MODULATION OF THE BASE EXCISION REPAIR (BER) PATHWAY IN THE TREATMENT OF GLIOBLASTOMA WITH RADIOTherapy

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“But I don't want to go among mad people,” Alice remarked.

"Oh, you can't help that," said the Cat: "we're all mad here. I'm mad. You're mad."

"How do you know I'm mad?" said Alice.

"You must be," said the Cat, "or you wouldn't have come here."

— Lewis Carroll, Alice in Wonderland
ABSTRACT

Glioblastoma is an aggressive brain cancer with a median survival rate of 14.6 months post diagnosis. Treatments for glioblastoma include surgery, radiotherapy, and chemotherapy with the alkylating agent temozolomide (TMZ). In 50% of patients, TMZ treatment is ineffective due to the reparative action of the protein O\(^6\)-MeG DNA methyltransferase (MGMT). The base excision repair (BER) pathway repairs the most common lesions caused by TMZ.

This work reports the characterization of several glioblastoma cell lines in terms of their repair status and sensitivity to traditional therapy of X-ray irradiation and TMZ. We find that the expression of BER proteins differed between cell lines, with alkyladenine-DNA-glycosylase (AAG) showing the greatest variation in expression. Sensitivity to TMZ and X-rays was MGMT dependent. Moreover, our results suggest that cell lines expressing higher AAG levels display increased sensitivity to X-rays and TMZ combination treatment in an MGMT independent fashion.

Pharmacological inhibition of BER enzymes AP-endonuclease (APE) and polymerase β (PolB) was examined, intending to enhance sensitivity of the glioblastoma cell lines to TMZ and X-ray or proton treatment. Methoxyamine (MX), an inhibitor of AP-endonuclease (APE) activity, leads to a modest increase in TMZ sensitivity. The combination of X-rays, MX and TMZ sensitised MGMT-negative cell lines, this was not seen in proton radiation. PolB inhibition greatly increased TMZ toxicity in conjunction with radiation in glioblastoma cell lines.

Proton irradiation systems were analysed and developed within this work, leading to a high-throughput broadbeam irradiation system. These methodologies lead to differences being detected in response to proton irradiation depending on the method used. This might in future, lead to further understanding of low-dose hypersensitivity.

In conclusion, the modulation of BER can enhance glioblastoma sensitivity to current treatment modalities, however, this is in an MGMT dependent fashion. These studies could provide insight for current clinical trials.
**ACKNOWLEDGEMENTS**

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ICRR Japan, 2015 “The modulation of BER in sensitising glioblastoma to radiotherapy” Poster presentation.


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# CONTENTS

## 1  INTRODUCTION TO ACCELERATORS IN MEDICINE

1.1 DEVELOPMENT OF CANCER ......................................................... 1

1.2 X-RAYS, DNA DAMAGE AND STRAND BREAKS ........................ 1

1.2.1 Types of DNA damage ..................................................... 2

1.3 APPLICATIONS OF ACCELERATORS IN MEDICINE ................... 2

1.4 HISTORY OF ACCELERATORS .................................................. 3

1.4.1 Cyclotrons ................................................................. 3

1.4.2 Synchrotrons ............................................................. 4

1.4.3 Linear Accelerators ...................................................... 5

1.5 HADRON THERAPY ............................................................... 6

1.5.1 Proton centres ............................................................ 7

1.6 MICROBEAMS ...................................................................... 8

1.6.1 Surrey Microbeam ......................................................... 8

1.7 DOSIMETRY ........................................................................ 9

## 2  GLIOBLASTOMA AND DNA REPAIR

2.1 INTRODUCTION ....................................................................... 11

2.2 GLIOBLASTOMA ..................................................................... 11

2.2.1 Glioblastoma Morphology ............................................... 12

2.2.2 Glioblastoma Subtypes .................................................... 12

2.2.3 Glioblastoma treatment ................................................... 15

2.3 THE EFFECTS OF RADIATION ................................................. 16

2.3.1 The importance of RBE and LET ...................................... 16

2.3.2 The case for proton therapy ............................................. 17

2.3.3 Types of damage caused by radiation .............................. 18

2.3.4 Double strand break signalling ...................................... 19

2.3.5 Repair pathways involved in radiation damage ................. 20

2.4 TEMOZOLOMIDE AND THE DNA .......................................... 24

2.4.1 Alkylating agents .......................................................... 24

2.4.2 Temozolomide discovery ................................................. 24

2.4.3 Repair of lesions caused by temozolomide ...................... 25

2.4.4 Limiting factors for efficacy of temozolomide ................. 29

2.5 SYNTHETIC LETHALITY .......................................................... 32

2.6 PHARMACOLOGICAL CONTROL OF BER .............................. 32

2.6.1 APE in the BER pathway ................................................ 32

2.6.2 How methoxyamine works ............................................... 34
2.6.3 Introduction to polymerase β................................................................. 36
2.6.4 XRCC1 ................................................................................................. 37
2.7 RESEARCH QUESTION ............................................................................. 38
2.7.1 Aims and objectives ............................................................................. 38
3 METHODS ..................................................................................................... 40
3.1 INTRODUCTION ....................................................................................... 40
3.2 METHODS ................................................................................................. 41
  3.2.1 Cell culture ......................................................................................... 41
  3.2.2 Western Blot ....................................................................................... 41
  3.2.3 Polymerase Chain Reaction .............................................................. 42
  3.2.4 Microarray data analysis .................................................................. 46
3.3 IRRADIATION PROTOCOL ...................................................................... 46
  3.3.1 X-ray protocol ................................................................................... 46
  3.3.2 Vertical proton irradiation ................................................................. 47
  3.3.3 Horizontal proton irradiation ............................................................. 48
3.4 CHEMOTHERAPY TREATMENT PROTOCOLS ...................................... 49
  3.4.1 Clonogenic assay .............................................................................. 49
  3.4.2 Viability assay ................................................................................... 49
  3.4.3 Cell growth assay ............................................................................. 49
  3.4.4 Flow cytometry ................................................................................ 50
  3.4.5 Activity assay ................................................................................... 50
  3.4.6 Assay to measure AAG activity ......................................................... 50
3.5 STATISTICS ............................................................................................... 53
  3.5.1 Combination indices ....................................................................... 53
4 THE CHARACTERISATION OF GliOBLASTOMA CELL LINES .................. 55
4.1 INTRODUCTION ....................................................................................... 55
4.2 AIMS AND HYPOTHESIS ....................................................................... 56
4.3 RESULTS .................................................................................................. 57
  4.3.1 AAG levels vary in different glioblastoma cell lines......................... 57
  4.3.2 AAG activity assay .......................................................................... 69
  4.3.3 High AAG levels don’t necessarily lead to an imbalance in BER .... 70
  4.3.4 Survival of glioblastoma cell lines after treatment is dependent on the expression of MGMT .......................................................... 71
4.4 SUMMARY ................................................................................................. 78
5 INVESTIGATING PHARMACOLOGICAL MODULATION OF BER WITH DIFFERENT TYPES OF RADIOThERAPY ................................................................. 81
5.1 INTRODUCTION ....................................................................................... 81
7.1.1 Naturally occurring BER protein expression levels can lead to an imbalance.................................139

7.1.2 BER imbalance only sensitizes cells when treated with a SN2 alkylation agent.................................................................141

7.1.3 X-rays and temozolomide are additive.........................................................143

7.1.4 MGMT status important in sensitizing cells to TMZ with MX and PA143

7.1.5 Protons are more cytotoxic than X-rays.......................................................145

7.1.6 Inhibiting BER in combination with radiotherapy and TMZ offers benefit only in MGMT negative cells..............................................145

7.2 Future work.....................................................................................................148

7.2.1 Conclusions..................................................................................................149

8 REFERENCES......................................................................................................150
<table>
<thead>
<tr>
<th>Equation</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dose rate;</td>
<td>48</td>
</tr>
<tr>
<td>2</td>
<td>Combination indices</td>
<td>54</td>
</tr>
<tr>
<td>3</td>
<td>Linear Quadratic (LQ):</td>
<td>74</td>
</tr>
<tr>
<td>4</td>
<td>Modified LQ:</td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td>Modified RCR equation:</td>
<td>101</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1 A summary of gene expression changes in subtypes of glioblastoma (Verhaak et al., 2010) ................................................................. 14

Table 2 Antibodies used in western blots ................................................................. 42

Table 3 SuperScript II reverse transcriptase reagent amounts - 1 .................. 43

Table 4 SuperScript II reverse transcriptase reagent amounts - 2 ............. 43

Table 5 Forward and reverse primers for mRNA detection of repair proteins ........ 45

Table 6 X-ray dose delivery of Gy and the monitoring units needed to achieve them .... 46

Table 7 Sequences of oligonucleotides used in experiments .......................... 51

Table 8 The difference in EC50 after treatment with TMZ in glioblastoma cell lines ... 74

Table 9 The output of the constants from fitting either the LQ model to the survival data. Y0 is survival at 0 Gy ........................................................................ 78

Table 10 A comparison of the expression levels of AAG and MGMT correlated to EC50s from different treatment modalities in glioblastoma cell lines .................. 79

Table 11 A comparison of AAG and MGMT status within cell lines and the combination indices derived from TMZ and X-rays .............................................. 94

Table 12 A comparison of relative biological effectiveness ..................... 110

Table 13 A comparison of the surviving fractions at 3 Gy after trimodal treatment. - significant figures test ................................................................. 110

Table 14 A comparison of the sensitizing enhancement ratio after a series of combinations of treatments. L= LN18, U = U87, A = A172 ................. 111

Table 15 SRIM calculations of the changing LET values as the air gap increases from accelerator window to cell dish ......................................................... 128

Table 16 Mean surviving fractions from 1 - 3 Gy for either vertical (V) or horizontal (H) radiation methods. With +/- standard deviation .................................. 134

Table 17 Parameters of the RCR equation fit to 2 Gy for vertical and horizontal irradiation systems ............................................................... 135

Table 18 Constants and goodness of fit for the RCR model vertical versus horizontal irradiation .............................................................................. 136
LIST OF FIGURES

FIGURE 1-1 OF A CYCLOTRON TAKEN FROM (REDFINE KNOWLEDGE, 2018) ............................................. 4

FIGURE 1-2 A SYNCHROTRON DIAGRAM TAKEN FROM (MACHINE DESIGN, 2018) ................................. 5

FIGURE 2-1 SPREAD-OUT BRAgg PEAK A) IT CAN BE SEEN THAT X-RAYS START TO MOVE THROUGH
Tissue with a higher energy that decreases as it reaches the target depth. However, proton beams have a Bragg peak that shows an increase in radiation dose/energy at the target depth, where the wave stops. In b) it can be seen that due to the lower radiation through the tissue and the Bragg peak seen at the target where the wave ends less radiation is seen in the healthy tissue past the tumour. ............................................. 18

FIGURE 2-2 NON-HOMOLOGOUS END JOINING DOUBLE STRAND BREAK REPAIR, LOW ACCURACY ASJOINS TWO DAMAGED ENDS TOGETHER, WITHOUT CHECKING THEY MATCH. TAKEN FROM (MLADENOV & I LiAKIS 2011) ................................................................................................................................................. 21

FIGURE 2-3 HOMOLOGOUS RECOMBINATION. DOUBLE STRAND BREAK REPAIR USING THE SISTER
CHROMATID TO ENSURE HIGHLY ACCURATE REPAIR. TAKEN FROM (MLADENOV & ILIakis 2011)
........................................................................................................................................................................ 23

FIGURE 2-4 MISMTACH REPAIR PATHWAY REPAIR OF INCORRECT DNA BASE MATCHING PREVENTING
POINT MUTATIONS. TAKEN FROM (JIRICNY 2006) ................................................................................................. 27

FIGURE 2-5 DNA DAMAGE REPAIR PATHWAYS INITIATED BY TEMOZOLOMIDE DAMAGE. O-MeG ADDUCTS ARE REPAIRED BY MGMT IF THE PROTEIN IS PRESENT, OTHERWISE MISMATCH REPAIR IS UTILISED. BASE EXCISION REPAIR REPAIRS THE OTHER ADDUCTS FORMED BY TMZ .......... 29

FIGURE 2-6 AAG, AAG 3-DIMENSIONAL STRUCTURE AND IT’S INTERACTION WITH DNA (RUBINSON ET
AL. 2016) .............................................................................................................................................................. 30

FIGURE 3-1 SCHEMATIC OF CELL SET OUT ON THE POLYPROPYLENE DISKS. THE DISKS ARE SET ON THE
XY STAGE AND THE BEAM COMES FROM UNDERNEATH THE CELLS ................................................................. 48

FIGURE 3-2 STRUCTURE OF OLIGONUCLEOTIDE SEQUENCES IN THEIR EXPECTED ORIENTATION BOUND
TO THE WELL OF A 96-WELL PLATE. STRUCTURE SHOWN IS SUBSTRATE FOR ALKYL ADENINE DNA
GLYCOSYLASE ....................................................................................................................................................... 53

FIGURE 4-1 DAMAGE REPAIR PATHWAYS INITIATED BY DAMAGE FROM TMZ. THE ENZYME CASCADE IS
INITIALISED BY AAG AND EACH ENZYME IS HELD IN CLOSE PROXIMITY TO THE DAMAGE BY XRCCI,
SCAFFOLDING PROTEIN. PARP SIGNALS A SSB HAS BEEN CREATED. O-MeG ADDUCTS ARE
REPAIRED BY MGMT IF THE PROTEIN IS PRESENT ............................................................................................... 58
FIGURE 4-2 BER repair mRNA expression levels. a) AAG mRNA expression levels. b) APE mRNA expression levels. c) PolB mRNA expression levels. d) Lic3 mRNA expression levels. All experiments were conducted on 3 separate mRNA extractions in at least triplicate on a minimum of n=3. T98G was always used as a reference sample and so remains at 1 relative expression, statistical significance was established by one way ANOVA .................................................. 60

FIGURE 4-3 Repair protein mRNA expression levels. All experiments were conducted on 3 separate mRNA extractions in at least triplicate on a minimum of n=3. T98G was always used as a reference sample and so remains at 1 relative expression, statistical significance was measured by one way ANOVA .................................................. 63

FIGURE 4-4 Western blot analysis of proteins important to TMZ. Show representative western blots of protein expression for A) AAG, B) APE and C) MGMT respectively. D, E and F show AAG, APE and MGMT protein expressions normalised over a minimum of 3 experimental replicates .......................................................... 68

FIGURE 4-5 Microarray analysis from GSE23806 using platform GPL570. Cell lines were A172: GSM587161, LN18: GSM587185, T98G: GSM587179, U87: GSM587180. All p values were normalised against T98G to make comparable to western blots and qPCR results .......................................................... 65

FIGURE 4-6 AAG activity in cell lines; a) LN18 and U251 and b) A172 and T98G. The nuclear fraction of the cell was extracted, purified and quantified. Normalised levels of protein were seeded into a 96 well plate containing fluorescent DNA containing AAG specific substrates. One biological repeat was used and the error is standard deviation .......................................................... 70

FIGURE 4-7 Survival curves after treatment with a range of mM doses of MMS for T98G, U87 and LN18. A minimum of 3 repeats in triplicate were conducted with two-way ANOVA statistics applied .......................................................... 71

FIGURE 4-8 GBM cell lines treated with a range of TMZ. a) Shows all five cell lines treated to 100 mM, no statistics were performed on this treatment. B shows MGMT negative cell lines. C shows MGMT positive cell lines treated up to 1000 mM. A minimum of n=3 on triplicate data, a two-way ANOVA was used and the curves fitted with the Hill model .......................................................... 73

FIGURE 4-9 Survival fractions after treatment with X-rays or X-rays and TMZ. a) LN18, b) T98G, c) A172, d) U251 and e) U87. Cell lines fitted with the best fit models for each cell line when treated with X-rays with and without 25 mM of TMZ. A minimum of 3 repeats in triplicate were performed and statistics were model fitting of LQ 77
FIGURE 5-1 The base excision repair pathway and inhibition with methoxyamine. The base excision repair pathway is initialised by AAG which removes the methylated base leaving an apurinic site. Methoxyamine binds to this site preventing APE from cutting this site and preventing the rest of the pathway from being activated. This will increase the number of apurinic sites leading to a build up of toxic intermediates and increasing the toxicity of other treatments such as TMZ and radiation.

FIGURE 5-2 Comparison of both X-rays and protons on the survival of glioblastoma cell lines. 3 cell lines; LN18 a), U87 b) and A172 c). Cell lines fitted with the RCR model. A minimum of 3 repeats in triplicate were performed.

FIGURE 5-3 Methoxyamine inhibition of APE1. a) Using a fluorescent assay with designer DNA with a fluorochrome attached, the number of incisions made to the DNA by increasing levels of APE enzyme was measured by the decrease in fluorescence as incised DNA containing the fluorochrome was washed away. b) The concentration of methoxyamine needed to inhibit APE and stop the incision of DNA, which leads to an increase in absorbance as less DNA containing the fluorochrome is washed away. A minimum of 3 biological repeats was performed.

FIGURE 5-4 Cytotoxicity of methoxyamine. a) LN18, b) T98G, c) A172, d) U251 and e) U87. Cells were seeded at a density of 5,000 cells per well. A range of MX concentrations were added to the cells and left for 48 hours. The cells ability to perform redox reactions was measured using a 1:10 dilution of alamar blue. Each graph represents a different cell line with a minimum of 3 repeats.

FIGURE 5-5 Viability of MGMT negative a) and positive b) cell lines after treatment with MX and TMZ. Cells were seeded at a density of 5,000 cells per well. Cells were treated with 500 mM of MX concentrations and either 75 mM of TMZ (a) for MGMT-negative cell lines, or 350 mM TMZ (b) for MGMT-positive cell lines and incubated for 48 hours. The cells ability to perform redox reactions was measured using a 1:10 dilution of alamar blue. A minimum of 3 repeats. Statistical significance was measured with a 2-way ANOVA.

FIGURE 5-6 Structure of polymerase beta and how pamoic acid binds to it. A) shows the whole protein with PA bound to the active site. B) shows just the amino acids within the active site where PA binds. Taken from (Hazan et al. 2008).

FIGURE 5-7 Cytotoxicity assay of glioblastoma cell lines treated with pamoic acid (PA). a) LN18, b) T98G, c) A172, d) U251 and e) U87. Cells were seeded at a density of 5,000 cells per well. A variation of PA concentrations were added to the cells and left
for 48 hours. The cells ability to perform redox reactions was measured using a 1:10 dilution of alamar blue. Each graph represents a different cell line with a minimum of 3 repeats. .................................................................................................................. 96

Figure 5-8 Viability of MGMT negative a) and positive b) cells after treatment with PA and TMZ. Cells were seeded at a density of 5,000 cells per well. Cells were treated with 100 mM of PA concentrations and either 100 mM of TMZ (a) for MGMT-negative cell lines, or 100 mM TMZ (b) for MGMT-positive cell lines, and left for 48 hours. The cells ability to perform redox reactions was measured using a 1:10 dilution of alamar blue. A minimum of 3 repeats. Statistical significance was measured with a 2-way ANOVA. .................................................................................................................. 98

Figure 5-5-9 Viability after MMS and inhibitor treatment on glioblastoma cell lines. a) LN18, b) T98G, c) A172, d) U251 and e) U87. Cells were treated with serum free media for and drugs for 1 hour, and then fresh media was added back in. Viability was measured with alamar blue 48 hours later. Experiments were plated in triplicate and with a minimum of an n=3. 2-way ANOVA measured statistical significance. .................................................................................................................. 100

Figure 5-10 Comparison of both X-rays and protons with TMZ and PA on the survival of glioblastoma cell lines. 3 cell lines; U87 a) X-rays b) protons, LN18 c) X-rays d) protons and A172 e) X-rays f) protons, were treated with 50 mM of PA, 25mM of TMZ, combination of both and either X-rays, left hand side, or protons, right hand side. Cell lines fitted with the RCR model. A minimum of 3 repeats in triplicate were performed. .................................................................................................................. 105

Figure 5-11 Comparison of both X-rays and protons with TMZ and MX on the survival of glioblastoma cell lines. 3 cell lines; U87, LN18 and A172 were treated with 50 mM of MX, 25mM of TMZ, combination of both and either X-rays, left hand side, or protons, right hand side. Cell lines fitted with the best fit models for each cell line and radiation type; RCR model for everything except A172 X-rays and MX. A minimum of 3 repeats in triplicate were performed. .................................................................................................................. 108

Figure 5-12 Number of colonies formed after treatment with PA. 4 cell lines; A172, LN18, U87 and U251 were treated with 50 mM of MX, a and b, or PA a and d, 25mM of TMZ and a combination of both, b and d. Cells were left to form colonies over 14 days until stained with crystal violet to count colonies. Results were compared to controls, left hand side. A minimum of 3 repeats were performed and 2-way ANOVA statistics were performed to look for significant changes in colony numbers. .... 114
Figure 5-13 Growth curves with and without treatment of inhibitors. A) shows proliferation of U87 over 5 days, 1*10^4 cells were seeded per well, and counted each day after treatment with either 50 mM of PA or MX. B) shows proliferation of U251 over 5 days, 1*10^4 cells were seeded per well, and counted each day after treatment with either 50 mM of PA or MX. A minimum of 2 repeats were conducted. Statistical significance was measured by 1-way ANOVA.

Figure 5-14 Cell cycle analysis of cells treated with either 25 mM of TMZ, 50 mM of PA, 50 mM of MX or combinations of in 3 cell lines; LN18, U87 and U251. There were 2 repeats on each day, with 2 days per cell line. Results were plotted as the mean of all results as percentage of cells in each phase.

Figure 6-1 Diagram of the two accelerator set ups at the ion beam centre. The top half of this figure shows the vertical beam line configuration. With a 90° bend in the beam line to allow the cells to be irradiated from underneath. This means that it is unknown how many cell layers are produced, and the cell thickness is increased. In the bottom half of the diagram a linear beamline without a bend leading to a horizontal irradiation system is used. The cells are irradiated without media whilst attached to the plastic wells and much thinner than the rounded vertical cells.

Figure 6-2 SRIM calculations for LET. Figure a) shows the change in LET through the distance of the cell for different stretches of air gap. Figure b) shows the mean LET dependent on the airgap from silicon window to polypropylene lid.

Figure 6-3 GAF Chromic radiated at 0.5 Gy through a 96 well plate, with the well bottoms removed. And a polypropylene lid applied. Beam homogeneity was checked through immediate colour change.

Figure 6-4 Analysis of RTQA Gaff Chromic colour change after irradiation with a 3.5 MeV proton beam. Experiments were repeated on each day of irradiations, with each “well” being measured for colour change N=6.

Figure 6-5 RCR Model fitting for clonogenic assay data for protons on 3 cell lines in either vertical irradiation or horizontal irradiation systems.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<td>AAG</td>
<td>Alkyladenine DNA glycosylase</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture centre</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated kinase</td>
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<tr>
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MBD1 - methylated DNA-binding domain 1

MGMT – O6 methylguanine- DNA- methyltransferase

MMR – Mismatch repair

MMS - Methyl methanesulfonate

mRNA – Messenger ribonucleic acid

MTIC - 5-(3-methyl)-1-triazen-1-yl-imidazole- 4-carboxamide

MX – Methoxyamine

NF1 – Neurofibromatosis type 1

NHEJ – Non-homologous end joining

N<sup>3</sup>-MeA – Nitrogen<sup>3</sup> methyl adenine

N<sup>7</sup>-MeG – Nitrogen<sup>7</sup> methyl guanine

OGG – 8-oxoguanine DNA glycosylase

O<sup>6</sup>-MeG - Oxygen<sup>6</sup>-Methyl guanine

PA – Pamoic acid

PARG – Poly(ADP-ribose) glycohydrolase

PARP – Poly (ADP – ribose) polymerase 1

PDFGRA – Platelet derived growth factor receptor a

PIXE – Proton induced X-ray emission

PolB – Polymerase β

PTEN – Phosphatase and tensin homolog

qPCR – quantitative polymerase chain reaction

RBE – Relative biological effectiveness

RBS – Rutherford Backscattering

RCR – Repair capability repair

ROS – Reactive oxygen species

RQ – Relative quantification

SER – Sensitizing enhancement ratio

SRIM – stopping and range of ions in matter
SSB – Single strand break
ssDNA – single strand DNA
TMZ – Temozolomide
TP53 – Tumour suppressor protein 53
UDG - Uracil – DNA- glycosylase
XRCC1 – X-ray repair cross complementing protein 1
1 INTRODUCTION TO ACCELERATORS IN MEDICINE

1.1 Development of cancer

Cancer can be defined as a disease caused by an uncontrolled division of abnormal cells causing a malignant growth or tumour (Hanahan, R. a Weinberg, et al. 2000). The cause of this unregulated cell division can be multifactorial but one of the major causes are from carcinogenic: reagents, chemicals, and agents which leads to mutations in the DNA, to either oncogenes or tumour suppressor genes, which can dysregulate the cell cycle pathways and cell death pathways (Hanahan & Weinberg 2011).

1.2 X-rays, DNA damage and strand breaks

X-rays were discovered in 1895 by Roentgen and the therapeutic potential for them was quickly realised (Kemp 1998). From the discovery, and the biological therapeutic advantages being understood X-rays were first used to treat cancer in America by Emil Grubbe in 1896.

There is debate over who invented the X-ray tube and and whether Coolidge or Grubbe conducted the first irradiations. X-ray tubes are vacuum tubes that convert electrical energy into X-rays, the invention of which is highly debated (Grubbé 1933). The tube contains an anode and cathode which the electrons (electron beam) flow between,
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

with X-rays being generated from the electrons bombarding into the anode, normally tungsten or copper. This continuous release of X-rays is due to bremsstrahlung.

1.2.1 Types of DNA damage

X-rays form many different types of DNA damage, these include; double strand breaks (DSBs), single strand breaks (SSBs) and crosslinks. A SSB is defined as a break in the backbone of the DNA on one side only and the strands have not separated. A cleavage on both sides of the DNA, within 10 bas pairs is characterised as a DSB, however the strands have not separated. These are often a direct effect of ionising irradiation. However Base methylation and crosslinking of bases are often caused by indirect effects; which is the creation of reactive oxygen species which then interact and damage the DNA.

The damage that occurs to DNA during X-ray irradiation repairs at different rates depending on the cell type. In rat splenic lymphocytes it is shown that the repair kinetics after irradiation with X-rays repair by 80% after 1 hr (Coogan et al. 1992). When human peripheral lymphocytes were irradiated with 4 Gy the repair kinetics were similar to rat lymphocytes and after 1 hr 80-90% of breaks were repaired (Boerrigter 1991). After 100 Gy dose of X-rays over 60% of single strand breaks (SSBs) can repair in drosophila (Oliver et al. 1990). These show fast repair kinetics, but does take into account both double strand breaks (DSBs) and SSBs.

However, other studies have shown that for DSBs in particular that 30 minutes after irradiation DSBs are 14 times higher than 24 hours later (Fleckenstein et al. 2011). In human lymphocytes, X-rays cause DSB foci of 11.66 foci per Gy per cell (Mandina et al. 2011). Different techniques of applying traditional radiotherapy, such as Intensity modulated radiotherapy (IMRT) and intensity modulated arc therapy (IMAT) produce a different number of DSB foci developing. Foci yields are higher in IMRT that in IMAT (Werbrouck et al. 2013).

1.3 Applications of accelerators in medicine

Since the development of accelerators there have been many other applications than just nuclear physics that they have been used for. One use of the utmost importance
is in health care. As will be discussed in detail below accelerators are used in medicine for many different applications. These include; radioisotope production, imaging and radiotherapy treatment. In this chapter we will focus on radiotherapy treatment.

1.4 **History of accelerators**

Accelerators were originally developed due to a lacking in understanding in nuclear physics. Ernest Rutherford urged the Royal Society in 1927 to develop a source of positive particles with higher energies. In 1929, in the Cambridge Cavendish laboratories, Cockcroft and Walton built the first accelerator and in 1932 they reported a nuclear reaction produced by a particle accelerator (Martins & Silva 2012). The accelerator used was an ion accelerating column connected to a voltage multiplying column, which was used to accelerate protons to 600 kV. The voltage multiplying circuits used in the design by Cockcroft and Walton were originally developed in 1920, they adapted this and it is called the Cockcroft-Walton machine (Livingston, 1980). The circuit works by charging capacitors in a parallel system and discharging them in series. The circuit used gave a four-fold voltage multiplication. The Cockcroft-Walton machine design was built all over the world, for example the one built in Rome by Amaldi which eventually managed to reach 1.1 MeV (Amaldi 2012).

1.4.1 **Cyclotrons**

A cyclotron works by providing a constant magnetic field, with a set of D-shaped electrodes driven by an RF source that alternates the fields constantly. This in turn speeds the particles in a spiralling outward motion. The kinetic energy of the particles can only increase when they are between the gaps of the two D shaped magnets/electrodes, the radius increases as the particle travels round the accelerator. As the particles accelerate their orbit increases as shown in figure 1-1. Lawrence realized that the cyclotron could be of use in the production of radioactive isotopes (Amaldi et al. 2010).
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

In 1938 a cyclotron was used to treat patients with cancer; the machine was 37 inches, produced neutrons and was run by Lawrence (Stone et al. 1940). The cyclotron ran 8 MV deuterons into a beryllium target producing the neutrons. Unfortunately when a machine was built which reacted 16 MV deuterons with beryllium, the effect on the healthy tissue was so severe that the approach was abandoned (Amaldi et al. 2010).

Radioisotopes were initially used in diagnostics with iodine \((^{131}\text{I})\) for imaging purposes (Silberstein 2012). In 2007 there were approximately 250 cyclotrons in use for the production of radioisotopes. These radioisotopes include C\(^{11}\), N\(^{13}\), O\(^{15}\) and F\(^{18}\) and are regularly used in PET scans (Denker et al. 2007). PET scans work by the radioisotopes emitting gamma rays within the body. The radioisotopes accumulate in the target tissue due to metabolism and bloody flow. The decay of the radioisotope results in the simultaneous release of an electron and gamma ray in opposite directions. Fluorine 18 is the most common tracer in oncology.

1.4.2 Synchrotrons

The design of the synchrotron accelerator came after the cyclotron, with the beam travelling in a circular motion. However, with a synchrotron the circular size is fixed. The magnetic field is applied externally at set points with the magnetic field strength increasing, leading to an increase in the acceleration of the particles. An example of a synchrotron is the large hadron collider at CERN.
The first synchrotrons were built for the purpose of studies in nuclear and particle physics. Two synchrotrons were built around the same time, from 1952-1954 Brookhaven built a 3 GeV synchrotron and Berkeley built a 6 GeV, both had different sized magnet apertures (Amaldi 2012).

Synchrotron radiation was also a problem for high speed synchrotrons. Synchrotron radiation was first observed in 1946 by John Blewett using a betatron of 100 MeV. The further uses of this radiation were realised, allowing it to be used for other applications in science such as by the Stanford Synchrotron Radiation Laboratory.

Synchrotrons in radiotherapy are used for the production of carbon ions. These synchrotrons are roughly 20-25 m diameter. Synchrotrons are also used for proton therapy, the size of these synchrotrons is around 6-8 m diameter (Amaldi et al. 2010).

1.4.3 Linear Accelerators

In the 1930s klystrons were invented by the Varian brothers which were improved upon by Ginzton (Freeman & Morris 2015). The magnetron was invented by Randall and Boot at the University of Birmingham (BOOT 1961). Both magnetrons and
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

Klystrons are RF cavities, with magnetrons having a broader spectrum. These inventions allowed linear accelerators to progress and be RF driven (Martins & Silva 2012).

In 1946, a linear accelerator (LinAcc) was built and tested by Fry at the Telecommunications Research Establishment, it was a 45 cm long 0.5 MeV linear accelerator. Further construction of these accelerators led to Fry building a 3 m long accelerator which reached 6 MeV (Martins & Silva 2012). Fry went on further to develop a LinAcc for medical x-ray use which was able to reach 8 MeV. The ministry of health decided that 4 MeV LinAccs for radiotherapy would be used. This was designed by Howard-Flanders, who developed the gantry in 1949. Ginztan and Kaplan built a medical LinAcc at Standford in 1954 which reached 6 MeV and was 1.65 m long (Martins & Silva 2012).

There are far more electron linear accelerators than proton accelerators, as cyclotrons are capable of accelerating protons for a lower price. The cost consideration is why many proton therapy centres use cyclotrons over LinAccs.

1.5 Hadron therapy

Hadron therapy is a type of radiotherapy involving the acceleration of hadrons which are particles formed from quarks, anti-quarks and gluons. Hadrontherapy has been considered as a new approach, however, in 1945 Lawrence and Wilson measured the stopping process in protons off the cyclotron in Berkeley and realised the potential it would have in therapy (Amaldi et al. 2010). The accelerators used in proton therapy are around 3-4 m diameter cyclotrons. Other than the initial findings of a proton beam’s stopping distances, hadron therapy has been a new development in radiotherapy. Proton therapy was originally used to treat the pituitary gland for breast cancer, with the work being conducted at the Berkeley facilities (Amaldi et al. 2010).
1.5.1 Proton centres

Currently there are 33 proton therapy centres in the world, with more currently in construction (Anderung 2012). Protons are advantageous to treating tumours due to the deposition of the dose within the target area, with a small tail of irradiation exiting the target area leading to a lower exit dose and less healthy tissue irradiated.

Proton therapy is further advantageous in comparison to X-ray irradiation due to the difference in energy deposition along the path, or track. X-rays deposit high levels of energy along the track, lowering the energy deposition the further through material it travels, the higher the energy deposition within tissue the higher the direct damage which occurs to the DNA due to the irradiation (Jäkel 2008). Direct DNA damage from high energy irradiation causes a clustering of damage at the Bragg peak, where the track is slowing and releasing the energy. This clustered damage causes a higher proportion of DSBs which if the damage is high enough, the cell cannot repair leading to cell death (Hill 1999). However, as the damage is deposited mainly in the Bragg peak (due to the stopping ions and energy deposition) for protons, this level of damage should not occur so greatly upon entry of the patient. It has been suggested that the track structure caused by the varying levels of linear energy transfer (LET), which is higher for slower moving particles like protons, is not a sufficient description of how radiation affects the DNA (Ballarini et al. 2008).

The relative biological effectiveness (RBE) is a measure for how different types and energies of irradiation produce a different biological effect. It is generally seen that the higher the LET the higher the RBE (Frankenberg 1994). However, the ion is important too, as heavy ions have a wider track structure. Heavy ions have high levels of energy deposition clustering in the DNA as well as being able to produce high-energy secondary electrons which can travel micrometres away from the main track causing additional damage to the DNA (Ballarini et al. 2008). Because of the combined effects of LET and how heavy the ion is, the RBE is the best explanation of how much damage a type of irradiation causes.
**Modulation of the Base Excision Repair (BER) pathway** in the treatment of glioblastoma with radiotherapy

### 1.6 Microbeams

The first microbeam was set up by Zirkle and Bloom using 2 MeV protons produced in a Van Der Graaff accelerator. Microappertures were manually positioned to be able to focus the beam to 0.5 µm to 5 mm. The beam was capable of depositing energy into the cells up to 2.5 µm. The first experiment looked at mitotic amphibian heart cells (Zirkle & Bloom 1953). This study shows how a microbeam can be used to focus on a small area of a cell, even in the 50’s to study the effects of radiation on just that area. Microbeam studies are mostly used for 3 reasons; irradiating specific parts of a cell to compare cellular response to damage, elucidating the role of Bystander effect, and to ensure each cell is irradiated rather than in broadbeam irradiation, where a cell being hit is reliant on poisson statistics (Prise et al. 2010). Microbeam studies can now focus on the difference in cellular response to radiation to the nucleus compared to the mitochondria, where the mitochondria spontaneously bursts and disappears (Walsh et al. 2017), and the long term effect of this can be elucidated. Studies looking into the bystander effect are also of importance. These studies ask if you miss a tumour cell with the irradiation, or a healthy one, can signalling cause the cell to die regardless i.e. does the non-hit cell also undergo damage from cellular signalling? (Bonner 2004)

1.6.1 Surrey Microbeam

Surrey University is home to the Ion Beam Centre, which hosts a 2 MV Tandetron accelerator with 5 beamlines. The accelerator itself is well described by Simon and colleagues (Simon et al. 2004), with two beamlines described, the microbeam line and the millibeam line, which are currently used for MeV single ion Mass Spectroscopy (SIMs), Rutherford backscattering (RBS) and proton induced x-ray emissions (PIXE). The scanning capabilities of the microbeam for RBS and PIXE was described and evaluated by Merchant for the in air rather than vacuum system (Merchant et al. 2005).

The current system for cell irradiation in place at the university of surrey is a scanning vertical microbeam line, where the design was first described by Kirkby and colleagues in 2007 (Kirkby et al. 2007). The system was designed to come off of the tandem accelerator in a lab 3 stories below and the beam bent at a 90° angle and focussed until it exits the beam line into the end-station where cell dishes are available to be irradiated. The initial design assumed that ion species ranging from protons and helium as well as O, C, N and Ar could be accelerated this distance. Since the conceptual ideas were
published, further work was completed on this beamline and cell irradiation systems were
designed. The beamline is described in detail by Merchant and co-workers, where they
describe the object and collimator aperture positions and capabilities along the beamline.
To ensure the correct dose is being given and no extra, a “beam switch” is used to deflect
the beam and scattered ions and stop further particles from entering the cells (Merchant
et al. 2009). The beam exits the line via a silicon nitride window and the stage controlling
the cells can be employed to insert the cells under the beam line remotely. Cells are placed
on polypropylene and fixed into metal dishes, as described by Jeynes and co-workers
(Jeynes et al. 2013). The physical limitations of a vertical beam that could be affected by
ground vibrations were dealt with by using a tripod stand, with sand as an insulator and
absorber (Merchant et al. 2012). Many of the studies successfully using this beam line look
into glioblastoma cell lines which are irradiated with both protons and alpha particles
(Barazzuol et al. 2012). As this system is set up to not only perform broadbeam
irradiation, but also single cell irradiation, studies using V79 Chinese hamster cells looked
into survival after irradiation on “broadbeam” or single cell targeting (Prakrajanga et al.
2013).

1.7 Dosimetry

When working with radiation for medical research the units Dosimetric
parameters of interest are the Gray (Gy) and Sievert (Sv). The absorbed dose is ... Sieverts
are the SI unit of radiation for the biological effect and refers to the effect of a joule of
energy to the receiver’s mass in kilograms. This is a measurement of stochastic health
risks from radiation and are generally used in accidental exposure to radiation. This is the
same for Gy, except it is the absorbed dose, rather than the effect of the dose. Gy are used
to measure the absorbed dose when treating a patient with radiotherapy in treatment
settings.

To work out the dose given in Gy from an accelerator, be it a medical device or
research, Equation 1 Dose rate;

\[
\text{Dose rate (Gy/s)} = 1.6 \times 10^{-9} \frac{L \Phi}{\rho}
\]

Where \(L\) is the LET (keV \(\mu\)m\(^{-1}\)), \(\Phi\) is the particle flux (particles per second) and \(\rho\)
is the density of the medium (g cm\(^{-3}\)). There are a multitude of ways to measure this
through simulations for the LET and for particle flux measuring devices include; ionisation
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

chambers, P-N diodes and calorimeters. The vertical microbeam beam tests the homogeneity with a scintillator, and beam flux is measured with a PIN diode (silicon p-i-n device) before the irradiation of cells (Jeynes et al. 2013).
2 Glioblastoma and DNA Repair

2.1 Introduction

This chapter will focus on the brain cancer glioblastoma and current treatments for this devastating disease. This will explore specifically, the damage which is caused to the cancer cell by the treatments and the way the cells repair this damage. There will be a particular focus on the DNA repair pathway base excision repair (BER), and how BER has been manipulated in current studies, as well as discussing drugs/inhibitors to modulate BER which are being tested as potential therapeutic tools in cancer treatment.

2.2 Glioblastoma

Glioblastoma multiforme is the most common form of brain cancer. The prognosis is poor with a 2 year survival rate of 7.7% as found by recent studies (Ahmadloo et al. 2013). Survival rates in the elderly are far more pessimistic with median survival being approximately 4-5 months (Scott et al. 2012). Recurrent glioblastoma has a median survival rate between 3-6 months (Vredenburgh et al. 2007). Surgical resection of this tumour is difficult due to its infiltrative nature and therefore the damage which could occur due to possible neural damage (Louis et al. 2007).
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

2.2.1 Glioblastoma Morphology

Glioblastoma is a brain cancer originating in glial cells; oligodendrocytes or astrocytes. Glioblastoma is most commonly found in men with a ratio of 3:2, male to female occurrence, from the ages of 40-70 years old (Jellinger 1978). It can occur anywhere within the CNS, but is most commonly found in the frontal and temporal lobes (Jellinger 1978). Glioblastomas can be primary or secondary by either developing spontaneously into a glioblastoma or from a recurring form of glioma tumour.

The hallmarks of cancer were defined by Hanahan and Weinberg who laid out 6 and then 11 traits common in all cancers (Hanahan, R. A. Weinberg, et al. 2000; Hanahan & Weinberg 2011). These include; evasion of apoptosis and cell death, self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential and sustained angiogenesis. In glioblastoma, tumours go through substantial angiogenesis, keeping the tumours with a high oxygen and nutrient supply. Ring growth structures are visualised in neuroimaging of glioblastoma due to heavy angiogenesis and distortion of the vasculature. Necrotic pseudopalisades are associated with an increase in micro vascularisation and are signs for a poor prognosis. The formation of pseudopalisades with microvasculature in glomeruloid appearance, signal a high growth phase within the tumour formation (Whittle et al. 2010). The pseudopalisades are often linked to high levels of necrosis (Jellinger 1978). The cells regularly have ill-defined cytoplasm and there are many mitotic figures (Jellinger 1978). The vasculature cells around the tumour tissues have sometimes been found to have higher levels of mitochondria than the cells in the blood vessels in normal areas (Coomberl et al. 1987). They also found that the junctions between cells in the interendothelial layer of the vasculature around some tumours were wide and distorted (Coomberl et al. 1987).

2.2.2 Glioblastoma Subtypes

As shown in one small case study of patients, the genetics of the DNA defects are very diverse for different patients and so treatment is hard to personalise (Hassler et al. 2006). Glioblastoma Multiforme have a number of subgroup divisions. These subgroups are proneural, mesenchymal, classical and neural, as identified by Pal (Pal et al. 2014). Proneural group occurred in much younger patients than the other groups and survived the longest, however, treatment saw no benefit to the survival rates. In the group
Chapter 2: Glioblastoma and DNA repair

Proneural, it was found that the tp53 gene is a tumour suppressor protein which has roles in activating DNA repair proteins, cell cycle and apoptosis (Romano et al. 2016). Proneural glioblastoma also regularly have mutations in other genes such as: IDH1, isocitrate dehydrogenase, changes the function of enzymes in the KREBs cycle to produce a mutogenic chemical which binds to the DNA and leads to abnormal cell growth (Cohen et al. 2013) and platelet derived growth factor receptor A (PDGFRA) which also leads to uncontrolled growth and invasion when heavily mutated (Pathania et al. 2017).

The mesenchymal group responded well to aggressive treatment. Mesenchymal glioblastoma had mutations in phosphatase and tensin homolog (PTEN), neurofibromatosis type 1 (NF1) and TP53. PTEN is a tumour suppressor gene which catalyses the dephosphorylation of the 3’ phosphate on the inositol ring in PIP3 during signal cascades, for example in cell cycle (Chu & Tarnawski 2004). NF1 regulates cell proliferation, differentiation, survival and growth through a signalling pathway common to them all (Helfferich et al. 2016).

Classical glioblastoma group of patients respond well to aggressive treatment and survive the longest due to the treatment given. Classical glioblastoma has high expression levels of EGFR which is a growth factor receptor, which leads to increased cell growth.

Neural glioblastoma was categorised as expressing gene types found on normal, noncancerous neurons. This type of glioblastoma was seen more commonly in the older patients and were found to respond well to treatments (Pal et al. 2014). These gene mutations show how the cancers hide from the immune system by disguising as neurons, neural type, or lead to excessive cell growth and proliferation.
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

<table>
<thead>
<tr>
<th>Glioblastoma subtype</th>
<th>Gene regulation changes</th>
<th>Cellular advantage</th>
<th>Effect of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proneural</td>
<td>p53, IDH1, PDGFRA</td>
<td>removal of cell cycle checks and inhibition to apoptosis, increased abnormal cell growth and increased invasion</td>
<td>No benefit from treatment.</td>
</tr>
<tr>
<td>Mesenchymal</td>
<td>PTEN, NF1, TP53</td>
<td>increased signalling to pass through the cell cycle, increased cell proliferation and differentiation, resistance to apoptosis</td>
<td>Responsive</td>
</tr>
<tr>
<td>Classical</td>
<td>EGFR</td>
<td>cell growth increased</td>
<td>Responsive and longest survival</td>
</tr>
<tr>
<td>Neural</td>
<td>NEFL, GABRA1</td>
<td>Neuron projection and filaments and GABA receptor (inhibitory signalling receptor)</td>
<td>Responsive</td>
</tr>
</tbody>
</table>

*Table 1 A summary of gene expression changes in subtypes of glioblastoma (Verhaak et al., 2010).*
2.2.3 Glioblastoma treatment

Although the morphology and the subtypes for glioblastoma is diverse a standard treatment regime is used. As can be expected the survival rate varies depending on patient age and treatment regimens (Stupp et al. 2005a). The standard treatment regimen as outlined by Stupp for glioblastoma includes surgery, radiotherapy and temozolomide (TMZ). The dose of radiotherapy traditionally used is 60 Gy administered in fractions of 2 Gy per day, 5 days per week, delivered by a linear accelerator (LinAcc). TMZ is concomitantly administered over a 4-week period at a dose of 200 mg/m$^2$ orally for 5 days (Stupp et al. 2001). Concomitant therapy such as this has increased the median survival rate from 12.1 months to 14.6 months (Stupp et al. 2005a)(Stupp et al. 2005b).

As the survival rate is still so low, different radiotherapy fractionation regimens have been examined, including an increase in the daily radiotherapy dose, or a reduction in the dosage per irradiation event whilst increasing the number of daily irradiation events administered (Balducci et al. 2014; Miwa et al. 2014; Kulshrestha et al. 2008). Neither of these made a significant difference to survival rates. It is of great importance to elucidate the best treatment delivery schedule for radiotherapy. This can be done via in silico, in vitro, in vivo or using epidemiological studies.

The majority of glioblastomas are treated with radiotherapy, for elderly patients trials involving radiotherapy, in comparison to resection alone, showed an increase in median survival by approximately 3.6 months (Arvold & Reardon 2014). However, few patients, about 30%, get the full prescribed dose often due to patients poor compliance (Ahmadloo et al. 2013). Small studies just looking at curative rates from radiotherapy show that median survival rates are 12 months (Lin et al. 2003). Temozolomide with radiotherapy provides the most effective care. Temozolomide alone in elderly patients has been shown to increase median survival from 3 months to 6.3 months (Gállego Pérez-Larraya et al. 2011).

Treatment with TMZ has also been extensively investigated as some patients show resistance to the cytotoxic effects of this chemotherapeutic. It has been shown that resistance to TMZ is due to the expression of the DNA repair protein O$^6$-methylguanine-DNA methyltransferase (MGMT) in some patients. MGMT repairs O$^6$-methylguanine (O$^6$-MeG), a toxic lesion caused by TMZ within the DNA, therefore preventing apoptosis from...
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

occurring (Explained further in section: 2.4.3). Due to this resistance it is imperative to manipulate the treatment plan, personalising the medicine for these patients. This has been suggested to be brought into hospitals in some countries (Wick & Platten 2014; McNamara et al. 2013).

2.3 The effects of radiation

2.3.1 The importance of RBE and LET

As cancers are currently being treated with different types of radiation to shrink the tumour mass it is useful to compare the different effects these radiation types have on the cells and specifically the DNA. Radiation types can be compared by looking at the linear energy transfer (LET). LET, as a rule of thumb, increases as the particle increases in atomic number. The LET defines the average energy locally imparted to the medium by a particle traversing a given distance (unit, keV/µm). The path that the particle travels is known as the track and the track structure is compared for different types of radiation and their energies (Hill 1999).

When treating patients with X-rays and protons the energy with which the particles are directed towards the tumour changes depending on how much material the particles have to travel through before hitting the tumour. In traditional X-ray therapy the treatment plan is often designed so the X-rays travel through the body at a multitude of angles to intersect and give a greater dose at the tumour and to minimise damage to the surrounding healthy tissue (Saunders 1985). This is important for X-rays as the energy deposition for X-rays is at its greatest within the first few cm of tissue, this then drops exponentially as it travels through, causing lower amounts of damage to the cell and specifically the DNA the further the X-ray travels (Saunders 1985). However, protons travel through material differently. A proton beam releases lower amounts of energy whilst travelling fast through material, however, as it slows, greater amounts of ionising energy are released in the material; this is seen as a sharp peak in energy deposition curves. This characteristic shape is known as the Bragg peak (Amaldi et al. 2010).

The LET, although very informative for the quality of the radiation, does not describe the biological effects of radiation (Waligórski et al. 2004). Particle therapy has
been shown to have a great biological effectiveness in comparison to traditional photon, X-ray, therapy. This change, due to the LET, has been termed the relative biological effectiveness (RBE). The RBE is defined as the ratio of absorbed dose of a reference radiation over the dose of a radiation under test to produce the same biological effect.

Although increasing LET is seen to give a higher RBE due to the deposition of energy, there is a point, 100 KeV/µM, where the increase in LET no longer affects the RBE as the energy deposited cannot cause any greater damage due to the intervals in which the energy is deposited is smaller than the size of the DNA and so a waste of energy expenditure (Paganetti et al. 2002).

2.3.2 The case for proton therapy

According to WHO 7.6 million people died of cancer in 2008 (World health organisation 2013). With statistics like this the importance of accelerators in medicine is understandable. Accelerators are used for radiotherapy which over 50% of cancer patients undergo (Delaney & Barton 2015; Barton et al. 2014). Linear accelerators are currently the standard for UK hospital radiotherapy, however, microtrons can be used also to provide x-rays and electrons (Amaldi 2012; Miwa et al. 2014). The x-rays are formed from an electron beam due to Bremsstrahlung, which is the production of a photon when an electron loses energy, as the electrons hit a heavy metal target. The accelerators used are often around 5-30 MeV capable accelerators with around 5000 in use around the world (Cleary et al. 2015). Other than the initial findings of proton beam’s stopping distances, hadron therapy has been a relatively new development in radiotherapy. Proton therapy was originally used to treat the pituitary gland for breast cancer, with the work being conducted at the Berkeley facilities (Amaldi et al. 2010).

The advantage of a proton beam is that far less energy is released until it reaches the target area, unlike x-rays as can be seen in Figure 2-1. This means that the tissue in front of the tumour has a lower amount of radiation deposited there and only the tumour is affected by the damage of the protons (McDonald & Fitzek 2010). In traditional radiotherapy, x-ray energy is deposited along the tissue until the wave stops, as the energy released through the tissue is similarly high until it comes to a stop, damage to healthy tissue occurs which can increase side effects such as secondary cancers. Where the proton beam stops is called the Bragg peak, as shown in Figure 2-1, this Bragg peak occurs due to
coulomb interactions within the target tissue (Liauw et al. 2013). The coulomb interaction causes the proton beam to slow down, as it does so more energy can be imparted to the atoms surrounding it, increasing the radiation which is why proton beams impart the majority of their energy at the target tissue.

![Figure 2-1 Spread-out Bragg peak](image)

**Figure 2-1 Spread-out Bragg peak** a) it can be seen that x-rays start to move through tissue with a higher energy that decreases as it reaches the target depth. However, proton beams have a Bragg peak that shows an increase in radiation dose/energy at the target depth, where the wave stops. In b) it can be seen that due to the lower radiation through the tissue and the Bragg peak seen at the target where the wave ends less radiation is seen in the healthy tissue past the tumour.

### 2.3.3 Types of damage caused by radiation

Traditional X-ray photons cause more single strand breaks (SSBs) than double strand breaks (DSBs) by direct and indirect interaction with the DNA within the cell. Indirect actions are mainly due to the formation of free radicals which damage the DNA (Yokoya et al. 2008). Direct mechanisms of DNA damage have been linked to the photo-electric effect, where electrons are produced within the cell along the track structure releasing energy onto the bonds of the DNA, breaking them and causing damage (Roots & Okada 1975). Further damage by radiation can be caused by the production of free radicals which can proteins proteins and other molecules, binding them to atoms on the DNA damaging either the sugar, base or phosphate backbone (Azzam et al. 2012). The amount of DSBs, which are more toxic than SSBs, formed from radiation depends on the cell type and nucleus size (Wéra et al. 2014). DSBs can be visualised and measured through an assay which binds to the DNA at H2AX histone which is phosphorylated at the site of damage (Yokoya et al. 2008), this is explained further later in this chapter. It has
been shown that as the level of LET increases the efficiency of SSB induction lowers (Jäkel 2008). It has however also been shown that with higher LET the rate of SSB repair is decreased in CHO cells (Olive & Banáth 1993). DSB formation has been measured to increase linearly with dose. DSB formation is lower than the formation of SSBs by a factor of 25 (Frankenberg 1994). Unlike SSBs, DSBs increase with increasing LET. Different studies report different amounts for the number of DSBs that are formed per Gy. Studies using the γ-H2AX assay show that between 11.6 and 14.7 foci form per Gy (Rogakou 1998; Redon et al. 2012; Mandina et al. 2011). This can be the difference of LET as well as limitations of the methodology to analyse DSBs.

2.3.4 Double strand break signalling

Histones play a crucial role in DNA structure, allowing the DNA to be super coiled or unwound. DNA is ravelled 1.7 times around the histone core, which is made up of 8 histones, repeating histone H2A, H2B, H3 and H4, this forms the nucleosome (Suto et al. 2003). The DNA is further compacted by a linker histone which compresses the nucleosome, this histone is H1.

There are 10 genes that code for the human H2A histones. The histone H2AX makes up 2-25% of the total H2A histones in the nucleosome (Fernandez-Capetillo et al. 2004). H2AX is considered a minor variant of the H2A histones due to the rarity of this polypeptide and it was discovered in the 1980s (Musgrave et al. 1991). Where H2AX differs from other histones in the H2A family is on the COOH-tail which becomes rapidly phosphorylated on Ser 139 upon DNA damage (Rogakou 1998), this post translational modification is highly conserved from yeast to humans (Redon et al. 2002).

H2AX has an important role in DNA repair signalling; DNA damage triggers a signalling cascade known as the DNA damage response (DDR) (Paull et al. 2000). The kinase family involved in the DDR is the phosphatidylinositol-3-kinase (PIKK) family of kinases, composed of ataxia telangiectasia mutated (ATM), ATM and Rad3-related (ATR), ATM related kinase (ATX) and DNA dependent protein kinase (DNA-PK), all being important during DNA damage signalling(Golding et al. 2012; Srivastava et al. 2009; Schultz et al. 2000; Burma et al. 2001; Riballo et al. 2004). It has been shown that H2AX is
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

phosphorylated by ATM after DSB (Burma et al. 2001) whereas ATR phosphorylates H2AX after a single strand break and during replication stress, DNA-PK phosphorylates H2AX when DNA fragments during apoptosis (Srivastava et al. 2009).

The γ-H2AX foci disappears as the repair pathways take place. The disappearance occurs as the H2AX histones becomes dephosphorylated by protein phosphatase-2A (Svetlova et al. 2010) and the nucleosome becomes reassembled (Suto et al. 2003). This gives a good representation of the repair kinetics within a cell after DSBs have occurred. As γ-H2AX foci appear at 1:1 at DSB locations, the foci start to appear a few seconds after damage but it can take up to 1 hour for foci to be fully visualised using immunohistochemistry (Hall 1985).

2.3.5 Repair pathways involved in radiation damage

There are two well recognised repair pathways which are of specific interest to radiotherapy, due to the importance of DSBs formed by radiotherapy; these pathways are non-homologous end joining (NHEJ) and homologous recombination (HR) (Kavanagh et al. 2013). These repair pathways are shown in Figure 2-2 and Figure 2-3. It has been shown that H2AX has a role in modulating non-homologous end joining (NHEJ) and homologous recombination (HR), although H2AX is not essential for these processes to occur (Petersen et al. 2001). The DSB is recognised by a complex of proteins known as MRN. MRN complex involves MRE11, RAD50 and Nijmegen breakage syndrome 1 (NBS1) proteins (Lavin 2007). It has been noted that depending on the phase of the cell cycle NHEJ or HR may or may not be used. HR is predominantly used to repair DSB damage during S and G2 phase (Allen et al. 2003; Aymard et al. 2014).

The major form of DSB repair can be utilised throughout all phases of the cell cycle. Non-homologous end joining is initiated when a heterodimer, formed by Ku70 and Ku80, bind to either end of the breakage site (Iyama & Wilson 2013). This binding recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and the resulting protein complex at the breakage site allows the strands to stabilise and align. Once the DNA is aligned and stable the DNA-PKcs autophosphorylates and dissociates from the complex, starting a signalling cascade which recruits the endonuclease artemis (Weterings & van Gent 2004). Artemis trims the 3’ and 5’ ends of the break creating an overhang to help
ligate the ends. PNKP and XRCC4-ligase IV remove phosphate groups attached to the overhangs before ligation takes place. DNA polymerases fill in any gaps that are missing in the homology of the overhangs, the DSB is ligated back together when XLF complexes with XRCC4-ligase IV and the DNA is sown back up (Iyama & Wilson 2013).

Figure 2-2 Non-homologous End Joining double strand break repair, low accuracy as joins two damaged ends together, without checking they match. taken from (Mladenov & Iliakis 2011)

Homologous recombination is utilised by the cell to repair DSBs during S and G2 phases of the cell cycle. Homologous recombination is an efficient repair mechanism as it employs the sister chromatid to accurately repair the lesion. The DSB is recognised by a complex of proteins known as MRN. MRN complex involves MRE11, RAD50 and Nijmegan breakage syndrome 1 (NBS1) proteins (Lavin 2007). MRE11 is the main catalytic component of this complex and acts as a sensor, as mentioned above, this complex also signals for protein kinase activation phosphorylating ATM at the site of the DSB (Lavin 2008; Iyama & Wilson 2013), and can act as an exo- and endonuclease (Dudás & Chovanec
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

2004). Single strand DNA (ssDNA) is created from 5’-3’ DNA end trimming by C-terminal binding protein-interacting protein (CtIP) and MRN when they associate (Sartori et al. 2008). During this step further trimming occurs due to exonucleases.

The next steps in HR is stabilisation of the ssDNA and signalling for checkpoint response. These steps are initiated by the binding of RPA stabilising the DNA and signalling for ATR and ATR-interacting protein to create the signal. BRCA2 promotes the replacement of RPA with RAD51. RAD51 is an essential part of the DSB repair as RAD51 filaments (from five RAD51 types) search for homology and strand invasion (Karpenshif & Bernstein 2012). Homologous recombination, after DNA stabilisation, forms a D-loop of DNA which is formed from the DNA being extended by polymerases. This D-loop is formed from the RAD51 filaments seeking homologous sequence with the ssDNA overhang invading the homologous double stranded DNA displacing the DNA and allowing the damaged DNA to be repaired correctly (Karpenshif & Bernstein 2012).

After the formation of the D-loop, homologous repair has two mechanisms that it can use to repair the DSB. A Holliday junction can be formed, or alternatively synthesis-dependent strand annealing (SDSA). A Holliday junction is formed when the second end of the damaged DNA is captured within the D-loop. This complex requires DNA nuclease and DNA helicases to be resolved. In SDSA the DNA is displaced and re-annealed to the other broken chromosome.
Radiation damage is also recognised by poly(ADP-ribose) polymerase 1 (PARP1) which ribosylates ATM to activate ATM in remodelling the chromatin (Haince et al. 2007). In human lymphoblasts PARP inhibition has been demonstrated to delay repair of DSBs (Haince et al. 2007). PARP is important in the recognition of breaks where alternative end-joining can be employed as a repair mechanism by the cell. PARP1 recognises the broken ends of the DNA in alternative end joining and through ribosylation can recruit ligase 3 to rejoin the breaks (Thompson 2012; Simsek, Brunet, et al. 2011). It has been shown that during this process the breaks are treated as single strand breaks during repair (Metzger et al. 2013). This can then lead on to late BER which involves DNA ligase 3 and XRCC1 to repair the nick in the DNA (Audebert et al. 2004). With this pathway, alternative end-joining is not as well studied as HR and NHEJ, however, it is vital to understand for this study.

Figure 2-3 Homologous Recombination. Double strand break repair using the sister chromatid to ensure highly accurate repair. Taken from (Mladenov & Iliakis 2011)
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

2.4 Temozolomide and the DNA

2.4.1 Alkylating agents

Alkylating agents are chemicals that react with biological molecules transferring an alkyl group. Alkylating agents can react with O- and N- groups on DNA bases which can disrupt the cell cycle due to the adducts caused (Kondo et al. 2010). There are different types of alkylating agents which describes the chemical structure and how it reacts with the DNA. There can be mono- and bifunctional alkylating reagents, which are named after how many reactive sites the alkylating agent has this can account for how many lesions will occur. Bi-functional alkylating agents can bind across the DNA and cause cross links (Fu, J. a. Calvo, et al. 2012). Alkylating agents can also have different types of nucleophilic substitution, this is denoted as either $S_N1$ or $S_N2$. $S_N1$ alkylating reagents mainly interact with the nitrogen in a carbon ring. However, the $S_N2$ reacts with not only the nitrogen in the ring, but also extra cyclic oxygen atoms. As alkylating agents interfere with the DNA they can be mutagenic, but, they can also be used as chemotherapeutics as they can put cells into cell cycle arrest (Wyatt & Pittman 2006).

2.4.2 Temozolomide discovery

Temozolomide (TMZ) is a derivate from imidiazotetrazinone and is pro-drug which is converted to the active metabolite 5-(3-methyl)1-triazen-1-yl-imidazole- 4-carboxamide (MTIC) (Denny et al. 1994). Studies showed that temozolomide has good bioavailability after oral consumption to all tissues, including the brain (Newlands et al. 1992). The good bioavailability can be explained due to the chemical properties of TMZ. TMZ is stable in acidic pH but becomes unstable from neutral to basic pH. This change in pH is what allows the drug to be taken orally and become active in the body, due to the change in pH from the stomach to other areas once absorbed. Conversely MTIC degrades into the reactive methyl agent in a pH which is more acidic than basic. This difference in stability and reactivity allows TMZ to not be destroyed by stomach acid, but only have a small window of reaction due to the pH changes (Newlands et al. 1997).

It has been shown that TMZ targets specific areas of the DNA. This unstable methylating agent is a major-groove methylating agent which reacts with the DNA in a base selective manor choosing the central guanine in a GGG sequence (Denny et al. 1994; Newlands et al. 1992). The methyl adducts occur here.
due to being the most electron dense area of DNA, which correlates to being the most basic. TMZ is a monofunctional alkylating agent, so does not cause cross links (Newlands et al. 1997). But it is an $S_N1$ alkylating agent and the methyl adducts occur on the N$^7$ atoms on guanine (70%), N$^3$ on adenine (9.2%), and O$^6$ on guanine (5%) (Chakravarti et al. 1991) as these functional groups are more accessible in the DNA’s major groove (Newlands et al. 1997).

Phase I and II studies run by Cancer Research Campaign showed that intravenous administration of TMZ had little effect whilst oral consumption gave good bioavailability at a dose of 200 mg/m$^2$ (Newlands et al. 1992). However, in practice the first dose is given at 150 mg/m$^2$ and if no toxicity through myelosuppresion is noted then the dose is increased to 200 mg/m$^2$. The toxicity of TMZ is evident in the myelosuppression with a nadir on the 22nd day of the 4 week cycle and through sickness and nausea. In in vitro studies it has been shown that TMZ has a half life of 1.24 hours in phosphate buffer solution (Newlands et al. 1997).

2.4.3 Repair of lesions caused by temozolomide

The most toxic TMZ-induced lesion, as mentioned above, is the O$^6$-MeG lesion. This lesion is the most toxic because it can lead to apoptosis induction (Newlands et al. 1992; Happold et al. 2012). In order to understand how the unrepaired O$^6$-MeG lesion leads to apoptosis induction, one needs to understand the DNA repair pathway mismatch repair (MMR) (Liu et al. 1999). MMR initiates by the recognition of a mismatched base and the pathway can be seen in Figure 2-4. The lesion is recognised by the mismatch repair (MMR) proteins in a heterodimeric complex; MSH2 with either MSH6 for MutS$\alpha$ (Drummond et al. 1995) or MSH3 for MutS$\beta$. MutS$\alpha$ recognises base-base mismatches and insertion-deletion loops, therefore is the important complex for recognition of O$^6$-MeG. The MutS$\alpha$ complex recognises that due to the methylation of guanine it can no longer provide 3 hydrogen bonds, but 2, and so should not be paired with cytosine. MutS$\alpha$ protein recognises the mismatch pair due to a contortion in the DNA and Van der Waal forces (Lamers et al. 2000). PCNA interacts with MSH2 to aide in DNA mispairing recognition (Lau & Kolodner 2003). EXO1 further interacts with MSH2, but to aid in excising the DNA in the 5’position (Zhang et al. 2005). RPA protects the gap in the DNA whilst polymerase delta is recruited to fill the gap with the correct base. The DNA is then ligated and sealed.
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

However, MMR proteins recognise that modified guanine should not be paired to thymine and recruit polymerases to the site for replacement of the mismatch with the correct base, cytosine. This cytosine is then replaced with thymine resulting in a futile mismatch repair loop leading to multiple breaks in the DNA strands and MutSα, part of the MMR pathway, signals for apoptosis (Hickman & Samson 1999).
Figure 2-4 Mismatch Repair pathway repair of incorrect DNA base matching preventing point mutations. Taken from (Jiricny 2006)

2.4.3.1 Base excision Repair

The additional lesions caused by TMZ are repaired by the base excision repair pathway (BER), shown in Figure 2-5. There is long patch BER (lp BER) or short patch BER (sp BER) which refers to the number of bases which need to be cleaved and replaced; sp BER refers to one base alone, but lp BER is when more than one nucleotide needs to be removed (Larsen et al. 2007). TMZ induced DNA damage is recognised by the enzyme alkyladenine-DNA-glycosylase (AAG) which is an initial enzyme in the BER cascade (Fishel et al. 2007; Zhang et al. 2012). AAG removes the lesions by rotating and cleaving the base leaving an apurinic/apyrimidinic (AP) site (Lau et al. 2000). This AP site is then hydrolysed at the phosphodiester backbone by an AP endonuclease which generates DNA ends as a 3’OH and a 5’ deoxyribose-5-phosphate (5’dRP) (Masuda et al. 1998). The 5’dRP is removed by the lyase activity of DNA polymerase β which also then replaces the missing
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

nucleotide (Sobol et al. 1996). DNA ligase I or III then ligates the new nucleotide leaving the DNA fully repaired.

Each intermediate step in BER can cause cytotoxic effects if left unrepaired so the enzymes have to be in close proximity to each other once the repair has been initiated (Fu, J. a. Calvo, et al. 2012). Studies on a scaffolding protein known as X-ray repair cross-complementing protein-1 (XRCC1) have shown that each enzyme in BER can bind to XRCC1, increasing their activity (Dianova et al. 2004) and keeping them in close proximity to lower the risk of interrupting the repair pathway (Mutamba et al. 2011). PARP is also important in BER as it acts as a sensor for SSBs, making PARP important also for radiation damage. PARP1 PARylates proteins and histones to signal a damaged site which allows the DNA to uncoil and the repair proteins are brought to the SSB (Campalans et al. 2013).
Figure 2-5 DNA damage repair pathways initialised by temozolomide damage. 
\(O^6\)MeG adducts are repaired by MGMT if the protein is present, Otherwise mismatch repair is utilised. Base excision repair repairs the other adducts formed by TMZ.

2.4.4 Limiting factors for efficacy of temozolomide

Temozolomide is not effective for all patients. As previously stated, the most toxic lesion is \(O^6\)-MeG which can be removed by the protein MGMT. The repair mechanism works by the \(O^6\)-MeG being transferred into the active site of MGMT where it interacts with cysteine 145, repairing the base and inactivating MGMT. Methylated MGMT is then flipped out of the DNA via the substrate bound minor groove, this releases MGMT so that it can be ubiquitinated and degraded via the proteasome (Srivenugopal et al. 1996). This reduces the cytotoxicity of TMZ and therefore can lead to resistance in some patients.

MGMT expression varies throughout different tissues and is controlled through epigenetic modification; the methylation of the promoter region of the gene transcribing MGMT. The MGMT gene is located on chromosome 10, 10q26. The promoter region can be hypermethylated at the CpG islands specifically on the cytosine and this inhibits the binding of transcription factors (Baylin et al. 2001). It has been shown that between 45-70% of high grade glioblastoma patients have the MGMT promoter methylated (Zhang et
al. 2012) which conveys sensitivity to TMZ. This resistance needs to be addressed, as well as the efficiency of GBM treatment, perhaps by manipulating other repair pathways.

2.4.4.1 AAG

Human alkyladenine DNA glycosylase (AAG) is a glycosylase within the BER repair pathway, which, as described above, is one of the first enzymes within this pathway (Engelward et al. 1997). AAG is also known as methylpurine DNA glycosylase (MPG) and alkylpurine-N-DNA-glycosylase (APNG), these names are associated with the adducts which the glycosylase recognise. AAG can excise many lesions; N³-Mea, N⁷-Meg, 1,N⁶-ethA (εA), 2,N³-ethG and hypoxanthine, but with different efficiencies.

![AAG](Image)

**Figure 2-6 AAG. AAG 3-Dimensional structure and it’s interaction with DNA (Rubinson et al. 2016)**

The ease with which AAG can remove the lesions differs depending on placement and specificity. For example hypoxanthine opposite thymine is removed faster than if opposite cytosine (Asaeda et al. 2000). Whereas, more specifically for adducts which are caused by S₇₁ alkylating agents, N⁷-Meg is removed 100 fold less efficiently than N³-Mea (Miao et al. 1998). Lau showed that once AAG has recognised its specific substrates, a Tyr-162 side chain of the protein is β-hairpin inserts into the minor groove of the DNA which slips the nucleotide into the enzymes active site (Lau et al. 2000).

The active site of AAG has been shown to be inhibited by magnesium (Lau et al. 2000). Some studies have looked at the repair kinetics of AAG for certain substrates have shown that they are consistent throughout the first 24 hours for the base removal of N⁷-Meg, with a half-life of 18 hours. But this study also showed that if AAG is knocked out then other enzymes will repair the lesions which AAG normally repairs, however the repair is at a slower rate (Smith & Engelward 2000).
AAG has been shown to be brought to the site of damage by methylated DNA-binding domain 1 (MBD1) which then represses transcription of the damaged gene by associating with the promoter of the gene. It was further shown that after alkylation damage by MMS this transcription repressing complex would be present on the damaged promoter, but MBD1 would dissociate after 3 hours, presumably for damage repair to ensue by AAG (Watanabe et al. 2003).

2.4.4.2 AAG and BER

The potential importance of AAG in the repair of TMZ lesions has been well acknowledged and hence studied. A study where AAG was overexpressed in breast cancer cell lines and subsequently treated with TMZ and a BER inhibitor, methoxyamine found that sensitivity to TMZ occurred when AAG was overexpressed by 4.8 fold (Rinne et al. 2004). However, the concentrations of TMZ used are not comparable to the concentration of drug found at the tumour site as (Ostermann et al. 2004) found that the concentration at the tumour site should be approximately 25-50 µM not 2 mM or more as used in this study (Rinne et al. 2004). Another study demonstrated that the overexpression of AAG in GBM caused a slight increase in sensitivity to TMZ; this effect was also potentiated by a BER inhibitor methoxyamine (MX), concluding high levels of AAG coupled with BER interruption can increase the efficacy of temozolomide through DNA glycosylation (Tang et al. 2011). Conflicting evidence for the role of AAG has also been elucidated in cells over expressing AAG as an increase in resistance to TMZ was found (Agnihotri et al. 2012).

These studies bring into question the role of AAG expression.

These conflicting results can be hard to explain further in terms of AAG expression within GBM patients. It has been shown in many patient based experiments that the overexpression of AAG is related to the increasing grade of GBM and can be related to poor treatment outcome (Liu et al. 2012). Therefore, it may not be the expression levels of AAG which should be manipulated to enhance TMZ sensitivity if AAG is high in all GBM tumours.

The effects of BER and MGMT as well as the effects of the inhibitors can be summarised in Figure 2-5. It is also necessary to remember the importance of the DSB
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

repair pathways for this study. Cell survival may also be decreased if DSB pathways are imbalanced for cell lines with certain repair protein expression profiles.

2.5 Synthetic Lethality

Synthetic lethality is when two genes, which normally do not lead to embryonic lethality when knocked out, do lead to lethality when combined. This can include genetic variations leading to lethality when combined. This was first described by Calvin Bridges (1922 the origin of variation) and then termed by Theodore Dobzhansky when working on Drosophila (1946). In cancer cell development often DNA damage response and repair enzymes become silenced, increasing the viability of the cancer cell as well as genomic instability (Hanahan and Weinberg 2011). PARP inhibitors have been found to be more effective in cancer cells with BRCA defects (Bryant 2005 and Turner et al 2004). This shows that inhibitors and genetic variation can be used together to increase toxicity in cancer cells. This is favourable to a combination of 2 inhibitors as it may be less selective to cancer cells.

2.6 Pharmacological control of BER

2.6.1 APE in the BER pathway

APE is a globular enzyme consisting of α/β folds with two domains and can be seen in Error! Reference source not found. As stated before, AP endonuclease (APE) is the second enzyme utilised in the BER after alkylation damage to N7G and N3A. APE is activated after apurinic/apyrimidinic site has been formed by a glycosylase enzyme, or a spontaneous event, has hydrolysed the N-glycosyl bond which attaches the base to the phosphate sugar backbone (Chohan et al. 2015; Talpaert-Borlé 1987). APE cleaves the backbone at the 5' leaving a deoxyribose phosphate and a 3'OH group. This is then the substrate which PolB interacts with continuing repair. It has been shown that APE can increase the binding affinity of PolB and accelerate the removal of the 5'dRP (Bennett et al. 1997). The C-terminus of the protein deals with repair activity, however the N-terminus deals with redox reactions with other proteins.
APE can also remove the 3’-phosphate damage produced by ionising radiation and reactive oxygen species through a phosphodiesterase activity (Barzilay et al. 1995). The activities of APE, endonuclease activity, phosphodiesterase and RNase H activity are all dependent on Mg+ ion in the active site (Barzilay & Hickson 1995). Due to these different roles, APE is also utilised after damage repaired by 8-oxoguanine glycosylase (OGG1). OGG1 is different to AAG as it not only has the glycolytic activity, but also an AP lyase activity which cuts the backbone at the 3’ position (Hedglin & O’Brien 2010). To allow the functional repair by POLβ APE utilises the phosphodiesterase activity removing the 3’ residues.

APE binds to either the 5’ or 3’ (for phosphodiesterase activity) of the AP site (Masuda et al. 1998; Strauss et al. 1997). APE binds to the DNA at both the major and the minor groove, forming an extrahelical structure allowing for the exclusion of normal DNA (Mol et al. 2000). APE stabilises the extrahelical structure and widens the minor groove (Mol et al. 2000).

2.6.1.1 The link of APE to cancer

APE expression levels have been shown to be elevated in some cancers including; cervical cancer, ovarian cancer, prostate cancers and germ cell tumours (Lu et al. 2001; Fishel et al. 2007; Herring et al. 1998; Kelley et al. 2001). Studies have found 18 polymorphisms of APE (Xi et al. 2004) with only 6 of the variants below 50% binding affinity (Xi et al. 2004). In a study by Kelley, they showed from tissue resection that within the tumour area there was an increase in the expression of APE, but not in the benign healthy tissue from the prostate (Kelley et al. 2001).

2.6.1.2 APE expression and resistance to treatment

Low APE1 levels measured in studies with mouse lymphoblastoid cells have shown a radioresistant trend (Chen et al. 1991). Ono et al., also found a non-significant correlation between APE levels and radioresistance (Ono et al. 1994). However, this finding was not replicated in human astrocytomas (Hughes-Davies et al., 1995). Furthermore, Herring and co-workers found no correlation with APE expression and radiosensitivity in cervical cancer (Herring et al. 1998). More recently siRNA for APE has been used to sensitise colorectal cancer cells both in vitro and in vivo, showing a good link...
between APE and radiation sensitivity in these cells (Xiang et al. 2008). This strengthens the argument to look into the protein expression levels for repair pathways in glioblastoma cells as part of treatment development. In head and neck cancers it has been shown that high expression of APE not only is associated with radioresistance, but also chemoresistance, when patients were treated with a range of chemotherapeutics. High APE expression lead to a very poor survival with all patients having passed at 60 months, however, mild expression of APE lead to 40% of patients surviving until 110 months, the end of the study (Koukourakis et al. 2001).

Chemo resistance due to APE expression has been seen in many different tumour types, with a variety of drugs. In non-small cell lung cancer it was shown that cisplatin resistance is associated with high expression of APE (Wang et al. 2009). Silber et al found that in glioma cell lines expression of APE lead to an increase in resistance to TMZ and BCNU, however, they also found that reactive oxygen species created during treatment lead to an increase in cell expression and activity of APE (Silber et al. 2002). In an experiment by Ono, using transfected rat glioma cells showed increased sensitivity to MMS and hydrogen peroxide in knockdown cells (Ono et al. 1994).

In an initial study into glial cell tumours, it was found that APE expression is increased in glial tumours (Bobola et al. 2001). AP activity levels were measured in glioma patients and correlated the activity to the overall survival and sensitivity to chemotherapy and radiotherapy. Bobola and coworkers found that an increase in activity lead to an increased hazard of tumour progression in grade III gliomas, however, this association was not found in grade IV (Bobola et al. 2004). To prove their theory, but using medulloblastoma and primitive neuroectodermal tumours they used tumour resected cells and knocked down APE. These knockdown APE cells were more sensitive to TMZ and BCNU, than cells transfected with antisense siRNA. They also linked APE expression in cells to time to tumour progression, however, this link was not statistically significant (Bobola et al. 2005).

### 2.6.2 How methoxyamine works

Methoxyamine (MX) is an APE inhibitor which is a small organic amine which reacts with the aldehyde group present on the deoxyribose after the base has been excised by a glycosylase or spontaneously removed (Talpaert-Borl & Liuzzi 1983). Liuzzi also
Chapter 2: Glioblastoma and DNA repair

found in further studies that MX does not inhibit the base excision performed by glycosylases, specifically uracil-DNA-glycosylase. As the AP-site then becomes stable MX has been tested to potentiate the effects of TMZ and other chemotherapeutics.

In vitro studies with MX have shown that it can impair the growth of osteosarcoma tumours and potentiate radiation and cisplatin effects in cell killing (Montaldi et al. 2014). In colon cancer cell lines it was shown that the addition of methoxyamine with temozolomide increased early apoptotic signs from TMZ treatment of DNA fragmentation 24 hours earlier than treatment with TMZ alone (Taverna et al. 2001). Specifically to this project it has also been used in the glioblastoma cell line LN428, which is resistant to TMZ due to positive expression of MGMT. Tang et al., found that as they increased the expression of AAG in LN428 cells, they became more sensitive to MX and TMZ combined (Tang et al. 2011). However, the LN428 cells were not sensitive at chemotherapeutically relevant doses of TMZ or MX. Although this study measured the levels of polymerase beta, MPG and PARP in an attempt to establish a link between efficacy of TMZ, MX and PARP inhibitors, however, these studies are interesting but do not help to establish feasible chemotherapeutic links and are so not applicable to clinic.

MX was brought into clinical trials for solid tumours and the first published results were in 2010 of the levels of methoxyamine found in plasma levels once treated orally daily for 5 days in 28 day cycles. It was found that the plasma levels increased over the days and cycles so that if given 100 mg/m² the plasma levels would increase from 152 ng/mL, 247 ng/mL to 225 ng/mL for day 1 of cycle, day 4 of cycle 1 and day 1 of cycle two respectively. All doses given orally leant to at least 50 ng/mL (Weiiss et al. 2010). This for an in vitro experiment would correlate to concentrations between 0.6 µM and 3 µM. Therefore, all experiments conducted in this study will be around these limits. Final results of the clinical trials were supposed to be correlated in April 2015, but no further results have been published as of yet.

A study to establish the AP site blocking function of MX and its role in combined toxicity with temozolomide was conducted by Yan and co workers (Yan et al. 2007). In this study, oligonucleotides were designed with uracil incorporated into the strands and treated with uracil DNA-glycosylase. The AP sites generated were then treated with methoxyamine and APE was added to find out how many AP sites were further processed, leading to a single strand break (SSB). They found that treatment of TMZ and MX (12.5
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

mmol/L) together mildly reduced the number of AP sites formed, however not significantly. They also showed that the addition of methoxyamine to treatment with temozolomide had an effect in cytotoxicity for the colon cancer cell line; SW480, however, did not have an effect on either human or mouse bone marrow cells (Yan et al. 2007).

To understand how methoxyamine and temozolomide work together a study was performed where Montaldi and Sakamoto-Hojo (2013) found that out of a cell panel including U87, U251 and T98G, only T98G was sensitized by methoxyamine in the presence of temozolomide. The effect was so dramatic it reduced the IC50 from approximately 3000 µM to 450 µM. Interestingly, they found that the increase in cell death due to the combination treatment was not due to apoptosis (Montaldi & Sakamoto-Hojo 2013).

2.6.3 Introduction to polymerase β

Polymerase β (POLB) is an enzyme in the polymerase family and as such inserts nucleotides into DNA specifically in BER (Sobol et al. 1996). It is also the third enzyme in the short-patch base excision repair pathway (sp-BER). It is a protein made up of 335 residues and it is 39kDa in weight. Polβ is formed from two subunits, an 8kDa subunit and a 31 kDa subunit. In vitro APE, the APsite and POLB form a ternery structure until the DNA is cleaved by APE, when POLB can bind to the DNA (Bennett et al. 1997). POLB can exhibit two enzymatic activities, traditional polymerase synthesis of DNA and a 5'dRp Lyase activity. It has been shown that these activities are performed by the two different subunits (Matsumoto & Kim 1995). The smaller 8 kDa subunit has the deoxyribose phosphate (dRP) lyase activity. Whereas the larger subunit consists of 3 subdomains; thumb, palm and fingers. The thumb domain binds to the DNA and connects to the smaller subunit. The palm domain contains the active site for polymerase activity and the fingers bind and select nucleotides (Beard & Wilson 2006). In the base excision repair pathway this enzyme is the penultimate enzyme which allows the DNA to be fully complimentary and just needs to be ligated, sealing the nicks where the new nucleotide has been inserted.

Kinetic studies have shown that the lyase activity of PolB is far more rapid than the nucleotide insertion (Prasad et al. 2010). In a study looking at enzyme kinetics it was found that in 10 seconds 37% of 5'dRP lesions were removed, this was analysed using gel electrophoresis and radiolabeling. However, in 20 seconds only 25% of nucleotides were
inserted into specially designed oligomers. This shows that the 5’dRP lyase function is faster. When they were measured in the same reaction after pre-treatment with UDG, leaving a 3’OH and a 5’dRP they found that after 10 seconds 46% of the lyase activity had occurred and 26% of the nucleotide insertion (Prasad et al. 2010).

The polymerization mechanism of POLB consists of four basic steps (Beard & Wilson 2006). First, POLB binds to one base gapped DNA to form a polymerase-DNA binary complex. These binary complex next binds a nucleotide, forming an enzyme-DNA-dNTP ternary complex. Once dNTP is bound in the active site, there is a rapid conformational change wherein the fingers domain rotates through the hydrophobic hinge region to close around the nucleotide. This movement initiates the nucleotidyl transferase activity in the active site, adding the nucleotide to the DNA strand. Lastly, in a likely rate-limiting step, the DNA product extended by one nucleotide is released from the polymerase generating an apo-enzyme that can complete the cycle again (Kim & Wilson 2012).

2.6.3.1 Polymerase β in cancer

POLB variants are linked to increasing likelihood of certain cancers, with 30% of tumours containing a mutated POLB (Starcevic et al. 2004). Some colon and prostate cancers have been linked to a mutated POLB (Dalal et al. 2005). In work on a gastric carcinoma cell line it was found that POLB mutations can lead to genomic instability (Lang et al. 2007), which can then lead to colon cancer progression (Nemec et al. 2012). Hong and co-workers found that an overexpression of POLB was correlated with poor prognosis in oesophageal cancers (Zhao et al. 2005). These studies show the importance of PolB in cancer progression due to an error prone pathway de-regulation and how it could be important as a method of inhibition for increased treatment sensitivity.

2.6.4 XRCC1

X-ray cross complementing group 1 (XRCC1) is a scaffolding protein which is recruited during BER (Caldecott 2003). XRCC1 was originally thought to be activated by PARP, which in turn brings Polymerase β and DNA ligase I/IIIα to the site of repair (El-Khamisy 2003), however, there is evidence that XRCC1 binds glycosylases (Campalans et al. 2005) and POLB (Dianova et al. 2004). Cells and organisms missing or with mutant
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

XRCC1 are more sensitive to ionising radiation, ROS, and alkylating agents. These cells with missing functional XRCC1 have an increased propensity for chromosomal aberrations, deletions and in null mice embryonic lethality (Tebbs et al. 1999). XRCC1 has been shown to have many single nucleotide polymorphisms, many of which are being linked to a high frequency of cancers such as breast cancer (Duell et al. 2001).

XRCC1 has no enzymatic activity, but has binding domains specific for the proteins within BER. The first binding domains found were for Polymerase β, PARP and DNA ligase III (Caldecott et al. 1996). XRCC1 also can be phosphorylated by Chk2, near the nuclear localisation signal (Goode et al. 2002). Dianova et al., found that the XRCC1 binds polymerase β at a site near the N-terminal and binds PARP at the BRCT1 and DNA ligase at the BRCT2 (Dianova et al. 2004). Recently, studies have shown that XRCC1 may also bind to the glycosylases in BER, which can increase the catalytic activity, in the case of AAG by five-fold (Campalans et al. 2005). These studies make the role of XRCC1 one which needs to be considered during this project.

2.7 Research Question

Can repair proteins within GBM cell lines specific for TMZ and radiotherapy DNA repair pathways be quantified? Can this quantification be used to create an imbalance through the use of inhibitors to increase the effect of cell killing by TMZ and radiotherapy? And what are the optimum doses of radiotherapy to use?

2.7.1 Aims and objectives

- To measure the levels of DNA repair proteins by either PCR or western blot relative to each other in established GBM cell lines.
- To measure cell survival of GBM after standard treatment with temozolomide and irradiation, specifically correlating the involvement of BER.
- To compare the survival of cells after treatment with inhibitors for BER. Specifically; Methoxyamine (MX) inhibiting AP Endonuclease and Pamoic acid (PA) inhibiting polymerase beta.
- Measure dosimetry of the proton beam accelerator to give the correct dose of radiation to compare with X-rays.
Chapter 2: Glioblastoma and DNA repair

**Hypothesis**

An imbalance in base excision repair will sensitise glioblastoma cells to chemotherapy and radiotherapy. Proton therapy will produce a higher cell killing effect, however, the sensitisation from BER inhibitors will be greatest with X-ray therapy due to the SSB DNA damage created.
3 Methods

3.1 Introduction

This chapter discusses the experimental design and the protocols employed to explore the DNA repair protein expression profiles in glioblastoma cell lines. The DNA repair pathway named base excision repair (BER) is of particular interest as the main treatment strategies for glioblastoma are the chemotherapeutic drug temozolomide and radiotherapy, which causes DNA damage that is repaired by BER. To further elucidate the role of BER in the treatment of glioblastoma, drugs/inhibitors that unbalance this pathway and are either already in clinical trials or being tested at the pre-clinical stage, were tested in an attempt to exploit genetic differences in cancer tumours to improve outcome after chemotherapy.

These results were then used to develop a model specific to the linear quadratic model for cells being treated with temozolomide in conjunction with radiotherapy. The different equations produced may be explained by the repair misbalances within the tumour.

This chapter further goes on to give details in cell culture, drug treatment and end-point assays used and finally data analysis methods are described.
3.2 Methods

3.2.1 Cell culture

Five established human glioblastoma cell lines (T98G, LN18, A172, U251, U87) were obtained from ATCC and grown in culture medium consisting of Dulbecco’s Minimum Essential Media (DMEM) (Sigma Aldrich- high glucose (4500g/L)) supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin, 4 mM L-glutamine, (Lonza). Cells were incubated at 37°C and 5% CO₂, with media changes every day. Once cells were 80% confluent they were passaged by exposing cells to 0.25% trypsin in 0.05 mM EDTA (Sigma Aldrich) until detached and then neutralised in media. These cell lines were chosen for their previous characterisation to different LETs by Barazzuol (2012). As well as being a mix of both oligodendrocyte and astrocyte derivatives.

3.2.2 Western Blot

Western blots were used to measure the amount of protein being expressed directly in the cells and could be normalised to loading controls and therefore comparable to each of the cell lines. The proteins are separated by size through a gel and then the protein of interest is specifically detected and visualised by using antibodies.

3.2.2.1 Preparation of protein

Cells were frozen in a pellet and stored at -20°C. Cells were lysed with a 1 x glycosylase buffer made of; 20 mM Tris at pH 7.6, 100 mM KCl, 5 mM EDTA, 1 mM EGTA and 5 mM β-mercaptoethanol using a 21g needle and syringe. Lysates were frozen and thawed 3 consecutive times to enhance cell lysis. Cells were then exposed to sonication for 4 pulses, each lasting 30 seconds. The samples were then centrifuged at 14000 rpm at 4°C and the supernatant was kept at -20°C.

The amount of protein per sample was determined using the Bradford assay (Bio-Rad) following the standard 96 well protocol. The plate was read at 595 nm.

3.2.2.2 Detection of protein

Standardised Bio-Rad gels, (mini-protean precast wells, 10 wells at 30 µl/well, BioRad) were loaded with 20 µg of protein per well. The gels were electrophoresed for an hour at a voltage of 150 V with running buffer (Tris-base 3.0 g, Glycine 14.4 g, 10 mL of
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

10% SDS and ddH₂O to 1 L). Proteins were transferred from the gels onto transfer membranes (mini nitrocellulose and PVDF, BioRad) using a Trans-Blot Turbo Transfer System (BioRad) for 30 minutes. The membranes were incubated for 2 hours at room temperature in 1% TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20) with 0.1% non-fat dry milk powder. The membranes were washed 3 times with 1% TBST. The concentration of primary antibody is shown in the below table which were made up in 1% TBST with 0.1% milk powder and incubated overnight at 4°C. Membranes were washed before a 1:10000 dilution of goat anti-mouse infrared dye labelled 800CW and goat anti-rabbit infrared dye labelled 650 CW (Li-Cor) was added for 30 minutes and incubated at room temperature, membranes were then washed before being read on the LiCor Odyssey which used lasers to detect the infrared fluorescence.

<table>
<thead>
<tr>
<th>Primary Ab</th>
<th>Dilution</th>
<th>Control Ab</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAG Mouse (Santa Cruz biotechnology, INC. sc-101237)</td>
<td>1:250</td>
<td>Beta-Actin Mouse (Abcam, ab6276)</td>
<td>1:5000</td>
</tr>
<tr>
<td>APE1 Rabbit (Cell Signalling Technology, #4128)</td>
<td>1:1000</td>
<td>Beta-actin Rabbit (Sigma, A2066)</td>
<td>1:5000</td>
</tr>
<tr>
<td>MGMT Rabbit (Cell Signalling Technology, #27395)</td>
<td>1:500</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2 Antibodies used in western blots

3.2.3 Polymerase Chain Reaction

Quantitative polymerase chain reaction (qPCR) was used to measure the amount of mRNA being transcribed for a particular gene during protein synthesis. This should give us a comparison between the transcribed levels and the translated, full protein, levels.

3.2.3.1 RNA extraction and first strand synthesis

Cell pellets were lysed using 5 ml of TRizol (Invitrogen) and chloroform to produce phase separation and to extract the RNA following the manufacturers protocol.
Chapter 3: Methods

Total RNA was measured for purity and RNA content using a “NanoDrop”. The RNA extracted from the cells was converted to complementary DNA (cDNA) using first strand synthesis kit SuperScript II Reverse Transcriptase (Life Technologies) following the standard protocol recommended by the manufacturer.

The following protocol was added to a 0.5 mL Eppendorf tube:

<table>
<thead>
<tr>
<th>Components for Primer mix</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>n L</td>
</tr>
<tr>
<td>Primers (forward and reverse)</td>
<td>1 µL of each</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>1 µL</td>
</tr>
<tr>
<td>DEPC water</td>
<td>Upto 10 µL</td>
</tr>
</tbody>
</table>

*Table 3 SuperScript II reverse transcriptase reagent amounts - 1*

For the control 1 µL of DEPC water was added instead of RNA. This was then heated to 65°C for 5 minutes, then cooled at 4°C for at least a minute using a thermo cycler.

The “reaction” mix was made following the instructions of the product, as shown in the table below. 9 µL was added of the reaction mix to the samples containing primers.

This mixture was then heated to 42°C for 2 minutes before 1 µL of the reverse transcriptase was added and the mixture is then heated to 42°C for 50 minutes, 70°C for 15 minutes and then it is chilled at 4°C. At this point 1 µL of RNAse H can be added and incubated with the mixture for 20 minutes at 37°C. A further control was made which did not include any reverse transcriptase.

<table>
<thead>
<tr>
<th>Components for Reaction Mix</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X Reverse Transcriptase Buffer</td>
<td>2 µL</td>
</tr>
<tr>
<td>25 mM MgCl</td>
<td>4 µL</td>
</tr>
<tr>
<td>0.1 M Dithiothreitol (DTT)</td>
<td>2 µL</td>
</tr>
<tr>
<td>RNAse OUT</td>
<td>1 µL</td>
</tr>
</tbody>
</table>

*Table 4 SuperScript II reverse transcriptase reagent amounts - 2*

A primer is a single stranded area of DNA which binds to single stranded DNA or RNA. In this reaction it binds to a poly(A) tail on the mRNA at the 3’ end and creating a 3’OH. The mRNA strand can then be turned into a cDNA strand by Reverse Transcriptase
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

separating the mRNA from other RNA and binding to it adding matching nucleotides, dNTPs, to the mRNA strand. Not only is cDNA more stable but it is used to amplify the genes of interest in quantitative real time PCR (q-RT-PCR). The DEPC water is specially treated to ensure no contaminating chemicals are included and that there is no RNA included as this could lead to amplification of foreign genes and not sample genes.

3.2.3.2 Quantitative real-time PCR

The cDNA that was formed from the first strand synthesis was used to amplify the gene of interest. The reactions were carried out within a 96-well fast reaction plate, in quadruplicate for each cell line. In these plates the genes of interest were amplified as well as a control transcript, which ideally should be ubiquitously expressed within the cells, for future experiments should not change expression levels after treatment. This adds not only a control but also allows for normalisation in case there is more cDNA in one sample than another, as it is not measured after first strand synthesis. A list of the primers used is displayed in the table below.

Each reaction contained 1 µL of cDNA, 10 µM of each the forward and reverse primers, 10 µL of Platinum SYBR Green qPCR SuperMix-UDG w/ROX (Life Technologies) and water q.s.p. a 20 µL reaction. The qPCR reactions were run in the QuantStudio 7 Flex Real-Time PCR System (Life Technologies). The settings for temperature cycling conditions were as follows: 50°C for 2 minutes, 95°C for 2 minutes, followed by temperatures cycling 40 times of 95°C for 15 seconds and 60°C for 1 minute. To finalise the reaction temperatures of 95°C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds, which allowed melt-curve analysis.
### Table 5 Forward and reverse primers for mRNA detection of repair proteins

The temperatures for qPCR allow the DNA to be manipulated by the enzymes that amplify the DNA. The initial temperature is to heat activate the DNA polymerase and enzyme which binds and reads the DNA. The high temperatures also cause the hydrogen bonds between the two strands of DNA to become denatured creating single stranded DNA. The single stranded DNA can have the primers for the specific proteins bind during the lower temperatures of the cycle. The DNA polymerase can extend the DNA at 60°C creating an exponential increase in the amount of DNA after each cycle.

SYBR green dye is a detection method for analysing how much DNA has been amplified by the q-RT-PCR. SYBR green dye is incorporated in double stranded DNA,

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MGMT</td>
<td>5’-CCT GGC TGA ATG CCT TCC AC - 3’</td>
<td>5’-GCA GCT TCC ATA ACA CCT GTC TG- 3’</td>
</tr>
<tr>
<td>XRCC1</td>
<td>5’- CGG ATG ACA CGG ACA GTG A -3’</td>
<td>5’- GAA GGC TGT GAC GTA TCG GAT G -3’</td>
</tr>
<tr>
<td>AAG</td>
<td>5’- CCC CGC AAC CGA GGC ATG TT -3’</td>
<td>5’- AGC AAG ACG CAA GCC CCG TC-3’</td>
</tr>
<tr>
<td>AAG, at another area on mRNA strand</td>
<td>5’ – CCC ACC ACT CCG GGC CCA TA -3’</td>
<td>5’- GGC TCC AGT GCT CGC AGC AA -3’</td>
</tr>
<tr>
<td>APEX</td>
<td>5’- CTG CTC TTG GAA TGT GGA TGG G -3’</td>
<td>5’- TCC AGG CAG TCT CTG AAG TTC A -3’</td>
</tr>
<tr>
<td>POL β</td>
<td>5’-TGC AGA GTC CAG TGG TGA CAT G -3’</td>
<td>5’- ATG AAC CTT TTG TAA CTG CTC CAC -3’</td>
</tr>
<tr>
<td>LIG 3</td>
<td>5’- GCT ACT TCA GCC GCA GTC TCA C -3’</td>
<td>5’- GCA GTG GTT TGC CTG TCT TGT TG -3’</td>
</tr>
<tr>
<td>LIG 1</td>
<td>5’ –TCA CAG AGG CTG AAG TGG CAA C -3’</td>
<td>5’- TCA GGC TCT GAA ACG CTT TCC G -3’</td>
</tr>
<tr>
<td>PARP</td>
<td>5’- CCA AGC CAG TTC AGG ACC TCA T -3’</td>
<td>5’- GGA TCT GCC TTT TGC TCA GCT TC -3’</td>
</tr>
<tr>
<td>PARG</td>
<td>5’- AGT GGC TTT GAA CTC CCA TTG AG -3’</td>
<td>5’- ACT TCT CCT GCT CGC AAA AGA TC -3’</td>
</tr>
<tr>
<td>Beta Actin</td>
<td>5’ – ATT GCC GAC AGG ATG CAG AA – 3’</td>
<td>5’- GCT GAT CCA CAT CTG CTG GAA -3’</td>
</tr>
</tbody>
</table>
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

between the two strands. As each new strand of DNA is created, more SYBR green dye binds to the double stranded DNA. This increase in DNA allows an increase of bound fluorescent dye which gives off an increased signal, this is measured after every cycle, hence it is measured in real time.

3.2.4 Microarray data analysis

Microarray data was taken from the online database under GSE 23806 (Schulte et al. 2011). In their samples of conventional cell lines they used 4 of the 5 in this project. Cell lines were A172: GSM587161, LN18: GSM587185, T98G: GSM587179, U87: GSM587180.

GEO 2 R extracted the p-values from the control sample for expression of the genes of interest, which were found through the platform; GPL570. All p values were normalised against T98G to make comparable to western blots and qPCR results.

3.3 Irradiation Protocol

3.3.1 X-ray protocol

X-ray irradiations were performed on a Gulmay machine at 250 kVp with a dose rate of 0.65 Gy/min (Royal Surrey County Hospital, Guildford, UK or Christie Hospital, Manchester, UK). Cells were exposed at room temperature to doses between 0.5 to 5 Gy using the monitor units in the table below. Cells were grown in 6-well plates and incubated for 5 h before irradiation.

<table>
<thead>
<tr>
<th>Dose (Gray)</th>
<th>Monitor units</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>49</td>
</tr>
<tr>
<td>1</td>
<td>99</td>
</tr>
<tr>
<td>2</td>
<td>197</td>
</tr>
<tr>
<td>3</td>
<td>296</td>
</tr>
<tr>
<td>4</td>
<td>394</td>
</tr>
<tr>
<td>5</td>
<td>493</td>
</tr>
</tbody>
</table>

Table 6 X-ray dose delivery of Gy and the monitoring units needed to achieve them
3.3.2 **Vertical proton irradiation**

Irradiations of cells by proton beam using a broad beam were performed on the vertical nanobeam (Wolfson Tower, Ion Beam Centre, University of Surrey). This is a 2 MV tandem accelerator which can produce ions at a range of energies (Merchant et al. 2012).

For these experiments the accelerator is set up to produce protons of 3 MeV energy. Irradiations were performed at 0.5 to 5 Gy according to a specific procedure (Jeynes et al. 2013). The dosimetry was verified using a pin diode which counts particles during irradiation and the beam homogeneity is measured by a scintillator, it should show a bell curve for a good clean beam. This was further verified using Gafchromic film. 15 µL of the cell suspension was pipetted onto 4µm thick polypropylene disks that were suspended in place by specially designed holders, as shown in Figure 3-1. The cells were diluted before irradiation to a concentration of 1*10^6 cells/ml leaving the 15,000 cells per droplet.
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

Figure 3-1 Schematic of cell set out on the polypropylene disks. The disks are set on the XY stage and the beam comes from underneath the cells.

To ensure correct dose the pin diode was used to measure the number of particles per 30 seconds and this was measured before each irradiation. This was divided by 30 to calculate the number of particles that travel through in one second, this is known as the fluence. This was then used to ensure the correct dose is given to the cells using the below formula.

\[
\text{Equation 1 Dose rate;}
\]

\[
\text{Dose rate (Gy/s) = 1.6 * 10^{-9} \frac{L \Phi}{\rho}}
\]

The LET is calculated from the energy of the particle at the centre of the cell layer with the units KeV/µm. Fluence is the number of particles travelling through the beam and is measured in particles/cm²/s. \( \rho \) is the cell density, which is set to 1. For more details see (Jeynes et al. 2013).

3.3.3 Horizontal proton irradiation

Horizontal irradiations became a necessity within this PhD project and the method was developed and tested within results chapter 6. However, the cells were irradiated within 96 well plates. Cells were seeded at a density 3 x 10⁴ cells/well and incubated for 24 hours before irradiations took place.
Chapter 3: Methods

3.4 Chemotherapy treatment protocols

Temozolomide (TMZ) (Sigma Aldrich), was bought in as a powder and made up to a stock concentration of 50 mM in dimethyl-sulfoxide (DMSO). All further dilutions were made in media from the stock solution.

Inhibitors; methoxyamine (MX) and pamoic acid (PA) (Sigma Aldrich) were dissolved in DMSO to a stock concentration of 100 mM. Working dilutions were then done in cell culture media.

3.4.1 Clonogenic assay

Clonogenic assays were used to detect the toxicity to the cells from either treatment with TMZ alone or after a combination of X-rays/protons with TMZ. Cells were seeded into 6 well plates and left to adhere for 24 hours. Cells were then exposed to either TMZ, inhibitors, irradiation or a combination of treatments. 24 hours after treatment cells had the media replenished with fresh, drug free media. Cells were then incubated for 14 days at 37°C and 5% CO₂ atmosphere. After 14 days media was removed and cells were fixed with 70% ethanol before being stained with 5% Crystal violet in PBS to allow colonies to be counted. Wells containing more than 20 colonies were counted.

3.4.2 Viability assay

The toxicity of inhibitors without further treatment was evaluated using viability assays in growing cells over time. Live cells were quantified in 96 well plates at different time points over a 72 hour period post-treatment. A non-toxic, non-fluorescent compound, Resazurin which is a blue dye which can be reduced within the cells to form a highly fluorescent red compound resorufin. Cells were plated and are incubated at 37°C and 5% CO₂ atmosphere for 24 hours. Cells were then incubated with inhibitors until 2 hours before they were due to be read where a 1:11 ratio of Alamar blue to media was incubated on the plate cells. After 2 hours the plate was read as according to manufacturer’s instructions, 560 nm. Fresh media was replaced on cells until 2 hours before the next time point.

3.4.3 Cell growth assay

To characterize cell growth, proliferation and doubling times in the presence of inhibitors and TMZ growth curves were assessed on two cell lines (U251 and U87). Cells
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

were seeded at a density of 2*10^4 cells/well and incubated for 24 hours in a 24 well plate with 1 mL of media or treatment (50 μM of PA or MX). Wells were trypsinised and cells counted at 24 hours' time points over 5 days. Each experiment was performed in triplicate.

3.4.4 Flow cytometry

To determine if the genotoxic treatment affected cell cycle progression flow cytometry was used. Cells were seeded at a density of 5*10^4 cells/well and incubated for 24 hours. Cells were then treated with the appropriate treatment protocol and harvested 72 hours later. Cells were fixed in 70% ethanol for 24 hours and then treated with RNase (100μg/mL). Cells were stained with propidium iodide (PI) in PBS (50 μg/mL). Cells were analysed using a BD canto II for DNA content, as described.

3.4.5 Activity assay

The following methods were provided by Eleanor Healing and Dr. Ruan Eliot who produced the procedure and completed the experiments

3.4.6 Assay to measure AAG activity

For all experiments, the initial oligonucleotide (HX02 or URA03; sequence information provided in Table 7) was diluted from 10 μM stock solutions into freshly prepared 0.1M bicarbonate buffer (pH 9.6) to a final concentration of 0.5 nM and 100μl of this solution was incubated in each well of a Nunc® Immobiliser™ amino 96-well plate overnight at 4°C. The plate was then washed with phosphate-buffered saline containing 0.1% v/v Tween-20 (PBST) and 100μl of a 0.5nM solution of the appropriate complementary oligonucleotide (Loop01T for Hx02, Loop01G for URA03; sequence information provided in Table 7) was added following dilution in hybridisation buffer (6x saline sodium citrate (SSC) buffer, 5 mM EDTA, 0.1% v/v Tween-20). Each plate was then sealed and heated to 95°C for ten minutes in a hybridisation oven, followed by gradual cooling to 80°C, maintenance at 80°C for ten minutes and then further gradual cooling to 21°C.
### Table 7 Sequences of oligonucleotides used in experiments.

1Flc indicates fluorescein; 2NH$_2$ indicates amino group modification; 3P indicates phosphorylation; 4lower case letters in the nucleotide sequence indicate nucleotides linked via phosphorothiate bonds which also serve as a two-nucleotide spacer between the plate and the start of the double stranded nucleotide complex.

To measure alkyl adenine DNA glycosylase (AAG) activity, plates containing HX02 annealed to Loop01T were treated with 0.05U T4 DNA ligase in 100 µl of T4 DNA ligase buffer (30 mM Tris-HCl pH 7.8, 30 mM NaCl, 10 mM MgCl$_2$, 10 mM DTT, 1 mM ATP) for one hour at 37°C. The plates were then heated to 65°C for a further 15 minutes and the hot liquid immediately decanted from the wells. Fresh DNA ligase buffer (100 µl) was added to each well and the plate heated to 65°C for a further 15 minutes, the liquid immediately decanted and the wells washed three times with PBST. This process produces double-stranded substrate containing a hairpin loop and one site of damage (Figure 2-1).

These substrates were then incubated with increasing concentrations of recombinant enzyme (hAAG1, from New England Biolabs) or cell extract (at a concentration of 10 µg/well) in an AAG reaction buffer (20 mM Tris-HCl pH 7.5, 100 mM KCl, 5 mM EDTA, 1mM EGTA, 5 mM β-mercaptoethanol, 2 µg/ml Herring Sperm DNA) for two hours at 37°C. Following the repair incubation, the contents of the wells were decanted and the wells washed three times with PBST. A volume of 150 µl of alkaline buffer (0.1x SSC, 0.1M NaOH) was added to the wells and the contents of the plate heated.
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

to 95°C for 10 minutes. The liquid was then decanted from the wells and the alkaline denaturation step repeated once more prior to three PBST washes.

To measure UNG activity, plates containing URA03 annealed to Loop01G were ligated and prepared in the same manner as the substrate for the AAG assay, to produce a hairpin loop structure containing a single uracil lesion in a U:G base pair within the double stranded region. The substrate was then incubated for one hour at 37°C with varying concentrations of recombinant UDG or 2.5μg/well of nuclear extract sample, both in DNA repair buffer (Tris-HCl pH 7.8, 100 mM NaCl, 0.4 mM EDTA, 3.4% v/v glycerol, 0.4 mM DTT, 2 mM MgCl₂, 1 μg/ml herring sperm DNA). Following the repair incubation, the wells were then subject to alkaline denaturation as in the AAG assay.

After the final denaturation step of each assay, fluorescein retained in the wells of the plates was quantified by colorimetric detection. Each well was incubated with anti-fluorescein antibody conjugated with horseradish peroxidase (HRP) (Abcam ab6656, Cambridge, UK) diluted 10,000-fold in PBST/1% w/v bovine serum albumin for one hour at 21°C. The liquid was decanted from the wells and the wells washed four times with PBST. TMB Microwell Peroxidase Substrate for HRP (KPL, Wembley, UK) was then added (100 μl/well) and incubated at 21°C for ten minutes. Reactions were stopped by addition 100μl 1M phosphoric acid to each well. Absorbance was read at 450nm using an Omega fluostar microplate reader (BMG labtech, Aylesbury, UK).
Chapter 3: Methods

3.5 Statistics

Assays were conducted in at least triplicate and statistics were performed on experiments with a minimum of 3 repeats on separate occasions. Error bars represent the standard error of the mean, unless stated otherwise. The linear quadratic model or the repairable-conditional repair model was used to analyse clonogenic data involving radiation. A 2-way ANOVA with Tukey post hoc test will be used to analyse the TMZ alone clonogenic data. 1-way ANOVA were used for western blots, qPCR, cell proliferation and growth assays.

3.5.1 Combination indices

The CI was calculated using the formula: where CA,X and CB,X are the concentration of drug A and B used together in combination to achieve cell death in this
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

case to a certain surviving fraction ICX,A and ICX,B are the concentrations of the single drugs to achieve the same effect, and surviving fraction.

Equation 2 combination indices

\[ CI = \frac{C_{AX}}{IC_{XA}} + \frac{C_{BX}}{IC_{XB}} \]

This equation was originally set out by Chou and Talalay to work out the interactions of chemicals in overall in biological settings. A CI of less than, equal to, or more than 1 indicates synergic, additive or antagonistic effect, respectively.
4 THE CHARACTERISATION OF GLIOBLASTOMA CELL LINES

4.1 Introduction

All cells constitutively express DNA repair proteins to deal with exogenous assaults on their DNA from chemical and physical agents such as pollution or tobacco smoke. Specifically, these DNA repair proteins function to repair damaged DNA which lowers genomic changes, chromosomal aberrations and prevents mutations. While some repair proteins can act alone, most repair proteins act concertedly in defined cellular pathways. However, there will be variation in the expression levels of DNA repair proteins amongst individuals’ due to genetic differences or epigenetic regulation. These differences in expression levels or mutations in DNA repair proteins can lead to cancer susceptibility and have further implications to cancer treatment. For example mutations in the BRCA1 or BRCA2 genes can lead to an increased likelihood of developing breast and ovarian cancer (Gudmundsdottir & Ashworth 2006). As mentioned previously in chapter 2.4 the expression levels of the repair protein O6-methylguanine DNA methyltransferase (MGMT) in glioblastoma particularly can lead to a resistance to the common chemotherapeutic modality temozolomide (TMZ) (Hermisson et al. 2006; Silber et al. 2012; Brandes et al. 2008). The expression changes in MGMT levels for glioblastoma are now being proposed as a biomarker for treatment modalities, so only MGMT negative patients would receive TMZ (Wick & Platten 2014). This is due to MGMT being able to remove the most toxic lesion caused by TMZ, O6-methylguanine, in a single enzyme pathway which stops apoptosis being initialised due to the cells inability to efficiently repair the lesion (Stupp et al. 2001), more detail of this is in chapter 2.4.3.
DNA repair protein expression levels can differ within the same individual, same tissue and for healthy and cancerous cells. In colon cancer, an increase in the base excision repair (BER) proteins 8-oxoguanine DNA glycosylase (OGG1) and poly(ADP-ribose)-polymerase 1 (PARP1) was seen in comparison to the healthy tissue (Dziaman et al. 2014). In glioblastoma, Liu and co-workers found an increase in the expression levels of alkyladenine DNA glycosylase (AAG) in correlation with severity and the grading of tumour that was different from the healthy tissue surrounding it (Liu et al. 2012).

The first part of this results chapter presents the results of measurements of gene expression levels of repair proteins using quantitative polymerase chain reaction (qPCR). Genes belonging to the base excision repair pathway were measured in particular as well as genes involved in the PAR signal cascade, PARylation and double strand break signalling. These measurements of basal level repair gene expression were then compared to the cell lines’ ability to repair DNA damage induced by temozolomide and radiation, the standard for glioblastoma treatment.

The qPCR results were statistically analysed using a one-way ANOVA after experiments were repeated at least three times. The effects of TMZ on cell survival were also analysed using two-way ANOVA after experiments were done in triplicate. For experiments involving radiation a three-way ANOVA was employed after 3 repeats in triplicate as well as fitting to either the linear quadratic (LQ) model.

4.2 Aims and Hypothesis

In this chapter the overall hypothesis was tested; that an imbalance in the BER capacity affects glioblastoma cells repair capability after treatment of temozolomide and irradiation. To test this hypothesis this chapter aims to reliably measure the expression levels of the proteins of the BER pathway basally expressed in these cell lines and robustly measure their survival to treatment.

To test this the following objectives were set:

- Discover the expression levels of repair proteins within 5 glioblastoma cell lines.
Chapter 4: The characterisation of glioblastoma cell lines

- Confirm these expression levels are consistent to the protein level and the activity level.
- Compare the survival of these cells after traditional treatment.

4.3 Results

4.3.1 AAG levels vary in different glioblastoma cell lines

Gene expression levels for different DNA repair proteins were measured in five glioblastoma cell lines to compare their background repair gene expression levels and evaluate if any deficiency in gene expression could potentially explain differential response in repair ability or response to potential treatment regimens.

The first repair pathway of interest evaluated was the BER pathway. The BER pathway can be seen in Figure 4-1 and is described in detail in chapter 2.4.3.1. The BER pathway is initiated by a DNA glycosylase which removes the damaged base by cleaving the glycosylic bond linking the base to the DNA sugar phosphate backbone, resulting in the formation of an abasic site. The glycosylase can either be monofunctional, performing this role only, or bi-functional, additionally cleaving the DNA sugar-phosphate backbone at the 3’OH position, resulting in the generation of a nick in the DNA strand (Wallace et al. 2013).

When monofunctional glycosylases initiate BER, the cleavage of the sugar phosphate backbone post damaged-base removal is done by an AP endonuclease (APE) enzyme (Chohan et al. 2015). This cleavage leaves a 5’dRP in the backbone of the DNA and a single strand break. Polymerase β (PolB) than removes the 5’dRP and the 5’OH and inserts the replacement base. The DNA is then fully ligated by DNA ligase III (Lig III/I) or I creating nick free correct DNA (Srivastava et al. 1998). This pathway is of particular interest as it affects the cells response to the majority of the lesions caused by TMZ on the DNA. Over 70% of the lesions in DNA caused by TMZ are repaired by the BER pathway (Zhang et al. 2012).
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

Figure 4-1 Damage repair pathways initialised by damage from TMZ. The enzyme cascade is initialised by AAG and each enzyme is held in close proximity to the damage by XRCC1, scaffolding protein. PARP signals a SSB has been created. O\(^6\)MeG adducts are repaired by MGMT if the protein is present.

In the treatment of glioblastoma, TMZ treatment is the first line treatment (Beauchesne et al. 2010), and a number of repair pathways are important in whether the patient's tumour will respond to the treatment (Happold et al. 2012). Some of these important proteins in the repair of damage caused by TMZ treatment are shown in Figure 4-1 these were measured for their mRNA expression levels in the 5 cell lines used within this study. As illustrated by Figure 4-2, the repair proteins involved in BER show a varied expression profile and it is clearly visible that the first protein within the BER pathway that deals with TMZ lesions, AAG, has highly variable mRNA expression in different cell
lines. The expression levels within T98G were used as a reference point in all measurements, and all measurements given are in relation to the expression level of 1 seen in T98G.

Our first finding using qPCR was of very high levels of variation within AAG expression were observed in cell lines, Figure 4-2 a. This was specifically found as high levels of AAG in LN18, ~12.85 relative quantification (RQ), and U87, ~15.83 RQ, when compared to the other cell lines, which displayed similar expression levels to the reference, T98G. The expression levels of AAG in these two cell lines, LN18 and U87, were statistically higher than the other cell lines (p <0.0001).

From this we further measured the rest of the BER proteins and found that there was variation in expression levels, however, it was lower than those seen in AAG levels. However, the cell line LN18 did display higher levels for all the BER proteins compared to the other cell lines. The expression levels for APE were highest in LN18, ~1.99 RQ, which was statistically higher than all other cell lines (p< 0.0001), seen in Figure 4-2 b. U87 and U251 both showed lower levels of APE mRNA, 0.5 and 0.526 RQ, respectively. As seen in Figure 4-2 c the expression level of U251 and U87 was statistically lower than the reference cell lines T98G (p < 0.0001). The expression levels for PolB were similar for all cell lines, with T98G and LN18 expressing 1 and ~1.2 RQ respectively, whilst the other cell lines were all below 1 for relative quantification. The difference in expression levels is largest between LN18 and U251, p > 0.0001, however, the differences seen between LN18 and U87 were still significantly different p > 0.0002. The expression levels of mRNA for Lig3 (Figure 4-2d) shows similar levels of expression between all cell lines except for LN18. The expression level of LN18 is ~2.16 RQ and statistically different from the expression seen in all the other cell lines with a p value of <0.0001. The variation between gene expression levels may seem large, however, the difference is most notable for the AAG gene, whilst the expression of the remaining BER genes is fairly comparable between cell lines.
Figure 4-2 BER repair mRNA expression levels. 

a) AAG mRNA expression levels. b) APE mRNA expression levels. c) PolB mRNA expression levels. d) Lig3 mRNA expression levels. All experiments were conducted on 3 separate mRNA extractions in at least triplicate on a minimum of n=3. T98G was always used as a reference sample and so remains at 1 relative expression, statistical significance was established by one way ANOVA.

As large variations were found in BER proteins, specifically in AAG, we went on to measure other repair proteins. As shown in figure Figure 4-1 another important protein in the repair of DNA after treatment with TMZ is MGMT. MGMT is a repair pathway in its own right, MGMT reverses the O6-methylguanine adduct by removing the methyl group from...
Chapter 4: The characterisation of glioblastoma cell lines

the damaged base. The qPCR results show that LN18 and T98G both express MGMT, which was expected, but U251 had low levels of mRNA detected. Low levels of MGMT expression was surprising as the regulation of the production of this protein is through methylation of the promoter (Costello et al. 1994) and the protein is either expressed or not.

Further proteins which are complementary but not directly involved in the repair of the DNA by BER include PARP, poly(ADP-ribose) glycohydrolase (PARG) and X-ray repair cross-complementing protein 1 (XRCC1) therefore the expression of these genes were also measured. PARP is important for single strand break (SSB) signalling whilst PARG catalyses the hydrolysis of the PARylation formed by PARP aiding in DNA damage repair. The expression levels of XRCC1, PARP and PARG are all similarly expressed across all 5 cell lines, in comparison to the BER proteins, as can be seen in Figure 4-3.

We found that the expression levels for XRCC1, Figure 4-3 b, are highest in LN18 and U87, ~1.59 RQ and ~2.0 RQ respectively in comparison to T98G. Which is significantly different from the other cell lines expression levels, with the most determinedly different expression levels between U87 and U251, p <0.0001, LN18 and U251, p = 0.0007, and U87 and A172, p = 0.0002. PARP and XRCC1 have tightly related roles due to PARP being closely associated to the stability of XRCC1 at the site of damage (El-Khamisy et al. 2003). However, XRCC1 recruitment to the site of damage and recruitment of proteins at BER specific damage, is independent of PARP1 (Campalans et al. 2013), however, PARP2 maybe able to take over this role. Whether this linked role of PARP and XRCC1 effects expression over healthy cell lines is unknown, but we found that only LN18 expressed high levels of PARP, ~1.7 RQ compared to a relative quantification of 1 to 0.5 and below for the other cell lines, Figure 4-3 c, however, how much protein is present is unknown. The largest difference is expression levels are between LN18 and both U251 and U87 (p<0.0001). PARG is involved in degrading the PARylation caused by PARP, signalling the SSB (Oka et al. 2006) and if this PARylation is not hydrolysed it can lead to a sensitivity in cytotoxicity from toxic agents (Koh et al. 2004), such as TMZ. There are very similar levels of PARG in all cell lines, but the highest levels of expression are seen in LN18 (~1.32 RQ) and U87 (1.52 RQ) Figure 4-3 d. Ligase III, which is a BER protein that predominantly ligates DNA damage occurring in the mitochondria (Simsek, Furda, et al. 2011), was found to be higher in the cell line LN18 (~1.5RQ) and U87 (~1.33RQ) as seen in Figure 4-3 e, but not to the same extent as the AAG protein overexpression in that cell line.
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

Although there are significant differences between cell lines in the expression levels for all these genes, the most extreme difference was in the expression levels of the AAG gene, where the cell lines LN18 and U87 had high AAG gene expression levels. However, the expression levels of the other genes in U87 were similar to the expression levels seen for the other cell lines. Due to the closely linked roles of BER with XRCC1, PARP, and PARG which, when repair is orchestrated to allow the enzymes to be in close proximity, lowering the time that toxic intermediates are left unrepaired less cell death should occur, however, such high levels of AAG in U87 could make this cell line more susceptible to chemotherapeutic agents than the others.
Chapter 4: The characterisation of glioblastoma cell lines

Figure 4-3 Repair protein mRNA expression levels. All experiments were conducted on 3 separate mRNA extractions in at least triplicate on a minimum of n=3. T98G was always used as a reference sample and so remains at 1 relative expression, statistical significance was measured by one way ANOVA.
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

QPCR results rely on the expression levels of mRNA within a cell, however, mRNA levels do not always correspond to the protein levels. In 2004 the human genome project was finalised and mapped the 2.85 billion base pairs within the DNA (Lander et al. 2001). There are ~200 cell types in the human body which, due to different methylation status of the histones and DNA, create different proteins to be translated and expressed and allow the cells to have different functions. There are approximately 20,500 proteins coded for within the human genome (Clamp et al. 2007) and these can be further modulated as a result of alternative splice variants and post-translational modifications which affect the function of the proteins. These effects can lead to different protein expression levels.

Furthermore, proteins are also regularly degraded within the cells through other mechanisms some of which are related to stress on the cells.

Because of the effects noted above, there have been a number of studies to look into the differences between mRNA expression levels and protein expression levels. One such study performed by Lundberg et al. in 2010 took 3 cancer cell lines; U251, A431 (squamous cell carcinoma) and U-2 OS (osteosarcoma) and compared the mRNA levels to the protein levels. They employed the use of RNA seq and found 11,575 genes, which were commonly expressed in all cell lines. To compare proteins levels a triple-SILAC method, stable isotope labelling by amino acids in cell culture, was employed in which mass spectrometry is utilised with triple peak detection locating proteins. This study found that only 5333 proteins were expressed across all cell lines, with 65% of these having similar expression levels to those found in the RNA seq with a difference of 2 fold expression change accepted by the authors (Lundberg et al. 2010).

As can be seen in Figure 4-4, 3 protein levels were measured by western blotting; namely, AAG, APE and MGMT, with normalisation to beta-actin levels, once normalised to beta actin levels the protein levels, were compared against T98G as 1 in a ratio of expression to facilitate comparison to the results obtained by qPCR. AAG showed the biggest differences in mRNA expression levels with all other expression levels or repair proteins being similar to those found in the qPCR for APE. MGMT is classically thought of as being either expressed or not, due to the epigenetic regulation of the protein (Costello et al. 1994). However, the qPCR results were not entirely consistent with this finding with U251 showing a low level of mRNA transcripts as being present for MGMT. The differences
in repair protein mRNA expression levels for AAG show that there is a many fold level of diversity between the 5 cell lines. However, this large fold change in expression levels of AAG when looking at the protein level show that LN18 has the highest expression of AAG, with a 4.8 fold increase from T98G levels. U87 was also found to have higher levels of AAG than the remaining 3 cell lines, however, this was not as high as when measured by qPCR and looking at the mRNA. LN18 and U87 always exhibited higher expression levels of AAG than the other cell lines whilst the APE expression levels showed that U87 had high levels of APE, which were significantly larger than the expression levels seen in LN18 and T98G. However, these protein results for the cell line U87, which displayed the highest expression levels for the APE protein, do not correlate with the expression levels of mRNA seen by qPCR. In the cell line U87, high APE levels found by western blot correlate well with a study by Naidu et al, 2010 which found that U87 cells had a higher expression than U251, which has been replicated here (Naidu et al. 2010).

Figure 4-4 Western blot analysis of proteins important to TMZ. Show representative western blots of protein expression for a) AAG, b) APE and c) MGMT respectively. D, e and f show AAG, APE and MGMT protein expressions normalised over a minimum of 3 experimental replicates.

A study by Liu et al, which investigated AAG expression levels in patients with different WHO graded gliomas, found a positive correlation between the AAG expression
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

level and the grading of tumours with higher graded tumours responsible for lower patient survival rates. As all of the cell lines in this research are derived from grade IV glioblastoma tumours, this study by Liu would suggest that all the cell lines express high levels of AAG in comparison to a grade I glioma (Liu et al. 2012). As Liu et al (2012) found high AAG levels correlated to a poor response to therapy it is reasonable to expect that the cell lines LN18 and U87 would have a lower survival after TMZ treatment than other cell lines, in an MGMT dependent fashion (Liu et al. 2012). This resistance to chemotherapeutics due to high AAG levels is supported by Agnihotri, who correlated an overexpression of AAG with resistance to TMZ (Agnihotri et al. 2012). However, this does not take in to consideration the whole pathway and the important balance that the other BER proteins play in successfully repairing the DNA base damage. As well as further differences between cell lines and whole human patients, which have more factors that will affect survival and tumour growth than just a single cell line.

A study (Schulte et al. 2011) was conducted comparing the expression profiles of stem-like glioma cells with traditional glioblastoma cell lines to look for differences in gene expression and compare expression with those in in vivo tumours. From this transcriptomic profile the authors wanted to find novel treatments which were important in real tumour situations for commonly expressed genes in both cell lines, stem-like glioma cell lines and tumours. To measure and compare the gene expression the authors employed microarray techniques to measure gene expression in a large set of glioblastoma cell lines, stem-like glioma cell lines and tumour tissue. This microarray data was then loaded into the GEO database for future researchers to use: this dataset is identified as GSE 23806. In the conventional cell line samples, they used 4 of the 5 that have been worked with in this study. These lines are A172, LN18, T98G and U87.

From this GSE set the expression data was extracted for the genes of interest. Taking the raw expression of the gene amplified on the microarray chip as provided by GEO 2 R expression profiles were normalized to T98G to have an expression of 1, Figure 4-5, so as to make all results comparable to the western blots and qPCR.

Controversially from this data set AAG levels are shown to have very little variation, unlike what was shown in qPCR and Western blot. There is a little variation in the rest of the BER proteins, however, it is lower than seen in our qPCR results. As can be
seen in Figure 4-5 a-d LN18 in this microarray had the highest level of all BER proteins, as seen in our qPCR results, except for the LIG3 results. The greatest difference in expression is seen in MGMT levels, Figure 4-5 e, which was expected, as non-expressing MGMT cell lines value were between 0.42 – 0.46 (U87 and A172 respectively), in comparison to MGMT expressing values of 1.3 – 1 (LN18 and T98G respectively).

Although it was interesting to compare the results found through qPCR and Western blot to microarray data available online, there are issues with trusting results from data available online. Within our study and this one the cell lines were cultured in the same conditions for both this current study and theirs, however, the authors did not provide information on where these cells were obtained from or if they were verified. Ours were all obtained from ATCC in August 2015. We take the values that we obtained as more important than those found in this microarray data, as these cells were specifically used for further
Figure 4-5 Microarray analysis from GSE23806 using platform GPL570. Cell lines were A172: GSM587161, LN18: GSM587185, T98G: GSM587179, U87: GSM587180. All p values were normalised against T98G to make comparable to western blots and qPCR results.

There are other proteins which are important to the survival of cells from radiotherapy treatment as well. In actual tumours are heterogenesis, and some studies predict to be filled with glioblastoma cancer stem cells. These studies have shown that the
predated glioblastoma stem like cells, marked by CD133 and nestin, are more radioresistant than the other cell lines (Ahmed et al., 2015 and Carruthers et al., 2015). And that this difference in radioresistance is linked to a lack of G2/M arrest as the cancer stem like cells have an increase in phosphorylation of ATM and Chk1 which this pathway affects mitosis through Cdc25c and cell cycle overall (Lui et al., 2018). One of these studies have found that the inhibition of CHK1, which is upregulated in these cell types, radiosensitizes glioblastoma cells, but not to the same extent in glioblastoma stem like cells (Ahmed et al., 2015). Other studies have shown that ATM kinase sensitises cancer stem cells to radiotherapy (Carruthers et al., 2015) and others have shown this is through a link with interleukin-6, preventing cell proliferation (Lim et al., 2018). Although the gene regulation for radioresistance is multifactorial, but is controlled or linked through the DNA repair and cell cycle systems.

4.3.2 AAG activity assay

We next wanted to understand how the mRNA expression level and the protein expression levels effects the activity levels of AAG within the cells. To do this collaborators, Eleanor Healing and Dr. Ruan Eliott, have devised an activity assay which works by measuring the release of fluorochromes attached to synthesized DNA containing hypoxanthine adducts which AAG can remove. As the hypoxanthine is removed the fluorescence decreases, showing an increase in activity. As expected from our results in qPCR and western blot, we found higher levels of activity within LN18 than in U251, seen in Figure 4-6 a. However, Figure 4-6 b shows the activity levels for A172 and T98G were measured on a different day, using a different standard curve and so cannot be directly compared with the results from LN18 and U251, but we can clearly see that the activity levels are very similar in these two cell lines (Figure 4-6).
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

Figure 4-6 AAG activity in cell lines; a) LN18 and U251 and b) A172 and T98G.
The nuclear fraction of the cell was extracted, purified and quantified. Normalised levels of protein were seeded into a 96 well plate containing fluorescent DNA containing AAG specific substrates. One biological repeat was used and the error is standard deviation.

4.3.3 High AAG levels don't necessarily lead to an imbalance in BER

The first thing we did to test whether the enzyme expression levels of BER actually led to an imbalance was use an alkylating agent which only causes damage repairable by BER. To eliminate the effects of MGMT on survival of cells after treatment with TMZ and to elucidate the role of AAG on cell survival after treatment with an alkylating agent, 3 cell lines were chosen to be treated with methyl methanesulfonate (MMS). MMS is an SN2 alkylator and therefore only adds methyl groups to nitrogen, which results in only the BER pathway being activated to repair this alkylation damage (Wyatt & Pittman 2006).

The three cell lines chosen were LN18, T98G and U87. T98G has the average expression of all the BER enzymes compared to the other cell lines as well as being used as the reference sample for the qPCR measurements. LN18 has a high expression of AAG and a higher expression of the other BER enzymes compared to the other cell lines. U87 has high levels of AAG, however, it has an average expression of other repair proteins. As the BER pathway is a multistep pathway which relies on each enzyme to be present and active, in turn, to repair the damage to the DNA caused by alkylating agents, higher
expression levels of singular enzymes in this pathway has been postulated to increase sensitivity to alkylating agents (Fu & Samson 2012). This is because each end-point product from the enzymes repairing the DNA in the BER pathway is toxic to the cell except the final ligation (Wyatt & Pittman 2006; Fu & Samson 2012). The potential for imbalance in BER for the cell line U87 due to the high levels of AAG and lower levels of other repair proteins, specifically PolB and PARP could lead to sensitization to MMS. When these cell lines are measured for BER sensitivity through treatment with MMS (Figure 4-7) it can be seen that the potential imbalance in U87 creates a sensitivity to MMS treatment. U87 is more sensitive to MMS, which is noticeable at 1.5 mM. However, this sensitivity is not statistically significant in comparison to T98G and LN18, even at 2 or 2.5 mM. T98G and LN18 have very similar sensitivities to treatment with MMS, and as mentioned are more resistant to MMS. This could be due to both cell lines having balanced BER enzyme level pathway. Even though LN18 displays high levels of AAG, it also has higher levels of all the other enzymes within the pathway too.

![Image](image.png)

**Figure 4-7 Survival curves after treatment with a range of mM doses of MMS for T98g, U87 and LN18. A minimum of 3 repeats in triplicate were conducted with two-way ANOVA statistics applied.**

### 4.3.4 Survival of glioblastoma cell lines after treatment is dependent on the expression of MGMT

#### 4.3.4.1 Survival after TMZ

We next wanted to understand how this BER imbalance affected glioblastoma cell lines response to standard treatment. As shown in Figure 4-8 the MGMT positive cell lines, namely LN18 and T98G, were more resistant to TMZ treatment compared to the MGMT negative cell lines, A172, U251 and U87, even at a low dose of temozolomide (100 μM). The predominant factor, within these cell lines, causing resistance to TMZ is MGMT. At low doses of TMZ the cell lines LN18 and T98G show some differences in sensitivity from each
other as LN18 appears mildly more sensitive to TMZ when examined up to a TMZ dose of 100 μM. However, at higher doses T98G is the most resistant to TMZ treatment of all the cell lines tested. As shown in the table below, these differences in dose sensitivities for LN18 and T98G lead to EC50, which is the effective concentration which kills 50% of the cells, to be 273 μM of TMZ for LN18 and 229.2 μM for T98G (Table 8).

Despite a similar EC50 for these two MGMT positive cell lines, if we examine the survival curves we note survival is similar up to a TMZ dose of 500 μM. From then on, there is a statistically significant difference in survival (p=0.035) and T98G is more sensitive to TMZ than LN18, up to a TMZ dose of 1000 μM. The high levels of AAG in LN18 could explain why it is more resistant to TMZ, as would be justifiable by Agnihotri and Liu’s findings however, the doses at which the cells become sensitive to TMZ are far higher than those used within a clinical setting and high or low AAG expression would not affect treatment outcome in MGMT positive patients.

When comparing the results of the MGMT negative cell lines, A172, U251 and U87, the cell line A172 was the most sensitive cell line, with only 10% of the originally plated cells forming colonies at 100 μM of TMZ. When looking at Table 8 it is clear that A172 also had the lowest EC50 (24.62 μM) however, we notice that the curves are similar until 25μM in TMZ sensitivity. Whereas, the EC50 for both U251 and U87 were similar; 48.19 and 45.18 μM of TMZ respectively. However, towards the high doses of the curves, 100 μM of TMZ, U251 is significantly more resistant to TMZ than A172 and U87 (p=0.047). At 75 μM of TMZ U251 is more resistant to TMZ than A172 (p=0.0051) and U87 (p=0.042). All of these cells would be sensitive to TMZ in a clinical setting. The BER pathway protein expression levels seemingly have no effect on the response of these cells to TMZ.
Figure 4-8 GBM cell lines treated with a range of TMZ. A shows all five cell lines treated to 100 μM, no statistics were performed on this treatment. B shows MGMT negative cell lines. C shows MGMT positive cell lines treated up to 1000μM. A minimum of n=3 on triplicate data, a two-way ANOVA was used and the curves fitted with the hill model.

Using survival curves shown above the EC50 values were then calculated for all cell lines in the presence of TMZ:
### Table 8 The difference in EC50 after treatment with TMZ in glioblastoma cell lines.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>EC50 of TMZ (μM)</th>
<th>Std. Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>A172</td>
<td>24.62</td>
<td>+/- 1.53</td>
</tr>
<tr>
<td>LN18</td>
<td>273</td>
<td>+/- 5.07</td>
</tr>
<tr>
<td>T98G</td>
<td>229.2</td>
<td>+/- 9.32</td>
</tr>
<tr>
<td>U251</td>
<td>48.19</td>
<td>+/- 3.99</td>
</tr>
<tr>
<td>U87</td>
<td>45.18</td>
<td>+/- 4.02</td>
</tr>
</tbody>
</table>

### 4.3.4.2 Survival after treatment with radiotherapy

Cells were characterised for their response to escalating radiation doses to determine a survival response to radiation. This allowed further characterisation of the cell lines in terms of their ability to repair and survive in light of the knowledge of the repair status of the cells. The surviving fractions were calculated and the data fitted to a model describing the curve. The cell lines survival were fitted using the LQ model Equation 3 with the alpha and beta ratios calculated.

**Equation 3 Linear Quadratic (LQ):**

$$SF = e^{-(\alpha D + \beta D^2)}$$

- \(D\) = dose
- \(\alpha\) = low dose surviving fractions (linear area of graph)
- \(\beta\) = higher dose surviving fractions (Quadratic area of graph)

This equation was further modified to take into account the cell death at 0 Gy when cells were treated with TMZ. This produced a further constant to analyse, to show the combined effect of TMZ and X-rays together, the \(Y_0\).
Chapter 4: The characterisation of glioblastoma cell lines

Equation 4 Modified LQ:

\[ SF = \gamma \times (e^{- (\alpha D + \beta D^2)}) \]

The LQ model describes the curve fitting as one exp. -\( \alpha D \) component and a \( \beta D^2 \) component. The \( \alpha D \) section of the equation describes the linear portion of cell killing, which represents single tracks throughout the cell causing lethal lesions which are repaired linearly (Brenner 2008). The \( \beta D^2 \) section describes when multiple tracks penetrate the nucleus and cause lethal lesions, leading to a quadratic section of the curve (Brenner 2008).

We can clearly see that the MGMT-positive cell lines, LN18 and T98G, unsurprisingly, the addition of TMZ treatment did not increase cell killing in combination with X-rays (Figure 4-9 a and b). This was expected as these cell lines are not sensitive to TMZ as shown above. The addition of the TMZ affected the parameter \( \gamma \) (survival at 0 Gy) of the MGMT-positive cell lines only slightly, 0.92 and 1.1 for LN18 and T98G respectively from a survival of 1, and not even to necessarily lower survival as shown in T98G. We next examined the alpha/beta ratio, which represents the dose at which the linear and the quadratic area of the graph is equal, but furthermore, low alpha/beta ratios are consistent with more radioresistant tissue (Brenner 2008). Most tumours show high alpha/beta ratio typically over 10 Gy (Williams et al. 1985). The alpha/beta ratios for LN18 were similar for both with (23.33 Gy) and without (21.43 Gy) TMZ (shown in Table 9). However, for T98G the doses were wildly different and negative, -28.46 and -60 Gy without and with TMZ respectively. Minus doses in Gy are nonsensical and therefore the alpha/beta doses for T98G are ignored. Neither cell line, T98G or LN18, reach 10% survival at 5 Gy. Neither LN18 or T98G appeared to display hypersensitivity at low doses. This was not expected as other studies have found that doses between 0.2-0.8 Gy show hypersensitivity in T98G (Short et al. 1999).

We next examined the MGMT-negative cell lines response to treatment of X-rays with and without TMZ, Figure 4-9 c-e. All MGMT-negative cell lines displayed sensitivity when treated with TMZ and X-rays in combination, with \( Y_0 \) values that were 0.74, 0.67 and 0.42 of the fraction was reduced when TMZ was added in A172, U251 and U87 respectively. With the addition of TMZ at 5 Gy the survival fraction for all cell lines was below 10%, with ~ 5% survival for U251 and U87, but 8% survival for A172. This is in
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

contrast to treatment with X-rays alone (5 Gy) where survival for all 3 cell lines is approximately at 15%. Hypersensitivity at low doses occurred in one of these cell lines, U87, this can be seen in the "dip" in the data points compared to the red curve at 0.5 Gy with TMZ and X-rays. The radiosensitivity of the cells was affected by the addition of TMZ in these cell lines, however, this was not predictable. This was measurable from looking at the change in the alpha/beta ratios, shown in Table 9 The output of the constants from fitting either the LQ model to the survival data. Y0 is survival at 0 Gy.
Chapter 4: The characterisation of glioblastoma cell lines

Figure 4-9 Survival fractions after treatment with X-rays or X-rays and TMZ.
a) LN18, b) T98G, c) A172, d) U251 and e) U87. Cell lines fitted with the best fit models for each cell line when treated with X-rays with and without 25 μM of TMZ. A minimum of 3 repeats in triplicate were performed and statistics were model fitting of LQ.
## Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

<table>
<thead>
<tr>
<th>Cell line</th>
<th>$\alpha$ (Gy$^{-1}$)</th>
<th>Std. error</th>
<th>$\beta$ (Gy$^{-2}$)</th>
<th>Std. error</th>
<th>$\alpha/\beta$ (Gy)</th>
<th>$\gamma$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A172</td>
<td>0.42</td>
<td>0.086</td>
<td>0.0094</td>
<td>0.026</td>
<td>44.68</td>
<td>1.00</td>
<td>0.94</td>
</tr>
<tr>
<td>A172 + TMZ</td>
<td>0.27</td>
<td>0.060</td>
<td>0.035</td>
<td>0.018</td>
<td>7.71</td>
<td>0.74</td>
<td>0.97</td>
</tr>
<tr>
<td>LN18</td>
<td>0.30</td>
<td>0.062</td>
<td>0.014</td>
<td>0.017</td>
<td>21.43</td>
<td>1.00</td>
<td>0.92</td>
</tr>
<tr>
<td>LN18 + TMZ</td>
<td>0.28</td>
<td>0.106</td>
<td>0.012</td>
<td>0.028</td>
<td>23.33</td>
<td>0.92</td>
<td>0.82</td>
</tr>
<tr>
<td>T98G</td>
<td>0.37</td>
<td>0.058</td>
<td>-0.013</td>
<td>0.014</td>
<td>-28.46</td>
<td>1.00</td>
<td>0.95</td>
</tr>
<tr>
<td>T98G + TMZ</td>
<td>0.36</td>
<td>0.048</td>
<td>-0.006</td>
<td>0.012</td>
<td>-60</td>
<td>1.11</td>
<td>0.97</td>
</tr>
<tr>
<td>U251</td>
<td>0.30</td>
<td>0.12</td>
<td>0.019</td>
<td>0.037</td>
<td>15.79</td>
<td>1.00</td>
<td>0.81</td>
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<tr>
<td>U251 + TMZ</td>
<td>0.16</td>
<td>0.14</td>
<td>0.047</td>
<td>0.040</td>
<td>3.40</td>
<td>0.67</td>
<td>0.82</td>
</tr>
<tr>
<td>U87</td>
<td>0.23</td>
<td>0.061</td>
<td>0.028</td>
<td>0.017</td>
<td>8.21</td>
<td>1.00</td>
<td>0.94</td>
</tr>
<tr>
<td>U87 + TMZ</td>
<td>0.25</td>
<td>0.11</td>
<td>0.015</td>
<td>0.030</td>
<td>16.67</td>
<td>0.42</td>
<td>0.84</td>
</tr>
</tbody>
</table>

Table 9: The output of the constants from fitting either the LQ model to the survival data. $Y_0$ is survival at 0 Gy.

## 4.4 Summary

Base excision repair is a multistep DNA repair pathway which is important in the treatment of glioblastoma as it repairs the most common lesions formed by the treatment with TMZ (Bobola et al. 2012). Expression levels have been monitored by other groups for individual repair proteins as a common part of elucidating the mechanisms for resistance to treatment to many cancers (Agnihotri et al. 2012; Naidu et al. 2010). No other studies report a range of glioblastoma cell lines that are assessed for levels of repair proteins specific to TMZ damage. These repair proteins were assessed to examine potential
Chapter 4: The characterisation of glioblastoma cell lines

differences in BER expression levels and if these differences affect the cell lines response
to TMZ and/or X-rays.

What was evident in this chapter is that these 5 cell lines had different expression
levels of proteins important to the repair of TMZ lesions on the DNA. Three cell lines
expressed mRNA levels of MGMT, as shown in the table below, however this was only
evident at the protein level in 2 of the cell lines, LN18 and T98G. MGMT levels had the
biggest effect on whether the cell line was sensitive to TMZ or not. AAG expression did not
seem to affect sensitivity to TMZ in the MGMT negative cell lines as A172 was the most
sensitive, with an EC50 of 24.62 μM. However, in combination treatment with X-rays and
TMZ U87 was the most sensitive cell line, with the EC50 dropping from 2.46 Gy to 0.059
Gy.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>MGMT expression</th>
<th>AAG expression</th>
<th>EC50 TMZ μM</th>
<th>EC50 X-rays Gy</th>
<th>EC50 combined Gy</th>
</tr>
</thead>
<tbody>
<tr>
<td>A172</td>
<td>0</td>
<td>1.704</td>
<td>24.62</td>
<td>1.766</td>
<td>1.247</td>
</tr>
<tr>
<td>LN18</td>
<td>0.986</td>
<td>12.880</td>
<td>273</td>
<td>2.046</td>
<td>1.952</td>
</tr>
<tr>
<td>T98G</td>
<td>1</td>
<td>1</td>
<td>229.2</td>
<td>2.377</td>
<td>2.604</td>
</tr>
<tr>
<td>U251</td>
<td>0.0342</td>
<td>0.736</td>
<td>48.19</td>
<td>2.423</td>
<td>1.841</td>
</tr>
<tr>
<td>U87</td>
<td>0</td>
<td>15.8262</td>
<td>45.18</td>
<td>2.461</td>
<td>0.0595</td>
</tr>
</tbody>
</table>

Table 10 A comparison of the expression levels of AAG and MGMT correlated to
EC50s from different treatment modalities in glioblastoma cell lines.

From the qPCR and western blot results, a potential imbalance in BER proteins
was noted in U87, with a balanced increased levels of BER protein expression found in the
cell line LN18. The other cell lines in the study (A172, T98G and U251) showed similar
expression levels for all other BER proteins. The other repair proteins of interest to TMZ
treatment were measured and found to be comparable in all cell lines, with the exception
of MGMT.

The cell lines’ sensitivity to TMZ treatment, measured as in changes in cell
survival in clonogenic assays, was shown to be dependent on the MGMT status, as
previously reported (Hermisson et al. 2006). However, the imbalance in BER was notable
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

when treated with MMS alone, an agent not resulting in O6-MeG adduct formation and therefore not requiring MGMT for repair and survival. With these MMS treatments, U87 was more susceptible to cell death than either LN18 or T98G.

All five cell lines had similar EC50 results from treatment with X-rays alone, however, when concurrently treated with TMZ it was shown that the EC50 was significantly reduced in U87, to a dose of 0.05947 Gy. This sensitivity could be due to the high expression levels of the initial enzyme AAG, however, not of the other enzymes, which could lead to a build up of AP sites. In comparison to the other cell lines, which were sensitised to TMZ when treated with X-rays as well, which included A172, LN18, and U251, this sensitisation lowered the EC50 for the combined treatment results. However, the decrease in combined EC50 for LN18 is not significant. However, T98G had an increase in the EC50. This increase in EC50 could be due to there being so very little difference between the survival curves with the addition of TMZ. But the increase in the EC50 is by 237 cGy, which is comparatively quite high. From this it can be clearly deduced that the MGMT status on the cell lines has the biggest effect on whether combination treatment would have any advantage in cell killing and therefore a consideration when treating patients.

The imbalances found within the BER pathway for U87 will be further examined in the next chapter, along with trying to create an imbalance in the other cell lines by inhibiting different enzymes within the BER pathway and measuring if the cell’s sensitivity to different treatments change accordingly.
5 INVESTIGATING PHARMACOLOGICAL MODULATION OF BER WITH DIFFERENT TYPES OF RADIOTHERAPY

5.1 Introduction

As shown in chapter 2.4.4, O⁶-methylguanine DNA methyltransferase (MGMT) positive cell lines are resistant to the chemotherapy used in treating glioblastoma. One of the most common ways to overcome this resistance is by combining chemotherapy with inhibitors for the proteins which regulate DNA damage, repair and structure. Poly (ADP-ribose) polymerase (PARP) inhibitors have recently shown promise in trials for ovarian and other cancers (Mirza et al. 2016). Currently PARP trials are ongoing for the treatment of breast cancer in combination with Herceptin, an antibody to human epidermal growth factor receptor which is overexpressed on some breast cancers this treatment triggers the immune system to attack the cancer cell, and temozolomide (TMZ) (Lord & Ashworth 2017) as well as being the subject of numerous publications (Weil & Chen 2012; Lord & Ashworth 2017). As the preferred, or first-line, treatment of glioblastoma is TMZ, which causes lesions on the DNA that are mostly repaired by the base excision repair (BER)
**Modulation of the Base Excision Repair (BER) pathway** in the treatment of glioblastoma with radiotherapy

pathway, modulating this pathway may lead to an increase in cell death due to disturbed and unfinished BER. Furthermore, as shown in chapter 2.5, there is a high variation in the expression level of BER enzymes in cell lines and, as studies have shown, in cancer patients (Leguisamo et al. 2017). This variation in expression levels combined with inhibiting certain enzymes within BER whilst treating with TMZ may lead to certain repair profiles being more susceptible to cell death with certain combinations of different BER inhibitors in conjunction with TMZ or radiotherapy and, if the survival differential is statistically significant, could be worth exploring further.

The BER pathway, as explained in detail in chapter 2.4.3.1, is a multi-enzyme repair pathway which is initiated by DNA glycosylases, shown in Figure 5-1. For some of the TMZ-induced base damage, the initiating glycosylase is alkyladenine DNA glycosylase (AAG) (Srivastava et al. 2009). In BER, each repair protein in sequence is held in close proximity to the DNA being repaired by the repair accessory protein X-ray repair cross-complementing protein 1 (XRCC1), which works as a scaffolding protein (Vidal et al. 2001). This highly organized coordination of the repair pathway is particularly important as the DNA repair intermediates generated at each repair enzymatic step, such as AP sites and 5’dRP, can lead to cell death if not swiftly resolved (Fu, J. a Calvo, et al. 2012).

Many inhibitors of the BER pathway are currently in clinical trials, including methoxyamine (MX) which was used in this study. MX inhibits AP endonuclease (APE) by binding to the abasic site formed by AAG activity on the methylated base resulting from TMZ treatment (Talpaert-Borlè 1987). The benefit of using MX is that it does not inhibit any other action of APE such as its role in metabolism (Evans et al. 2000). The blocking of abasic sites accessibility by MX leads to an increase in unrepaired AP sites as shown in Figure 5-1. Since AP sites are mutagenic, cytotoxic and can lead to single strand break (SSB) and double-strand break (DSB) formation during replication, MX treatment should lead to detrimental consequences. Therefore, treatment with MX in combination with TMZ or irradiation could lead to an increase in SSBs and a corresponding increase in cell death.
Chapter 5: Investigating pharmacological modulation of BER with different types of radiotherapy

Figure 5-1 The base excision repair pathway and inhibition with methoxyamine. The base excision repair pathway is initialised by AAG which removes the methylated base leaving an Apurinic site. Methoxyamine binds to this site preventing APE from cutting this site and preventing the rest of the pathway from being activated. This will increase the number of Apurinic sites leading to a build up of toxic intermediates and increasing the toxicity of other treatments such as TMZ and radiation.

The second inhibitor used in this study was pamoic acid (PA), which is a polymerase beta (PolB) inhibitor. PolB is also the next enzyme in the BER pathway after APE, has two roles in repairing the DNA comprising:

- a lyase activity of the 5'dRP, created after the incision to the backbone of the DNA by APE
- a gap-filling role (Sobol et al. 1996) which places new nucleotides into the strand of DNA filling the gap

This is discussed in greater detail in chapter 2.6.3.1 There are a few published studies on the potential benefits of blocking PolB for the treatment of multiple cancers but most publications, whilst providing some evidence of the value of further investigation, did not document successes in in vitro situations (Wallace et al. 2013; Jaiswal et al. 2011; Asagoshi et al. 2010). One of the limiting factors for such studies is the lack of specificity of PolB inhibitors combined with low binding affinities. This has led to a concerted effort in recent years into producing better inhibitors for PolB (Gao et al. 2008). However, despite these limitations, we felt that researching the role of PolB inhibition in treatment of
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

glioblastoma with TMZ and irradiation would be useful in identifying potential benefits for further study in this area and may also help elucidate a role for unbalanced BER repair enzymes to be manipulated in personalised treatments.

This chapter focuses on work with inhibitors to measure toxicity of the inhibitor alone, at varying doses, when used to treat cells. Following this, the additional cytotoxicity in combination treatment was analyzed for the case of inhibitor and TMZ alone and with X-rays/protons and TMZ in combination. These combination studies were intended to clarify the role of BER and BER imbalance in treatment for glioblastoma. Currently there is very little research available on the effects of inhibiting APE1 and even less on PolB under the influence of different radiation types. The majority of the research studies available on inhibiting APE1 or PolB is on cells over- or under-expressing BER proteins and their response to alkylating agents (Yan et al. 2007; Montaldi et al. 2014; Taverna et al. 2001; Hazan et al. 2008; Barakat et al. 2012; Yang et al. 2010).

5.1.1 Aims and hypothesis

This chapter is focused on the testing of the hypothesis mentioned previously that an imbalance in the BER capacity affects glioblastoma cells repair capability after treatment. To further test this hypothesis this chapter aims to take the cell lines previously measured for their BER capability to be further modulated with inhibitors for BER to see if this creates a greater imbalance sensitizing cells to traditional treatment; X-rays and TMZ as well as protons.

To test this, the following objectives were set:

- Discover if short term treatment with BER inhibitors leads to cellular toxicity
- Identify if there is an interaction between BER inhibition and TMZ in cell lines with different basal levels of BER proteins
- Evaluate the combined effect of X-rays and TMZ or Protons and TMZ on glioblastoma cellular survival, in presence or absence of BER modulation via inhibitors.
- Establish if this BER imbalance produced from inhibitors affects cell growth and cell cycle.
Chapter 5: Investigating pharmacological modulation of BER with different types of radiotherapy

5.2 **Protons cause a greater cell killing effect in glioblastoma cell lines than X-rays.**

Before comparing the interaction of different drugs in combination with radiation and temozolomide, two different type of radiation were directly compared. From this later in the chapter the combination of drugs with these different radiation types will be compared to measure if they lead to greater levels of cell death due to the difference in DNA damage and therefore, potential synerism from DNA repair pathways.

When comparing clonogenic survival 3 cell lines were chosen; LN18, U87 and A172, due to the vastly differing repair capabilities measured in chapter 4. LN18 has high expression levels of MGMT and AAG. U87 has high expression levels of AAG but does not express MGMT at all. And finally A172 does not express MGMT and has average levels of AAG. However these proteins should not affect the cell lines response to radiation damage, as they are responsible for repairing damage created by temozolomide and other alkylation agents. However, radioresistance is brought on from a plethora of different reasons. These are often interlinked to the cell cycle, DNA DSB repair pathways and signalling proteins (Krause et al., 2017).

What we can see from figure ... below that each cell lines was more sensitive to proton irradiation than X-rays. In LN18 and U87 there was visible hypersensitivity brought on from proton irradiation, which was not produced from X-ray irradiation. These comparisons will be drawn on further in figure 5.9.
Figure 5-2 Comparison of both X-rays and protons on the survival of glioblastoma cell lines. 3 cell lines; LN18 a), U87 b) and A172 c). Cell lines fitted with the RCR model. A minimum of 3 repeats in triplicate were performed.

5.3 Short-term treatment of methoxyamine on glioblastoma cell lines does not lead to toxicity alone, but can be synergistic with TMZ in BER imbalanced cells

5.3.1 Inhibiting APE1

We first wanted to check the binding affinity of MX to abasic sites, so an assay was developed by the research team, Ms Eleanor Healing and Dr Ruan Eliot, which measures the release of fluorochromes attached to synthesized DNA containing abasic sites. The fluorochrome is released upon addition of the purified enzyme, APE, as a result of cleavage of the sites. As the abasic sites are cut by APE, fluorochrome attached to the
Chapter 5: Investigating pharmacological modulation of BER with different types of radiotherapy

DNA may then be washed off which lowers the absorbance reading from the plate. Blocking of the abasic sites by MX inhibits the action of APE resulting in increased absorbance/fluorescence.

From this we found in Figure 5-3 a) that 0.3 U/100μL of purified APE lowers absorbance from 0.45 to 0.2 Abs at 450 nm demonstrating that recombinant APE enzyme is added to the modified DNA, cleavage at the abasic site occurs lowering fluorescence. Using this assay, it can be seen in Figure 5-3 b) when 32 pM/μL of MX was added to 0.25pM of abasic site-containing DNA, it bound to approximately 100% of the abasic sites thus fully inhibiting APE1. (0.33U/100μL of APE1 was added within all of the experiments with MX.) These assays show that MX does bind to DNA in an in vitro situation. Note, however, that this does not, and cannot, answer the question of whether the MX is taken up in to the nucleus to bind to the DNA.
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

5.3.2 Methoxyamine toxicity

To further study the cellular effects of MX but without any other treatment we measured the cytotoxicity of MX in the 5 glioblastoma cell lines which were categorised in the previous chapter for their basal levels of BER proteins and response to standard treatment modalities. The experimental design is described fully in the methods chapter, in summary the cells were measured for growth and viability in the presence of a range of

![APE1 standard curve](image1)

![Methoxyamine concentration curve](image2)

Figure 5-3 Methoxyamine inhibition of APE1. a) Using a fluorescent assay with designer DNA with a fluorochrome attached, the number of incisions made to the DNA by increasing levels of APE enzyme was measured by the decrease in fluorescence as incised DNA containing the fluorochrome was washed away. b) the concentration of methoxyamine needed to inhibit APE and stop the incision of DNA, which leads to an increase in absorbance as less DNA containing the fluorochrome is washed away. A minimum of 3 biological repeats was performed.
Chapter 5: Investigating pharmacological modulation of BER with different types of radiotherapy

doses of MX. As can be seen in Figure 5-4 doses ranging from 50 μM to 10 mM had little effect on the viability of the cell lines. However, at 50 mM there was a drop in viability in the majority of cell lines apart from U87. This drop in viability, however, could have been due to the increase in acidity in the media of the cells. The pH of the MX at stock concentration and at 10 mM were measured to have a pH of 6. Because of this acidity further experiments were limited to a maximum dose of 1 mM. The lack of toxicity from MX correlates with a study by Montaldi and co-workers in which fibroblast cells were cultured with MX treatment and left for 72 hours to measure viability, with doses ranging from 0.75-15 mM (Montaldi et al. 2014).
Figure 5-4 Cytotoxicity of methoxyamine. a) LN18, b) T98G, c) A172, d) U251 and e) U87. Cells were seeded at a density of 5,000 cells per well. A range of MX concentrations were added to the cells and left for 48 hours. The cells ability to perform redox reactions was measured using a 1:10 dilution of alamar blue. Each graph represents a different cell line with a minimum of 3 repeats.

As TMZ is the standard of care for glioblastoma, a combination of inhibitor and TMZ was assessed for overall toxicity over a 48 hour period. The concentration of TMZ...
used for this study was 25 μM as this is the amount found within the plasma of patients during clinical treatments. It was found that the addition of TMZ with MX showed no effect in cell growth or induction of cell death to any of the cell lines (data not shown).

As a combination of MX with 25μM TMZ had little to no effect in terms of cellular survival, higher doses were chosen according to the sensitivity of the cells due to the MGMT status. During 14 day clonogenic assays, the EC50 (TMZ) was found to be below 50 μM for MGMT-negative cell lines and lower than 300 μM for MGMT-positive cell lines, however as these toxicity assays are over a shorter term, higher doses were used of 75μM and 350 μM for MGMT-negative and -positive cell lines respectively. The MGMT-negative cell lines all show in Figure 5-5 only a minimal increase in cytotoxicity when treated at 75 μM of TMZ and 500 μM of MX. U251 and U87 when treated concomitantly with TMZ and MX each had a 9% mean decrease in survival rate. The survival of A172 decreased by 11% with the addition of MX. None of these results were found to be statistically significant (2-way ANOVA).

The MGMT-positive cell lines both showed a statistically significant effect of the MX & TMZ combination with a drop in survival by 11.4% for LN18 and T98G by 10% (Figure 5-5). As the levels of AAG are different in these cell lines this indicates that the effect of inhibiting APE1 and sensitising cells to TMZ is not AAG dependent. Whilst MGMT status affected the cell lines sensitivity to TMZ, in all cases MX did further sensitisate the cells to TMZ.

In this study lower doses of MX were used in comparison to studies by other researchers (Montaldi et al. 2014; Agnihotri et al. 2014) to take into account the results from a clinical trial into MX in the plasma after treatment of patients. This trial found that levels of MX in the μM range, rather than mM range, were found in the blood and as such any experiments which would be looking for a correlation between drug interactions for clinical usage, we felt, should be conducted at clinically relevant doses. Montaldi et al., (2013) treated T98G cells with a combination of MX and TMZ. The dose of MX remained fixed at 20 mM, and although not clinically relevant, found that MX did sensitize these cells to the TMZ (Montaldi & Sakamoto-Hojo 2013).
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

Figure 5.3.3 Viability of MGMT negative a) and positive b) cell lines after treatment with MX and TMZ. Cells were seeded at a density of 5,000 cells per well. Cells were treated with 500 μM of MX concentrations and either 75 μM of TMZ (a) for MGMT-negative cell lines, or 350 μM TMZ (b) for MGMT-positive cell lines and incubated for 48 hours. The cells ability to perform redox reactions was measured using a 1:10 dilution of alamar blue. A minimum of 3 repeats. Statistical significance was measured with a 2-way ANOVA.

5.3.3 An AAG mediated effect with methoxyamine and temozolomide

To further understand the interactions between TMZ and MX in the different cell lines the combination indexes (CI) were analysed. This is because the combined cytotoxicity of TMZ and MX appears to affect glioblastoma cells in an MGMT expression dependent fashion. CI values probe the interactions between multiple drugs using a mathematical analysis, developed by Ting-Chao Chou (Chou 2010). The analysis leads to
the quantitative description of drug-drug interactions as either; synergistic (CI <1), additive (CI=1-1.2) or antagonistic (CI>1.2) (Chou 2010).

Interestingly, the cell line which showed synergism over a large range of doses was U87, 25 μM (low) and 100 μM (high) of TMZ with 500 μM of MX. The CI values are 0.88 and 0.83 at low and high doses of TMZ respectively, as can be seen in Table 11. This synergism means that the effect of the two drugs in combination shows more than an additive effect of cell killing and is therefore synergistic. This cell line expresses high levels of AAG which could suggest that the interaction between TMZ and MX is synergistic when high levels of AAG are expressed in the cells, however, there may be other unmeasured factors at play here.

U251 and LN18 also both show synergism at higher doses of TMZ, 100 μM and 500 μM respectively. For LN18, the CI number was 0.46 (highly synergistic) which might be explained as follows; as the levels of TMZ are increased the protective role of MGMT is negated in the LN18 cell line. As this occurs the potential role for high AAG levels, leading to an increase in abasic sites, teams with the action of MX blocking these sites and this is, in this way, a synergistic interaction between TMZ and MX.

U251 showed synergy with a CI of 0.45 even though it does not display high levels of AAG. This is not consistent with the hypothesis that high levels of AAG leads to greater cytotoxicity when cells are co-treated with TMZ and MX. However, this synergism is only apparent at doses higher than would be available in blood plasma, and the other cell lines may also display synergism at higher levels of TMZ than were tested.

In colon cancer cells the interaction between TMZ and MX showed constant synergism over a range of doses (Liu et al. 1999). The distinction between the two cell lines used in the Liu et al. study were whether they were MMR proficient or deficient and therefore TMZ resistant or sensitive. Only the MMR proficient cell line was potentiated to the effects of TMZ by MX. This indicates that another mechanism or repair pathway is important in sensitising the interaction, BER being the most logical pathway due to amount of damage that can be repaired by BER caused from TMZ. However, Tang et al,
found that the increase in expression levels in PolB removes the MX potentiation to TMZ in certain cell lines over-expressing AAG (Tang et al. 2011).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>AAG</th>
<th>MGMT</th>
<th>Dose (TMZ)</th>
<th>CI</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>A172</td>
<td>Average</td>
<td>No</td>
<td>25 uM</td>
<td>2.16098</td>
<td>No</td>
</tr>
<tr>
<td>LN18</td>
<td>High</td>
<td>Yes</td>
<td>25 uM</td>
<td>6.72698</td>
<td>No</td>
</tr>
<tr>
<td>T98G</td>
<td>Average</td>
<td>Yes</td>
<td>25 uM</td>
<td>58715.1</td>
<td>No</td>
</tr>
<tr>
<td>U251</td>
<td>Average</td>
<td>No</td>
<td>25 uM</td>
<td>5.24399</td>
<td>No</td>
</tr>
<tr>
<td>U87</td>
<td>High</td>
<td>No</td>
<td>25 uM</td>
<td>0.87568</td>
<td>Synergistic</td>
</tr>
</tbody>
</table>

A172
| Average | No   | 100 uM | 1.70520 | No        |
| LN18     | High  | Yes   | 500 uM   | 0.45957  | Synergistic|
| T98G     | Average | Yes  | 500 uM   | 1204.13  | No         |
| U251     | Average | No   | 100 uM   | 0.44651  | Synergistic|
| U87      | High   | No   | 100 uM   | 0.83420  | Synergistic|

Table 11 A comparison of AAG and MGMT status within cell lines and the combination indices derived from TMZ and X-rays.

5.4 Short term treatment with inhibitors leads to toxicity lowering cell viability at high doses

5.4.1 Pamoic acid toxicity

Besides MX, another inhibitor was examined, as the effect of inhibiting other enzymes within the BER pathway was also of interest. Pamoic acid (PA) is an inhibitor which binds to the 8 kDa domain of PolB, as can be seen in Figure 5-6. It is soluble in an aqueous buffer allowing for additional analysis with nuclear magnetic resonance spectroscopy (NMR) (H.-Y. Hu et al. 2004).

The study conducted by Hazan and his team used AutoDock 3.0.5 to investigate the binding structure of PA to the 8 kDa domain of polymerase beta. Different structures have been ranked by AutoDock according to their binding affinity with the protein and
around of the 100 lowest energy structures were noted for further investigations. When AutoDock force fields have been used, the best ligands were selected, with binding energies from -9.58 kcal/mol to -8.96 kcal/mol. These numbers, representing free energy binding and final intermolecular energy respectively, illustrate the suitable attachment between ligand and active site (Hazan et al. 2008). This study indicates the area in which PA bound to PolB inhibiting its action.

Figure 5-6 Structure of polymerase beta and how pamoic acid binds to it. A) shows the whole protein with PA bound to the active site. B) shows just the amino acids within the active site where PA binds. Taken from (Hazan et al. 2008)

The cytotoxicity of PA was measured in the 5 cell lines used throughout this work. The experimental design is described in the methods chapter, however; briefly the cells were measured for growth and viability in the presence of a range of doses of PA. As can be seen in Figure 5-7 a-e. with doses ranging from 100 μM to 1 mM, all of the cell lines were sensitive to PA in short term toxicity testing with alamar blue. This could be due to a multitude of reasons, including the specificity of the PA to PolB rather than other polymerases and the importance of PolB to general cell cycle and DNA repair.
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

Figure 5-7 Cytotoxicity assay of glioblastoma cell lines treated with pamoic acid (PA). a) LN18, b) T98G, c) A172, d) U251 and e) U87. Cells were seeded at a density of 5,000 cells per well. A variation of PA concentrations were added to the cells and left for 48 hours. The cells ability to perform redox reactions was measured using a 1:10 dilution of alamar blue. Each graph represents a different cell line with a minimum of 3 repeats.
5.4.2 48 hour measurement of temozolomide toxicity

As TMZ is the standard of therapy for glioblastoma, in this study we chose a treatment combination of PA with TMZ which was examined for overall toxicity after treatment over a 48 hour period. As this was examined for MX and TMZ studies above, similar doses were used, as this allows for a direct comparison between the effects of either MX or PA with TMZ. Examining both PA and MX interactions with TMZ can help elucidate if inhibition of one enzyme over the other could lead to a better cell killing effect, in general or in different genetic profiles.

In this study a direct comparison of cell lines being treated with 100 μM of TMZ with and without 100 μM of PA was examined (Figure 5-8). Although all of the cell lines were more sensitive to TMZ in the presence of PA this was not found to be statistically significant. The mean changes in the MGMT-negative cell lines (a) were; 11.6%, 28.7% and 11.7% for A172, U87 and U251 respectively however, the large error bars due to repeats make these changes not statistically significant. The large effect seen in U87 with combination treatment may be due to the high AAG expression however more repeats would be needed to confirm this.

MGMT-positive cell lines were also treated with 350 μM of TMZ in the presence of PA (Figure 5-8 b). These cell lines also experienced a decrease in viability however this was not significant. LN18 had a reduction in viability of 7.9% and T98G was reduced by 9%.
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

5.4.3 When stimulating the BER pathway only for repair inhibitors had a small effect potentiating effect in imbalanced cell lines

We then wanted to test how the inhibitors affected the different cell lines when the BER pathway was challenged by treatment with a BER-specific alkylator. To do this, methyl methanosulphonate (MMS) was used. MMS is an SN₂ alkylator which adds methyl groups to nitrogen resulting in only the BER pathway being activated to repair the alkylation damage (Wyatt & Pittman 2006). It creates lesions on the DNA distributed as 11% 3-methyladenine (N³-MeA) and 0.3% O⁶-methylguanine (O⁶-MeG). This change in
damage distribution, in comparison to TMZ, means that the O\textsuperscript{6}-MeG lesion is not of concern and therefore the MGMT status and MMR pathways will not affect cell death. Since O\textsuperscript{6}-MeG lesions are not formed at any significant level, this means that the BER pathway is examined only.

We found that in all cell lines the addition of an inhibitor increased sensitivity of the cells to MMS-induced toxicity (Figure 5-5-9). In MGMT-positive cell lines, LN18 a) and T98G b), and MGMT-negative A172 c) and U251 d), MX increased cell death induced by MMS treatment. However, the error bars overlap hence the mean difference between MMS alone or MMS with PA or MX is not statistically significant. Studies conducted on PA and MMS have previously used higher doses of PA, which we found to be cytotoxic, which lead to a greater sensitization to MMS (H.-Y. Hu et al. 2004).

The MGMT-negative cell line U87 e), which displayed high levels of AAG was more acutely sensitised to MMS by both inhibitors in comparison to the other cell lines. In this cell line PA sensitised U87 to MMS more than MX, specifically at doses 1 mM (p = 0.0096) and 2 mM (p= 0.0077) of MMS. The increase in MMS sensitivity from both inhibitors could be explained by the high AAG levels, leading to an increase in BER intermediates which are further prohibited from repair activity by either inhibitor.
Figure 5. Viability after MMS and inhibitor treatment on glioblastoma cell lines. a) LN18, b) T98G, c) A172, d) U251 and e) U87. Cells were treated with serum free media for and drugs for 1 hour, and then fresh media was added back in. Viability was measured with alamar blue 48 hours later. Experiments were plated in triplicate and with a minimum of an n=3. 2-way ANOVA measured statistical significance.

5.5 MGMT-negative cell lines are potentiated to X-rays and Protons by imbalancing BER through inhibitors.

5.5.1 Clonogenic Assay for combined treatment of X-rays with and without temozolomide and inhibitors

We next wanted to compare the effects of the inhibitors with or without TMZ in the presence of either X-rays or protons. To do this three cell lines were selected; LN18, U87 and A172 chosen because these three cell lines had the widest variation in genetic background for BER and MGMT expression.
Chapter 5: Investigating pharmacological modulation of BER with different types of radiotherapy

All experiments included radiation with and without TMZ and no treatment at all as the base/control and to allow comparison between the different treatments. X-rays versus protons are compared directly.

Survival data for the next section was fitted with either the linear quadratic (LQ) model (Equation 4) or the repair capability repair (RCR) model (Equation 5). Both with a modified constant $\gamma$ at the start to describe where the line cuts the Y axis, how the other treatment affects the 0 Gy dose. These values are further analysed later.

**Equation 5 Modified RCR equation:**

$$S(D) = \delta \ast (\exp \ast (\alpha \ast D) + \beta \ast D \ast \exp(-C \ast D))$$

Where $\delta$ is a modification constant to adjust the curves to cross the y axis where the data starts rather than at 1. This is important for treatment to cells at the 0 Gy with temozolomide (TMZ). With the parameters being equivalent to no damage, repair and misrepair. No damage to cells ($e^{-\alpha D}$) and the cells which were able to repair themselves ($\beta De^{-\beta D}$) are parameters which are described within the equation as a whole (Brahme & Lind 2010). However the last section of the equation, which specifies repair is characterised by a misrepair section also ($e^{-\beta D}$). This means that at higher doses the amount misrepaired and ultimately leading to cell death increases. We used this equation to fit the data as we first noted a difference in the curves produced from vertical protons to horizontal protons which would not allow the commonly used linear quadratic equation (LQ) to fit all the data.

The RCR model is a new model used to fit survival curves which is appropriate to accommodate hypersensitivity at low doses of irradiation (Brahme & Lind 2010). The model chosen to fit the data depended on the $r^2$ value for each model and the general appearance of the line produced, this is further explained in chapter 6.2.3.1. The RCR model was of particular importance in this chapter as we see low-dose hypersensitivity, which the RCR model can fit better (Nilsson 2004).
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

5.5.2 Pamoic acid:

The first experiments involved X-rays treatment with or without TMZ and PA; it can be seen that in all of the cell lines, survival increased when treated with PA and X-rays in combination compared to X-rays alone or other combinations, Figure 5-10 Comparison of both X-rays and protons with TMZ and PA on the survival of glioblastoma cell lines. 3 cell lines; U87 a) X-rays b) protons, LN18 c) X-rays d) protons and A172 e) X-rays f) protons, were treated with 50 μM of PA, 25μM of TMZ, combination of both and either X-rays, left hand side, or protons, right hand side. Cell lines fitted with the RCR model. A minimum of 3 repeats in triplicate were performed. However, this observation does not hold true when treated with protons and inhibitors, as A172 had higher levels of survival when treated with protons alone.

In the MGMT-positive cell line, LN18 Figure 5-10 c and d, it can be seen that concomitant treatment of TMZ with radiation and PA leads to more cells surviving than with radiation and/or TMZ alone. This is seen in both X-ray treated and proton treated cells. Although protons lead to an increase in cell death in comparison to X-rays, the addition of either chemotherapeutic provided no benefit to the radiation treatment regime. X-rays, have a lower cell killing effectiveness than protons but with both radiation modalities the addition of chemotherapy showed no increase in cell death. The combination of treatments led to an increase in survival fraction of X-rays at 2 Gy; 47.6%, 50.8%, 81% and 72.4% for X-rays alone, with TMZ, with PA, and with TMZ and PA respectively. These results show that the addition of PA greatly increases the number of surviving colonies. The surviving fractions at 2 Gy for protons were very similar; 24.7%, 26.7%, 27.6% and 27.9% for protons alone, with TMZ, with PA, and with TMZ and PA respectively. This shows that, although protons are more toxic to the LN18 cell line, the combination of treatments cause higher numbers of surviving colonies.

When examining the proton irradiation protocol for the LN18 cell line, Figure 5-10 d, it is observed that LN18 displayed hypersensitivity to all treatment combinations. This is low dose hypersensitivity resulting in a much steeper reduction in cell survival at 0.5 Gy when treated with protons than would be expected when compared to higher doses. This hypersensitivity in LN18 is most prominent in treatment with protons alone, with a survival fraction of 28%, however treatment with protons, TMZ and PA combined,
although displaying hypersensitivity, had a surviving fraction of 44%. This hypersensitivity is not seen when cells are treated with X-rays and is therefore a proton irradiation-specific effect. These results also illustrate that the addition of TMZ to the MGMT-positive cell line LN18 increases the fractions of cells surviving to treatment.

In the MGMT-negative cell lines, U87 a and b and A172 e and f, the addition of PA, as mentioned above, did lead to a higher colony-forming percentage of cells than the control. However, the combination of PA, TMZ and irradiation led to an increased level of cell death in both cell lines after X-rays treatment, but only in U87 after proton irradiation. The greatest difference in survival is seen at 0.5 Gy for both cell lines between X-rays alone and X-rays with PA; 75.4% and 137% for U87, and 82.7% and 145% for A172. As both of these cell lines are MGMT-negative they are sensitive to treatment with TMZ. Both U87 and A172 are further susceptible to the combined treatment of X-rays, TMZ and PA together. The greatest range in survival fractions is at 2 Gy where for A172 the percentage survived with X-rays alone was 71.7%, X-rays and TMZ combined was 44.9% whereas when treated with X-rays, TMZ and PA the mean survival was 25.4% and the surviving fractions at 2 Gy for U87 were 54.1% for X-rays alone, 29.1% for X-rays and TMZ combined and 21.9% for X-rays, TMZ and PA. In these treatment regimes, the addition of TMZ reduced the survival of each cell line by 26.8% for A172, and 25% for U87. But when PA was added in combination the reduction of survival dropped further by 19.4% for A172 and 7% for U87. This difference with the addition of PA was not expected as U87 already has an imbalance in the BER enzyme expression levels, and as such we expected it to be more susceptible to inhibiting PolB than A172.

All cell lines treated with protons exhibited low dose hypersensitivity. In addition it was clear that the hypersensitivity to proton irradiation shifted and continued into higher doses, than the normal under 1 Gy, in particular treatment combinations. This shift in hypersensitivity could be due to the methylation status and expression of MGMT which could lead to further sensitisation by PA and protons combined. The hypersensitivity observed in A172 shifted from the peak of the trough being at 0.5Gy when treated with TMZ and protons to 1.1 Gy when treated with TMZ, PA and protons. At 0.5 Gy the differences between protons alone, with TMZ or with TMZ and PA are 54.3%, 12.2% and 11.6% respectively for A172.
**Modulation of the Base Excision Repair (BER) pathway** in the treatment of glioblastoma with radiotherapy

For U87 the surviving fractions are 32.2% for protons alone, 15% for with TMZ and 17% for TMZ and PA together at 0.5 Gy. These surviving fractions show that A172 was more sensitive to combination therapy than U87. Unlike A172, U87 when treated with 0.5 Gy of protons, TMZ and PA combined, was no further sensitised than when treated with protons and TMZ alone.

At 2 Gy, the differences between surviving fractions for treatment with TMZ and TMZ with PA are 8.3% and 0.7% for U87 and A172 respectively. Clearly, for A172, the increased radiation dosage shows a diminishing effect and, for both cell lines, virtually disappear by 3 Gy.

The results demonstrate that proton irradiation lowers cell survival far more effectively than X-rays and both of the MGMT-negative cell lines would experience greater cell death from the combination of protons with TMZ and PA at lower radiation levels. The specific nuances of the response of each cell line in the presence of the different radiation types could be due to the AAG expression level differences and the type of damage the radiation types predominantly cause but, as only BER has been measured and no other repair proteins, this is speculative and has not been rigorously tested.
Chapter 5: Investigating pharmacological modulation of BER with different types of radiotherapy

Figure 5-10 Comparison of both X-rays and protons with TMZ and PA on the survival of glioblastoma cell lines. 3 cell lines; U87 a) X-rays b) protons, LN18 c) X-rays d) protons and A172 e) X-rays f) protons, were treated with 50 μM of PA, 25μM of TMZ, combination of both and either X-rays, left hand side, or protons, right hand side. Cell lines fitted with the RCR model. A minimum of 3 repeats in triplicate were performed.
5.5.3 Methoxyamine

We next wanted to compare the cellular effects of APE1 inhibition with MX, when combined with radiation and TMZ. The survival curves for the cell lines treated with combinations of radiation, TMZ and MX are presented below in Figure 5-11.

The graphs show that the MGMT-negative cell line, LN18 Figure 5-11 c and d, showed increased cell survival when treated with radiation and MX in comparison to radiation alone with surviving fraction differences of 26% (X-rays) and 21% (protons).

For A172 and U87, the combination of MX, TMZ and radiation together lead to a decrease in cell survival in comparison to other treatment combinations. At 2 Gy of X-rays the percentage increase in cell death from TMZ treated to TMZ and MX treated is 7% (U87) and 11.5% (A172). When treated with protons the difference in survival between TMZ and TMZ with MX is much larger in U87 than A172; 6% compared to 1.3% at 3 Gy. Therefore, it is clear that the addition of MX to a combination of TMZ and radiation increases cell death rates except in LN18.

LN18, which is MGMT-positive, is not sensitive to the addition of TMZ or MX or a combination of the two. However, treatment with protons affect the cells survival at lower doses than expected, with the LN18 cell line exhibiting low-dose hypersensitivity at 0.5 Gy, which is not the case for X-rays. Therefore, in this MGMT-positive cell line, combination therapy which exploits the BER pathway does not increase cell death, and in patients could just lead to further toxicity of healthy cells. However, protons lead to a greater cell killing ability, as at 2 Gy the cell death fraction was 24% compared to 47% for X-rays.

The radiosensitivity shown in U87 and A172 is seen in other cell lines when MX is added to irradiation treatment (Oleinick et al. 2016). In small-cell lung cancer cell lines the addition of 20 mM of MX treatment to two cell lines mildly increased radiosensitisation when treated concurrently with gamma-rays up to a dose of 6 Gy. However, LN18, the MGMT positive cell line, regardless of the combinations of treatments used, showed little difference from the treatments in terms of cell survival. This seems to suggest that the
Chapter 5: Investigating pharmacological modulation of BER with different types of radiotherapy

MGMT status and not the status of the BER enzymes, are of more importance when examining radiosensitivity caused by BER inhibitors in the treatment of glioblastoma.

As seen in all cell lines and treatments, protons lower cell survival far more effectively than X-rays. With this in mind, U87 and A172 show a greater relative biological effect upon the addition of MX, TMZ and X-rays together, as the potential increase in single strand breaks and strain on single strand break repair mechanisms leads to an increase in cell death higher than other treatment regimes when treated with X-rays. However, when cells are treated with protons the additional benefit to treatment with TMZ and MX is very low. As mentioned above, no treatment combination provides a clear advantage for MGMT-positive LN18, other than protons over X-rays.
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

Figure 5-11 Comparison of both X-rays and protons with TMZ and MX on the survival of glioblastoma cell lines. 3 cell lines; U87, LN18 and A172 were treated with 50 μM of MX, 25μM of TMZ, combination of both and either X-rays, left hand side, or protons, right hand side. Cell lines fitted with the best fit models for each cell line and radiation type; RCR model for everything except A172 X-rays and MX. A minimum of 3 repeats in triplicate were performed.
5.5.3.1 Mathematical analysis of the effects modulation of BER during irradiation

We next wanted to understand the effect of the different radiation types on the overall survival of the cells. The relative biological effectiveness (RBE) is a ratio defining how two different sources of radiation compare by dose for equivalent cell killing. This measure shows how effective the type of radiation is at damaging DNA and leading to cell death. The higher the ratio the more damage the radiation causes. Any end point can be taken to derive the RBE; survival at 50%, number of H2AX foci, etc.

Protons are considered to be 10% more effective than photons traditionally however different models are used to measure the reliability of this and many papers find variations in this ratio (Paganetti et al. 2002; Ding et al. 2015). The endpoint chosen for the RBE and the cell line, determined how high the ratio was for the effect of protons in comparison to photons. Meta-analysis studies (Carabe et al. 2013) have shown that as \( \alpha/\beta \) ratios increase RBE decreases as Table 12 and Table 13 below show, this theory holds true for RBE\(_{20}\). However, RBE\(_{50}\) gives very high ratios and is thought to be due to the hypersensitivity seen at low doses.

The RBE\(_{20}\) ratio increases in each cell line as the \( \alpha/\beta \) ratio decreases though not in an obvious relationship. What the RBE\(_{20}\) shows is that the response of LN18 to a higher LET radiation type, protons, is less strong than the sensitivity to protons from U87 and A172. This could be due to a number of reasons, either the size of the nucleus or the repair proteins present within the cells.
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

<table>
<thead>
<tr>
<th>X-rays/Protons</th>
<th>RBE 50</th>
<th>RBE 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN18</td>
<td>5.326</td>
<td>1.026</td>
</tr>
<tr>
<td>U87</td>
<td>6.635</td>
<td>1.985</td>
</tr>
<tr>
<td>A172</td>
<td>1.900</td>
<td>1.989</td>
</tr>
</tbody>
</table>

*Table 12 A comparison of relative biological effectiveness.*

To further elucidate how PA and MX can affect the cell lines response to radiation damage with or without the addition of TMZ the survival fraction at 3 Gy were compared. However, without the addition of TMZ in some of the cell lines, an inhibitor and radiation could lead to higher surviving fractions and therefore not be beneficial to patient prognosis. Comparing the differences between the radiation type and inhibitor type, Table 13 shows the surviving fractions for 3 Gy. MX is the better sensitizer in combination with TMZ and radiation in all cell lines and likewise, in all cell lines, protons produce a greater cell killing effect.

<table>
<thead>
<tr>
<th>Surviving fraction at 3 Gy</th>
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<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>A172</td>
</tr>
<tr>
<td>LN18</td>
</tr>
<tr>
<td>U87</td>
</tr>
</tbody>
</table>

*Table 13 A comparison of the surviving fractions at 3 Gy after trimodal treatment.* - significant figures test
Chapter 5: Investigating pharmacological modulation of BER with different types of radiotherapy

We furthered our understanding of these interactions by looking at the sensitizing enhancement ratio (SER) of TMZ with MX, PA and alone, Table 14. The addition of TMZ is most effective in the MGMT-negative cell lines at sensitizing the cells to the radiation with treatment of either X-rays or protons (U87 4.835 and 3.019, A172 4.293 and 208.507 for X-rays and protons respectively). The addition of TMZ and inhibitors sensitized A172 to both radiation types (MX; 15.171 and 13.154 and PA: 10.480 and 13.723 for X-rays and protons respectively). However, the sensitization of U87, to either TMZ or radiation, by the inhibitors was so strong that the 0 Gy was below 50% survival. This enhanced sensitisation could be due to the already imbalanced BER pathway within this cell line.

<table>
<thead>
<tr>
<th></th>
<th>with TMZ</th>
<th>With MX</th>
<th>With PA</th>
<th>with MX and TMZ</th>
<th>with PA and TMZ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SER$_{50}$ X-rays</td>
<td>SER$_{50}$ Protons</td>
<td>SER$_{50}$ X-rays</td>
<td>SER$_{50}$ Protons</td>
<td>SER$_{50}$ X-rays</td>
</tr>
<tr>
<td>L</td>
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<td>0.948</td>
<td>0.631</td>
<td>0.658</td>
</tr>
<tr>
<td>U</td>
<td>-</td>
<td>-</td>
<td>1.095</td>
<td>1.656</td>
<td>0.845</td>
</tr>
</tbody>
</table>

*Table 14 A comparison of the sensitizing enhancement ratio after a series of combinations of treatments. L = LN18, U = U87, A = A172.*

Vermeulen and co-workers found that knocking out PolB and inhibiting APE1 with MX increased radiosensitivity greatly when blocking each enzyme individually (Vermeulen et al. 2007). Unfortunately blocking both APE and PolB was not performed within these studies and could be a point for future work to try and elucidate the role of BER in glioblastoma resistance to common treatment modalities.

One of the striking features of these graphs was the increase in proliferation when the cells were exposed to the inhibitors alone with no other treatment. This observation led us to further investigate to see if there were any negative effects from treatment with inhibitors alone.

Natalie Mayhead - August 2018
5.6 Treatment with BER inhibitors promotes increased cell growth.

During the previous experiments, results obtained after treatment with inhibitors led to some questions being raised about cell growth under the influence of these inhibitors such as: Does inhibiting AP site repair of PolB action lead to an increase in cell growth? Do the number of colonies forming increase? And does combined treatment with TMZ and BER inhibitors lower colony formation? To answer this the growth and proliferation of the cell lines was measured when treated with the inhibitors alone and with TMZ (Figure 5-12). Treatment with MX did not significantly increase or decrease the number of colonies formed on any of the cell lines. The proliferation of A172, LN18 and U251 was decreased by MX by 19, 2 and 15% respectively, except for U87, where there is a clear increase in colonies formed by 23%. However, when treated with PA, all cell lines except for U251 showed a large increase in colony numbers. The mean increase in number of colonies formed is 90, 40 and 47% for A172, LN18 and U87 respectively whilst the colony formation decreased for U251 by 10%.

The next question to answer was this; when 50 μM of MX was added to 25μM of TMZ, was the growth of the cells increased from treatment with TMZ alone? If so, then this is clearly not a good treatment pathway to pursue under clinically relevant treatment conditions. We found in Figure 5-12 b, for U251 and LN18 the growth under treatment with MX and TMZ showed increases over treatment with TMZ alone of 20 and 9% respectively. Both of these cell lines have a balanced expression of BER proteins, however LN18 expresses MGMT protein, removing the main toxic effects of TMZ, and U251 expresses MGMT mRNA although this does not affect the toxic lesion formation from TMZ. U87 shows a non-significant decrease (5%) whilst A172 also shows a decrease (4%). U87 does display an imbalance in the BER pathway due to high levels of AAG whereas A172 has a balanced BER pathway. Neither cell lines express any MGMT protein or mRNA.

When growth under the influence of PA and TMZ was analysed (Figure 5-12 d) it was found that U87 had a decrease in cell growth and proliferation under the influence of PA and TMZ combined (11%) in comparison to TMZ alone. This could be due to the imbalance in expression of BER proteins. A172 and LN18 showed increases in cell growth.
Chapter 5: Investigating pharmacological modulation of BER with different types of radiotherapy

and proliferation under the influence of TMZ and PA in combination in comparison to treatment with TMZ alone of 9 and 22% respectively. U251 displayed a similar growth profile with TMZ and PA however there was a mild increase in colony formation of 3% in comparison to TMZ alone.

In PA experiments, A172 treated with TMZ alone and TMZ and PA both showed significant changes when compared to PA alone (p=0.0001 for both) with a decrease in cell colonies formed. U87 treated with PA was significantly different to treatment with TMZ alone (p=0.0009) and TMZ with PA (p = 0.0001). For A172 and U87 there is an increase in colony formation when treated with PA alone than when treated with TMZ or a combination (Figure 5-12). This means that if PA, or another POLB inhibitor, was brought into clinical trials, dosing times and regimens would have to be examined carefully to ensure that they did not lead to an increase in tumour mass. For MX, which is in clinical trials (Gordon et al. 2013) only U87 showed an increase in colony formation. The reason for this increase in colony formation should be examined further as MX is in clinical trials and treatment regimes should be examined very carefully, whilst investigating the protein profile which could cause this, to ensure harm is not occurring to the patients. However, in treatments with MX and TMZ on A172 and U87 it was shown that both cell lines decreased in proliferation significantly when compared to TMZ alone or MX alone, p values are 0.0052/0.0043 and <0.0001 respectively.
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

Figure 5-12 Number of colonies formed after treatment with PA. 4 cell lines; A172, LN18, U87 and U251 were treated with 50 μM of MX, a and b, or PA a and d, 25μM of TMZ and a combination of both, b and d. Cells were left to form colonies over 14 days until stained with crystal violet to count colonies. Results were compared to controls, left hand side. A minimum of 3 repeats were performed and 2-way ANOVA statistics were performed to look for significant changes in colony numbers.

As the growth profiles of these cells showed an increase when treated with inhibitors alone and the colony staining and size also showed an increase in intensity, the next question was whether or not this was due to an increase in the size of the cells, the number of cells present or, alternatively, to an increase in DNA within the cells as the cells are proliferating faster hence there is more DNA for the crystal violet to bind to

5.6.1 Growth curve after treatment with 50 μM of each inhibitor

We next went on to analyse two cell lines for their growth rate, U87 and U251. These were chosen due to their vastly different colony forming responses to the addition of PA and MX during clonogenic analysis. Whilst their responses were different, the expression level of BER proteins were also different, with U87 expressing high levels of AAG and average levels of the other proteins, whilst U251 expressed average levels of all
Chapter 5: Investigating pharmacological modulation of BER with different types of radiotherapy

BER proteins. Neither cell line expressed MGMT however this is not expected to affect their response to inhibitors as the inhibitors do not affect the O^6-MeG repair.

In Figure 5-13 below we can see that the two cell lines were treated with either PA or MX at 50 μM and incubated on the cells until they were harvested to be counted for proliferation. What is immediately clear is that U87 and U251 have very different growth profiles with U87’s growth plateauing at day 3 after it has doubled whilst U251 continues doubling.

U251 grew at similar rates regardless of whether it was treated with an inhibitor or not. This similarity in growth was in agreement with the results found in the clonogenic assays and by day 5 there were approximately 20 *10^4 cells per mL in each treatment group. Whether treated with MX or left alone, U87 had produced approximately 9*10^4 cells per mL but when treated with PA the number of cells by day 5, per mL, was 20 *10^4 cells. The number of cells in the U87 cohort treated with PA was significantly greater than the cell numbers in the MX or no treatment group, p value of 0.0021. This increase in cell number is greater than what was found in the clonogenic assay experiments, however the clonogenic assay was looking at the ability of cells to form colonies and not how they were proliferating within these colonies.
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

Figure 5-13 Growth curves with and without treatment of inhibitors. a) shows proliferation of U87 over 5 days, \(1 \times 10^4\) cells were seeded per well, and counted each day after treatment with either 50μM of PA or MX. B) shows proliferation of U251 over 5 days, \(1 \times 10^4\) cells were seeded per well, and counted each day after treatment with either 50μM of PA or MX. A minimum of 2 repeats were conducted. Statistical significance was measured by 1-way ANOVA.

5.6.2 Cell cycle

The observed increases in proliferation and increased clonogenic ability when cells are treated with inhibitors was further examined by identifying which stage of the cell cycle the cells were in 72 hours after treatment, Figure 5-14. This was then compared to the cell cycle profile of cells following treatment with TMZ and inhibitors, Figure 5-14.

In normal cell cycle analysis of untreated control cells, the majority of cells are in growth with one DNA copy (G1/G0) (60%, 46% and 53% for LN18, U87 and U251 respectively), with less cells passing through synthesis (S) phase or Growth with two copies of DNA in to mitosis (G2/M) which we can see in Figure 5-14. The cells in these
Chapter 5: Investigating pharmacological modulation of BER with different types of radiotherapy

phases is expected as the cells are not synchronized and are cycling through growth phases where they spend the majority of time in G₁. Upon treatment with TMZ there was a change in the predominant phase of the cell they were in, as cells were arresting in S (9% increase for U251) and G2/M phase (37.6% and 18.9% increase for LN18 and U87).

When the cells were treated with inhibitors, the cell cycle distribution of the cell lines shifted from the large G1/G0 of the control to an increase in the number of cells in S phase. This S phase arrest was seen in all cell lines with both inhibitors. In clonogenic assays, LN18 forms a similar number of colonies after MX treatment and an increase after PA, whereas when U87 is treated with either inhibitor more colonies are formed and U251 experiences a small decrease in colonies after treatment with either inhibitor. These trends were also evident in the growth curves. An increase in the percentage of cells in S phase could indicate either an increase in DNA replication and an increased rate of proliferation or, alternatively, an arrest in DNA replication, leaving the cells arrested and unable to progress into mitosis. LN18 and U251 had the largest increase in cells in the S phase; 39.5% and 33.5% in LN18 when treated with PA and MX respectively and 30.7% and 26% in U251 when treated with PA and MX respectively.

When TMZ and inhibitors were combined the cell lines reacted differently and the response to each inhibitor also varied; LN18 when treated with a combination of TMZ and PA showed a similar cell cycle distribution to the treatment with PA alone. When treated with TMZ and MX the cell cycle distribution mimics that of treatment with TMZ alone however the proportion of cells in G2/M phase is increased in the TMZ + MX treatment by 52.3% over the control.

The cell cycle phase distribution in the cell line U87 shifted after treatment with TMZ and MX concurrently and displays an increase in G₂/M (32.2% over control). This treatment appears to create a much greater G₂/M phase shift than when treated with either TMZ, which looks like MX is potentiating the effect of TMZ on the cells to increase this G₂/M phase. This combination of TMZ and MX together leads to a much greater response than either drug does on the cell cycle alone. When U87 is treated with TMZ and PA concurrently the cell cycle profile is very similar to the treatment of PA alone. This is because TMZ and PA as well as PA alone leads to an arrest in the S/early G₂ (40.5% and 43% for PA and TMZ PA together, respectively).
Cell cycle changes after U251 is treated with inhibitors produce a similar response to the shift seen in U87, however the arrest in S phase is greater. However, treatment with TMZ and PA led to an increase in cells in G2/M phase (45% increase). Whereas the G2/M phase arrest when treated with PA alone was 19%. This G2/M phase was larger when U251 was treated with TMZ (26.5%), these results show that PA enhances the effect on the cell cycle produced by TMZ to the G2/M phase.
Figure 5.14 Cell cycle analysis of cells treated with either 25 µM of TMZ, 50 µM of PA, 50 µM of MX or combinations in 3 cell lines; LN18, U87 and U251. There were 2 repeats on each day, with 2 days per cell line. Results were plotted as the mean of all results as percentage of cells in each phase.

5.7 Summary

The results presented in this chapter showed the effects of inhibiting different BER repair proteins in five glioblastoma cell lines (A172, LN18, T98G, U251 and U87). Cells were evaluated for their ability to repair and grow under the influence of these inhibitors of APE1 and PolB. Neither inhibitor was found to be toxic at low doses, which were later selected for the further studies.

MGMT negative cell lines, A172 and U251, were not sensitive to the combination of temozolomide with the BER inhibitors. However, U87 at most doses was shown to have a synergistic relationship with MX and TMZ (CI value). This could indicate an imbalance in
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

BER due to the high AAG levels and the imbalance being further exacerbated by the inhibitor, leading to an increase in the formation of single strand breaks through inhibiting repair of damage, this could explain lower levels of cell survival. This theory is further supported by the analysis of the treatment of all five cell lines with MMS, indicating that the MGMT status no longer matters as repair of the alkylation damage induced, is carried out by the BER process. For the U87 cell line, BER inhibition led to a significant reduction in cell survival to MMS treatment. We propose U87 has a BER imbalance due to high levels of AAG but not of the other downstream BER proteins. In contrast, LN18 should not display BER imbalance since it expresses high levels of all the BER proteins.

In this study the efficacy of protons was confirmed to create a greater level of cell death in comparison to X-rays. Three cell lines (A172, LN18 and U87) were chosen to compare the effects of concurrent treatments of an inhibitor with TMZ and an irradiation type, selected due to their very differing genetic profiles for BER proteins. With both inhibitors it was found that the major protein influencing cell radiation sensitisation was the MGMT status and not the perceived imbalance in BER and therefore inhibiting either APE1 or PolB in MGMT negative cells, could lead to an increase in cell death with irradiation and TMZ.

Although in treatments of TMZ with inhibitors the biggest effects in increasing cell death was seen in the MGMT positive cell lines, LN18 and T98G, the EC50 was not reduced sufficiently for this to be clinically relevant and therefore, when concurrent treatment regimes with radiation were conducted, no increase in cell death was seen as only clinically relevant doses were studied.

Treatment with low doses of the inhibitors led to an increase in cell proliferation in the cell line U87, suggesting BER inhibition was mitogenic in this cell line. However, when also treated with TMZ, significant toxicity was seen and BER inhibition sensitised the cell lines to TMZ-induced cytotoxicity. In contrast, the LN18 cell line had a small increase in colonies formed when treated with PA alone, and a slight decrease when treated with MX, however, a combination of treatments had little to no effect on survival even when combined with radiotherapy; this shows that MGMT status still has the greatest effect on survival even when the BER pathway is inhibited. At clinically relevant
Chapter 5: Investigating pharmacological modulation of BER with different types of radiotherapy

doses only the cell line with an imbalanced BER (U87) was sensitised to combination treatment.

These findings, in conjunction with the proliferation studies showing U87 proliferating faster when treated with PA and forming more colonies when treated with MX, raises some concerns for the current clinical trials in MX. However in vitro studies are not directly comparable to full systems and therefore clinical trials, but the treatment regime, timings and overall effect on cancer cell growth could lead to a poorer prognosis than if the patients were treated with TMZ and radiotherapy alone.

The results showing the interaction between PA, TMZ and radiation were unexpected; knocking out PolB, which is more efficient at blocking its role than inhibiting it, showed an increase in radiosensitivity in a study performed on MEF cells (Vermeulen et al. 2007). However, this increased radiosensitivity did depend on the confluence of the cells being irradiated. Confluent cells were found to be more radiosensitive when PolB was knocked out. This was not tested in our study as cells were already plated at the correct numbers for the colony-forming assay before irradiation. This difference in confluence could explain the difference in results as well as the difference in cell lines used as higher doses of irradiation need higher numbers of cells to be plated.

The experiments into cell cycle were enlightening to show arrest, or maybe potential changes in the growth phase due to inhibitors, however, we note that this experiment could have been strengthened by staining with yo-pro for apoptosis, PI for a live/dead indicator and Hoescht or Draq5, that can be taken up by live cells, to measure DNA content. This would have strengthened the experiment such that not only the cell cycle stage could be measured, but also whether this had led to cell death and further the percentage of cell death per cell line and treatment. This is important as the cells may have arrested in a particular stage of the cell cycle, but would be able to amend the damage and continue replication. There may also be a shift in cell cycle due to an increase in proliferation.
6 Dosiometry on an external beam line for cellular radiation experiments

6.1 Introduction

Cellular radiation with protons at the University of Surrey has traditionally been conducted using the Ion Beam Centre's Vertical microbeam line (Jeynes et al. 2013). There are many positives to irradiating cells using this method, most notably the following: Due to the positioning of the beam the cells are covered in media and are thus maintained in a homeostatic environment during irradiation lowering cellular stress. There are also a number of limitations including:

- cells are not in growth phase as they cannot attach to the polypropylene sheet they are irradiated on.
- it is a low throughput system which means that, for multiple experiments and different treatments, it can need quite a lot of beam time to cover all the irradiation conditions.

To overcome the above drawbacks we decided to explore a new methodology for high throughput studies at the ion beam centre, named HOBBIT.
Figure 6-1 Diagram of the two accelerator set ups at the ion beam centre. The top half of this figure shows the vertical beam line configuration. With a 90° bend in the beam line to allow the cells to be irradiated from underneath. This means that it is unknown how many cell layers are produced, and the cell thickness is increased. In the bottom half of the diagram a linear beamline without a bend leading to a horizontal irradiation system is used. The cells are irradiated without media whilst attached to the plastic wells and much thinner than the rounded vertical cells.
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

As can be seen in figure ...the new methodology the same accelerator was used, however the beamline, which is more commonly used for external beam MeV SIMS, was adapted for use as a broadbeam cellular irradiation station (Merchant et al. 2009). To do this the stage was reprogrammed for correct dosage delivery by changing the movement protocol and blocks were put in place to hold cell dishes. The experimental design was modelled in simulations to work out LET and correct dosing. Gaf Chromic RTQA paper was used to measure beam homogeneity to ensure correct, expected dosage was delivered.

6.1.1 Aims and hypothesis

In a change from the overall hypothesis this chapter details methodology development for a new radiation method. The methodology devised and described in this chapter contributes to the overall results for the hypothesis. However, the aim for this chapter was to devise a new cellular irradiation mechanism, using a different beamline off of the same accelerator. To do this a number of objectives were set:

- Calculate the LET that the cells will receive using this beam line.
- Simulate scatter of particles from the proton beam into the cellular radiation dish
  - Measure the beam homogeneity using RTQA Gafchromic.
- Evaluate and compare the surviving fractions of cells between vertical beam irradiations and horizontal.

6.1.2 Dosimetry

The absorbed dose is measured in Grays (Gy), where one Gy is one Joule of radiation energy absorbed by 1Kg of matter (units J/Kg). A Joule is a unit of energy and can be defined in multiple ways but is the work required to produce one watt of power for one second. To work out the dose given in Gy from an accelerator, be it a medical device or research, the equation used is Equation 1 Dose rate;

\[
\text{Dose rate (Gy/s)} = 1.6 \times 10^{-9} \frac{L \Phi}{\rho}
\]

Where \(L\) is the LET (keV \(\mu\)m\(^{-1}\)), \(\Phi\) is the particle flux (particles per second) and \(\rho\) is the density of the medium (g cm\(^{-3}\)). To use this calculation in determining the dose, we first have to work out the LET through the cells.
6.1.3 Beam stability and homogeneity

To ensure a stable dose distribution to the cells, the beam homogeneity needs first to be measured and then adjusted. This is so that while the beam scans the plate, the dose the cells receive remains consistent. Noting the fluctuation of current through the accelerator during the start and end of the irradiation period, allows for that error to be taken into account. In addition, the step movement of the cell dish holder also had to be set to ensure that the beam was covering the whole cell area and didn't leave gaps or overlap. To measure these things RTQA was employed for its fast colour change and accessibility.

6.2 Results

6.2.1 LET

The LET was calculated using the stopping and range of ions in matter (SRIM) program. SRIM is designed specifically for working out stopping distances and can be used for LET calculations through different materials and is a program which allows you to set up the dimensions and materials of your target area, however it does not allow you to define the geometry of the detector/target area. With SRIM you can simulate the LET changes through the materials and how far the beam will travel through them. With SRIM, the thickness of the material layers is described in angstroms and then the beam energy, ion type and direction can be simulated allowing the LET to be calculated. SRIM is user friendly and hence a collection of different set ups were simulated to measure the change in LET through the target.

The first setup parameter to determine was how far the accelerator window would be from the lid on the plates that contained the cells. In figure Figure 6-2 a) it shows that the air gap used has an effect on the change in LET initially, with a linear trend depending on the gap increase.

In figure Figure 6-2 b) the air gap effect on the LET within the cellular target show that the change in initial LET due to the air gap has a minimal effect on the LET within the cells and the trend in energy loss is comparable. Due to configurational implications within the actual set up, a distance of 2 mm from exit window to the lid was utilised. The LET starts at 11.3 keV/µm and ends at approximately 11.45 keV/µm. We
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

therefore chose an LET to calculate the dose in Gy given as 11.32 keV/µM. The LET changes are documented precisely in Table 15 below.

Figure 6-2 SRIM calculations for LET. Figure a) shows the change in LET through the distance of the cell for different stretches of air gap. Figure b) shows the mean LET dependent on the airgap from silicon window to polypropylene lid.

To calculate the mean LET throughout the cell using SRIM, the LET was taken at different entrance, exit and midpoint and then used to integrate the LET throughout the whole cell. This integrated LET can then be used to calculate the dose given over a set time. These values are displayed in Table 15 below for different distances from the window of the accelerator to the lid of the plate.
<table>
<thead>
<tr>
<th>Air Gap (µm)</th>
<th>0</th>
<th>250</th>
<th>500</th>
<th>600</th>
<th>700</th>
<th>1000</th>
<th>1500</th>
<th>2000</th>
<th>2500</th>
<th>3000</th>
<th>3500</th>
<th>4000</th>
</tr>
</thead>
</table>

| Mean Between 500-700µm | 65.91330 | 66.28018 |
| Mean Between 1mm and 4mm | 11.17175 | 11.23393 |
| Mean Between 1mm and 4mm | 10.89720 | 10.95770 |
| Mean Between 1mm and 4mm | 11.27340 | 11.33296 |
| Mean Between 1mm and 4mm | 11.39963 | 11.47246 |
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

Table 15 SRIM calculations of the changing LET values as the air gap increases from accelerator window to cell dish.

6.2.2 Beam homogeneity and stability

When designing an irradiation system capable of delivering reliable dosimetry, one of the important factors is to ensure the beam does not overlap or misses sections of the area being irradiated. To measure dose delivery GafChromic was used, which is a radiocromic material which has water equivalent dosimetry. GafChromic is comprised of an outer layer of lithium 10,12-pentacosydiayonate micro-crystalline monomers between clear polyester sheets. During irradiation photo-induced polymerisation occurs forming rod shapes as the absorbed dose increases, as does the polymerisation and colour formation. The polymerisation reaction which occurs when the material is exposed to radiation is independent of the energy and can be used over a range of MV beams. There are many different types of GafChromic some of which have high resolution, however, the film used in this case, RTQA, has a lower resolution but is accurate at low doses, important for this experiment.

The control of the radiation delivery was through a “rooks move” control of the end-station, which brought the plate and each well, in front of the beam window at a constant rate, to ensure consistent delivery to all cells. The stepwise movement to ensure broadbeam irradiation by the pencil beam was tested at a number of different steps: 1.5, 1.6, 1.7 μM, a representation is shown in Figure 6-3. As can be seen, the steps of the “rooks move” movement on the end-station were initially too big, resulting in the GafChromic forming striations over the area irradiated, as some parts of the film were not irradiated. As the colour change was instantaneous the stepping action was able to be monitored and corrected at the time of irradiation before cellular radiations had taken place. This was measured at the start of each day of experiments. The gap and colour was not analysed in these films.
Beam homogeneity, correct scanning for dose delivery and beam stability over different days was measured using Gaf Chromic. The methodology used to analyse the RTQA was adapted from a paper by Thomas and Warrington. This paper found that the error is largest in the smaller doses, however, the differences within sensitivity are greatest at lower doses, below 8 Gy (Thomas & Warrington 2006). This is why RTQA was selected for these studies.
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

Therefore, for the analysis of the colour change due to polymerisation the red channel only was analysed on imageJ, adapted from methodology due to Thomas and Warrington in their investigative research into the use of GafChromic in X-ray measurements (Thomas & Warrington 2006). What needs to be taken into account when measuring the variation in dose over different days, is that there is not a linear relationship between colour formation and Gy. As we can see in Figure 6-4 the change in colour increases over dose, as expected, however, not linearly. The highest level of variation over radiations was at 2 Gy with a standard deviation of 1.09. The standard deviation was much lower for the other doses for colour saturation. These high numbers of replicates with low levels of variation within the repeats makes us feel certain that the stability of the beam, its homogeneity in one run and dose replication is accurate.
6.2.3 **Comparison of vertical beam irradiations with HOBBIT irradiations**

The vertical beam line that was used at Surrey has been previously well documented for cellular radiation set up (Jeynes et al. 2013) and used to research radiation in cellular models (Barazzuol et al. 2013). The main use of the vertical beam line was for broadbeam irradiation (Wéra et al. 2014; Jeynes et al. 2013). As detailed in the paper by Jeynes and co-workers, once the current was measured, the timings were changed to give different doses to different droplets on one dish, which meant the current and therefore particle flux remained constant. However, during development of the HOBBIT system it was felt that as the current was readily changed and beam stability was reliable, due to a shorter beamline than the vertical beamline, the time would be held constant. The importance of this extends to ensuring the cells were out of media for the same amount of time. During horizontal beam irradiations the current was changed between doses and the time of irradiation remained the same, increasing the number of particles that the cells are exposed to as the dose increases. Therefore, the only change between radiation styles is time, media coverage and cell angle.

---

![RTQA colour development after irradiation](image)

*Figure 6-4 Analysis of RTQA Gaff Chromic colour change after irradiation with a 3.5 MeV proton beam. Experiments were repeated on each day of irradiations, with each “well” being measured for colour change N=6.*
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

The survival curves produced from vertical and horizontal radiations were fitted with a modified RCR equation Equation 5.

\[
S(D) = \delta \ast (\exp(-\alpha \ast D) + \beta \ast D \ast \exp(-C \ast D))
\]

Where \( \delta \) is a modification constant to adjust the curves to cross the y axis where the data starts rather than at 1. This is important for treatment to cells at the 0 Gy with temozolomide (TMZ). With the parameters being equivalent to no damage, repair and misrepair. No damage to cells \((e^{-\alpha D})\) and the cells which were able to repair themselves \((\beta De^{-\alpha D})\) are parameters which are described within the equation as a whole (Brahme & Lind 2010). However the last section of the equation, which specifies repair is characterised by a misrepair section also \((e^{-\alpha D})\). This means that at higher doses the amount misrepaired and ultimately leading to cell death increases. We used this equation to fit the data as we first noted a difference in the curves produced from vertical protons to horizontal protons which would not allow the commonly used linear quadratic equation (LQ) to fit all the data.

Horizontal protons produce a very obvious low-dose hypersensitivity in the cell lines LN18 and U87 and, after treatment with TMZ, in A172 (Figure 6-5). This hypersensitivity is not evident in cells treated by vertical proton beam irradiations. At 0.5 Gy, without TMZ, in LN18 the survival is 0.90 for vertical and 0.29 for horizontal beam. However, at 1 Gy, the survival fraction is very similar as it has plateaued for horizontal beam irradiation, but has reduced greatly for vertical beam irradiation to 0.243. This difference in sensitivity at low-doses was unusual and the potential reasons for it are explored below in the discussion. As mentioned above, the only differences in how the cells were irradiated were whether they were covered in media or not, the time of irradiation and confluence/attachment of the cells.

After the low-dose of 0.5 Gy, both the vertical and horizontal irradiations produced very similar survival fractions in LN18 and A172, as shown in Table 16. U87 only had a similar survival after 3 Gy and it would be recommended to irradiate up to higher doses to check for survival fraction differences there. At 2 Gy, LN18 had between 20-25% survival and A172 survival was approximately 30%. At 3 Gy the survival fraction
reduces by a further 0.1 for each cell line, this incremental decrease in cellular survival then appears to mimic a linear quadratic survival.

Figure 6-5 RCR Model fitting for clonogenic assay data for protons on 3 cell lines in either vertical irradiation or horizontal irradiation systems.
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

<table>
<thead>
<tr>
<th>Dose</th>
<th>LN18 V</th>
<th>LN18 H</th>
<th>A172 V</th>
<th>A172 H</th>
<th>U87 V</th>
<th>U87 H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.243 +/- 0.023</td>
<td>0.243 +/- 0.093</td>
<td>0.437 +/- 0.083</td>
<td>0.398 +/- 0.164</td>
<td>0.778 +/- 0.042</td>
<td>0.388 +/- 0.109</td>
</tr>
<tr>
<td>2</td>
<td>0.201 +/- 0.050</td>
<td>0.247 +/- 0.062</td>
<td>0.294 +/- 0.121</td>
<td>0.322 +/- 0.101</td>
<td>0.541 +/- 0.054</td>
<td>0.301 +/- 0.087</td>
</tr>
<tr>
<td>3</td>
<td>0.165 +/- 0.073</td>
<td>0.136 +/- 0.046</td>
<td>0.238 +/- 0.095</td>
<td>0.192 +/- 0.050</td>
<td>0.320 +/- 0.061</td>
<td>0.243 +/- 0.051</td>
</tr>
</tbody>
</table>

Table 16 Mean surviving fractions from 1 - 3 Gy for either vertical (V) or horizontal (H) radiation methods. With +/- standard deviation.

6.2.3.1 RCR parameter comparison of vertical versus horizontal irradiation

To directly compare the effects of the different angles of radiation and the general setup, the constants from the RCR fitting were analysed. Studies have shown that the equation could relate to cells that were not damaged from radiation ($e^{-\alpha D}$) and the cells which were able to repair themselves ($\beta De^{-\alpha D}$) (Brahme & Lind 2010). Experiments with glioma cell lines MO59K (DNA protein kinase subunit c (DNA PKC) efficient) and MO59J (DNA PKC deficient) found that, at low doses, Non-homologous end joining (NHEJ) is the main repair pathway and at higher doses it is homologous recombination (HR). Misrepair ($e^{-\alpha D}$), due to the error-prone NHEJ repair mechanism, causes lowers survival at low doses leading to low-dose hypersensitivity, whereas at higher doses both repair pathways work together to repair the DNA damage, however the damage is greater so an increase in cell death still results(Brahme & Lind 2010).
Chapter 6: Dosimetry on an external beam line for cellular radiation experiments

To avoid the low-dose hypersensitivity issue for horizontal irradiation, we used the RCR model parameters displayed in Table 18 and calculated, at a dose of 2 Gy the damaged, undamaged and misrepaired components for vertical and horizontal irradiation of the various cell lines, shown in Table 17. What we can see straight away from the table is that vertical irradiations lead to a higher fraction of cells being left undamaged in all cell lines. This is most evident in the U87 cell line, where the undamaged fraction goes from 1 for vertical beams to 0.29 for horizontal irradiation. Also strongly evident is a decrease in fraction of cells able to repair from the damage caused from horizontal irradiations.

Furthermore, there is also a decrease in the fraction of cells misrepaired when horizontally irradiated. In LN18 the fraction of misrepaired cells goes from 0.69 to 0.01. This decrease in misrepair could explain how the surviving fractions remain similar between the two irradiation protocols. This analysis highlights a big difference in damage occurring to cell lines, which would be interesting to further investigate, but also suggests that the cells have different repair capacity for DSBs between the radiation types.

Although there are studies which justify the parameters of the RCR falling into either undamaged, repaired and misrepaired, for specific cell lines, this may not be the case here. It seems nonsensical to have a negative proportion of cells repaired, however, the negative parameter fits and describes the curve accurately. The negative “repaired” parameter describes the recovery after low dose hypersensitivity, before the continued cell death, represented by the exponential “misrepaired” section.

<table>
<thead>
<tr>
<th></th>
<th>2Gy</th>
<th>undamaged</th>
<th>repaired</th>
<th>misrepaired</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$e^{-\alpha D}$</td>
<td>$\beta De^{-\beta D}$</td>
<td>$e^{-\gamma D}$</td>
</tr>
<tr>
<td>A172 V</td>
<td>0.999</td>
<td>-0.699</td>
<td>0.552</td>
<td></td>
</tr>
<tr>
<td>A172 H</td>
<td>0.346</td>
<td>-0.0452</td>
<td>0.0167</td>
<td></td>
</tr>
<tr>
<td>LN18 V</td>
<td>0.999</td>
<td>-0.658</td>
<td>0.686</td>
<td></td>
</tr>
<tr>
<td>LN18 H</td>
<td>0.292</td>
<td>-0.0596</td>
<td>0.0105</td>
<td></td>
</tr>
<tr>
<td>U87 V</td>
<td>1</td>
<td>-0.498</td>
<td>0.769</td>
<td></td>
</tr>
<tr>
<td>U87 H</td>
<td>0.294</td>
<td>-0.0376</td>
<td>0.00487</td>
<td></td>
</tr>
</tbody>
</table>

Table 17 Parameters of the RCR equation fit to 2 Gy for vertical and horizontal irradiation systems.

Some constraints were put on the constants produced from the curve fitting for the proton irradiations. As there were multiple parameters and a low number of data

Natalie Mayhead - August 2018
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

points it leads to a high degree of freedom for the fitting which allows the equation to fit easily without logical constants being produced. We therefore constrained the alpha parameter to only produce positive constants. This is because irradiation of cells will cause a decrease in cellular survival. This could have been corrected for by creating more data points to higher Gy; as the dose increases the c section of the graph would lead to lower and lower repair, we therefore constrained the parameters for c to also produce positive constants. What we can tell from looking at the constants is that the alpha for vertical irradiations is much lower than those for horizontal irradiations. The larger the alpha value the lower the step in the equation leading to a more gradual change in gradient on the curve. As beta increases, this effects the curve linearly with an increase in survival, which constrains c to be positive to lead to an overall decrease in the curve. As the beta values are negative, this forces the curve to decrease and a smaller c value is needed to describe the overall decrease in survival.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>α</th>
<th>β</th>
<th>c</th>
<th>r2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A172 V</td>
<td>0.0000004579</td>
<td>-0.6328</td>
<td>0.297</td>
<td>0.77</td>
</tr>
<tr>
<td>A172 H</td>
<td>0.5303</td>
<td>-1.356</td>
<td>2.047</td>
<td>0.99</td>
</tr>
<tr>
<td>LN18 V</td>
<td>0.0002327</td>
<td>-0.4793</td>
<td>0.1882</td>
<td>0.57</td>
</tr>
<tr>
<td>LN18 H</td>
<td>0.6158</td>
<td>-2.836</td>
<td>2.278</td>
<td>0.93</td>
</tr>
<tr>
<td>U87 V</td>
<td>1.22E-16</td>
<td>-0.3238</td>
<td>0.1314</td>
<td>0.93</td>
</tr>
<tr>
<td>U87 H</td>
<td>0.6124</td>
<td>-3.859</td>
<td>2.662</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Table 18 Constants and goodness of fit for the RCR model vertical versus horizontal irradiation

6.3 Discussion

In this chapter we aimed to set up a new methodology for radiation of cells in a high-throughput manner. After discovering the LET using SRIM and checking the beam homogeneity and stability using RTQA, the physical data was collected and analysed. From the analysis of the RTQA we can see that beam stability was good over individual irradiations as well as over different days. This led us to have faith in our system and use it to irradiate cells. A comparison of the two different radiation methods was undertaken. This mostly looked at the difference in shape produced after irradiation, as well as a comparison of the means at 1-3 Gy. This showed that although horizontal irradiation
produced low dose hypersensitivity, the overall survival means were similar and within reasonable error margin of each other. Due to the overall similarity in results, it was concluded that a reliable and accurate dose delivery method had been produced. This method of high-throughput horizontal irradiations was used in the proton results seen in chapter 5.

Cells covered in media replicate in vivo conditions as tumours would have a constant stream of nutrients whilst being irradiated within the body. Cells that are entirely dry could implode due to osmosis, leading to an increase in cell death if the cells are left fully dry for too long. Even in the preparation of cells for dry conditions where the cells are fixed, they need to be slowly dried with increasing alcohol concentrations (Ring et al. 2011). Studies have shown that cellular levels of ROS are higher in cell culture in comparison to in vivo situations due to the culture methodology, including the media (Halliwell 2003). Radiation causes the formation of ROS through radiolysis of water (Azzam et al. 2012) and therefore the irradiation of cells within media could lead to an increase in ROS formation which could damage the DNA further. This assault on the cells, from media radiolysis as well as background induction of ROS, could lead to an increase in cell death in cell culture. However, this potential source of cell death is not realised or does not contribute to the overall cell enough as irradiation of the cells without media has higher levels of cell death especially at low doses.

The time of irradiation between doses remained the same for the horizontal irradiation but varied for the vertical beam irradiation of cells, the current and therefore particle flux, was not always the same for the vertical irradiations over different days. The current for each dose, although changed between doses, was kept constant for the horizontal method over different days. This constant change should lower the difference and variability which is clearly seen at low doses on the horizontal irradiation system, however it could cause an effect as the amount of particles entering the cell area is double in the same space of time as the dose before.

The final variable in these different irradiation systems was the confluence of cells and their attachment. Cells on the vertical beam are irradiated within a droplet, detached from the surface of the radiation dish (polypropylene surface), whereas cells irradiated in the HOBBIT system are attached to the plastic well and are approximately 70% confluent. However, in the dosimetry for the vertical beamline described by Jeynes
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

(2013), although the cells are balled up and should be 10µM rather than the calculation they chose for flattened cells of 6µM. This fault in their dosimetry however would not explain the low dose hypersensitivity seen in the HOBBIT system. One of the potential mechanisms of bystander effect is the creation of reactive oxygen species (ROS) (Shao et al. 2005), which the cellular gap junction is important for the communication of this damage and to the transmission of the ROS (Shao et al. 2003). However, in treatment with drugs, confluence is shown to lead to resistance in breast carcinoma cells from doxorubicin (Fang et al. 2007).

Without further testing, we cannot be sure why low-dose hypersensitivity occurs in cells irradiated on the horizontal system and not on the vertical system. However, confluence may be the biggest factor, due to the spread of ROS and other cell signalling chemicals.
7 DISCUSSION

The current survival rate for glioblastoma is only 14.6 months when treated with both temozolomide (TMZ) and X-rays (Stupp et al. 2005a). In Iran only 7.7% of diagnosed cases survive two years post-diagnosis between the periods of 1990-2008 (Ahmadloo et al. 2013). As such, resistance to current treatment modalities needs to be assessed (Lee 2016) and therapeutic improvements need to be made. In this study, we aimed to understand further mechanisms in resistance with a view to find potential areas to exploit in the current treatment.

To do this we measured repair enzymes for base excision repair (BER), O6-methylguanine DNA methyl transferase (MGMT) and accessory proteins needed for the repair of TMZ-induced damage. From this it was tested whether or not these expression levels correlated to the cellular repair proficiency after treatment with TMZ and irradiation.

7.1.1 Naturally occurring BER protein expression levels can lead to an imbalance

Studies have shown that faulty proteins, due to mutations leading to misfolding and functional inadequacy are correlated with different cancer incidence (Koukourakis et al. 2001; Gudmundsdottir & Ashworth 2006; Lavin 2008). Of these mutations, many of the short patch (sp)-BER proteins have been linked to different cancers; colon (Nemec et al. 2012), breast (Duell et al. 2001) and non-small cell lung cancer (Zienolddiny et al. 2006).

However, it has also been shown that in some cancers there is a correlation between prognosis of cancer and expression of BER proteins (Liu et al. 2012; Leguisamo et al. 2017;
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

Dziaman et al. 2014). Some studies in cancer patients go on to link expression to resistance or sensitisation to treatment. For example APE expression in head and neck cancers to chemoresistance (Koukourakis et al. 2001), and variant expression of XRCC1 leading to radiosensitivity (Patrono 2014). This link to prognosis many researchers have postulated by many researchers that an imbalance in BER pathway proteins would lead to sensitization to chemotherapeutics such as temozolomide (Fu, J. a Calvo, et al. 2012; Engelward et al. 1996; Pegg 2011).

An imbalance in BER has previously been created by knocking-out alkyladenine-DNA-glycosylase (AAG) in mouse models which has lead to a decrease in sensitivity to methylmethanesulfonate (MMS) (Engelward et al. 1997; Calvo et al. 2013a). This imbalance in BER lowers the initiation of removal of methylated base damage, and therefore, lowers the amount of toxic intermediates being formed such as AP sites and further down/along the pathway, SSBs. Furthermore, it has been found that over-expressing AAG in breast cancer cells sensitizes them to TMZ (Rinne et al. 2004), as well as in colo-rectal cancer cells (Leguisamo et al. 2017), and ovarian cancer (Fishel et al. 2007).

Notably suppressing APE1, a protein further down in the repair pathway, leads to sensitizing cells to the alkylating agents: MMS, TMZ and carmustine (BCNU) (Silber et al. 2012; Ono et al. 1994).

In this research, the aim was to determine if cell lines had a naturally occurring imbalance in BER, and whether this could be correlated to the cell lines response to treatment, which has not been previously done. Here in, we showed that BER was naturally imbalanced in one cell line, U87, and another cell line showed potential to be imbalanced, LN18. A potential imbalance was determined as the AAG expression levels in LN18 were high, but the expression of the other repair proteins for BER was also higher than measured in the other cell lines. The other cell lines however had a coordinated expression of the BER proteins.

The determination of whether a cell line had an imbalanced or coordinated BER pathway was confirmed through quantitative polymerase chain reaction (qPCR), western blots, activity assays and response to MMS. Through qPCR and western blot experiments it was found that AAG levels varied among cell lines, with highest levels shown in LN18 and U87. LN18 also was shown to have an increase in AAG activity in comparison to U251. It
was felt that if this variation is not accompanied by compensatory changes in levels of enzymes downstream in the pathway, BER imbalance could occur. High levels of AAG in LN18 were previously found and correlated with TMZ resistance (Shao et al. 2015). Two cell lines out of the glioblastoma panel express MGMT; LN18 and T98G, which has also previously been shown in other research (Barazzuol et al. 2012). The differences in expression of MGMT and AAG/BER proteins allowed a good comparison for the role of MGMT and AAG in the treatment of GBM with TMZ and inhibitors.

7.1.2 BER imbalance only sensitizes cells when treated with a SN2 alkylating agent

SN2 alkylating agents such as MMS do not form O\textsuperscript{6}-MeG adducts, and therefore negate the role of MGMT within the cells and the mismatch repair (MMR) efficiency when cells are treated with this agent. Therefore, to elucidate the role of BER alone, and establish if these cell lines exhibit a BER imbalance, three cell lines were chosen for studying the effect of MMS on survival. Specifically, these cells were LN18, T98G and U87. LN18 showed slightly higher levels of all BER proteins and therefore there is a potential for the BER pathway to be imbalanced. T
304er were high levels of AAG in U87, but not other BER proteins leading to an imbalance of the repair, and potentially an increase in the formation of abasic sites. It was shown in this experiment that U87 was more sensitive to MMS than LN18 or T98G. When AAG only is overexpressed and cells are treated with MMS they develop an increased sensitivity to the cytotoxic effects (Calleja et al. 1999; Rinne et al. 2004; Calvo et al. 2013b). Other studies have modulated the AAG expression, leaving the expression of other BER proteins normal, an imbalance in BER is created, and from this we draw the assumption that LN18 may not have an imbalanced BER.

After these measurements were collated we further confirmed that MGMT had the greatest effect on the cell killing proficiency of TMZ, this was demonstrated in the clonogenic assays. MGMT expression protects cells from TMZ induced O\textsuperscript{6}-MeG related apoptosis (Gaspar et al. 2010; Kaina et al. 2007). In this study it was shown that MGMT expression increased the EC50 for LN18 and T98G cells in comparison to other cell lines by approximately 10 fold.

These results showing that the sensitivity to TMZ is solely or predominantly due to MGMT status agrees with other studies; in the study by Hermisson et al., they showed that MGMT expressing cell lines only become sensitive to TMZ effects at doses over 500µM
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

(Hermisson et al. 2006). However, the fold change between the cell lines used in this study, and others have been collated in meta analysis to show that T98G and U87 differ from 5 to 100 fold difference, due to MGMT status (Lee 2016). Although Lee showed the EC50 varied considerably for all cell lines, and each one in this study was mentioned in the meta analysis, what was constant was that MGMT expression was the major determining factor in a higher or lower EC50 for glioblastoma cell lines treated with TMZ (Lee 2016).

The amount of TMZ in blood plasma after treatment in patients has been shown to be around 25-65 µM. In this study we showed therefore that clinically relevant doses of TMZ would not affect MGMT positive glioblastoma cell lines as the EC50 for LN18 and T98G is over 100 µM. This shows that clinically relevant doses of TMZ would not affect GBM patients who express MGMT and therefore they should not be treated with TMZ. Unless significant sensitization can be made with combination treatment for these patients without increasing toxicity in healthy cells, TMZ should not be used.

The MGMT negative cell lines were compared for their TMZ sensitivity, and for BER protein balance. A172 has been shown to be extremely sensitive to TMZ, even in comparison to U251 and U87, the other MGMT negative cell lines. This finding has been shown in other studies, however, it was then correlated with a lack of AAG expression (Agnihotri et al. 2012). Agnihotri measured AAG expression through western blot and immunohistochemistry whereas in this study AAG expression was measured through qPCR, western blot, and activity assays; further testing was done through analysis of microarray data found online to add justification to the results found experimentally. Note that, the cell line, A172, may have been from different sources actually providing different base lines leading to these differences. Whatever the cause for the difference in expression data, both studies found A172 to be the most sensitive cell line. Agnihotri reasoned that within different cell lines that the effects of MGMT and AAG contribute equally to TMZ resistance. To do this he overexpressed AAG in A172, which he found increased resistance to TMZ. Studies using other types of cancer cells showed that in breast cancer cells, AAG sensitised cells to TMZ (Rinne et al. 2004) and in resistant ovarian cell lines over expression of AAG led to a massive increase in sensitivity to TMZ (Fishel et al. 2007). However, in glioma cells, overexpression of AAG leads to only a mild sensitization to TMZ (Tang et al. 2011). The difference in the studies cited above and this one is that the sensitization to TMZ through AAG levels are all manipulated through over expression levels which are higher than would be found naturally; and as such the sensitization is
Chapter 7: Discussion

much greater than would be seen in clinical samples or unmodified cell lines. This may help to explain why U87 is not more sensitive than A172, as although the expression levels are greater in U87, they are not over expressed. Furthermore, there are other repair pathways involved in TMZ sensitization, such as MMR, which were not measured.

Other studies have also shown A172 to have high levels of AAG with some studies suggesting that a high AAG expression has been linked as a hallmark of GBM (Liu et al. 2012; Tang et al. 2011). This shows that although a BER imbalance sensitizes cells to MMS, the interaction of MMR on the O6-MeG adduct formed by TMZ could create or exhibit a greater influence to cell death and therefore the sensitization of cells to TMZ after MGMT expression.

In this study we find that MGMT status affects glioblastoma cell lines sensitization to treatment more than BER status. The most sensitive cell line to TMZ was A172 which does not have an imbalance in BER that we could detect or high AAG levels.

Therefore, we accept our null hypothesis that a natural imbalance in BER without modulation does not sensitize glioblastoma cells to TMZ treatment. U87, which not only has a high AAG level but, due to the median expression of other proteins, would then have an imbalance in BER which, if our theory was right, would have led to a greater sensitivity to TMZ treatment (Fu, J. a. Calvo, et al. 2012). This was not seen and we therefore conclude that the treatment with TMZ is not sensitized by BER imbalance or high AAG levels.

7.1.3 X-rays and temozolomide are additive

For MGMT positive cell lines there is no combined effect of treatment with TMZ and X-rays (Barazzuol et al. 2012). As no effect is seen, the recommendations made by other researchers (Chen et al. 2013; Wick et al. 2012; Stupp et al. 2009) for using MGMT as a biomarker for whether to treat a patient with TMZ or a different chemotherapeutic is also recommended from the outcome of this work. In MGMT negative cell lines the addition of TMZ increased cell killing greatly. In patients who do not express MGMT within the tumours this treatment modality is currently the best course of treatment.

7.1.4 MGMT status important in sensitizing cells to TMZ with MX and PA

We next modulated BER through the use of inhibitors at clinically relevant doses for methoxyamine (MX) and similarly low doses for pamoic acid (PA). These studies
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

aimed to try to create an imbalance in cell lines, except in U87 where we aimed to further deregulate the coordination of BER to increase sensitivity to TMZ, as well as to ascertain if the deregulation of BER leads to sensitization of glioblastoma to current treatment which could be used clinically. MX is currently in clinical trials (Yang et al. 2012) as it binds to AP-sites, blocking their repair (Talpaert-Borlè 1987). However, due to the poor specificity, PA (Hazan et al. 2008) or other PolB inhibitors are not (H.-Y. Hu et al. 2004).

At clinically doses close to the EC50 of TMZ and MX, MGMT-positive cell lines were sensitized to TMZ (T98G and LN18). These findings for T98G agree with others studies (Montaldi & Sakamoto-Hojo 2013), however, the sensitization is not great. This low level sensitisation is seen in other studies, however they find that over expression of AAG and treatment of MX leads to even further sensitization (Tang et al. 2011). We do not see an increase in sensitization of LN18 to TMZ due to its high AAG expression levels, however this could be due to a number of reasons; The AAG levels in LN18 are not modulated, unlike the studies above, and much lower levels of MX are used in this study, to keep it clinically relevant. However, we did find that the interaction of TMZ and MX in the cell line LN18 was synergistic.

The effect of MX on sensitizing the MGMT-negative cell lines to TMZ was not significant. Although there was a decrease in viability after treatment with MX and TMZ. This is similar to results found from knocking out APE and treating with TMZ in U87, however, Montaldi found knocking down APE and treating with TMZ led to an increase in SSBs (Montaldi et al. 2015). However, we did find that the AAG highly expressing cell line, U87 had TMZ and MX interact synergistically to cause cell death. In the cell line U251 higher doses of TMZ and MX lead to a synergistic interaction, and this was not due to AAG status. These findings have not been found before, and the further sensitization of MGMT-negative cells could still be of importance to help increase survival rates in MGMT-negative patients too.

The combined treatment of PA and TMZ lead to a non-significant increase in sensitivity in all cell lines. In TMZ resistant colon cancer cells, PA sensitises cells to the effects of TMZ (Jaiswal et al. 2009). Studies have shown that inhibiting PolB sensitises cells to TMZ, however, an increase in AAG expression did not further enhance the cells sensitization to TMZ from PolB inhibition (Gregory C. Stachelek, Shibani Dalal, Katherine
A. Donigan & Joann B. Sweasy 2010). The greater sensitization seen in the study by Gregory may be due to the difference in PolB inhibitor used. One study shows that the greatest sensitization to alkylating agents would be to inhibit, through knockout and inhibition, both APE1 and PolB (Horton & Wilson 2007) and as such this could be explored further in an attempt to understand the effect of BER on TMZ resistance.

The MGMT status of the cells affects whether or not they will be sensitised by the inhibitor to TMZ. This could be due to the fact that the levels of unrepaired BER intermediates is still too low in comparison to the more toxic O\textsuperscript{6}-methylguanine adducts, which lead to apoptosis in MGMT negative cells but are removed in MGMT-positive cells. Another repair pathway which needs to be taken into account whilst correlating repair of these cell lines is MMR as the MGMT-negative cell lines repair the O\textsuperscript{6}-methylguanine adducts through this pathway, and could lead to further differences in sensitivity.

7.1.5 Protons are more cytotoxic than X-rays

Within the investigation into dysregulation of BER through the use of inhibitors, different types of radiation and the effects they have on cell death fractions were also studied. It is well known that protons cause more damage than X-rays (Goodhead 2006). Due to the difference in damage from different radiation sources relative biological effectiveness (RBE) is taken into account in treatment planning for protons (Paganetti et al. 2002; Carabe et al. 2013; Burigo et al. 2014).

We found hypersensitivity to protons was found at low doses in all cell lines, which were potentiated by the addition of TMZ and inhibitors together. This furthers the case for use of protons in the treatment of hard to treat cancers, as well as adding validity and importance to the building of the 2 proton therapy centres in the UK. The potential reasons for this are discussed further on in this chapter.

7.1.6 Inhibiting BER in combination with radiotherapy and TMZ offers benefit only in MGMT negative cells

MGMT does not have a role in the toxicity of damage caused by radiation as radiation does not cause O\textsuperscript{6}-MeG lesions. Studies have previously found that overexpressing APE in U251 and U87 leads to an increase in radioresistance (Naidu et al. 2010). AP-sites are formed from radiation damage making MX a good drug for not just co-treatment with TMZ, but also with radiation.
In this study low doses of radiation were used and it was found that MX produced a better cell killing response at 3 Gy. Osteosarcoma cells are sensitized to $\gamma$-radiation by MX treatment at doses from 2-6 Gy (Montaldi et al. 2014). We found that MX led to sensitization in the MGMT-negative cell lines to X-rays. Furthermore, it has been shown that non-small cell lung cancer cells are sensitised to $\gamma$-irradiation through 3mM MX treatment (Oleinick et al. 2016). The results for U87 being sensitized to X-rays from MX are comparable to results showing an increase in DNA damage and slow repair after MX treatment and X-rays (Neshasteh-Riz et al. 2008). In this study it was further found that the combination of TMZ, MX and X-rays in MGMT-positive cell lines led to an increase in sensitisation, that was so great for U87, it could not be calculated from the data points collected. The enhanced sensitization to treatment to MX, TMZ and X-rays in U87 could be linked to the high AAG expression and lack of MGMT.

When the radiation source was protons, the cellular response to MX was different, with U87 not being sensitized at all, whilst A172 was. However, treatment with MX and TMZ with protons do not produce sensitization or an increase in cell death.

The response of LN18 with radiation, TMZ and inhibitors was not significantly different to any other combination. Therefore we conclude that at clinically relevant levels MGMT expression affects the cell lines sensitization to trimodal therapy. But furthermore, the effect of cell killing is not enhanced by radiation and MX. However, this is not the case when the radiation source was protons in the treatment of LN18, as protons alone lowered survival more than combination therapies.

The role of PolB in radiation damage repair is important as radiation can cause single strand breaks which are then repaired by PolB through BER. However, damage by radiation causes predominantly double strand breaks, more so when treating with protons, and as such these double strand breaks are repaired predominantly by NHEJ or HR (Gerelchuluun et al. 2015; Hunt et al. 2013).

In experiments with PA it was found it can lead to a decrease in cell viability therefore low doses were used within the rest of the studies conducted. PA binds to the
Chapter 7: Discussion

8kDa domain on PolB which binds to single stranded DNA and has a d’RP lyase activity (Beard & Wilson 2006). PA is not a very specific PolB inhibitor, hence there is a push to develop improved versions (Barakat et al. 2012; Gao et al. 2008). A study by another lab showed that inhibiting PolB with PA led to a decrease in DNA synthesis (H. Y. Hu et al. 2004), perhaps through unspecific binding to polymerases involved in DNA synthesis, which could explain the short term toxicity of the cell lines in increasing presence of PA. Because of this, low doses of PA were used in these studies.

In this study it was shown that PA and X-rays lead to an increase in cell survival. There was little difference between proton and PA survival fractions. This was unexpected as PolB knock-outs increased radiosensitivity (Vermeulen et al. 2007). Of course, due to the low specificity of PA to PolB, these studies are not easy to compare. However, the combination of PA, TMZ and radiation increased cell death in MGMT-negative cell lines but not MGMT positive LN18.

During this study we found that the interaction between radiation and inhibitors in MGMT-negative cell lines is worth further investigation. Both inhibitors increased the sensitivity of the cell line to the radiation but, in conjunction with TMZ the enhancement was so great in U87 that a ratio could not be calculated. The cause of this difference in reaction would be important for the current clinical trials in MX, and could be tested in individuals with the best genetic chance to experience benefit from this treatment combination. The increase in sensitivity in combination with TMZ and radiation could be associated with the high AAG levels and imbalanced BER. We therefore conclude that in glioblastoma cells with a propensity to have an imbalanced BER, if further deregulated with BER inhibitors, specifically MX, the combination of TMZ and radiation enhances cell death rates in MGMT negative cells.

We further elucidated the role of these inhibitors in potentiating cell growth in certain cell lines when cells were treated with inhibitors alone and is critical for these patients in current clinical trials. Conversely, other studies have shown that MX reduces cell growth and viability, in osteosarcoma cell lines (Montaldi et al. 2014), which, in this work, is seen in every cell line but U87. This difference in response to viability could be due to BER expression, however, this would need to be further tested to draw conclusions. These questions should be answered before further clinical trials. Regardless of how these inhibitors affect cell lines alone, when trimodal treatment is utilized in both MGMT
negative cell lines, there was an increase in cell death, and further investigation could discover what type of cell death the cells undergo.

The different radiation methodologies showed a difference in response at low doses. With low doses of horizontal protons displaying low dose hypersensitivity, whilst vertical irradiation did not show this. As discussed in chapter 6.2.3.1 low dose hypersensitivity has been linked to repair capacity (Chalmers et al. 2004; Daşu & Denekamp 2000). As well as cell cycle arrest and apoptosis (Marples 2004; Enns et al. 2004). However, in these experiments a difference in repair capacity leading to changes in cell cycle distribution response to apoptosis is unlikely, and other mechanisms should be explored.

### 7.2 Future work

This work characterised how MGMT-positive cell lines cannot be sensitized to treatment at clinically relevant doses, despite potential natural, non-modified BER imbalances. In our work with protons we showed that the interaction between a combination of drugs and protons in certain cell lines lead to much lower relative biological effect then when treated with X-rays. It was shown that MX has an overall better inhibitory effect leading to an increase in sensitization to TMZ and radiation in MGMT negative cell lines. However, neither inhibitor or treatment combination lead to an increase in cell death for MGMT-positive cell lines. Furthermore, AAG levels or BER imbalance may have had an influence in sensitivity in trimodal treatment in an MGMT dependent manner. From this further questions were raised which could be addressed.

Within this work we started correlating survival after treatment with TMZ to the expression level of BER proteins. This, however, is not the only repair pathway of interest when treating with TMZ. As mentioned in the introduction and through-out this work, the BER pathway repairs the majority of the adducts formed by TMZ treatment, however, the suicide enzyme, MGMT, repairs the lesion on the O6-guanine and mismatch repair incorrectly processes this damage. This lesion if attempted to be repaired through the MMR pathway, leads to apoptosis due to a futile repair cycle leading to double strand breaks and cellular exhaustion leading to apoptosis. In chapter 4.3.1, MMR proteins were evaluated from microarray data from other studies only, and as such cannot be reliably traced to the same cell lines used in this study. As such, for future works we would
measure the expression levels of MMR proteins, and try to correlate this with the MGMT-negative cell lines sensitivity to TMZ alone.

One of the interesting results of the inhibitor studies was that when cells were treated alone there was an increase in colonies formed, cell growth and cell cycle. As this was further investigated it was found that inhibitors alone created a shift in the cell cycle, leading to an arrest in S/G2/M. This should be further examined, as MX is currently in clinical studies a few questions should be answered; is this a problem with the timing of the delivery of the inhibitor and as such would these effects be seen if MX, TMZ and radiation are given in too long a time frame. To measure this staggered time points could be taken on differing treatment regimens to measure the following end points; Survival via clonogenic assay and proliferation via Bromine deoxyribonucleotide Uracil (BrdU).

Furthermore, from this line of study the cell cycle measurements were very interesting and needs to be further tested. The work with inhibitors and protons needs to be further explored by looking into the changes in cell cycle in combination treatment. Furthermore to this measuring for cell cycle check point kinases and their regulation to confirm where in the cell cycle the cells are arresting.

7.2.1 Conclusions

Despite BER imbalances naturally occurring in glioblastoma cell lines, these imbalances had little effect on the response of the cells to treatment. The modulation of the BER pathway during treatment with TMZ and radiation only caused an increase in cell death in MGMT-negative cell lines, but no improvement was shown in MGMT-positive cell lines. To increase the efficacy of treatment regimes in glioblastoma for MGMT-positive cell lines a different style of treatment should be used, especially as clinical trials found that inhibiting MGMT with O6-benzylguanine lead to an increase in toxic side effects.
Modulation of the Base Excision Repair (BER) pathway in the treatment of glioblastoma with radiotherapy

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168

Natalie Mayhead - August 2018
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Natalie Mayhead - August 2018
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Natalie Mayhead - August 2018 175
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