles (Silverman et al., 2005), the stability and structural integrity of certain proteins were vulnerable to degradation (Shabihkhani et al., 2014, Shao et al., 2012). This may have contributed to the difference in results. Therefore, we believe that the original sample results represented the actual level of procaspase-3, which later were included for current statistical analysis. For the expression of procaspase-9 ($p = 0.8906$), XIAP ($p = 0.1244$), Smac ($p = 0.3017$) and Apaf-1 ($p = 0.4602$), there were no significant differences identified between the primary and recurrent tumour samples.
Figure 4.12 Densitometry analysis of the expression of procaspase-9, Apaf-1 and XIAP

33 primary tumour samples are indicated as black, 15 recurrent tumour samples are indicated as dark grey while HeLa control is in white. All patient samples (in grey and black bars) generally displayed low basal expression of procaspase-9, Apaf-1 and XIAP when compared to the HeLa cell line. Mann-Whitney test; p > 0.05.
Figure 4.13: Densitometry analysis of the expression of Smac and procaspase-3

33 primary tumour samples are indicated as black, 15 recurrent tumour samples are indicated as dark grey while HeLa control is in white. A statistical analysis indicated significantly higher expression of procaspase-3 in primary tumour samples (p= 0.0311). Mann-Whitney test; p > 0.05.
An analysis of procaspase-3 expression level in some patient tumour samples was repeated and compared with the previous result from Murphy et al., 2013. The original sample results were shown to have a higher expression of procaspase-3 when compared to the repeat sample results.
4.2.2.3 Single proteins analyses are not a useful prognostic marker of PFS in GBM patients

Having identified the basal protein expression levels of studied proteins, next, we explored the prognostic value of each of the proteins. Therefore, for the analysis, the protein value was separated into two groups (high expression versus low expression). Tumours with an expression score less than the median expression score of all tumours are considered as having a low level of basal protein expression, or tumours with an expression score more than the median expression score of all tumours are considered as having a low level of basal protein expression. We did not observe any significant differences in PFS Kaplan-Meier curves when comparing patients with high (> median) versus low (≤ median) protein expression. The log-rank tests are: procaspase-3 (p = 0.37), procaspase-9 (p = 0.11), XIAP (p = 0.93), Smac (p = 0.78) and Apaf-1 (p = 0.64) (Figure 4.15 - 4.16).

Together, the results indicate that single protein analysis may not be a powerful prognostic marker of PFS in GBM patients. Having found the results, we undertook further analysis of the five key proteins and compared these results with the output of an experimentally validated, mathematical model of the caspase execution network previously developed by our group (APOPTO-CELL).
Figure 4.15 Single proteins analysis are not prognostic markers of disease progression

Kaplan-Meier estimates for procaspase-3 (A), procaspase-9 (B), and XIAP (C) comparing patients with low (≤ median) and high (> median) protein expression. No statistically significant differences in PFS were observed among groups for any of the protein (Log-rank test, p > 0.05).
Figure 4.16 Single proteins analysis are not prognostic markers of disease progression

Kaplan-Meier estimates for Smac (A), and Apaf-1 (B) comparing patients with low (≤ median) and high (> median) protein expression. No statistically significant differences in PFS were observed among groups for any of the protein (Log-rank test, p > 0.05).
4.2.2.4 The APOPTO-CELL model suggests that the cellular susceptibility to undergo caspase cleavage and activation is impaired in recurrent tumour samples

We previously showed a variation in protein concentrations within primary and recurrent tumour samples. Next, we sought to examine the potential of a mathematical model of caspases activation previously developed in our laboratory, APOPTO-CELL, could be utilised to predicts cellular susceptibility to undergo caspase cleavage and activation in primary and recurrent tumour samples. We hypothesized that the ability of GBM cells to activate apoptosis following caspase-dependent mitochondrial release of cytochrome c and Smac would be better captured by the interactions of the involved proteins rather than solely by single proteins.

Absolute protein amounts of procaspase-3, procaspase-9, XIAP, Apaf-1 and Smac (in µM) in GBM patient samples were calculated in relation to published protein concentrations of HeLa. Table 4.5 showed the calculated protein amounts, which served as an input into APOPTO-CELL. Figure 4.17 showed a representative of the modelled output for caspase-3 substrate cleavage, as generated by APOPTO-CELL. From 33 primary tumour samples, 94% (31/33) of those were capable of activating a sufficient level of executioner caspases. From 15 recurrent tumour samples, only 73% (11/15) were capable of activating a sufficient level of executioner caspases. Furthermore, of the tumour samples that failed to reach sufficient substrate cleavage within the simulation time of 60 min, all were also unable to do so at a simulation time of 100 min.

Collectively, the APOPTO-CELL output indicated a trend towards impaired apoptosis execution in recurrent tumour samples. Having found these results, next, we wanted to examine if the APOPTO-CELL could provide a tool to predict the suitability of targeted therapeutics (XIAP antagonist) in patient tumour samples.
Table 4.5 Protein concentrations of procaspase-9, procaspase-3, Apaf-1, Smac and XIAP within patient tumour samples

<table>
<thead>
<tr>
<th>Patient tumour samples</th>
<th>Procaspase-3 [µM]</th>
<th>Procaspase-9 [µM]</th>
<th>XIAP [µM]</th>
<th>Smac [µM]</th>
<th>Apaf-1 [µM]</th>
</tr>
</thead>
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<tr>
<td>HeLa</td>
<td>0.12000</td>
<td>0.03000</td>
<td>0.06300</td>
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<td>Patient tumour samples</td>
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<td>Procaspase-9 [µM]</td>
<td>XIAP [µM]</td>
<td>Smac [µM]</td>
<td>Apaf-1 [µM]</td>
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<tr>
<td>P23</td>
<td>0.017</td>
<td>0.002</td>
<td>0.002</td>
<td>0.114</td>
<td>0.101</td>
</tr>
</tbody>
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Figure 4.17 The APOPTO-CELL outputs for patient tumour samples

A representative of modelled output for caspase-3 substrate cleavage as generated by APOPTO-CELL. Optimal substrate cleavage (80%) should occur within 60 min after the onset of cyt-c release. A few patient samples did not achieve the targeted substrate cleavage, even at 100 min time frame. Data input as absolute protein concentrations [µM] based on established protein expression profiles (Figure 4.11).
4.2.2.5 Application of XIAP antagonist in APOPTO-CELL as targeted alternative treatment

In this study, we leveraged APOPTO-CELL to identify optimal candidates for XIAP antagonism by mimicking the administration of increasing concentrations of Smac at a physiologically relevant dosage (0 - 1 µM). We assessed the potential therapeutic effect by monitoring the likelihood of the cells to undergo caspase activation as a function of the XIAP antagonist dose.

A representative of APOPTO-CELL output of patient samples were presented in Figure 4.18 and 4.19. Similar to GBM cell lines, the patient samples showed variable sensitivity to XIAP antagonist at 1 µM. We observed three response patterns: non-improvers, non-responders and responders. The non-improvers group (n = 42) were previously predicted to reach sufficient substrate cleavage with standard treatment and showed minimal benefit from addition of XIAP antagonist. The non-responders group (n = 1; P18) remained resistant to apoptosis even after supplementation with the highest dose of XIAP antagonist. Interestingly, the responders group (n = 5; #441, #22, #1393, #811 and P12) showed a potential benefit with this personalised treatment regime, rendering this sub-group a suitable target population.
Addition of XIAP antagonist at different concentrations (0 - 1 µM) was performed and the outputs were calculated for the time (min) needed for optimal 80% caspase-3 substrate cleavage. Two primary tumour samples (#441 and P12) that previously failed to reach sufficient substrate cleavage were able to do so following addition of XIAP antagonist at 1 µM.

Figure 4.18 XIAP antagonist accelerates substrate cleavage in primary tumour samples
Figure 4.19 XIAP antagonist accelerates substrate cleavage in recurrent tumour samples

Addition of XIAP antagonist at different concentrations (0 - 1 μM) was performed and the outputs were calculated for the time (min) needed for optimal 80% caspase-3 substrate cleavage. At 1 μM, of four recurrent tumour samples (#22, #1393, #811 and P18), only P18 sample still fail to achieve the targeted substrate cleavage.
A Kaplan-Meier curve showed that the predicted times to 80% caspase-3 substrate cleavage vary significantly between primary and recurrent tumour samples (Log-rank, $p = 0.045$; Figure 4.20). We also look at the median time needed to reach sufficient substrate cleavage following in silico administration of 1 µM of XIAP antagonist compared to respective controls. For primary tumour samples, the median time needed with and without addition of XIAP antagonist were 6 and 12 min, respectively (Log-rank, $p < 0.0001$). For recurrent tumour samples, the median time needed with and without addition of XIAP antagonist were 6 and 27 min, respectively (Log-rank, $p = 0.0072$).

Collectively, analysis of the data showed that the ability to undergo apoptosis is impaired in recurrent compared to primary tumour samples. Our findings also suggest that this systems modelling approach could benefit low risk patients who do not need a more aggressive regime from those at high risk who are likely to either be sensitive or likely to remain resistant to treatment. Having found the result, next, we wanted to see if the systems modelling could serve as a prognostic tool of 7-months PFS in glioblastoma patients.
Figure 4.20 The predicted time to sufficient substrate cleavage in primary and recurrent tumour samples as calculated from APOPTO-CELL output

The calculated five protein expression levels in 48 tumour samples (33 primary and 15 recurrent samples) were used as an input for the model. Kaplan-Meier curve showed that, within 60 min time frame, the predicted times to 80% caspase-3 substrate cleavage vary significantly between primary and recurrent tumour samples \((p = 0.045)\), and between tumour samples after addition of XIAP antagonist (primary samples with and without addition of XIAP antagonist \((p < 0.0001)\); recurrent samples with and without addition of XIAP antagonist \((p = 0.0072)\)). Log-rank test; \(p < 0.05\).
**4.2.2.6 System modelling approach is a prognostic tool of 7-months progression-free-survival in glioblastoma patients**

As previously classified (Murphy et al., 2013), any patient who had a PFS time of greater than 7 months was considered a long-term PFS, while any patient who had a PFS time of less than 7 months was considered a short-term PFS. We hypothesised that the long-term PFS patients were capable of activating sufficient caspase-3 substrate cleavage quicker than the short-term PFS patients.

From 48 tumour samples that were analysed, 29 patients were considered as having a long-term PFS and 19 patients were considered as having a short-term PFS. APOPTO-CELL predictions for long-term PFS patients were 96.6% (28 of 29 patients) (i.e. capable of activating sufficient levels of executioner caspases). Of the 19 patients with short-term PFS, five were also accurately predicted by APOPTO-CELL (i.e. incapable of activating sufficient levels of executioner caspases). Overall, the APOPTO-CELL model had a predictive rate of 70% as the model’s substrate cleavage profiles correlated with PFS time in 33 of 48 patients analysed.

Kaplan-Meier analysis showed that long-term PFS patients had a significantly shorter time to reach 80% substrate cleavage than the short-term PFS patients (Log-rank, $p = 0.025$) (**Figure 4.22**). The result indicated that system-level analysis that accounts for the network of interactions and the relative abundance of the species can serve as a prognostic tool of 7-months PFS in GBM patients.
The APOPTO-CELL model was utilised to calculate whether the model prediction could serve as a prognostic tool of 7-months progression-free-survival in glioblastoma patients.
Concentrations of procaspase-3, procaspase-9, Smac, XIAP and Apaf-1 were used as an input for the model. Kaplan-Meier curve showed that the predicted times to achieve 80% caspase-3 substrate cleavage were significantly shorter ($p = 0.025$) for patients with long-term PFS times (median 18.4 min) compared to patients with short-term PFS times (median 24.6 min). Log-rank test; $p < 0.05$. 

Figure 4.22 APOPTO-CELL output in patient tumour samples with long-term and short-term progression-free-survival (PFS) time
4.3 Discussion

This study analysed five basal proteins concentration levels from sixteen GBM cell lines and forty-eight tumour samples from a cohort of GBM patients to classify proteins biomarkers involves in caspase-dependent mitochondrial cell death, followed by an integrated systems analysis of the interactions of these proteins using a mathematical model, APOPTO-CELL. The apoptosome formation is a signal of downstream effector caspases activation (Cullen and Martin, 2009). Caspase-3 can form a dependent feedback on the apoptosome, further generating the active caspase-9 form (Twiddy and Cain, 2007). In caspase-9 deficient animals or cells with mutant form of caspase-9, the apoptosome formation is compromised, which blocks activation of caspases-3 and -7 (Gyrd-Hansen et al., 2006). Since the role of caspase-9 is important in cytochrome c mediated apoptosis, in this study, our results showed a low procaspase-9 expression in GBM patient samples, and both low procaspase-9 and Apaf-1 expression in GBM cell lines. This may account, in part, for the observed apoptosis resistance in GBM as it could result in reduced apoptosome formation (Murphy et al., 2013).

During analysis of GBM cell lines, we wanted to correlate the data generated from the APOPTO-CELL with previous *in vitro* MTT studies. We found that all commercially available GBM cell lines that were sensitive to TMZ treatment *in vitro* were accurately predicted to achieve an early substrate cleavage within a 60 min time frame. Meanwhile, 7 of 10 patient derived GBM cell lines that did not show significant reduction of cell survival, also failed to reach the targeted substrate cleavage, while the other three cell lines were able to do so, but at a later time point compared to the commercial cell lines. With a predictive rate of 81.3%, the accuracy of APOPTO-CELL model in explaining the TMZ response in GBM cell lines is a useful tool for future studies of GBM.

The patient derived GBM cell lines generally showed a trend of XIAP overexpression. Assuming that MOMP and cytochrome c release has occurred, the deceleration of caspase-3 substrate cleavage could be explained by the inhibitory effect of XIAP. We further extended the APOPTO-CELL model in
GBM by implementing XIAP antagonist and mimicking the behaviour of Smac to predict the effect of this therapy on the likelihood of the cells to activate executioner caspase-3. Addition of XIAP antagonist at 1 µM significantly shortened the time needed to achieve optimal substrate cleavage compared to respective controls. While this system analysis of GBM cell lines was consistent with the previous experimental study by our group (Rehm et al., 2006, Schmid et al., 2012), however, the model did not accurately predict the *in vitro* responses following TMZ plus Birinapant treatment (*Table 4.3*).

The important limitation to this system modelling APOPTO-CELL is mainly that the model predicts effector caspase activation and substrate cleavage but not explicitly cell death or survival. Cells that are lacking cytochrome c could not activate caspases in response to mitochondrial pathway stimulation (Yeh et al., 2000). However, the model is based on the assumption that MOMP occurs following apoptotic stimulation, thus calculated the time from cytochrome c release to 80% caspase-3 substrate cleavage. While caspase-3 is the main executioner caspase involved during intrinsic apoptosis pathway activation (Mcllwain et al., 2015), caspase-6 and-7 are also greatly involved and in some cells, caspase-7 can propagate further caspase activation events in this cascade when caspase-3 is absent or disabled (Cullen and Martin, 2009). In GBM cell lines that were treated with immunosuppressant FTY720, a fully cleaved caspase-6 and only partial activation of caspase-3 were found (Sonoda et al., 2001). Similarly, Jiang and colleagues found that several GBM cells do not express caspase-3 and this may be a common finding among other neoplasms. When the cell lines were treated with *bcl-2/bcl-xL* antisense nucleotides, they found caspase-7 and -6 were cleaved to produce the active form, but not caspase-3. In particular, the U87 cell line displayed significant basal levels of caspase-3, but western blot failed to show any evidence of caspase-3 cleavage. Therefore, they suggested that there might be other preferential patterns of caspase activation in glioma (Jiang et al., 2003). The differences in caspase preferences in GBM cells could explain the difference between the *in vitro* results and the APOPTO-CELL output. The results from Jiang *et al.* (2003) were consistent with our U87 cell line data, in which we found caspase-dependent cell death *in vitro* even though caspase-3 cleavage
was not detected by western blot analysis; this confirms that caspase-3 is not significant for apoptosis induction in this cell line.

Furthermore, while the APOPTO-CELL capture the process of apoptosis-dependent caspase activation following MOMP, the cells still die independent of caspases. Apoptotic cell death process requires energy, and this comes from a constant elevation of cytosolic ATP (Zamaraeva et al., 2005). In the event of a rapid decline and ATP production becomes insufficient, the cells manipulated the event by generating reactive oxygen species (ROS), contributing to the demise of the cell (Kroemer and Martin, 2005). Also, when MOMP-inducing stimuli are not dominant, is blocked by caspase inhibitors or extensive cellular stress occur, autophagy may also play as a death executioner, inducing autophagy-mediated cell death (Gozuacik and Kimchi, 2004, Sui et al., 2013). Functional interplay is also common, such as an increase of ROS represents a cellular stress signals for the initiation of autophagy (Dewaele et al., 2010). Other protein mediators that are released during MOMP include apoptosis-inducing factor (AIF) and Endonuclease G, whereby upon released, are translocated to nucleus (Chipuk and Green, 2005) causing DNA cleavage (Prabhu et al., 2013). Towards the end, when mitochondria becomes defective and dysfunctional, PINK1/PARKIN pathway is activated and mitophagy; a selective process that mediates clearance of damaged mitochondria starts to work (Kubli and Gustafsson, 2012, Ashrafi and Schwarz, 2013).

Lastly, caspase-independent cell death following signalling from death receptors is a well elucidated mechanism of death almost certainly distinct from MOMP-dependent event (Degterev et al., 2005). The IAP antagonist, apart from inhibiting XIAP activity for effective intrinsic cell death, also exerts its function through downregulation of cIAP1 and cIAP2. Downregulation of cIAP prevents the ubiquitination of RIP1. While this will allow the formation of RIP1/FADD/caspase-8 for caspase activation and apoptosis (Wagner et al., 2013), it also leads to necroptosis, a programmed and regulated form of necrosis that is dependent on RIP1 and/or RIP3 activity (Vanden Berghe et al., 2014). In the absence of sufficient caspase-8 activation (Vandenabeele et al., 2010), RIP1 can form a necosome complex with FADD and RIP3 which in
turns initiates necroptotic cell death (Fulda, 2013). Downstream signaling of necroptosis has been proposed via an increase in ROS production (Zhang et al., 2009) or activation of autophagy (Ch'en et al., 2008, Christofferson and Yuan, 2010). Therefore, the mechanism of IAP antagonist does not equate with cell death via apoptosis but also with necrosis and necroptosis. In addition, differences with regards to treatment response in vitro and APOPTO-CELL is likely due to the fact that the APOPTO-CELL model does not take into account other proteins that could be influencing the cell death mechanism but considers only the intrinsic apoptotic events that occur subsequent to MOMP. Most importantly, the model only includes XIAP protein concentration as part of mathematical modelling but in vitro, the effect of Birinapant has specificity for cIAP1 and to the lesser extent, XIAP. Therefore, the addition of XIAP antagonist may accurately predict the ability of resistant GBM cell lines to execute sufficient caspase-3 substrate cleavage, but does not predict the survival of the cells.

GBM patients routinely undergo surgical resection, but patients will eventually relapse. Second line therapy is a formidable challenge complicated by upregulation of growth factors and anti-apoptotic signalling, leading to the development of new resistance mechanisms (Burton et al., 2002, Wick et al., 2011). Besides, TMZ therapy itself leads to genetic changes in post-TMZ GBMs, making recurrent GBMs more resistant to second-line therapy (Yip et al., 2009). The clinical characteristic in the cohort of GBM patients and their relation to PFS were assessed in this study. We found no association between PFS and gender and MGMT promoter status. The MGMT promoter was initially recognized following a trial of TMZ and radiotherapy in primary GBM patients, where patients with methylated MGMT promoter had a significant response to treatment, with better overall survival and PFS (Stupp et al., 2005a). However, this finding was not always replicated in subsequent studies (Costa et al., 2010). Evidence is growing that molecular subtypes of GBM influence the prognostic ability of MGMT promoter status (Brennan et al., 2013).

Our study sought to determine potential alterations in five key apoptosis execution proteins and examined its prognostic value. Our results demonstrated
no correlation of single proteins with patients PFS, hence is not a useful prognostic marker. A previous study attempted to identify other protein markers such as FasL, Fas, cleaved caspase-3, and cleaved caspase-8, but only found it was a negative prognostic indicator for patient survival in the absence or low expression of cleaved caspase-8 (Saggioro et al., 2014). Even though cleaved caspase-3 was known to be higher in GBM than normal glial tissues (Saggioro et al., 2014), other anti-apoptotic proteins, such as XIAP were also upregulated (Lopez et al., 2012). Previous studies also looked at the upregulation of certain microRNAs (Bo et al., 2015), and other genetic alterations (Virk et al., 2015) likely to contribute to further GBM recurrences. We observed a trend of increased XIAP and decreased pro-caspase-3 in recurrent tumour samples. Pro-caspase-9, a crucial upstream activator of cytochrome c-mediated caspase activation was not elevated in recurrent tumour samples. Moreover, since the patients with recurrent tumours were more likely to have received chemo- and radiotherapy, the cytotoxic effect through apoptotic mechanism could also result in an increased migration of subpopulation of tumour cells in which caspase activation was too low to induce cell death (Gdynia et al., 2007).

Previous study by our group has demonstrated that in HeLa cervical cancer cells, continuous elevation of XIAP levels delayed substrate cleavage (Rehm et al., 2006). Using system modelling, we could successfully predict which patients could be sensitised to TMZ treatment by correlating with tumour cells ability to activate intrinsic apoptotic cell death. At the same time, in tumour cells that showed a delay or unable to activate intrinsic apoptotic cell death, these patients could be personalised to other agents, such as XIAP antagonist. Indeed, upon simulation with XIAP antagonist, APOPTO-CELL provided guidance as to whether or not tumour cells could re-establish caspase-3 activity. Extension of the mathematical model to include the effect of XIAP antagonism identified a subset of patients that may indeed benefit from such a treatment. This data shows that mathematical modelling approaches can identify patients at higher risk, and forecast, on a case-by-case basis, whether or not therapeutic intervention could help the tumour cells to re-enter apoptosis.
We also undertook an analysis of APOPTO-CELL calculation of tumour cells’ ability to activate caspase-3 substrate cleavage and correlate with 7-month PFS. We found that those patients categorised as long-term PFS could activate sufficient substrate cleavage in a significantly shorter time frame compared to short-term PFS. Our system modelling carries a prediction rate of 70%, suggesting that those patients who were categorised into long-term PFS were those who demonstrated a better treatment response to standard TMZ therapy and radiotherapy. At the same time, those patients who were categorised into the short-term PFS time were not able to respond to these agents due to effective resistance mechanism within tumour cells.

Taken together, APOPTO-CELL model is a useful tool in defining caspase activation in cancer cells (Schmid et al., 2012, Rehm et al., 2006), with limitations applying to GBM. While the model is a predictive tool of TMZ response in vitro, we do not see a correlation following addition of IAP antagonists, particularly in patient derived GBM cell lines. This suggests that other pathway might be activated independent of MOMP-dependent caspase-3 activation. In clinical practice, Birinapant is tested in other types of cancer (Amaravadi et al., 2015, Noonan et al., 2015), but not yet in GBM patients. We believe that addition of IAP antagonists, on a case-to-case basis can help the tumour cells to enter apoptosis. This in future can be a useful tool to stratify patients that require additional chemotherapeutic agent during or after the standard TMZ therapy and radiotherapy. The data obtained in the previous (Chapter 3) and current chapter was the initial experimental design combining TMZ and Birinapant. With the completion of in vitro study, Birinapant was further investigated in in vivo small animal models. Since the combination treatment has not previously been reported, a pilot study was designed in small cohort of animals to determine toxicity of Birinapant alone and in combination with TMZ in non-tumour bearing animals (Chapter 5), prior to use of these agent in clinically relevant orthotopic GBM models.
CHAPTER 5:

*In vivo* toxicity of oral delivery of TMZ in combination with intraperitoneal administration of Birinapant in a small animal model
5.1 Introduction

5.1.1 Toxicity studies

Since the combination treatment of TMZ and Birinapant has not previously been assessed in vivo, the aim of this chapter was to determine the maximum tolerated dose (MTD) and uncover the toxicity profiles of these agents, in pre-clinical animal models. Three important aspects for detecting the adverse effects observed during a toxicology study are haematology, clinical chemistry assays, and histopathology evaluations of tissues collected at necropsy (Guarino, 2009). Within this chapter, the above effects were of particular interest due to previous findings that showed TMZ and Birinapant as a single agent can cause abnormal blood profiles. Full blood count (FBC) is one of the most commonly performed investigations to detect general health of animals. An analysis of the haematopoietic system provides an overall assessment of the circulating blood, focusing on erythrocytes, leukocytes and clotting ability. The enumeration of peripheral blood reticulocytes is often performed to obtain information about the functional integrity of the bone marrow (Riley et al., 2001). A reduction in red and white blood cell count may indicate a defect in bone marrow activity (Stock and Hoffman, 2000) (Figure 5.1). The bone marrow is the primary target of toxicity for many chemotherapy agents, such as TMZ, and myelosuppressive effect commonly occur, both in patients and rodents (Thomas et al., 2013, Stedt et al., 2013, Tsujiuchi et al., 2014).

During the past 25 years, cytokines have become an important pleiotropic regulator of the immune response (Schulte et al., 2013), and play a vital place as diagnostic, prognostic and therapeutic agents in human disease (Dinarello, 2007). Birinapant can stimulate an immune response. As mention previously, Birinapant induces the degradation of IAPs, which in turn can lead to the activation of the non-canonical NFκB pathway and increased production of TNFα. TNFα drives a variety of cellular responses, including inflammatory responses (Varfolomeev and Ashkenazi, 2004, Erickson et al., 2013), which are needed for an effective immune response to pathogens. However, overstimulation aggravates further release of pro-inflammatory cytokines (e.g.,
Interleukins, Interferon-γ) and chemokines (Erickson et al., 2013), leading to potential multi-systemic toxicity (Tarrant, 2010). An influx of leukocytes during tissue injury (Moser and Loetscher, 2001, Lebre et al., 2005), is detected from evidence of neutrophilia (raised neutrophil) during haematological investigation.
Figure 5.1 Major development stages of blood cells

All development stages of blood cells are formed in the bone marrow. A blood stem cell goes through several steps to become a red blood cell, platelet, or white blood cell.
Within this chapter, analysis of biochemistry profiles is performed for the particular organ of interest such as liver, pancreas and kidney. Drug-induced liver injury is a major cause of attrition in preclinical and clinical drug development, and when a drug reaches the market (O'Brien et al., 2006). The most commonly evaluated marker of drug-induced liver injury in preclinical and clinical studies is alanine transaminase (ALT) (Yang et al., 2009). However, recently, in rats, glutamate dehydrogenase (GDH) has been shown to be a more effective biomarker of acute hepatic injury than ALT, aspartate aminotransferase (AST) and ALP, based primarily on its large increase following hepatocellular injury, high tissue specificity, and prolonged persistence in the blood following injury (O'Brien et al., 2006). Evaluation of liver profiles in our study is of interest due to the finding that both TMZ and Birinapant treatment can cause liver injury in patients. In the US, TMZ is included as one of the antineoplastic agents to cause drug-induced liver disease (Chalasani et al., 2008). In a randomised phase II trial, of 85 patients with glioblastoma treated with TMZ, ALT or AST elevations occurred in 6% of patients overall (Clarke et al., 2009). Similarly, mini case reports have reported TMZ-induced liver injury in GBM patients (Neyns et al., 2008, Dixit et al., 2011). For Birinapant, safety data is available from 16 acute myeloid leukaemia (AML) subjects who received Birinapant as a single agent in a Phase 1/2 clinical trial; the study reported elevated AST and ALP as a treatment related adverse event (http://ir.tetralogicpharma.com/secfiling.cfm?filingid=1047469-14-2620&cik=1361248).

Evaluation of pancreatic injury is also of an interest due to findings that TMZ or Birinapant treatment can cause elevated lipase levels in patients. In a phase II study of sorafenib plus daily TMZ in adults with recurrent GBM, 8 of 32 patients experienced grade 2 to 4 toxicity, with symptomatic elevation of lipase (Reardon et al., 2011). In another study combining bevacizumab and TMZ in adults with recurrent GBM, pancreatitis was reported as grade 4 toxicity (Desjardins et al., 2012). Similar events were observed in patients treated with Birinapant. Tetralogic Pharmaceutical, the manufacturing company of Birinapant, has reported a grade ≥ 3 toxicity associated with elevated serum amylase and
lipase in patients who received Birinapant treatment (http://ir.tetralogicpharma.com/events.cfm).

In this study, lipase level were measured using DGGR-lipase, which has previously been validated in dogs (Graca et al., 2005) and cats (Oppliger et al., 2014) but never in rodents. DGGR is cleaved by lipase, resulting in an unstable dicarboxylic acid ester, which is spontaneously hydrolyzed under alkaline pH to yield glutaric acid and methylresorufin, an indigo chromophore with peak absorption at a wavelength of 580 nm. The rate of methylresorufin formation is directly proportional to the lipase activity in the sample (Oppliger et al., 2013, Panteghini et al., 2001).

5.1.2 Aims

Our first aim was to determine the MTD for the combination treatment of TMZ and Birinapant in rats. Our second aim was to assess the toxicity profile of TMZ and Birinapant as monotherapies and as a combination therapy, via weekly tail vein blood sampling. Our third aim was to evaluate whether the DGGR-assay for lipase can be used as a suitable marker for detecting acute pancreatic injury in rats. Our fourth aim was to evaluate whether GDH is a superior marker than ALP for detecting acute liver injury in rats. Our final aim was to compare the blood results and correlate them with the histopathology analysis using Haematoxylin and Eosin (H&E) stain.
5.2 Results

5.2.1 *In vivo* dose escalation study of systemic oral administration of TMZ in combination with intraperitoneal administration of Birinapant in athymic nude rats

As Birinapant has never been administered with TMZ, it was important to determine whether the animals would tolerate the combination of these drugs. Thus, initially a dose escalation study to determine the MTD using athymic rats (n = 5 animals) was performed. For each animal, the TMZ dose was maintained at 25 mg/kg for 7 consecutive days via oral gavage (p.o.), while each animal received 9 doses of Birinapant, given intraperitoneally (i.p.) every 3 days *(Figure 5.2A)*. The Birinapant treatment was carried out on the same week as TMZ. Treatment commenced on one animal, whereby this animal received a starting dose of Birinapant of 10 mg/kg. Following completion of TMZ administration (7 days) and 3 doses of Birinapant (10 mg/kg), if the animal did not show signs of toxicity, the next animal received an incremental dose of 5 mg/kg. This treatment pattern continued until the dose of Birinapant reached 25 mg/kg. Following completion of TMZ and three doses of Birinapant at 25 mg/kg, the rat became lethargic and agitated. Even though the weight loss did not reach 15%, we considered this event as a side effect of the treatment. The dose was then reduced to 20 mg/kg on this animal until the end of the study.

These findings demonstrated that the targeted dose for Birinapant, when used in combination with TMZ, is 20 mg/kg as this animal displayed no significant change in general condition or body weight during the 9 dose treatment regimen *(Figure 5.2B and C)*. Using these doses of TMZ (25 mg/kg) and Birinapant (20 mg/kg) as monotherapies and as combination therapies, we next performed tail vein blood sampling to assess their haematological and biochemistry profiles.
Figure 5.2 *In vivo* dose escalation study of systemic oral administration of TMZ in combination with intraperitoneal administration of Birinapant in athymic rats

All animals received TMZ at 25 mg/kg (diluted in 10% DMSO/D-PBS) for 7 consecutive days and 9 doses of Birinapant given every 3 days (in 20% captisol/H₂O). Birinapant treatment commenced on the same week as TMZ, with one animal receiving a starting dose of 10 mg/kg. Following completion of TMZ and 3 doses of Birinapant, if the animal did not show signs of toxicity, the next animal received an increasing dose of 5 mg/kg. The maximum tolerated dose of Birinapant achieved in combination with TMZ was 20 mg/kg (A). Weight of each athymic rat was monitored daily until treatment completion (B). Percentage of body weight gain of rat during treatment is shown (C). Data are expressed as mean ± S.E.M (one-way ANOVA and Tukey’s post-hoc test).
5.2.2 Change of Birinapant dose in Sprague Dawley rats

From the established doses of TMZ and Birinapant, next, we sought to determine the haematological and biochemistry profiles of TMZ and Birinapant as monotherapies and as combination therapies. **Figure 5.3A** shows the schematic diagram of the treatment schedule. In this study, female Sprague Dawley rats were assigned to four groups (n=5 animals per treatment group). The control animals received both vehicle agents via p.o. (DMSO/H₂O) and i.p. (Captisol/H₂O) injection. Animals in the TMZ group were administered 25 mg/kg p.o. (in 10% DMSO/D-PBS) for seven days. Animals in the Birinapant group were administered 20 mg/kg i.p. (in 20% captisol/H₂O) every three days (9 doses in total). Animals in the combined therapy group received both treatments at these doses. As before, Birinapant treatment commenced the same week as TMZ.

To minimise the stress and physiological variables in experimental rats, handling and blood sample collections were performed by the same researcher. A week prior to the experiment, each rat was handled daily by the researcher, including placing the rat in a restrainer device to acclimatize. IV canulation of the lateral tail vein was performed in the morning while the rat was in the restraining device, without anaesthesia or food and water deprivation. Rats were monitored daily for any evidence of toxicity. Following treatments, the animals in the Birinapant group that were scheduled to receive nine doses were halted after the sixth dose, as the animals could not tolerate the treatment. After the fifth dose of Birinapant, 2 of 5 animals from Birinapant group showed signs of acute toxicity which were followed by another 2 animals after the sixth dose, all requiring euthanasia (**Figure 5.3B**). The rest of the animals showed no evidence of toxic effect.

**Figure 5.4** shows the weight of each rat per treatment group. The rats in the vehicle and TMZ group showed a steady increase in weight with no side effects from the treatment (**Figure 5.4 A, B**). A decrease in body weight (<15% from the baseline) was observed in Birinapant and combination treated animals following the second dose of Birinapant. When compared to control animals, a
more significant decrease in body weight was found in the Birinapant treated animals than in the combination treated animals, which was observed from day 5 ($p < 0.05$) (Figure 5.4 C-E). However, the weight returned to baseline over the course of treatment, but the minimal gained increase in weight, especially in Birinapant treated animals, indicated that the animals in this group could not tolerate the drug at this dose. The association of the body weight changes was consistent with the toxic effect of Birinapant, where the animals in this group showed signs of reduced activity and lethargy (C). The animals in the combination group surprisingly tolerated the doses and were alive after the sixth dose of Birinapant, with no clinical signs of toxicity (D, E). Following these events, the collected blood sample was analysed in order to determine the organs involved during the toxic event.
Figure 5.3 Schematic diagram of an *in vivo* treatment for the toxicity study

Twenty Sprague Dawley rats (n = 5 animals per treatment group) were randomised into four groups. The control animals received both vehicle agents. Animals in the TMZ group were administered the drug p.o. at 25 mg/kg for seven days. Animals in the Birinapant group were administered the drug i.p. at 20 mg/kg every three days (9 doses in total). For animals in the combined therapy group, the treatments consist of both TMZ and Birinapant (A). After the fifth dose of Birinapant, 2 of 5 animals from the Birinapant group showed sign of acute toxicity followed by another 2 animals after the sixth dose, all requiring euthanasia (B). All animals were euthanised two week post treatment onset.
Figure 5.4 Body weight changes during *in vivo* toxicity study

Weight of each Sprague Dawley rat (n=5 animals per treatment group) administered with Vehicle (A), 25 mg/kg/day for 7 days of TMZ (B), 20 mg/kg/day every 3 days (6 doses) of Birinapant (C), and combination of TMZ and Birinapant (D). All animals treated with vehicle or TMZ showed no clinical signs of toxicity. When compared to control animals, a significant decrease in body weight in Birinapant than combination treated animals was observed from day 5. Body weight in Birinapant treated animals remained significant at day 8 post treatment, while combination treated animals steadily increased (E). Data are expressed as mean ± S.E.M (one-way ANOVA and Tukey’s post-hoc test, *p* < 0.05 versus control group).
5.2.3 TMZ treatment induces myelosuppression while Birinapant targets circulating haemoglobin

In this study, four sets of blood samples were collected; 1) Pre-treatment- the bloods were collected one week before any treatment commenced, 2) Week 1- the bloods were collected at the end of the first week of treatment, 3) Week 2- the bloods were collected at the end of the second week of treatment and 4) Recovery- the bloods were collected one week after the completion of all treatments. There were no samples collected for rats in the Birinapant group from Week 2, as the animals were euthanised during the second week of treatment due to acute toxicity. Statistical differences were compared between the treated and control animals at the indicated week. The results of each parameter analysed were referred to the normal ranges from Petterino et al. (2006). They evaluated the clinical haematology and chemistry data in control Sprague Dawley rats in particular, the differences between male and female animals for all parameters, and the differences between the two major intra-sex groups at 4 and 13 weeks of the study. Since the age of animals used in the current study was 8-10 weeks old, the results were referred to normal ranges at 13 weeks. Of note, several values were not analysed in their studies, including reticulocyte and lipase.

Figure 5.5 shows the results of reticulocytes and haemoglobin for each group. As expected from the previous reported study (Kourelis et al., 2015), TMZ activity was consistent with reticulocytopenia, a reduction of reticulocyte count. The suppression of bone marrow was significant during the treatment (week 1) (p < 0.01), which then resolved on the subsequent week. A similar result was observed in the combination group at Week 1 (p < 0.01), which was resolved by Week 2. However, reticulocytopenia was again detected on Recovery (p < 0.05). For Birinapant, the treatment had no significant effect on bone marrow (Figure 5.5 A). Next, when we analysed the haemoglobin level, the animals treated with TMZ were normal (134-179 g/L), while Birinapant treatment were significantly decreased (p < 0.05). A decreased level of haemoglobin in the combination group was observed from Week 1 until Recovery (Figure 5.5 B).
Four sets of blood samples (two in the Birinapant group) were collected (n = 5 animals per group). TMZ treatment mainly affects erythrocyte production in bone marrow, causing reticulocytopenia. The reticulocyte count was normal in the Birinapant group (A). For the haemoglobin count, a normal level was observed in the TMZ group, while the Birinapant and combination group were significantly decreased. A decreased level of haemoglobin in the combination group was observed from Week 1 till Recovery (B). Data are expressed as mean ± S.E.M (one-way ANOVA and Tukey’s post-hoc test, *p < 0.05, **p < 0.01, and ***p < 0.001; versus control group of indicated week).
5.2.4 TMZ and Birinapant combined therapy prevents Birinapant-induced acute neutrophilia

In this study, we analysed four sera; WBC, neutrophils, platelets, and lymphocytes (Figure 5.6). The animals in the TMZ group had neutropenia and lymphocytopenia on Week 1 ($p < 0.01$), which were normalised by Recovery. A variation in platelets was observed, with increased platelets on Week 1 and Recovery ($p < 0.05$), and decreased platelets on Week 2 ($p < 0.01$). Animals in the Birinapant group had increased WBC with significant neutrophilia ($p < 0.01$), suggesting a treatment-induced inflammatory response. In addition, there were lymphocytopenia ($p < 0.05$), with a normal platelet count. For the combined treatment, there was a trend towards leukocyt- , neutro-, thrombocyto- and lymphocytopenia, in particular during Recovery.
Figure 5.6 Levels of WBC, neutrophils, platelets and lymphocytes following treatment with TMZ and/or Birinapant

Four sets of blood sample (two in Birinapant group) were collected (n=5 animals per group) in the study. Analysis were performed on white cell count (A), neutrophil (B), platelets (C) and lymphocytes (D). The TMZ group showed evidence of myelosupression, with neutropenia and lymphocytopenia. A similar effect was observed when the treatment was combined. Animals treated with Birinapant however, showed a significantly increased neutrophils count (neutrophilia), with lymphocytopenia. Data are expressed as mean ± S.E.M (one-way ANOVA and Tukey’s post-hoc test, *p < 0.05, **p < 0.01, and ***p < 0.001; versus control group of indicated week).
5.2.5 Birinapant induces acute pancreatitis and liver injury

To examine the biochemistry profiles of Sprague Dawley rats treated with TMZ and/or Birinapant, we analysed the biochemistry profile of three organs: the pancreas (lipase), the liver (GDH and ALP) and the kidney (calcium, magnesium, phosphate urea, creatinine and creatinine kinase). Due to its known sensitivity and specificity, DGGR-assay was used to measure lipase levels to detect if there was evidence of acute pancreatic injury. Figure 5.7 shows results for lipase and glucose levels. The animals in the TMZ and combination group had a normal lipase level. However, this level was significantly higher in the Birinapant group (p < 0.05), suggesting acute pancreatitis (Figure 5.7A). For glucose, a normal level was observed in the TMZ and Birinapant group. However, animals in the combination group developed hyperglycemia during Recovery (p < 0.001) (Figure 5.7B).

Next, analysis of the liver profile was performed for GDH and ALP (Figure 5.8). Both levels were normal in the TMZ group, while the animals in the combination group had a normal GDH but significantly low levels of ALP (p < 0.05). The animals in the Birinapant group however, showed evidence of acute liver injury with a significant level of GDH (p < 0.05) but a normal level of ALP.

Next, analysis of kidney profiles were performed. All animals had a normal calcium and magnesium level (Figure 5.9 A, B). For phosphate, a significantly high level was observed in the combination group on Week 2, which was back to normal at Recovery (Figure 5.9C). Figure 5.10 shows analysis of urea, creatinine and creatinine kinase. Animals in the Birinapant and combination group had a normal creatinine but a significant increase in urea (p < 0.05) (Figure 5.10 A), suggesting mild dehydration. Both urea and creatinine were normal for the TMZ group (Figure 5.10 A, B). For creatinine kinase, the level in the combination groups were significantly decreased during treatment, with further decreases observed at Recovery (p < 0.001) (Figure 5.10 C).

Following completion of this study, necropsy was performed and the organs of interest were kept in formalin and later analysed using H & E stain technique.
Four sets of blood sample (two in Birinapant group) were collected (n=5 animals per group) in the study. The lipase levels in the TMZ and combination group were normal, while a significantly high level was observed in the Birinapant group (A). For glucose, a normal level was observed in the TMZ and Birinapant group. Animals in the combination group showed significant hypoglycemia on Week 2, but later developed hyperglycemia on Recovery (B). Data are expressed as mean ± S.E.M (one-way ANOVA and Tukey’s post-hoc test, *p < 0.05, and ***p < 0.001; versus control group of indicated week).
Four sets of blood samples (two in Birinapant group) were collected ($n = 5$ animals per group) in the study. The GDH levels in the TMZ and combination group were normal while a significantly high level was observed in the Birinapant group (A). For ALP, normal levels were observed in the TMZ and Birinapant groups. Animals in the combination group showed a significantly low level of ALP on Week 1 and 2 (B). Data are expressed as mean ± S.E.M (one-way ANOVA and Tukey's post-hoc test, *$p < 0.05$ and **$p < 0.01$; versus control group of indicated week).
Figure 5.9 Levels of calcium, magnesium and phosphate following treatment with TMZ and/or Birinapant

Four sets of blood sample (two in Birinapant group) were collected (n=5 animals per group) in the study. All animals had a normal calcium (A) and magnesium level (B). For phosphate, a significantly high level was observed in the combination group on Week 2, which later was back to normal on Recovery (C). Data are expressed as mean ± S.E.M (one-way ANOVA and Tukey’s post-hoc test, *p < 0.05 versus control group of indicated week).
Figure 5.10 Levels of urea, creatinine and creatinine kinase following treatment with TMZ and/or Birinapant

Four sets of blood sample (two in Birinapant group) were collected (n=5 animals per group) in the study. Animals treated with TMZ had a normal urea level, while animals treated with Birinapant had significantly increased urea levels. A significant level was also observed in the combination group, particularly on Week 2 and Recovery (A). All animals had a normal creatinine level (B). For creatinine kinase, a significantly decreased level was observed in the combination group, while normal levels were observed in the TMZ and Birinapant group (C). Data are expressed as mean ± S.E.M (one-way ANOVA and Tukey’s post-hoc test, *p < 0.05 and **p < 0.01; versus control group of indicated week).
5.2.6 Histopathological changes associated with Birinapant treatment

Microscopically, control rats revealed no significant abnormalities in the liver, spleen and pancreas. The rat receiving TMZ treatment had features consistent with the control animals. The splenic tissue was analysed for the control and TMZ treated rats, which showed a normal pathology (Figure 5.11A, B). However, within the spleens of rats treated with Birinapant only and in combination with TMZ, there was a reduction in white matter with diffuse loss of the marginal zones (Figure 5.11C, D); the marginal zone is a passage for most arterial blood to enter the spleen and is central for trapping of particulate antigen and is well equipped with marginal zone macrophages (MZMs), a small subset of specialized splenic macrophages that immunoregulate and control the pathogen entering the spleen from circulation (McGaha et al., 2011). Loss of marginal zone represents a depletion of MZM, which in turn leads to uncontrolled release of proinflammatory cytokines and limits the successful control of infection (Aichele et al., 2003, McGaha et al., 2011).

Histopathology of the liver shows occasional parenchymal mononuclear cell infiltrations, with normal architecture in the control, TMZ and combination group (Figure 5.12A, B, D). However, in the Birinapant group, there was diffuse hepatocyte hypertrophy. Hepatocyte hypertrophy is a typical response to drug induced enzyme induction within liver cells. In addition, there was occasional periportal mononuclear cell infiltration and individual cell necrosis of leucocytes within hepatic sinuses (Figure 5.12C).

Pancreatic tissue was analysed for Control, TMZ and Birinapant rats. Both Control and TMZ rats showed normal histology (Figure 5.13A, B). However, in Birinipant rats, between lobules and within the mesentery, there was prominent vascular congestion, recent haemorrhage and extensive oedema. This was accompanied by a mixed inflammatory cell infiltration (lymphocytes, plasma cells, macrophages), and there is individual cell necrosis. Overall, this suggested acute inflammation in the (peri)pancreatic region (Figure 5.13C).
Figure 5.11 Haematoxylin and Eosin stain analysis of splenic tissue

Representative images of splenic tissues of animals in control (A), TMZ (B), Birinapant (C) and combination group (D) are shown. Animals in the control and TMZ group had normal splenic tissue while the Birinapant and combination groups had a diffuse loss of the marginal zones. Magnification 10x; scale bar = 25 µm.
Figure 5.12 Haematoxylin and Eosin stain analysis of liver tissue

Representative images of liver tissues of animals in control (A), TMZ (B), Birinapant (C), and combination groups (D) are shown. Histopathology of the liver shows occasional parenchymal mononuclear cell infiltration, with normal architecture in the control, TMZ and combination group. Hepatocyte hypertrophy (arrow) was found in the Birinapant group. Magnification 40x; scale bar = 25 µm.
Figure 5.13 Haematoxylin and Eosin stain analysis of pancreatic tissue

Representative images of pancreatic tissue of animals in Control (A, B), TMZ (C, D) and Birinapant rats (E, F) are shown. A normal histopathology specimen was found in the Control and TMZ rats. The pancreatic tissue in the Birinapant-treated rat was associated with prominent vascular congestion (arrow), extensive oedema and mixed inflammatory cell infiltration (dash arrow), suggesting acute inflammation. Magnification 10x (A, C and E) and magnification 20x (B, D and F); scale bar = 25 µm.
5.3 Discussion

In initial experiments, we investigated the MTD for TMZ and Birinapant in athymic nude rats. During this study, there was no evidence of side effect or toxicity attributed to any of the agents that were administered. The TMZ dose was maintained at 25 mg/kg, even though there were a few papers that used a higher dose up to 100 mg/kg in rat models (Wei et al., 2013). We justified the dosage based on the current treatment of GBM (as per Stupp protocol), which states that the safest dose for adjuvant TMZ treatment is 150 mg/m$^2$ and should only be increased so long as there is no haematologic toxic effects (Stupp et al., 2005b). Based on the dose translation from human to animal studies, the dose in humans is equivalent to 25 mg/kg in rats (Freireich et al., 1966, Reagan-Shaw et al., 2008). Therefore, together with TMZ, the dose escalation study was designed by increasing the concentration of Birinapant to where the toxicity became evident. Birinapant has previously been used in mice in vivo. The dosage ranges from 15 mg/kg (6 doses), 30 mg/kg (9 doses) to a maximum of 60 mg/kg (8 doses) (Ma et al., 2012, Krepler et al., 2013, Eytan et al., 2015). Given the equivalent surface area dosage conversion factors (Freireich et al., 1966, Reagan-Shaw et al., 2008), the appropriate dose the rats should receive is half the dose of the mice. When combined with TMZ, we found the highest tolerated dose for Birinapant was 20 mg/kg (9 doses in total).

In a small study of animal models, once the dosage is established, it is sufficient to start an efficacy or survival study (in this case, the in vivo mouse GBM model). However, we have expanded this study by investigating the haematological and biochemistry profiles using the immunocompetent Sprague Dawley rats, a widely used animal model for toxicity studies (Erickson et al., 2013, Wong et al., 2012). During the study, we found that Sprague Dawley rats could not tolerate the same doses that were given to athymic rats. This was an unexpected clinical finding as previously, the same dosages were administered in Athymic rats and the animals demonstrated no evidence of toxicity. Furthermore, this was only evidenced in animals treated with Birinapant only, but not the combination group.
We speculated that Birinapant treatment had aggravated a systemic inflammatory response in the Sprague Dawley rats, which was consistent with our haematology and biochemistry result. Since this effect was not evident in the athymic rats, we believe that an immune response played a role in the demise of these animals. To some extent, the inflammatory response was prevented by TMZ that have immune inhibitory effects, specifically via selective toxicity to proliferating lymphocytes and inhibition of differentiation of immune effectors (Roos et al., 2007, Alvino et al., 1999). Given the toxicological hazards identified in this study, we believe that careful selection of the animal used is crucial to toxicology assessment (Erickson et al., 2013).

It has previously been shown that bone marrow cells are particularly vulnerable to TMZ due to lower MGMT activity than tumours, resulting in myelosuppression (Gander et al., 1999, Gerson, 2002). Haematologic toxicities can occur in as much as 14% of patients, thereby preventing the administration of the intended cumulative dose (Mutter and Stupp, 2006). These percentages are very low when compared to the study reported by Gupta et al. (2013); they showed that in adult patients with GBM who received TMZ, the overall incidence of significant acute haematologic toxicity was 40%. In the current study, the effect of TMZ was similar to those observed in patients. Myelosuppressive effect, as evident by reticulocytopenia, was evident in TMZ treated animals (Figure 5.5 and 5.6). However, animals treated with Birinapant displayed decreased haemoglobin levels, while the combination treated animals were decreased in both parameters. These data demonstrate a specificity of the each drug on the RBC. Birinapant treatment affects the haemoglobin circulating in the peripheral blood without affecting the erythrocyte production in bone marrow. However, with persistent reduction of haemoglobin from continuous treatment, the bone marrow had to increase the production of erythrocyte to a certain stage that it became exhausted, and the decreased bone marrow activity was evident during Recovery.

While inhibition of IAPs has been of interest therapeutically due to findings that it can enhance cell death in vitro, either by promoting the intrinsic or extrinsic pathways (Chapter 1.5.3 and 1.5.4), inhibition of IAP proteins has been shown
to work differently in vivo; for example, it has been shown that IAP inhibition in cancer cells can lead to anti-tumoural activity via stimulation of the immune system (Lecis et al., 2013). Even though this response could help to kill tumour cells, healthy tissues are also exposed to the effect of IAP antagonist. The acute inflammatory process is characterized by rapid recruitment of granulocytes (i.e. neutrophils, eosinophils and basophils) to the inflammatory site (Maskrey et al., 2011). Monotherapy treatment with Smac mimetic has previously been shown to promote inflammation and immunogenic cell death in vivo (Lecis et al., 2013). In this study, we observed significant findings from the haematological analysis, particularly in the Birinapant group where increased neutrophils level was observed, while others were normal (Figure 5.6). Furthermore, in the combination group, the level was initially high (Week 1) but on the subsequent weeks was decreased. This was contributed to by the effect of TMZ in inducing lymphopenia and myelosuppression (Sengupta et al., 2012), hence preventing the inflammatory response aggravated by Birinapant treatment.

From the biochemistry profiles, we also found an increase in GDH levels in the Birinapant group, while others remained normal (Figure 5.8). The ALP level however, remained normal in this group. In the introductory section, we discussed the clinical significance between GDH and ALP and recent data have shown GDH to be a more effective biomarker for acute hepatic injury (O'Brien et al., 2002). Our result is in agreement with this finding and we believe that the animals treated with Birinapant developed acute liver injury; this was supported by the histopathological changes found in the liver tissue (Figure 5.12), likely as a result of systemic inflammatory response.

On literature review, we came across two papers that described quite similar events (Wong et al., 2012, Erickson et al., 2013). The authors used the Smac mimetic, GDC-0152, and performed the study on female Sprague Dawley rats (as per current experiment); they found that this molecule induced activation of the inflammatory response with elevated plasma cytokines and chemokines. TNFα increases the phagocytic potential of neutrophils while stimulating the expression of adhesion molecules on endothelial cells, thereby enhancing the
movement of leukocytes out of the circulatory system towards the site of injury or infection (Wong et al., 2012). It also elicits an increased production of monocytes/macrophages from bone marrow. These haematological investigations are consistent with our findings, which showed an increased level of neutrophils, with decreased levels of lymphocytes. The toxicity profiles were systemic and involved lung and liver injury, both of which were attributed to acute inflammatory responses of the immune system and confirmed from histopathological examination.

Since the observation that IAP antagonists, such as Birinapant, allow for non-canonical NFκB activation, it also leads to spontaneous production of cytokines from certain cell types, both in vitro as well as in vivo (Kearney et al., 2013). It may be very useful in cancer cells overexpressing IAPs, where an increase in local TNFα would be essential for any antitumour effects of IAP antagonists. Furthermore, others have postulated that in vivo TNF originates primarily from the host rather than from tumour cells (Lecis et al., 2013). Therefore, normal cells are also subject to the effects of IAP antagonism and widespread NF-κB activation can result in adverse events due to a TNFα driven inflammatory response (Wong et al., 2012). This is potentially a significant complicating factor regarding the use of Birinapant as a single agent. However, combining Birinapant with chemotherapy agents that have immunosuppressive effects, such as TMZ, may be a useful strategy to minimize the inflammatory effect of Birinapant.

Pancreatic damage causes a release of pancreatic lipase that is stored in the pancreatic acinar cells into circulation, and serum lipase activity becomes useful in the diagnosis and evaluation of pancreatitis (Steinhauer et al., 2002). Amylase is another serum marker and most commonly analysed together with lipase (Mitchell et al., 2003). However, since plasma lipase is more sensitive and specific than plasma amylase (Smotkin and Tenner, 2002), our interest is only towards lipase levels. This study found that rats treated with Birinapant developed acute pancreatitis, which was confirmed from a marked increase in lipase levels and supported by histopathological changes in the pancreatic tissue (Figure 5.7 and 5.13). The DGGR assay, though it has never been
reported on before in rodents, seemed to be very sensitive in detecting pancreatic injury.

So far, we have shown that TMZ and Birinapant single or combination treatments affect several organs, such as the liver and pancreas as well as bone marrow. However, these treatments only showed a moderate effect on kidneys (Figure 5.9 and 5.10). The calcium, magnesium and creatinine were normal throughout the treatment. Similarly, the urea level also reached significance in the Birinapant (7.62 mM) and combination group (6.6 mM on Week 2, 8.45 mM on Recovery); however, these were within normal limits (6.9 – 30.5 mM). For creatinine kinase, there was no standard value reported by Petterino et al. (2006). Nevertheless, in our study, we did find decreased creatinine kinase in the combination group with further decreases observed towards Recovery, with levels (284.3 U/L) five times lower than the vehicle group (1394 U/L). It has previously been suggested that worsening creatinine kinase is associated with decreased muscle mass and muscle strength, and from reduced physical activity caused by illness or advanced age (Baird et al., 2012). Nevertheless, all animals in this group did not have significant changes in body weight and appeared well every time the animals were assessed. Perhaps changes in body weight are not sufficient to indicate general wellbeing, and while creatinine kinase is more clinically relevant, these two together may provide a better observation.

In summary, within the current chapter, important findings were identified. We showed that a dose escalation study is important when different drugs are combined in order to evaluate the safety, efficacy and accurate estimation of the maximum target dose. Toxicity studies are also warranted since the observed normal physiological status of the animals may not reflect the blood profiles or histopathological analysis. We showed that the two drugs used in this study have organ specificity; TMZ is mainly bone marrow specific with myelosuppressive effects, while Birinapant preferably targets circulating haemoglobin. From the observed increased neutrophils, GDH and lipase levels, Birinapant, in this study, aggravated an inflammatory response and the effects were systemic. When the two drugs were combined, the immunosuppressive
effect from TMZ attenuated and saved the animals from an inflammatory response induced by Birinapant. The results of this study also confirmed that GDH is a superior marker than ALP when detecting acute liver injury. For pancreatic injury, DGGR assay is a potential marker for detecting lipase levels in rodents. Finally, great caution is needed when selecting the animal and strain for toxicity studies due to different geneticity, sensitivity and host immune responses, especially when the drugs used in the study can potentially stimulate inflammatory responses. Upon completion of the toxicity study, we next move on to the orthotopic rat and mouse GBM models, where the efficacy of TMZ and Birinapant treatment was investigated.
CHAPTER 6: 

*In vivo* validation of surgical resection model

&

Combination effect of TMZ and Birinapant in a clinically relevant intracranial mouse model of GBM
6.1 Introduction

6.1.1 *In vivo* orthotopic GBM model

Surgery is generally the first step in GBM patients, prior to chemo- and radiotherapy treatment. It is also the most effective treatment for reducing tumour size and relieving brain pressure. Despite many preclinical studies, most *in vivo* GBM models do not mimic the clinical scenario of surgical debulking and instead focus on treating solid intact intracranial tumours (Kauer et al., 2012). Advancement in *in vivo* GBM research has led to the application of surgical resection in small animal models, such as in rats and mice. The difficulty with this approach is the minimal visualization of the tumour due to the small animal brain size. This limitation can be improved using microsurgical techniques and specialised dissecting microscopes that rely on fluorescence to visualize the tumour for resection (Akbar et al., 2009, Zhang et al., 2006). At the time of tumour resection, delivery of drugs, such as TMZ or microspheres containing carboplatin, to the tumour bed can be given, which has been proven to have a better effect on survival of the animals in this group (Emerich et al., 2000, Akbar et al., 2009).

We carried out two pilot studies of GBM surgical resection models prior to investigating the efficacy of TMZ and Birinapant treatment in an orthotopic GBM model. The first surgical resection model was performed in rats (Sweeney et al., 2014). U87luc cell were implanted in athymic rats, and weekly bioluminescence imaging (BLI) was performed to assess the tumour growth. When the tumour reached the exponential growth phase, animals were randomised into the resection and non-resection group (n = 5 animals per group). Animals that underwent tumour resection achieved a gross total resection (GTR) rate of 75%, which conferred a significant survival advantage compared to the non-surgical group. This technique successfully recapitulated the clinical GBM resection strategy.

In light of the success of this procedure in rats, we next determined whether this surgical resection technique could be performed in mice. This was the first
attempt by our group to use mice for this procedure; however, others have reported successful implementation of this procedure in mice. Kauer et al. (2012) performed tumour resection in U87 cells transduced with lentiviral and fluorescent proteins. At the time of tumour implantation, a cranial window was created, and tumour resection was performed using fluorescence intravital microscopy. This maximized the percentage of tumour resected, with 80% reported to be achievable (Kauer et al., 2012). Another group used U87GFP-Fluc cells; in this study, tumour resection was guided by amino levulinic acid (5-ALA), and intraoperative images were continuously captured to access the accuracy of the surgical resection. Post-operative BLI showed this technique enabled 92% tumour resection compared to 76% tumour resection using the standard non-guided technique (Hingtgen et al., 2013). Recently, a similar technique comparing fluorescence-guided surgery and standard bright light surgery was reported and showed significant survival benefit compared with earlier techniques (Momiyama et al., 2013). To assess their resection techniques, all of the above-mentioned studies used commercially available the U87 GBM cell line, which are known to be highly tumourigenic with well-circumscribed tumour growth patterns observed in vivo; hence, use of U87 cells provides some advantage for increasing the percentage of tumour being resected.

To allow for identification of tumour growth and calculation of percentages of tumour resection, BLI technique can be applied (Chapter 1.11.1). For this to work, the GBM cell line selected for this study had to be transfected with luciferase survive and grow within rats and mice brains while having a diffusely infiltrating tumour phenotype closely related to the human pathology. For this reason, U87RGFP1uc-2 and RN11uc cells were used in this study. These cell lines were first assessed in vitro using TMZ and Birinapant as single and combination therapies (Chapter 3).

U87RGFP cells were previously established through serial in vivo selection from a relatively non-invasive tumour cell mass in order to identify genes broadly responsible for invasive properties of GBM. Following intracranial injection of GFP-expressing U87 cells into immuno-compromised mice, GBM cells, which
invaded out of the tumour mass, were collected. Upon antibiotic selection in vitro, GFP-positive cells were expanded and re-injected into animals in order to further select highly invasive U87 cells. A similar procedure was undertaken with tumour mass-derived cells in order to identify gene expression differences between the non-invasive and highly invasive sub-populations of tumour cells (Figure 6.1). Based on microarray gene expression screening, the gene that encodes p75 neutrotrophin receptor (p75NTR) was identified as the most over-expressed gene in the invasive cell populations (Johnston et al., 2007). The U87RGFP-luc2-based model was developed by our group to facilitate sensitive, reproducible, BLI that could be applied to pre-clinical GBM therapeutic studies (Jarzabek, 2012). Figure 6.2 shows representative images of U87RGFP-luc2 cells implanted in NOD/SCID mice and the BLI graphs showing an increase in tumour growth over time; this supports evidence that NOD/SCID mice are an immunologically compatible graft-host.
Figure 6.1 Serial *in vivo* selection is used to isolate an invasive GBM population of cells from a non-invasive U87 cell line

The non-invasive human GBM U87 cell line stably expressing GFP (U87GFP) was implanted into the brains of SCID mice. Four to six weeks later, mice were sacrificed. The ipsilateral side of the brain (containing a grossly visible tumour) was separated from the contralateral side (containing only isolated invasive GBM cells). Both populations were grown in culture. The non-invasive (tumour) and highly invasive GBM cells were re-implanted into additional mice, and the process was repeated to select for increasingly non-invasive or invasive GBM cells. RNAs extracted from the resulting invasive and tumour populations were used to prepare labelled cDNA that was hybridized to oligonucleotide microarrays. Adapted from (Johnston et al., 2007).
Figure 6.2 *In vivo* tumour growths in U87RGFP-luc2 intracranial GBM model assessed using bioluminescence imaging

U87RGFP-luc2 cells have previously been established in NOD/SCID mice. Bioluminescent images of a representative mouse at each time point until *ex vivo* settings showed increased tumour growth, which supports evidence of an immunologically compatible graft-host (A). Tumour bioluminescence was quantified using Living Image software and plotted over time in logarithmic scale for each mouse (B) and as a mean ± S.E.M (n = 5 animals) (C).
6.1.2 Aims

The aim of this study was to investigate the application of BLI in assessing tumour volume in an *in vivo* intracranial xenograft rat and mouse GBM model. Our first objective was to establish a surgical resection model in intracranial rat GBM using the U87RGFP-luc2 cells, followed by survival analysis comparing the resection and non-resection groups. Our second objective was to extend the surgical resection model to an intracranial mouse GBM model using the RN1luc cells, followed by survival analysis. Our third objective was to design an *in vivo* survival study to assess the potential therapeutic benefit of the Smac mimetic, Birinapant, both alone and in combination with TMZ in tumour bearing mice.
6.2 Results

6.2.1 U87RGFP-luc2 tumours develop spontaneous regression when implanted in athymic rats

In order to monitor tumour growth non-invasively using BLI, tumour cells are required to express the luciferase enzyme. Prior to commencement of in vivo studies, luciferase expression of U87RGFP-luc2 cells was first evaluated in vitro. Dilutions of cells (5 x 10^2 to 1 x 10^4) were seeded in wells of a clear bottom 96-well black plate. Following addition of luciferase substrate, D-luciferin (150 µg/ml), plates were imaged using the IVIS Spectrum scanner. An increase in bioluminescent signal was observed with increasing numbers of U87RGFP-luc2 cells (Figure 6.3).

In this study, Foxn1^rnu^ T-cell deficient athymic rats (n = 10 animals) were inoculated with 1 x 10^6 of U87GFP-luc2 cells. From day 7-post tumour inoculation, tumour growth was assessed via BLI. Figure 6.4 shows three representative animals following BLI. All tumours were detected from the bioluminescent signals for the first two weeks. However, a decrease in bioluminescent signal was then observed, and by eight weeks post tumour implantation, the bioluminescent signal could not be detected at all, and all animals were at 100% survival. All animals were sacrificed and ex-vivo brain imaging showed a similar result.

All brain specimens were kept in formalin and stained with haematoxylin and eosin (H&E). Figure 6.5 shows a comparison between two animals based on the H&E stains. Histopathology in Rat 2, in comparison to Rat 7, shows a discrete single tumour nodule was observed in Rat 7 in the meninges in the longitudinal fissure between the cerebral hemispheres. On higher magnification, the tumour is composed of tightly packed cells in a fine vascular stroma. Cells appear mononuclear with a pleomorphic nucleus and abundant cytoplasm but indistinct cell borders. There is also occasional single cell necrosis. Similarly in Rat 7, previous track from needle site injection was observed to pass beyond the third ventricle.
Next, we sought to confirm whether the tumour is present in these specimens. **Figure 6.6 A and B** shows a representative photomicrograph of the brain stained with H&E. As the tumour cells were previously transfected with firefly luciferase (luc), the same nodule identified on the H&E was stained with anti-luciferase antibodies (**Figure 6.6 C, D**). There were minimal firefly luciferase-expressing tumour cells found on this nodule. Moreover, two of the animals had a normal histology even though earlier BLI images (first two week-post tumour implantation) showed bioluminescent signal activity.

We then examined other areas of the brain section to identify the presence of tumour cells. In two animals, the tumour cells were identified intraventricularly. In rat 3 (**Figure 6.7 A, B**), an increased cellularity in the proximal dorsal third ventricle was identified involving blood vessels of the choroid plexus. Furthermore, in rat 4 (**Figure 6.7 C, D**), within the choroid plexus of the fourth ventricle, there was mild focal mononuclear cell infiltration. In both cases, a mixture of inflammatory and neoplastic cells was noted. Based on these findings, it was deemed that a surgical resection model in rats using U87RGFP-luc2 cells could not be performed.
Figure 6.3 *in vitro* assessment of luciferase expression in U87RGFP-luc2 cells

U87RGFP-luc2 cells were previously transduced with the firefly luciferase gene-encoding viral supernatant, and the luciferase expression profiles were checked using IVIS spectrum. The cells were seeded in a clear bottom black 96-well plate at a density ranging from $5 \times 10^2$ to $1 \times 10^4$ cells (100 µl/well). The BLI was obtained following addition of 100 µl of D-luciferin (150 µg/ml). Results showed a stable increase in bioluminescent signal with increasing number of cells per well.
Figure 6.4 *In vivo* tumour growth of U87RGFP-luc2 in athymic rats assessed using bioluminescence imaging (BLI)

Anatomic locations of the xenograft and tumour volumes were monitored weekly with BLI. Images show representative animals with variable bioluminescent signals. Successful detection of the tumour was evident in all animals up to two weeks post tumour inoculation. A decreased in bioluminescent signal was observed during the following weeks, and no signal was detected in *ex-vivo* imaging.
Figure 6.5 Representative photomicrograph of brain stained with haematoxylin and eosin (H&E)

H&E analysis was examined to identify the presence and characteristics of U87RGFP-luc2 cells in rats. Histopathology in Rat 2 (A, B), in comparison to Rat 7 (C, D), shows a discrete single tumour nodule in Rat 4 in the meninges along the longitudinal fissure between the cerebral hemispheres. Previous track from needle site injection was observed to pass beyond the third ventricle (C, dashed arrow). Magnification 5 x (A and C) and magnification 10 x (B and D); scale bar = 100 µm.
Figure 6.6 Representative photomicrograph of brain stained with haematoxylin and eosin (H&E) and immunohistochemistry (IHC)

H&E stained section of Rat 4 identifies a discrete densely cellular nodule within the meninges composed of tightly packed cells with moderately pleomorphic nuclei (A, C). IHC stain section with antiluciferase antibodies of the same section shows minimal positive luciferase-expressing tumour cells (B, D). Magnification 5 x (A and C) and magnification 10 x (B and D); scale bar = 100 µm.
H&E analysis was examined to compare the histological changes in the brain between rats inoculated with U87RGFP-luc2 cells. Different locations and abnormalities were detected between animals, whereby in Rat 3, the tumour was evident by increased cellularity around a blood vessel in the third ventricle accompanied by speckles of lymphoid cells (A, B). In Rat 4, mild focal mononuclear cell infiltration and occasional cell necrosis was evident in the choroid plexus of the 4th ventricle (C, D). Magnification 20 x (A and C) and magnification 60 x (B) and magnification 40 x (D); scale bar = 100 µm.
6.2.2 Development and validation of surgical resection model for intracranial xenograft mouse GBM

6.2.2.1 *In vitro* assessment of luciferase expression in RN1luc cells

In order to establish and validate a surgical resection mouse GBM model and to monitor tumour growth based on BLI, RN1luc cells were used. Prior to commencement of *in vivo* studies, luciferase expression of RN1luc cells was first evaluated *in vitro*. Cells diluted to various concentrations (5 x 10^2 to 1.5 x 10^4) were seeded into wells of a clear bottom 96-well black plate. Following addition of luciferase substrate, D-luciferin (150 µg/ml), plates were imaged using the IVIS spectrum scanner. Results show a low luciferase expression in RN1luc cells with minimal increase in bioluminescent signal at higher seeding densities (*Figure 6.8 A*). Using a black 24-well plate, the same method was used and the number of cells plated was increased from 2 x 10^5 to 2 x 10^6 cells. When compared to U87RGFP-luc2 cells (*Figure 6.3*), a higher number of RN1luc cells was required to produce luciferase expression (*Figure 6.8 B*).
Figure 6.8 *In vitro* assessment of luciferase expression in RN1luc cells

RN1luc cells were previously transduced with the firefly luciferase gene-encoding viral supernatant, and the luciferase expression profiles were checked using IVIS spectrum. The cells were seeded into clear bottom black 96-well plate at a density ranging from $5 \times 10^2$ to $1.5 \times 10^4$ cells (100 µl/well). The BLI was obtained following addition of 100 µl of D-luciferin (150 µg/ml). Results show a low luciferase expression in RN1luc cells with a minimal increase in bioluminescent signal at higher seeding densities (A). The same method was repeated using a black 24-well plate; the number of cells plated was increased from $2 \times 10^5$ to $2 \times 10^6$ cells (500 µl/well). A stable increase in bioluminescent signal was observed with increasing seeding densities (B).
6.2.2.2 Assessment of RN1luc tumour growth using BLI

In this study, NOD/SCID mice (n = 10 animals) were inoculated with 5 x 10⁵ RN1luc cells. From day 7-post tumour inoculation, tumour growth was assessed via BLI, and randomization was performed at day 63. Mice were randomised into surgical and non-surgical groups (n = 5 mice per group) (Figure 6.9 A). Mice in the surgical group underwent attempted resection of the tumour, with modifications made to our previously established procedure (Sweeney et al., 2014). First, during the procedure, the initial location of the burrhole from previous tumour inoculation could not be identified as the burrhole was fully closed. Therefore, a new burrhole was made based on the recent BLI on each mouse. Second, the tumour surgical plane was difficult to locate due to the nature of RN1luc cells being invasive and not well circumscribed; hence, apart from a slight bulging of the brain (identified as tumour mass), differentiation between normal brain and tumour tissue was indistinguishable. Third, due to the smaller size of the mouse cranium and subsequently the tumour size, minimal extension of the burrhole was made (< 4 mm); hence, cranial defect did not require a repair. All animals survived from the second procedure with no general anaesthetic complications.

In order to obtain baseline tumour bioluminescence once surgical resection was performed and to compare bioluminescent signal before and after the resection, animals were imaged prior and after surgery. As shown in the representative images of resection and non-resection animals, a decrease in tumour bioluminescent signal was observed in the resection group (Figure 6.9 B).
Intracranial inoculation of RN1luc GBM cells was performed, and randomization was initiated (63 days following inoculation) when the tumour reached the exponential growth phase, as assessed by weekly BLI, where n = 5 mice per group (A). Bioluminescent images of representative mice from the resection and non-resection groups are shown (B).

Figure 6.9 Intracranial surgical resection model using RN1luc cells in NOD/SCID mice
6.2.2.3 Surgical resection of RN1luc tumour does not enhance survival in mouse GBM model

Gross total resection for each animal was established by calculating the percentage reduction of tumour volume using immediate post-operative BLI. In this study, we calculated an average gross total resection rate of 59%, with the highest being 85%. Despite the observed immediate reduction of bioluminescent signal after tumour resection, the tumour cells replicated and increased in volume to nearly the same as pre-resection bioluminescent signal (Figure 6.10 A, B). Upon observation of disease symptoms, including weight and activity loss, animals were euthanized. This step was considered a “death event” in Kaplan-Meier survival analysis, which revealed surgery did not confer survival advantage (Log-Rank p = 0.6432). The median survival of animals in the resection and non-resection groups was 81 days and 80 days, respectively (Figure 6.11).
Attempted tumour resection was performed in animals in the resection group. Pre-resection bioluminescent signal was set to 100% and gross total resection for each animal was established by calculating the percentage reduction of tumour volume on immediate post-operative BLI (A). Tumour volumes were followed-up until the animals succumbed to the disease. Despite the observed immediate reduction in bioluminescent signal, the tumour cells replicated and increased in volume to nearly the same as the pre-resection signal. Bioluminescent signals of representative mice from the resection and non-resection groups are shown in (B).

Figure 6.10 *In vivo* tumour bioluminescence to determine the gross total resection rate in resection group
Surgical resection of the tumours did not confer a survival advantage when compared to the non-resection group ($p = 0.6432$, Log-Rank test).

Figure 6.11 Kaplan-Meier survival curves: Resection vs. Non-resection groups
6.2.2.4 Haematoxylin and eosin (H & E) staining

In order to confirm RN1luc cells, during surgical resection of the tumour, the specimen were collected for histopathological analyses. At the end of the study, brains were fixed and routine H&E staining was performed. Microscopically, a representative of fragment taken from the resected specimen confirmed evidence of neoplastic cells, appeared as a cluster of tightly packed cells, which is densely cellular in morphology (Figure 6.12 A). Of the same animal, a similar population of tumour cells were identified on ex-vivo brain specimen (Figure 6.12 B). The tumour cells, when compared to specimens from the non-resection group (Figure 6.12 C), showed a similar finding of non-encapsulated densely cellular, high mitotic index mass. No histological differences were found between groups.
Figure 6.12 Detection of RN1luc cells in surgical resection model of intracranial mouse GBM

H&E images of a representative mouse brain showing evidence of densely cellular tumour cells (arrow) from the post-surgical resection specimen (A). Similar cellular morphology and high vascularity of tumour stroma (arrowhead) were found in both the surgical (B) and non-surgical resection specimens (C). Magnification 20 x (A) and 40 x (B and C); scale bar = 100 µm.
6.2.3 *In vivo* effects of Birinapant alone and in combination with TMZ in an intracranial mouse GBM model

6.2.3.1 Treatment planning and assessment of tumour volume using bioluminescence imaging (BLI)

The main objective of using xenograft models is to mimic the dynamic and heterogeneous characteristic of GBM, thus accurately mimicking the human disease. Based on previously described studies (*Chapter 3*), Type C RN1Iuc cells that stably expressed luciferase were next selected to determine whether sensitization could be achieved *in vivo*, following orthotopic intracranial tumour cell delivery.

In this study, 50 NOD/SCID adult female mice were inoculated with 5 x 10⁵ RN1Iuc cells. To ensure our treatment commenced when tumours reached the exponential growth phase, we first monitored tumour growth in all animals using BLI (*Figure 6.13 A*). At day 63 post tumour inoculation, mice were randomised into five groups (control, vehicle, TMZ, Birinapant and combination group where n = 10 mice per treatment group. Control animals in this study were untreated. Animals in the vehicle and TMZ groups received treatment via oral gavage. TMZ was given at 25 mg/kg (formulated in 10% DMSO in sterile D-PBS) daily for seven consecutive days, while vehicle was formulated without TMZ. Animals in the vehicle and Birinapant group received treatment via an i.p. injection. Birinapant was given at 20 mg/kg (formulated in 20% captisol in sterile H₂O) every three days for 9 doses in total, while vehicle was formulated without Birinapant. The combination group received both TMZ and Birinapant treatments (*Figure 6.13 B*). Animals were sacrificed when signs of any distress or weight lost were evident and median survival time for each group was calculated.

The randomization and treatment started 63 days following tumour inoculation, reflecting the slow growth process of RN1Iuc cells compared to other GBM cells (Dinca et al., 2007). *Figure 6.14A* presents study time points for drug combination treatments with weekly BLI performed. Birinapant treatment had no
impact on tumour growth as reflected by an increase in bioluminescence signal similar to the Vehicle group (p = 0.2420). In contrast, TMZ and TMZ + Birinapant significantly inhibited tumour growth (p < 0.0001 for both). TMZ + Birinapant treatment did not have a significantly greater tumour growth inhibitory effect when compared with TMZ, even after adjustment for multiple testing (p = 0.9116); however, significant effect was observed when TMZ and TMZ + Birinapant were compared with Birinapant (p < 0.0001 for both) (Figure 6.14 B). Nevertheless at > 49 days post treatment, tumour growth inhibition was greatest in the TMZ + Birinapant group, suggesting a later progression in tumour burden for the combined treatment, although this effect was not significant. Figure 6.14C shows images of tumour growth over time in a representative animal at 35 days (vehicle and Birinapant) and 84 days (TMZ and TMZ + Birinapant) post treatment commencement.
Intracranial inoculation of RN1luc GBM cells was performed on 50 NOD/SCID mice, and luciferase activity was monitored weekly with BLI using IVIS Spectrum. Randomisation into five treatment groups (n = 10 mice per group) and logarithmic mean BLI signal ± S.E.M. were plotted over time (A). Treatment was initiated 63 days after tumour inoculation (day -63). On day 0, animals were randomized into five groups (control, vehicle, TMZ, Birinapant and combination group). Control animals in this study were untreated. Animals in Vehicle and TMZ groups received the treatment via oral gavage at 25 mg/kg (formulated in 10% DMSO in sterile D-PBS) daily from day 1 to 7. Animals in the vehicle and Birinapant groups received the treatment via i.p. injection at 20 mg/kg (formulated in 20% capotsol in sterile H₂O) every three days (9 doses in total). The combination group received both treatments (B).
Figure 6.14 In vivo tumour bioluminescence following treatment with TMZ and/or Birinapant in intracranial mouse GBM model

Weekly in vivo tumour bioluminescence in each treatment group was assessed before and after treatment (A). The results matrix for comparison of tumour bioluminescence between groups is shown (B). Error bars represent mean relative tumour bioluminescence ± S.E.M. *Statistically significant using a Bonferroni-adjusted significance level of 0.5% (p < 0.005). Images show tumour growth over time in a representative animal at 35 days (vehicle and Birinapant) and 84 days (TMZ and TMZ + Birinapant) post treatment commencement (C).
6.2.3.2 Combination of Birinapant and TMZ improves survival of mice with GBM

The results of the survival analysis show that animals treated with TMZ and the combination therapy had a statistically significant longer survival benefit, with median survivals times of 161.5 days and 173.5 days, respectively, compared to vehicle group (92.5 days; p < 0.0001 for both comparisons) (Figure 6.15 A, B). This was an unexpected and a significant finding that despite resistant activity to TMZ and the combination treatment in vitro, the RN1luc cells responded to these agents in vivo. Comparing the median survival times between animals in the single agent treatment group with vehicle animals, we found no significant difference between animals treated with Birinapant compared to vehicle (p = 0.042), suggesting that the survival benefit of the animals was due to tumour sensitisation with TMZ treatment. Although addition of Birinapant to TMZ treatment showed a modest median survival advantage of 12 days (p = 0.0293) compared to TMZ animals, this was not statistically significant after adjustment for multiple testing.
Figure 6.15 *In vivo* effect of TMZ and/or Birinapant treatment on tumorigenesis and survival of mice in an intracranial GBM model

Kaplan-Meier curves were used to describe the survival times and log-rank tests used to compare treatment groups (A). The results matrix with median survival times (diagonal entries) and p-values from the log-rank tests (off-diagonal entries) (B). Disease-related symptoms following euthanasia of animals were considered as a 'death event' for survival analysis. *Statistically significant using a Bonferroni-adjusted significance level of 0.5% (p < 0.005).
6.2.3.3 Haematoxylin and eosin (H & E) staining

Microscopically, the characteristics of the RN1luc tumour cells are similar across all treatment groups and with previous surgical resection specimen (Figure 6.12). Tumour tissue continued to be present and displayed all the characteristics of the untreated and vehicle treated tumours. A representative of the H&E staining is shown in Figure 6.16. Within the cerebral hemispheres, midbrain and extending to the brain stem (favouring the right side), there is a non-encapsulated, at times expansile, and at other times invasive, densely cellular mass composed of tightly packed cells with pleomorphic medium to large nuclei (oval, elongated polygonal). Nuclei have a dense stippled chromatin, through which single nucleoli are occasionally seen. There is prominent nuclear hyperchromasia with a prominent mitosis in some fields. Single cell necrosis is also noted and the tumour stroma is quite vascular (Figure 6.16 A, B).

The invasive nature of these cells was evident on this staining, whereby the tumour cells invaded the corpus callosum and into the meningeal spaces. The extension of tumour cells to the contralateral brain tissue caused compression of the adjacent area (Figure 6.16 C). Moreover, ventricular distortion, both dilation and compression (unilateral right ventricle) was noted (Figure 6.16 D).
Figure 6.16 Representative photomicrograph of brain stained with haematoxylin and eosin (H&E)

Characteristic of RN1luc cells following treatment with TMZ and/or Birinapant were similar across all treatment groups. The tumour cells were highly invasive, as evidenced by contralateral extension (black arrow) and into meningeal space (arrow head) (A). The tumour was highly packed with abnormally shaped vessels (white arrow) (B). Ventricular distortion was noted (dashed arrow) (C), and at higher magnification, showed evidence of tumour cells intraventricularly (D). Magnification 10 x (A and C), 20 x (D) and 40 x (B); scale bar = 100 µm.
6.3 Discussion

In this study, we established three different \textit{in vivo} animal models and assessed the advantages of applying BLI to determine tumour volume and tumour growth. The use of BLI was helpful for identifying the U87RGFP-luc2 cells that showed tumour regression and measuring the tumour volume of RN1luc cells postsurgical resection and after treatment therapy with TMZ and Birinapant.

BLI provides an advantage for detecting successful tumour inoculation, which is evident when tumours become detectable after only a few days post-surgery. The decline in signal intensity on day six is reproducible and most likely due to loss of tumour take by some of the implanted cells (Puaux et al., 2011). This period is the important time for the surviving tumour cells to establish nourishment, whereby the cells will actively migrate towards and divide adjacent to pre-existing capillaries. Later, the tumour cells will erode into capillaries, which is followed by angiogenesis (Folkman, 1972, Folkman, 2002, Yamada et al., 2004). An increase in bioluminescent signal is expected after this period followed by a steady increase in tumour burden until the animals ultimately succumb to the disease (Abdelwahab et al., 2011). Earlier, using U87RGFP-luc2 cells in athymic rats, we observed a decrease in bioluminescent signal, and by 8 weeks post tumour implantation, the signal could not be detected, and all animals were at 100\% survival. It is tempting to speculate on the mechanism of tumour disappearance. Athymic rats are used in standard intracranial GBM models. Previously, successful tumour growth has been observed using U87MG-luc2 and G55 GBM cells, demonstrating adaptation of these cells to the microenvironment within the rat brain cavity (Rubenstein et al., 2000, Sweeney et al., 2014). We determined that our results could be due to two possible reasons: (1) variability in the implantation procedure of U87RGFP-luc2 cells; or (2) characteristics of the cells may induce incompatible immunological tumour-host interactions.

Less precise techniques can result in tumour cells being delivered to wide areas of the brain, ventricles, subarachnoid space or even extradurally, which will have a significant effect on the rate of tumour growth (Yamada et al., 2004).
Leakage of cells due to: (1) animals not properly anaesthetised at which point the animal may move during cell inoculation, (2) improper cleaning of the syringe needle at which point the viable tumour cells may attach along and outside the needle, or (3) removal of the needle too quickly after cell inoculation, may cause ectopic growth of tumour cells. In this case, the finding of tumour cells outside the brain (such as in our case along the meninges) demonstrated the importance of ensuring consistent stereotactic coordinates are achieved during cell inoculation.

During cell infusion, it is also important not to puncture the ventricles; the lateral ventricles are most commonly punctured when the burrhole is made too medial from the bregma, while the third ventricle can be punctured if the needle is advanced too deep from brain surface (Abdelwahab et al., 2011). The latter could also occur due to error in estimating the position of the stereotactic syringe holder and syringe needle when it touches the brain’s surface. This may have happened in our study, as one of the H&E stained showed that the needle track was beyond the third ventricle. This also implies that the cancer cells may have accidently been injected into the ventricles, as evidenced by the presence of tumour cells within the ventricles and extending into the brain parenchyma (Figure 6.5).

Our second assertion is potential incompatible immunological tumour-host interactions. The development of in vivo GBM models is based on two methods: (1) immunologically compatible tumour cells injected into a receptor animal (allograft); and (2) non-immunologically compatible tumour cells injected into immune-deficient animals (xenografts). In the latter case, animals can be congenitally immunosuppressed (athymic animals, like Rowett rats and nude mice) or they can be immunosuppressed using anti-rejection drugs, like cyclosporine (Miura et al., 2010, Jacobs et al., 2011). Spontaneous regression is defined as “the partial or complete disappearance of a malignant tumour in the absence of treatment or in the presence of therapy considered inadequate to exert a significant influence on the disease (Everson, 1967). It is a rare but known event, generally restricted to tumours that are highly immunogenic (Bodey, 2002).
Rats produce strong immune responses if implanted tumour cells are not immunologically compatible. For example, C6 glioma cells can grow invasively in Wistar rats (Jacobs et al., 2011). However, inoculation of C6 glioma in thymectomy Sprague-Dawley rats can induce activation of the immune system, as evidenced by a persistent influx of CD8+ cytotoxic leukocytes and macrophages into tumour tissue. With continuing tumour regression, it becomes obvious that the level starts to drop, reaching near baseline values (Vince et al., 2004). Furthermore, the humoral and cell-mediated host immune responses against the implanted tumour could activate cytotoxic T cell reactions that are sustained by the release of cytokines, IL-2, and TNFα, and the maintenance of natural killer cell/monocyte-dependent nonspecific responses (Kausalya et al., 1994, Khar and Anjum, 2001).

Despite having non-functional T cells, the athymic rats used in this study still have intact B lymphocytes and NK cells. B lymphocytes are recruited into the brain tumour microenvironment, which aggravates the production of various cytokines and immune-mediated GBM regression (Gonzalez et al., 1998, Harvey et al., 2007, Candolfi et al., 2011). Moreover, the B-BB that protects foreign antigens in the brain from immune attack is injured during tumour implantation (Pollack and Lund, 1990). Any surgery, such as neurotransplantation, breaks the B-BB and introduces foreign antigens into the brain, which may affect immune response and graft survival (Sloan et al., 1991). Since it takes 12 days for the B-BB to be re-established after transplantation (Borlongan et al., 1996), the transplanted cells could be exposed to the immune system via the blood during that time.

Following this disappointing result in rats, we set out to repeat this procedure in a mouse model of GBM tumour resection. Others have reported that surgical resection in intracranial mouse models of GBM provides survival advantages following tumour resection (Bello et al., 2002, Kauer et al., 2012). In our study, there was no noticeable survival benefit between the resection and non-resection groups, with median survival of 81 days and 80 days, respectively. Following this, we reviewed the type of GBM cells used as well as the surgical resection technique that was implemented in previous studies with rodents. So
far, there are nine papers published that relate to GBM resection models, with four performed in rats (Emerich et al., 2000, Zhang et al., 2006, Akbar et al., 2009, Sweeney et al., 2014) and five performed in mice (Bello et al., 2002, Kauer et al., 2012, Momiyama et al., 2013, Hingtgen et al., 2013, Redjal et al., 2015). In our study, there are several reasons that may contribute to the difficulty in achieving a higher average gross total resection. First, we examined previously reported characteristics of GBM cells and tumour formation in an in vivo surgical resection model. The majority of studies used U87 cells for their model, as it has been proven to form a well circumscribed, round, and bulky tumour in vivo, while the rest used rat glioma cells, which produces similar tumour formation (Huszthy et al., 2012). This allows for a better differentiation of the tumour cavity compared to our GBM cells (RN1luc), which has a poorly defined tumour margin. Using a standard microscope, we achieved an average gross total resection rate of 59%, which is comparable to some studies that used advance fluorescence intravital microscopy to achieve 60% gross total resection in mice bearing small tumours but less than other studies that achieved ~ 80% total resection in mice bearing large tumours that were easier to visualise (Kauer et al., 2012).

Second, a slower growth rate of RN1luc tumours was observed following intracranial inoculation, hence the tumour resection could only be performed 63 days post tumour implantation. This is in line with previously published data, which has demonstrated that invasive GBM cells are characterized by decreased proliferation rate in comparison to tumour bulk cells. A diffusely infiltrating and relatively slow growing tumour phenotype is likely more closely related to the human pathology (Molina et al., 2010). By this stage, the previous burrhole made was fully healed and closed, making it difficult to precisely locate the previous tumour site. In contrast, other groups performed the tumour resection as early as day 7 after tumour implantation (Zhang et al., 2006) and at the latest at day 28 (Sweeney et al., 2014). Given these differences, difficulties related to achieving a better average gross total resection becomes understandable. Perhaps for future studies, GBM cells with an invasive tumour phenotype should not be selected for surgical resection models, or if they are used, a better microsurgical technique and microscope, e.g. fluorescence
microscopy, should be used in order to precisely locate the tumour and the
tumour margin.

For each experiment that was presented earlier, the number of cells implanted
was the same but the bioluminescent signal achieved was different. This
variability could be due to several reasons, such as differential quenching of the
photon flux from tumours growing at different depths within the brain. Therefore,
it is important to verify the presence of an intracranial tumour once cells have
been inoculated in mice as well as standardise the mean tumour volume for
each group prior to commencement of treatment. In our study, we performed
weekly BLI to determine the growth pattern of tumours until they reached the
exponential growth phase. Determining an appropriate number of animals per
group can further minimize the standard error. The tumour volume in each
group, as calculated by the bioluminescence signal, was consistent with the
survival analysis. In TMZ and TMZ + Birinapant treated animals, the
bioluminescence signals remained at a low threshold, suggesting growth
inhibition of tumours following treatment that correlated with prolonged survival
benefit when compared to the control, vehicle, and Birinapant animals.

Animal models of human GBM play an important role in pre-clinical neuro-
oncology. The study of tumorigenesis and the evaluation of new therapies for
GBM require accurate and reproducible brain tumour animal models, which
ideally should recapitulate key features of the human disease (Candolfi et al.,
implantation of GBM cells, as described in this study, recapitulates a more
physiological microenvironment for tumour growth, making subcutaneous
implantation an imperfect model (Dinca et al., 2010). While many ongoing
(pre)clinical trials for Birinapant, either alone or in combination with other
chemotherapy agents, were conducted on non-CNS cancers, we elucidated the
role of Birinapant as a targeted therapy for CNS cancer. From the observed
Type C response pattern in vitro, to our surprise, the RN1luc cells exhibited
sensitivity and antitumour activity to TMZ and TMZ plus Birinapant treatment in
vivo. Animals in the TMZ group showed a significantly increased median
survival benefit of 69 days compared to the vehicle group, while the combination therapy gave an additional 12 day advantage.

We suspect that the discrepancy with our current study could be due to two reasons. First, the TMZ treatment given in vitro and in vivo could affect the DNA methylation activity in GBM cells. While RN1luc cells were treated with TMZ in vitro as a single dose, in vivo treatment was given over a seven day dosage, which could potentially change the concentration of TMZ exposed to the tumour. Moreover, RN1luc cells are unmethylated (Tivnan et al., 2014), and yet in vivo treatment showed a surprising sensitivity to TMZ. This was in consistent with previous report that observed that MGMT expression changes in response to TMZ treatment (Kitange et al., 2009). This resulted in the inhibition of tumour growth and greater tumour cell death, as shown by the lack of an increase in bioluminescent signals during the first 49 days post-treatment. Secondly, culturing the cells as a monolayer (2D, adherent) could limit the efficacy of chemotherapeutics agents compared to culturing them as spheroids (3D, neurosphere). Even with the improved monolayer technique, as applied in this study (described by Pollard et al., 2009), some important signals and key regulators needed for drug uptake by the cells occur naturally within the in vivo microenvironment.

The result of this study addresses the heterogeneous effect observed from cell culture techniques to inside the brain microenvironment is much more complex than expected. Although the literature is still lacking relevant studies with regards to the effect of TMZ treatment on tumour stroma cells (Jones and Holland, 2012), it is possible that the stroma cells are also important for drug action in vivo. The effects of Birinapant single agent treatments in vivo were less pronounced, as predicted from our in vitro studies. We cannot fully exclude that Birinapant has limited B-BB permeability, preventing the molecule to reach its target. However, a role for TMZ in increasing the permeability of the B-BB to allow co-treated drugs to reach the tumour cells has been reported (Riganti et al., 2014). This could explain the tendency towards an increased survival advantage in the TMZ plus Birinapant group. The discrepancies we observed between our in vitro and in vivo findings highlight the importance of careful
experimental approach/design when it comes to the preclinical testing of new therapeutics.
CHAPTER 7:
General Discussion
7.1 Current application of TMZ and Birinapant treatment in *in vitro* and *in vivo* studies in glioblastoma

Targeting anti-apoptotic proteins has emerged as a new tool to overcome the TMZ resistance. This comes from the observed cancer cells that acquire resistance to apoptosis by highly expressing anti-apoptotic proteins, such as Bcl-2 (Hassan et al., 2014), and IAPs family members (Fulda, 2014). A high level of IAPs has been linked to a defect in apoptosis, therapy resistance and poor prognosis (LaCasse et al., 2008, Gillissen et al., 2013). Within the current studies, combination of TMZ and Birinapant were experimented, both in *in vitro* and *in vivo* models.

*In vitro* data presented in Chapter 3 reveals the potential benefit of combining an IAP antagonist Birinapant with TMZ. The result of MTT assay mainly revolved on distinct differences between the commercially available and patient derived GBM cell lines. Importantly, the commercially available GBM cell lines were considered TMZ-sensitive while patient derived GBM cell lines were considered TMZ-resistance. Birinapant-sensitive cell lines showed a mixture of both. Birinapant treatment shows in general a high specificity towards cIAP1 protein in all cell lines tested except for U251 showing complete degradation in response to the TMZ and TMZ plus Birinapant treatment. Furthermore, the effect of molecular heterogeneity on therapeutic response was evident, and identifiable from three principle response patterns, with Type A cells showing sensitivity in response to TMZ, with minimal sensitisation following addition of Birinapant; Type B cells showing responses to Birinapant but no further sensitization with TMZ; and Type C cells that showed no significant cell death or moderately enhanced cell death in the combined treatment paradigm.

Application of computational APOPTO-CELL model in GBM by focusing on analysing the ability of the cells to activate sufficient caspase-3 substrate cleavage following MOMP and cytochrome-c release was presented in Chapter 4. Again, the differences between the commercially available and patient derived GBM cell lines were observed. All commercially available GBM cell lines that were TMZ-sensitive were correctly predicted to achieve an early
substrate cleavage within the 60 min simulation time frame, while 70% of patient derived GBM cell lines that were TMZ-resistant also failed to reach sufficient substrate cleavage within the 60 min simulation time frame. This corresponds to a predictive rate of 81.3%. High levels of XIAP control caspase activation and substrate cleavage (Rehm et al., 2006), but this activity was abolished over simulation with XIAP antagonist. Nevertheless, compared to other cancer cells that showed correlation with the in vitro data (Schmid et al., 2012); the model did not accurately predict the in vitro responses following TMZ plus Birinapant treatment. We stipulated that these were due to either activation of other caspases following MOMP independent of caspase-3, caspase-independent cell death following MOMP, or caspase-independent cell death following signalling from death receptors independent of MOMP.

Next, analysis of patient tumour samples explains the difference between protein concentrations of primary and recurrent tumour samples, in that the primary tumour samples were capable to activate sufficient caspase-3 substrate cleavage at a shorter time frame when compared to recurrent tumour samples. This perhaps contributed to a higher expression of procaspase-3 level in primary tumour samples. Addition of XIAP antagonist demonstrated a potential benefit of this personalised treatment regime, mainly to the responders group. We believe that addition of IAP antagonists, on a case-to-case basis can help the tumour cells to enter apoptosis. When looking at patients PFS, single proteins analysis is not a useful prognostic marker. However, using the APOPTO-CELL approach, Kaplan-Meier analysis showed that the system level analysis could serve as a predictive tool of 7-months PFS.

From the in vitro experiment, the effect of TMZ and Birinapant treatment are examined in vivo, starting at the toxicology of these drugs (Chapter 5). From the reviewed role of IAP antagonist to potentiate the activity of cytokines, we stipulated that the demise of the animals in Birinapant group at some stage were due to acute pancreatic injury, which triggered systemic inflammatory response affecting other organs, such as liver and spleen. This was proven with significant elevation of lipase, GDH, and marked neutrophilia. The histopathological findings were also consistent with the blood results. A
surprising result was that the event only occurred in Birinapant treated animals but not in the combination group. Data analysis confirmed that the immunosuppressive effect of TMZ contributes to the attenuation of the inflammatory response aggravated by Birinapant. Other findings that were found include the use of GDH as a marker of liver injury, and DGGR-lipase for pancreatic injury. The result is in keeping with previously reported use of GDH as a superior biomarker to ALP in detecting acute liver injury in rats (O'Brien et al., 2002). Our study also demonstrated the first time the DGGR assay has been utilised in rodents and from the observed sensitivity; we recommend that the DGGR assay is an innovative method to evaluate pancreatic lipase activity in small animal study.

An obvious deficiency of the in vitro culture than pre-clinical animal models is the failure to account for the microenvironment (Shankavaram et al., 2012, Hickman et al., 2014), which can significantly influence tumour cell biology and may thus affect the molecular determinants of treatment response (Unger et al., 2015). In Chapter 6, using the BLI technique, application of an orthotopic rats and mouse GBM were used to validate the surgical resection model in small animals, followed by the efficacy of TMZ plus Birinapant treatment. The first experiment (Figure 6.4) represents an example of tumour cells disappearance over time, which was picked up by weekly BLI. The second experiment (Figure 6.9) represents the useful application of BLI in detecting the tumour growth after surgical resection, while the third experiment (Figure 6.14) represents the useful of BLI in monitoring the tumour volume following treatment. A somewhat surprising finding was that the treatment sensitivity of RN1luc cells to TMZ in vivo, as indicated by significant survival benefits (161.5 days) of the animals as compared to the vehicle group (92.5 days), while initial in vitro experiment showed resistance to treatment. The TMZ plus Birinapant treatment only showed a modest median survival advantage of 12 days when compared to TMZ group, which was not significant.
7.2 Strengths and limitations of the current study

In *in vitro* studies, we used a large panel of GBM cell lines and compared the treatment response on the commercial (n = 6) and patient-derived (n = 10) cell lines. Our study displayed a heterogeneous response, consistent with a high degree of genetic instability, which generates cellular heterogeneity within each GBM cells (Nieto-Sampedro et al., 2011). The result allows for categorisation of different response patterns according to sensitivity to TMZ and/or Birinapant treatment. The focus of the study was to look at the apoptotic cell death activity. The most prominent signature found in this study was that the ability of GBM cells to activate the apoptosis is dependent on the activation of both intrinsic and extrinsic pathways. The initiator caspase-8 is important in that activation of this protein is correlated with downstream activation of caspase-3, with increased apoptotic cell death. Birinapant, by inhibiting cIAP proteins, alters the NFκB signalling from pro-survival to pro-death (Fox and MacFarlane, 2016). By doing so, assembly of the cytosolic complex RIP1/FADD/caspase-8 drives caspase-8 activation (Wang et al., 2008). It has previously been found that in GBM cells, Smac mimetic treatment was not associated with increased TNFα production (Wagner et al., 2013). Furthermore, crosstalk between apoptosis, necro(pto)sis and autophagy could occur within the cells following treatment, which could limit the validity of our data interpretation. In future, it would be interesting to look at other cell death pathway activation following Smac mimetic treatment in GBM.

The *in vitro* study did not have an equal number of the commercial and patient-derived cell lines. Similarly, the primary (n = 33) and recurrent (n = 15) patients tumour samples were also at unequal numbers. This could effect the statistical calculations for the APOPTO-CELL model. Furthermore, due to limited quantity of tumour lysates, the western blot analysis was performed once and some of the tumour samples were excluded if actin was not detected. Therefore, the number of tumour samples available for analysis was smaller than what we originally had in our facility.
Our toxicity study was the first study to investigate the treatment effect on haematological and biochemistry profiles of Sprague-Dawley rats following treatment with TMZ and/or Birinapant. It came as a surprise the hazardous effect of Birinapant in inducing acute pancreatic and liver injury, along with an acute inflammatory response. As many pre-clinical toxicity research performed is based on the body weight changes or clinical status of animals, this comes as a less accurate measurement to determine the toxicity of the drugs. Furthermore, since clinical trials in humans also involve analysing the haematology and biochemical profiles, it is ideal that when we speak of animals predicting human response in drug testing, this should include analysis of the same approach. As shown previously (Lecis et al., 2013, Wong et al., 2012), Smac mimetic treatment works differently in vivo. Overstimulation of TNFα and other cytokines stimulated the immune system (Kearney et al., 2013), which resulted in the demise of Birinapant-treated animals. The limitation of this study was the lack of capacity to further evaluate the treatment effect on the cytokine overstimulation. This could however, be a potential target for future study, particularly once it is known that combination with TMZ can attenuate the Birinapant-related side effect.

Neuroimaging of small animal model is increasingly emerging as a critical tool existing at the interface between pre-clinical and clinical translational research. BLI undoubtedly represents a valuable imaging tool for non-invasive assessment of tumour growth and monitor any change in tumour volume following treatment of xenograft (subcutaneous or orthotopic) tumours in rodents (O'Farrell et al., 2013). Despite our successful bioluminescence signal monitoring, others have reported that at early time points following tumour implantation, false bioluminescent signal can be generated by the presence of intra-parenchymal haemorrhages and extensive hydrocephalus at the site of injection (Jarzabek, 2012). Therefore, our group has implemented the following steps that could help to prevent some of the limitations of BLI in clinically relevant GBM models: (1) use of a standardised stereotactic coordinate during intracranial implantation of tumour cells, (2) a stable luciferase expression of the GBM xenograft system, (3) in vivo expansion of the tissue material in order to maintain histological traits of the original patient tumour (when GBM biopsy
derived model is used), (4) analysis of the exponential phase of tumour growth by bioluminescent signal quantification, and (5) assessment of xenograft volumes (Jarzabek, 2012, Jarzabek et al., 2013). Using these steps, improvement in pre-clinical models that is more closely representative of human disease can be achieved.

In an orthotopic mouse GBM model, while the RN1luc cells were resistant to TMZ treatment in vitro, in vivo studies showed sensitisation to TMZ treatment hence, a significant median survival benefit. Unfortunately, in vivo treatment with Birinapant did not show a significant effect on reduction of tumour growth or survival of the animals. Therapeutics resistance for treating GBM might not be due to an intrinsic resistance of the tumour to therapy, but due to the inability of the chemotherapeutic agents to penetrate the CNS, specifically the B-BB (Yung et al., 2014). The B-BB maintains homeostasis between the blood circulation and the CNS such that essentially 100% of large-molecule pharmaceutics and more than 98% of small molecules cannot cross this barrier (Weiss et al., 2009, Madsen and Hirschberg, 2010, Hauptman, 2011, Zhang et al., 2015). A wide range of lipid-soluble molecules can diffuse though the B-BB and enter the brain passively (Liu et al., 2004). Molecule that have a high polar surface area and a molecular weight in excess of 450 Da also appear to restrict B-BB permeability (Abbott et al., 2010). With this fact, the Birinapant treatment in GBM is at a disadvantage due to high molecular weight of 806.94 Da. This could also be the reason for the observed reduction of anti-tumour effect in our in vivo study (Chapter 6).

Another reason was due to the presence of ATP-binding cassette (ABC) efflux transporters in the B-BB that prevents systemically administered drugs from entering the targeted site (Loscher and Potschka, 2005, Nieto-Sampedro et al., 2011, Rama et al., 2014). ABC transporters belong to a superfamily of membrane pumps such as p-glycoprotein (Pgp), the multidrug resistance proteins (MRPs) and breast cancer resistance protein (BCRP), which catalyse the ATP-dependent transport of various endogenous compounds and xenobiotics out of the cell (Loscher and Potschka, 2005, Golebiewska et al., 2013, Rama et al., 2014). Glioma cells frequently overexpressed these proteins
In an immunohistochemistry study of 33 GBM samples, a significantly increased MRP5 holds a prognostic significance and is correlated with poor PFS (Alexiou et al., 2012). Recently, our group examined the effect of ABC transporters inhibitions on GBM cell lines response to chemotherapy drugs. Reversan-mediated inhibition of MRP1 and Pgp led to significant enhancement of TMZ, vincristine and etoposide-induced cell death in primary and recurrent GBM cell lines (Tivnan et al., 2015). This finding shows a barrier to the chemotherapy drugs in CNS treatment, whereby in an attempt for efficient drug delivery to target tumour cells, not only do they have to pass through the B-BB but also be able to escape the overly expressed efflux transporter protein on the cell membranes.

The discrepancies we observed between our *in vitro* and *in vivo* findings highlight the importance of careful experimental designs when it comes to the preclinical testing of new therapeutics. Particularly for Birinapant that has a large molecular weight; it may not be beneficial for treatment of CNS malignancy. However, targeting the resistance of Birinapant or other chemotherapeutic agents to pass through B-BB is an interesting potential target. One promising method to maximise therapeutic drug delivery, especially for a large or hydrophobic molecule is using particular nanoparticles (NPs) (Gao et al., 2014, Stephen et al., 2014). NPs can deliver potent doses of therapeutic agents with slow release of drug into tumour cells (Manzoor et al., 2012), which results in significantly improved specificity and reduced toxicities to cancer cells (Jain and Stylianopoulos, 2010, Davis et al., 2008). Birinapant plus NPs is a potential study to enhance the efficacy of therapeutic response in GBM.
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Publications


Abstract Presented


The 29th Annual Meeting of the British Society of Toxicological Pathology (BSTP) held jointly with the Association of Comparative Clinical Pathology (ACCP) and the Minipig Research Forum (MRF), UK (poster presentation, 14th November 2014) Zakaria Z, O'Brien PJ, O'Brien MF, Miller I, Byrne AT, Prehn JHM. The DNA-alkylating, anticancer-drug Temozolomide protects rats
from dose-limiting pancreatic toxicity produced by the Smac-mimetic, anticancer-drug TL32711.


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