Poly-ADP-Ribose-Polymerase as a Therapeutic Target in Paediatric Diffuse Intrinsic Pontine Glioma and Paediatric High Grade Astrocytoma

by

Yevgen Chornenkyy

A thesis submitted in conformity with the requirements for the degree of Master of Science Laboratory Medicine and Pathobiology University of Toronto

© Copyright by Yevgen Chornenkyy 2015
Poly-ADP-Ribose-Polymerase as a Therapeutic Target in Paediatric Diffuse Intrinsic Pontine Gliomas and Paediatric High Grade Astrocytoma

Yevgen Chornenkyy

Master of Science

Laboratory Medicine and Pathobiology
University of Toronto

2015

Abstract

Paediatric high-grade astrocytomas (pHGA) and diffuse-intrinsic-pontine-gliomas (DIPG) are devastating paediatric malignancies for which there are no effective therapies. Previous evidence found Poly-ADP-Ribose-Polymerase 1 (PARP1) over-expressed in DIPG and pHGA. Immunohistochemical and western-blot analysis was performed on pHGA and DIPG patient tumour samples and cell lines to establish PARP1 expression. Three PARP inhibitors, Veliparib, Olaparib, and Niraparib, were used to study PARP inhibition in multiple pHGA and DIPG cell lines and intracranial xenografts. PARP1 was expressed in the majority of pHGA and DIPG patient samples and cell lines and PARP inhibition in vitro resulted in growth arrest and/or apoptosis. Niraparib was the most effective monotherapeutic agent. Niraparib reduced the rate of DNA repair and sensitized cells in vitro to ionizing radiation (IR). In vivo, Niraparib when combined with IR, inhibited PARP1 and extended survival by 40%. Therefore, PARP1 may serve as a potential therapeutic target in pHGA and DIPG.
Acknowledgments

The past two years at the Hawkins Laboratory have been an amazing, exciting, and an educational experience. This experience would not have been possible without the people that I met and interacted with on a daily basis. These individuals offered insight, guidance, and support when I encountered challenges during my project, and celebrated the fruits of hard labour when my experiments worked and my project moved forwards.

The biggest thank you goes to Dr. Cynthia Hawkins, and my committee members, Dr. Uri Tabori, and Dr. Jane McGlade, for their indispensable mentorship, guidance, and critique of my work. By being part of my committee and working together Dr. Hawkins, Dr. Tabori, and Dr. McGlade demonstrated an amazing propensity to orient novice students, like myself, in the right direction.

The second biggest thank you goes to Dr. Sameer Agnihotri who taught me how to prioritize my time in the lab, critically evaluate my work, and think for myself. Dr. Agnihotri constantly challenged, and continues to challenge me with new ideas. He pushed me beyond my limits and for that I am grateful. I personally believe he deserves a medal of honor for putting up with me on a regular basis.

I would like to give many thanks to the following members of the Hawkins Lab, both past and present, for their wonderful help: Dr. Sanja Pajovic, Dr. Pawel Buczkowicz, Patricia Rakopoulos, Mark Barszczyk, Andrew Morrison, Scott Ryall, Dr. Man Yu, Dr. Robert Siddaway, and Stephie Leung. I am also very grateful for the help provided by Kevin Wang, Daniel Picard, Brian Golburn, and Dr. Vijay Ramaswamy. Finally I would like to thank my family for their support and patience.
Table of Contents

Acknowledgments.............................................................................................................. iii
Table of Contents.............................................................................................................. iv
List of Figures ...................................................................................................................... vii
Abbreviations.................................................................................................................... viii

Chapter 1 Introduction ..................................................................................................... 1
  1 Cancer Biology ............................................................................................................. 1
    1.1 Hallmarks of Cancer ............................................................................................... 1
  2 DNA Damage – Sources and Responses ...................................................................... 2
    2.1 Three Sources of DNA Damage ........................................................................... 2
    2.2 Cellular Responses to DNA Damage .................................................................. 3
    2.3 Base Excision Repair ......................................................................................... 3
    2.4 Homologous Recombination .............................................................................. 5
    2.5 BRCA1, BRCA2 and PARP1 – A Complicated Relationship ............................. 7
    2.6 Non-Homologous End Joining ........................................................................... 8
    2.7 The Plot Thickens – The Tale of Two Non-Homologous End Joining Mechanisms ... 9
  3 Poly-ADP-Ribose Polymerase ..................................................................................... 10
    3.1 One Big Happy Family – The PARP Superfamily .............................................. 10
    3.2 Structure of Poly-ADP-Ribose Polymerase 1 ...................................................... 11
    3.3 Function of Poly-ADP-Ribose Polymerase 1 ....................................................... 12
      3.3.1 PARP1, DNA repair, and Genomic Stability ............................................ 15
      3.3.2 PARP1 in Transcription and Inflammation ............................................. 18
      3.3.3 PARP1 and Metabolism ........................................................................... 18
  4 Poly-ADP-Ribose Polymerase as a Therapeutic Target ........................................... 19
    4.1 PARP Inhibitors – Structure and Function ....................................................... 19
4.2 PARP Inhibition – Chemosensitization? ................................................................. 21
4.3 PARP Inhibition – Radiosensitization? ................................................................. 21
4.4 PARP Inhibition – Monotherapy? ........................................................................... 22
5 Paediatric High-Grade Astrocytoma and Diffuse Intrinsic Pontine Glioma ................. 24
  5.1 Background Information .......................................................................................... 24
  5.2 Known Genetic and Molecular Alterations in Paediatric High Grade Astrocytoma and
      Diffuse Intrinsic Pontine Glioma ............................................................................ 25
  5.3 Pre-Clinical Studies of Paediatric High-Grade Astrocytoma and Diffuse Intrinsic
      Pontine Glioma ....................................................................................................... 26
6 Hypothesis .................................................................................................................... 27
Chapter 2 Methods ........................................................................................................ 29
7 Methods ....................................................................................................................... 29
  1.1 Cell Culture ............................................................................................................. 29
  7.1 Clinical Poly-ADP-Ribose Polymerase Inhibitors ..................................................... 30
  7.2 MTT Cell Proliferation ............................................................................................ 30
  7.3 Colony Forming Assay ........................................................................................... 30
  7.4 Cell Counts and Trypan Blue Cell Viability .............................................................. 30
  7.5 Immunofluorescence ............................................................................................... 30
  7.6 Western Blotting and Densitometry ....................................................................... 31
  7.7 Immunohistochemical Staining and Scoring ........................................................... 31
  7.8 PARP1 Silencing .................................................................................................... 32
  7.9 Cell Cycle Analysis .................................................................................................. 32
  7.10 Evaluation of PARP Inhibition in Combination With Stereotactic Cortical Radiation
      in an Orthotopic Model of Paediatric High Grade Astrocytoma............................... 32
  7.11 Statistics .............................................................................................................. 33
Chapter 3 Results ............................................................................................................ 34
8 Results .......................................................................................................................... 34
8.1 Poly-ADP-Ribose Polymerase 1 is expressed in Paediatric High Grade Astrocytoma and Diffuse Intrinsic Pontine Glioma Patient Samples and Patient Derived Cell Lines, and PARP1 Loss Reduces Proliferation. ................................................................. 34

8.2 Compared to Veliparib and Olaparib, Niraparib Monotherapy is More Effective at Reducing Tumour Cell Growth. ......................................................................................... 37

8.3 Niraparib Monotherapy Induces DNA Damage and Reduces Cell Proliferation. ..........39

8.4 Induction of Growth Arrest and Apoptosis after Niraparib Treatment is Cell Type Specific ................................................................................................................................. 40

8.5 Low Dose Niraparib Results in Persistence of Radiation Induced DNA Damage and Sensitizes Tumour Cells to Ionizing Radiation. ................................................................. 42

Chapter 4 Discussion and Future Directions ................................................................................................................................................................................................. 46

9 Discussion and Future Directions ................................................................................................................................. 46

9.1 Treating Paediatric High Grade Astrocytoma \textit{in vitro}: Niraparib Inhibits Growth ..........46

9.2 Treating Paediatric High Grade Astrocytoma \textit{in vitro}: ......................................................... 46

9.3 Treating Paediatric High Grade Astrocytoma \textit{in vivo}: Niraparib Extends Survival ..........48

9.4 Conclusions ................................................................................................................................. 49

9.5 Future Directions .......................................................................................................................... 49

10 References .................................................................................................................................51
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Cartoon demonstrating the three categories of DNA damage and some examples of each category.</td>
</tr>
<tr>
<td>2</td>
<td>Base Excision Repair</td>
</tr>
<tr>
<td>3</td>
<td>Homologues Recombination</td>
</tr>
<tr>
<td>4</td>
<td>Simple overview of non-homologous-end joining</td>
</tr>
<tr>
<td>5</td>
<td>Schematic of NHEJ pathways including D-NHEJ and B-NHEJ</td>
</tr>
<tr>
<td>6</td>
<td>Protein Structure of Poly-ADP-Ribose Polymerase 1</td>
</tr>
<tr>
<td>7</td>
<td>PARP1 Function and Regulation</td>
</tr>
<tr>
<td>8</td>
<td>PARP1 role in Double Strand Repair</td>
</tr>
<tr>
<td>9</td>
<td>Chemical structures of PARP inhibitors</td>
</tr>
<tr>
<td>10</td>
<td>PARP1 is present in pHGA and DIPG patient samples</td>
</tr>
<tr>
<td>11</td>
<td>PARP1 is expressed in pHGA and DIPG derived cell lines and PARP1 loss affects cell proliferation</td>
</tr>
<tr>
<td>12</td>
<td>Effects of PARP inhibitors on PARP activity and cell growth</td>
</tr>
<tr>
<td>13</td>
<td>Niraparib treatment increases DNA damage and decreases and proliferation.</td>
</tr>
<tr>
<td>14</td>
<td>Induction of apoptosis and cell cycle arrest after Niraparib treatment is cell type specific</td>
</tr>
<tr>
<td>15</td>
<td>Low doses of Niraparib reduces the rate of DNA repair</td>
</tr>
<tr>
<td>16</td>
<td>Low doses of Niraparib reduces the rate of DNA repair and sensitizes cells to ionizing radiation</td>
</tr>
<tr>
<td>17</td>
<td>Niraparib in combination with ionizing radiation extends survival</td>
</tr>
</tbody>
</table>
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\gamma$H2AX</td>
<td>Phosphorylated (Ser139) histone variant 2AX</td>
</tr>
<tr>
<td>3-AB</td>
<td>3-aminobenzamide</td>
</tr>
<tr>
<td>ALT</td>
<td>Alternative lengthening of telomeres</td>
</tr>
<tr>
<td>AP</td>
<td>Apurinic/apyrimidinic</td>
</tr>
<tr>
<td>APE</td>
<td>Apurinic/apyrimidinic Endonuclease</td>
</tr>
<tr>
<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
</tr>
<tr>
<td>ATR</td>
<td>Ataxia telangiectasia Rad3-related</td>
</tr>
<tr>
<td>AURKB</td>
<td>Aurora Kinase B</td>
</tr>
<tr>
<td>BER</td>
<td>Base excision repair</td>
</tr>
<tr>
<td>BLM</td>
<td>Bloom’s syndrome helicase</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer 1, early onset</td>
</tr>
<tr>
<td>BRCA2</td>
<td>Breast cancer 2, early onset</td>
</tr>
<tr>
<td>BRCT</td>
<td>BRCA1 C-terminus domain</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CDK4</td>
<td>Cyclin dependent kinase 4</td>
</tr>
<tr>
<td>Chk1</td>
<td>Checkpoint kinase 1</td>
</tr>
<tr>
<td>Chk2</td>
<td>Checkpoint kinase 2</td>
</tr>
<tr>
<td>CtIP</td>
<td>C-terminal-binding protein 1</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle media</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA-PKcs</td>
<td>DNA-dependent protein kinase catalytic subunit</td>
</tr>
<tr>
<td>EXO1</td>
<td>Exonuclease 1</td>
</tr>
<tr>
<td>ds</td>
<td>double strand or double stranded</td>
</tr>
<tr>
<td>DSB</td>
<td>Double stranded breaks</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epithelial growth factor receptor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin-fixed paraffin-embedded</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HeLa</td>
<td>Cell line derived from Henrietta Lacks’ uterine adenocarcinoma</td>
</tr>
<tr>
<td>HR</td>
<td>Homologous recombination</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High-mobility-group box 1</td>
</tr>
<tr>
<td>HDR</td>
<td>Homology driven repair</td>
</tr>
<tr>
<td>HJ</td>
<td>Holliday junctions</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing radiation</td>
</tr>
<tr>
<td>IUR</td>
<td>Internal untranslated region</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Kiel-clone 67</td>
</tr>
</tbody>
</table>
Ku70/XRCC6 X-ray repair cross-complementing protein 6

Ku80/XRCC5 X-ray repair cross-complementing protein 5

LOH Loss of heterozygosity

LPS Lipopolysaccharide

MPG Methylpurine-DNA Glycosylase

MRE Meiotic recombination 11 homolog A

MRN Meiotic recombination 11 homolog A/RAD50/Nibrin complex

MTT (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide)

NER Nucleotide excision repair

NHEJ Non-homologous end joining

B-NHEJ Backup non-homologous end joining

D-NHEJ DNA-PKcs dependent non-homologous end joining

NAD Nicotinamide adenine dinucleotide

NF-κB Nuclear factor kappa-light-chain-enhancer

NBS1 Nibrin

NOD Non-obese diabetic

NSG NOD/SCID/Gamma immunodeficient mice

OS Overall survival

PAR Poly-ADP-ribose

PARylation Poly-ADP-ribosylation
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PARP1</td>
<td>Poly-ADP-ribose Polymerase 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBZ</td>
<td>Poly(ADP-ribose)-binding zinc finger motif</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell antigen A</td>
</tr>
<tr>
<td>PDGFRA</td>
<td>Platelet derived growth factor receptor α</td>
</tr>
<tr>
<td>PDGFB</td>
<td>Platelet derived growth factor beta</td>
</tr>
<tr>
<td>PFS</td>
<td>Progression-free survival</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator-activated receptor gamma co-activator 1- alpha</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIKK</td>
<td>phosphatidylinositol 3-kinase-like kinase</td>
</tr>
<tr>
<td>PNK</td>
<td>Polynucleotide 5’hydroxyl kinase</td>
</tr>
<tr>
<td>Pol β</td>
<td>DNA polymerase Beta</td>
</tr>
<tr>
<td>Pol μ</td>
<td>DNA polymerase Mu</td>
</tr>
<tr>
<td>Pol λ</td>
<td>DNA polymerase Lambda</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPA</td>
<td>Replicating protein A</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate - polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>SIRT1</td>
<td>Sirtuin 1</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>ss</td>
<td>single stranded</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue micro array</td>
</tr>
<tr>
<td>TMZ</td>
<td>Temozolomide</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor - alpha</td>
</tr>
<tr>
<td>XRCC1</td>
<td>X-ray repair cross-complementing protein 1</td>
</tr>
<tr>
<td>XRCC4</td>
<td>X-ray repair cross-complementing protein 1</td>
</tr>
</tbody>
</table>
Chapter 1 Introduction

"That which does not kill us makes us stronger."
– Friedrich Nietzsche

1 Cancer Biology

1.1 Hallmarks of Cancer

According to the somatic mutation theory, tumorigenesis in humans is a multistep process involving genetic alterations that drive transformation of normal human cells into malignant derivatives (1). Depending on the nature of genetic alteration, a human cell may obtain a growth advantage leading to its progressive conversion into a cancerous cell. Two leading edge reviews by Hanahan and Weinberg proposed that a normal human cell must acquire certain hallmarks to be considered cancerous (1, 2). Underlying these eight hallmarks are two enabling characteristics – tumour-promoting inflammation and genomic instability.

Cellular systems preserving genomic integrity safeguard the DNA to ensure low mutation rates, and high fidelity during transfer of genetic information to daughter cells (1). Mutations impairing genomic integrity can result in accumulation of activating mutations in oncogenes and loss-of-function mutations in tumour suppressors – moving the cell towards a malignant phenotype. The malignant cell may further suppress genomic stability to gain a survival advantage through numerous opportunistic mutations even though there is a severe cost to its own somatic fitness (3). High level of genomic instability allows the cancer cell to rapidly adapt to changing environments, however it is also its Achilles heel. With the majority of genomic integrity networks in disarray, the cancer cell becomes dependent on a handful of DNA repair pathways that keep it on precipice of a genomic catastrophe. Therapies, such as radiation or chemotherapy, that induce additional DNA damage, combined with small molecular inhibitors that target the remaining DNA repair pathways can be used to push the cancer cell over the threshold and into oblivion – cell death.
2 DNA Damage – Sources and Responses

2.1 Three Sources of DNA Damage

DNA molecules are under constant assault by a variety of agents and processes that can be divided into three categories. First, the replication of DNA sequences by DNA polymerases during S phase of the cell cycle is subject to low but nevertheless significant level of error (27). This occurs when incorrect nucleotides are incorporated instead of their complementary counterparts. Second, in the absence of replication errors or attack by mutagenic agents, nucleotides within the DNA undergo spontaneous chemical changes, which alter the base sequence and the information stored within the DNA molecule (28). Finally, DNA molecules may be attacked by various molecules, including those generated endogenously by normal metabolism as well as agents of exogenous origin that are introduced from outside of our bodies (29).

Sources of DNA Damage

1. Replication Errors
   - Strand slippage
   - Strand mispairing

2. Spontaneous Nucleotide Changes
   - Depurination
   - Depyrimidation
   - Deamination

3. Endogenous and Exogenous Agents
   - X-rays
   - UV radiation
   - Reactive oxygen species
   - Chemical Agents

Figure 1 – The three categories of DNA damage and some examples of the causative agents of each category.
2.2 Cellular Responses to DNA Damage

The mammalian cell contains a plethora of pathways for detecting and repairing different types of DNA lesions. For the sake of simplicity, DNA repair pathways can be divided into two categories. The first category consists of DNA repair pathways that use a template, which can be a sister chromatid or the opposite strand of the DNA; these are termed homology driven repair (HDR) pathways. Base excision repair (BER) and homologous recombination (HR) are the relevant examples of this category (35). The second category consists of DNA repair pathways that do not require a template and make use of random regions of homology at sites of DNA damage in order to tether the broken strands to one another. A great example of a DNA repair mechanism that falls into the latter category is non-homologous end joining (NHEJ) (35).

2.3 Base Excision Repair

The fundamental purpose of BER is to remove chemically altered bases that produce small distortions in the DNA double helix (larger helix distorting modifications are removed by nucleotide excision repair [NER]). Some of the common chemical modifications repaired by BER include alkylated nucleotides, spontaneous deaminations of cytosine to uracil, and of methylated cytosine (5-methyl-cytosine) to thymine. The specificity of BER depends on enzymes that recognize the modified nucleotide and cleave the covalent bond attaching the nucleotide to the deoxyribose sugar – DNA glycosylases. For example 3-methylpurine-DNA-glycosylase recognizes and cleaves N₃ methyladenine residues (adenine methylated at the N3 position), uracil-DNA-glycosylase removes uracil from DNA, and T:G glycosylase removes thymine produced by deamination of 5-methyl-cytosine (12, 112, 113). After a specific DNA glycosylase recognizes and cleaves the base from the sugar, apurinic/apyrimidinic endonuclease (APE) and apurinic/apyrimydinic (AP) lyase completely excise the base from the DNA helix (Figure 2). Following this, Poly-ADP-Ribose Polymerase 1 (PARP1) detects and binds to the single stranded (ss) DNA break, auto-modifies itself with Poly-ADP-Ribose (PAR) and recruits X-ray repair cross-complementing protein 1 (XRCC1) that acts as a scaffold for DNA Polymerase Beta (Pol β) and DNA Ligase I. Together these proteins replace the missing nucleotide and ligate the DNA (31).
Figure 2 - Base Excision Repair After the nucleotide, in this case guanine, has been modified by methylation it distorts the DNA helix. Upon detection of the distortion caused by the methylation, 3-methylpurine-DNA-glycosylase (MPG) cleaves the covalent bond attaching the nucleotide base to the deoxyribose sugar creating an abasic site. Next, apurinic/apyrimidinic endonuclease (APEX) cleaves the 5’ phosphodiester bond and apurinic/apyrimidinic (AP) lyase cleaves the 3’ phosphodiester bond releasing the deoxyribose sugar. Following this PARP1 recognizes the ssDNA break and binds to it via its zinc fingers located on its N-terminal. When PARP1 is bound to the DNA it activates its enzymatic activity automodifying itself with poly-ADP-ribose. This attracts XRCC1, a scaffolding protein, as well as DNA polymerase beta (Pol β) and DNA Ligase III. Pol β repairs the DNA using the complementary strand as a template and DNA ligase III ligates the repair. (Figure Adapted from Oka S, Hsu CP, Sadoshima J. Regulation of cell survival and death by pyridine nucleotides. Circulation research 2012. 1115: 611-627.)
Homologs of BER are found in *S. cerevisiae* and bacteria indicating that BER process is highly conserved through evolution and BER has been demonstrated to be an absolutely essential process as mouse Polβ or XRCC1 knockouts die at embryonic stages (31, 32, 33). From a clinical perspective, the BER pathway is essential for cancer cells as it repairs DNA damage induced by oxidizing and alkylating agents – the majority of chemotherapeutic drugs used currently in the clinic (34). As such, targeting members of BER is an ideal therapeutic strategy for cancer therapy.

2.4 Homologous Recombination

Homologous Recombination (HR) repairs DSBs and intranast cross-links, preserves replication forks, and maintains telomeres, (48). Therefore mutations in genes encoding the enzymatic steps of HR produce drastic consequences to the cell (Figure 3). HR is initiated after ionizing radiation (IR) or another agent produces DSB in the DNA molecule. Bloom’s syndrome helicase (BLM) relieves helical tension and unwinds the DNA strands to facilitate extensive 5' to 3' end-processing by meiotic recombination 11 (MRE)/RAD50/Nibrin1 (NBS1) (MRN) complex and exonuclease 1 (EXO1). The resulting 3' ssDNA tails are bound by replicating protein A (RPA) to remove any secondary DNA structure. Following this RAD51 replaces RPA, an action mediated by RAD22, RAD55, and RAD57. The resulting nucleoprotein filament, consisting of RAD51-coated-DNA, searches for and invades a homologous sequence forming a D-Loop intermediate. DNA polymerase then extends from the 3’ of the invading strand using the homologous strand as a template. The second DSB end is “captured”, by RAD51 and its associated members, by annealing to the extended D loop. This forms two crossed strands or Holliday junctions (HJ) that can be resolved by either a cross over or non-cross over mechanism. In meiotic cells the cross-over mechanism is preferred but in somatic cells non-cross over mechanisms occur the majority of the time (48, 49, 50). In addition to RAD22, RAD55, and RAD57, breast cancer 2, early onset (BRCA2) has been shown to displace RPA and assist RAD51 in coating the processed ssDNA. HR is predominantly active in late S and G2 phases of the cell cycle for several reasons. First, HR requires a template to work and this template consists of sister chromatids. Second, during S and G2 phases there is an active transcription of HR related genes. Finally, CDK1 positively and negatively regulate DNA repair by phosphorylating some of HR proteins (49). For example, it has been shown that during the M phase CDK
mediated phosphorylation of serine 3291 of BRCA2 blocked its interaction with RAD51 (51, 52).

**Figure 3 - Homologous Recombination (HR)** Homologous recombination operates in S and G2 phases of the cell cycle and repairs DSBs in DNA. DNA repair by HR is high fidelity however it may result in gene conversions and lead to LOH. See text for detailed explanation of HR. Only defining features of HR are shown.
2.5 BRCA1, BRCA2 and PARP1 – A Complicated Relationship

The discovery that mutations in BRCA1 or BRCA2 are synthetically lethal with PARP inhibition generated a lot of enthusiasm in the field of cancer research, however the exact mechanism behind this synthetic lethality remains unclear (53). BRCA1 is a large protein that is expressed during S and G2 phases of the cell cycle, and upon DNA damage it co-localizes with RAD51 and other proteins involved in DSB repair (54, 55, 56). While the role of BRCA1 and 2 is mostly associated with HR-mediated DSB repair, BRCA1 was also shown to participate in NER and NHEJ (57, 58, 59). While PARP1 is involved in HR, NER, and NHEJ the role of PARP1 is best characterized in ssDNA break repair, and it has been shown that PARP1 and PARP2 activity is critical for efficient processing of ssDNA breaks (60, 61). The standard model to explain the sensitivity of BRCA1 and 2 mutant cells to PARP inhibitors invokes the effect of PARP inhibitors and subsequent accumulation of large amounts of stalled replication forks and DSBs which are lethal to HR-defective cells (62). Additionally, PARP1 and PARP2 were shown to bind to and become activated by stalled replication forks during S-phase and PARP1/2 activity is required for efficient repair (63). Furthermore, PARP1/2 depletion results in reduced recruitment of MRN complex to collapsed replication forks, a complex that BRCA1 has been shown to interact with to regulate end resection during S-phase HR (64). Taken together there may be a functional interaction between BRCA1/2 and PARP1/2 during end processing at stalled replication forks. While BRCA1 has a much broader role in DNA repair, the loss of BRCA1 there may put a greater reliance on PARP1 for DNA repair. Therefore this may be the reason why PARP1 inhibition is so effective in BRCA1/2 cancers.
2.6 Non-Homologous End Joining

Double stranded breaks (DSB) can occur when DNA encounters IR and reactive oxygen species (ROS), failure to repair DSB can result in chromosome breakage, cell death, onset of cancer, and defects in the immune system of higher vertebrates (36). When cells encounter DSBs they undergo cell death, however sometimes a cell will undergo a temporary growth arrest to repair damaged DNA. DSB mediated cell cycle arrest is initiated by members of phosphatidylinositol 3-kinase-like kinase (PIKK) family, ataxia telangiectasia mutated (ATM), ataxia telangiectasia Rad3-related (ATR), and DNA-dependent protein kinase catalytic subunits (DNA-PKcs), by phosphorylating p53, Chk1, and Chk2 (37). Normally DSBs are repaired by error free HR, however if HR pathway is compromised or the cell is not in S or G2 cell cycle phase DSB will be repaired by NHEJ – direct ligation of two ends of dsDNA at the breakpoint (Figure 4) (36). Since NHEJ can lead to loss of nucleotides at the site of DSBs, it contributes to loss of genomic integrity (38).

**Figure 4 – Simple overview of non-homologous-end joining (NHEJ).** NHEJ brings the ends of the broken DNA molecule together through the formation of a synaptic complex and ligates the broken ends of DNA together. The process is initiated when the Ku70/Ku80 - heterodimer binds to each DSB end and recruits DNA-PKcs. Auto-phosphorylation of DNA-PKcs affects conformation and dynamics of the DSB, and allows processing enzymes to access the DNA. There are two ways to process the ssDNA overhangs 1) synthesize a complementary strand using the ssDNA overhand, 2) resect the ssDNA overhang to create a blunt ends. NHEJ can use terminal deoxynucleotidyl transferase (TdT), DNA pol μ or λ for the former, and Artemis for the latter approach. Additionally, polynucleotide kinase (PNK) and APE can be recruited to phosphorylate the 5’ end and cleave the 3’ phosphoglycolate, respectively. Interestingly, processing enzymes involved in NHEJ are not unique to NHEJ and are used in other processes such as DNA synthesis. Finally, DNA Ligase IV and XRCC4 are recruited to the complex to ligate the two strands together successfully repairing the broken DNA molecule. (Figure adapted from: Weterings E, Chen DJ. The endless tale of non-homologous end-joining. Cell Res. 2008;18(1):114-24).
2.7 The Plot Thickens – The Tale of Two Non-Homologous End Joining Mechanisms.

While NHEJ, also known as D-NHEJ because of its dependency on DNA-PKcs, was thought to be the only pathway responsible for direct ligation of two broken DNA ends, data obtained from Ku or DNK-PKcs depleted cervix adenocarcinoma and adult GBM cells, HeLa and MO59J, respectively, suggest that a backup version of NHEJ (B-NHEJ) exists (Figure 5) (39, 40, 41). DSB repair without DNA-PKcs, Ku70 or Ku80, raised a question about which DNA Ligase is involved in this novel mechanism. To answer this question Wang el al. used extract fractionation and immunodepletion protocols to show that the majority of DNA end joining activity in extracts of HeLa cells derived from DNA Ligase III (42). The implication of DNA Ligase III in B-NHEJ raised the question whether known partners of this protein are also involved in B-NHEJ. PARP1 is one of these proteins and is involved in BER, NER, and single strand repair, however its role in DSB repair remains incompletely characterized (39). Studies have demonstrated that PARP1 is likely a member of B-NHEJ. Using a cell free system it was demonstrated that PARP1 binds to DNA ends in direct competition with Ku. However, in irradiated cells the higher affinity of Ku for DSB limits PARP1’s contribution to DSB repair (43, 44, 45). Additionally PARP1 has been shown to be recruited for DSB repair in absence of Ku (46). These studies suggest that PARP1 is a candidate component for B-NHEJ. Furthermore, using protein fractionation protocols histone H1 was also identified as a putative member of B-NHEJ and was shown to enhance DNA end joining and increase PARP1 activation (47). From a clinical perspective, the NHEJ pathway is

![NHEJ of IR-Induced DSBs](image)

Figure 5 - Schematic of NHEJ pathways including D-NHEJ and B-NHEJ. See text for more details. Only defining components of each pathway are shown.
essential as it repairs DNA damage induced by IR – the conventional therapy used for cancer treatment. As such targeting members of NHEJ is an ideal therapeutic strategy for cancer therapy.

3 Poly-ADP-Ribose Polymerase

3.1 One Big Happy Family – The PARP Superfamily

The characterization of the human PARP family of proteins was based on an exhaustive search of the non-redundant protein database (NCBI) using the human PARP1 catalytic domain (GenBank XP_037275 residues 796-1014) (65). This search uncovered seventeen putative PARP homologues that could carry out poly(ADP-ribose) polymerase activity. PARP1, the founding member, has been most extensively studied and is implicated in DNA repair. PARP1 uses nicotinamide adenine dinucleotide (NAD) to synthesize linear or multi-branched polymers of ADP-ribose on to arginine, glutamic acid or aspartic acid residues of acceptor proteins. This post-translational modification produces an area of negative charge causing the protein to function differently. The role of PARP1 in facilitating DNA repair has been demonstrated by the generation of several independent knock out mouse models (73). These animals show hypersensitivity to IR and alkylating agents at the cellular and whole-animal level. Unexpectedly, these animals showed protection against various inflammatory processes such as cerebral and cardiac ischemia and resistance to septic shock (65, 74, 75). PARP2 was discovered because fibroblasts derived from PARP1−/− mice contained residual PARP activity. After more characterization it was found that PARP2 preferentially modifies histone H2B while PARP1 modifies histone H1. Like PARP1, PARP2 also interacts with XRCC1, DNA Pol β, and DNA Ligase III. However, unlike PARP1, PARP2 preferentially binds to DNA gaps not DNA nicks. The role of PARP2 in DNA repair was also established after it was determined that PARP2 knockout mice exhibited reduced ability to repair DNA damage after IR and alkylating agents (65). PARP3 was found to be the core component of the centrosome, most often located at the daughter centriole during cell cycle (76). PARP3 also interacts with PARP1 at the centrosome, indicating a link between DNA damage repair and mitotic fidelity checkpoints. PARP4, also known as VPARP, is the largest PARP protein (192.6 kDa). PARP4 is found to be associated with vault particles – cytoplasmic riboprotein complexes (114). While the exact function of vault particles remains unknown, vault particles consist of three components: major vault protein,
telomerase associated protein 1, and untranslated vault RNA (77). PARP5a, also known as tankyrase1 (TRF1-interacting-ankyrin-related ADR ribose polymerase), has been shown to be implicated in telomere elongation and insulin signaling (78, 79). The function of PARP7, also known as TiPARP, still remains unknown, however it has been shown to be involved in T-cell function and its over-expression is associated with tumourigenesis. PARP7, PARP11, and PARP14 contain a WWE domain occurring in classes of proteins associated with ubiquitination. PARP10 is also associated with ubiquitination as it contains an ubiquitin interaction domain (80). PARP10 and PARP15 also have a motif specific for binding to RNAs with the RNA recognition motif found in a variety of RNA-binding proteins, including heterogeneous nuclear ribonucleoproteins, proteins implicated in regulation of alternative splicing, and protein components of small nuclear ribonucleoproteins. PARP9 has been recently discovered in patients with certain types of diffuse large B-cell lymphomas. PARP9 has been shown to be over-expressed in these malignancies and is implicated in B-cell migration (81). Overexpression of PARP9 increases the rate of migration of B-cell lymphoma transfectants, suggesting that the risk-related protein may promote the dissemination of high-risk diffuse large B-cell lymphomas. As it is shown, the PARP superfamily consists of many proteins with different cellular localizations and functions.

3.2 Structure of Poly-ADP-Ribose Polymerase 1

PARP1, the founding family member, is responsible for the synthesis of the majority of PAR in eukaryotic cells and after the histones, is the most abundant nuclear protein (66). The gene is located on chromosome 1q41-42 and the 113-kD human PARP1 (PARP1) protein is organized into at least six domains, four of which have well-defined functions (Figure 6). Domain A in the N-terminal region is the DNA-binding domain and its affinity for damaged DNA is regulated by two zinc-finger motifs which are sufficient to target the entire protein to the damaged DNA (67). The two PARP1 zinc-finger motifs are unique as they recognize altered DNA structures rather than specific sequences: they are known to recognize DNA nicks, overhangs, blunt ends, and other forms of damage (67, 68, 69). The B domain contains a bipartite nuclear localization signal (NLS) and a caspase-3 cleavage site. The auto-modification domain D contains a BRCA1 C-terminus (BRCT) motif via which PARP1 participates in various protein–protein interactions. The domain F is the catalytic C-terminal region (65). This domain can be reduced to just a 40-kDa C-terminal polypeptide without losing the basal catalytic activity (70). Little is known about
the function of the C and E domains. However, a third zinc domain in hPARP1 has recently been characterized in the C domain (71, 72). This region is thought to modulate the N-terminal-to-C-terminal communication that leads to the DNA-dependent activation of PARP1 (71). Although this zinc ribbon domain does not bind DNA directly, it is necessary for DNA-stimulated activation of the full-length enzyme (72).

Figure 6 - Protein Structure of Poly-ADP-Ribose Polymerase 1 The domain structure of PARP1, showing the DNA-binding, the automodification and catalytic domains (domains A–F). The PARP signature sequence (in dark blue within the F catalytic domain) is the most conserved between the PARPs. Zn I and Zn II: Zinc-finger motifs. Zn III: Zinc ribbon domain. NLS nuclear localization signal; BRCT: BRCA1 carboxy terminus; NLS nucleolar localization signal

3.3 Function of Poly-ADP-Ribose Polymerase 1

PARP1 protein is expressed from the ADPRT gene that is under constitutive expression depending on the context, type of tissue, or cell. ADPRT expression seems to be controlled by ETS transcription factor interacting with multiple ETS-binding motifs in the ADPRT promoter region (83). Poly-ADP-ribosylation (PARylation) is post-translational modification of PARP1 (auto-PARylation) and of other acceptor proteins (trans-PARylation) on glutamate, aspartate, and lysine residues. The ADP-ribose polymer is formed by sequential attachment of ADP-ribosyl moieties from NAD$^+$ and can be up to 200 units long and contain multiple branch points, thus representing a large accumulation of negative charge (Figure 7) (82). Addition of poly-ADP-ribose (PAR) as a post-translational modification is most often associated with cellular stress signaling. PARylation of acceptor proteins can affect DNA damage response, chromatin dynamics, and necrosis induced inflammation (116). In the context of DNA damage, auto-PARylation of PARP1 produces high density of negative charge at the DNA break site that simultaneously repels PARP1 from the DNA and recruits XRCC1 that acts as a scaffold for other
members of BER (65). Additionally, PARP1 also trans-PARylates histone H1 at sites of DNA damage and through negative-negative charge repulsion causes H1 to come off the DNA and loosen the chromatin, making it accessible to DNA repair proteins (116). In addition to its role in modifying histone H1, PARylation and subsequent inhibition of histone demethylase, JARID1B, helps maintain levels of H3K4me3 – a histone modification associated with active promoters. This allows RNA Polymerase II recruitment, binding, and transcription of genes (117). Also it...

**Figure 7 – PARP1 Function and Regulation.** PARP1 can be regulated through multiple pathways that either activate it or inhibit it. PARP1 can also post-translationally modify itself and other proteins by using NAD$^+$ as its substrate to long chains of poly-ADP-ribose.
has been discovered that PARylation of high-mobility-group box 1 (HMGB1) protein in response to genotoxic stress produces negative charge repulsion between HMGB1 and DNA causing HMGB1 to dissociate from condensed chromatin, translocate to the cytoplasm, and leak out of necrotic cells. Extracellular HMGB1 then acts as a pro-inflammatory signal inducing macrophage activation (115). PARP1 is responsible for the majority (80%-90%) of PARP activity in the cell, while the remainder is predominantly PARP2.

In addition to post-translational modifications of proteins, PARP1 also interacts directly with proteins and this interaction can be either dependent or independent on its PARylation activity. Using immunoprecipitation and mass spectrometry Isabelle et al. demonstrated that PARP1 from human neuroblastoma (SK-N-SH) and HeLa cervical carcinoma cell lines interacts with proteins involved in RNA metabolism, DNA repair and apoptosis, glycolysis, and cell cycle (128). Another study by Rossi et al. using a similar approach found that PARP1 interacts with proteins within the mitochondria, specifically mitofillin (129). Interestingly both studies reported this interaction. However, neither of these studies used a PARP inhibitor to evaluate whether these interactions were PARP activity dependent. Building on this, work by others characterized three motifs located on proteins involved in non-covalent binding to PAR. Pleschke et al. found a conserved PAR binding motif on DNA damage checkpoint proteins that is 20-amino acids long and contains two conserved regions – cluster rich in basic amino acids, and a hydrophobic region with basic residues. (130). Some proteins that contain this motif include: p53, p21, XPA, MSH6, and DNA Ligase III. The second PAR binding motif is 180 amino acids long and was shown to reside within the macro domain (131). This domain is particularly interesting as it is conserved across yeast, viruses, and eukaryotes and is found in histones and other proteins involved in chromatin metabolism (132, 133, 134). Some proteins containing a macro domain include: YBR022W (yeast), histone-macro-H2A (mammals), and envelope proteins (severe acute respiratory corona virus). The third PAR binding motif, named poly(ADP-ribose)-binding zinc finger motif (PBZ) , was recently discovered by Ahel et al. (135). This motif is present in aprataxin PNK-like factor (APLF), a nuclease involved in dsDNA break repair, and checkpoint protein with FHA and RING domains (CHFR). Ahel et al found that the function of CHFR in antephase checkpoint was abrogated upon treatment with a PARP inhibitor, providing strong evidence that this PAR binding motif is dependent on the presence of PAR.
While there are many studies characterizing proteins that interact with PAR, much fewer studies in comparison focus on PARP1-protein interactions that occur independently of PARP1’s enzymatic activity, however several studies have began to explore this area. Using an immunoprecipitation approach, Aguilar-Quesada et al. demonstrated that ATM directly interacts with PARP1 in G361, a human melanoma cell line (136). Under normal cell growth conditions PARP1 was immunoprecipitated with ATM, however the amount of PARP1 that was pulled down with ATM increased after 10 Gy of IR. This suggests that this interaction may be dependent on PARP1’s catalytic activity – something that the authors demonstrated later in their paper. However, the authors never elaborate why PARP1 was pulled down with ATM during normal growth conditions, was this dependent on PARP1’s basal catalytic activity or on direct protein-protein interactions? Using co-immunoprecipitations Park et al. demonstrated that PARP1 directly interacts with rotavirus nsP3 in a neuronal cell culture, and this interaction is independent of PARP1’s catalytic activity (137). During viral infection the rotavirus nsP3 protein shuts off of cellular protein synthesis causing the cellular machinery to focus on translating viral proteins (138). Park et al. explained this interaction by suggesting that PARP1 may play a role in regulating viral RNA synthesis in infected neuronal cells. While the study by Park et al. provided new knowledge of PARP1 protein interactions, it did not identify what region of the PARP1 protein is required to interact with nsP3. A very recent study by Miyazaki et al. demonstrated that PARP1 interacts with three prime repair exonuclease 1 (TREX1), a 3’-5’ DNA exonuclease (139). The authors used deletion/truncation mutants and identified that only the first two zinc fingers of PARP1 (amino acids 1-203) were required for this interaction. This finding is unique in light of other potential protein-protein interacting regions present in PARP1. The main function of TREX1 is to digest cytosolic ssDNA to prevent activation of cell-intrinsic responses to immunostimulatory DNA, however TREX1 also translocates into the nucleus where it has been shown to interact with PARP1 suggesting that it may be involved in BER. PARP1 protein interaction network is complex as it composed of both protein interactions that are dependent on PARP1 enzymatic activity and those not-dependent on enzymatic activity, future studies will shed more light on PARP1 protein-protein interactions.

3.3.1 PARP1, DNA repair, and Genomic Stability

In the past two decades many studies have been conducted using PARP inhibitors, expression of dominant negative PARP1, PARP1 knockdowns, ADPRT gene disruption, and production of
mouse models (84). These studies have firmly established that PARylation is involved in cellular recovery from cytotoxic insults of DNA alkylating agents, reactive oxygen species (ROS), and IR – establishing PARP1 as a “survival factor”. The involvement of PARP1 in BER (Figure 2), B-NHEJ, and HR (Figure 8) further supports the notion that PARP1 is an important DNA repair enzyme critical to maintaining genomic stability and facilitating DNA repair. Additionally, studies looking at more detail into PARP1 function found a 20 amino acid PARylation sequence in proteins involved in DNA damage repair and cell cycle check points such as p53, p21, XPA, MSH6, DNA Ligase III, XRCC1, Pol β, Pol μ, DNA-PKcs, Ku70, NF-κB, BRCA1 and BRCA2 and telomerase (85). This suggests that PARP1 directly affects these proteins in carrying out their normal functions when a cell is exposed to genotoxic stress. Taken together this suggests that PARP1 and PARylation are involved in cell survival, genomic maintenance, and genomic stability.
Figure 8 – PARP1 role in DNA Double Strand Break Repair. Rapid association of Ku70/80 to DSBs promotes NHEJ (aka D-NHEJ) (A). Alternatively to D-NHEJ, MRN, which is initially recruited to DSBs by PARP1 in competition with Ku70/80, mediates initial stages of DSB resection together with C-terminal-binding protein 1 (CtIP) and BRCA1 to promote HR in S and G2 phases (B). CtIP and MRN in G1 carry out some DSB resection can as a result of alt-NHEJ (aka B-NHEJ). During M and G1 phases HR cannot occur therefore majority of DSB repair depends on D-NHEJ or PARP1 mediated B-NHEJ (C). Please see text for more details. (Figure adapted from: Ciccia et al. The DNA damage response: Making it safe to play with knives. Mol Cell Rev 2010. 40: 179-204.)
3.3.2 PARP1 in Transcription and Inflammation

Since PARylation is involved in the regulation of chromatin structure it has been postulated that it could have an impact on gene expression, causing PARP1 to act as a transcriptional regulator. In the last decade it has been demonstrated that PARP1 is capable of physical and functional interaction with specific transcription factors such as AP-2, DF1-4, E47, NF-κB, p53, PC1, Oct1, RXR, TEF-1 and YY2 (84). Depending on the gene and the context in which the interaction was studied, PARP1 can have a negative or a positive effect on transcription of the target gene. The involvement of PARP1 with NF-κB, as a cofactor, suggested that PARP1 may have a function in immune and inflammatory responses and this was confirmed with Adprt1-null mice. These mice experienced a reduced lipopolysaccharide (LPS) induced increase in tumour necrosis factor – alpha (TNF-α) dependent inducible nitric oxide synthase expression and nitric oxide levels. Additionally these mice had an amazing protection from endotoxin shock. It was additionally shown that surprisingly the co-transactivation effect of PARP1 on NF-κB was independent of PARP1 catalytic activity and was instead dependent on the ability of PARP1 and NF-κB to form a stable nuclear complex (86). Another role for PARP1 in inflammation and immunity was demonstrated when it was discovered that PARP1 is a co-activator of CXCL1, a gene encoding melanoma growth-stimulatory protein that is strongly expressed during inflammation (87). Upon deeper study it was found that CXCL1 is regulated through several cis-acting elements including a NF-κB element and an adjoining IUR element. Interestingly PARP1 was identified as an IUR binding protein.

3.3.3 PARP1 and Metabolism

While PARP1 has been historically described as a DNA repair enzyme, PARP1-null mice display enhanced energy expenditure (88). This effect may result from an increase in SIRT1 activity since SIRT1 can regulate energy expenditure by deacetylating and activating master transcriptional regulators of oxidative phosphorylation, such as peroxisome proliferator-activated receptor gamma co-activator 1- alpha (PGC-1α) and forkhead (FOXO) family of transcription factors (89). When myocytes from PARP1-null mice were analyzed, there was a marked deacetylation of PGC-1α and FOXO1, which was linked to increased biogenesis and higher oxidative profile of muscle fibers (88). Furthermore, PARP1-null mice also contained an
increased amount of brown adipose tissue, rendering PARP1-null mice more able to maintain body temperature when exposed to cold (90). PARP1-null mice were also found to have an increased glucose clearance in response to an insulin tolerance test, an effect that is believed to be caused in part by increased oxidative profile of muscle fibers (89, 90). Other investigators found that PARP1 has a role in islet β cell physiology as PARP inhibitors improved diabetes mellitus in partially de-pancreatized rats by promoting faster β cell regeneration and normalization of blood glucose (91). Also it was demonstrated that PARP1-null mice were also resistant to the development of streptozotocin-induced diabetes and maintain normal pancreatic insulin content and islet morphology (92).

The role of PARP1, while mostly studied from a DNA damage repair perspective, is ubiquitous in other cellular processes such as transcription, inflammation, and metabolism. While using PARP inhibitors may be an effective therapeutic strategy for inhibiting DNA damage there should also be consideration of other consequences that PARP inhibitors may have on cells, tissue, and the entire body.

4 Poly-ADP-Ribose Polymerase as a Therapeutic Target

4.1 PARP Inhibitors – Structure and Function

The substrate for PARP, NAD+, contains a nicotinamide moiety recognized by PARP’s catalytic domain (124) (Figure 9). PARP inhibitors contain a region mimicking nicotinamide that allows them to compete with endogenous NAD for PARP binding. Nicotinamide, a byproduct from the conversion of NAD into chains of PAR, was the first PARP inhibitor identified in 1971 (125). The second-generation PARP inhibitors were identified by empirical screening of large chemical libraries for compounds containing the nicotinamide moiety (126). Third generation of PARP inhibitors, like Veliparib, Olaparib, and Niraparib were shown to have increased specificity for PARP1 (127). Of the three PARP1 inhibitors mentioned, only Veliparib is currently used in clinical trials for paediatric gliomas, emphasizing the need to evaluate Olaparib and Niraparib in paediatric brain tumour models (clinicaltrials.gov).
Figure 9 - Chemical Structures of PARP inhibitors. Chemical structures of Veliparib (ABT-888), Niraparib (MK-4827), Olaparib (AZD2281), and Nicotinamide adenine dinucleotide (NAD). The nicotinamide moiety is highlighted in red. Adapted from: Murai J, Huang SN, Das BB, et al. Trapping of PARP1 and PARP2 by Clinical PARP Inhibitors. Cancer Res. 2012;72(21):5588-99.
4.2 PARP Inhibition – Chemosensitization?

In 1980, a group led by Sydney Shall published a paper in Nature demonstrating that PARP inhibitor, 3-aminobenzamide (3-AB), enhanced cytotoxicity of an alkylating agent, dimethyl sulphate, and prevented rejoining of DNA strand breaks in L1210 mouse leukemia lymphoblast cells (93). 3-AB is an analogue of nicotinamide, a by-product of NAD+ mediated PARylation, and is therefore a weak inhibitor. Furthermore, like 3-AB majority of PARP inhibitors at the time were competitive inhibitors, acting through the catalytic domain of the enzyme and therefore having no selectivity for different members of the PARP superfamily. Due to their lack of specificity and potency the first generation of PARP inhibitors never entered clinical studies. Using rational drug design (based on X-ray crystallography of PARP inhibitors in complex with PARP proteins) more potent and specific inhibitors were identified. These inhibitors were also found to sensitize animal models of cancer to conventional anti-cancer therapies. Miknyocksli et al demonstrated that CEP-6800 potentiated antitumor activity of TMZ, irinotecan, and cisplatin in tumour xenografts of U251MG (adult GBM), HT29 (colon carcinoma), Calu-6 (non-small cell lung carcinoma), respectively (94). Tenori et al showed that GPI-15427 enhanced anti-tumour activity of TMZ against melanoma, adult GBM, and lymphoma (95). Additionally Calabrese et al found an improved therapeutic index with AG14361, an inhibitor 1000 times more potent that 3-AB, in combination with TMZ, irinotecan, and radiation in a human colon xenograft model (96). While initially the enhancement of anti-tumour activity of TMZ by combination with PARP inhibitors was attributed to blocking of BER that removes N-methylpurine lesions, recent evidence demonstrating that PARP1 is involved in BER, NHEJ, and HR complicates this mechanism and broadens the application of PARP inhibitors to other forms of anti-tumour therapies. Due to positive preclinical results, the first phase I clinical trial of a PARP inhibitor was carried out between 2003 and 2005 using AGO14669 in combination with TMZ in patients with advanced solid tumours (97). Thirty-three patients were enrolled, the combination was well tolerated, and pharmacodynamic assessments were used as a proof of principle since patients receiving the combined therapy showed an increase in ssDNA breaks.

4.3 PARP Inhibition – Radiosensitization?

In addition to chemosenzititation early experimental evidence by Noel et al. demonstrated that PARP inhibitors radiosensitized rodent cells to IR, however their effect in human cells was
marginal (98). To determine the discrepancy between these observations, a panel of human and mouse cells were assessed, using 4-ANI as the PARP inhibitor. This group observed that radiosensitization depended on the percentage of cells engaged in DNA replication. These results were confirmed using synchronized HeLa cells. Specifically, radiosensitization was shown to occur in the S phase of the cell cycle. A study by Gordon et al. examined the effect of PARP1 inhibition relative to PARP1 silencing on IR induced break repair in synchronized, isogenic HeLa cells (99). It was found that PARP1 inhibition prevented the recruitment of XRCC1, slowed down single stranded break repair, and induced persistent accumulation of PARP1 and proliferating cell antigen A (PCNA) at sites of DNA damage. The authors concluded that this accumulation of DNA repair enzymes on the DNA hindered the recruitment of other downstream effectors and induced further DNA damage by collision with replication forks during S phase, forming dsDNA breaks. Also these authors observed that PARP1 silencing made cells hypersensitive to IR (99). The evidence that PARP inhibition or PARP silencing enhances radiosensitivity in rapidly dividing tumours has been supported by other studies using different PARP inhibitors and different models. For example, Dungey et al. demonstrated that Olaparib radiosensitized human adult GBM cell lines via a replication dependent mechanism that generated persistent dsDNA breaks (100). While it is well established that PARP inhibitors are radiosensitizers the mechanism is not very clear. It is unknown whether this sensitivity is due to compromised ssDNA break repair that results in accumulation of dsDNA breaks, or whether dsDNA break repair is also impaired. This information suggests that PARP1 is a potential therapeutic agent. Furthermore, PARP inhibition has been explored in adult GBM, and there is an urgent need to study whether PARP inhibition is an effective therapeutic approach in pHGA and DIPG.

4.4 PARP Inhibition – Monotherapy?

In 2005 it was demonstrated for the first time that PARP inhibitors may be used as monotherapy in the context of already compromised HR pathway due to BRCA1 or BRCA2 mutations (101, 102). These authors concluded that in HR deficient cells the dsDNA break repair pathway was compromised making these cells more sensitive to blockage of ssDNA break repair by inhibiting PARP activity. The hypothesized mechanism behind this sensitivity is that blocking ssDNA break repair produces dsDNA breaks at replication forks. While normal cells have functional HR repair pathways, dsDNA repair pathways in tumour cells are compromised due to loss of
BRCA1 or BRCA2 wt alleles. These findings made the concept of synthetic lethality very popular in cancer biology, a concept stating that a mutation in either of the two genes individually has no effect, however combining both mutations results in cell death. While BRCA1 and BRCA2 loss demonstrated that synthetic lethal interactions can be taken advantage of for devising anti-cancer therapies they also hinted that there may be other synthetic lethal interactions. A recent study by Murai et al. used a genetic screen in DT40 cells and found that loss of DNA Ligase IV, ATM, XRCC1/2/3, USP1, FANCC, FEN, XPA and many other DNA repair pathway genes are synthetically lethal when combined with Veliparib, Olaparib, or Niraparib (103). Additionally, Mendes-Pereira et al. demonstrated that mutations in PTEN make cells more sensitive to PARP inhibitors (105).
5 Paediatric High-Grade Astrocytoma and Diffuse Intrinsic Pontine Glioma

5.1 Background Information

Brain tumors account for 20% of all neoplasms in children, and are the largest group of solid tumors that develop in childhood (4). Among these tumours, paediatric supratentorial high-grade astrocytoma (pHGA) and diffuse intrinsic pontine glioma (DIPG) comprise 8-12% and 15-20% of all childhood brain tumours, respectively (5, 6). The histologically diverse group of pHGA consists of anaplastic astrocytoma (AA) (World Health Organization [WHO] grade III) and glioblastoma multiforme (GBM) (WHO grade IV). pHGA commonly arise in the supratentorial area, however they can also originate from the cerebellum. Studies comparing expression profiles of pHGA with normal developing brain found enriched signatures of ganglionic eminences that are present in late embryonic state and early fetal stages of development, these regions are believed to give rise to cortical interneurons and neuroprogenitors (17). pHGA are commonly diagnosed by MRI and biopsy. The majority of diffuse intrinsic pontine gliomas (DIPG) (85-90%) are malignant intrinsic infiltrative AA and/or GBM of the pons that are not amendable to resection. DIPG expression signatures overlap with hindbrain developmental structures at later developmental time points (17, 18). Interestingly, this data points to a different cell of origin for these tumour types. As with pHGA diagnosis is based on MRI findings, however biopsy is not indicated due to the high-risk location of the tumour.

The mainstay of therapy for pHGA involves maximum safe surgical resection followed by radiation therapy (RT) and has a 5-year progression free survival (PFS) of 18% (7). Due to the toxicity of RT, it is only administered to patients over three years of age. Several clinical trials (CCG943, CCG945, SJHG98) examined combinations of chemotherapeutic agents (prednisone, vincristine, lomustine, and temozolamide) with surgical resection and RT, however little to no survival benefit was observed (7, 8, 9,10). In contrast to pHGA, survival for DIPG has not changed in decades and the medial survival after diagnosis remains less than 1 year (6). As with pHGA RT forms the mainstay of treatment. Currently no chemotherapeutic agent or regimen has influenced survival. Dismal survival rates of pHGA and DIPG and lack of effective chemotherapeutic regimens highlight the need for more effective and specific therapeutic approaches.
5.2 Known Genetic and Molecular Alterations in Paediatric High Grade Astrocytoma and Diffuse Intrinsic Pontine Glioma

In the past decade it has been established that while there are genetic and molecular similarities between paediatric high-grade gliomas and their adult counterparts, the mechanisms of tumorigenesis in children are quite different (11, 16). Focal amplifications (43%), mutations (18-26%), deletions (3-64%), and fusions (8%) in epidermal-growth-factor-receptor (EGFR) gene, EGFR, are more common in adults than in children. However amplifications or mutations in platelet-derived-growth-factor-receptor-α (PDGFRA) gene, PDGFRA, are more common in children (15%-39% vs 11-26%). This suggests that PDGFRA pathway is more important for driving gliomas in the paediatric setting and points to potential therapeutic targets (19, 20).

Genetic lesions in phosphoinositide 3-kinase 3-kinase (PI3K) regulatory subunit (PIK3R1) and catalytic subunit (PIK3CA) occur in adult GBM, pHGA, and paediatric DIPG, albeit at different frequencies. In adults, mutations in PIK3R1 and PIK3CA occur at a frequency of 6-11% and 7-21%, respectively, and in pHGA and DIPG mutations in the regulatory domain occur at a similar frequency as in adults (5-12%). In contrast, catalytic domain mutations in children are less frequent in cortical HGA (5%) but more frequent in DIPG (15-25%) (19, 20). This suggests that there may be an age dependent and location dependent selection for mutations in the PI3K pathway. Knowledge that PI3K pathway is affected in these cancers gives new applications for inhibitors targeting the PI3K pathway.

Alterations in genes coding for cell cycle effectors have also been observed in adult and paediatric high-grade astrocytomas. Focal deletions and inactivating mutations of RB1 occur at approximately similar frequencies in adults and children (3-4% vs 0-3% and 10% vs 7-9%, respectively). In contrast to retinoblastoma, other members of the cell cycle pathway appear to be affected more frequently in adult than paediatric patients. For example, focal deletions in CDKN2A and CDKN2B (62% vs 10-19%) and amplifications in CDK4 (13-18% vs 3-4%) are more frequent in adults. Interestingly mutations in TP53 are more frequent in paediatric than adult patients (34-37% to 20-29) (19, 20). These findings suggest that lesions in cell cycle effectors are more important in driving adult GBM, nevertheless targeting these lesions can benefit both adult and paediatric patients.
The most striking difference between adult and paediatric high-grade astrocytoma, in the context of genetic alterations, are the recurrent hotspot mutations found in genes coding for histones H3.3 and H3.1. Non-synonymous mutations changing lysine to a methionine at position 27 (K27M) in H3F3A and HIST13B have been found in DIPGs and tumours of other midline structures such as the thalamus and spinal cord. While the majority of K27M mutations affect H3.3 (65%) some mutations affecting H3.1 have also been reported (15%) (13, 19). By contrast, mutations in H3F3A that alter glycine to valine or arginine at position 34 (G34R or G34V), are restricted to hemispheric HGA (13, 19). While our understanding of histones in tumour biology remains incomplete, the frequency of histone mutations in paediatric brain cancers suggests that epigenetics play an important role in tumourigenesis.

In addition to mutations in canonical cancer pathways, several studies also highlighted that pHGA and DIPG have extensive alterations in DNA damage repair pathways that may affect their sensitivity to conventional therapies thus making them ideal candidates for small molecular inhibitors (12, 14, 21, 22). Using single nucleotide polymorphism (SNP) arrays and pathway analyses of DIPGs, our group found deletions and losses of heterozygosity (LOH) in genes coding for members of various DNA repair pathways. Deletions or LOH were found in genes involved in homologous recombination (HR), non-homologous-end-joining (NHEJ), nucleotide excision repair (NER), and base excision repair (BER), suggesting that DIPG have high levels of genomic instability and compromised ability to repair their DNA (11). In addition to finding deletions and LOH in DNA repair genes, Zarghooni et al found that PARP1 was gained in 3 DIPG cases and stained positive in 6 DIPG cases (4 strong, 2 weak) (11). Interestingly, Barton et al found that PARP1 is expressed in other tumours of the central nervous system (CNS), and Smith et al observed that PARP1 protein expression correlates with survival in pHGA (23, 24). This preliminary data suggests that targeting members of DNA damage repair, especially PARP1, may be an effective therapeutic approach in DIPG and pHGA.

5.3 Pre-Clinical Studies of Paediatric High-Grade Astrocytoma and Diffuse Intrinsic Pontine Glioma

There have been a limited number of preclinical studies regarding pHGA and DIPG due to a lack of in vitro and in vivo models and rational targets. However, in light of recent advances in our understanding of the molecular and genetic pathways responsible for driving pHGA and DIPG,
research studies are beginning to move towards targeted therapies. In one study by our group, Aurora Kinase B (AURKB) was found to be over-expressed in 6/9 DIPG and 8/11 pHGGs. Treatment of patient-derived DIPG and pHGG cells with AURKB inhibitors, Reversine or VX-680, resulted in growth arrest, cell cycle aberrations, polyploidy, and resulted in a significant decrease of colony formation in all treated cells (15). As amplifications in cyclin dependent kinase 4 (CDK4) gene, CDK4, have been previously reported in paediatric high-grade gliomas, a study by Barton et al evaluated PD-0332991, a novel CDK4/6 inhibitor, in a platelet derived growth factor B (PDGF-B) driven Ink4a-ARF−/− or p53−/− mouse model of brain stem glioma. Inhibition of CDK4/6 with PD-0332991 induced cell cycle arrest in the PDGF-B;Ink4a deficient model both in vitro (61.22% in G0/G1) and in vivo (75% reduction of cells in M phase), however PDGF-B; p53−/− model was mostly resistant to treatment with PD-0332991. Additionally when PD-0332991 was combined with a single 10 Gy dose of radiation it extended overall survival (OS) by a median of 10 days (25). Another study found that MK-1775, a Wee1 kinase inhibitor, when combined with gemcitabine, carboplatin, and cisplatin selectively abrogated the G2/M checkpoint in in vitro and in vivo models of p53 deficient DIPG and attenuated tumour growth and extended survival (26). Finally, recent work by our group identified a novel ATM-3-methylpurine-DNA Glycosylase (MPG) axis that is responsible for resistance to alkylating agent, temozolomide (TMZ), in pHGA (14). Knockdown of BER, NHEJ, and NER members in two in vitro models of pHGA showed significant growth attenuation (>50%) when combined with 100 mM TMZ relative to normal human astrocytes and neuronal stem cell controls. Although these studies demonstrate that inhibition or loss of specific proteins in cell cycle check point or DNA damage repair pathways attenuate pHGA or DIPG growth, respectively, none of these studies to date have demonstrated whether small molecular inhibitors of BER repair can sensitize in vitro and in vivo models of pHGA and DIPG to radiation attenuation.

6 Hypothesis

There is an unmet need for better therapeutic targets in pHGA and DIPG due to their dismal clinical outcome and limited number of available treatment strategies. PARP inhibition has been demonstrated in cell and animal models of other adult cancers, suggesting that PARP inhibition may effectively inhibit growth, however, a lack of preclinical models addressing pHGA and DIPG thus far has hampered extensive study of this therapeutic target. It is my hypothesis that
PARP inhibition sensitizes paediatric high-grade gliomas to conventional therapies by inhibiting DNA repair.

**Aim 1: Evaluate whether PARP inhibitors are effective as monotherapeutic agents**

**Obj. 1:** Characterize PARP protein levels in pHGA and DIPG cell lines and patient samples

**Obj. 2:** Test the effects of PARP inhibition as a monotherapy *in vitro*

**Aim 2: Evaluate whether PARP inhibitors sensitize cells to radiation**

**Obj. 1:** Test whether PARP inhibitors reduce rate of IR induced DNA repair and sensitize cells to radiation.

**Obj. 2:** Test PARP inhibitors in an orthotropic mouse model with and without radiation.
Chapter 2 Methods

“You know my methods, Watson. There was not one of them which I did not apply to the inquiry. And it ended by my discovering traces, but very different ones from those which I had expected.” - Sherlock Holmes (The Crooked Man)

7 Methods

1.1 Cell Culture

Normal human astrocytes (NHA), paediatric supratentorial high-grade astrocytoma cell lines (SJG2, SF188, KNS42, 464, and 626), and paediatric diffuse intrinsic pontine glioma cell lines (DIPGM, DIPG58, DIPG4) were used to study the effects of PARP inhibition in vitro. NHAs were used as a control to test for cytotoxic effects of PARP inhibitors in non-tumour cells. SJG2 were used because of their ability to grow as xenografts in NSG mice. SJG2 and SF188 contain p53R273C mutation, SJG2 is PTEN<sup>-/-</sup> and SF188 is PTEN<sup>wt</sup>, also SJG2 and SF188 contain CDKN2A/B/C deletions. KNS42 and DIPG58 contain H3.3G34R and H3.3K27M mutations, respectively. DIPG58 contains R175H mutation. Cell lines 464, 626, DIPG58, and DIPG4 were low passage primary cell lines. DIPG4 contains H3.1K27M mutation. NHA (Lonza) were grown in Clonetics<sup>TM</sup> AGM<sup>TM</sup> Astrocyte Growth Medium (Lonza), supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). SJG2, SF188, and KNS42 cell lines were derived from supratentorial paediatric high-grade astrocytoma and grown in Dulbecco’s modified eagle media (DMEM) (Wisent) supplemented with 10% FBS, and 1% penicillin/streptomycin (Invitrogen). 464, 626, and DIPG Monje, DIPG 58, DIPG4 cells were derived from paediatric-supratentorial high grade astrocytoma and diffuse intrinsic pontine glioma, respectively. The five aforementioned cell lines were grown as spheres in stem cell media containing Neurocult NS-A Basal Medium (Human Stem Cell Technologies), supplemented with L-glutamine (2mM) (Invitrogen), antibiotic/antimycotic (1X) (Invitrogen), N2 supplement (1%) (Stemcell Technologies), B27 supplement (2%) (Gibco), 75ng/mL Bovine Serum Albumin (BSA) (company), human epidermal growth factor (hEGF) (20ng/ml) (Invitrogen) and human basic fibroblast growth factor (hFGF) (20ng/ml) (Biosource). All cell lines were incubated at 37°C and 5% CO2. All cells were incubated at 5% CO2 and 37°C.
7.1 Clinical Poly-ADP-Ribose Polymerase Inhibitors

Veliparib (Sellekchem), Olaparib (Sellekchem), and Niraparib (ChemiTech), and were reconstituted in dimethyl sulfoxide (DMSO) prior to treatment of cells at designated concentrations.

7.2 MTT Cell Proliferation

MTT Cell Proliferation Kit I (Roche) was used to evaluate the effects Veliparib, Olaparib, and Niraparib on cell proliferation. Cells were seeded in triplicate at the following densities: NHA, 7000 cells/well; SJG2, SF188, KNS42, 462, 626, 3000 cells/well; DIPG Monje, DIPG58, DIPG4, 8000 cells/well. Cells were allowed to adhere overnight. The following morning media was added containing either DMSO vehicle control or designated concentration of PARP inhibitor. On the seventh day cells were fixed, according to manufacturer’s instructions, and analyzed at 592nm.

7.3 Colony Forming Assay

Briefly, 500 SJG2, SF188, KNS42 cells were seeded in 6-well dishes in triplicate, treated with DMSO or indicated concentration of PARP inhibitors, and left to grow for two weeks. Media was removed, cells were washed with PBS, fixed with 4% Paraformaldehyde (VWR), and stained with crystal violet solution for five minutes. Cells were washed and colonies were counted. To evaluate the radiosensitizing effects of PARP inhibition, cells treated with DMSO control, IR, Niraparib, or Niraparib administered two hours before IR.

7.4 Cell Counts and Trypan Blue Cell Viability

To evaluate the effect of Niraparib on cell number and viability, direct cells counts (Vi Cell XR cell counter, Beckman Coulter) and cell viability (trypan blue) measurements were taken. Briefly, 5e5 SJG2 and SF188 cells were seeded in 10cm² petri dishes in triplicate, treated with DMSO or indicated concentration of Niraparib and counted on day three.

7.5 Immunofluorescence

Immunofluorescence was performed as previously described. Briefly, 1x10⁵ cells were seeded on glass coverslips and left overnight to attach. DIPG58 cells were plated on poly-L-ornithine
(Sigma)/laminin (Sigma) coated coverslips to aid in attachment. The following day cells were treated with either vehicle, irradiated, or incubated with a PARP inhibitor for a designated amount of time. Cells were then fixed with 4% paraformaldehyde, permeated with 0.5% Triton-X, and blocked with 5% BSA at 37°C for 1 hour. Slides were then incubated with primary antibody overnight at 4°C. Primary antibody concentrations were as follows: γH2AX (1:1000, Millipore), Ki67 (1:50, Dako). After incubation slides were washed with TBS and incubated with a fluorescent conjugated antibody specific for the primary antibody for 1h at room temperature in a dark environment (Fluorescein isothiocyanate (FITC) conjugated antibody 1:200; Life Technologies). Slides were mounted on DAPI containing VECTASHIELD Mounting Medium (Vectorlabs, H-1200) and sealed with clear nail polish. Images were captured using a spinning disk confocal microscope and quantified by ImageJ.

7.6 Western Blotting and Densitometry

Cells were lysed with standard PLC lysis buffer containing protease and phosphatase inhibitors (Sigma-Aldrich). Protein concentration was determined using the bicinechoninic acid (BCA) assay (Pierce Chemical Co.). Lysates containing 30 ug total protein were loaded onto 10% or 12.5% SDS-PAGE gels and electrophoresed. Proteins were then transferred onto PVDF membranes (NEN Research Products) using a semidry transfer apparatus (Bio-Rad). Membranes were probed for varying proteins overnight in 5% BSA. Antibodies were as follows: GAPDH (1:1000; Cell Signalling), beta-actin (1:10,000; Sigma), PARP1 (1:1000; Cell Signalling), PAR (1:1000, Millipore). After incubation, membranes were washed 3 times for 20 minutes with TBS with 0.1% Tween 20 and incubated with horseradish peroxidase–conjugated antibodies specific for the primary antibody (BioRad Laboratories). Binding was detected using Chemiluminescence Reagent Plus (PerkinElmer). ImageJ software was utilized to quantify images.

7.7 Immunohistochemical Staining and Scoring

Paraffin-embedded blocks were cut into 5-µm sections and were dewaxed in xylene followed by rehydration in a standard alcohol series. Antigen retrieval was achieved by pressure cooking for 20 minutes in citrate buffer (pH 6.0), followed by blocking of endogenous peroxidase in 0.3% H2O2. The PARP1 antibody (1:500 dilution, Abcam) was added and incubated overnight at 4°C. Detection used biotinylated secondary antibodies for 30 minutes, the ABC reagent kit (Vector Labs), and DAB chromagen (Vector Labs). Sections were counterstained in hematoxylin (Fisher
Scientific Inc.) for 30 seconds; dehydrated in 70%, 80%, and 100% ethanol; briefly washed in xylene; and mounted in Permount (Fisher Scientific Inc.). H&E sections were stained using standard protocols (Eosin Yellowish Solution, 1% w/v; Fisher Scientific Inc.). Two observers blinded to clinical data scored all slides for both intensity (negative, weak or strong) and distribution (0-25%, 25-50% or >50%) (Concordance: 94%). If distribution was greater than two and intensity greater than one a core was considered positive. If two of three cores were positive, a tumour was considered positive for PARP1.

7.8 PARP1 Silencing

Lyophilized PARP1 Smartpool siRNA (Thermo Scientific) and scrambled RNA control (Thermo Scientific) were reconstituted according to manufacturer’s instructions. 20nM of scrambled and PARP1 siRNA were used to treat cells. Cells were incubated with siRNA and scrambled control for a period of five days. Cells were collected for Western blot analysis on day two, three, and five.

7.9 Cell Cycle Analysis

Following 72-hour treatment with either DMSO or Niraparib, 1x10^6 cells were washed in ice-cold PBS and fixed with ice-cold 80% ethanol for 30 minutes at 4°C. Cells were then resuspended in RNase A for 5 minutes, followed by incubation with propidium iodide and NP-40 for 30 minutes. Cells were then filtered through 85 µm Nitex mesh and analyzed via flow cytometry for the population of cells in each stage of the cell cycle. FlowJo was used to analyze data.

7.10 Evaluation of PARP Inhibition in Combination With Stereotactic Cortical Radiation in an Orthotopic Model of Paediatric High Grade Astrocytoma

Stereotactic-guided intracranial implantation in NSG mice were performed by injecting 50,000 SJG2 cells into the frontal cortex (Coordinates: X=-1.0, Y=1.5, Z=2.4, with Bregma serving as reference point). Drugs were administered by IP injections on the fifth day post surgery. Experimental arms included Saline (vehicle), Niraparib 50mg/kg daily for two 5-day cycles, 2Gy radiation once a day for two 5-day cycles (total of 20Gy), or Niraparib administered 6 hours
before radiation. Mice were sacrificed upon signs of sickness. Animal use protocols (AUPs) were approved by the University Health Network (UHN) animal care committee (ACC).

7.11 Statistics

All experiments were done in triplicate. Mean and SEM is used where appropriate. Two tailed T-tests, ANOVA, Linear regression, and Kaplan-Meier analyses were used. ANOVA for multi-group comparisons was followed by a post-hoc Dunnett’s test or a post-hoc Tukey’s test where appropriate. Statistics were completed with GraphPad Prism 6.0. *, **, ***, **** Denotes significance of p<0.05, p<0.01, p<0.001, p<0.0001. Error bars represent SEM.
Chapter 3 Results

"Data! Data! Data!" he cried impatiently. "I can't make bricks without clay." – Sherlock Holmes (The Adventure of the Copper Beeches)

8 Results

8.1 Poly-ADP-Ribose Polymerase 1 is expressed in Paediatric High Grade Astrocytoma and Diffuse Intrinsic Pontine Glioma Patient Samples and Patient Derived Cell Lines, and PARP1 Loss Reduces Proliferation.

PARP1 protein levels in pHGA and DIPG patient tumour samples were evaluated using tissue micro arrays (TMA), which were constructed from formalin-fixed-paraffin-embedded pHGA and DIPG samples obtained from The Hospital for Sick Children Pathology Department. Each TMA contained tissues from different parts of normal brain as negative controls. While normal neo-cortex brain tissues were negative for PARP1 protein expression non-malignant cerebellum showed weak staining (data not shown). Immunohistochemical analysis demonstrated PARP1 protein expression in 28/33 pHGA and 13/17 DIPG (Figure 10A). In the majority of pHGA and DIPG cases, PARP1 protein demonstrated nuclear localization. PARP1 protein levels were also characterized by Western blot in three sets of tumour-to-normal DIPG autopsy samples (Figure 10B). After conducting densitometry analysis, two of three pairs had higher PARP1 levels in tumour relative to normal tissue control (Figure 10C). As majority of patient tumour cases examined expressed PARP1 we characterized established and primary pHGA and DIPG cell lines to establish an in vitro model system. Western blotting and densitometry demonstrated high PARP1 expression in all cell lines tested except two pHGA cell lines – 462 and 626 (Figure 11A and B). Prior to evaluating the effect of PARP inhibitors we tested whether loss of PARP1 by siRNA would detrimentally affect cell growth. Knockdown of PARP1 in SJG2 significantly reduced proliferation validating our hypothesis that PARP1 is a potential target (Figure 11C and D).
Figure 10 – PARP1 is present in pHGA and DIPG patient samples. Immunohistochemical staining for PARP1 in pHGA and DIPG TMAs with a hematoxylin and eosin counter stain demonstrating variable expression (A). Expression and densitometry of PARP1 in normal-to-tumour pairs obtained from three DIPG patients (B, C). All images were taken at a 10X magnification. *** Denotes significance p<0.001. Error bars represent SEM. (B) and (C) consist of a minimum of three biological replicates. N= normal tissue, T= tumour tissue.
Figure 11 - PARP1 is expressed in pHGA and DIPG derived cell lines and PARP1 loss affects cell proliferation. Western blot (A) and densitometry (B) of PARP1 demonstrating variable expression in NHA, pHGA, and DIPG cell lines. Western blot (C) of PARP1 showing effective protein knockdown and cell count (D) of SJG2 at various time points after an incubation with 20 nM PARP1 siRNA and scrambled control. *, **, *** Denotes significance of p<0.05, p<0.01, p<0.001. Error bars represent SEM. In (B) densitometry analysis was conducted on three biological replicates, in (C) densitometry analysis was conducted on three technical replicates.
8.2 Compared to Veliparib and Olaparib, Niraparib Monotherapy is More Effective at Reducing Tumour Cell Growth.

We decided to evaluate the utility of monotherapy with three clinically relevant PARP inhibitors, Veliparib, Olaparib, and Niraparib, using the MTT proliferation and colony formation assays. Non-immortalized fetal normal human astrocytes (NHA) were used to test for potential toxic effects of PARP inhibitors, no reduction in proliferation was detected (Figure 12B). While all PARP inhibitors tested inhibited PARP1 activity, only Olaparib and Niraparib significantly reduced PARP activity and cell proliferation (Figure 12A and B). Niraparib was the most effective monotherapeutic agent in the majority of the cell lines tested. Niraparib generally reduced cell proliferation at doses ≥2uM, while Olaparib reduced cell proliferation at doses ≥5uM. These results suggest that inhibition of PARP activity does not correlate with the ability of an inhibitor to affect cell proliferation. Next we examined our data for an association between PARP1 protein levels and response to PARP inhibitors (Figure 12C). We did not find significant trends between cell proliferation and PARP1 protein levels. To corroborate the results obtained from MTT proliferation we evaluated clonogenic growth of SJG2 and SF188 cells after treatment with different doses of Veliparib, Olaparib, or Niraparib (Figure 12D, E). The colony-forming assay demonstrated that Niraparib is more effective at reducing tumour cell growth relative to Olaparib or Veliparib. As our results indicate that Niraparib was more effective than Olaparib or Veliparib, we conducted all further experiments with Niraparib.
Figure 12 - Effects of PARP inhibitors on PARP activity and cell growth

Western blot of total cell PAR in SJG2 cells after 6h treatment with Niraparib, Olaparib, Veliparib (location of auto-PARylated PARP1 is indicated by arrow) (A). MTT relative absorbance values across multiple cell lines after a 7-day incubation with Veliparib, Olaparib, or Niraparib. Each heatmap box represents the average of at least three independent experiments (B). MTT relative absorbance values across multiple cell lines after a 7-day incubation treated with 5uM Veliparib, Olaparib, or Niraparib plotted against PARP1 protein levels of each cell line (C). Clonogenic survival of SJG2 and SF188 cells after incubation of SJG2 or SF188 in indicated doses of Veliparib, Olaparib, or Niraparib (D and E). *, **, ***, **** Denotes significance of p<0.05, p<0.01, p<0.001, p<0.0001. Error bars represent SEM. Results in (A) and (B) consist of three biological replicates. Results in (D) and (E) represent three technical replicates.
8.3 Niraparib Monotherapy Induces DNA Damage and Reduces Cell Proliferation.

We postulated that Niraparib might be reducing viability by inhibiting endogenous DNA repair that could lead to increased DNA damage and reduced proliferation. To determine the acute effects of Niraparib on DNA damage and cell proliferation, SJG2, SF188, KNS42, and DIPG58 cells were incubated with 5μM of Niraparib for 24h. Levels of DNA damage and percentage of proliferating cells were evaluated using phosphorylated (Ser139) histone variant 2AX (γH2AX) and Kiel-clone 67 (Ki-67), as markers of DNA damage and proliferation, respectively (Figure 13A, B, C, D). Across all cell lines tested, Niraparib significantly increased γH2AX levels and reduced ki67 positivity.

Figure 13 - Niraparib treatment increases DNA damage and decreases proliferation. Immunofluorescent staining for γH2AX and ki67 in SJG2, SF188, KNS42, and DIPG58 cells after a 24-hour incubation with 5μM Niraparib. DAPI was used as a nuclear counterstain. (A, C) Quantification of γH2AX and ki67 in SJG2, SF188, KNS42, and DIPG58 (B, D). All images were taken at 63X magnification. *, **, ***, **** Denotes significance of p<0.05, p<0.01, p<0.001, p<0.0001. Error bars represent SEM. V = Vehicle N = 5μM Niraparib. Minimum of three technical replicates were completed, at least 100 cells were counted from each replicate.
8.4 Induction of Growth Arrest and Apoptosis after Niraparib Treatment is Cell Type Specific

DNA damage may result in apoptosis and/or growth arrest. SJG2 and SF188 cells were treated with 5uM on Niraparib for 72h. While Niraparib significantly reduced total cell number in both SJG2 and SF188 cells, trypan blue viability was only significantly reduced in SJG2 cells (Figure 14A, B). PARP1 can be cleaved by caspase 3/7 during the latter stages of the apoptotic cascade (123). In SJG2 but not SF188 cells treatment with Niraparib caused a significant increase in cleaved PARP1 levels indicating that SJG2 cells were undergoing apoptosis (Figure 14C, D). In addition to apoptosis Niraparib induced significant S phase and G2/M arrest in SJG2 and SF188 cells, respectively (Figure 14E, F). Our results also demonstrate an increase in Sub-G1 content in SJG2 cells. While presence of small fragmented DNA is not specific to apoptosis as it can also indicate necrotic cell death, when combined with cleaved PARP and trypan blue assays it suggests that SJG2 cells were likely undergoing apoptosis.

Because SJG2 and SF188 cells responded differently to Niraparib, we decided to explore this further with IR. We used IR because we wanted to evaluate whether this difference between apoptosis and growth arrest in SJG2 and SF188 cells was specific to PARP inhibition or a general approach the cells utilized when they encountered DNA damage. SJG2 and SF188 cells were treated with increasing doses of IR and analyzed after 72h for total cell number and cell viability via Trypan Blue (Figure 14G, H). IR significantly reduced viability of SJG2 cells but not of SF188. Nevertheless, in both cell lines ionizing radiation significantly reduced proliferation. This suggests that SJG2 and SF188 have different cellular mechanisms for responding to DNA damage, whether a PARP inhibitor or IR induces that damage.
Figure 14 – Induction of apoptosis and cell cycle arrest after Niraparib treatment is cell type specific. Direct cell count and Trypan Blue cell viability of SJG2 and SF188 cells after being treated with 5uM Niraparib for 72h (A, B). Western blot for cleaved PARP1 in SJG2 and SF188 after being treated with 5uM Niraparib for 72h (C, D). Propidium Iodide DNA staining analysis in SJG2, SF188, cells, respectively, after 72h incubation with 5uM Niraparib (E, F). Direct cell count and Trypan Blue cell viability of SJG2 and SF188 cells at 72h after being treated with indicated doses of ionizing radiation (G, H). *, **, ***, **** Denotes significance of p<0.05, p<0.01, p<0.001, p<0.0001. Error bars represent SEM. All experiments were done using at least three technical replicates.
8.5 Low Dose Niraparib Results in Persistence of Radiation Induced DNA Damage and Sensitizes Tumour Cells to Ionizing Radiation.

Since PARP is involved in repair of DSB we hypothesized that PARP inhibition would inhibit IR induced DNA damage repair and sensitize cells to radiation. Since we previously demonstrated that Niraparib at 5uM induced apoptosis and growth arrest we used 1uM to evaluate radiosensitization to avoid confounding our experiment with apoptosis and/or growth arrest. To study the effect of Niraparib on persistence of DSBs in pHGA, the presence of γH2AX foci was assessed by immunofluorescence. In pHGA cell lines, SJG2, SF188, and KNS42, rapid formation of γH2AX foci was observed minutes after ionizing radiation (Figure 15, Figure 16A, B, C). A six-hour pretreatment with 1uM Niraparib did not significantly increase the background levels of DNA damage. One hour after irradiation, more γH2AX foci were observed in Niraparib pretreated cell relative to cells treated with radiation alone. In SJG2 and KNS42 this difference persisted up to 72 hours after radiation. To evaluate the effects of Niraparib and radiation on survival and proliferation of pHGA cells a colony-forming assay was used. A six-hour pretreatment with 1uM Niraparib was significantly more effective at reducing clonogenic survival when combined with 2Gy of ionizing radiation relative to Niraparib or radiation alone. (Figure 16D).

**Figure 15 - Low doses of Niraparib reduces the rate of DNA repair.** Representative example of SJG2 cells after immunofluorescent staining for yH2AX with DAPI as a counterstain after 1uM Niraparib, 2Gy gamma radiation, or 2Gy radiation pre-treated with 1uM Niraparib. All images were taken at 24X magnification.
Figure 16 – Low doses of Niraparib reduces the rate of DNA repair and sensitizes cells to ionizing radiation. A, B, C Fold change of γH2AX foci per nuclei in SJG2, SF188, KNS42 cells, respectively. E Clonogenic survival of SJG2, SF188, and KNS42 cells after 2 Gy of gamma radiation alone or in combination of 1μM Niraparib administered prior to radiation. *, **, ***, **** Denotes significance of p<0.05, p<0.01, p<0.001, p<0.0001. Error bars represent SEM. Minimum of three technical replicates were completed. For immunofluorescent quantification analysis at least 100 cells were counted from each replicate.
Niraparib in Combination with Radiation Prolongs Survival of pHGA Orthotopic Mouse Model.

We hypothesized that Niraparib in combination with ionizing radiation will increase survival. To test this we used a SJG2 orthotopic mouse model, previously used by our group, due to its reliable tumour formation. (28). We also confirmed tumour formation by histopathological examination (Figure 17C). We used a small pilot experiment (n=3) to ensure that SJG2 cells retain PARP1 expression in vivo and to see if Niraparib was capable of penetrating into the tumour and inhibiting PARP activity. Previous studies demonstrated that 50mg/kg doses of Niraparib, alone or with radiation, were well tolerated in mice and when combined with radiation achieved the most significant tumour growth reduction (119, 120). Furthermore the clearance rate of Niraparib is 36 hours in humans and 24 hours in mice (118) (119). Taking this into account we chose to administer either saline or Niraparib at a dose of 50mg/kg via IP injections.

For our pilot studies Niraparib was administered (1 dose/day x 3 days) after mice developed tumours and were sacrificed at 6 hours after the last administration of Niraparib. We treated the mice for three days to ensure that Niraparib had sufficient time to penetrate the blood brain barrier and establish a steady-state equilibrium within the tumour mass. Tumours and contralateral normal brain tissue was harvested and analyzed by Western blot for PARP1 protein levels and PARP activity (Figure 17A, B). To test the in vivo effect of Niraparib either alone or in combination with IR, mice were randomized into four treatment arms: vehicle (n=5), Niraparib (50mg/kg) (n=5), 20 Gy IR (2Gy/fraction/day) (n=4; n=5 were injected but one mouse did not develop a tumour), and Niraparib+IR (50mg/kg+20Gy; Niraparib was administered by IP 6h before IR) (n=7, n=10 were injected; 1 mouse died during surgery; 2 mice developed eye irritation and were sacrificed) (Figure 17D). Based on power calculations we chose to use a minimum of 5 mice in each arm as this would provide sufficient power to detect a difference of 5 days with a standard deviation of 4 days. The niraparib+IR arm initially contained 10 mice to account for potential losses as combining IP injections with IR is very stressful for the mice. Compared to vehicle treated mice IR and Niraparib treated mice demonstrated significant increase in survival (25 days vs 27.5, 32, respectively). However, mice that were pre-treated 50mg/kg Niraparib before each fraction of IR received the longest survival benefit (~doubling of overall survival p<0.001). It is important to note that two of the nine mice in the Niraparib+IR arm exhibited significant eye irritation after combined treatment.
Figure 17 – Niraparib in combination with ionizing radiation extends survival.
Western blot of PARP1 and PAR in normal mouse brain (NRML) obtained from the contralateral hemisphere and tumor (TUMOR) tissue (A). Western blot of PARP1 and PAR in tumour tissue obtained from mice treated with three rounds of saline or 50mg/kg of Niraparib (B). Hematoxylin and eosin stain of tumour produced by intracranial injection of 50,000 SJG2 cells (C). Kaplan Meier survival curve comparing survival of an orthotopic xenograft model after treatment with vehicle, 50mg/kg Niraparib, 20 Gy, or a combination of 20Gy and 50mg/kg of Niraparib (D). All images were taken at a 24X magnification. *, **, ***, **** Denotes significance of p<0.05, p<0.01, p<0.001, p<0.0001. Error bars represent SEM. (A) and (B) experiments were conducted on at least three technical replicates.
Chapter 4 Discussion and Future Directions

“Having gathered these facts, Watson, I smoked several pipes over them, trying to separate those which were crucial, from others which were merely incidental.” - Sherlock Holmes (The Crooked Man)

9 Discussion and Future Directions

9.1 Treating Paediatric High Grade Astrocytoma in vitro: Niraparib Inhibits Growth

Using immunohistochemistry and Western-blotting we demonstrated that PARP1 is expressed in pHGA and DIPG patient samples, thus suggesting that PARP1 may be a potential therapeutic target in these cancers. Previous immunohistochemical analyses by our group demonstrated PARP1 expression in 54% of DIPG analyzed (6/11; 4 high, 2 low) (11). Others groups demonstrated that in pHGA TMA cohorts, PARP1 positivity can range from 28.1% to 100% (22, 24, 106). In our TMA patient cohort, PARP1 expression was found in more pHGA than DIPG cases, 84% vs. 76%, respectively. We corroborated our clinical data by further characterization of DIPG normal-tumour patient samples, five pHGA, and three DIPG cell lines. We observed that PARP1 expression was found in the majority of samples tested and in SJG2 loss of PARP1 by siRNA negatively affected cell growth.

9.2 Treating Paediatric High Grade Astrocytoma in vitro:

We demonstrated that while all PARP inhibitors sufficiently inhibit PARP activity not all PARP inhibitors are able to reduce tumour cell growth when used in a monotherapeutic fashion. Similar to our results, Jelinic et al. also demonstrated that Olaparib was more effective than Veliparib at reducing cell growth in vitro (107). We further validated our MTT proliferation results using colony-forming assays in two cell lines. Additionally we found that Niraparib, when used at 5uM, induced formation of DNA damage and reduction of ki67 staining across multiple cell lines. To further explore the effects of Niraparib we used SJG2 and SF188 because of their similar doubling rate. Initially we believed that differences in sensitivity to Niraparib and Olaparib were attributed to PARP1 protein expression levels. However, after correlating PARP1
protein levels across multiple cell lines vs. reduction of proliferation due to PARP inhibitors we did not find any trends. Our results suggest that while there is no correlation between PARP1 protein levels and PARP inhibitor cytotoxicity, some level of PARP1 protein must to be present in order for the inhibitors to have their effect. When we tested three PARP inhibitors as monotherapy in NHA, cells without any PARP1 protein, no reduction of proliferation by MTT was detected. Furthermore, in a study by Murai et al, PARP1 silencing by siRNA protected DT40 chicken lymphoma cells from Niraparib and Olaparib. Data by Murai et al suggests that Niraparib is specific for PARP1, and that PARP1 must be present for the inhibitors to function (103). It is likely that the relationship between PARP1 protein levels and inhibitor cytotoxicity is more complex than we assumed and depends on other factors such as the efficacy of other DNA damage repair pathways in the cell.

While evaluating Veliparib, Olaparib, and Niraparib as monotherapy, we found that Olaparib and Niraparib, but not Veliparib, reduced cell proliferation by MTT. Murai et al have also demonstrated that Niraparib and Olaparib, but not Veliparib reduce reduced cell growth and Chang et al found that Olaparib but not Veliparib was capable of inducing apoptosis in a panel of breast cancer cell lines (103, 109). Murai et al found that certain PARP inhibitors, Niraparib and Olaparib, are capable of forming PARP1-DNA damage complexes that are thought to be cytotoxic to dividing cells (103). His group postulated that PARP inhibitors have a dual mechanism of action by inhibition of induced DNA repair and production of DNA damage by forming endogenous PARP1-DNA complexes.

We noticed from our MTT proliferation and clonogenic growth assays that Niraparib was much more effective at inhibiting the growth of SJG2 cells than SF188 cells and decided to evaluate this in more detail. It is interesting that induction of apoptosis and/or growth arrest was not dependent on mechanism of DNA damage (ie: by Niraparib or IR) but was instead a general response that the cell line had to DNA damage. Radiation is known to induce DSBs in DNA and it was interesting that SF188 cells were resistant to IR (even at 10Gy) and Niraparib – suggesting that Niraparib may be causing DNA damage by producing DSBs. This is plausible as the recent finding by Murai et al found that Niraparib induced formation of PARP1-DNA complexes that were cytotoxic in proliferating cells because during collisions with replication forks these complexes produced DSBs (103). The different responses observed between SJG2 and SF188 to Niraparib may also be attributed to their PTEN status – SJG2 are PTEN−/−, while SF188 are...
PTEN\textsuperscript{wt}. It has been reported that cancer cells lacking PTEN are more sensitive to PARP inhibitors because of down regulation of Rad51. (121 122).

PARP1 is well known for its role in repair of ssDNA and dsDNA breaks (111). We found that Niraparib sensitized cells to IR induced DNA damage by preventing DNA damage repair and reduced clonogenic growth. Across all cell lines tested, 6 hour pretreatment with Niraparib (1uM) reduced the rate of IR induced DNA damage repair and significantly decreased clonogenic growth with combined with radiation. Previous work by Cloos’ group found that Olaparib (4uM) reduced the rate of IR induced DNA damage repair in several paediatric brain tumour cell lines (21). Both, the Cloos’ group and ours used SF188 and KNS42 paediatric GBM cells and while data by Cloos’ shows that Olaparib at 4uM effectively reduced the rate of IR induced DNA damage repair and clonogenic survival when combined with radiation, we found that Niraparib at 1uM has the same effect in both cell lines and was more effective at reducing clonogenic growth. Therefore, our data suggests that Niraparib is a better radiosensitizer compared to Olaparib.

9.3 Treating Paediatric High Grade Astrocytoma \textit{in vivo}: Niraparib Extends Survival

Currently, there have been no studies showing an effective response to Niraparib and IR in an \textit{in vivo} model of pHGA. Furthermore, after obtaining promising \textit{in vitro} results showing that Niraparib was an effective both as a monotherapeutic and a radiosensitzing agent we proceeded to test the effectiveness \textit{in vivo}. Since efficacy of Niraparib depended on the presence of PARP1 protein we confirmed that our orthotopic pHGA model using SJG2 cells retained PARP1 expression and PARP1 activity \textit{in vivo}. We then proceeded to evaluate whether Niraparib was able to cross the blood brain barrier and penetrate the tumour mass. PARP activity within the tumour was completely abolished after three administrations of 50mg/kg of Niraparib. To test the effect of Niraparib \textit{in vivo} we proceeded to treat mice implanted with SJG2 cells with either saline, as vehicle control, Niraparib, IR, or Niraparib+IR. Niraparib and IR when used alone extended survival by 28% and 10%, respectively. These results suggested that tumour growth was attenuated by increasing the burden of DNA damage, however the tumours were likely capable of repairing this DNA damage and thus causing mice to die. Pre-treatment of tumours with Niraparib prior to radiation extended survival by 40% suggesting that persistent levels of
unrepaired DNA damage attenuate tumour growth. To the best of our knowledge our study is the first to demonstrate that Niraparib is capable of crossing the blood brain barrier and extending survival of an orthotopic model of pGBM.

9.4 Conclusions

In conclusion, our study provides \textit{in vitro} and \textit{in vivo} evidence that Niraparib is more effective when used as a radiosensitizer compared to monotherapy in models of pHGA and DIPG. However, we encountered some radiation toxicity of normal tissue, likely due to radionsensitizing effects, in two of our nine mice when Niraparib was combined with IR. While the Niraparib+IR treatment arm extended survival the most, more work should be done to optimize the dose of Niraparib and IR to avoid toxicity. Our findings are significant for two reasons. First, as some paediatric brain tumours show radioresistance, Niraparib can be used to sensitize these tumours to radiation. Second, we demonstrated that there is a survival benefit with Niraparib alone, albeit not as pronounced. This finding is useful because Niraparib can be used alone if its combination with IR is too toxic thus still providing a survival benefit without the toxicity associated with conventional IR therapy. This is in line with the current results of a Phase I clinical trial of monotherapeutic applications of Niraparib in patients with breast and ovarian cancers showing that it was well tolerated even at high doses (118). While our results are encouraging, more work is needed to evaluate different chemo- and radio-sensitization properties of Niraparib. For example, since PARP1 is involved in BER, PARP inhibitors should be evaluated in combination with TMZ using \textit{in vitro} and \textit{in vivo} models of pGHA and DIPG. Furthermore, since we are proposing the use of Niraparib in a paediatric population more information about Niraparib’s long-term effects should be taken into consideration if this drug is to be used for treating childhood cancers.

9.5 Future Directions

Regarding PARP inhibition in vitro, better understanding of the mechanism should be obtained as to why some cells undergo apoptosis while other undergo growth arrest when treated when PARP inhibitors, and whether a growth arrest phenotype can be manipulated into that of apoptosis. A good way to tease apart the mechanism would be to use a proteomic micro array that contains probes (antibodies) for apoptosis related pathways (this kit is available from Cell Signaling). After identifying which pathways are used to avoid apoptosis, inhibitors or siRNA of
certain effectors of these pathways can be used in attempts to sensitize resistant cells to PARP inhibitors. A more costly approach would be to use a siRNA screen to identify targets that when knocked down in resistant cell lines induce apoptosis. However, the limitation of the siRNA screen is two fold. First, there is no way to know whether the knockdown caused apoptosis because that protein was in fact involved in resistance or that protein simply sensitized the cells to DNA damage. The second limitation is that majority of these large throughput screens use MTT, MTS or Alamar blue as a read out. All of these dyes are based on mitochondrial metabolic activity and not on the actual growth arrest or apoptosis of cells. If the read out can be adapted to using trypan blue, or caspase assay, more pertinent information can be obtained. Furthermore, these experiments should be repeated as newer, more potent, PARP inhibitors become available. Also, combining Niraparib with other conventional therapies such as temozolomide should be evaluated in the paediatric setting. There is an urgent need to develop more preclinical animal models of pHGA and DIPG to test therapeutic responses and gain more insight regarding tumour biology. These animal models should be clinically and biologically relevant and be used to validate the effects of Niraparib and other novel PARP inhibitors developed in the future. As such the limitation of the in vivo portion of this project work is the use of only one orthotopic mouse model. Further studies should test this using different orthotopic mouse models as well as genetic models of pHGA and DIPG.
Chapter 5 References

"It is of the highest importance in the art of detection to be able to recognize, out of a number of facts, which are incidental and which vital. Otherwise your energy and attention must be dissipated instead of being concentrated." - Sherlock Holmes (The Reigate Puzzle)

10 References


63. Yang, Y.G., Cortes, U., Patnaik, S., et al. Ablation of PARP1 does not interfere with the
repair of DNA double-strand breaks, but compromises the reactivation of stalled replication forks. *Oncogene* 2004. 23, 3872–3882


100. Dungey FA, Loser DA, Chalmers AJ. Replication-dependent radiosensitization of human


MK-4827 together with Radiation as a Novel Therapy for Metastatic Neuroblastoma. 
*Anticancer Res* 2013 (762) 755-762.


