Overcoming resistance to targeted and non-targeted therapies with combi-molecules designed to target PARP

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A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

ABSTRACT

Targeting cancer cells at multiple signaling pathways has become the most common approach to sensitize them to chemotherapy. It is in this context that our laboratory developed the combi-targeting concept, that seeks to design molecules to modulate different targets. Here, to overcome resistance to both targeted and non-target therapies, we investigated two major strategies: a) enhancing the potency of a chemotherapeutic agent by combining a DNA damaging agent with a DNA repair inhibitor in a single agent, and b) targeting the DNA repair inhibitor to a receptor overexpressed in cancer cells. In the context of these strategies, we demonstrated the feasibility of two classes of molecules: one designed to target PARP and DNA, with EG22 and ZSM02 as lead molecules, and one engineered to target PARP and EGFR, with ZSMR06 as a lead. Our first prototype PARP-DNA combi-molecule, EG22, showed similar inhibition of PARP as its corresponding naked PARP inhibitor, 4-amino-1,8-naphthalimide (ANI), in a PARP assay. It showed anomalously high levels of DNA damage when compared with temozolomide alone or temozolomide + ANI. It showed selectivity towards BRCA2 mutant cells and greater potency against MGMT positive cells, which are resistant to temozolomide. EG22 was more potent than temozolomide alone and its corresponding combination with ANI. Confocal microscopy analysis showed that it was primarily localized in the nucleus, which may explain its strong DNA damaging potential and growth inhibitory potency. While EG22 was a good prototype to demonstrate the feasibility of PARP-DNA combi-molecules, it was too unstable to be considered for further development. We therefore designed ZSM02 as a stabilized prodrug of EG22 and demonstrated that it has the same growth inhibition profile as the latter. In light of clinical trials demonstrating the toxicities of combinations of PARP inhibitors and DNA damaging agents, we decided to expand this work to the targeting of PARP inhibitors to EGFR, a validated cancer target. The first

EGFR-PARP combi-molecule, ZSMR02, was a strong PARP but a poor EGFR inhibitor. We then optimized this type of combi-molecule to finally produce a balanced EGFR-PARP combi-molecule, ZSMR06. The results showed that: (a) it is capable of inducing a dose-dependent inhibition of PARP in isolated enzyme assay, (b) it induced a dose-dependent inhibition of EGFR in an isolated kinase assay, (c) it showed a dose-dependent inhibition of EGFR phosphorylation and downstream signaling in whole cell assay, (d) it was selectively potent towards BRCA2 mutant and EGFR-overexpressing cell lines, (e) it was extremely potent, with activities superior to that of olaparib or gefitinib alone and their corresponding equimolar combinations in a panel of solid tumour cell lines. The significantly superior activity of ZSMR06 compared with the equimolar combination of olaparib + gefitinib indicated that perhaps the combi-molecule was acting through a unique mechanism of action. Indeed, it has proven highly effective in downregulating BRCA1 levels, which perhaps suggests that it is able to promote DNA repair deficiency in the cells. These results *in toto* suggest that this new combi-molecule could be developed as a single drug modality emulating the combination of PARP and EGFR inhibitors with the added benefit of being targeted to EGFR-expressing tumour cells. Further pre-clinical work is required to advance this new type of molecule to clinical trials.

RÉSUMÉ

Cibler les cellules cancéreuses par leurs multiples voies de signalisation est devenu l'approche la plus commune pour sensibiliser les tumeurs à la chimiothérapie. C'est dans ce contexte que notre laboratoire a mis au point le concept de fusion moléculaire multiciblée, nommé ‘’combi-ciblage’’. Ce concept se définit par la conception de molécules capables de moduler différentes cibles. Dans le cadre de cette thèse, pour surmonter la résistance aux thérapies ciblées et non ciblées, nous avons étudié deux stratégies : a) l'amélioration de l'activité d'un antinéoplasique, par la combinaison d'un agent endommageant de l'ADN avec un inhibiteur de réparation de l'ADN en une seule entité, et b) le ciblage du même inhibiteur de réparation d’ADN à un récepteur surexprimé dans les cellules cancéreuses. Dans le cadre de ces stratégies, nous avons démontré la faisabilité de deux classes de molécules : l'une visant à cibler le PARP et l'ADN, avec EG22 et ZSM02 en tant que molécules phares, et l'autre conçue pour cibler le PARP et l’EGFR, avec ZSMR06 comme molécule phare. Notre premier prototype de molécule PARP-ADN, EG22, a induit une inhibition de PARP aussi forte que 4-amino-1,8-naphthalimide (ANI), un inhibiteur de PARP déjà connu. Nous avons découvert que cette molécule peut infliger des niveaux anormalement élevés de lésions dans l'ADN par rapport à temozolomide, un médicament clinique connu pour sa capacité d’endommager l’ADN, et par rapport à sa combinaison avec ANI. EG22 a montré une sélectivité envers les cellules BRCA2 mutantes et une plus grande efficacité contre les cellules exprimant la protéine de réparation de l’ADN, MGMT, qui est responsable de la résistance liée au traitement du cancer avec le temozolomide. Nous avons démontré que EG22 est plus puissant que temozolomide lui-même ainsi que sa combinaison avec ANI. L'analyse par microscopie confocale montre que la molécule est principalement localisée dans le noyau, ce qui peut expliquer sa puissante capacité à endommager l'ADN et celle d’inhiber la croissance cellulaire. Malgré que nous ayons confirmé

que EG22 soit un excellent prototype pour démontrer la faisabilité des molécules PARP-ADN, son instabilité dans le milieu physiologique à compromis sa progression vers des étapes ultérieures de développement. Par conséquent, nous avons envisagé une stratégie de stabilisation qui consistait à acétyler son azote N3, donnant ainsi lieu à la formation de ZSM02. Par la suite, nous avons démontré que ZSM02 a le même profil d'inhibition de croissance que EG22, ce qui suggère qu'elle pourrait être sa prodrogue. Dans le contexte des résultats clinique rapportant la toxicité des combinaisons des inhibiteurs de PARP avec la chimiothérapie, nous avons redéfini notre stratégie vers le développent d'inhibiteur de PARP ciblé contre un biomarqueur exprimé dans plusieurs types de cancer : l'EGFR. La première molécule PARP-EGFR, ZSMR02, était un excellent inhibiteur de PARP mais un médiocre inhibiteur de l'EGFR. Nous avons donc dû l'optimiser pour finalement produire une molécule PARP-EGFR de ciblage équilibré ZSMR06. Nos résultats nous ont permis de démontrer que: (a) ZSMR06 est capable d'induire une inhibition de manière dose-dépendante de PARP dans un test enzymatique et cellulaire, (b) elle a induit une inhibition de manière dose-dépendante de l'EGFR dans un test de kinase et de phosphorylation cellulaire, (c) cette inhibition s'est traduite en celle des voix de signalisation associées à l'activation de l'EGFR (ERK, AKT), (d) plus important encore, ces mécanismes moléculaires se sont traduits en un ciblage sélectif des cellules BRCA2 mutées et de celles surexprimant l'EGFR. La capacité d'inhibition de croissance cellulaire était nettement supérieure à celle de la combinaison de deux antinéoplasiques cliniques l'olaparib et le gefitinib, démontrant ainsi que la molécule seule est supérieure à la combinaison de deux agents cliniques et que peut-être elle agit selon un mécanisme d'action unique. En effet, on a découvert que ZSMR06 est capable d'induire une très forte réduction de BRCA1, ce qui suggère qu'elle est peut-être capable de générer une déficience en réparation de l'ADN dans les cellules. Ces résultats suggèrent que cette nouvelle molécule pourrait

être développée comme un agent capable de mimer la combinaison d'inhibiteurs de PARP et d'EGFR avec l'avantage d'être ciblées aux cellules tumorales surexprimant l'EGFR. Nos résultats suggèrent que des études précliniques plus avancées pourraient faire progresser cette molécule vers la clinique.

ACKNOWLEDGEMENT

Since my youngest age, I have always been curious about the inner mysteries of the human body. I never had a doubt that research would one day be an integral part of my life. My PhD was an interesting journey and was not at all what I expected to be. It taught me way more than critical thinking and producing results. I had the chance to evolve as a person by becoming stronger, more confident and extremely independent. I owe all these wonderful changes mainly to my supervisor, Dr. Jean-Claude, but also to our collaborators, my thesis committee members, colleagues and family.

Dr. Jean-Claude not only gave me the opportunity to instigate my first steps in the biomedical research field, but also provided me with tremendous amount of great opportunities. I started my journey as a volunteer in his lab over a year before the beginning of my PhD. He taught me that the most important part of research, is knowledge. Since then, reading was the primary source of my research progression. Dr. Jean-Claude also allowed me to explore different research areas and patiently initiated me to the world of medicinal chemistry, which allowed me to complement my pharmacology background. Furthermore, he allowed me to broaden my scientific knowledge and refine my skills by giving me the opportunity to go abroad and learn new methods. What I am the most grateful of, is his belief in me. Dr. Jean-Claude allowed me to become completely independent and develop my own ideas.

I would like to thank my thesis committee members and collaborators for always encouraging me for my progress and helping me evolve. My meetings and discussion with them were contributed in interesting ideas to be explored.

Of course, no work in complete without team work! I was extremely lucky to have the best

colleagues, some of them became true friends. Along with my family, they provided me with the support and strength to overcome all the challenges encountered during my doctoral studies. Without them, I would definitely not be completing one of my biggest achievements so far.

Although this journey has come to an end, this was only the first chapter of a series. I will forever be grateful for all your love, patience and support!

Thank you!

CONTRIBUTIONS OF AUTHORS

This manuscript-based thesis is composed of three manuscripts and the contributions of each author are stated below.

CHAPTER 2: ^{15}N-, ^{13}C- AND ^{1}H-NMR SPECTROSCOPY CHARACTERIZATION AND GROWTH INHIBITORY POTENCY OF A COMBI-MOLECULE SYNTHESIZED BY ACETYLATION OF AN UNSTABLE MONOALKYLTRIAZENE

This paper was published in Molecules in July 19th, 2017. 22.7: 1183.

I synthesized the stable form of this combi-molecule to yield ZSM02 and crystallized it. I also synthesized the labelled compounds and performed the NMRs, and mass spectrometry of the labeled compounds. I interpreted the data with the help of Elliot Goodfellow. I carried out the biological assay and helped with the preparation of the manuscript. Elliot Goodfellow synthesized the prototype combi-molecule, EG22, and helped with the preparation of the manuscript. Dr. Robin Stein performed the HMBC and HSQC NMR of the non-labelled compound. Dr. Robin Rogers and Dr. Steven P. Kelley performed the crystallography of ZSMR02. Dr. Bertrand Jean-Claude overlooked the proceedings of the experimental work and revised the manuscript.

CHAPTER 3: A TYPE I COMBI-TARGETING APPROACH FOR THE DESIGN OF MOLECULES WITH ENHANCED POTENCY AGAINST BRCA1/2 MUTANT- AND O6-METHYLGUANINE-DNA METHYLTRANSFERASE (MGMT)- EXPRESSING TUMOUR CELLS

This paper was published in BMC Cancer in August 11th, 2017. 17.1: 540.

I have participated in the design, execution and the interpretation of the experiments in this paper. I have significantly contributed to the writing of the manuscript and handled all the referee-

requested revisions. Elliot Goodfellow synthesized one of the combi-molecules, EG22. Dr. Bertrand Jean-Claude overlooked the proceedings of the experimental work and revised the manuscript.

CHAPTER 4: A NOVEL COMBI-MOLECULE ENGINEERED TO TARGET THE PUTATIVE SYNTHETIC LETHAL INTERACTIONS BETWEEN THE EPIDERMAL GROWTH FACTOR RECEPTOR (EGFR) AND POLY(ADP-RIBOSE)POLYMERASE (PARP)

This manuscript is currently being finalized for submission.

Martin Rupp and I designed and synthesized ZSMR02, 03, 04 and 06 molecules. I designed all the experiments involving, growth inhibition, and the study of EGFR and PARP inhibitory arms of the molecules. I am grateful for having the opportunity to fully lead the decision process involving the progression of this work. I have significantly contributed to the writing of the manuscript. Martin Rupp significantly contributed in the design and synthesis of the molecules and he also trained me in western blots. Dr. Stochaj performed all the western blots and immunofluorescence experiments for the evaluation of homologous recombination deficiency and also significantly contributed in the decision process regarding these experiments. Dr. Junko Murai and Dr. Pommier evaluated the PARP trapping potential of the lead compound, ZSMR06. Dr. Bertrand Jean-Claude made all the necessary comments and revised the manuscript.

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LIST OF ABBREVIATIONS

γH2AX – Phosphorylated Histone 2AX

3AB - 3-aminobenzamide

5-FU – 5-fluorouracil

53BP1 - p53-Binding Protein 1

APE1 - Apurinic/apyrimidinic (AP) endonuclease

ART - ADP-ribosyltransferases

ATM - Ataxia-Telangiectasia Mutated

ATR - Ataxia-Telangiectasia Mutated-Related

ATP – Adenosine Triphosphate

BAP1 - BRCA1 associated protein-1

BARD1 - BRCA1-Associated Ring Domain Protein 1

BER – Base Excision Repair

BRCA1/2 – Breast Cancer gene 1/2

BRCT - BRCA1 C-Terminal domain

CDK – Cyclin-Dependent Kinase

CML – Chronic Myelogenous Leukemia

COX – Cyclooxygenase

CPD - Cyclobutane Pyrimidine Dimers

CSA, CSB - Cockayne syndrome A and B

CtIP - CtBP-interacting protein

DAG - 1,2-diacylglycerol

DNA – Deoxyribonucleic Acid

DSB – Double Strand Break

DSS1 - Deleted in Split-hand/split-foot Syndrome

EGF – Epidermal Growth Factor

EGFR – Epidermal Growth Factor Receptor

ER – Endoplasmic Reticulum

ERK – Extracellular signal-Regulated Kinase

FEN1 – Flap Endonuclease 1

FDA – Food and Drug Administration

HER2 – Human Epidermal Growth Factor Receptor-2

HMBC - Heteronuclear Multiple Bond Correlation spectroscopy

HR – Homologous Repair

HSQC - Heteronuclear Single Bond Coherence spectroscopy

IC_{50} - Inhibition Concentration (Inhibit Cellular Proliferation by 50%)

IFN-γ - Interferon gamma

IP3 – 1,3,5-triphosphate

IP-3R – 1,4,5-Triphosphate Receptor

JAK – Janus Kinase

MAPK – Mitogen Activated Protein Kinase

MEK - MAPK/Extracellular signal-regulated Kinase

Met – Hepatocyte growth factor receptor

MGMT – O^6-Methylguanine DNA Methyltransferase

MMR – Mismatch repair

MRN - Mre11-Rad50-Nbs1 complex

mTOR – Mammalian Target of Rapamycin

NAD+ - Nicotinamide adenine dinucleotide

NER – Nucleotide Excision Repair

NHEJ – Non-homologous End-Joining

NFκ-B – Nuclear Factor Kappa B

NLS - Nuclear Localization Sequence

NSCLC – Non-Small Cell Lung Cancer

OB - Oligonucleotide-Binding domains

PA - Phosphatidic Acid

PALB2 - Partner and localizer of BRCA2

PAR - poly (ADP-ribose) chain

PARP - Poly(ADP-ribose) polymerase

PCNA - Proliferating cell nuclear antigen

Pgp – P-glycoprotein

PKB – Protein Kinase B (commonly referred to as Akt)

PI3K – Phosphotidylinositol-3-Kinase

PIP3 – Phosphotidylinositol-3,4,5-triphosphate

PLCγ - Phospholipase C

PTB – Phosphotyrosine Binding

PTEN – Phosphatase and Tensin Homolog

RAP80 - Receptor-Associated Protein 80

RFC – Replication Factor C

RNA – Ribonucleic Acid

RPA – Replication Protein A

RTK – Receptor Tyrosine Kinases

SCD - SQ/TQ Cluster Domain

SH1/2/3/4 – Src Homology

SRB – Sulforhodamine B

STAT – Signal Transducers and Activators of Transcription

TDP1 - Tyrosyl-DNA-phosphodiesterase I

TGF-α - Transforming growth factor alpha

TKI – Tyrosine Kinase Inhibitors

TMZ – Temozolomide

TNBC – Triple Negative Breast Cancer

VEGFR - Vascular Endothelial Growth Factor Receptors

XPG - Xeroderma Pigmentosum Complementation Group G

XRCC1 - X-ray repair cross-complementing protein 1

CHAPTER 1: INTRODUCTION

1.1 Preface

Cancer drug development has greatly evolved since world war II. During that time, scientists observed a great depletion of bone marrow and lymph node cells after exposure to mustard gas. Replacing the sulfur atom by a nitrogen led to a new compound termed nitrogen mustard, which was shown to be active on transplanted lymphoid cells in a mouse model. After observing regression of the cancer, they tested the compound on one patient with non-Hodgkin's lymphoma and saw great results [1-3]. About two years later, the next anti-cancer agent, a folic acid antagonist, was developed. The folic acid analogs, such as methotrexate, showed undeniable remission in children with leukemia [4, 5]. The antifolates inspired further development of drugs that inhibited adenine metabolism. These agents are known as thiopurines and were then used in the treatment of acute leukemia [6, 7]. In the same decade, the first drug for solid tumours was developed. Heidelberger and colleagues synthesized a molecule with a fluorine atom in the 5-position of uracil base, known as 5-fluorouracil (5-FU) [8], which is now the main treatment for colorectal, breast and head and neck cancers [9]. Later, in the 1950s and 1960s, some antibiotics were found to have antitumour effects. The first antibiotic used in the treatment of cancer was actinomycin D that showed remarkable results in pediatric tumours [10]. These results then stimulated interest in the development of other antitumour antibiotics that are commonly used today, such as doxorubicin, epirubicin, daunorubicin, etc [11].

In the early 1960s and after many observations that single agent therapies did not lead to full remission, the first drug combination was used in the treatment of testicular cancer. They used different combinations of alkylating agents, antimetabolites and antitumours antibiotics, that showed significantly improved results [12]. Since then, several combinations have been tested and many are still used in the clinical management of numerous tumours. In 1963 vinca alkaloids, used

as antimitotic agents, were isolated and tested in Hodgkin's disease. Mouse models of Hodgkin's lymphoma injected with vinca alkaloids showed significant tumour regression. Similar results were observed in clinical studies [13]. Later in the 60s, another promising drug, ibenzmethyzin or procarbazine, was developed for the treatment of Hodgkin's lymphoma and showed favorable results [14, 15]. The chronology of all major development in cancer treatment is depicted in figure 1.1. All the anticancer agents developed prior to the 1990s were not designed to block cancer specific targets and thus caused wide variety of side effects. This has initiated the search for oncogenic targets and eventually the development of targeted therapies. The first successful targeted therapy, gleevec (imatinib mesylate), was first reported in 1996. This report described an extraordinary decrease in cellular proliferation in tumour chronic myelogenous leukemia (CML) cells carrying the Philadelphia chromosome that encodes a fusion protein, bcr-abl [16]. Gleevec then received the Food and Drug Administration (FDA) approval in 2001 for the treatment of treatment of chronic myelogenous leukemia [17]. Since then, major discoveries regarding deregulated oncogenic targets in different cancers have emerged and led to the design and development of many tyrosine kinase inhibitors. These small molecule inhibitors targeting various cancers include: gefitinib [18] and erlotinib [19] for epidermal growth factor receptor (EGFR), crizotinib for ALK and hepatocyte growth factor receptor (Met) [20], Sorafenib for Raf family kinases [21], vemurafenib for BRAF [22], etc.

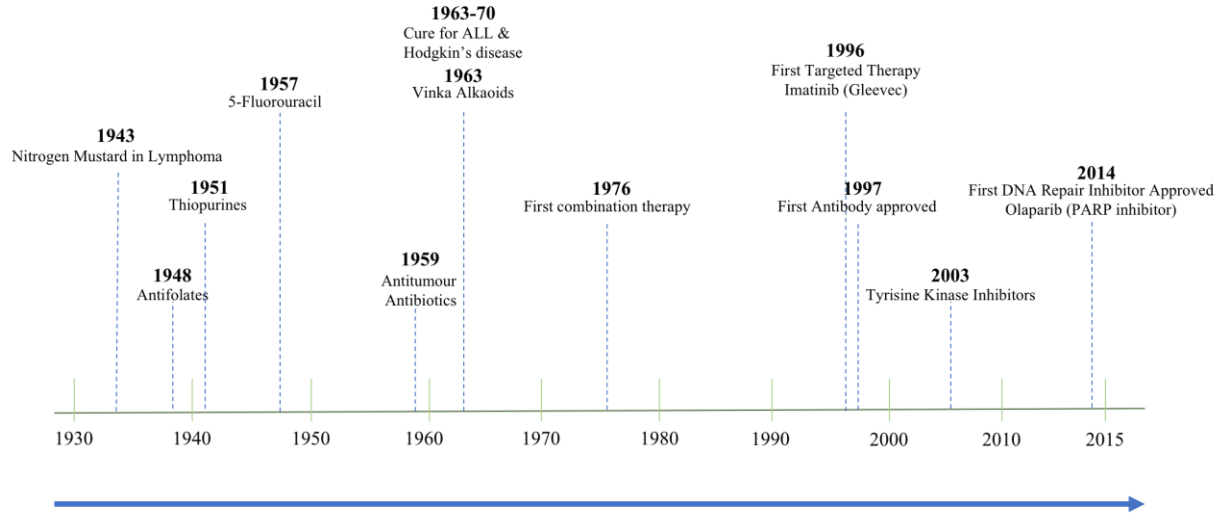


Figure 1.1. Historical overview of key dates in cancer drug development from the first non-targeted chemotherapy, through the first combination therapy, to finally the first approved targeted therapy.

Unfortunately, the development of these targeted therapies did not translate into significant clinical outcomes. Diminished efficacy in the clinic is mostly due to acquired resistance mechanisms. From the beginning of cancer drug development, the adaptive character of cancer cells made cancer treatment with a single agent quite challenging. For this reason, the idea of combination therapy or polypharmacology has emerged. Targeting cancer cells from multiple angles is then key. It is therefore crucial to direct cancer drug research towards the development of multitargeted drugs that are able to target cancer cells from multiple angles. With this idea in mind, our laboratory developed the combi-targeting concept, which involved single molecules (**I-Tz**) composed of two distinct targeting arms (e.g. **I** targeting EGFR and **Tz** targeting DNA). The two arms are connected by either a stable linker or a hydrolysable linker. Both arms can influence two cellular targets: inhibition of a kinase and damage DNA [23, 24]. This thesis focuses on the design, synthesis, and

elucidation of the mechanism of action of combi-molecules inhibiting a DNA repair with or without classical cytotoxic lesion.

The scope of the work presented herein is multidisciplinary and covers multiple aspects of drug discovery and development that converge into the discovery of a novel class of drugs. Prior to discussing the results obtain from this work, it is important to review some key aspects of cancer treatment considered to be relevant to this work. Our work being related to synthetic lethality, we will cover the principles of DNA repair, the key biomarker BRCA1/2, the role of poly(ADP-ribose)polymerase (PARP) and corresponding inhibitors. Our novel targeting approach being directed at the epidermal growth factor receptor (EGFR), we will review herein its structure and function and previous strategy developed in our laboratory to targeted the latter receptor.

1.2 DNA repair pathways

1.2.1 Overview

As depicted in figure 1.2, there are five basic DNA repair pathways and each of them repairs specific DNA damage [25, 26]. The base excision pathway (BER) repairs single strand breaks involving non-bulky base lesions caused by reactive oxygen species and alkylating agents [26]. Nucleotide excision repair (NER) engages in the recognition and elimination of bulky helix disturbing DNA lesions caused by UV irradiation and chemotherapeutic cross-linking agents [27]. As for double strand breaks, they are repaired by the homologous recombination repair (HR) or the non-homologous end-joining (NHEJ). HR is an error-free repair, which uses the homologous non-damaged sister chromatid as a template. In contrast, NHEJ joins the ends of the damaged DNA without using a homologous template, which makes the pathway error-prone. Double strand breaks are the most lethal type of DNA damage as a single unrepaired DSB can be sufficient to lead to cell death [28]. Thus, double strand break repair is critical for cell survival. Another DNA

repair pathway is the direct repair. It involves the direct reversal the damaged base without the excision or replacement of the base. This repair is performed by a single enzyme known as O^6-methylguanine-DNA methyltransferase (MGMT) [29]. The last DNA repair pathway is the mismatch repair (MMR). It eliminates errors such as mismatched bases, deletions and insertions [30].

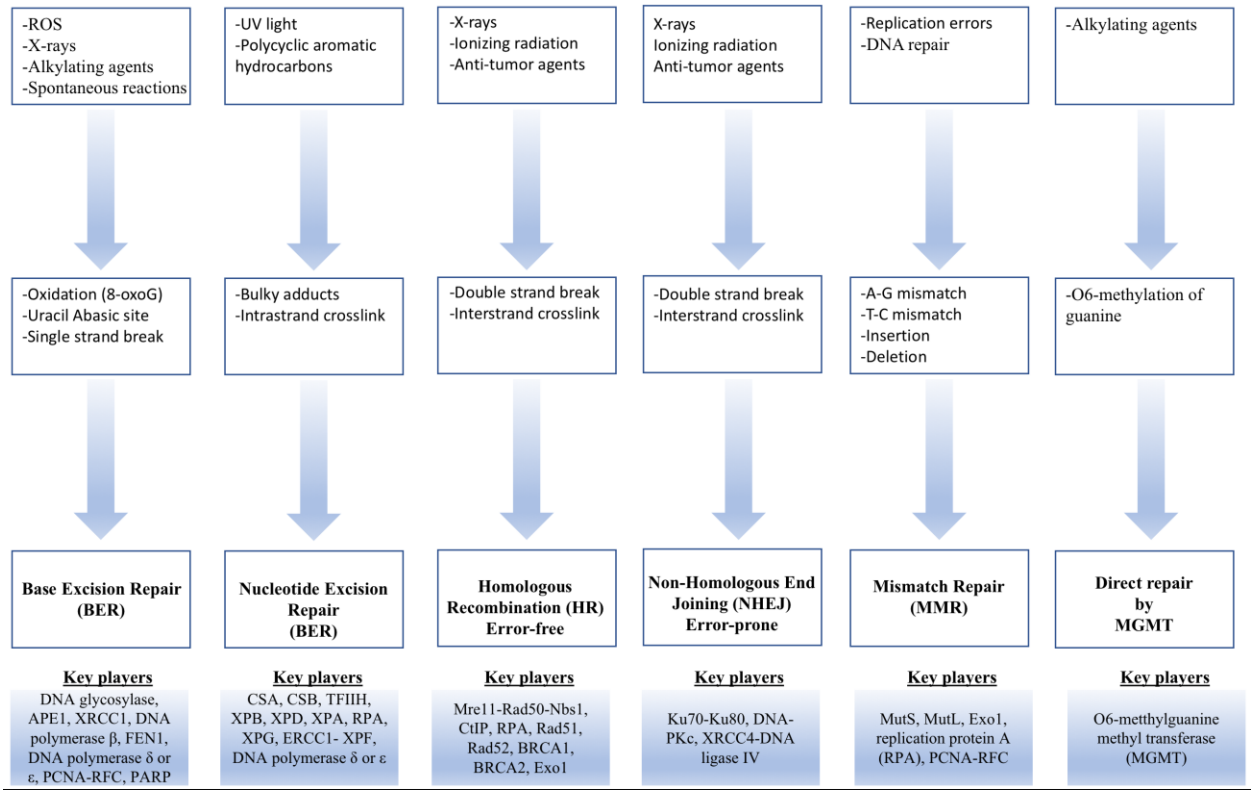


Figure 1.2. The six DNA repair pathways. Six different DNA repair pathways are triggered specific lesions caused by DNA damaging agents. Those pathways resolve the DNA damage by recruiting key DNA repair proteins.

1.2.2 BER pathway

The BER pathway is the main mechanism of DNA repair involving damaged bases [31]. Here we will only summarize the major step involved in the BER mechanism. More details will be given,

where the role of PARP, which is the main topic of this thesis, will be extensively covered. As outlined in figure 1.3, the pathway starts with lesion specific glycosylases that remove the damaged base. These enzymes mainly cleave the bond between the base and its deoxyribose, leading to a AP site [32]. Subsequently, AP sites are repaired by apurinic/apyrmidinic endonuclease 1 (APE1), which hydrolyzes the phosphodiester backbone at the $5'$-position, thereby creating a single strand break [33]. The DNA single strand break is then detected by poly(ADP-ribose) polymerase (PARP1), which acts as a molecular sensor of DNA strand breaks. PARP then binds to DNA and catalyzes the formation of a poly(ADP-ribose) (PAR) chain and transfers it to itself and many other protein substrates. The transfer of the PAR chain allows for recruitment of key repair proteins, such as X-ray repair cross-complementing protein 1 (XRCC1). The transfer of the negatively charged PAR chain onto PARP1 itself is also important for the dissociation of PARP1 from DNA to allow the recruited repair proteins to have access to the DNA damage site [34]. XRCC1 is a molecular scaffold protein whose main function is the assembly of key enzymes involved in the DNA single strand break repair. The recruited enzymes include: DNA glycosylases, DNA polymerase β, APE1, ligase III, PNKP, Tdp1, and APTX [35]. The nucleotide synthesis and ligation can follow two different sub-pathways: short-patch and long patch (41). The short patch BER involves the replacement of a single nucleotide by DNA polymerase β and is then ligated by DNA ligase III and XRCC1. The long patch BER involves the replacement of several nucleotides and required many proteins for the process such as: DNA polymerase δ or ε for DNA synthesis, PCNA (proliferating cell nuclear antigen) for elongation, RFC (replication factor-C) for PCNA loading, FEN1 (flap endonuclease-1) for overhang cleavage, and DNA ligase I for ligation [36].

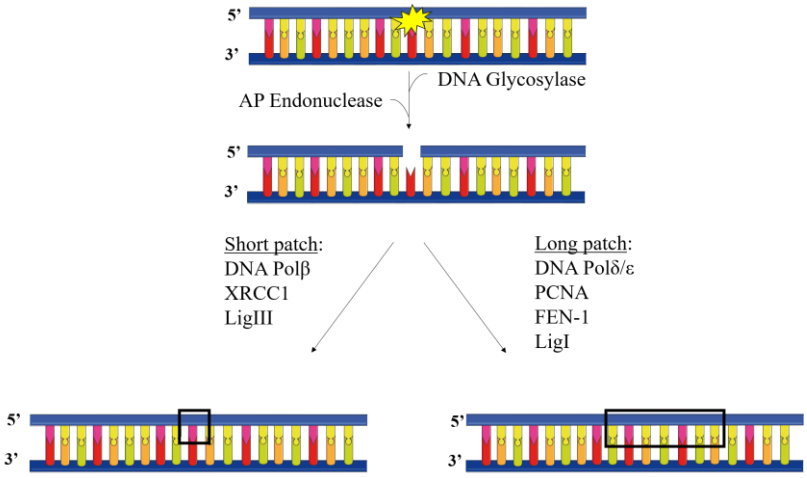


Figure 1.3. The base excision repair (BER) pathway. The single DNA strand break repair pathway, BER, involves the repair of damaged bases by either simply replacing a nucleotide, or by replacing several nucleotides.

1.2.3 NER pathway

The NER pathway is the main mechanism of DNA repair involving the repair of bulky adducts, such as cyclobutane pyrimidine dimers (CPD) caused by UV exposure, and interstrand cross links after platinum- and mustard-based treatments [37]. As depicted in figure 1.4, the NER pathway is composed of two subpathways: the global genome NER (GG-NER), which is involved in repairing DNA damage through the genome and the transcription-coupled NER (TC-NER), which is specifically involved in repairing DNA damage in actively transcribed genes. Both pathways contain the same steps leading to DNA repair but differ in their DNA damage recognition steps, where the damage recognition protein complex for GG-NER is XP complementation group C/Rad23homolog B/Centrin-2 (XPC/HR23B/CEN2) [38], and for TC-NER is simply RNA polymerase II followed by Cockayne syndrome A (CSA) and B (CSB) proteins [39, 40]. After DNA damage recognition, a multi-subunit protein complex and the multi-functional transcription factor TFIIH is recruited on site, which allow ATP-dependent helicases, XPB and XPD, to unwind

the DNA helix to form a ~30 nucleotide bubble flanking the lesion. The unwinding of DNA allows the protein XPA to recruit many replication protein A (RPA), which serves as DNA single strand stabilization. Subsequently, two structure-specific endonucleases XPG (3' specific) and XPF/ERCC1 (5' specific) are recruited to cleave the damaged nucleotides and neighboring sites. This then signals DNA polymerase δ or ε to add the missing nucleotides using the other strand as a template, and the gaps are closed by DNA ligase [37].

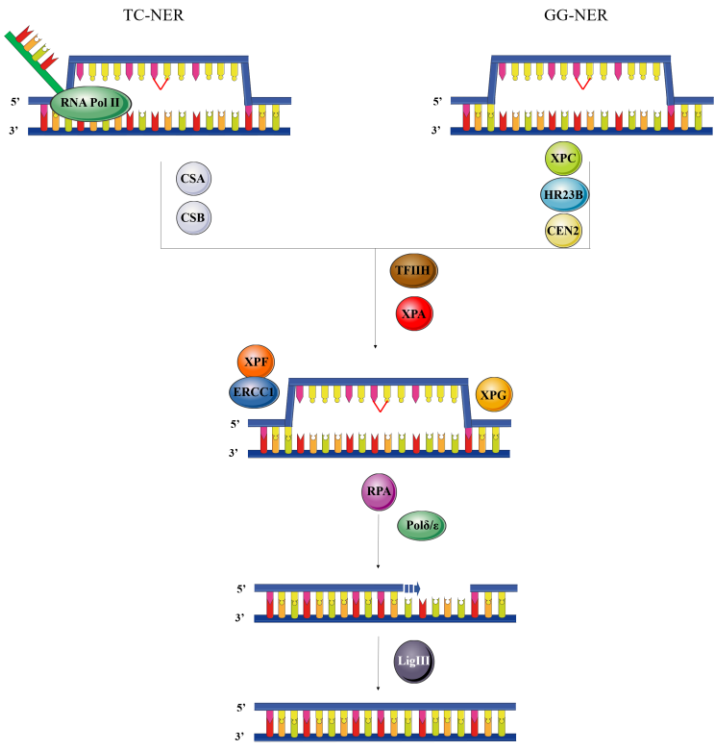


Figure 1.4. The nucleotide excision repair (NER) pathway. The single DNA strand break repair pathway, NER, involves the repair of bulky adducts due to UV or polycyclic aromatic hydrocarbons exposure. The global genome NER (GG-NER) repairs DNA damage through the genome, and the transcription-coupled NER (TC-NER) repairs DNA damage in actively transcribed genes.

1.2.4 HR pathway

HR is one to two mechanisms for DNA double strand break (DSB). This repair mechanism is error-free as it uses the sister chromatid as a template to repair the damaged strand [41]. Only the major steps will be described in this section as more details will be given in section 1.3.2. As outlined in figure 1.5, following DNA double strand break, the heterotrimeric MRN complex (Mre11-Rad50-Nbs1) acts as a double-strand break sensor, which then recruits ataxia-telangiectasia mutated (ATM) on site [42]. ATM then phosphorylates and activates BRCA1, which will allow the formation of the Ctip-MRN-BRCA1 complex [43]. This protein complex is involved in the resection of 5'-ends on both sides of the DSB, leaving 3'-overhangs of single-strand DNA [44]. The 3'-overhangs are then stabilized by replication protein A (RPA), which will later be replaced by RAD51 [45]. Subsequently, BRCA1 will form a complex with PALB2 and BRCA2, the latter mediates the RAD51 filament formation on 3'-overhangs coated with RPA [46]. The newly RAD51-coated overhangs search for a DNA sequence homologous to the damaged sequence. Once the right DNA sequence is located, RAD51 invades the sister chromatid and DNA replication is initiated by DNA polymerase η and followed by ligation by DNA ligase I. These last steps lead to a four-way junction structure termed Holliday junction [47]. The Holliday junction can be finished resolved in three ways: by 'dissolution', symmetrical cleavage, or asymmetric cleavage [48].

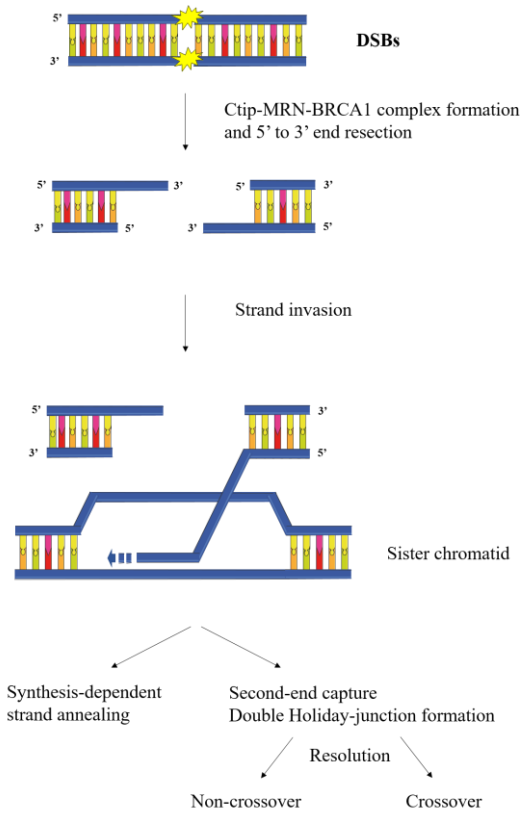


Figure 1.5. Homologous recombination (HR). The high-fidelity DNA double strand break repair pathway (HR) involves many steps leading to the use of a DNA template from the sister chromatid to insure and error-free DNA repair.

1.2.5 NHEJ pathway

NHEJ is the other repair mechanism for DNA DSB. As opposed to HR which is error-free, this repair mechanism is error-prone as it directly ligates the broken ends and does not use any template [49]. NHEJ is composed of two sub groups: classical or canonical and alternative NHEJ. Classical NHEJ, which is shown in figure 1.6, begins with DNA DSB recognition and binding of both Ku70 and Ku80 to the broken ends of DNA. This Ku heterodimer forms a circular shape to allow a stable complex with DNA [50]. Following the formation of the Ku-DNA complex, DNA-dependent protein kinase (DNA-PKcs) is recruited on site and displaces Ku inwards to allow DNA-PKcs to

access DNA DSB ends. Once DNA-PKcs is in contact with DNA, it activates its catalytic kinase activity [51, 52]. Depending on the type of break, DNA may require some processing before being ligated. One of the processing enzymes is Artemis. Artemis is recruited and phosphorylated by DNA-PKcs, which then resects DNA from 5′ to 3′ [51]. The DNA gaps left after resection are then re-synthesized by DNA polymerases, Pol μ and Pol λ (77). Finally, the DNA ends are ligated by DNA ligase IV in complex with XRCC4 (82). The alternative NHEJ (a-NHEJ) takes over when cells are deficient in XRCC4 or DNA ligase IV [53]. A-NHEJ is mostly dependent on PARP1 for detecting the strand breaks and recruiting DNA repair proteins and the MRN-CtIP complex for DNA resection. The gap are filled by DNA polymerase θ and ligated by DNA ligase III [54].

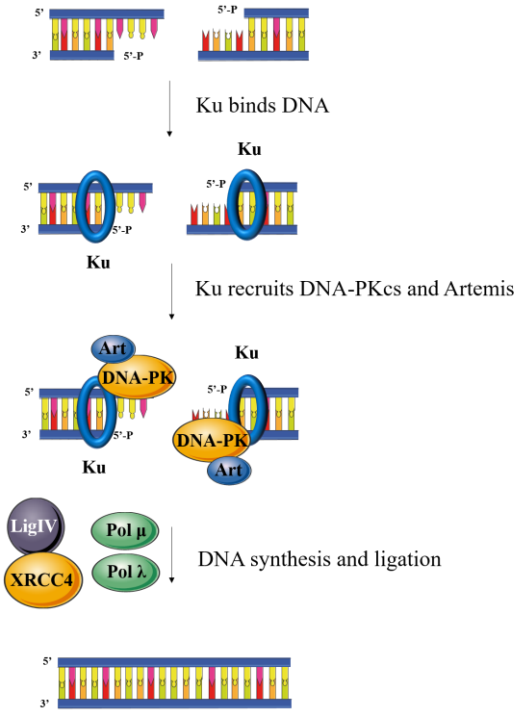


Figure 1.6. Non-homologous end joining (NHEJ). The error-prone DNA double strand break repair pathway (NHEJ) simply involves the joining of the two broken stands without the use of a template.

1.2.6 MMR

The MMR pathway is key in the repair of base-base mismatches and insertion/deletion mispairs occurring during replication, recombination, or DNA repair. The main MMR steps are shown in figure 1.7. There are two major protein complexes involved in MMR: MutS, which recognizes the mismatched bases, and MutL, which initiates the repair [30, 55]. MutS is further subdivised into MutSα, which is a heterodimer of MSH2 and MSH6, and MutSβ, which is a heterodimer of MSH2 and MSH3. MutSα is involved in the recognition of 1 or 2 mismatched nucleotides, whereas MutSβ recognizes longer patches of mismatched bases. MutL exists as several structural variants: MutLα, MutLβ, or MutLγ. MutLα is composed of a heterodimer of MLH1 and PMS2, MutLβ of MLH1 and PMS1, and MutLγ of MLH1 and MLH3 [30, 55]. MutS, MutL and DNA then form a complex which initiates the exonuclease-mediated degradation of damaged DNA [56]. It has been shown that MutLα has a PCNA/replication factor C (RFC)-dependent endonuclease activity [57]. This allows for the $5'$ to $3'$ strand excision followed by strand stabilization by RPA, clamping by PCNA, replication by DNA polymerase δ, and ligation by DNA ligase I [30, 55].

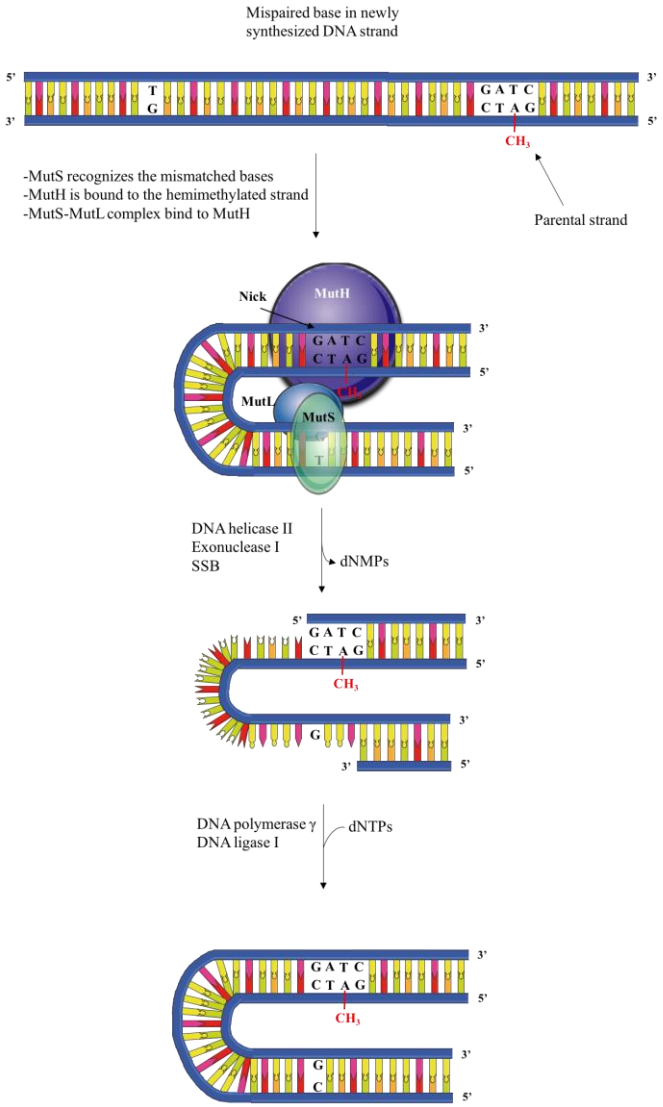


Figure 1.7. Mismatch repair (MMR) pathway. This repair pathway involves the recognition of mismatched bases during replication. MutH is essential in recognizing the parental strand and direct the MutS-MutL complex, bound to the mismatched bases, to resect nucleotide of the newly synthesized strand only.

1.2.7 Direct repair by MGMT

Direct repair by MGMT, as opposed to other DNA repair pathways, involves only one single enzyme. As illustrated in figure 1.8, MGMT simply removes the methyl group from the O^6 position of guanine. The removal of the methyl group from guanine is irreversible. The transfer occurs

when the alkyl group is positioned in such a way that allows the histidine and glutamic-acid residues to form a hydrogen bond with the O^6 and the alkyl group, leading to the transfer of the methyl group onto the cysteine residue. The now methylated MGMT is then then ubiquitinated and targeted for degradation, hence is named a ‘’suicide enzyme’’ [29, 58].

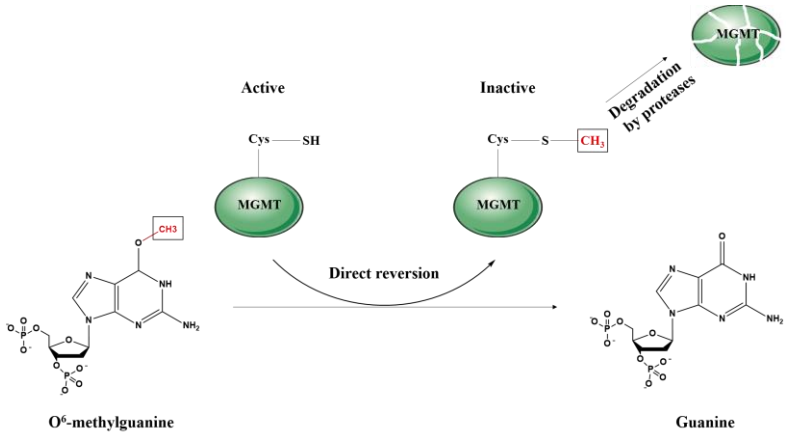


Figure 1.8. Methylguanine methyltransferase (MGMT) enzyme mechanism of DNA repair. MGMT is known as a suicide enzyme. Once the enzyme displaces the methyl group from the methylated base to its cysteine residue, it is ubiquitinated and targeted for proteasome degradation.

1.3 Targeting DNA repair proteins

It has been shown that deficiencies in any DNA repair pathways lead to genetic instability and tumor formation [59]. Furthermore, since DNA repair pathways also repair DNA damage due to chemotherapeutic drug and radiotherapeutic treatments, it can lead to resistance [60-62]. Thus, one way to target cancer and/or overcome chemoresistance is to target DNA repair proteins. Certain tumours harbor mutation in one or more DNA repair genes leading to DNA repair deficiency. For example, mutations in the MLH1, MSH2, MSH6 or PMS2 are deficient in the MMR pathway [63]. Likewise, mutations in the BRCA1 or BRCA2 genes lead to a deficiency in HR pathway [64]. These DNA repair deficiencies and others can be pharmacologically exploited to specifically target cancer cells.

1.3 BRCA1 and BRCA2 discovery, structure and function

1.3.1 BRCA1/2 discovery and structure

Germline mutations in BRCA1and BRCA2 (BRCA1/2) are associated with an increased risk of developing breast, ovarian, prostate and pancreatic cancer [65-67]. A long road in genetic studies led to the discovery of these mutations. The first insight into susceptibility genes in familial breast cancer were reported in 1990, where chromosome q17 was shown to be the common denominator. It is characterized by autosomal dominant inheritance with incomplete penetrance [68]. BRCA1/2 is autosomal dominant as this gene is located in a non-sex-chromosome and only one of the two genes is necessary to increase the lifetime risk of developing cancer. Incomplete penetrance of this gene refers to the fact that some individuals will not develop cancer even though they carry the mutated gene on one of the allele. In most familial breast and ovarian tumours, loss of heterozygosity was often observed, which lead to labelling BRCA genes as a tumor suppressors [69, 70]. Four years later, the gene on chromosome q17 was identified as BRCA1 [71]. Since then, many different germline mutations, few sporadic mutation and epigenetic silencing have been discovered linking them to cancer susceptibility [72-74].

As illustrated in figure 1.9, BRCA1 is a protein composed of four major domains: a zinc-finger domain, a BRCA1 serine domain, and two BRCA1 carboxy-terminal repeat motif (BRCT) domains. The BRCA1 protein also comprises a nuclear localization signal as well as a nuclear export signal [75, 76]. It was shown that the zinc finger is essential in interacting with associated proteins such as BRCA1-associated RING Domain protein 1 (BRAD1). Such complex was found to be critical in BRCA1 tumour suppression [77]. This domain is also part of the catalysis of protein ubiquitination in conjunction with ubiquitin E3 ligases [78]. The BRCA serine domain is the region of the protein where the phosphorylation occurs. ATM and ATR are kinases, which upon DNA damage, are activated and in turn phosphorylates BRCA1. Importantly, this domain is the main

site for high rates of mutation [43, 75]. The two BRCT regions are located at the C-terminus of the BRCA1 protein. This domain is involved in DNA repair, transcription regulation and tumor suppressor functions [79]. In short, upon DNA damage, the BRCT domain interacts with various proteins to control the cell cycle. The most common mutation in this domain is a missense mutation, which leads to a loss of control of the cell cycle [80].

Since BRCA1 mutation was only found in 45% of hereditary breast cancers, it was suggested that there might be another breast cancer susceptibility gene. In 1995, the BRCA2 gene was identified at chromosome 13q12.3 [81, 82]. As depicted in figure 1.9, BRCA2 protein is composed of three major domains: a BRC repeat, three oligonucleotide-binding (OB) domains, and a tower domain [83]. The BRC repeat is in the middle of the BRCA2 protein. This domain is essential for binding to RAD51, a protein involved in DNA recombination [84-87]. OB domains are known to be essential for BRCA2 binding to DNA. More specifically, OB1 binds single strand of DNA and the DSS1 protein, which is involved in the stabilization of BRCA2. OB2, which has a tower shaped domain, and OB3 are key to a crucial DNA binding [83, 88].

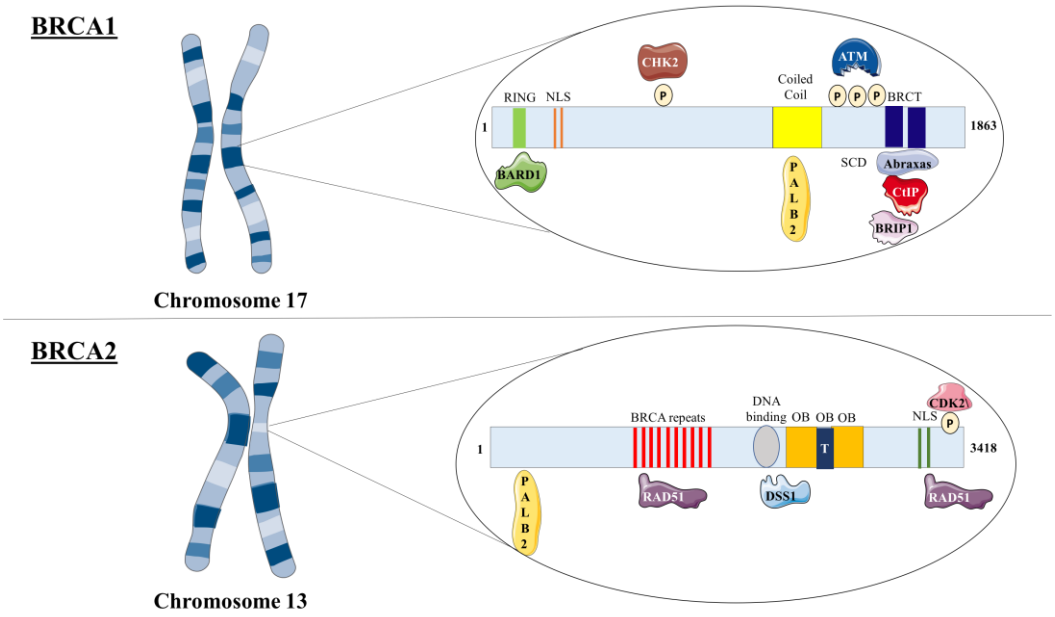


Figure 1.9. BRCA1 and BRCA1 gene structure and some of their interacting partners. A) The BRCA1 N terminus includes a RING domain. This domain allows the association of BRCA1 with BRCA1-associated RING domain protein 1 (BARD1) and a nuclear localization through the nuclear localization sequence (NLS). The domain neighboring the RING domain is a CHK2 phosphorylation site. The coiled-coil domain is the binding region for the partner and localizer of BRCA2 (PALB2). The SQ/TQ cluster domain (SCD) is the ataxia-telangiectasia mutated (ATM) phosphorylation site. Finally, the BRCT domain serves as a binding site for ATM-phosphorylated abraxas, CtBP-interacting protein (CtIP) and BRCA1-interacting protein C-terminal helicase 1 (BRIP1). **B)** The BRCA2 N terminus is the region where PALB2 binds. BRCA2 also contains eight BRCT repeats which allow RAD51 to bind. The BRCA2 DNA-binding domain contains a helical domain (H), three oligonucleotide binding (OB) folds and a tower domain (T). This region allows BRCA2 to bind single- and double-stranded DNA and complexes with deleted in split-hand/split-foot syndrome (DSS1) for protein stability. Finally, the BRCA2 C terminus is composed of an NLS and a cyclin-dependent kinase (CDK) phosphorylation site.

1.3.2 Role in DNA repair

One of the early events after a DNA DSB is the phosphorylation of the H2AX histone. Some studies have shown that BRCA1 co-localized with phosphorylated H2AX (γH2AX), which cause chromatin remodeling [89]. BRCA1 is thought to recruit kinase to phosphorylate histone H2AX, which in turn will mediate further DNA damage signaling [90].

BRCA1/2 involvement in HR is shown in figure 1.10. Upon DNA damage, BRCA1 is hyperphosphorylated and moves to the site of the replication fork [91, 92]. BRCA1 can be phosphorylated at different locations by different kinases. For example, DSBs caused by ionizing radiation recruits and activates ataxia-telangiectasia mutated (ATM) that binds to BRCA1 and phosphorylates it [93, 94] at the Ser1387 [95]. On the other hand, upon UV exposure, ATM-related kinase (ATR) mainly phosphorylates BRCA at the Ser1457 residue [95]. CHK2, a kinase involved in cell cycle control, also phosphorylates BRCA1 at Ser988 after ionizing radiation exposure [96, 97]. Other sites of BRCA1 have been shown to be phosphorylated upon DNA damage, but little is known about their role in BRCA1 function [93, 94].

Some studies show the co-localization and co-immunoprecipitation of BRCA1 with the Rad50-Mre11-NBS1 (MRN) complex [98, 99]. The Mre11 acts as a nuclease that resects the 5' ends of the DSB to produce single stranded DNA tracks [100]. In short, BRCA1 acts as a regulator of the complex [101] by binding to DNA and inhibiting Mre11 activity when necessary [102]. Other studies show the involvement of BRCA1 and BRCA2 in the homologous recombination process by forming a complex with RAD51 and other proteins [103, 104]. RAD51 is involved in the recombination during the DSB repair [105]. By coating single-stranded DNA, it forms a nucleoprotein filaments, which then invades a homologous DNA in the sister chromatid for an error-free repair [106, 107]. BRCA1 also plays an indirect role in DNA damage. It has been shown

that BRCA1 forms a complex with c-Abl, which is disrupted by ATM upon radiation-induced DNA DSB. Once the BRCA1-c-Abl complex is disrupted, c-Abl is activated and is involved in transcription regulation and DNA repair [108].

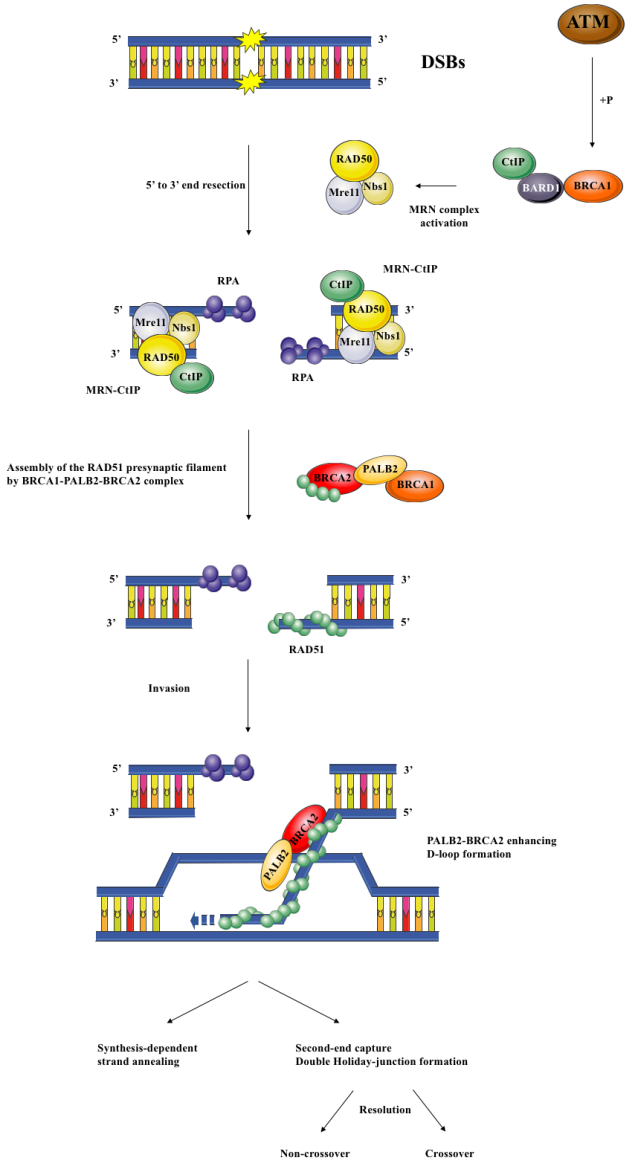


Figure 1.10 BRCA1 and BRCA2 functions in HR. Following DNA DSB, BRCA1 is rapidly phosphorylated by ATM/ATR, which allows the recruitment of the MRN complex through interaction with CtIP proteins. This protein complex initiates resection of the ends in the 5' to 3'

direction. RPA then binds the 3' ssDNA overhangs to stabilize them. BRCA1 recruits BRCA2 through PALB2 interaction, which allows BRCA2 to interacts with RAD51 proteins. Subsequently, RAD51 binds and displace RPA to initiate the RAD51-dependent strand exchange.

1.3.3 Role in transcription regulation

BRCA1 has been shown to regulate many genes involved in DNA damage response. MacLachlan *et al.* [109] showed that BRCA1 stabilizes p53 and together, they coactivate the transcription of genes that involved in both direct DNA repair and cell cycle arrest. Their study also shows that although p53 is involved in the transcription of genes triggering apoptosis, BRCA1 is not. Some studies show that BRCA1 acts as a co-repressor with ZBRK1. ZBRK1 is a transcription factor that specifically binds to the DNA sequence GGGXXXCAGXXXTTT. This sequence is present in the promoter region of many genes, such as p21, GADD45, and EGR1. Together, BRCA1 and ZBRK1 were shown to repress the transcription of those genes [110, 111]. Ouchi *et al*. [112] studied the role of BRCA1 as a transcription activator of IFN-γ target genes. They showed that BRCA1 and STAT1 interact and that together they co-activate the transcription of the p21WAF1 gene, a cyclin-dependent kinase inhibitor, to ultimately modulate cell growth.

1.3.4 Role in cell cycle

Upon DNA damage, BRCA1 is shown to be involved in cell cycle regulation by activating all cell cycle checkpoints: G1/S, S-phase and G2/M checkpoints. Indeed, Fabro *et al.* [113] showed that after ionizing radiation or UV exposure, G1/S checkpoint is triggered when BRCA1 is phosphorylated by ATM or ATR, which then allows BRCA1 to associate with BARD1 to ultimately regulate p53 phosphorylation on serine-15 position. Phosphorylation of p53 then acts as a key component in the transcription of the cyclin dependent kinase (CDK) inhibitor p21, which is directly involved in G1/S arrest. BRCA1 has also been shown to be involved in the S-phase and

G2/M checkpoints, but the precise mechanism of action is still not clear [114]. The BRCA1-BRAD1 complex is also able to interact with DNA topoisomerase 2-binding protein 1 (TOPBP1), which is essential in the S phase checkpoint regulation [115, 116]. Similarly, BRCA1 interacts with receptor-associated protein 80 (RAP80) [117] and a coiled-coil domain–containing protein (CCDC98) [118] to act as a G2/M checkpoint control.

1.3.5 Role in ubiquitination

In the RING finger domain of BRCA1, there are helices at the main portion involved in the BRCA1-BARD1 complex. This complex is now well studied and its main function is for its E3-ubiquitin ligase activity [119]. The heterodimerization of the two proteins is essential for the proper function of the ubiquitin ligase activity [120]. The mechanism of action of the E3-ubiquitin ligase is done via the essential interaction with E2 ubiquitin-conjugate enzyme [121]. The BRCA1 and BARD1 complex also impacts their cellular localization. Indeed, upon dimerization, the nuclear export sequence (NES) of both BRCA1 and BARD1 become hidden, which leads to the nuclear retention of the two proteins [122, 123]. Some of the targets ubiquitinated by the BRCA1 E3 ligase include histone protein H2A, CtIP, estrogen receptor-alpha, and progesterone receptor [124-127].

On the other side of the spectrum, there is a deubiquitinase, BRCA1 associated protein 1 (BAP1), involved in chromatin remodeling by deubiquitinating histone H2A [128] and modulation of the E3-ubiquitine ligase activity of the BRCA1-BARD1 complex by deubiquitinating BARD1 [129]. BAP1 has been shown to be involved in DNA damage response of double strand breaks [128] and in cell cycle [130, 131]. In fact, BAP1 has been shown to have a role in tumour growth suppression and is now known as a tumour suppressor gene [132].

1.3.6 Role in apoptosis

The role of BRCA1 in apoptosis is not clear. Wang *et al.* [133] studied the function of BRCA1 as a function of its cellular localization. Indeed, many groups studied the BRCA1 shuttling between the nuclear and cytoplasmic region [134-136]. As mentioned earlier, the BRCA1 gene contains two nuclear localization signals, which influences its nuclear localization [137]. The BRCA1 gene is also composed of two nuclear export sequences (NES), which allows the transport of BRCA1 from the nucleus to the cytoplasm [138]. The signal of these sequences can be compromised when BRCA1 interacts with certain proteins. As mentioned in the previous section, the BRCA1-BARD1 complex leads to the hiding of the NES of BRCA1, which prevents BRCA1 from leaving the nuclear compartment [135, 136]. Other studies show that BRCA1 is able to bind to p53, which in this case leads to BRCA1 export to the cytoplasm [134]. Wang *et al.* [133], in 2010, showed that not only BRCA1 export out of the nucleus prevents BRCA1 from functioning in its DNA repair roles, but they also show that BRCA1 cytosolic localization might be involved in apoptosis following DNA damage [133]. A few years later, Hedgepeth *et al.* [139] showed that BRCA1 binds to the inositol 1,4,5,-triphosphate receptor (IP3R) and enhances the binding of the latter to its substrate, leading to apoptosis. IP3R is a calcium channel in the endoplasmic reticulum (ER) membranes and is known to be involved in gene expression, metabolism, apoptosis, etc [139].

1.4 BRCA1/2 levels and cancer

Worldwide, breast cancer is the most commonly diagnosed cancer and the leading cause of death in women [140]. In the developed world, ovarian cancer is the fourth leading cause of cancer [141]. Among the multiple risk factors, a BRCA1/2 mutation represents an increase risk in developing breast and/or ovarian cancer. Statistics show that on average 12% of women will develop breast cancer at some point [142]. However, having a BRCA1 mutation increases the chances of

developing cancer up to 55 to 65% and 45%for BRCA2 mutations by age of 70 [143, 144]. About 1.3% of women will develop ovarian cancer [142], with up to 39% risk for women with BRCA1 mutation and 11 to 17% for women with BRCA2 mutations [143, 144]. Mutations in BRCA1/2 also increases the risks of other cancer in women such as, fallopian tube and peritoneal cancers [66, 145]. These mutations can also increase the risk for male breast cancer to 1.2% for BRCA1 mutation and 6.8% for BRCA2 mutation [146]. Similar risks are reported for the development of prostate cancer with mutations in these genes [147]. In both men and women, BRCA1/2 mutations increase the risk of developing pancreatic cancer [148].

Individuals who inherit a BRCA1 or BRCA2 mutation are usually heterozygous, where only one of the two alleles is defective. Overtime, these people can acquire a mutation in the second allele, which leads to genomic instability and eventually cancer [149]. BRCA-associated cancers are about 90% hereditary, where the other 10% are due to rare sporadic, promoter methylation, etc [150]. Familial BRCA deficiency is largely cause by frameshift mutations leading to a truncated and non-functional BRCA protein [151]. The population genetics of BRCA1 and BRCA2 varies largely on geography, showing a loss of genetic variation known as the founder effect [152]. Indeed, most BRCA1/2 mutation are specific to certain countries (e.g. Israel) and are also found in populations/communities outside of their country of origin (e.g. Ashkenazi jews) [153].

Although very rare, non-functional BRCA1 or BRCA2 proteins can also be due to a somatic mutation [72, 150, 154, 155]. A study done at university of Texas M.D. Anderson Cancer Centre showed that out of all the patients with BRCA1/2 mutations, 42.9% had a somatic mutation in the BRCA1 gene and 28.6% in the BRCA2 gene. They also showed a strong positive association between BRCA1 mutation and TP53 mutation [74]. Similarly, other studies reported rare somatic mutations in the BRCA1 gene in unselected breast and ovarian cancer patients [156-158].

Decreased BRCA1 expression is also seen in promoter hypermethylation. Many studies have shown methylation of this gene [150, 159]. In short, BRCA1 transcription is partially regulated by the methylation of the promoter gene, which leads to gene silencing [160]. The silencing of BRCA1 leads to tumours that are deficient in homologous recombination and are as sensitive to DNA damaging agents and PARP inhibitors as BRCA1/2 mutant tumours [74]. Another type of silencing involves the overexpression of miR-182, a key player in the downregulation of BRCA1 [161]. In the case of BRCA2 silencing, Hughes-Davies *et al.* [162] showed that the EMSY gene, which is amplified exclusively in some sporadic breast cancer and high-grade ovarian cancer, binds in a region within BRCA2 protein and downregulates its activity.

1.5 Poly(ADP-ribose)polymerase

1.5.1 Overview

The history of Poly(ADP-ribore)polymerase (PARP) started in 1963, when Chambon *et. al.* [163] first discovered the poly(ADP-ribose) polymer. After many years working on the determination of the molecular structure of poly(ADP-ribose) and its associated enzymes, they synthesized PARP inhibitors to study the function of the enzyme. Knowing that nicotinamide is a by-product of the enzyme, the first inhibitors synthesized were based on the benzamide system due to it close structural resemblance to nicotinamide [164, 165]. In 1980, Durkacz *et al.* [166] reported for the first time that the PARP inhibitor 3-aminobenzamide (3AB) enhanced the cell killing effect of dimethyl sulfate *in vitro*. This work led to the first hypothesized function of PARP, which is its involvement in DNA damage response [167-169]. Similar results were observed in an *in vivo* experiment conducted by Sakamoto *et al.* [170] where they showed that benzamide increased the cytotoxicity of bleomycin, and therefore increased its antitumour activity. Thereafter, PARP inhibitors were presented as novels molecules to be used to enhance the effect of chemotherapy

and radiotherapy [167, 171]. This is in line with the fact that chemo- and radio-therapy damage DNA and that PARP inhibitors inhibit the base excision repair and probably other DNA repair pathways involved in the repair of such damage.

Later in the mid-1990s, more potent PARP inhibitors were developed, NU1025 and NU1064, that were more soluble and over 200 times more potent than 3AB [172]. Next in line with PARP discoveries was the first crystal structure of the active site of chicken PARP-1 [173]. This work led to a better understanding of the inhibitor binding to the active site and allowed the development of a new generation of PARP inhibitors [174, 175]. This initiated a whole new era for PARP inhibitors with very successful pre-clinical testing, leading to the very first clinical trial, which was performed in combination with temozolomide [176].

The next major discovery was the use of these successful PARP inhibitor in the context of synthetic lethality. Studies showed the exceptional synergistic potency of PARP inhibitor in BRCA1/2 deficient tumours. Their working theory was around the fact that, in dividing, unrepaired single-strand breaks, due to PARP inhibition, accumulate to become double-strand breaks. These double strand breaks which are normally repaired by the HR pathway, are left unrepaired due to BRCA1/2 deficiency. Consequently, unrepaired double-strand DNA breaks lead to apoptosis and cell death [59, 177].

The next sections will describe in more details the structure and function of PARP-1 and PARP2, and briefly mention the other members of the superfamily. Then, a full description of the two mechanism of action of PARP inhibitors will be discussed before introducing the various PARP inhibitors in the clinic, and finally discuss the acquired mechanism of resistance to this new therapy.

1.5.2 PARP structure and function

ADP-ribosyltransferases (ARTs), or PARP superfamily, gathers 17 or more known members with an ability to transfer the ADP ribose group from nicotine adenine dinucleotide (NAD+) onto specific proteins, which leads to proteins modulation [178-180]. It has been shown that only 6 of these have poly (ADP-ribose) polymerase (PARP) activity allowing them to form polymers of ADP-ribose [181]. From these, only three PARPs (PARP1, PARP2 and PARP3) have terminal DNA binding domains allowing them to make contact with damaged DNA [182] For the purpose of this thesis, I will focus on PARP1 and briefly describe the other members of the PARP superfamily.

PARP1 is localized in the nucleus. As depicted in figure 1.11, it structure is essentially composed of fours domains: the DNA binding domain, the automodification domain, the WRG domain/catalytic domain. The DNA binding domain is at the N-terminus of the protein and contains three zing fingers. This domain is involved in detecting and binding DNA strand breaks [183, 184]. The automodification domain is responsible for transferring the poly(ADP-ribose) chain onto itself [185]. Within this domain, a BRCT domain, which is also found on other DNA repair proteins, allows the recruitment of downstream partners [186]. Although PARP-1 has been shown to interact and activate effectors involved in chromatin remodeling, transcription and telomere maintenance, it main role is to detect DNA strand breaks and initiate the DNA repair cascade [187]. Upon DNA strand break, PARP1 act as a sensor and its DNA binding domain binds to the broken DNA [188]. DNA binding initiates the synthesis of the poly(ADP-ribose) chains by increasing its catalytic activity 10- to 500-fold and transfers it to proteins from the DNA repair machinery [189, 190]. In order to amplify the signal at the site of DNA break, it has been shown that the PARylation is also able to recruit more PARP1 (see figure 1.12) [191]. However, in order for DNA repair proteins to access the site of DNA break, PARP has to dissociate from the DNA.

This is done by the auto-ribosylation, which makes PARP1 negatively charged; since DNA is negatively charged, PARP1 is repelled by the DNA [192].

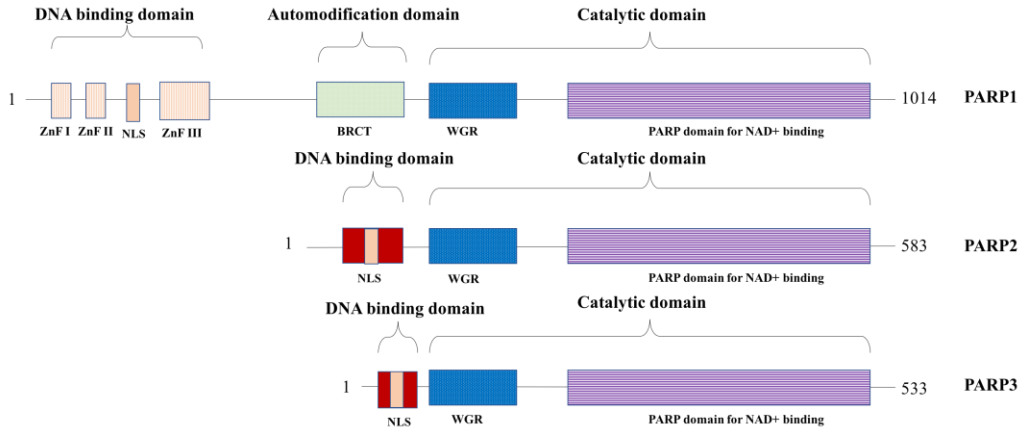


Figure 1.11. Structure of PARP1, PARP2 and PARP3. PARP-1 contains DNA binding domain, an automodification domain and a catalytic domain. Zinc finger motifs (ZnF) is the sensor part of the protein and allow binding to the damaged DNA. This leads to the activation of the catalytic domain, which synthesizes poly(ADP-ribose) (PAR) chains. PARP-2 has a similar structure but is lacking zinc finger and an automodification domain. Its function is overlapping with those of PARP1, as both play a role in the base excision repair, nucleotide excision repair, homologous recombination and non-homologous end joining. PARP3 is composed of the same domains than PARP2, but its functions differ as it has been shown to be strictly involved in non-homologous end joining. NLS, nuclear localization sequence; BRCT, BRCA1 carboxy-terminal repeat motif; WGR, WGR motif.

The role of PARP1 was difficult to assess at first. Although cell-based assay showed an increased sensitivity to ionizing radiation and alkylating agents when the cells were PARP1 deficient, PARP1 knockdown mice were viable [193]. Further studies showed a double knockdown of both PARP1 and PARP2 being lethal. This led to the discovery of a redundant pathway with PARP2,

which takes over when PARP1 is absent [194]. Although PARP1 is not essential for DNA repair, it is known to increase the rate of DNA repair by recruiting DNA repair proteins on site. Most DNA repair proteins interact with PARP through the PAR chain. However, some proteins such as tyrosyl-DNA phosphodiesterase 1 (TDP1) and XRCC1 interact directly with PARP 1 and 2 [195-197]. TDP1 is a DNA repair protein involved in the repair of DNA damage triggered by topoisomerases I and II [198]. TPD1 interacts with PARP1 regardless of the PAR formation [195]. XRCC1, a DNA repair protein, which role is to increase and improve the recruitment and activity of other DNA repair proteins involved in the single strand break repair [35]. Although XRCC1 directly interacts with PARP1/2 and therefore does not need PAR formation, the rate of its recruitment is increased when PAR is formed [196, 197]. The role of PARP in base excision repair is depicted in figure 1.12. Once PARP1 sensed the DNA break, produced its PAR, and recruited DNA repair players, PAR is quickly degraded by poly(ADP-ribose) glycohydrolase (PARG) [199] to allow DNA repair protein complexes to disassemble after DNA repair [200].

Although its main role remains in the BER pathway, PARP-1 also plays a role in double strand break repair, in both HR and NHEJ. In HR, PARP1 has been shown to increase the DNA repair activity by recruiting key players such as BRCA1, which heterodimerizes with BARD1. The heterodimer then interacts with PARP1 through the C-terminal BRCT domain of BARD1 and PAR [201]. PARP1 also recruits RNA-binding motif protein, X-linked (RBMX), which is tought to regulate BRCA2 expression at the mRNA level. As mentioned earlier, BRCA2 is a key player in HR [202]. PARP1 is also thought to be involved in the NHEJ pathway. As mentioned in the DNA repair pathways section, NHEJ is composed of two subpathways: the canonical NHEJ (C-NHEJ) and the alternaternative NHEJ (A-NHEJ). There is very little evidence that PARP1 is involved in or stimulates C-NHEJ [203]. There is more evidence of the involvement of PARP1 in

the A-NHEJ, where PARP1 has been shown to recruit the XRCC1-DNA ligase III complex [53, 204, 205]. PARP1 is also shown to be involved in the NER pathway, where PARP1 binds to lesions formed by UV exposure [206]. In short, PARP1 interacts, recruits or stabilizes some key players in this pathway such as the Cockayne's syndrome complementation group proteins CSB [207], the Xeroderma pigmentosum complementation group proteins XPC and XPE/ DDB2, and the chromatin remodeler ALC1 [208].

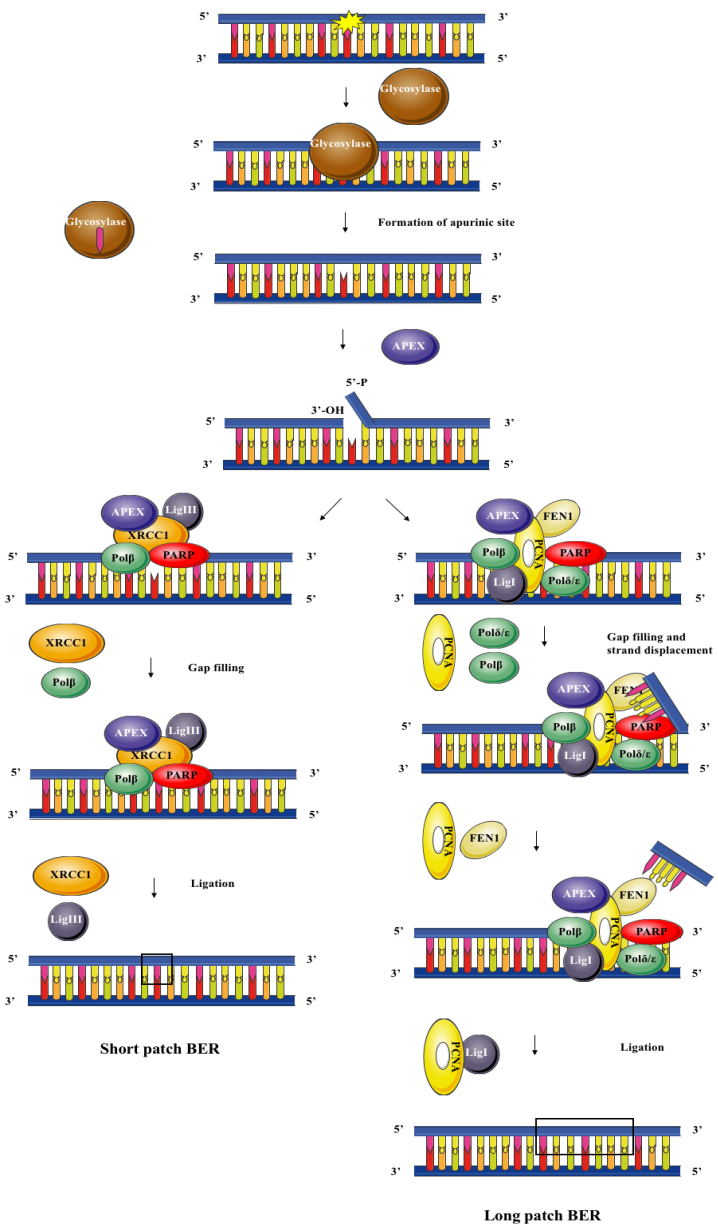


Figure 1.12. **The role of PARP-1 in base excision repair (BER) pathway.** BER is the DNA repair mechanism, which is involved in the repair of single strand breaks caused by oxidation or alkylation. BER is initiated when a lesion specific DNA glycosylase removes the damaged base. PARP senses the strand break and recruits other DNA repair proteins. BER can be processed by two sub-pathways: the short-patch where a single nucleotide is replaced, and the long-patch where 2-13 nucleotides are replaced.

As depicted in figure 1.11, PARP2 structure is very similar to that of PARP-1 but is lacking the zinc fingers and automodification domain [209]. As mentioned earlier, the discovery of PARP2 was revealed after *PARP1*$^{-/-}$ mouse cells were exposed to DNA damaging agents and produced PAR chains in the absence of PARP1 [209]. Although the produced PAR chain was similar that of PARP1, PARP2 is only involved in 10-25% of PARP1 activity and is shown to interact with the same protein targets as PARP1 [210]. These findings *in toto* suggest that PARP2 acts as a redundant protein in DNA repair when PARP1 is absent. Furthermore, since studies on *PARP2*$^{-/-}$ mice showed different phenotypes than *PARP1*$^{-/-}$, PARP2 may have other functions than the ones overlapping with PARP1 [211].

PARP3 is similar in structure as PARP1/2 with some differences in the DNA binding domain [212]. PARP3 is preferably activated by double strand break and results in monomeric ADPribosylation or short PAR chains as opposed to longer and more complex one produced by PARP1 and PARP2 [213]. Rouleau *et al.* [214] showed that PARP3 forms a complex with DNA-PKcs, PARP-1, DNA ligase III, DNA ligase IV, Ku70, and Ku80, suggesting that this PARP is involved in DNA repair pathway. Other studies showed that PARP3 depletion led to a compromised NHEJ double strand break repair after radiation, bleomycin and etoposide treatment [215, 216].

The other PARP members are not involved in the DNA damage response and are described in Table 1.

Table 1. The 17 members of the PARP super family, their subcellular localization and functions

PARP Family Member	Alternative Name	Subcellular localization	Function
PARP1	N/A	Nucleus	-DNA strand break sensor -DNA damage response in base-excision repair (BER), nucleotide excision repair (NER), homologous recombination (HR), and non-homologous end joining (NHEJ) -Chromatin remodeling
PARP2	N/A	Nucleus	-DNA damage response; overlapping functions with PARP1 -Developmental and immunological functions
PARP3	N/A	Nucleus, cytoplasm	-DNA damage response in non-homologous end joining (NHEJ)
PARP4	Vault-PARRP (vPARP)	Cytoplasm, Nucleus	-Roles in multidrug resistance, nucleo-cytoplasmic transport, formation of ribonucleoparticules, signal transduction pathways and immune response
PARP5A	Tankyrase 1	Nucleus, cytoplasm	-Telomere homeostasis, mitotic spindle formation, and vesicle trafficking
PARP5B	Tankyrase 2	Nucleus, cytoplasm	-Telomere homeostasis, mitotic spindle formation, and vesicle trafficking -Caspase-independent cell death through the loss of mitochondrial potential
PARP6		ND	-Negative regulator of cell proliferation through cell cycle regulation
PARP7	TiPARP	ND	-Selective transcriptional repressor of aryl hydrocarbon receptor (AHR) -IFN gene stimulation
PARP8	N/A	Centrosome, nuclear envelope	Maintenance of nuclear envelop structure
PARP9	BAL1	Nucleus, cytoplasm	-Transcriptional regulation in response to immunoregulatory cytokines -IFN gene stimulation
PARP10	N/A	Nucleus, cytoplasm	-Regulation of cell proliferation, cell survival, transformation or viral infection -binding partner of the proto-oncoprotein c-Myc -IFN gene stimulation
PARP11	N/A	Centrosome	-ND
PARP12	ZC3HDC1	Nucleus	-Antiviral activity -IFN gene stimulation
PARP13	ZC3HAV1, ZAP	Cytoplasm, (nucleus)	-Antiviral activity -IFN gene stimulation
PARP14	BAL2, COAST6	Nucleus, cytoplasm	-Transcriptional regulation in response to immunoregulatory cytokines -IFN gene stimulation
PARP15	BAL3	Nucleus, cytoplasm	-Transcriptional regulation in response to immunoregulatory cytokines -IFN gene stimulation
PARP16	N/A	Nucleus, RE membranes	-Component of the nuclear trafficking machinery -Regulate the unfolded protein response (UPR)

1.5.3 PARP inhibitors mechanism of action

During the development of PARP inhibitors, it has always been thought that the only mechanism of action of the small molecules inhibiting PARP was through the inhibition of the formation of the poly(ADP-ribose) polymerase chain. As mentioned previously, upon DNA damage, PARP detects the lesion and activates its catalytic domain to hydrolyze NAD+ to produce PAR. AutoPARylation of PARP1 or PARP2 and the transfer of PAR to chromatin protein recruits essential DNA repair proteins.

There are many PARP inhibitors currently tested in clinical trials with olaparib approved for the treatment of germline BRCA mutated advanced ovarian cancer and BRCA or ATM mutated metastatic castration resistant prostate cancer [217, 218]. Although all these small molecule inhibitors are great PARP catalytic inhibitors with IC50 values in the low nanomolar range, they have a different effect on PARP1 deleted cell. It has been shown that they sensitize $Parp1^{+/+}$ cells more when combined with alkylating agents than $Parp1^{-/-}$ cells [219]. These findings *in toto* suggest that PARP inhibitors may have another mechanism of action distinct from the inhibition of the catalytic domain, depicted in figure 1.13.

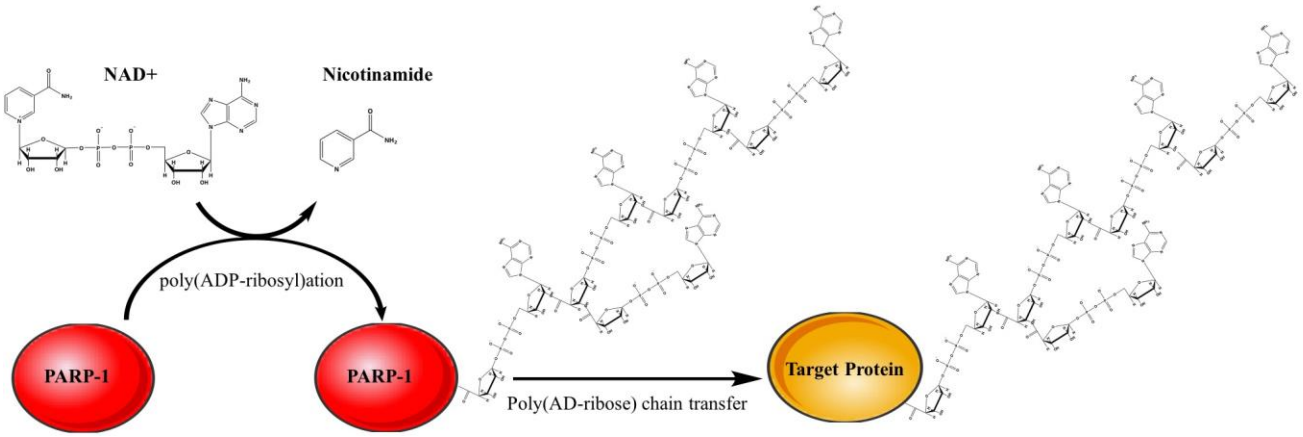


Figure 1.13. Poly(ADP-ribosyl)ation. The formation of the poly(ADP-ribose) chain from binding of NAD+ in the nicotinamide pocket by PARP-1 and the subsequent transfer of the chain onto target protein, such as XRCC1.

A new mechanism of action of PARP inhibitors was first proposed by Strom *et al.* in 2011 [220], which states that upon PARP binding of the small molecule inhibitor, PARP1 is trapped on DNA. In 2012, Murai *et al.* [219] gave the first evidence of this new function of PARP inhibitors. Through fluorescence anisotropy DNA-binding assay, they showed that different PARP inhibitors can have a similar inhibition of catalytic activity but their ability to stabilize PARP-DNA complex can significantly vary. In short, in this assay, the DNA single strand break is labeled with AlexaFluor488. The broken strand rotates fast giving a low fluorescence anisotropy reading. Once PARP binds to the DNA lesion, the rotation slows down to give a higher fluorescence anisotropy reading. In untreated cells, the presence of NAD+ will lead to autoPARylation, which will cause the dissociation of PARP and DNA complex. In cells treated with PARP inhibitors, the PARylation is inhibited and the PARP-DNA complex is stabilized. Furthermore, PARP inhibitor binding to the NAD+ pocket can also allosterically change PARP conformation and thus augment PARP binding to DNA [219]. They also showed that the bulkier the PARP inhibitor, the stronger the

PARP-DNA complex. These studies concluded by showing that, at equal catalytic inhibition potency, a strong PARP-DNA trapping is the primary cytotoxic mechanism of action.

Along with the discovery of this new function of PARP inhibitors, the same group studied in 2014 [221] the combination potency of PARP inhibitor with different DNA targeted agents based on their PARP trapping or catalytic inhibition. They showed that catalytic PARP inhibitors are highly synergistic in combination with camptothecin, a topoisomerase I inhibitor, whereas PARP inhibitors with strong DNA trapping are more potent in combination with alkylating agents such as temozolomide [221]. Although these findings could be helpful to rationalize PARP inhibitors combinations in the clinic, *in vivo* tolerability of these treatments should be considered. In 2015, Hopkins *et al.* [222] showed that PARP inhibitors with high DNA-trapping activity are more toxic in combination with temozolomide. Despite the great *in vivo* results with DNA-trapping in combination with alkylating agents, the reduced therapeutic index emerged as an important drawback.

1.5.4 PARP inhibitors in the clinic

In 2008, the first phase I clinical study of a PARP inhibitor, rucaparib, was done in combination with temozolomide in patient with advanced solid tumours. The results showed that the combination was well tolerated and there was evidence of increased DNA damage in the combination when compared to temozolomide alone [176]. Although PARP has been studied for decades, it was not until 2005 that two different groups showed the selective potency of PARP inhibitor as a single agent in HR defective cells [59, 177]. This new discovery initiated over 200 clinical trials involving PARP inhibitors as a single agent or in combination with chemo- or radiotherapy. A repository of these trials can be found in the following website: www.clinicaltrials.gov. In fact, nine PARP inhibitors are currently studied in early and late phases

of clinical trials. In 2014, olaparib received an accelerated FDA approval as a monotherapy for the treatment of patients with germline BRCA-mutated advanced ovarian cancer previously treated with three or more lines of chemotherapy [217]. This approval was based on the encouraging results obtained in a phase II trial. The patients treated with olaparib twice daily showed an objective response rate (ORR) of 21.7 to 50% in all cancer types harboring BRCA1/2 mutations (breast, ovarian, pancreatic, and prostate), with little side effects [223].

In January 2016, olaparib was granted the Breakthrough Therapy Designation (BTD) for the treatment of metastatic castration-resistant prostate cancer with BRCA1/2 or ataxia telangiectasia mutated (ATM) mutations [218]. This approval was again a result of spectacular outcome in the TOPARP trial (phase II). The response rate was as high as 33%, which included patients with mutation in BRCA1/2, ATM, Fanconi's anemia genes, and Chk2. These patients experienced very little side effects [224, 225]. In December 2016, another PARP inhibitor, rucaparib, received accelerated FDA approval for the treatment of germline or somatic BRCA-mutated advanced ovarian cancer previously treated with three or more lines of chemotherapy. Clinical trial with rucaparib showed an overall response of 54% [226]. Very recently, the FDA approved for the first time a PARP inhibitor, Niraparib, in the treatment of non-BRCA1/2 mutated tumours. Niraparib was granted Fast Track, Priority Review and Breakthrough Therapy designation, as well as Orphan Drug designation specifically for treating recurrent epithelial ovarian cancer after the groundbreaking result of the NOVA trial [227]. In this study, the primary outcome, the progression-free survival (PFS), was shown to be significantly higher in the niraparib-treated cohort regardless of the BRCA1/2 status [228]. It is now approved as a maintenance treatment for patients with recurrent epithelial ovarian, fallopian tube, or primary peritoneal cancer who are in

complete or partial response to platinum treatment [227]. The crystal structure of olaparib bound to PARP-1 protein is depicted in figure 1.14.

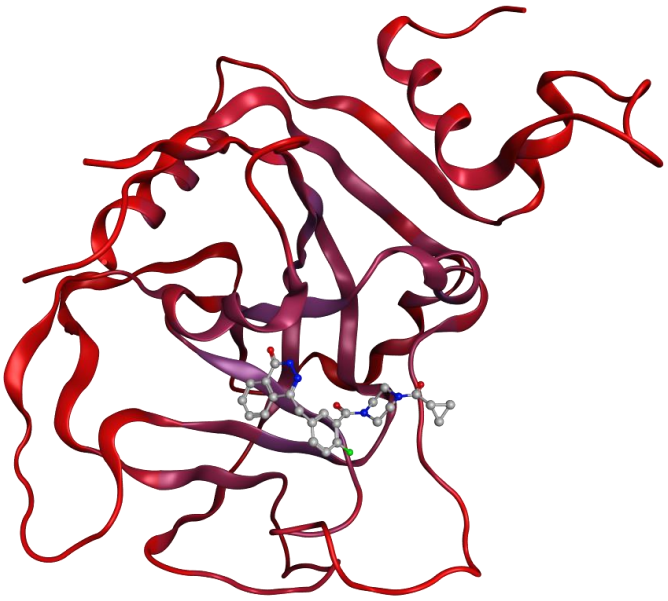


Figure 1.14. Crystal structure of olaparib bound to the nicotinamide pocket in the ribbon structure of PARP-1 protein. Downloaded from Protein Data Bank (PDB code: 5DS3 Crystal structure of constitutively active PARP-1) [229].

Although the first clinical trial combining rucaparib and temozolomide showed very little toxicity, other studies showed myelosuppression. In 2013, a phase II study showed that rucaparib in combination with temozolomide in metastatic melanoma caused myelosuppression in 54% of the patients [230]. Other studies showed similar results when using other PARP inhibitors, veliparib [231] and INO101 [232], in combination with temozolomide. Combinations with other chemotherapeutic agents were also explored. Olaparib was also combined with cisplatin and gemcitabine [233] and veliparib with topotecan, which once again, showed dose-limiting toxicity of myelosuppression [234]. For all these cases, the doses were de-escalated to reach the maximum

tolerated dose (MTD) for the combination. Likewise, increased toxicity was also described in studies combining olaparib with dacarbazine and paclitaxel [235, 236]. These results are similar to those obtained with another DNA repair protein inhibitor (i.e. methylguanine methyltransferase (MGMT) inhibitor), O^6-benzyl guanine and lomaguatrib [237-239]. More combinations of PARP inhibitor with chemotherapy are currently ongoing and can be found on www.clinicaltrials.gov. In conclusion, although PARP inhibitors show little toxicity as single agents, their combination with chemotherapy leads to increased myelosuppression.

PARP inhibitors are also combined with other therapeutic agents such as vascular endothelial growth factor receptor (VEGFR) inhibitors. It has been previously shown that, in hypoxia, the HR pathway is impaired, where both BRCA1 and RAD51 are downregulated [240-242]. Similarly, inhibition of VEGFR results in downregulation of both BRCA1 and BRCA2 involved in HR, leading to a contextual synthetic lethality with PARP inhibition [243]. Phase I and II clinical trials combining olaparib and cediranib (VEGFR inhibitor) showed dose-limiting hematologic toxicities and evidence of encouraging preliminary efficacy results in ovarian cancer patients [244-246].

As mentioned earlier, PARP inhibitors have also shown great potential as radiosensitizers *in vitro* and *in vivo*. Only two phase I clinical trials have been completed so far involving the combination of the PARP inhibitor veliparib and radiation. Both studies show the same low toxicity profile as veliparib alone and encouraging efficacy results in brain metastasis and in advanced solids tumours [247, 248]. There are several other clinical trials currently ongoing evaluating the combination of veliparib and olaparib with either radiotherapy or chemoradiotherapy.

1.5.5 PARP inhibitor resistance

There are currently four known mechanisms of resistance to PARP inhibitors. As depicted in figure 1.15, the main one is the development of BRCA1/2 secondary mutations, which leads to a functional protein and therefore restoration of homologous recombination. Another one is the upregulation of multidrug efflux transporter P-glycoprotein (Pgp), which is a common cause of resistance to many therapeutics. Two more mechanisms have been recently revealed. One such mechanism is the loss of p53-binding protein 1 (53BP1) in BRCA1 mutant, leading to a partial restoration of HR, and loss of PARP-1 expression. *In vitro* and *in vivo* data have accumulated to show BRCA1/2 restoration as a mechanism of resistance. In 2008, the two first published studies were performed in cell lines in which PARP inhibitor resistance has been induced with continuous stepwise exposure to increasing concentration of PARP inhibitors [249, 250]. Interestingly, some of these secondary mutations led to an almost full-length BRCA2 protein, and RAD51 foci formation, which is a hallmark of HR competence. This was the first proof of PARP resistance mechanism through the restoration of full length protein and HR [249]. Moreover, the other group also found that, after ionizing radiation exposure, the PARP inhibitor-resistant clones were able to form RAD51 foci. Another interesting finding by the same group is that PARP inhibitor-resistant clones also became resistant to cisplatin [250]. Restoration of BRCA1 was also seen in ovarian cancer cell lines and conferred resistance to platinum compounds treatment [251]. *In vivo* data also showed restoration of BRCA2 due to secondary mutation. This study looked at cells derived from a patient with a BRCA2 mutation and sensitivity to cisplatin. After two rounds of cisplatin, 5-fluorouracil and chlorambucil therapy, the patient relapsed and was no longer responding to cisplatin treatment. After relapse, cells were derived from the ascites, and showed a secondary mutation in the BRCA2 gene, leading to its restoration [252]. There are also few clinical cases that show PARP inhibitor resistance due secondary mutation leading to restoration of BRCA1/2.

Norquist *et al.* [253] showed that patients responding to the PARP inhibitor olaparib have an inherited mutation and the patients not responding to olaparib had a secondary mutation on BRCA1/2 that restored its function. Another study published by Barber *et al.* [254] showed secondary mutations of BRCA2 leading to its restoration and resistance to PARP inhibitors. This study described patients with BRCA2 mutations, who were treated first with radiotherapy and/or chemotherapy and then treated with olaparib. After an initial response to the PARP inhibitor, their tumour relapsed and stopped responding to the treatment. Exome sequencing revealed secondary mutation in the BRCA2 gene leading to a fully functional BRCA2 protein [254]. Unfortunately, in all these studies, whether they were clinical or pre-clinical, the cells, animals or patients were first treated with rounds of chemotherapy before being treated with PARP inhibitors. To our knowledge, there are no studies looking at PARP inhibitor resistance with only PARP inhibitor treatment as the only treatment modality.

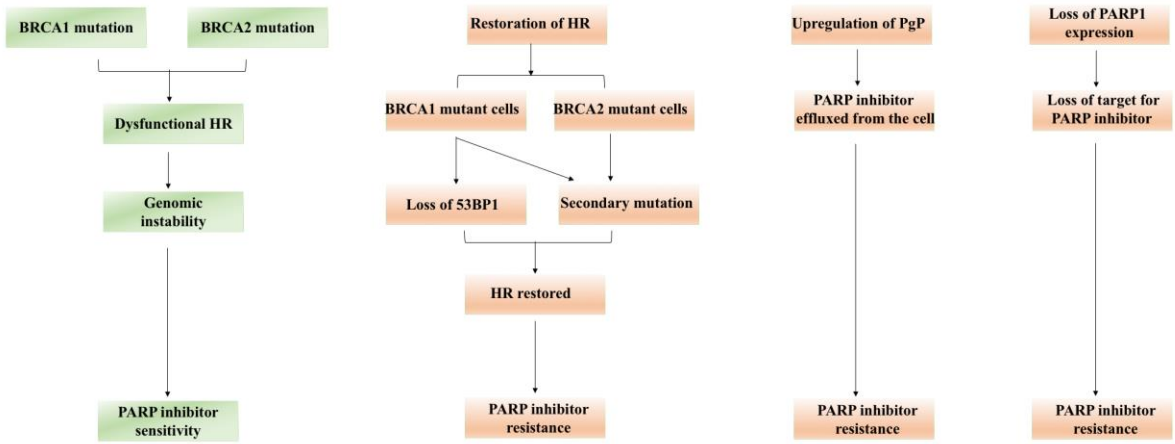


Figure 1.15. Molecular routes of PARP inhibitor sensitivity and resistance. Knowing the mechanism of PARP sensitivity in the context of homologous recombination (HR) deficiency, various mechanisms of resistance have been studied. One of the best studied mechanism of resistance to PARP inhibitors is the restoration of BRCA1/2 due to secondary mutations. HR is

also controlled by another protein, 53BP1, which promotes NHEJ rather than HR. A loss of 53BP1 will then favor HR. A familiar mechanism of drug resistance is the upregulation of the efflux pump, P glycoprotein, which pumps the PARP inhibitor out of the cell preventing it to reach its target. The last mechanism of resistance is the loss of the drug target, PARP1.

The second mechanism of resistance is the loss of 53BP1, a protein involved in DNA double strand break repair and cell cycle checkpoints. In a normal cell overcoming double strand breaks, the balance between the error-free repair, HR, and the error-prone repair, NHEJ, rely on two proteins: BRCA1 and 53BP1. As mentioned in previous sections, BRCA1 promotes HR. Conversely, 53BP1 is responsible for the initiation of the error-prone pathway. In the case of BRCA1 mutant tumours, 53BP1 takes over and promotes NHEJ [255]. The loss of 53BP1 leads to an increase in HR even in BRCA1 mutant cells, promotes RPA phosphorylation and rescues BRCA1-deficient cells from dying, thereby leading to resistance to both PARP inhibitors and DNA damaging agents [255-257]. It has also been shown that loss of 53BP1 is seen in some triple negative breast cancer and BRCA1/2-mutant tumours, which leads to a decreased metastasis-free survival [256].

Another mechanism of resistance to PARP inhibitors is the upregulation of multidrug efflux transporter P-glycoprotein (Pgp), which pumps the drug out of the cells. An *in vivo* study showed that long term exposure to olaparib led to an increase in Abcb1a or Abc1b genes expression that encode P-glycoproteins. The same study showed that sensitivity to olaparib is restored when combined with the P-glycoprotein inhibitor tariquidar [258].

The last mechanism of PARP inhibitor resistance is the cellular levels of the target, PARP1. Pettitt *et al.* [259] studied the identification of determinants involved in cancer drug toxicity and resistance. They showed that PARP1 is essential for olaparib activity and that toxicities arising

from this treatment is specific to its target. They also discuss the correlation between PARP inhibitor response and PARP1 levels, where low PARP1 levels lead to PARP inhibitor resistance [259]. Loss or lower levels of PARP1 as a mechanism of PARP inhibitor resistance has not been observed yet in the clinic and should be explored further.

1.6 Synthetic lethality

Loss of BRCA1/2 is highlighted in triple-negative breast, high grade serous ovarian, pancreatic and prostate cancers. As an attempt to find a treatment for these advanced disease, research led to the concept of synthetic lethality. Synthetic lethality arises when the mutation of gene A or B alone do not affect the viability of the cell but when both are mutated, it leads to cell death (summarized in figure 1.16). An obvious case of synthetic lethality is that of cells expressing the mutant forms of BRCA1 and BRCA2 [59, 177]. Loss of BRCA1/2 functionality impairs the DSB repair process. On the other hand, the base excision repair protein (PARP) takes over and compensates for this deficiency. Thus, concomitant loss of function in the BRCA1 or 2 genes and the inhibition of PARP induces significant genomic instability that ultimately leads to cell death. This situation is produced by using PARP inhibitors to block its function in the context of BRCA1/2 deficiency [59, 177].

SSBs are the most common DNA aberrations and are repaired by BER, NER, and MMR by using the undamaged complementary strand as a template for the repair. The BER pathway is the main pathway for SSBs and involves PARP. As mentioned in the previous sections, PARP is the SSB sensor, which upon DNA break detection, binds to DNA and catalyzes the formation of a poly (ADP-ribose) chain of varying length and complexity. PARP will transfer this chain to itself and other acceptor proteins, such histones, to allow chromatin relaxation and leave the access to repair proteins. Other ADP-ribose chain acceptors include other DNA repair proteins such as DNA ligase

III, DNA polymerase β, and XRCC1 [260]. Once the DNA strand break is repaired, the poly (ADP-ribose) chains are degraded by poly(ADP-ribose) glycohydrolase (PARG) [200].

SSBs that are not repaired lead to replication-associated DNA lesions that are normally repaired by HR. BRCA1 and BRCA2 play an important role in repair of DSBs by HR. Normal cells can repair DSBs by sister chromatid exchange, which is error free. However, loss of BRCA function guides cells towards another option, non-homologous end-joining (NHEJ), which is error prone and leads to genomic instability. BRCA deficient cells lead to two possible outcomes: either die due to accumulative DNA damage, or survive with DNA mutations, the latter leading to an increase risk of cancer [260].

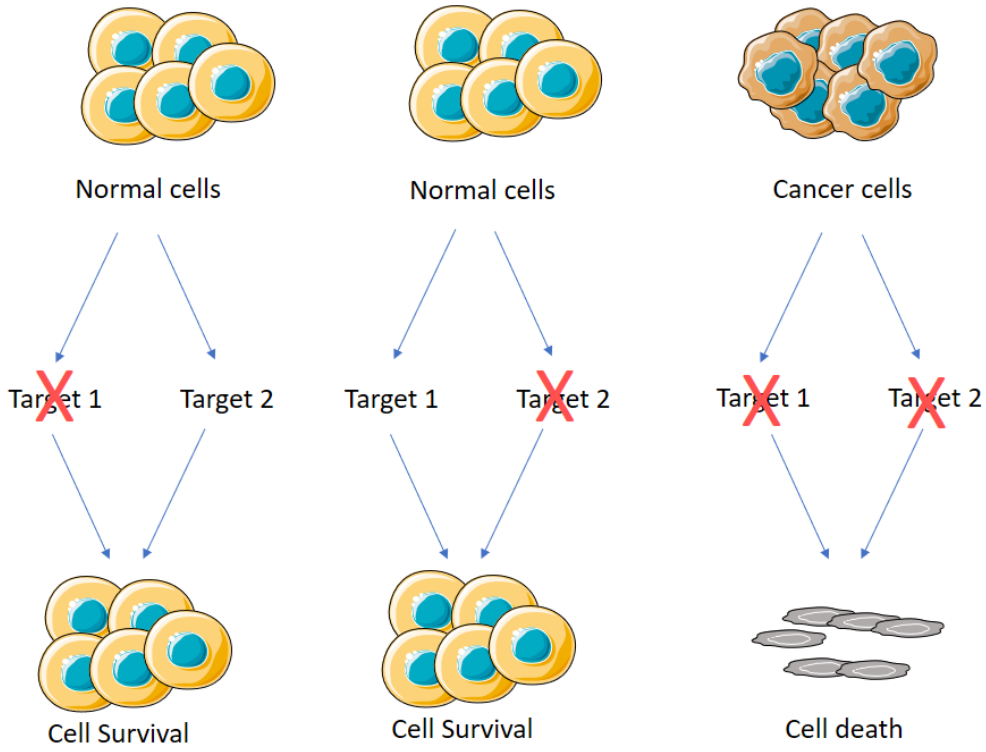


Figure 1.16. The synthetic lethality concept. Synthetic lethality arises when the mutation or inhibition of target 1 alone does not affect the viability of the cell because target 2 is able to take over. Same end point is reached when target 2 is mutated of inhibited. However, when both targets

1 and 2 are unable to keep homeostasis due to a mutation or pharmacological inhibition, the cells are unable to compensate and leads to cell death.

1.6.1 Other PARP- induced synthetic lethality targets

PARP inhibition is not only synthetically lethal with BRCA1/2 mutation, but also with HR deficiencies. Other synthetic lethal targets includes genes directly involved in the HR pathway or DNA damage signaling [261]. Proteins and enzymes that are essential for HR and can be synthetically lethal with PARP inhibition include, RAD54 [262], RAD51, DSS1 [263], RPA1 [264], ATR [265], ATM [266], NBS1 (part of the MRE11/RAD50/NBS1 complex) [267], CHK1 [268] and CHK2 [269]. Also, it has been shown that even genes deletions involved in the Fanconi Anaemia pathway such as, FANCD2, FANCA and FANCC, lead to HR deficiency and therefore PARP inhibition sensitivity [270].

Other possible targets for PARP inhibitor are genes that indirectly regulate HR. For example, the gene EMSY is a negative regulator of BRCA2 that prevents its activation. Accordingly, its overexpression represses HR [271]. Another indirect player of HR is the phosphatase and tensin homolog (PTEN), a negative regulator of the phosphoinositide-3 kinase (PI3K) pathway, and one of the most mutated gene in cancer. PTEN has been previously shown to control RAD51 expression [272] and cell cycle checkpoints [273]. Its indirect involvement in HR and cell cycle regulation is probably the reason behind its synthetic lethality with PARP inhibition [274]. Another indirect regulator of HR is PI3K, whose inhibition has been shown to not only downregulate HR by decreasing BRCA1/2 levels, but also increase PARP activity. The downregulations of BRCA1/2 is thought to be due to ERK phosphorylation, which activates the transcription factor ETS1 [275].

As mentioned in section 1.7.4, PARP inhibition is synthetically lethal in combination with vascular endothelial growth factor receptor (VEGFR) inhibitors. As a result of hypoxia, key DNA repair proteins are repressed [276, 277]. Some of the proteins downregulated during hypoxia include NBS1, part of the MRN (MRE11–RAD50–NBS1) complex [278], RAD51 and BRCA1 [242, 279], RAD52 [280], RAD54 [281], BRCA2 [241], etc. Similar to hypoxia, VEGFR inhibitors inhibit blood vessel formation and therefore deprive tumours cells from nutrients and oxygen, creating a hypoxic environment [282]. Inhibition of VEGFR leads to HR deficiency by downregulating BRCA1 and BRCA2, leading to a contextual synthetic lethality with PARP inhibition [243]. There are other synthetic lethality interactions that are shown to be induced between the epidermal growth factor receptor (EGFR) itself or its downstream signaling cascade and PARP inhibitors. This contextual synthetic sickness will be discussed in section 1.7.6.

1.7 EGFR involvement in cancer

1.7.1 Overview

EGFR is a glycosylated transmembrane receptor tyrosine kinase (RTK) composed of an extracellular binding a receptor tyrosine kinase (RTK), a transmembrane and a cytoplasmic domain [283, 284]. The EGFR superfamily includes EGFR (also known as Her1 or ErbB1), Her2 (ErbB2), Her3 (ErbB3) and Her4 (ErbB4). Upon ligand binding, EGFR is activated and stimulates various downstream signaling pathways leading to cell growth, proliferation, differentiation, migration, invasion, metastasis and survival [285].

As depicted in figure 1.17, solid tumors are characterized by overexpression and/or mutation of growth factor receptors, such as EGFR. Implicated in the signaling pathway of EGFR are Ras/Raf/MEK/MAPK, PI3K/Akt, STAT, and PLCγ. It has been shown that inhibition of EGFR leads to a decrease in the activation of Erk1/2, which reduces proliferation. Also, the inhibition of

this tyrosine kinase receptor increases the signaling molecule p27 and/or p21, which causes a decrease in angiogenesis and cell cycle arrest. Attenuation of Akt, and reduced Bcl-2 or Bad activation due to this same EGFR inhibition induced increased cell death. Small molecules designed to inhibit EGFR (e.g. gefitinib/iressa and tarceva) are the two drugs now clinically used for this purpose. A significant body of evidence accumulated to show that overexpression of the ligand and ligand-independent receptor activation are implicated in gliomas, melanoma, breast, head and neck, lung, ovarian, prostate, colon carcinomas [286].

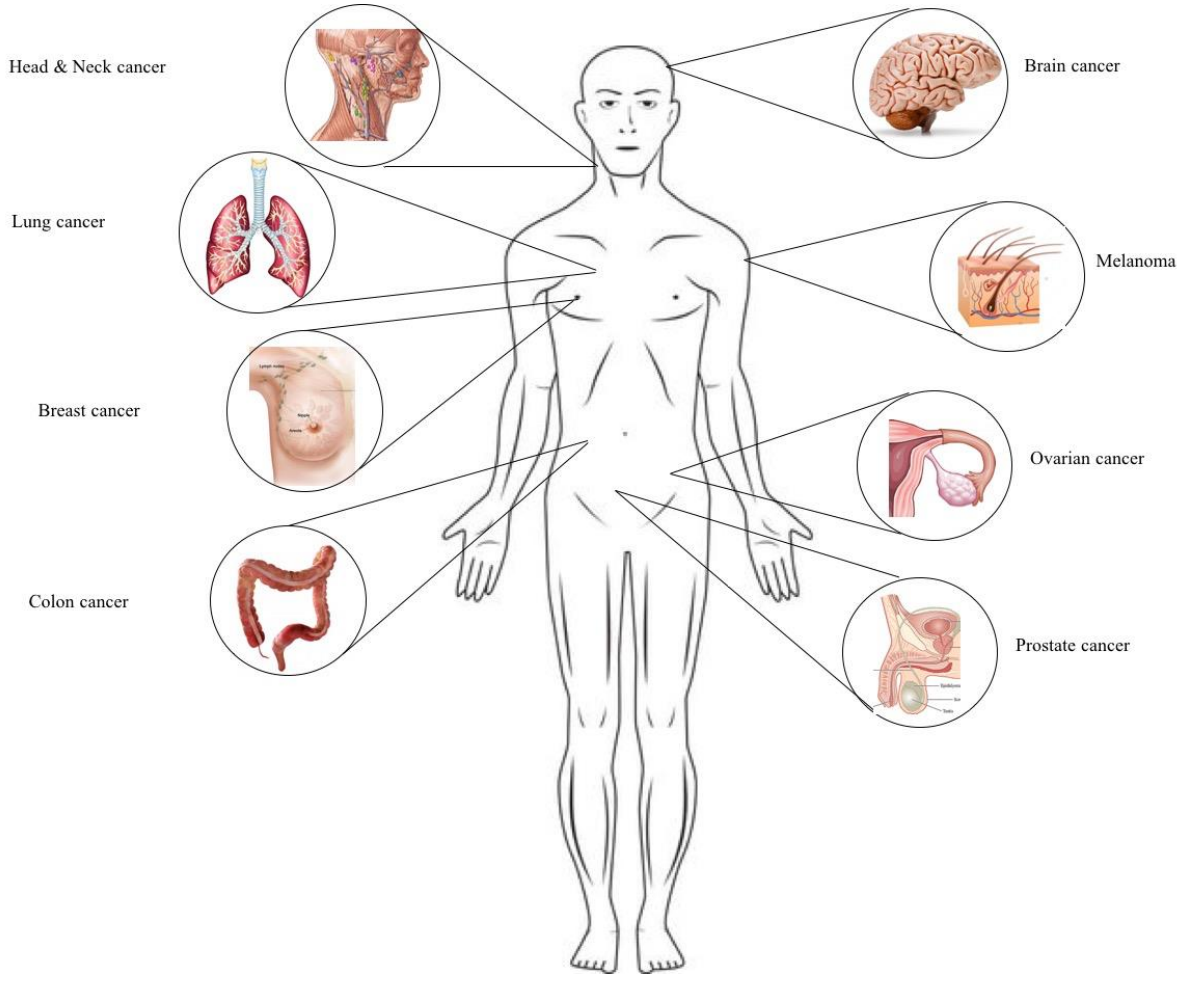


Figure 1.17. EGFR deregulation in different human cancers. The EGFR deregulation, which include its overexpression or mutation, is implicated in many solid tumours such as: gliomas, breast, ovarian, and colon carcinomas.

1.7.2 EGFR structure

The extracellular portion of EGFR is composed of four domains: L1, CR-1, L2 and CR-2 domains. Domains L1 and L2 form the ligand binding region, while the CR-1 and CR-2 domains are involved in the dimerization of the receptor. Upon ligand binding, EGFR has been shown to either homodimerize or heterodimerize with other members of the EGFR superfamily [287, 288]. As a transmembrane receptor, EGFR is also composed of a transmembrane domain, which is mainly α-helical. This domain is mainly for anchorage to the cellular membrane. The juxtamembrane region has important functions involved in the regulation of the receptor, such as: downregulation, ligand-dependent internalization, association with proteins, etc [289]. Following the juxtamembrane is the kinase domain. As for other kinases, ATP-binding region lies between the N-terminal and C-terminal lobes [290]. The last EGFR domain is the carboxy-terminal tail, which comprises of tyrosine residues involved in phosphorylation-EGFR-mediated signal transduction. When phosphorylated, this region serves as a docking site of various signaling proteins involved in that signal transduction. Other residues within this domain are known to be involved in downregulation processes and endocytosis [291].

There are seven ligands that are known to bind to EGFR: the epidermal growth factor (EGF), transforming growth factor (TGF)-α, heparin binding epidermal growth factor (HB-EGF), amphiregulin, betacellulin, epiregulin and epigen [292-296]. The activation of the receptor begins with the bonding of a ligand between domains L1 and L2 in one single monomeric receptor [297-299]. This event will then lead to a conformational change in the extracellular region, where the CR-1 from the ligand-activated EGFR monomers interact with each other, dimerize and ultimately activate the kinase domain [300]. Once the kinase domain is activated, ATP binds between the C-lobe and the N-lobe. Only the C-lobe is involved in protein substrate interaction [301]. ATP binding triggers autophosphorylation of tyrosine residues in the C-terminal tail of EGFR, which

leads to protein docking. A signaling cascade is then initiated [302].

EGFR associates with signaling proteins in three different ways. The first physical association is an indirect one and is done at the C-terminus of EGFR, where it recruits SH2 (Src homology region 2) or PTB (phosphotyrosine-binding) domains. These domains are small protein segments that facilitate protein-protein interactions involved in the signal transduction pathways [303]. The second mode of recruitment is a direct one, where signaling proteins associate directly with the C-terminus of EGFR [304-307]. The third association is done through a heterodimer partners from other EGFR superfamily members, where the recruitment of some signaling proteins is preferentially done by an EGFR heterodimer as opposed to an EGFR homodimer [308].

1.7.3 EGFR signaling

EGFR stimulation leads to the activation of a diverse array of signaling pathways, leading to different functions (illustrated in figure 1.18). Although these pathways are regulated by other redundant signaling pathways, here they are described as EGFR pathways. One of the pathways activated by EGFR is the Mitogen Activated Protein Kinase (MAPK) pathway. The key players of this cascade are Ras, Raf, MEK, and ERK1/2 [309, 310]. When EGFR is activated, it recruits the adaptor protein Grb2, which is bound to the Ras exchange factor Sos, leading to the activation of Ras [311]. Grb2 can bind to the active EGFR either directly (via Y1068 and Y1096)[312] or indirectly, through the tyrosine phosphorylated Shc [313]. When activated, Ras then phosphorylates the serine/threonine kinase Raf-1 [314], which activates MEK and a series of intermediate kinases. This pathway ultimately leads to the phosphorylation, activation, and nuclear translocation of Erk-1 and Erk-2 to regulate transcription factors [315] involved in cellular growth, proliferation, differentiation, migration and apoptosis [309, 312].

Another key player in EGFR signaling pathway is c-Src. c-Src plays a dual role, where it can either be activated by EGFR [316], or it can phosphorylate EGFR in an EGF-independent manner [317, 318]. The most studied roles are the ones involving c-Src phosphorylating and activating EGFR. When c-Src phosphorylates EGFR on Y845, EGFR then activates the STAT5b pathways, which is involved in cell proliferation [319], or activate a key player in cell survival, cyclooxygenase-II (COX-II) [320]. c-Src is also required for EGFR activation, which then induces Zn2+-mediated Ras activation [321]. Another role of c-Src is the phosphorylation of EGFR at Y920, which recruits p85, a PI3K regulatory subunit [304]. It is also involved in the MAPK pathway by phosphorylating Shc, required for Grb2-SOS binding and activation. c-Src can also prevent the degradation of EGFR by phosphorylating Cbl, which is involved in EGFR endocytosis and proteasomal degradation [322].

EGFR also activates the JAK/STAT pathway, that regulates cell cycle progression, differentiation and survival [323]. Signal transducer and activators of transcription (STATs) are signal transducer protein that were first discovered in the context of cytokine receptor signaling pathways [324, 325]. There are seven STAT genes in total: STAT1, 2, 3, 4, 5s, 5b, and 6, with STAT1, 3 and 5 implicated in the EGFR signaling [326-328]. Their structure comprises an oligomerization domain, a DNA binding domain, and a SH2 domain [329]. Typically, downstream of cytokine receptor, STAT protein activation is mediated by JAK kinases. Upon ligand binding and activation of the cytokine receptor, JAK is recruited to the receptor. Since JAK lack a SH2 or SH3 domain, it binds to the proline-rich region of the cytokine receptor through its homology domain. This interaction between the receptor and JAK allows for phosphorylation of the receptor and the recruitment of STAT proteins [328]. To be functional, STAT proteins require not only phosphorylation, but also dimerization with other activated STAT proteins prior to translocation to the nucleus and binding

to DNA [330, 331]. The activation of STAT downstream of EGFR is slightly different than that of cytokine receptor. JAK is not implicated in the activation of STAT proteins downstream of EGFR. Also, it appears that not only STAT proteins do not bind to the C-terminal phosphotyrosine residues of EGFR, but they have also been shown to be constitutively associated with EGFR [332, 333]. Since JAK is not required for STATs activation, STAT activity exclusively depends of EGFR stimulation [327]. Another possible path for STAT activation downstream of EGFR is through c-Src. As mentioned above, EGFR can activate c-Scr, which then can activate STAT proteins [299].

EGFR is also involved in phospholipid metabolism, and can activate three enzymes involved in these pathways: phospholipase C (PLC), phosphatidylinositol-3-kinase (PI3K), and phospholipase D (PLD). PLD is the enzyme responsible for the production of choline and phosphatidic acid (PA) from the hydrolysis of phophatidylcholine [334]. PA is not only one of the main components in lipid metabolism [335], but it is also involved in mitogenesis by positively regulating the mammalian target of rapamycin (mTOR) [336]. The second enzyme activated by EGFR, PLC, catalyzes the hydrolysis of PtdIns(4,5)-P2 (PIP2) to produce 1,2-diacylglycerol (DAG) and inositol 1,3,5-trisphosphate (IP3). The former is involved in the activation of the serine/threonine kinase PKC (role in the MAPK and JNK pathways) [337, 338], and the latter in the regulation of intracellular calcium stores [339]. The third enzyme, PI3K, is involved in many cellular processes including proliferation, survival, adhesion, and migration [340]. PI3Ks are organized into three classes but only class Ia is activated by EGFR. The activation of PI3K is mediated by phosphorylated EGFR and the p85 subunit of PI3K via the SH2 domain [341]. As mentioned above, p85 binds preferentially to ErbB3 rather than EGFR. However, EGFR/ErbB3 heterodimer is essential. c-Src can also activate indirectly PI3K by phosphorylating EGFR [304]. Activation of

PI3K leads to the phosphorylation of phosphatidylinositol-4,5-phosphate (PIP2) to phosphatidylinositol-3,4,5-phosphate (PIP3). This step of the pathway is tightly regulated by the phosphatase and tensin homolog (PTEN), which dephosphorylates PIP3 to generate back PIP2 and shuts off the signal. The activation of the pathway leads to translocation of Akt to the plasma membrane, which is further activated by the protein kinase PDK-1 [342]. mTORC2 then phosphorylates Akt at a second site, which is required for the complete activation of Akt [343]. In turn, Akt phosphorylates numerous downstream players, which leads to cellular growth, proliferation, metabolism, survival and apoptosis [342] [344].

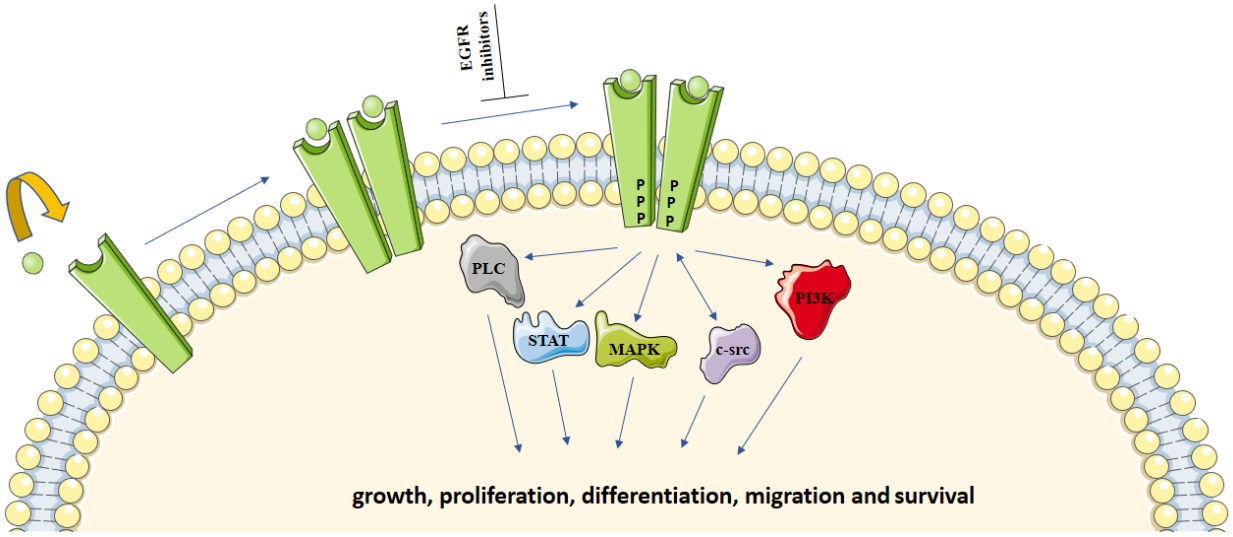


Figure 1.18. The epidermal growth factor receptor (EGFR) activation and inhibition. Upon ligand binding, EGFR dimerizes and autophosphorylates itself, leading to the activation of many downstream signaling pathways resulting in proliferation, migration, survival, etc. Small molecules inhibitors of EGFR bind to the ATP binding pocket preventing autophosphorylation and activation of EGFR and downstream signaling.

1.7.4 EGFR mutations and overexpression

Because of all the functions of EGFR mentioned above, such as growth, proliferation, invasion and survival, it is easy to see how deregulation of this RTK can be involved in cancer progression and poor prognosis [345, 346]. EGFR deregulations can be classified into many groups including, mutations, gene amplification, transcriptional hyperactivation, etc [286]. Most solid tumors are characterized by EGFR gene amplification typically leading to EGFR protein overexpression, which is the most common EGFR deregulation in cancer [347, 348]. Solid tumours that are recognized for EGFR overexpression include breast, lung, head and neck, pancreatic, brain, prostate, gastric and ovarian, bladder, etc [286]. Importantly, EGFR overexpression has also been seen in the absence of gene amplification [347, 348]. High EGFR activity can also be the result of the upregulation of ligands (e.g. TGF-α) production by the stroma or the tumour itself, leading to the activation of EGFR [286]. EGFR has also been shown to dimerize with other receptors (e.g. EGFR vIII and HER2) to potentiate cancer progression. The cooperation of EGFR with EGFRvIII leads to the phosphorylation of STAT proteins and potentiate malignant transformation in GBM [349]. EGFR and HER2 heterodimers result in synergistic activity in bladder cancer cells proliferation [350]. Similar results were seen in breast and pancreatic cancer progression [351].

Another form of EGFR deregulation is mutation in the receptor. The EGFRvIII variant exhibits a deletion in exons 2-6 in the extracellular domain. This variant does not need a ligand for activation as the receptor is constitutively active [352]. This mutation is found in more than 50% of GBM with EGFR alterations [353], and is also seen in other solid tumours such as breast, ovarian and lung cancer [354]. The EGFRvIII receptor produces only a weak signal, but this signal lasts longer since there is no ligand-dependent receptor internalization. As mentioned previously, EGFRvIII

also forms homodimers with the wild-type receptor and heterodimers with other receptor of the same family, which results in constitutive activation of theses receptors too [355].

Other EGFR mutations are found in a subtype of lung cancer, non-small cell lung cancer (NSCLC), which represents about 75-80% of all lung cancer cases [356]. Since 2004, few groups have reported new EGFR mutations in NSCLC patients [357-359]. These mutations involved the deletion of amino acids 746–750 in exon 19 (d746-750), and a point mutation in exon 21 (L858R), which are in the tyrosine kinase domain of EGFR. These ‘’gain-of-function’’ mutations lead to a constitutively active receptor, which is associated with tumor cell dependence on the mutated kinase for proliferation [360, 361]. Activating EGFR mutations have also been reported in few other tumour types such as: colon, esophagus, pancreas and the salivary gland cancers [362-364].

1.7.5 EGFR inhibitors in the clinic

In the 1980s, many groups reported that EGFR overexpression is observed in a large fraction of solid tumours. For this reason, dysregulated EGFR expression and signaling has been hypothesized to be key in cancer progression [365-370]. Furthermore, the idea of ‘’oncogene addiction’’, first proposed by Bernard Weinstein, described that tumour growth may be dependent on a single oncogene, which when blocked, can lead to tumour growth inhibition and cell death [371]. For these reasons, investigators worked on the development of antibodies targeting the extracellular domain of EGFR, thus preventing ligand binding and activation of the receptor [372]. Mendelsohn and colleagues [372, 373] developed the first anti-EGFR monoclonal antibody mAb225 (C225), now known as cetuximab, which showed promising antitumor activity *in vitro* and *in vivo*. Following its promising activity, cetuximab was approved by the Food and Drug Administration (FDA) in 2004 for the treatment of colorectal cancer and in 2011 for the treatment of head and neck cancer.

Small-molecule tyrosine kinase inhibitors against EGFR were also developed and investigated. EGFR inhibitors can be regrouped into three classes: first, second and third generation. The first generation of selective EGFR inhibitors include gefitinib and erlotinib, which are reversible binders to the ATP binding domain [374]. Gefitinib was first approved by the FDA in 2003 for the treatment of locally advanced or metastatic NSCLC after failure of both platinum-based and docetaxel chemotherapies [18]. Unfortunately, only a subset of patients with NSCLC responded to the treatment. Later on, it was shown that NSCLC tumours harboring specific activating mutation (i.e. deletions in exon 19 (EGFRdel19) and exon 21 L858R mutation (EGFRL858R)) were responding very well to small-molecule EGFR inhibitors [357-359]. These led to many successful phases II and III clinical trials [375-383], to finally result in erlotinib and gefitinib FDA approval in 2013 and 2015 respectively as first line monotherapy of NSCLC harboring EGFRdel19 and/or EGFRL858R [384, 385].

Although the first generation of EGFR inhibitors came as a breakthrough for a subtype of NSCLC, acquired resistance to these treatments emerged quite rapidly. Disease progression occurs on average 12 to 16 months after the initiation of the treatment [379, 380, 386]. The main mechanism of acquired resistance is the secondary EGFR mutation, EGFR T790M, which is observed in over 60% of patients who developed resistance to these agents. This mutation occurs at a different site in exon 20, and involves an increased affinity for ATP [387, 388]. This suggests that ATP binding is favored over EGFR inhibitors, that lost binding affinity to the ATP site. Other resistance mechanism to first generation EGFR inhibitors include amplification of Met [389, 390], histologic transformation [391], HER2 amplification [392], BRAF mutations [393], KRAS mutations [394], and PTEN mutations [395]. These mechanisms of resistance stimulated forward the development of the second generation of EGFR inhibitors, afatinib, neratinib and dacomatinib. These inhibitors

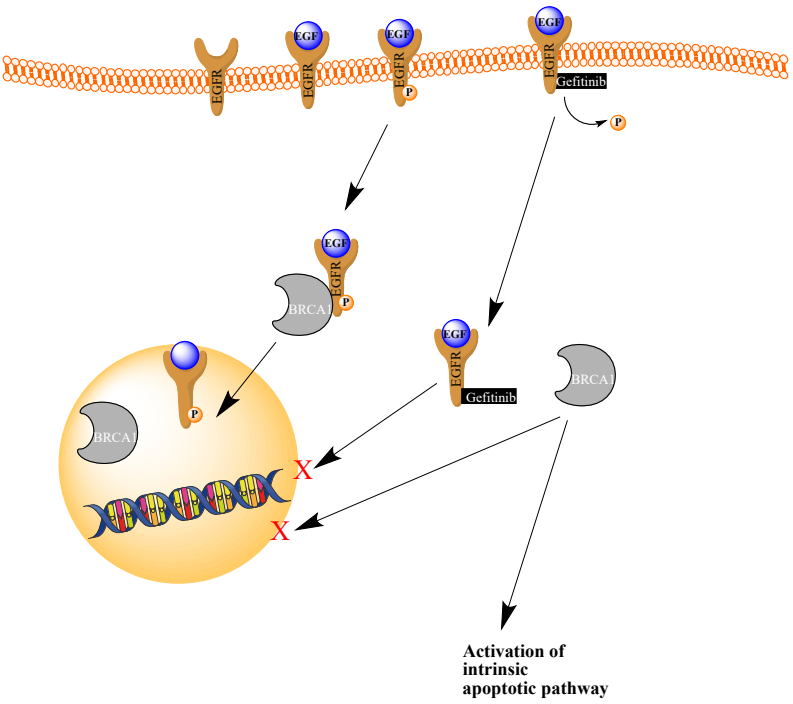
bind irreversibly to the ATP-binding pocket, leading to a longer inhibition of EGFR, and they are therefore more potent than the first-generation inhibitors [396]. Although it has been shown that afatinib can inhibit EGFR T790M *in vitro*, the dose required to inhibits it in humans cause high skin and gastrointestinal toxicities [397]. In 2014, a trial showed great efficacy of the combination of afatinib and cetuximab in lung cancer tumours with or without T790M mutation [398].

Because of the lack of available treatment against EGFR T790M mutants and the dose-limiting toxicities of the second-generation of EGFR inhibitors, a third generation of EGFR inhibitors, AZD9291 and rociletinib, was developed [399, 400]. Not only these drugs are potent on both the common EGFR mutation and the resistant mutation T790M, they also have significantly less side effects than the second-generation of EGFR inhibitors [401, 402]. Although there is a significant decrease in skin and gastrointestinal toxicities, grade 3 hyperglycemia was observed in few patients treated with rociletinib, which can be overcome easily with metformin treatment. Unfortunately, over time, acquired resistance to AZD9291 soon developed [403, 404]. The resistance is due to the C797 mutation, which is thought to prevent drug binding to the ATP-binding pocket [404, 405]. This mutation can also occur in the absence of the T790M mutation, which leaves them sensitive to the first- and second-generation of EGFR inhibitors [405, 406]. Interestingly, no resistance to rociletinib has been reported so far [403].

1.7.6 Synthetic lethal interactions between PARP and the EGFR pathway

Recent studies showed that EGFR and PI3K are also involved in the regulation of BRCA1. Nowsheen and colleagues [407] showed that EGFR regulates HR-mediated repair. Indeed, they showed through immunoprecipitation experiments in triple negative breast cancer (TNBC) cell lines (estrogen, progesterone and HER2 receptor negative) that EGFR and BRCA1 form a complex in the cytosol and by inhibiting EGFR, this complex is disrupted. They also show that the cytosolic

interaction between EGFR and BRCA1 is essential for the transport of both proteins into the nucleus. Figure 1.19 summarizes the contextual synthetic lethality described by Nowsheen *et al*., which shows that entrapping BRCA1 in the cytosol not only prevents it from playing its role in HR, but it is also able to trigger the intrisic apoptotic pathway [407]. Another study shows similar results, where inhibition of EGFR leads to an increase in γH2AX levels (marker for DNA DSB), decrease in RAD51 levels and BRCA1 cytoplasmic retention leading to HR deficiency [408]. In 2011, Burga *et al.* [409] showed a strong negative correlation between EGFR and BRCA1 in TNBC. Certainly, they showed that by downregulating BRCA1, EGFR was upregulated. In 2015, Kumaraswamy *et al.* [410] studied the functional role of BRCA1 in EGFR regulation. Their proposed mechanism of action is that BRCA1 binds to MIR147A promoter to activate the transtription of microRNA146A, which then donwregulates EGFR expression. From their studies, the combination of PARP and EGFR inhibitors resulted in promising tumour growth inhibition both *in vitro* and *in vivo*.



Fugure 1.19. Contextual synthetic lethality between EGFR, BRCA1, and PARP1. BRCA1 and EGFR form a complex allowing them to go to the nucleus. Inhibition of EGFR disrupts the EGFR-BRCA1 complex preventing both proteins from going inside the nucleus. BRCA1 being trapped into the cytoplasm, leads to deficiency in homologous recombination (HR) and activates the intrisic apoptotic pathway. Deficiency in HR sensitizes the cell to PARP inhibition.

Another EGFR family member has been shown to be involved in a synthetic lethality context with PARP inhibitors. Nowsheen *et al.* [411] showed that HER2 overexpression led to PARP inhibitor sensitivity in a DNA repair-independent fashion. In this study, PARP inhibition led to a decrease in the transcription faction, NF-kB, only in HER2-dependent manner. Downsteam of EGFR, PI3K and PTEN are also implicated in the synthetic lethality with PARP inhibitors and were described in section 1.6.

1.8 Temozolomide and its resistance mechanism

1.8.1 Temozolomide mechanism of action

DNA alkylating agents such as temozolomide and mitozolomide have showed, with clear evidence, high potency in both melanoma and glioma. They damage DNA by adding alkyl groups to DNA [412]. Unable to be repaired, this damage induces apoptosis, thereby killing the tumour cells. Although these alkylating agents may modify DNA at different sites, they preferably target the N^7 position of guanine and adenine, the O^6 position of guanine, or the N^3 of adenine. While these chemotherapeutic agents are efficient in damaging DNA, the base excision repair pathway readily repairs their adducts. Thus, this repair mechanism decreases the cytotoxic potential of the alkylating agent. It has been estimated that only 5 to 10% of the methylation caused by the DNA-damaging agent yield O^6-methylguanine methylation. If this adduct is not removed before cell division, the DNA mismatch repair (MMR) will be triggered, leading to futile attempts to repair the damage and ultimately apoptosis [413].

O^6-Methylguanine-methyltransferase (MGMT) is an enzyme that acts at the O^6 position of guanine. It repairs the damage that is caused by alkylating agent capable of inducing O^6 alkylation. Although MGMT is expressed ubiquitously in healthy human tissues, resistance to alkylating agents arises in tumour cells expressing MGMT. This repair enzyme specifically removes the methyl group from the O^6 position of guanine by transferring it to an internal cystein residue [412, 413]. This transfer inactivates the enzyme. A *de novo* synthesis is therefore required to replenish the cell. By targeting the tumour with alkylating agents, the activity of this enzyme will eventually saturate leading DNA damage unrepaired. In the following replication of the DNA, the modified guanine residue will preferably couple thymine; this will recruit the MMR pathway Since the MMR pathway only corrects the DNA damage in the newly synthesized daughter strand, the

template will remain impaired. In some study, it has been hypothesized that the previously stated pathway will go through an extra cycle to try repair the mismatch vainly. This repair attempt will leave the DNA with a double-strand break, which leads to cell death through the apoptotic pathway [413]. In order for alkylating agent to achieve a high therapeutic activity, ideally the tumour cells should contain low levels of MGMT and a functional MMR system [413].

1.8.2 Temozolomide resistance and attempts to overcome resistance

MGMT, with its ability to directly remove O^6-methylguanine adducts caused by temozolomide (TMZ), represents the major mechanism of resistance to the latter drug [414]. To enhance response to TMZ treatments, few MGMT inhibitors such as, O^6-benzyl guanine (O^6-BG) and O^6-(4-bromothenyl)guanine (lomeguatrib), have been developed. These molecules act as a substrate for MGMT and are used to deplete MGMT in the cell prior to TMZ treatment [415-417]. Specifically, O^6-BG transfers the benzyl moiety to the cysteine residue 145 in the active site of MGMT, causing it to be ubiquitinated and degraded [417]. Pretreatment with O^6-BG leads to the enhancement of TMZ activity *in vitro* and *in vivo* [418]. As for lomeguatrib, it acts in a similar way. It transfers the bromoethenyl group to the active site cysteine, and leads to MGMT ubiquitination and degradation [419, 420]. Promising preclinical data let to the initiation and completion of a Phase I clinical trial combining lomeguatrib and TMZ [239]. A randomized Phase II clinical trial of 100 patients with metastatic melanoma showed no difference between the efficacy of lomeguatrib + TMZ and TMZ treatment alone in terms of response rates and median time to disease progression due to the lack of a lasting MGMT depletion [421]. Other clinical trials used a different treatment schedule and doses for lomeguatrib and showed effective MGMT depletion in melanoma, CNS, prostate and colorectal tumours [422, 423]. Unfortunately, although these results translated into effective antitumour activity, deleterious side effects (e.g. myelosuppression) are a major deterrent in the

use of this type of agents. Unfortunately, in these approaches MGMT depletion is not tumour selective and is concomitant with bone marrow cell killing, thereby leading to acute myelototoxicity [424].

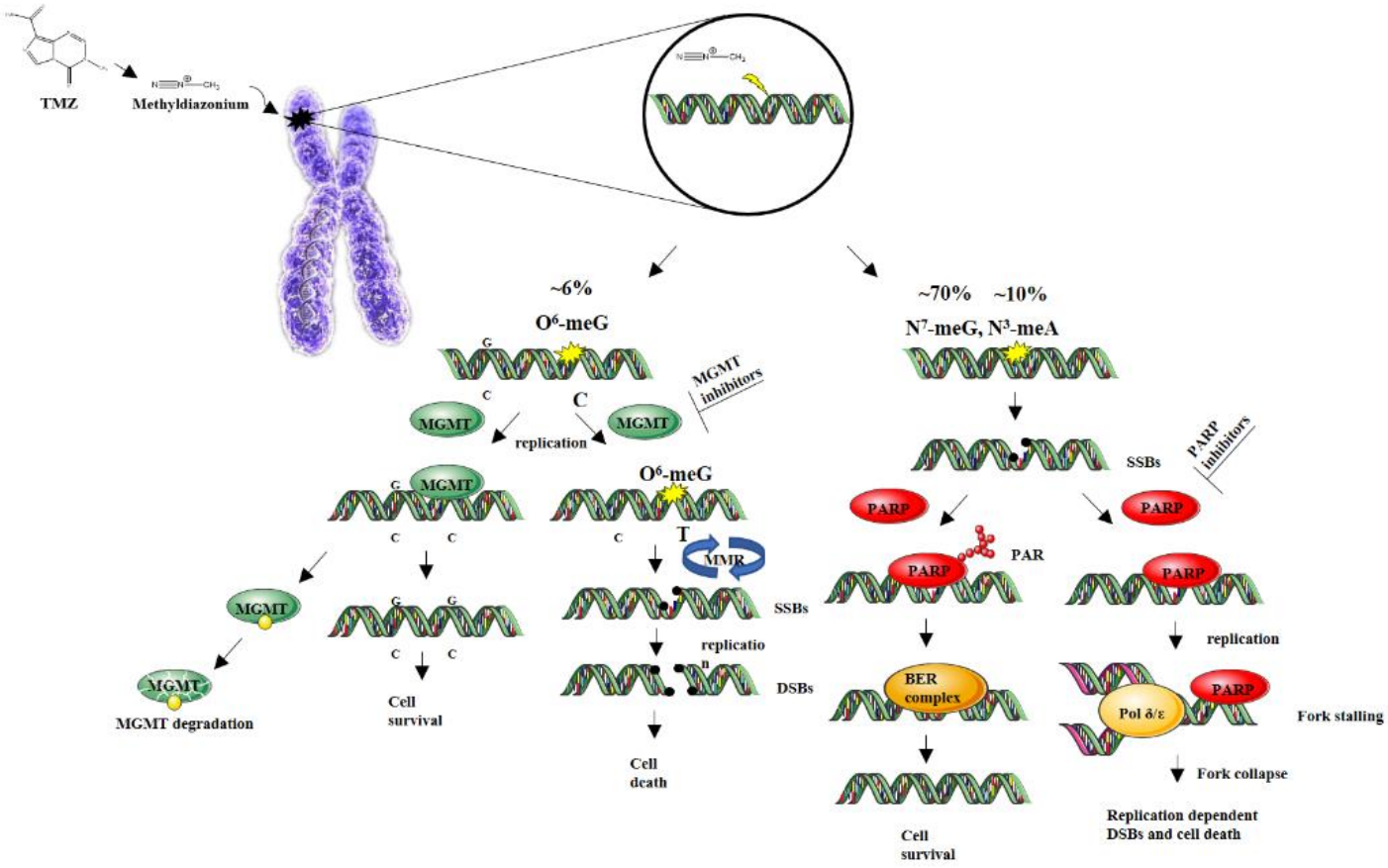


Figure 1.20. The two DNA repair pathways involved in repairing lesions caused by the alkylating agent temozolomide. The clinical alkylating agent temozolomide induces three types of lesions, including: O^6-methylguanine, N^7-methylguanine and N^3-methyladenine. Cytotoxicity only arises from unrepaired O^6-methylguanine lesions, which is usually repaired by the enzyme methylguanine methylatransferase (MGMT). The other two types of lesions are repaired by the base excision repair (BER) pathway. To overcome resistance to TMZ, inhibitors of both repair pathways were developed, MGMT inhibitors and PARP inhibitors.

Although the O^6-methylguanine is the main cytotoxic adduct and the N^7-methylguanine and N^3-methyladenine are rapidly repaired by the BER pathway, inhibiting the latter pathways enhances TMZ cytotoxicity (illustrated in figure 1.20) [425, 426]. The mechanism by which PARP inhibitors potentiate TMZ is due to the accumulation of single strand breaks. Indeed, by inhibiting PARP, the N^7-methylguanine and N^3-methyladenine lesions are cleaved, forming single strand breaks. The inhibited PARP can no longer synthesize the PAR chain and therefore the BER pathways is not initiated. Over few DNA replications, single strand breaks are accumulated and are converted into doubles strand breaks [427]. The combination of different PARP inhibitors with TMZ were tested in the clinic and showed promising potentiation of TMZ in a wide range of cancers [230, 231, 428]. It is important to note that the combination of PARP inhibitors with TMZ was well tolerated after a dose adjustment [176, 232].

1.9 Cancer drug development: from monotherapy to polypharmacology

Drug discovery has been governed by the design and development of very selective compounds targeting a single component of the disease [429]. In the early 1960s and after many observations that single agent therapies did not lead to full remission, the first drug combination was used in the treatment of the testicular cancer. They used different combinations of alkylating agents, antimetabolites and antitumour antibiotics, that showed significantly improved results [12]. Since then, the use of chemotherapeutic cocktails for many cancer types has been the standard therapy (described in table 2).

Cancer is an extremely heterogenous group of diseases and each organ-specific cancer comprises of various subgroups with distinctive molecular signatures. There have been many groundbreaking discoveries regarding molecular signatures in cancers, which allowed the development of targeted therapies. However, single drug therapies are rarely successful due to the complexity of redundant

mechanisms in cancer cells. Today, cancer treatment involves a multi-targeting approach, which can employ three possible strategies: (a) the combination of multiple individual drugs, (b) single pill formulations with multiple drugs, and (c) single drugs with multi-targeted properties [429]. The challenge in using chemotherapeutic cocktails is the increase in toxicities due to drug interactions. Similarly, combining different inhibitors into a single pill may be challenging as the different drugs in the pill may have a different pharmacology, which can impact their efficacy [429]. For these reasons, the best way to approach polypharmacology is the development of single drugs able to target two or more pathways in the cell, which would reduce pharmacological and toxicity concerns [429]. Current multitargeted therapies include small molecule kinase inhibitors, such as sorafenib and sunitinib that were initially developed as single kinase inhibitors and were later found to be multi-targeted [430, 431].

The rational development of multi-targeted therapies relies on the discovery of synthetically lethal targets, redundancy or compensatory signaling pathways and mechanisms of resistance [429]. Using this principle, we chose to develop rationally designed multitargeted molecules that are able to exploit the concept of synthetic lethality, mechanisms of resistance, and the overexpression of EGFR. The next section reviews such an approach termed ‘’combi-targeted concept’’ developed in our laboratory.

Table 2. Drug combination cocktails currently used in the clinic.

Cancer type	Drug cocktail	Acronym	Reference
Breast Cancer	Cyclophosphamide, methotrexate, 5-fluorouracil	CMF	[432]
	Doxorubicin, cyclophosphamide	AC	[433]
Hodgkin's disease	Mustine, vincristine, procarbazine, prednisolone	MOPP	[434, 435]
	Doxorubicin, bleomycin, vinblastine, dacarbazine	ABVD	[434, 435]
Non-Hodgkin's lymphoma	Cyclophosphamide, doxorubicin, vincristine, prednisolone	CHOP	[436]
Germ cell tumour	Bleomycin, etoposide, cisplatin	BEP	[437]
Stomach cancer	Epirubicin, cisplatin, 5-fluorouracil	ECF	[438, 439]
	Epirubicin, cisplatin, capecitabine	ECX	[439]
Bladder cancer	Methotrexate, vincristine, doxorubicin, cisplatin	MVAC	[440]
Lung cancer	Cyclophosphamide, doxorubicin, vincristine	CAV	[441]
Colorectal cancer	5-fluorouracil, folinic acid, oxaliplatin	FOLFOX	[442]
	5-fluorouracil, folinic acid, irinotecan	FOLFIRI	[442]

1.10 The combi-targeting concept

As described earlier, anti-cancer drug therapies have been developed to target various tyrosine kinases, DNA repair proteins, etc. This search for specific inhibitor was intended to avoid the multiple side effects caused by unselective DNA-damaging agents. Despite this effort to develop a targeted therapy, tumour cells grow with alternative compensatory mechanisms to survive the effect of drugs, or otherwise the ability to modulate the downstream signaling to compensate for the inhibition of some pathways. These inconveniences such as toxicities and resistance have

directed researchers towards the development of a novel strategy that targets divergent cellular components. This strategy not only targets structurally unrelated targets of a synergetic effect, but also reduces adverse effects due to redundancy [443]. The combi-targeting concept is described as a strategy that seeks to make molecules with a specific receptor affinity, which is also capable of further degrading into to two molecular species: one of which acts as an inhibitor of the target receptor, and the other is capable of damaging DNA of the same cells. This dual acting molecule is therefore able to induce significantly persistent antiproliferative activity in cells expressing these receptors. Since DNA targeting agents usually lack selectivity and therefore cause severe toxicity, combining an EGFR blocker and a DNA alkylating agent would not only have a dual effect on the tumor but has also the potential to induce selective targeting, thereby reducing toxicity [444].

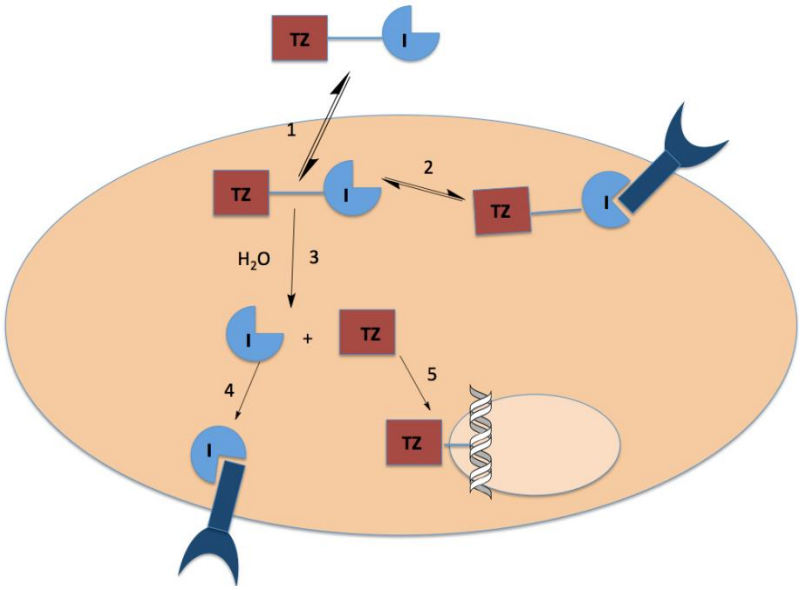


Figure 1.21. The combi-targeting concept. I' represent the EGFR inhibitor and TZ the DNA damaging portion of the molecule. From the extracellular compartment, the molecule diffuses through the membrane into the cytosol to inhibit EGFR with its intact form. It will then be hydrolyzed into its two separate moieties I' and TZ. I' inhibits EGFR and TZ damages DNA.

Our Laboratory was the first to develop this novel class of compounds termed "combi-molecules" (recently reviewed by Sun *et al.)*[445], depicted in figure 1.21. A combi-molecule is a single entity (**I-Tz**) composed of two distinct targeting arms (e.g. **I'** targeting EGFR and **Tz** targeting DNA). The two arms are connected by either a stable or a hydrolysable linker. Both arms can influence two cellular targets: inhibition of a kinase and damage of DNA. Combi-molecules are categorized as type I, type II, or type III, as illustrated in figure 1.22 [446-448]. Type I combi-molecules have a labile linker, which under physiological condition, release their two active arms in the cell (**I + Tz**). **I'** is designed to inhibit biological targets (e.g. EGFR) and **Tz** to damage DNA. As for type-II combi-molecules, they carry a stable non-hydrolazable linker. They exert their binary inhibitory properties as an intact structure (**I-Tz**) within the cell [23, 446, 448-455]. More recently, our laboratory has developed type III combi-molecules that merge the concept of both type I and type II. Type III combi-molecules are not only able to target and inhibit two different kinases with its intact form, but also to be hydrolyzed to the two kinase inhibitors. The rationale behind this new type of combi-molecules is that in case the hydrolysis is slow inside the tumour cell, the intact structure can still inhibit both kinases [456].

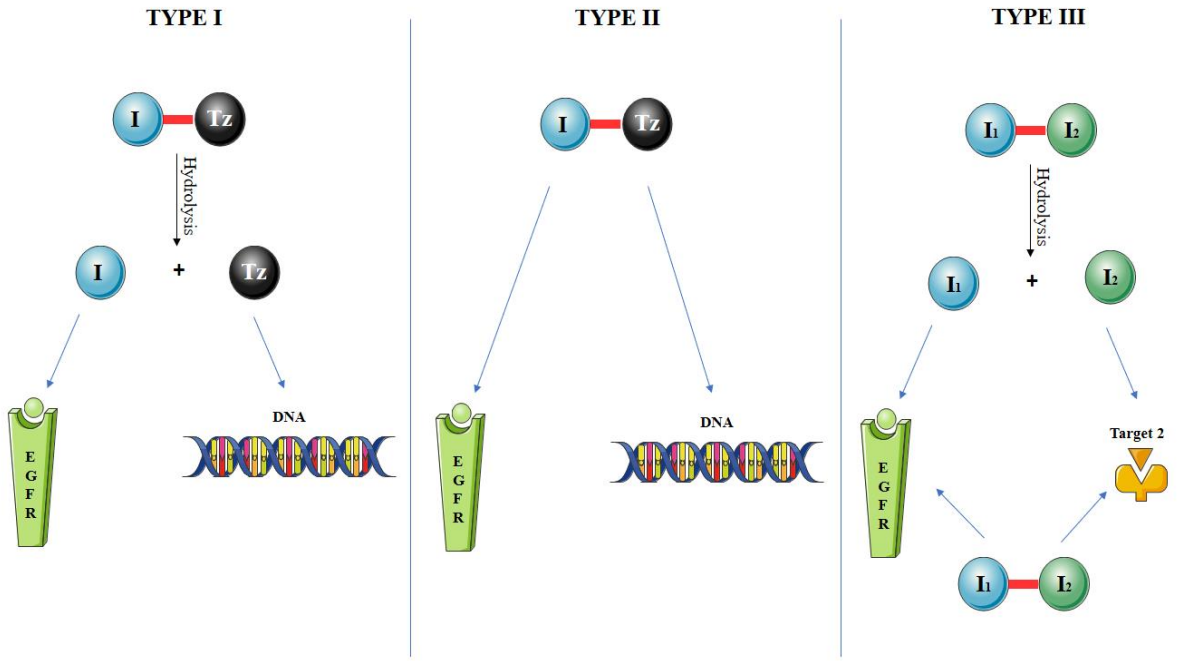
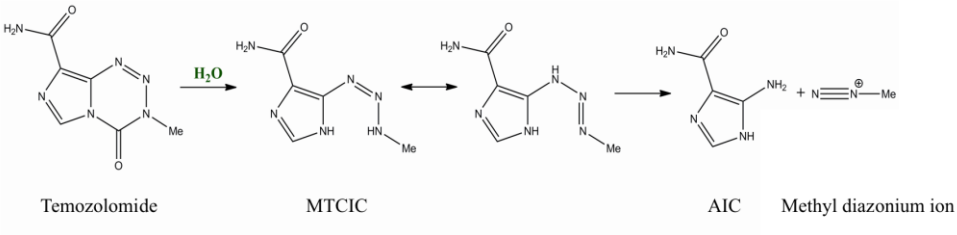


Figure 1.22. The different types of combi-molecules. Type I combi-molecules hydrolyze under physiological conditions to release two active moieties targeting two independent biological entities. Type II combi-molecules are very stable, do not hydrolyze under physiological conditions and are able to interact with two different targets. Type III combi-molecules are able to act as both type I and type II combi-molecules. Not only each moiety is able to inhibit their respective targets while the molecule keeps its intact form, it is also able to hydrolyze and release the two active species to allow them to act independently.

1.10.1 Type I combi-molecules

The first combi-molecule ever synthesized was a type I combi-molecule. This molecule SMA41 published in 2001, was designed to hydrolyze under physiological conditions to release an active EGFR inhibitors and a DNA damaging agent. The first DNA damaging warhead used in combi-molecules, the methyldiazonium ion, is the same as the one released by the clinically approved alkylating agent temozolomide (Scheme 1.1). As mentioned previously, these new molecules are

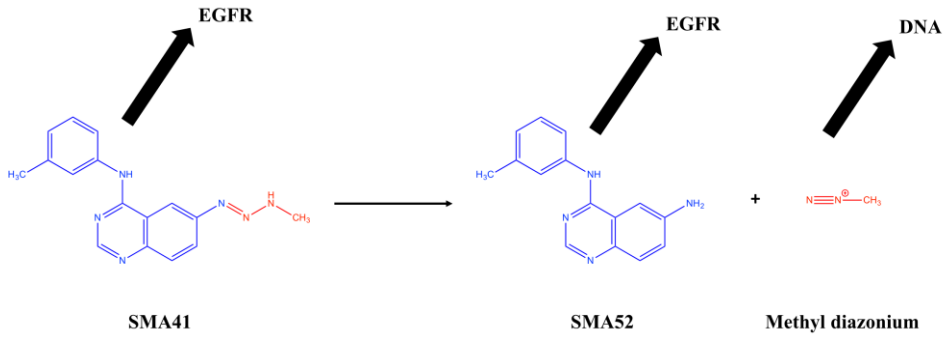
designed to have all the benefit of targeted therapy and non-targeted therapy (i.e. spare healthy cells and being aggressive respectively), while overcoming their weaknesses (i.e. resistance and toxicity respectively). Solid tumors are characterized by overexpression and/or mutation of growth factor receptors, such as EGFR. This receptor is involved in many tumorigenic pathways, such as: apoptosis, proliferation, invasion and migration. Based on this rationale, our laboratory specialized in combining a non-targeted therapy to a targeted therapy to enhance potency and reduce toxicities. Mechanistic studies of such compounds showed inhibition of all downstream signaling pathways and induction of DNA damage. Most importantly, these combi-molecules were selectively potent in EGFR-expressing cells and abrogated chemoresistance.



Scheme 1.1. Temozolomide hydrolysis. The clinical alkylating agent, temozolomide, under physiological conditions, is able to hydrolyze to release its active species, the methyl diazonium ion.

Combi-molecules are based on a rational design using structure-activity relationships (SAR) to optimize the binding of the molecule to its target. SAR studies have shown that the aniline moiety of the inhibitor is essential in binding to the hydrophobic region of the ATP-binding pocket of EGFR [457]. In the quinazoline backbone, a H_2O-mediated hydrogen bond between N3 has been shown and Thr-766 and the N1 atom acts as a hydrogen bond acceptor from Met-769. Further molecular modeling studies showed that the 6- and 7-positions of that backbone allows structural changes without major loss of affinity to EGFR [458]. Our laboratory then designed the first

prototype of EGFR-DNA combi-molecule, SMA41, based on the quinazoline system and a triazene moiety at the 6-position (Scheme 1.2). The combi-molecule not only showed promising inhibition of EGFR in an *in vitro* kinase assay and its phosphorylation in a whole cell assay, but it also induced DNA damage. SMA41 showed superior potency when compared to SMA52, the naked EGFR inhibitor, or TEM, the DNA damaging agent [23].

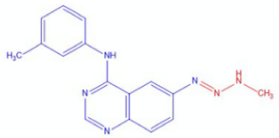
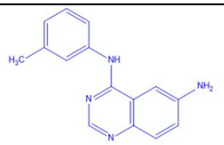
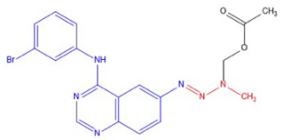
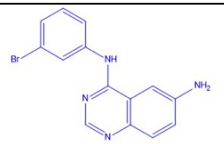
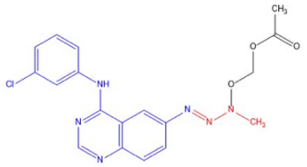
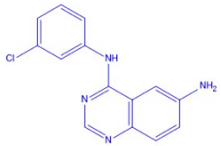
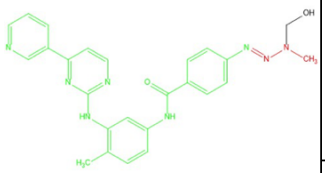
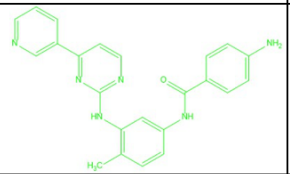


Scheme 1.2. The type I combi-molecule SMA41 targeting EGFR with its intact form, which can hydrolyze to release the EGFR inhibitor (SMA52) and the DNA-damaging agent (methyldiazonium)

Although the design and development of SMA41 showed that a DNA-damaging species could be grafted on to the quinazoline backbone without losing the inhibitory property of either moiety, a few questions remained unanswered. SMA41 being a type I combi-molecule and therefore is hydrolysable, we were unsure as to whether the molecule hydrolyzed outside or inside the cell. The methyl diazonium part of molecule was then labelled with ^{14}C, while the EGFR inhibitory portion is fluorescent. While the ^{14}C-labelled DNA damaging agent was found to be distributed everywhere in the cell and alkylated DNA, RNA and proteins, the EGFR inhibitory arm was preferentially located in the perinuclear region. This led to a better understanding as to how combi-molecules cause higher levels of DNA damage than temozolomide. It was suggested that since the

EGFR targeted combi-molecule locates in the perinuclear region, it therefore localizes the DNA damaging arm in closer proximity to the nucleus [459]. The subcellular localization of these molecules was also studied in a different way by Todorova *et al*. who [460] synthesized this molecule with a green fluorescent tag on the DNA damaging portion, taking into account that the EGFR inhibitor is already blue fluorescent. This study clearly showed that both moieties are inside the cell in the perinuclear region. Other published type I combi-molecules are listed in Table 1.3.

Table 1.3. Type I combi-molecules designed and synthesized in our laboratory

Type I Combi-molecules name	Combi-molecule type	Structure	Arm 1	Arm 2	Reference
SMA-41	**EGFR-DNA**		 EGFR inhibition	 DNA damage	[23]
RB24	**EGFR-DNA**		 EGFR inhibition	 DNA damage	[446]
ZRS1	**EGFR-DNA**		 EGFR inhibition	 DNA damage	[444]
ZRCM5	**BcrAbl-DNA**		 Bcr-Abl inhibition	 DNA damage	[458]

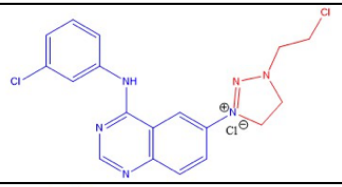
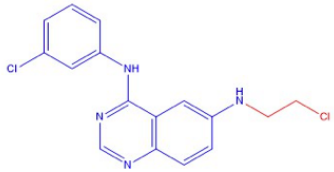
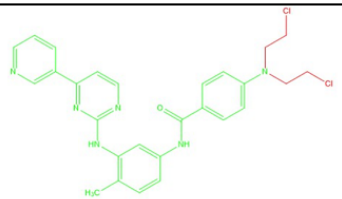
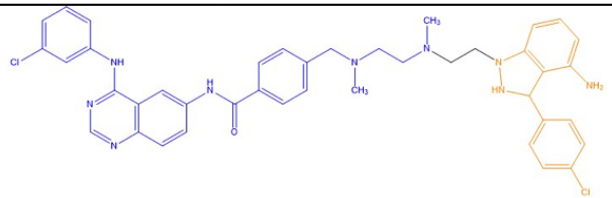
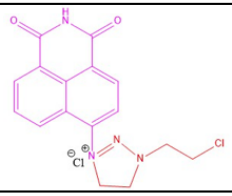
1.10.2 Type II combi-molecules

The first type II combi-molecule, JDD35, was composed of an EGFR inhibitor and a DNA damaging agent with a stable linker between them. It was the first balanced type II combi-molecules, with strong inhibition of EGFR and strong DNA damaging potential [24]. These results lead to the development of many other type II combi-molecules. ZR2003, another EGFR-DNA combi-molecule, was the first type II combi-molecule to be tested *in vivo.* Despite its extraordinary potency in multiple *in vitro* assays, its potency in two *in vivo* models showed very little response. Further studies showed that its tumour penetration was consistently lower than gefitinib, a clinical EGFR inhibitor [461].

The same type II concept was used to design the first BcrAbl-DNA combi-molecule, AK04. This molecule combined the structure of gleevec, a clinical Bcr-Abl inhibitor used in the treatment of leukemia, and chlorambucil, a clinical alkylating agent used in the treatment of lymphomas. AK04 was not only a good inhibitor of Bcr-Abl phosphorylation and a great alkylator, but it was also very selective towards Brc-Abl positive cells [454].

A different kind of type II combi-molecules were synthesized 2009, where for the first time, a combi-molecule, SB163, is composed of two kinase inhibitors, EGFR-c-Src. SB163 was developed based on a rational design using molecular modeling studies of known X-ray structures of EGFR and c-Src inhibitors. SB163 was developed to induce tandem blockade of both EGFR and c-Src due to their redundant signaling pathways involved in invasion and metastasis. For the c-Src inhibitor backbone, a 7-phenyl-pyrazolopyrimidine moiety was selected and, for the EGFR inhibitory arm, a quinazoline moiety was used. SB163 showed a dose dependent inhibition of both kinases in *in vitro* kinase assay, but showed imbalanced targeting [462]. All type II combi-molecules mentioned in this section are listed in Table 1.4.

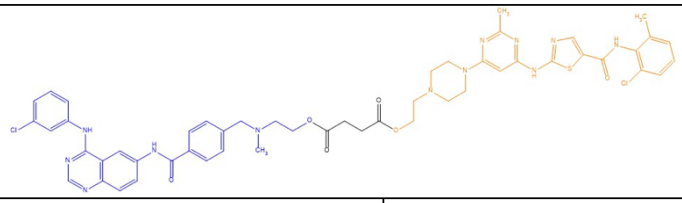
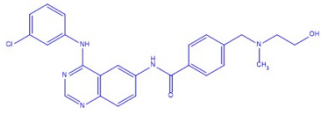
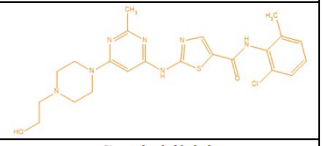
Table 1.4. Type II combi-molecules designed and synthesized in our laboratory

Type II Combi-molecules name	Combi-molecule type	Structure	Reference
JDD36	**EGFR-DNA**		[24]
ZR2003	**EGFR-DNA**		[459]
AK04	**BcrAbl-DNA**		[451]
SB163	**EGFR-c-Src**		[460]
EG40	**PARP-DNA**		[461]

1.10.3 Type III combi-molecules

Type III combi-molecules have been designed to have both the ability to act as a type I and a type II combi-molecule. The first and only type III, AL776, was designed to target EGFR and c-Src (Table 1.5). In its intact structure, AL776 is able to modulate both targets and also hydrolyze and release two active kinase inhibitors. The idea underlying the design of a combi-molecule targeting two kinases was based on the signaling redundancy between multiple kinases found in refractory tumours. This is known to cause resistance to targeted therapy. Although AL776 was able to inhibit both c-Src and EGFR *in vitro* and *in vivo,* for unknown reason, this new strategy did not translate into tumour shrinkage *in vivo* [456].

Table 1.5. Type III combi-molecules designed and synthesized in our laboratory

<table>
<tr><th>Type III Combi-molecules name</th><th>Combi-molecule type</th><th colspan="2">Structure</th><th>Reference</th></tr>
<tr><td rowspan="5">AL776</td><td rowspan="5">EGFR-c-Src</td><td colspan="2">  </td><td rowspan="5">[453]</td></tr>
<tr><td>Arm 1</td><td>Arm 2</td></tr>
<tr><td>  </td><td>  </td></tr>
<tr><td>EGFR inhibition</td><td>c-Src inhibition</td></tr>
</table>

RESEARCH OBJECTIVES

We have now entered the genomic era. In contrast to the traditional one-size-fit-all approach to therapy of cancer, we are moving towards more targeted and personalized treatment modalities. In this context, the investigation of specific biomarkers for treatment indication for advanced cancer has taken the center stage. It is in this context that the work in this thesis was oriented. We primarily considered to develop molecules that can enhance the potency of agents designed to be specific for BRCA1/2 mutations, which is now a clinical biomarker in ovarian cancer and now in prostate cancer. Furthermore, work in this context was directed at enhancing potency in tumours expressing MGMT, a specific biomarker associated with resistance to temozolomide. Discoveries from the latter work inspired us to pursue a daunting challenge of targeting inhibitors in the context of BRCA1/2 to a more boarder biomarker of EGFR, which is overexpressed in most solid tumours.

In this thesis, we sought to overcome resistance to PARP inhibitors in BRCA1/2 mutant tumours and overcome resistance associated to the drug temozolomide due to MGMT expression through: (a) a unimolecular approach to the design and development of PARP-DNA combi-molecules, (b) the optimization of the stability of molecules obtained towards objective (a) under physiological conditions, (c) expansion of the use of PARP inhibitors beyond BRCAness by targeting them to EGFR-expressing cells.

The thesis focuses on the development of new strategies using PARP inhibition going from non-targeted to more targeted PARP inhibitors. The new non-targeted PARP inhibitors were designed to anchor a DNA damaging agent into the nucleus in order to enhance DNA damage in temozolomide resistant cells. The novel targeted PARP inhibitor uses an EGFR inhibitor as the targeted warhead to selectively inhibit the growth of EGFR expressing cells.

Work under the aims of this thesis involved the development of synthetic strategies to achieve the isolation of the complex dual targeted molecules, optimized their stability, and molecular modeling was used to simulate their binding to their cognate target. Molecular analyses were performed to investigate their mechanisms of action in murine and human tumour cell carrying specific biomarkers to the resulting molecules. The complex mechanism of action of these molecules on cells was investigated by dissecting signaling response to the effect of each arm of these combi-molecules, namely, DNA damage and PARP inhibition, as well as EGFR targeting and PARP inhibition. The pursuit of these objectives led to the unpredicted discoveries described in this thesis. The very specific objective of this thesis are described below.

Objective 1: To optimize the stability of the first successful prototype PARP-DNA combi-molecules (Chapter 2).

Objective 2: To elucidate the mechanism of action of the first stable PARP-DNA combi-molecule in the context of BRCAness and MGMT-mediated resistance to temozolomide (Chapters 3).

Objective 3: To design and optimize a series of PARP-EGFR combi-molecules, elucidate their mechanism of action and study their potency in a panel of EGFR-expressing cells (Chapter 4).

The achievement of these objectives reported in this thesis have given original contributions to knowledge which are discussed in Chapter 5.

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CHAPTER 2: ^{15}N-, ^{13}C- AND ^{1}H-NMR SPECTROSCOPY CHARACTERIZATION AND GROWTH INHIBITORY POTENCY OF A COMBI-MOLECULE SYNTHESIZED BY ACETYLATION OF AN UNSTABLE MONOALKYLTRIAZENE

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Keywords:

NMR, ^{1}H, ^{13}C, ^{15}N, Naphthalimides

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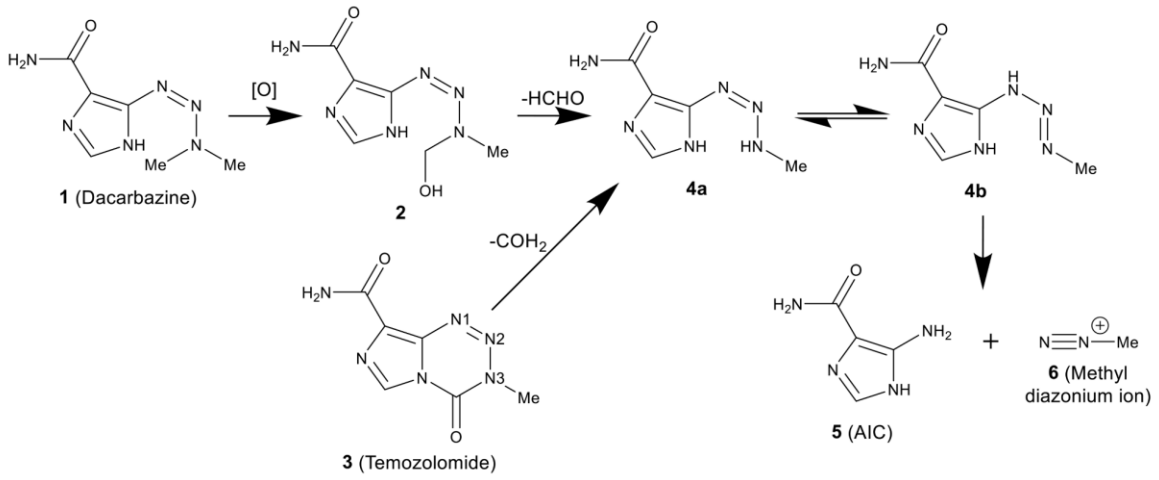
2.1 Abstract

The compound studied, namely EG22 (**8a**), is an open-chain 3-alkyl-1,2,3-triazene termed "combi-molecule" designed to inhibit poly(ADP-ribose) polymerase (PARP) and damage DNA. To delay its hydrolysis, acetylation of N3 was required. Being a monoalkyl-1,2,3-triazene, EG22 could assume two tautomers in solution or lose nitrogen during the reaction, thereby leading to several acetylated compounds. Instead, one compound was observed and to unequivocally assign its structure, we introduced isotopically labeled reagents in its preparation, with the purpose of incorporating ^{15}N at N2 and ^{13}C in the 3-methyl group. The results showed that the 1,2,3-triazene moiety remained intact, as confirmed by ^{15}N-NMR, coupling patterns between the ^{15}N-labeled N2 and the ^{13}C-labeled methyl group. Furthermore, we undertook heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond coherence (HMBC) experiments that permitted the detection and assignment of all four nitrogens in ZSM02 (**9a**) whose structure was further confirmed by X-ray crystallography. The structure showed a remarkable coplanarity between the N-acetyltriazene and the naphtalimide moiety. Thus, we unequivocally assigned **9a** as the product of the reaction and compared its growth inhibitory activity with that of its precursor, EG22. ZSM02 exhibited identical growth inhibitory profile as EG22, suggesting that it may be a prodrug of EG22.

Keywords: ^{15}N-NMR; 1,2,3-triazene; temozolomide; monoalkyltriazene; 4-Amino-1,8-naphthalimide

2.2 Introduction

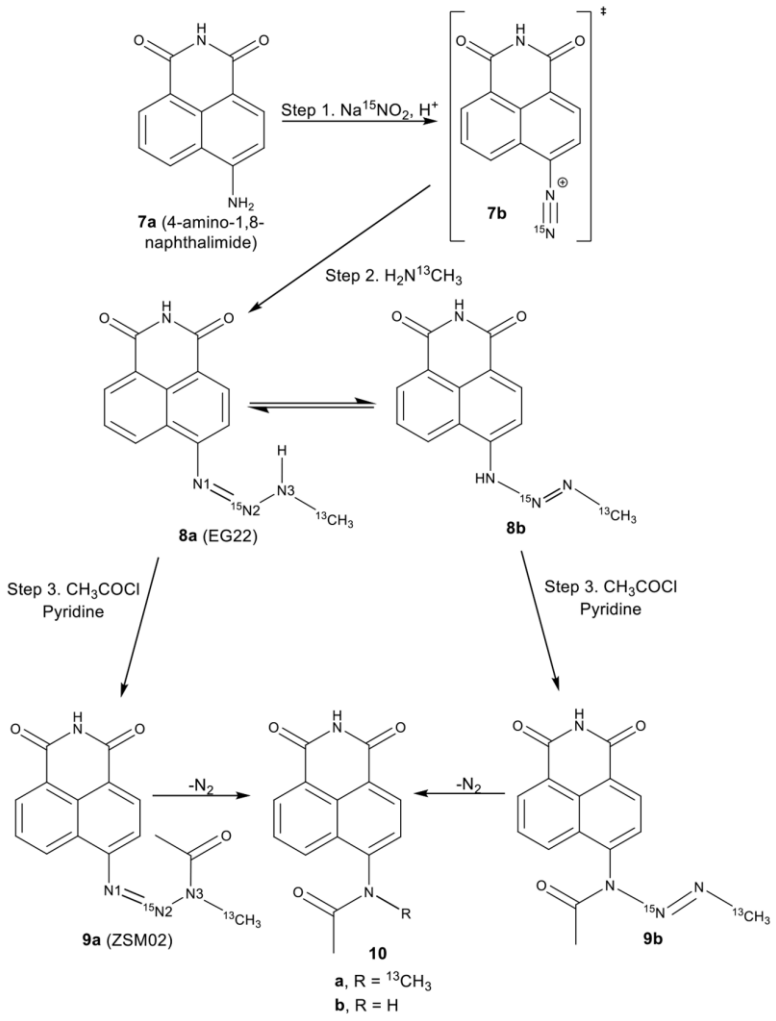
The alkyltriazenes are amongst the oldest classes of alkylating agents used in the clinic for the treatment of cancer. One such agent, dacarbazine (**1**, Scheme 1), has been used in the clinical management of malignant melanoma for more than 35 years [1–3]. As depicted in Scheme 1, in vivo, dacarbazine (**1**) is metabolized into the monoalkyltriazene **4a** that is further hydrolyzed to give the methyl diazonium species that alkylates DNA [4]. It is also known to be the species released from the hydrolysis of Temodal, a potent clinical agent used in the clinical management of glioblastoma [5]. Monoalkyltriazene **4a** is in equilibrium with its corresponding tautomer **4b**, which upon hydrolytic cleavage, generates aminoimidazole carboxamide **5** and a methyldiazonium species **6** [6] that reacts with DNA in the cells to generate many adducted bases, including N3-methyladenine, N7-methylguanine, N7-methyladenine and *O*-6-methylguanine adducts [2,7,8].



Scheme 1. Metabolic pathway for dacarbazine and temozolomide [1–3,8].

The mechanism of hydrolysis of monoalkyltriazenes of type **4a**, which leads to an aromatic amine (e.g., **5**) and a DNA alkylating agent, has inspired the development by our group of a new tumour targeting approach termed “combi-targeting” [9–11]. This approach seeks to design molecules termed “combi-molecules” to behave as bioactive species on their own and to further

be hydrolyzed into other bioactive species. Agents that require hydrolysis to generate bioactive species are termed type I combi-molecules [12] and those that do not require hydrolysis to exert multiple activities, type II [13]. Recently, such a type of molecule 6-(3-Methyltriaz-1-en-1-yl)-1H-benzo[de]isoquinoline-1,3(2H)-dione, EG22 (8a, Scheme 2), was designed to behave as an inhibitor of a DNA repair protein termed "poly(ADP-ribose) polymerase" (PARP) and a DNA alkylating agent. EG22 (**8a**) was successfully synthesized and shown to possess dual PARP and DNA targeting properties [14]. However, its rate of hydrolysis under physiological conditions was too fast and we believed that this could compromise its activity in vivo. Thus, in order to stabilize the combi-molecule, we investigated means to delay its hydrolysis under physiological conditions. Here we report on the unequivocal characterization of 6-(3-Acetyl-3-methyltriaz-1-en-1-yl)-1H-benzo[de]isoquinoline-1,3(2H)-dione, ZSM02 (**9a**), the first prototype of a masked form of EG22 (**8a**), using ^{1}H-, ^{15}N- and ^{13}C-NMR of its isotopically labelled form. We hypothesized that if ZSM02 (**9a**) is a prodrug of EG22 (**8a**), its growth inhibitory potency should be the same as EG22 (**8a**). Therefore, we compared their activity in human breast, colon and brain cancer cell lines.



Scheme 2. Synthesis of ZSM02 (**9a**) and possible by-products.

2.3 Results and Discussion

2.3.1 Stability of EG22 in DMSO

The synthesis of non-isotopically labeled EG22 (**8a**) was reported elsewhere [14]. As shown in Figure 1, the first evidence of its instability was obtained by monitoring the appearance of a peak corresponding to the shielded proton *ortho* to the amino group of 4-amino-1,8-naphthalimide (ANI, **7a**) and the disappearance of a deshielded doublet of the aromatic ring of EG22 (**8a**) in wet DMSO-d_6 at room temperature. Indeed, after a two-day period, EG22 (**8a**) was almost fully

converted to of 4-Amino-1,8-naphthalimide, ANI (**7a**) and the decomposition $t_{1/2}$ under these conditions was 12.6 h (Figure 2).

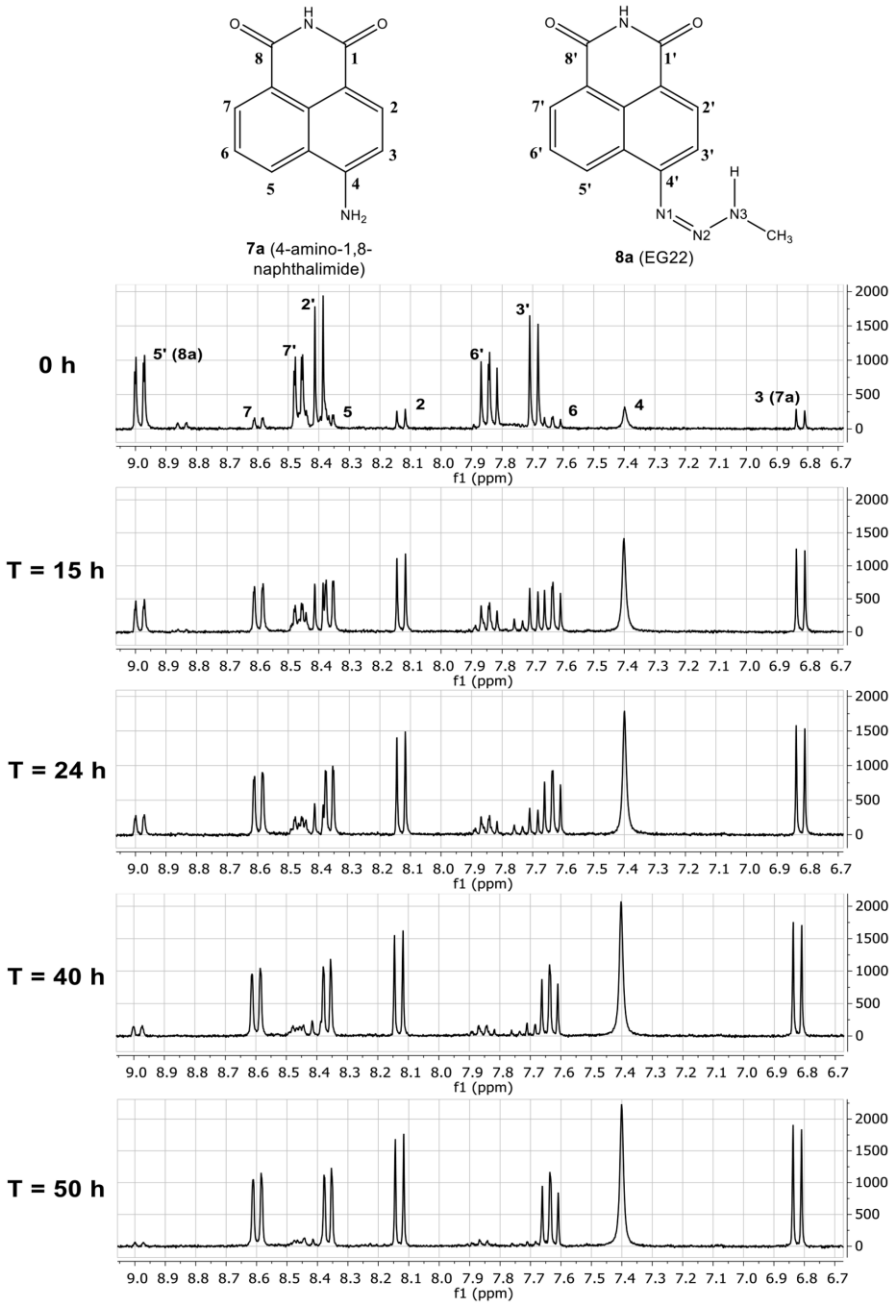


Figure 1. ^{1}H-NMR spectra of EG22 (**8a**) showing slow conversion to ANI (**7a**) (0–50 h) in non-dried DMSO-d_6.

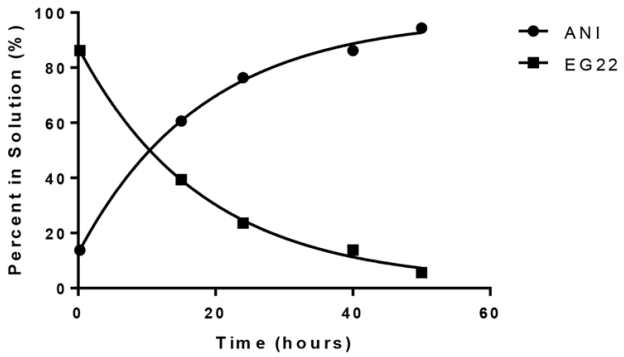
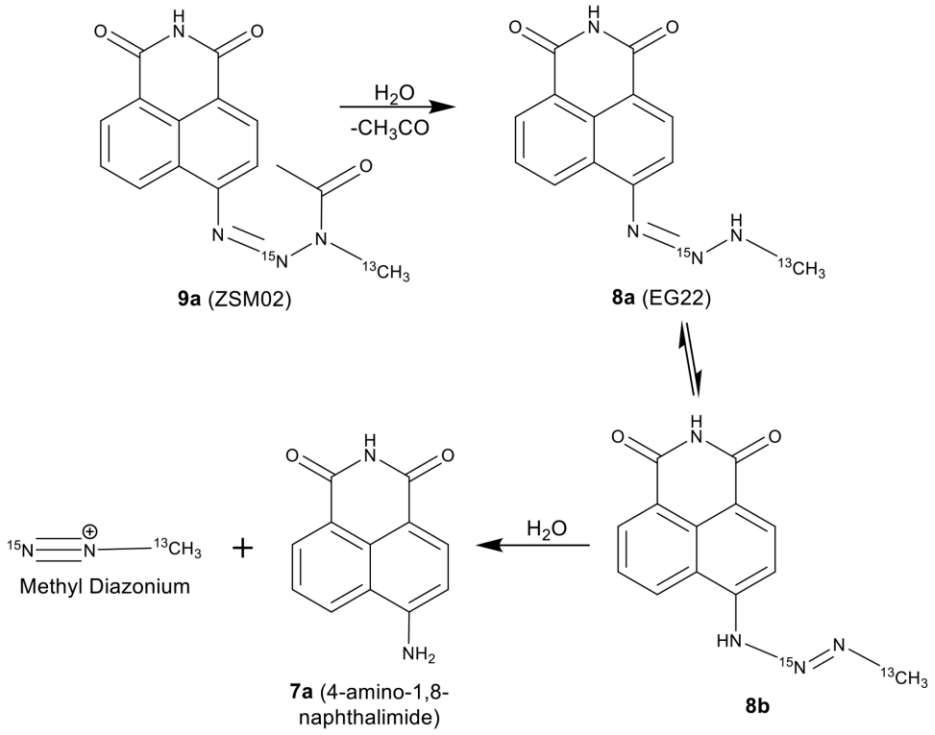


Figure 2. Formation of 4-Amino-1,8-naphthalimide, ANI (**7a**) in DMSO over time using ANI/(ANI + EG22) percent peak ratio and decay curve for EG22 (**8a**) in DMSO over time using EG22/(ANI + EG22) percent aromatic peak ratio.

The rate determining step of the hydrolysis of monoalkyltriazenes has been shown to be the protolysis of their non-conjugated tautomer [15]. Therefore, we believe that this conversion is catalyzed by trace amount of water in DMSO-d_6 and the mechanism of degradation is primarily based on the cleavage of the non-conjugated tautomer **8b** (Scheme 3).



Scheme 3. Deacetylation of ZSM02 (**9a**) and protolysis of EG22 (**8a**) to regenerate ANI (**7a**) and create a methyl diazonium species.

Accordingly, we surmised that the hydrolysis of EG22 (**8a**) could be delayed by acetylating N3, thereby shifting the overall rate determining step to that of the slow cleavage of the N3–CO bond. The fact that EG22 (**8a**) can assume the two tautomeric forms outlined in Schemes 2 and 3 in organic solutions and since acetylation of each tautomer would lead to different structures of the same mass or resulting from loss of N_2, a confusing NMR spectrum was expected for the reaction products. Surprisingly, the synthesis led to one major product, the exact structure of which remained elusive. Thus, we undertook an isotopic labeling study involving ^{15}N and ^{13}C labeling of the 3-methyl-1,2,3-triazene moiety.

2.3.2 Isotopic labelling and NMR spectroscopy

As outlined in Scheme 2, the incorporation of the isotopes proceeded by substituting two reactants for their isotopically labelled counterparts in the synthesis of EG22 (**8a**): (a) ^{15}N sodium nitrite to be incorporated in the in situ generated diazonium salt and (b) ^{13}C methylamine for addition to the latter under basic conditions. Having shown that EG22 (**8a**) can be converted to ANI (**7a**) and considering that it perhaps exists as two tautomers in solution, we expected products resulting from: (i) a direct reaction of the acetyl chloride on the N3 of the triazene moiety, leading to the desired structure **9a**, (ii) an acetylation of the non-conjugated isomer **8b** to produce **9b**, (iii) an acetylation of ANI (**7a**) resulting from the decomposition of EG22 (**8a**) or the loss methyl diazonium from **9b** to give **10b**, or (iv) loss of nitrogen from **9a** and **9b** to give **10a** [16].

^{1}H-NMR analysis of the product (Figure 3a) showed an interesting coupling pattern for the 3-methyl group, which appeared as a doublet ($^{1}J_{HC}$ = 142 Hz), indicating that it is coupled with ^{13}C [17]. Since we expected this coupling to be consistent with the presence of the ^{13}C labeled methyl, we further analyzed the product by ^{13}C-NMR.

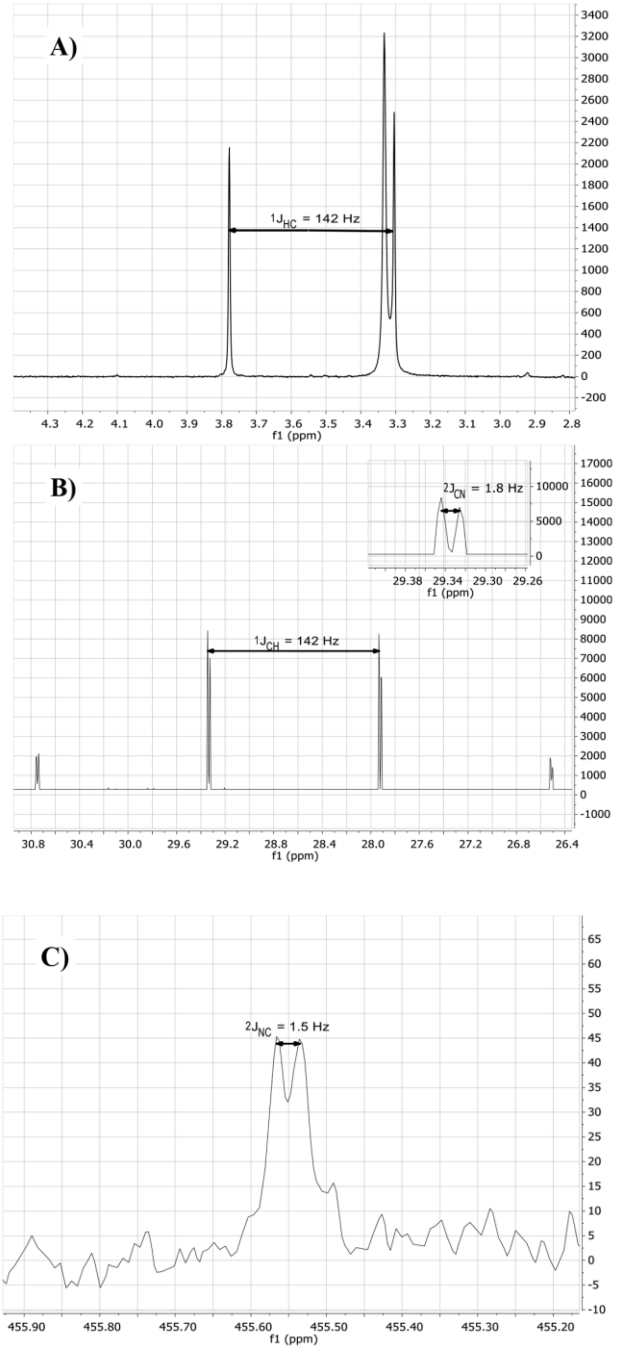
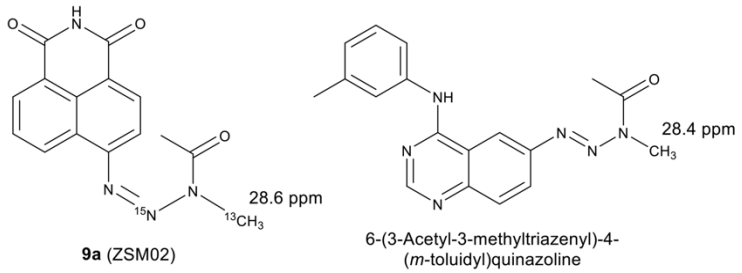


Figure 3. (**A**) ^{1}H-NMR spectrum of ^{13}C and ^{15}N labelled ZSM02 (**9a**) in non-dry DMSO-*d*$_6$ displaying the $^{13}CH_3$ doublet ($^1J_{CH}$ = 142.2 Hz). (**B**) Proton coupled ^{13}C-NMR spectrum of ^{13}C and ^{15}N labelled ZSM02 (**9a**) ($^1J_{CH}$ = 142 Hz and $^2J_{CN}$ = 1.8 Hz). (**C**) Proton decoupled ^{15}N-NMR spectrum of ^{13}C and ^{15}N labelled ZSM02 (**9a**) ($^2J_{CN}$ = 1.5 Hz).

Interestingly, the results showed that the ^{13}C methyl peak appeared at 28.6 ppm, as a quartet of doublets ($^{1}J_{CH}$ = 142 Hz, $^{2}J_{CN}$ = 1.5 Hz) (Figure 3b), indicating that it does not only couple with its directly bound hydrogen but also with the central nitrogen N2 of the triazene chain.

In order to further ascertain the presence of the ^{15}N label of the central nitrogen, a full ^{15}N-NMR spectrum was acquired in decoupled mode. ^{15}N-NMR analysis showed a peak at 455.6 ppm (ammonium scale) or 75 ppm (converted to the nitromethane scale) that showed up as a doublet ($^{2}J_{NC}$ = 1.8 Hz) as a result of coupling with the isotopically labeled ^{13}C (Figure 3(c)). The observed shift for N2 is in agreement with previous reports by our group showing that the N2 in 1,2,3-triazene containing molecules is in the +70 ppm range [18,19].

The ^{15}N-NMR and its corresponding coupling with the ^{13}C-labeled carbon allowed to rule out structures **10a** and **10b** (Scheme 2). Our data is consistent with the presence of an intact 1,2,3-triazene chain in the structure, as in **9a** and **9b**. The ^{13}C shift of the ^{13}C-labeled methyl group in the non-conjugated tautomer is known to be considerably deshielded (e.g., 54 ppm for 3-methyl-l-*p*-tolyl-triazene) [20]. Our observed shift (28.6 ppm) is significantly more shielded and is consistent with that of a similar *N*-methylacetyltriazene previously synthesized by our group [21]. This allowed to rule out **9b**. As depicted in Scheme 4, in 6-(3-acetyl-3-methyltriazene)-4-(*m*-toluidyl)quinazoline that carries a similar $NNN(CH_3)COCH_3$ moiety, the ^{13}C shift of the methyl group (28.4 ppm) is almost identical to that in ZSM02 (**9a**) (28.6 ppm).



Scheme 4. Comparison of ZSM02 (**9a**) to previously synthesized *N*-methylacetyltriazene. The respective N3 methyl shifts are 28.6 ppm and 28.4 ppm.

2.3.3 HMBC and HMQC analysis

While the ^{15}N labeling led to the determination of the presence and chemical shift of the central nitrogen, absence of coupling with the other nitrogens, N1 and N3 in the chain, did not allow us to infer on the presence of the latter two nitrogens. Likewise, the nitrogen of the naphthalimide system could not be detected from the experiment. Therefore, we undertook connectivity studies using heteronuclear multiple bond correlation (HMBC) and heteronuclear single bond coherence (HSQC) to indirectly detect the latter natural abundance nitrogens. Indeed, HSQC experiment showed a sharp peak for the NH of the naphthalimide ring (Figure 4) at 167 ppm (−213 ppm, nitromethane scale). As depicted in Figure 5, HMBC analysis further confirmed the presence of N1, N2, and N3 at 393.4 (13 ppm), 455.8 (75 ppm), and 217.4 ppm (−163 ppm), respectively. Therefore, these experiments allowed us to confirm the complete nitrogen content of the molecule. Our NMR data in toto confirm that structure **9a** (ZSM02), is the product of the reaction.

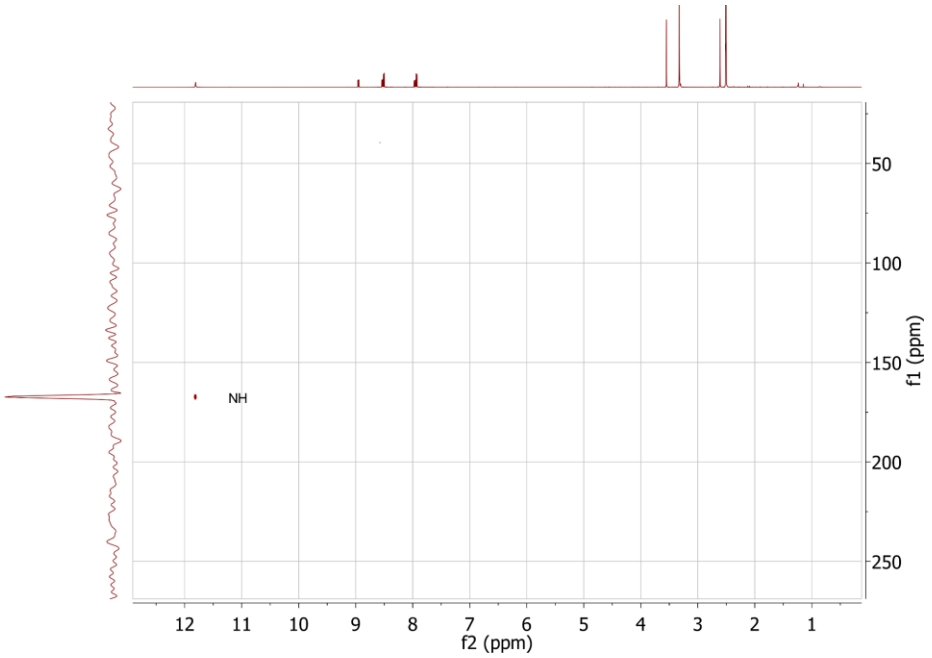


Figure 4. heteronuclear single bond coherence (HSQC) experiment showing a sharp peak for the NH of the naphthalimide ring of compound **9a**.

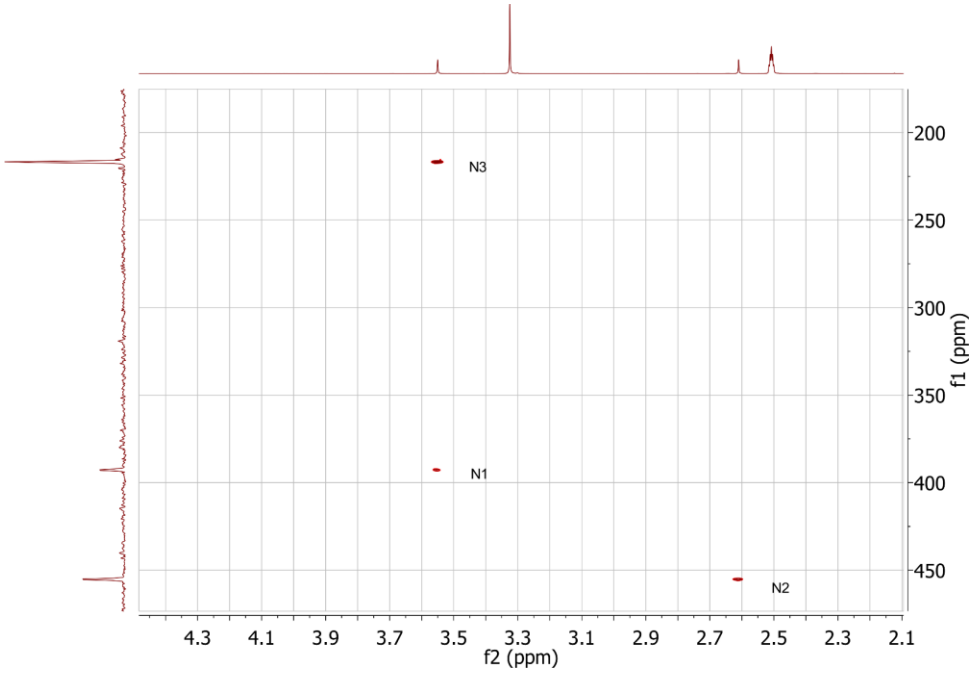


Figure 5. Heteronuclear multiple bond correlation (HMBC) experiment showing peaks corresponding to N1, N2 and N3 of the triazene chain of compound **9a**.

2.3.4 Determination of the 3D structure

In order to determine the three-dimensional structure of the molecule, we sought to crystallize f **9a**, but all attempts via conventional methods failed. A dimethylformamide (DMF)/hexane biphasic approach led however to needles that lent themselves to diffraction. The crystal structure was identified as a DMF hemisolvate with two unique molecules of **9a** and one molecule of DMF in the asymmetric unit (Figure 6). As depicted in Figure 4, the results showed that the complete *N*-acetyl triazene moiety was almost completely coplanar with the naphthalimide rings. The least square planes of the acetyl and napthalimide groups were 5.8° and 9.8° for molecules A and B, respectively (as labeled in Figure 4). Acetylation of the nitrogen did not lead to a significant elongation of N2–N3 bond (N4A–N3A: 1.36(2) Å; N4B–N3B: 1.34(2) Å), which is consistent with the observed coplanarity and perhaps the electron donating character of the *N*-acetyltriazene moiety. Indeed, NMR analysis showed that the proton attached to C11B (Figure 4) is more shielded than the one at C8B in naphthalimide ring. Reported lengths for non-acetylated open-chain triazenes are in the range of 1.30 Å [22].

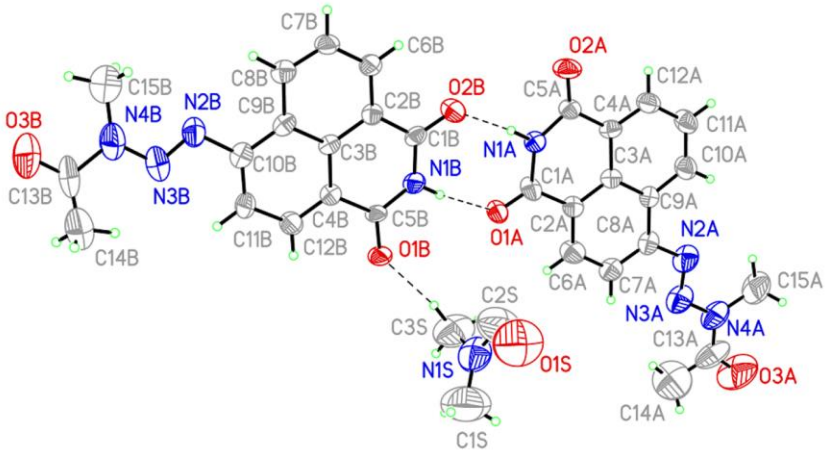


Figure 6. X-ray crystallographic structure of compound **9a**. Non-hydrogen atoms drawn as ellipsoids at the 50% probability level and hydrogen atoms as circles of arbitrary radii.

2.3.5 Biological activity

Having ascertained the structure of ZSM02 (**9a**), we further analyzed its effect on cell growth in comparison with its precursor, EG22 (**8a**). We surmised that if ZSM02 (**9a**) is converted into EG22 (**8a**) intracellularly, their growth inhibitory profile should be similar. Indeed, as shown in Figure 7, using the Sulforhodamine B (SRB) growth inhibitory assay [23], we confirm that ZSM02 (**9a**) and EG22 (**8a**) exhibit similar growth inhibitory profiles. Further work on the mechanism of action of both molecules is reported elsewhere [14].

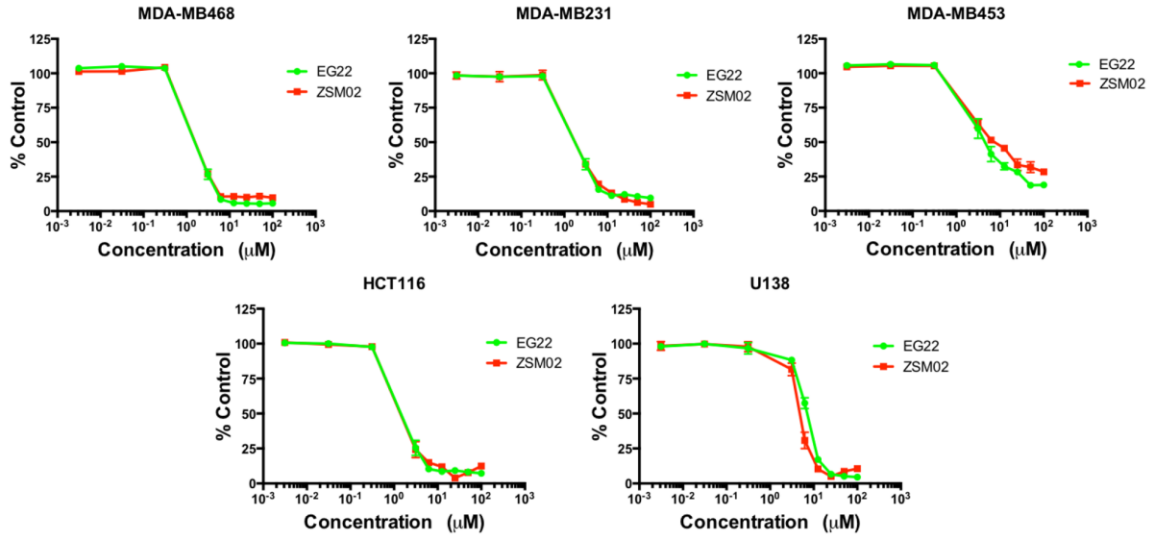


Figure 7. Growth inhibition activity of compounds **8a** (EG22) and **9a** (ZSM02).

2.4 Material and Methods

2.4.1. General Information

4-Amino-1,8-naphthalimide (ANI) was purchased from Ark Pharm (Arlington Heights, IL). All other chemicals were purchased from Sigma-Aldrich.

2.4.2 Chemical Synthesis

6-(3-Methyltriaz-1-en-1-yl)-1H-benzo[de]isoquinoline-1,3(2H)-dione **(8a):**

The methyltriazene compound **8a** was synthesized as described in Scheme 2. Briefly, 4-amino-1,8-naphthalimide (ANI, **7a**) (1 eq., 0.236 mmol) was dissolved in concentrated trifluoroacetic acid and was cooled to −5 °C for 15 min. The ^{15}N labeled sodium nitrite (2 eq., 0.472 mmol) in a clear solution was then added dropwise. Once diazotized, ^{13}C labelled methylamine hydrochloride (3 eq., 0.708 mmol) was dissolved in water and added slowly dropwise thereafter. Upon reaction completion, the solution was neutralized with a saturated solution of sodium bicarbonate and left to precipitate for an hour. The mixture was then filtered and the precipitate collected and dried. ^{1}H-NMR (300 MHz, DMSO-d_6) δ ppm 11.61 (s, 1H, NH), 11.44 (q, 1H, J = 3.6 Hz, $NHCH_3$), 8.97 (dd, 1H, J = 8.4 Hz, 0.9Hz, ArH), 8.46 (dd, 1H, J = 7.2 Hz, 1.2 Hz, ArH), 8.39 (d, 1H, J = 8.1 Hz, ArH), 7.83 (t, 1H, J = 8.0 Hz, ArH), 7.69 (d, 1H, J = 8 Hz, ArH), 3.26 (dd, 3H, J =139.3 Hz, 4.2 Hz, $NH^{13}CH_3$).

6-(3-Acetyl-3-methyltriaz-1-en-1-yl)-1H-benzo[de]isoquinoline-1,3(2H)-dione **(9a):**

The acetylated compound **9a** was synthesized as described in Scheme 2. Briefly, 3 mL of anhydrous pyridine was flash frozen using liquid nitrogen. Once completely frozen, acetic anhydride (10 eq, 1.97 mmol) was introduced and flash frozen using liquid nitrogen. A total of 50 mg of **8a** (EG22) in Scheme 2 (1 eq, 0.197 mmol) was added as a powder. The reaction was allowed to reach a temperature of −5 °C for 30 min and then reach room temperature slowly for 2 h. Once the reaction was complete, the pyridine was azeotroped with toluene. The resulting solid was collected and dried. ^{1}H-NMR (300 MHz, DMSO-d_6) δ ppm 11.83 (s, 1H, NH), 8.96 (dd, 1H, J = 8.4 Hz, 0.8 Hz, ArH), 8.54 (dd, 1H, J = 7.2 Hz, 1.2 Hz, ArH), 8.51 (d, 1H, J = 8.0 Hz, ArH), 7.96 (t, 1H, J = 8.0 Hz, ArH), 7.94 (d, 1H, J = 8 Hz, ArH), 3.54 (d, 3H, J = 142.2 Hz, $N^{13}CH_3$), 2.60 (s, 3H, $COCH_3$). ^{13}C-NMR (300 MHz, DMSO-d_6) δ ppm 172.96, 163.71, 148.35, (2C) 130.56, 130.53, 129.78, 129.69,

127.72, 127.57, 122.74, 122.18, 114.33, 28.63 (qd, J = 142 Hz, 1.8 Hz, $^{15}NN^{13}CH_3$) and 22.03. ^{15}N-NMR (30.4 MHz, DMSO-d_6) δ ppm 455.55 (d, J = 1.5Hz, $^{15}NN^{13}C$). ESI m/z 297 (MH^-).

2.4.3 NMR Acquisition

The ^{1}H- and ^{13}C-NMR spectra were acquired at ambient temperature on a Mercury 300 spectrometer (Varian/Agilent, city, state abbrev if USA, country) equipped with an ATB probe. Concentration of samples were 1 mg/mL in DMSO-d_6. The relaxation delay was 1 s after a 45 degrees pulse and acquisition time of 1042 s. For the ^{1}H-NMR spectrum, 256 scans were collected and for the ^{13}C coupled spectrum 6800 scans were collected. Both spectra were acquired with NOE. The spectral width was 18,832 Hz, 39,248 points were collected and zero filled to 256K points before Fourier transformation for a digital resolution of 0.14 Hz. No apodization was used.

The ^{15}N-NMR spectrum was obtained at ambient temperature on a Varian/Agilent VNMRS 500 spectrometer equipped with a dual broadband probe. Concentration of the sample was 5 mg/mL in DMSO-d_6 and was referenced using ^{15}N ammonia as an external standard and for conversion to the nitromethane scale the following equation was used: δ(nitromethane) = δ (ammonia) – 380.3. The relaxation delay was 3 s after a 20-degree pulse and acquisition time of 1.6 s. A total of 1064 scans were collected. The spectrum was acquired with NOE. The spectral width was 25,000 Hz, 80,004 points were collected and zero-filled to 512K points before Fourier transformation for a digital resolution of 0.10 Hz.

All spectra of the non-isotopically labeled compounds were acquired on an AVIIIHD spectrometer (Bruker, city, state abbrev if USA, country) operating at a 1H frequency of 500.3 MHz using a BBFO + SmartProbe (Manufacturer, city, state abbrev if USA, country). Around 2 mg of ZSM02 (**9a**) were dissolved in 1 g DMSO-d_6. The ^{1}H spectrum was acquired in 72 scans using an acquisition time of 3.7 s and a recycle delay of 1 s. The ^{13}C spectrum was acquired using

power gated WALTZ decoupling (Manufacturer, city, state abbrev if USA, country) in 6144 scans with a recycle delay of 2 s (total experimental time 5.5 h). The COSY spectrum was acquired in 20 min using a spectral width of 10 ppm in each dimension, 2048 points in the direct dimension, and 256 points in the indirect dimension. Two ^{13}C-HSQC spectra were acquired, one with a spectral width in the indirect dimension of 165 ppm and 64 points (4 min) and the other centered on 129.5 ppm with a spectral width of 8 ppm and 512 points (30 min). The ^{13}C HMBC spectrum was acquired using a spectral width in the indirect dimension of 65 ppm centered at 143 ppm using 384 points (one hour). The ^{15}N-HSQC spectrum was acquired in 1.25 h using optimization for a 90 Hz *J* coupling. The ^{15}N-HMBC spectrum was acquired in 8.5 h using 16 scans per increment and 512 increments, in 8.5 h, with delay times optimized for a 1.6 Hz *J* coupling.

2.4.4 X-ray crystallography

Data collection was performed on a Bruker D8 Venture diffractometer equipped with a Photon 100 area detector (Bruker-AXS, Madison, WI, USA). Yellow, crystalline needles were isolated by inspection under microscope. Although the crystals were well-formed and transparent, they showed signs of polycrystallinity including uneven extinction under polarized light and the ability to bend. Diffraction was unexpectedly weak for crystals of their size, and cooling the crystals to low temperature appeared to result in even weaker diffraction. This behavior suggested the crystals may have been aggregates of much smaller crystallites which remained fairly well aligned at room temperature but lost their alignment under the strain of thermal contraction on cooling. Ultimately, the best data was collected using graphite monochromated Mo-Kα radiation from a microfocus source on a crystal measuring 0.22 × 0.08 × 0.01 mm using shutterless scans at a rate of 120 s/degree. A full hemisphere of unique data was collected out to 0.80 Å resolution using scans about the omega and phi axes. Unit cell determination, data collection, data reduction, and absorption

correction were performed using the Bruker Apex3 software suite (version 2015-R7; Bruker-AXS, Madison, WI, USA) [24].

The structure of **9a** was solved and its space group determined by the iterative dual space approach implemented in the program SHELXT [25]. The absolute configuration of the crystal structure could not be determined from Mo Kα data. Non-hydrogen atoms were refined anisotropically by full-matrix least squares refinement against F^2. Hydrogen atoms were placed in calculated positions, and their coordinates and thermal parameters were constrained to ride on the carrier atom. Least squares refinement was done using SHELX v.2014 [26] implemented in the Bruker SHELXTL software suite [27].

Diffraction from this crystal becomes essentially indistinguishable from noise at resolutions beyond 1 Å, so the precision on bond distances is low (approximately 0.02 Å). The structural features of interest—the 3-D configuration of the molecule and the distinction between single, double, and aromatic bonds by length—can all still be unambiguously detected at this resolution. Additionally, most of the reflections lie in the 1.0–0.8 Å resolution range, so the global values for R_{int}, wR_2 are high while the ratio of observed to unique reflections is low. Values that are constrained to resolutions containing observed reflections, such as R_1, are in acceptable ranges. While these deficiencies could be solved by using a larger crystal or a more powerful X-ray source, the refinement presented here represents the limit of what could be achieved with available facilities and is chemically reasonable and fully consistent with other experimental characterization.

Crystal data for **9a**·0.5DMF (ZSM02, **9a**): $C_{16.5}H_{15}N_{4.5}O_{3.5}$ (Mw = 332.33 g/mol), monoclinic, space group *Pc*, *a* = 13.856(3) Å, *b* = 4.9842(9) Å, *c* = 23.836(5) Å, β = 100.651(6)°, α = γ = 90°, V = 1617.8(5) $Å^3$, Z = 4, T = 298(2) K, λ(Mo Kα) = 0.71073 Å, Dcalc = 1.364 mg/m^3, F(000) =

694, independent reflections 24669/6617 (R_{int} = 0.2463), 2.494° < 2θ < 26.435°, the final R_1 was 0.1434 (I > 2σ(I)) and wR_2 was 0.3900 (all data).

The supplementary crystallographic data for compound **9a** can be obtained in Supplementary Materials and from the Crystallography Open Database (COD no. 3000124) free of charge at http://www.crystallography.net/ cod/.

2.4.5 Cell Culture

HCT116 colon cancer cell line was kindly provided by Moulay Alaoui-Jamali (Segal Cancer Centre and Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, QC, Canada). The MDA-MD468 breast cancer cell line was bought from the ATCC (Manassas, VA, USA). MDA-MB453 and MDA-MB231 were a generous gifts of Suhad Ali (the Research Institute of the McGill University Health Centre, Montreal, QC, Canada). U138 glioblastoma cell lines were kindly given by Siham Sabri (the Research Institute of the McGill University Health Centre, Montreal, QC, Canada). Media preparation was supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, gentamicin sulfate, amphotericin B and ciprofloxacin. All the reagents used in the preparation of the media were purchased from Wisent Inc. (St-Bruno, QC, Canada). The cells were grown in a humidified incubator with a stable temperature of 37 °C and CO2 level of 5%.

2.4.6 Growth Inhibition Assay

Cells were plated in 96-well plates (Corning Inc., Corning, NY, USA) at 3000–10,000 cells/well in 100 µL medium/well. They were then treated, 24 h later, with a wide range of drug concentrations (0.0031 µM to 800 µM). The treatment was done in triplicate for 5 days in the incubator. Following the drug treatment, the cells were fixed with 50 µL per well of cold TCA (50%) for 2 h at 4 °C, rinsed, dried well and stained with 50 µL sulforhodamine B (SRB) (0.4

g/100 mL). Subsequently, the SRB was rinsed with 1% acetic acid, and allowed to air-dry overnight. Finally, the dye was solubilized with Tris base (10 mM, pH 10–10.5). Absorbance readings of the solubilized dye were recorded on a ELx808 microplate reader, BioTek (Winooski, VT, USA) at an optical density of 492 nm. The results were analyzed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) to derive a dose-response curves and the IC50. Each experiment was carried out four times, in triplicate.

2.5 Conclusion

We successfully acetylated the 1,2,3-methyl triazene **8a**. Subsequently, we unequivocally assigned the structure of the resulting compound **9a** (ZSM02), using ^{15}N isotopic labeling, HMBC, HSQC and X-ray crystallography. Finally, a biological assay showed similar growth inhibitory potency for both compounds **8a** (EG22) and its acetylated form, **9a**. These results in toto suggest that **9a** could be a prodrug of **8a.** A detailed study on the hydrolysis of **8a** and **9a** under physiological conditions and on their biological effects as combi-molecules, is reported elsewhere [14].

Supplementary Materials:

Table S1. Crystal data and structure refinement for s1_a.

Acknowledgements: We thank the Canadian Institutes of Health Research (CIHR) for financial support. We are grateful to the McGill NMR Lab and the McGill University Health Center Drug Discovery platform for NMR access. Zhor Senhaji Mouhri thanks the Fonds Quebecois de Recherche sur la Sante (FQRS) for a Doctoral Research Award.

Author Contributions: Elliot Goodfellow synthesized the prototype combi-molecule, EG22, and helped with the preparation of the manuscript. Zhor Senhaji Mouhri synthesized the stable form of this combi-molecule to yield ZSM02 and crystallized it. She also synthesized the labelled compounds and performed the NMRs, and mass spectrometry of the labeled compounds. She interpreted the data with the help of Elliot Goodfellow. She carried out the biological assay and helped with the preparation of the manuscript. Dr. Robin Stein performed the HMBC and HSQC NMR of the non-labelled compound. Dr. Robin Rogers and Dr. Steven P. Kelley performed the crystallography of ZSMR02. Dr. Bertrand Jean-Claude overlooked the proceedings of the experimental work and revised the manuscript.

Conflicts of Interest: All authors declare no conflict of interest.

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2.7 Supplementary Material

Table S1. Crystal data and structure refinement for 9a (s1_a).

Identification code	s1_a
Empirical formula	C16.50 H15 N4.50 O3.50
Formula weight	332.33
Temperature	298(2) K
Wavelength	0.71073 A
Crystal system, space group	Monoclinic, Pc
Unit cell dimensions	a = 13.856(3) A alpha = 90 deg. b = 4.9842(9) A beta = 100.651(6) deg. c = 23.836(5) A gamma = 90 deg.
Volume	1617.8(5) A^3
Z, Calculated density	4, 1.364 Mg/m^3
Absorption coefficient	0.099 mm^-1
F(000)	694
Crystal size	0.220 x 0.080 x 0.010 mm
Theta range for data collection	2.494 to 26.435 deg.
Limiting indices	-17<=h<=17, -6<=k<=5, -29<=l<=29
Reflections collected / unique	24669 / 6617 [R(int) = 0.2463]
Completeness to theta = 25.242	99.8 %
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	6617 / 380 / 448
Goodness-of-fit on F^2	1.025
Final R indices [I>2sigma(I)]	R1 = 0.1434, wR2 = 0.3046
R indices (all data)	R1 = 0.2820, wR2 = 0.3900
Absolute structure parameter	-1.0(10)
Extinction coefficient	n/a
Largest diff. peak and hole	0.496 and -0.345 e.A^-3

Table 2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters (A^2 x 10^3) for s1_a.
U(eq) is defined as one third of the trace of the orthogonalized Uij tensor.

	x	y	z	U(eq)
O(1B)	5468(8)	8450(20)	3932(5)	52(3)
O(1A)	4950(8)	5270(20)	5243(4)	46(3)
O(2B)	6562(8)	10350(20)	5795(4)	46(3)
O(2A)	6144(9)	6780(20)	7102(5)	61(3)
O(3B)	9638(15)	22510(40)	2351(8)	135(8)
C(14A)	800(20)	-7160(60)	5075(13)	126(9)
N(1A)	5499(8)	5920(20)	6184(5)	36(3)
N(1B)	6040(9)	9510(30)	4855(5)	39(3)
N(2A)	2365(12)	-3250(30)	6357(7)	62(4)
N(2B)	8630(10)	17750(30)	3685(6)	55(4)
N(3A)	1869(11)	-4470(30)	5927(7)	64(4)
N(3B)	8518(11)	18320(30)	3161(6)	60(4)
N(4B)	9163(12)	20140(30)	3043(7)	68(4)
N(4A)	1231(11)	-6300(30)	6071(8)	70(4)
C(1B)	6598(10)	10830(30)	5279(7)	32(3)
C(1A)	4922(12)	4680(30)	5724(7)	41(4)
C(2A)	4284(11)	2500(30)	5900(6)	36(3)
C(2B)	7239(10)	12980(30)	5140(6)	33(3)
C(3B)	7265(9)	13470(30)	4553(6)	26(3)
C(3A)	4261(10)	2050(30)	6477(6)	31(3)
C(4B)	6674(10)	11940(20)	4123(6)	29(3)
C(4A)	4947(11)	3420(30)	6924(6)	36(3)
C(5B)	6021(11)	9890(30)	4284(6)	34(3)
C(5A)	5598(11)	5530(30)	6762(6)	35(3)
C(6B)	7821(11)	14450(30)	5552(7)	43(4)
C(6A)	3670(13)	1200(30)	5477(8)	52(4)
C(7B)	8428(12)	16430(30)	5406(7)	43(4)
C(7A)	3024(12)	-730(30)	5625(7)	46(4)
C(8A)	2995(11)	-1340(30)	6193(7)	39(3)
C(8B)	8504(11)	16920(30)	4851(7)	39(3)
C(9B)	7892(10)	15440(30)	4401(7)	32(3)
C(9A)	3628(10)	20(30)	6629(6)	31(3)
C(10B)	7930(11)	15840(30)	3821(7)	37(3)
C(10A)	3651(13)	-470(30)	7212(7)	49(4)
C(11A)	4275(13)	930(30)	7634(7)	50(4)
C(11B)	7321(12)	14310(30)	3406(7)	45(4)
C(12B)	6694(12)	12370(30)	3569(7)	42(4)
C(12A)	4921(12)	2800(30)	7481(7)	46(4)
C(13B)	9075(16)	20710(40)	2451(9)	75(5)
C(13A)	687(16)	-7620(50)	5664(12)	85(6)
O(3A)	159(14)	-9530(40)	5720(9)	135(7)
C(14B)	8340(20)	19510(60)	2070(11)	120(10)
C(15A)	1140(17)	-6760(40)	6688(10)	90(7)
C(15B)	9911(18)	21390(50)	3492(10)	100(8)
C(3S)	3741(19)	3800(50)	3790(12)	110(8)
N(1S)	2870(15)	5630(40)	3741(9)	90(5)

O(1S)	2165(18)	8550(50)	4178(10)	160(9)
C(2S)	2850(30)	6850(70)	4190(13)	135(11)
C(1S)	2210(30)	5480(80)	3240(15)	169(14)

Table 3. Bond lengths [A] and angles [27] for s1_a.

O(1B)-C(5B)	1.252(17)
O(1A)-C(1A)	1.190(17)
O(2B)-C(1B)	1.263(17)
O(2A)-C(5A)	1.180(17)
O(3B)-C(13B)	1.24(2)
C(14A)-C(13A)	1.46(4)
N(1A)-C(1A)	1.378(19)
N(1A)-C(5A)	1.373(18)
N(1B)-C(1B)	1.326(19)
N(1B)-C(5B)	1.370(18)
N(2A)-N(3A)	1.277(19)
N(2A)-C(8A)	1.40(2)
N(2B)-N(3B)	1.263(18)
N(2B)-C(10B)	1.437(19)
N(3A)-N(4A)	1.36(2)
N(3B)-N(4B)	1.34(2)
N(4B)-C(13B)	1.42(2)
N(4B)-C(15B)	1.48(3)
N(4A)-C(13A)	1.29(3)
N(4A)-C(15A)	1.52(3)
C(1B)-C(2B)	1.47(2)
C(1A)-C(2A)	1.51(2)
C(2A)-C(6A)	1.36(2)
C(2A)-C(3A)	1.40(2)
C(2B)-C(6B)	1.36(2)
C(2B)-C(3B)	1.427(19)
C(3B)-C(9B)	1.404(19)
C(3B)-C(4B)	1.411(18)
C(3A)-C(9A)	1.43(2)
C(3A)-C(4A)	1.460(19)
C(4B)-C(12B)	1.34(2)
C(4B)-C(5B)	1.462(19)
C(4A)-C(12A)	1.37(2)
C(4A)-C(5A)	1.48(2)
C(6B)-C(7B)	1.38(2)
C(6A)-C(7A)	1.40(2)
C(7B)-C(8B)	1.37(2)
C(7A)-C(8A)	1.39(2)
C(8A)-C(9A)	1.40(2)
C(8B)-C(9B)	1.44(2)
C(9B)-C(10B)	1.41(2)
C(9A)-C(10A)	1.41(2)
C(10B)-C(11B)	1.40(2)
C(10A)-C(11A)	1.39(2)
C(11A)-C(12A)	1.39(2)
C(11B)-C(12B)	1.40(2)
C(13B)-C(14B)	1.37(3)
C(13A)-O(3A)	1.22(2)
C(3S)-N(1S)	1.50(3)

N(1S)-C(2S)	1.24(3)
N(1S)-C(1S)	1.36(3)
O(1S)-C(2S)	1.27(3)

C(1A)-N(1A)-C(5A)	132.1(13)
C(1B)-N(1B)-C(5B)	126.3(13)
N(3A)-N(2A)-C(8A)	111.7(15)
N(3B)-N(2B)-C(10B)	114.1(15)
N(2A)-N(3A)-N(4A)	113.3(16)
N(2B)-N(3B)-N(4B)	113.3(16)
N(3B)-N(4B)-C(13B)	114.0(18)
N(3B)-N(4B)-C(15B)	122.6(16)
C(13B)-N(4B)-C(15B)	123.4(17)
C(13A)-N(4A)-N(3A)	117.9(19)
C(13A)-N(4A)-C(15A)	120.7(17)
N(3A)-N(4A)-C(15A)	121.4(15)
O(2B)-C(1B)-N(1B)	121.8(13)
O(2B)-C(1B)-C(2B)	119.5(14)
N(1B)-C(1B)-C(2B)	118.6(14)
O(1A)-C(1A)-N(1A)	122.5(15)
O(1A)-C(1A)-C(2A)	124.8(15)
N(1A)-C(1A)-C(2A)	112.7(14)
C(6A)-C(2A)-C(3A)	121.8(14)
C(6A)-C(2A)-C(1A)	117.2(15)
C(3A)-C(2A)-C(1A)	120.7(14)
C(6B)-C(2B)-C(3B)	119.7(13)
C(6B)-C(2B)-C(1B)	122.1(14)
C(3B)-C(2B)-C(1B)	118.2(13)
C(9B)-C(3B)-C(4B)	119.6(13)
C(9B)-C(3B)-C(2B)	120.1(13)
C(4B)-C(3B)-C(2B)	120.2(12)
C(2A)-C(3A)-C(9A)	119.3(13)
C(2A)-C(3A)-C(4A)	120.7(13)
C(9A)-C(3A)-C(4A)	119.7(13)
C(12B)-C(4B)-C(3B)	120.9(13)
C(12B)-C(4B)-C(5B)	119.7(13)
C(3B)-C(4B)-C(5B)	119.4(13)
C(12A)-C(4A)-C(3A)	118.0(14)
C(12A)-C(4A)-C(5A)	122.7(14)
C(3A)-C(4A)-C(5A)	119.2(13)
O(1B)-C(5B)-N(1B)	119.0(13)
O(1B)-C(5B)-C(4B)	123.8(14)
N(1B)-C(5B)-C(4B)	117.2(12)
O(2A)-C(5A)-N(1A)	123.2(15)
O(2A)-C(5A)-C(4A)	122.7(15)
N(1A)-C(5A)-C(4A)	114.1(13)
C(2B)-C(6B)-C(7B)	120.6(15)
C(2A)-C(6A)-C(7A)	118.9(16)
C(8B)-C(7B)-C(6B)	121.9(15)
C(8A)-C(7A)-C(6A)	121.8(16)
C(7A)-C(8A)-C(9A)	119.3(14)
C(7A)-C(8A)-N(2A)	123.4(15)
C(9A)-C(8A)-N(2A)	117.3(15)
C(7B)-C(8B)-C(9B)	119.3(14)
C(10B)-C(9B)-C(3B)	119.3(13)
C(10B)-C(9B)-C(8B)	122.4(14)
C(3B)-C(9B)-C(8B)	118.2(14)

C(8A)-C(9A)-C(10A)	123.1(15)
C(8A)-C(9A)-C(3A)	118.9(14)
C(10A)-C(9A)-C(3A)	118.0(14)
C(9B)-C(10B)-C(11B)	119.3(14)
C(9B)-C(10B)-N(2B)	117.7(14)
C(11B)-C(10B)-N(2B)	123.0(14)
C(11A)-C(10A)-C(9A)	121.8(16)
C(12A)-C(11A)-C(10A)	119.6(16)
C(10B)-C(11B)-C(12B)	120.3(16)
C(4B)-C(12B)-C(11B)	120.6(16)
C(11A)-C(12A)-C(4A)	122.8(15)
O(3B)-C(13B)-C(14B)	127(2)
O(3B)-C(13B)-N(4B)	113(2)
C(14B)-C(13B)-N(4B)	118.9(19)
O(3A)-C(13A)-N(4A)	126(3)
O(3A)-C(13A)-C(14A)	113(2)
N(4A)-C(13A)-C(14A)	120(2)
C(2S)-N(1S)-C(1S)	132(3)
C(2S)-N(1S)-C(3S)	112(3)
C(1S)-N(1S)-C(3S)	115(2)
N(1S)-C(2S)-O(1S)	116(3)

Symmetry transformations used to generate equivalent atoms:

Table 4. Anisotropic displacement parameters (A^2 x 10^3) for s1_a.
The anisotropic displacement factor exponent takes the form:
-2 pi^2 [h^2 a*^2 U11 + ... + 2 h k a* b* U12]

	U11	U22	U33	U23	U13	U12
O(1B)	58(7)	55(7)	38(6)	2(5)	-3(5)	-33(6)
O(1A)	67(7)	41(6)	33(5)	-10(4)	15(5)	-28(5)
O(2B)	70(8)	32(6)	37(5)	0(4)	8(5)	-16(5)
O(2A)	78(8)	56(8)	42(6)	-5(5)	-4(5)	-32(6)
O(3B)	152(15)	152(15)	104(12)	51(10)	34(10)	-60(12)
C(14A)	150(20)	83(18)	141(14)	-12(12)	24(13)	-19(17)
N(1A)	48(7)	23(7)	38(5)	-6(4)	10(5)	-9(5)
N(1B)	42(7)	33(7)	39(5)	7(4)	1(5)	-15(5)
N(2A)	67(8)	42(8)	79(9)	-12(6)	21(7)	-23(6)
N(2B)	55(8)	60(8)	53(7)	10(6)	18(6)	-6(6)
N(3A)	63(8)	41(8)	91(9)	-15(6)	22(7)	-16(6)
N(3B)	72(8)	57(8)	57(7)	14(6)	32(6)	12(6)
N(4B)	73(9)	63(9)	74(8)	23(6)	30(6)	5(7)
N(4A)	60(9)	50(9)	101(10)	-9(7)	17(7)	-22(7)
C(1B)	23(7)	31(7)	40(6)	6(5)	0(5)	0(5)
C(1A)	64(9)	25(7)	36(6)	-14(5)	19(5)	-8(6)
C(2A)	44(7)	25(7)	39(6)	-8(5)	12(5)	-1(5)
C(2B)	39(7)	24(6)	37(6)	-4(4)	8(5)	-3(5)
C(3B)	28(6)	10(6)	38(5)	-1(4)	4(4)	4(4)
C(3A)	36(7)	18(6)	36(5)	0(4)	2(5)	2(5)
C(4B)	38(7)	11(6)	36(6)	-2(4)	7(5)	1(5)
C(4A)	35(7)	34(7)	37(6)	-3(5)	-1(5)	-5(5)

C(5B)	40(7)	27(7)	32(6)	4(5)	-4(5)	-15(6)
C(5A)	43(8)	26(7)	37(6)	-8(5)	6(5)	-2(5)
C(6B)	50(8)	40(8)	37(7)	0(5)	4(6)	-18(6)
C(6A)	67(9)	40(9)	48(8)	-5(6)	7(6)	-17(7)
C(7B)	53(9)	37(8)	35(7)	-2(5)	-1(6)	-18(7)
C(7A)	47(8)	37(8)	56(7)	-11(6)	11(6)	-4(6)
C(8A)	46(8)	20(7)	54(7)	-5(5)	15(5)	-4(5)
C(8B)	48(8)	21(7)	47(6)	1(5)	4(6)	-6(6)
C(9B)	33(7)	17(6)	47(6)	2(4)	6(5)	3(5)
C(9A)	35(7)	18(6)	39(6)	1(4)	9(5)	6(5)
C(10B)	37(7)	25(7)	50(6)	8(5)	10(5)	9(5)
C(10A)	59(10)	47(9)	42(7)	2(5)	13(6)	-12(7)
C(11A)	68(10)	42(9)	40(8)	4(6)	11(7)	-10(7)
C(11B)	52(8)	41(8)	45(8)	8(6)	14(6)	-12(7)
C(12B)	51(9)	36(8)	39(7)	1(5)	7(6)	-5(6)
C(12A)	53(9)	49(9)	36(7)	-5(6)	3(6)	-12(7)
C(13B)	90(12)	76(12)	73(9)	25(7)	53(8)	9(8)
C(13A)	60(11)	56(11)	133(12)	-18(9)	0(9)	-21(8)
O(3A)	122(13)	101(12)	172(16)	-4(10)	5(12)	-63(11)
C(14B)	143(18)	140(20)	83(13)	31(11)	35(11)	-43(16)
C(15A)	88(16)	77(15)	110(12)	-6(9)	31(10)	-42(12)
C(15B)	99(14)	117(18)	87(12)	10(11)	27(10)	-24(13)
C(3S)	109(14)	87(15)	130(20)	-48(13)	10(13)	-18(11)
N(1S)	98(12)	72(12)	103(13)	-29(9)	28(9)	-27(8)
O(1S)	162(18)	153(19)	161(19)	-29(15)	21(15)	42(14)
C(2S)	150(19)	140(20)	107(15)	-43(13)	11(13)	36(17)
C(1S)	140(20)	220(30)	135(17)	-46(18)	-15(15)	0(20)

Table 5. Hydrogen coordinates (x 10^4) and isotropic displacement parameters (A^2 x 10^3) for s1_a.

	x	y	z	U(eq)
H(14A)	1117	-5470	5047	189
H(14B)	164	-7151	4832	189
H(14C)	1190	-8574	4957	189
H(1AA)	5866	7173	6091	43
H(1BA)	5653	8312	4948	46
H(6BA)	7808	14120	5935	52
H(6AA)	3679	1574	5096	62
H(7BA)	8795	17461	5694	52
H(7AA)	2603	-1631	5337	56
H(8BA)	8947	18187	4765	47
H(10A)	3235	-1765	7318	59
H(11A)	4260	617	8017	60
H(11B)	7332	14582	3021	54
H(12A)	6289	11367	3291	51
H(12B)	5356	3661	7769	56
H(14D)	7944	18425	2274	180
H(14E)	8618	18397	1813	180
H(14F)	7933	20860	1856	180
H(15A)	1781	-7028	6915	135
H(15B)	743	-8321	6713	135

H(15C)	838	-5226	6827	135
H(15D)	10255	22762	3324	150
H(15E)	10369	20054	3664	150
H(15F)	9594	22181	3778	150
H(3SA)	3530	2069	3639	165
H(3SB)	4047	3630	4184	165
H(3SC)	4204	4537	3577	165
H(1SA)	2559	5390	2927	254
H(1SB)	1797	7033	3199	254
H(1SC)	1818	3895	3242	254

Connecting Text 1

The work described in the previous chapter conclusively showed the feasibility of a molecule containing PARP targeting arm and a triazene based DNA targeted molecule. As described, the monoalkyl triazene moiety despite being a precursor to the DNA alkylating portion was too unstable to warrant further investigation. Therefore, stabilization by acetylation was required. The full characterization of the resulting compound, ZSM02, unequivocally confirmed its structure. Having successfully demonstrated the feasibility of molecules with PARP and DNA targeted arm with EG22 and successfully stabilized it by synthesizing ZSM02, we undertook a comparative study to determine whether the stabilized form recapitulate the effect of EG22. The approach we chose to study was to compare the rate of stability of EG22 with that of ZSM02 in serum containing medium at 37ºC. In order to compare their potency, we selected a specific cell panel with containing cell lines with BRCA2 mutation in order to assess their PARP targeting potency, and given that PARP plays a significant role in DNA repair in the BER pathway, we thought of interest to determine whether the new class of compound would be efficacious in MGMT positive cells. As mentioned earlier, classical alkylating agent of the triazene class, such as temozolomide, induce O6-methyl guanine and N^7-methyl guanine lesion which is a type of lesion that is often repaired by the MGMT and BER mechanism respectively. We surmised that by blocking the repair of the N^7 lesion, resistance mechanism evoked by MGMT could be bypassed. Therefore, cells with MGMT expression are good models for evaluating the biological effects of such compounds.

CHAPTER 3: A TYPE I COMBI-TARGETING APPROACH FOR THE DESIGN OF MOLECULES WITH ENHANCED POTENCY AGAINST BRCA1/2 MUTANT- AND O6-METHYLGUANINE-DNA METHYLTRANSFERASE (MGMT)- EXPRESSING TUMOUR CELLS

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3.1 Abstract

Background

Mutations of the DNA repair proteins BRCA1/2 are synthetically lethal with the DNA repair enzyme poly(ADP-ribose) polymerase (PARP), which when inhibited, leads to cell death due to the absence of compensatory DNA repair mechanism. The potency of PARP inhibitors has now been clinically proven. However, disappointingly, acquired resistance mediated by the reactivation of wild type BRCA1/2 has been reported. In order to improve their efficacy, trials are ongoing to explore their combinations with temozolomide (TMZ). Here, in order to enhance potency in BRCA1/2-mutant cells, we report on the design of single molecules termed "combi-molecules" capable of not only inhibiting PARP but also damaging DNA like TMZ, which is known to induce a large number of DNA adducts. The majority of these lesions are processed through PARP-dependent base-excision repair machinery. Paradoxically, the least abundant lesion, the O6-methylguanine adduct is the most cytotoxic. Its repair by the O6-methylguanine DNA methyl transferase (MGMT) confers robust resistance to TMZ. Thus, we surmise that a combi-molecule designed to generate the same DNA adducts as TMZ, with an additional ability to block PARP, could induce BRCA1/2 mutant selective potency and a growth inhibitory profile independent of MGMT status.

Methods

The hydrolysis of EG22 and its stabilized form ZSM02 was analyzed by HPLC and fluorescence spectroscopy. Growth inhibitory potency was determined by SRB assay. PARP inhibition was determined by an enzyme assay and DNA damage by the comet assay. Subcellular distribution was visualized by confocal microscopy.

Results

Studies on EG22 showed that: (a) it inflicted anomalously higher levels of DNA damage than TMZ (b) it induced PARP inhibitory potency in the same range as ANI, a known PARP inhibitor (IC50 = 0.10 μM) (c) it showed strong potency in both BRCA1/2 wild type and mutated cells with 6-fold selectivity for the mutants and it was 65–303-fold more potent than TMZ and 4–63-fold than ANI alone and 3–47-fold than their corresponding equimolar combinations and (d) its potency was independent of MGMT expression.

Conclusion

The results in toto suggest that a combi-molecular approach directed at blocking PARP and damaging DNA can lead to single molecules with selective and enhanced potency against BRCA1/2 mutant and with activity independent of MGMT, the major predictive biomarker for resistance to TMZ.

Keywords

Chemoresistance, Temozolomide, MGMT, BRCA1/2 reactivation, PARP inhibitor, Combi-targeting, DNA repair, 1,2,3-methyltriazene

3.2 Background

Over the past decade, a new strategy to target DNA repair deficiency has progressed to clinical trials: synthetic lethality. The concept of synthetic lethality applies to a situation where mutation of gene A or B alone does not affect the viability of a cell. However, mutation of both genes leads to cell death [1, 2, 3, 4]. A typical case of synthetic lethality is that of cells expressing the mutant BRCA1 or 2. Loss of BRCA1/2 functions impair the DNA repair process. On the other hand, the base excision repair protein PARP is critical for compensating for the loss of BRCA1/2 by providing an alternative DNA repair function to the cells. Thus, concomitant loss of function of the BRCA1/2 genes and PARP induces significant genomic instability and this ultimately leads to cell death [1, 2, 4]. This situation is produced by using inhibitors to block PARP function in BRCA1/2 mutant cells. Thus, PARP inhibitors selectively kill tumour cells with disordered expression of BRCA1/2 (mutation or loss) [1, 4]. Olaparib, the first PARP inhibitor approved in the clinic has proven effective in the treatment of ovarian tumours characterized by BRCA1/2 mutations [5, 6, 7] and many other trials are ongoing to demonstrate the potency of other PARP inhibitors in BRCA1/2 tumours [8]. Disappointingly, clinical trials revealed that some patients become resistant to PARP inhibitors and this is believed to be due to genetic reversion that corrects the original BRCA1- or 2-inactivating mutation [9, 10]. Therefore, strategies to augment the potency of the approach in BRCA1/2 mutant cells are urgently needed. Here we surmised that a small molecule capable of not only blocking PARP, but also damaging DNA, would be a more effective agent against BCRA1/2 mutants than a PARP-specific inhibitor. The design of such a type of molecule was based upon a principle developed by our group termed: “the combi-targeting concept”, which, as outlined in Fig. 1, postulates that a small molecule AB kept small enough to be bound to its target T and capable of generating, upon hydrolysis, another inhibitor A of the same

target + another bioactive molecule B (e.g a DNA damaging species), should induce greater potency than its single targeted counterpart. Importantly, we surmised that due to its targeted property, such a type of molecule could also be more potent than combinations of the two agents A (inhibitor) + B (DNA damaging species) or their corresponding analogues with identical mechanisms action [11, 12]. As depicted in Fig. 2, the molecules that requires hydrolytic cleavage to exert its activity is termed: "type I combi-molecules" as opposed to type II combi-molecules that do not require hydrolytic cleavage. Here we design a combi-molecule to inhibit PARP and to release a DNA damaging species (methyldiazonium), the same agents known to be responsible for the cytotoxicity of temozolomide (TMZ) [13, 14] (Fig. 2).

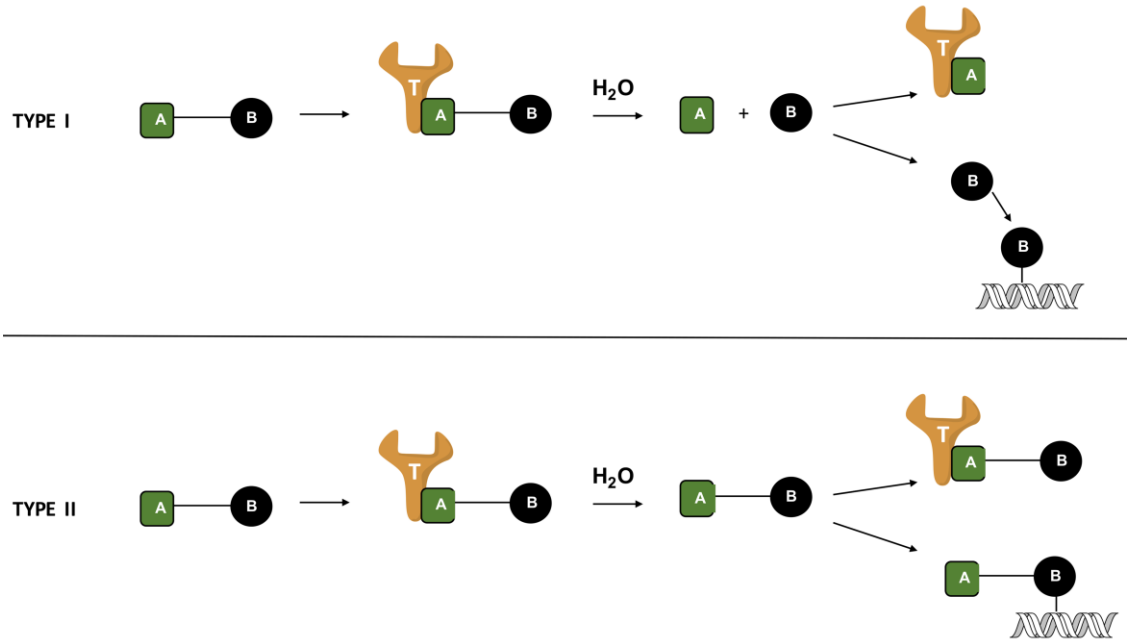

Figure 1. Schematic representation of type I and type II combi-molecules. Upon entering the cells, type I combi-molecules are able to bind and inhibit their target as intact molecules. In the cells, the molecule is hydrolyzed to release an inhibitor 'A' and a DNA damaging agent 'B'. Type II combi-molecules enter the cells and are able to inhibit their target and damage DNA without hydrolysis. Inhibition of the target can synergize with the effects of the DNA damaging species.

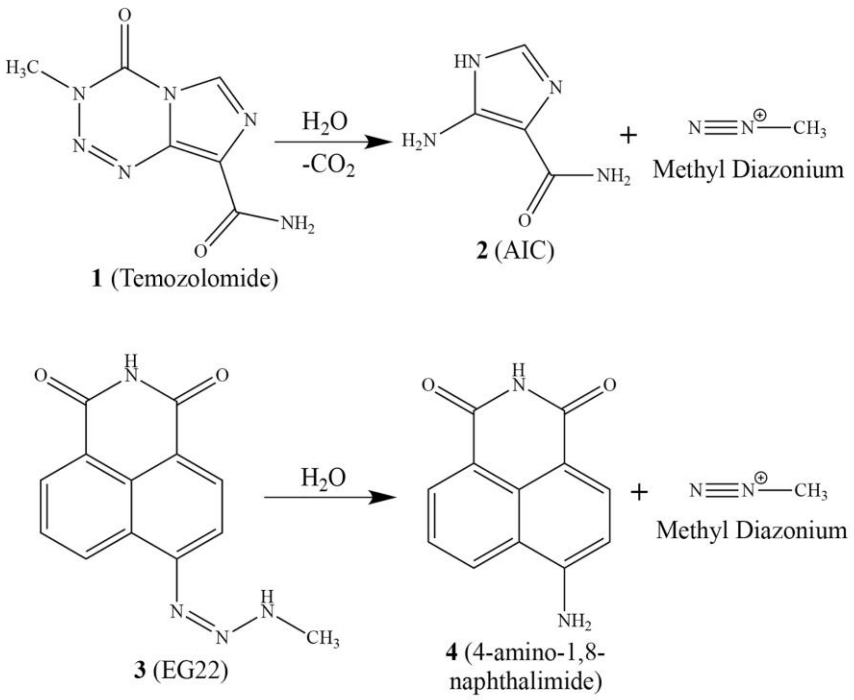

Figure 2: top: hydrolysis of temozolomide to generation inactive AIC and methyl diazonium ion; bottom: hydrolysis of EG22 to regenerate PARP-4-ANI, the naked PARP inhibitor, and the same methyl diazonium species as temozolomide.

On the other hand, because of the sensitivity of BRCA1/2 mutant cells to DNA damaging agents, the most studied combinations designed to enhance the potency of PARP inhibitors involve alkylating agents like TMZ, a second generation alkyltriazene that is used in the treatment of glioblastoma and melanoma [15, 16, 17]. The hydrolysis of TMZ under physiological conditions leads to 5-aminoimidazole-4-carboxamide (AIC) and a methyldiazonium ion (Fig. 2) that reacts with DNA to create N3-methyladenine, N7-methylguanine, N7-methyladenine and O6-methylguanine adducts [18]. The clinical potency of TMZ is significantly affected by the expression O6-methylguanine DNA methyl transferase (MGMT) [14, 19], a DNA repair enzyme that removes the methyl group from guanine by transferring it to its own cystein residue [14, 20].

The other types of lesions induced by TMZ (e.g. N7-methylguanine and N3-methyladenine) are processed by the base excision repair machinery, in which PARP plays a central role. It has already been shown that in MGMT-proficient cells, PARP inhibition sensitized cells to TMZ [21, 22, 23] and this was believed to be due to the cytotoxic effects of unrepaired alkylated bases other than O6-methylguanine. Accordingly, given that the mechanism of cell-killing by the designed combi-molecule in BRCA1/2 depends on PARP inhibition, we also sought to determine whether the MGMT status of the cells would influence the potency of these dual PARP-DNA targeting combi-molecules.

To achieve synthetic lethality–directed combi-molecules, we exploited the chemistry of open-chain and cyclic 1,2,3-triazenes, which has led to the synthesis of the potent clinical alkylating agent TMZ. The hydrolysis of both open-chain or cyclic triazene ultimately leads to the formation of an aromatic amine and a DNA alkylating species [24]. Thus, we designed EG22 to contain a hydrolabile 1,2,3-triazene link that masks a PARP inhibitor, 4-amino-1,8-naphthalimide (ANI) and a methyldiazonium species (Fig. 2). Here we report on the synthesis and the dual targeting properties of EG22, the first open-chain and dual targeted PARP-DNA combi-molecule ever synthesized. Furthermore, since the hydrolysis of EG22 was rather fast under physiological conditions, we also report herein the synthesis and growth inhibitory profile of its acetylated form designed to delay its hydrolysis, thereby stabilizing it under physiological conditions.

3.3 Material and methods

3.3.1 Chemicals and reagents

ANI was purchased from AstaTech Inc. All the chemical reagents and solvents were purchased from Sigma Aldrich Canada.

3.3.2 Chemistry

6-(3-Methyltriaz-1-en-1-yl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (3):

EG22 (**3**) was synthesized as described in Fig. 3. The synthesis of its ^{15}N and ^{13}C–labeled form for purpose of characterization was reported elsewhere [25]. Briefly, 4-amino-1,8-naphthalimide (ANI, **4**) (50.0 mg, 1 eq, 0.236 mmol) was dissolved in concentrated trifluoroacetic acid (5 mL) and the resulting solution cooled to -5 °C for 15 min. An aqueous solution (1 mL) of sodium nitrite (32.5 mg, 2 eq, 0.472 mmol) was subsequently added dropwise and the solution kept at −5 °C for 15 min, thereafter, methylamine (40% *v*/v) (0.122 mL, 6 eq, 1.41 mmol) was added dropwise. The solution was subsequently neutralized with a saturated solution of sodium bicarbonate and the precipitate that formed collected and dried overnight in vacuo to give **2** as a brown powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.59 (s, 1H, NH), 11.42 (q, 1H, J = 4.0 Hz, $NHCH_3$), 8.98 (dd, 1H, J = 8.5 Hz, 1.3 Hz, ArH), 8.47 (dd, 1H, J = 7.2 Hz, 1.2 Hz, ArH), 8.40 (d, 1H, J = 8.1 Hz, ArH), 7.84 (t, 1H, J = 7.9 Hz, ArH), 7.69 (d, 1H, J = 8.1 Hz, ArH), 3.26 (d, 3H, J = 3.9 Hz, CH_3NH). ESI m/z 253.0732 (MH^-).

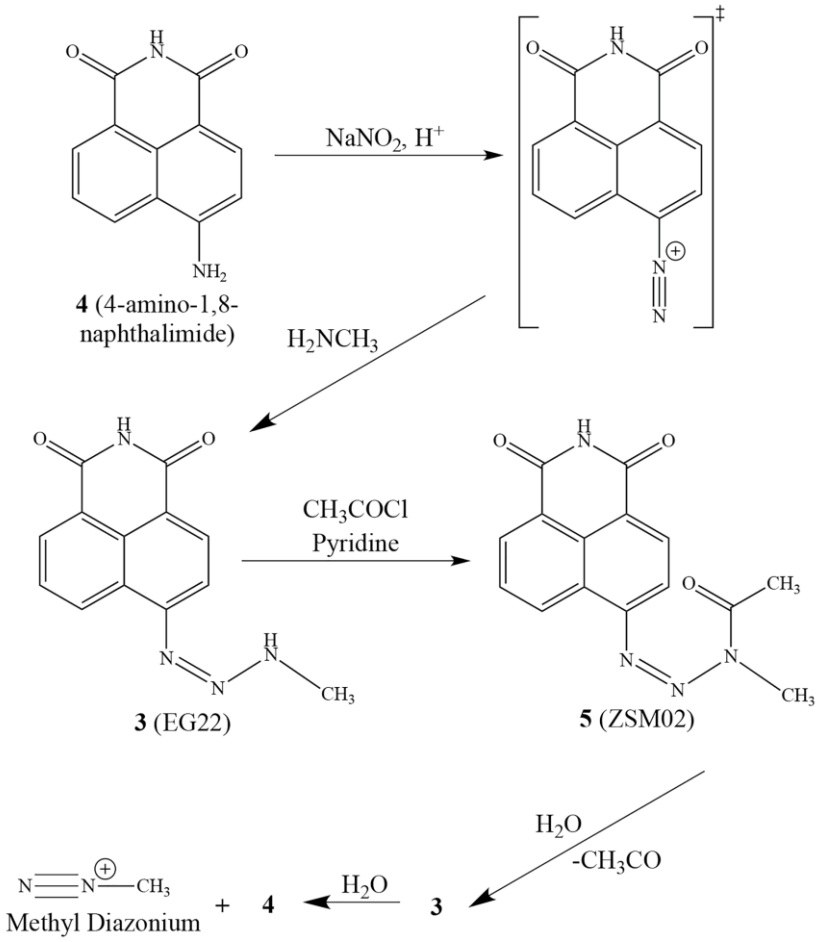

Figure 3: Synthesis of ZSM02 from the acetylation of EG22 and its hydrolysis under physiological conditions

6-(3-Acetyl-3-methyltriaz-1-en-1-yl)-1H-benzo[de]isoquinoline-1,3(2H)-dione (5):

The acetylated compound ZSM02 (**3**) was synthesized as depicted in Fig. 3 and methods for the preparation of its isotopically labeled form for purpose of characterization was reported elsewhere [25]. Briefly, anhydrous pyridine (3 mL) was flash frozen in liquid nitrogen. Acetic anhydride (0.186 mL, 10 eq, 1.97 mmol) was introduced all at once thereafter. The triazene (**2**) in Fig. 3 (50.0 mg, 1 eq, 0.197 mmol) was added as a powder. The reaction was allowed to reach a

temperature of -5 °C for 30 min and then reached room temperature slowly for 2 h. Once the reaction was complete, the pyridine was removed using toluene to create an azeotrope and the resulting solid collected, dried and purified by preparative HPLC (acetonitrile/water: 50/50). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 11.83 (s, 1H, NH), 8.96 (dd, 1H, J = 8.5 Hz, 1.2 Hz, ArH), 8.54 (dd, 1H, J = 7.3 Hz, 1.2 Hz, ArH), 8.51 (d, 1H, J = 7.9 Hz, ArH), 7.96 (t, 1H, J = 7.9 Hz, ArH), 7.94 (d, 1H, J = 8.0 Hz, ArH), 3.54 (s, 3H, CH_3N), 2.60 (s, 3H, CH_3CO). ^{13}C NMR (400 MHz, DMSO-d_6) δ ppm 172.96, 163.71, 148.35, (2C) 130.56, 130.53, 129.78, 129.69, 127.72, 127.57, 122.74, 122.18, 114.33, 28.17 and 22.04. ESI m/z 297 (MH^-).

3.3.3 Cell culture

VC8, VC8-BRCA, and V79 Chinese Hamster Lung cells were generously provided by Dr. Bernd Kaina (Institute of Toxicology, Mainz, Germany). T98 glioblastoma cell lines were kindly given by Dr. Siham Sabri (the Research Institute of the McGill University Health Centre, Montreal, Canada). A549 (ATCC® CCL-185™), DU145 (ATCC® HTB-85™), and A427 (ATCC® HTB-53™), was purchased from ATCC. A427-MGMT was obtained by stable transfection of A427 with MGMT viral vector in our lab [26]. All cell lines were maintained in in DMEM media from Wisent Bio Products. Media preparation was supplemented with 10% Fetal Bovine Serum (FBS), 12 mL HEPES, 5 mL L-glutamine, 500 µL of gentamicin sulfate, 250 µL of fungisome, and 170 µL of ciprofloxacin. All the bio-products used in the preparation of the media were purchased from Wisent Inc. The cells were grown in Thermo Scientific™ BioLite Cell Culture Treated Flasks cell cultured treated polystyrene flasks, which are placed in an incubator with a stable temperature of 37 °C and CO_2 level of 5%. The media of each flask was changed when necessary and cell passaging was performed at 85 and 95% confluence.

3.3.4 In Vitro Growth Inhibition Assay

Growth inhibitory potency was evaluated using the SRB assay [27]. Briefly, cells were plated in 96-well in triplicate and treated with drugs (0.078 μM to 100 μM) 24 h after seeding. Following drug treatment, the cells were fixed using 50 μl of cold TCA (50%) for 1 h at 4 °C, washed five times with tap water, and stained for 30 min at room temperature with SRB (0.4%) in acetic acid (0.5%). The plates were subsequently rinsed five times with acetic acid (1%) and allowed to air dry. The resulting purple residue was dissolved in Tris base (200 μl, 10 mM), and optical densities read on a ELx808 BioTek microplate reader. IC_{50} values were determined using the GraphPad Prism software.

3.3.5 In Vitro PARP assay

The Trevigen HT Universal Colorimetric PARP assay kit with histone-coated strip well was used as per protocol provided by the vendor. Briefly, 50 μl per well of 1X PARP buffer was added to the strip well to rehydrate the histones and the plate was subsequently incubated at room temperature for 30 min. The solution was aspirated and replaced with a dose range of EG22 or ANI (10^{-6} to 100 μM) in triplicate. PARP enzyme (0.5 Unit/well) and a PARP cocktail were added to the appropriate wells containing the inhibitor. A negative control was prepared without PARP to determine the background absorbance, and a positive control without the inhibitor for a 100% reference point. After a 60-min incubation time, the strip wells were washed twice with 1X PBS + 0.1% Triton X-100 (200 μl/well) followed by 2 washes with 1X PBS. Some diluted Strep-HRP was then added after the washing and incubated for 60 min. Finally, a pre-warmed TACS-Sapphire colorimetric substrate was added to each well, in the dark, for 15 min at room temperature, after which the reactions were stopped by adding 0.2 M HCl. Optical densities at 450 nm were recorded on ELx808 Biotek microplate reader. The results were analyzed using

GraphPad Prism software to derive a dose-response curve and the IC_{50}values. The PARP assay was performed twice, in triplicate.

3.3.6 Alkaline comet assay for DNA damage quantification

Cells were plated in 6-well plates (Corning Inc.) at 200,000 cells/well in 2 mL medium/well. They were allowed to attach for 24 h and then treated with a wide range of drug concentrations (0, 6.25, 12.5, 25, 50. and 100 μM). The cells were exposed to the drugs (EG22, TMZ, and ANI + TMZ) for 2 h, harvested with trypsin EDTA, centrifuged and subsequently resuspended twice in PBS. The cell suspensions were mixed in low melting point agarose (0.75% in PBS) at >37 °C in a 1:10 dilution. The gels were cast on GelBond Film (Lonza, Switzerland) using gel casting chambers and allowed to solidify before being placed into a lysis buffer [2.5 M NaCl, 0.1 M tetrasodium EDTA, 10 mM Tris-base, 1% (*w*/*v*) N-lauryl sarcosine, 10% (*v*/v) DMSO, and 1% (*v*/v) triton X-100, pH 10.0]. They were subsequently kept at 4 °C overnight, rinsed with distilled water and immersed in a second lysis buffer [2.5 M NaCl, 0.1 M tetrasodium EDTA, and 10 mM Tris-base, pH 10.0] for 60 min at 37 °C after which they were gently rinsed with distilled water, incubated in alkaline electrophoresis buffer [0.3 M sodium hydroxide, 0.1 M tetrasodium EDTA, 7 mM 8-hydroxyquinoline, 0.2% (*v*/v) DMSO, pH 13.0] for 30 min at room temperature, and electrophoresed at 20 V, 400 mA for 20 min. Thereafter, they were gently rinsed with distilled water and placed in 10 M ammonium acetate for 30 min. Finally, the gels were soaked in 100% ethanol for 2 h, dried overnight, and stained with SYBR Gold (1:10,000 dilution of stock) (Molecular Probes, Eugene, OR) for 20 to 60 min. Comets were visualized at 400X magnification and DNA damage was quantified using Comet Assay IV software to calculate tail moments.

3.3.7 Live Cell Confocal Microscopy

The V79 cell line was plated at 60–70% confluence in petri dishes, allowed to adhere overnight, and treated with 25 μM EG22, ANI and ZSM02 for 2 h. After treatment, cells were washed with PBS, a drop of DAPI (NucBlue® Live ReadyProbes® Reagent, ThermoFicher Scientific) was added and 3-D images were taken with the appropriate filter. Only the image corresponding to the equatorial plan of the cells was used to visualize cellular distribution.

3.3.8 Kinetics of the hydrolysis of EG22 and ZSM02

The rate of hydrolysis of EG22 under physiological conditions was measured using a Spectra Max Gemini plate reader. The compound was dissolved in a minimum volume of DMSO and diluted with DMEM supplemented with 10% FBS. The solution was incubated in a 96-well plate at 37 °C in the ELISA reader and readings were taken over a period of 1 h. The excitation wavelength was 444 nm and emission 538 nm [27, 28]. The half-life was estimated from the formation of ANI using first order kinetics, one-phase exponential decay. (GraphPad software, Inc., San Diego, CA).

The stability of ZSM02 under physiological conditions was studied by HPLC, Agilent technologies. The compound was dissolved in minimum volume of DMSO and diluted with DMEM supplemented with 10% FBS. The solution was incubated at 37 °C and 100uL was collected at various time points: 0 min, 2 h, 4 h, 6 h, 12 h, 24 h, 48 h, 60 h, and 72 h. The drug was extracted from the media with 100 μL of methanol, centrifuged at 13,000 rpm for 1 min, after which the supernatant was collected and evaporated. The extraction was performed three times and after being dried in vacuo overnight, the resulting extract was reconstituted in 100 μL of methanol for HPLC analysis using a 150 mm × 4.6 mm ODS-3 (C18 column, 5 μm pore size) (Canadian Life Science). The absorbance was detected at 460 nm and the half-life estimated from the formation of ANI using first order kinetics analysis.

3.3.9 Statistical analysis

Data were analyzed with Student's two-tailed t-test or one-way ANOVA, using GraphPad Prism 5.0 software (GraphPad Prism, San Diego, CA). $P < 0.05$ was defined as statistically significant.

3.4 Results

3.4.1 Chemistry

The proof-of-principle of the approach was first achieved by the synthesis of EG22, which proceeded according to Fig. 3. Using a known PARP inhibitor containing an aromatic amino group, 4-amino-1,8-naphtalimide (ANI), we designed EG22 to carry a 1,2,3-triazene moiety, which upon hydrolysis would regenerate ANI intact, while concomitantly releasing the DNA alkylating methyl diazonium ion, the latter species being identical to the one released by the clinical drug TMZ [13, 14]. EG22 was synthesized by diazotizing the amino group with sodium nitrite and adding methylamine under basic conditions. It was used as our first prototype to study the dual targeting of PARP and DNA with a single molecule in tumour cells. While EG22 was a useful probe for the combi-targeting of PARP and DNA, it was hydrolyzed too rapidly under physiological conditions (Fig. 4). Thus, we sought to delay its hydrolysis by acetylating its N3 nitrogen in pyridine cooled with liquid nitrogen prior. The unequivocal characterization of the resulting compound (ZSM02) by isotope labeling and heteronuclear NMR (^{13}C, ^{15}N) are reported elsewhere [25]. The structure was also confirmed by mass spectrometry, with a molecular ion at 296, consistent with its molecular weight.

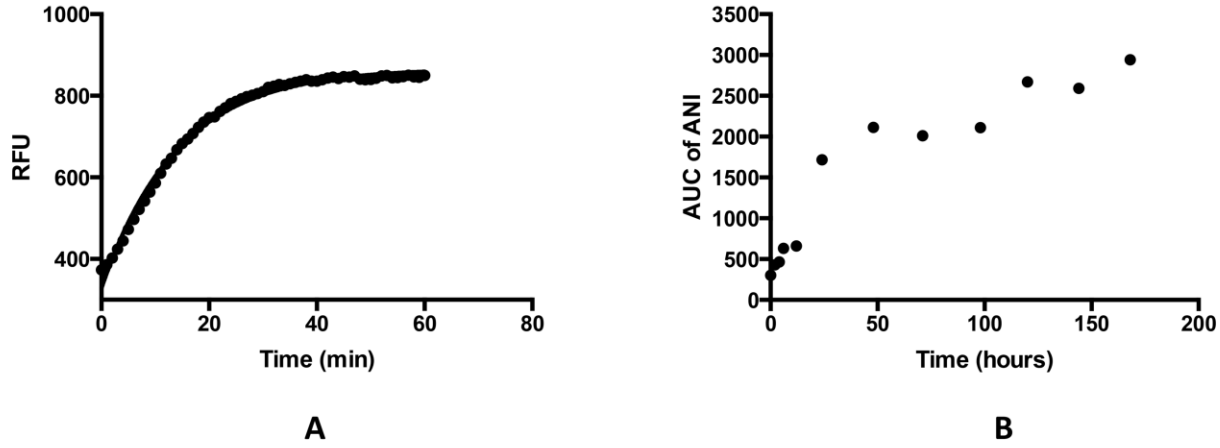

Figure 4. Hydrolysis of EG22 and ZSM02 in serum-containing medium. **a** The solution of EG22 was kept at 37 °C in the fluorescence reader and an intensity curve automatically generated at the maximum emission wavelength corresponding to ANI (538 nm) ($t_{1/2}$ = 9.76 min); **b** ZSM02 was dissolved in a minimum volume of DMSO and diluted with DMEM supplemented with 10% FBS. The solution was incubated at 37 °C and 100 µL aliquots were analyzed by HPLC as described in Material and Methods. ZSM02 was slowly converted to ANI with $t_{1/2}$ greater than 24 h.

3.4.2 Dual PARP-DNA targeting properties of EG22

In order to verify whether EG22 could modulate its two targets (i.e. PARP and DNA), a PARP and a comet assay were performed to determine its ability to inhibit the function of PARP and to induce DNA damage, respectively. The known PARP inhibitor ANI could induce PARP inhibition in our assay with an IC_{50} of 0.11 µM, which is consistent with literature value (IC_{50}: 0.16 µM) [29]. Our results showed that under the conditions of the assay, EG22 could induce a dose-dependent inhibition of PARP with an IC_{50} = 0.10 µM, which was in the same range as that of ANI (Fig. 5).

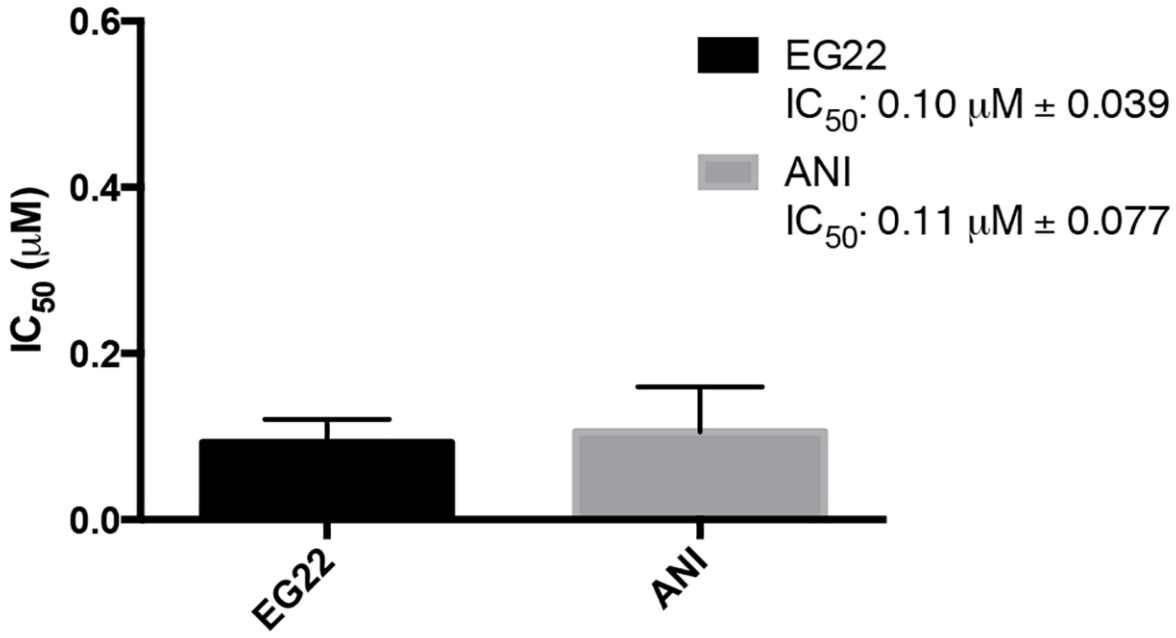

Figure 5. Enzymatic assay test for PARP inhibition by measuring the incorporation of biotinylated poly(ADP-ribose) onto histone proteins by the PARP enzyme. This allowed the determination of the IC_{50} value of our new PARP-DNA combi-molecule, EG22. The Trevigen HT Universal Colorimetric PARP assay kit with histone-coated strip wells was used and dose response curves analyzed with the Graphpad Prism software. The results showed that EG22 was capable of inducing a dose-dependent inhibition of PARP with IC_{50} = 0.1 μM.

In order to determine whether EG22, a monomethyltriazene that like TMZ ultimately releases the methyl diazonium species, could induce DNA damage in tumour cells, we used the microelectrophoresis comet assay. The results showed that EG22 induced significantly higher levels of DNA damage than TMZ in a Chinese Hamster lung cancer BRCA2-mutant VC8 and proficient tumour cells V79 cells (Fig. 6). Interestingly, the levels of DNA damage induced by EG22 were significantly higher than those generated by the clinical drug TMZ. Since TMZ is the

prodrug of the same alkylating species as EG22, the levels of the induced DNA damage appear anomalously high.

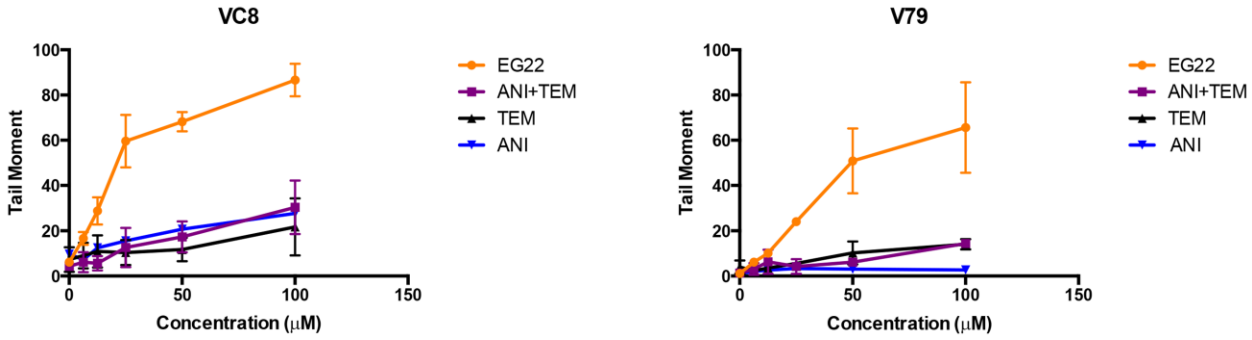

Figure 6. Anomalously strong DNA damaging potential of EG22 after a 2 h drug treatment as compared with temozolomide (TMZ) and ANI + TMZ in the Chinese lung cancer cell lines VC8 and V79. The cells were exposed to the drugs (EG22, TMZ, ANI and ANI + TMZ) for 2 h, and subsequently harvested with trypsin EDTA, centrifuged and resuspended twice in PBS. Comet assay was performed as per Materials and Methods. Comets were visualized at 400X magnification and DNA damage measured as tail moments using Comet Assay IV software

3.4.3 BRCA1/2 response profile

In order to verify whether the new combi-molecule could target BRCA1/2 mutants, we analyzed the potency of EG22 against the pair of Chinese Hamster lung cancer cell line: with V79, a BRCA1/2-proficient and the other VC8 BRCA1/2-mutant. The results showed that like the naked PARP inhibitor ANI, EG22 selectively killed the mutant forms (Fig. 7). Furthermore, in order to further ascertain the BCRA1/2 mutant selectivity of the approach, growth inhibition studies were performed in an isogenic context with VC8 cells (non-transfected) and VC8-BRCA (transfected with wild type BRCA2 gene). The results showed that EG22 was selectively more potent against the non-transfected VC8 cells (17-fold, $p < 0.001$) (Fig 7).

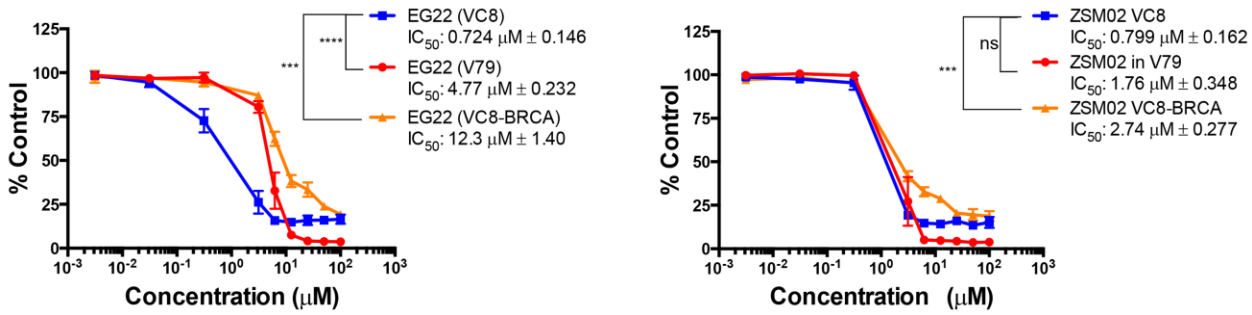

Figure 7. Dose response curve obtained from growth inhibition with EG22 in a panel of Chinese Hamster Lung cancer cell lines. V79 cells express wild type BRCA2.The VC8 cell line expresses mutant BRCA2, and VC8-BRCA is transfected with the wild type BRCA2 gene. EG22 was significantly more potent against the BCRA2 mutant cell line ($p < 0.0001$). ZSM02 did not show significant selectivity for VC8 when compared with V79. However, a 3-fold selectivity ($p < 0.001$) was apparent when compared with its isogenic VC8-BRCA counterpart. *** $p < 0.001$, ****$p < 0.0001$, ns. Not Significant

One of our goals was to verify whether the combi-molecule induced enhanced potency in the BRCA1/2 mutant. To this end, we compared the potency of EG22 with that of ANI, which is deprived of DNA alkylating functions. Importantly, the combi-targeted approach enhanced the potency of ANI in the BRCA2 mutants by 4-fold ($p < 0.001$, Table 1). It also enhanced ANI's potency by 8-fold in the cells transfected with the wild type BRCA2 gene, which is an important advantage under conditions where resistance is associated with restoration of wild type BRCA1/2 [9, 10].

Table 1. Potency of EG22 on BRCA2-proficient and mutant Chinese lung cancer cell lines (IC_{50} μM)

	VC8	VC8-BRCA	V79
BRCA2 status	-	+	+
EG22	0.724 ± 0.146	12.3 ± 1.40	4.77 ± 0.232
ZSM02	0.799 ± 0.162	2.74 ± 0.277	1.76 ± 0.348
ANI	2.99 ± 0.567	>100	>100
TMZ	61.1± 12.3	>800	433.3 ± 55.9
ANI+TMZ	2.30 ± 0.552	83.5 ± 7.45	60.5 ± 10.6

3.4.4 Relationship with MGMT status

To answer the question as to whether MGMT could affect the potency of EG22, we tested its potency in a panel of cells with known MGMT status, including an isogenic pair of human lung cancer cell line, A427 and A427 MGMT cell lines (Table 2). The results showed that MGMT expression did not affect the potency of EG22, indicating, that perhaps its ability to block PARP may enhance the cytotoxicity of DNA adducts other than O6-methylguanine in the cells. Unlike ANI + TMZ or TMZ alone, growth inhibition assays showed consistently strong potency of EG22 throughout the panel of MGMT positive cell lines. Indeed, EG22 was more than 13 to 47-fold more potent than the ANI + TMZ combination ($p < 0.001$), and 100- to 303-fold more potent than TMZ in the panel of cell lines. This shows that EG22 is capable of overcoming resistance to TMZ in the presence of MGMT.

Table 2. Potency of EG22 and ZSM02 on MGMT-proficient and deficient human tumour cells (IC_{50} μM)

	A427	A427 MGMT	A549	T98	DU145
MGMT status	-	+	+	+	+
EG22	3.8 ± 0.12	2.9 ±0.21	1.6 ±0.16	1.7 ± 0.16	6.0 ±0.55
ZSM02	2.9 ± 0.49	3.7 ± 0.23	2.0 ± 0.50	4.3 ± 1.6	4.7 ± 0.11
ANI	>100	>100	>100	>100	>100
TMZ	34 ± 4.0	305 ± 11	337 ± 28	516 ± 72	598 ± 59
ANI+TMZ	5.0 ± 0.82	41 ± 1.1	47 ± 11	80 ± 10	78 ± 4.6

3.4.5 Potency of the combi-molecular approach in comparison with 2-drug combinations

Importantly, the growth inhibitory potency of EG22 was 3-fold greater than that of the combination of ANI + TMZ against VC8 (mutant form), 13-fold in V79 (wild type) and 7-fold in VC8-BRCA, leading to an evidence of the ability of the combi-targeting approach to illustrate the principle underlying "the whole being greater than the sum of the part" (Fig. 8). The marked superiority of EG22 when compared with ANI + TMZ was further confirmed in a panel of established prostate, brain, and lung cancer cell lines (Fig. 9).

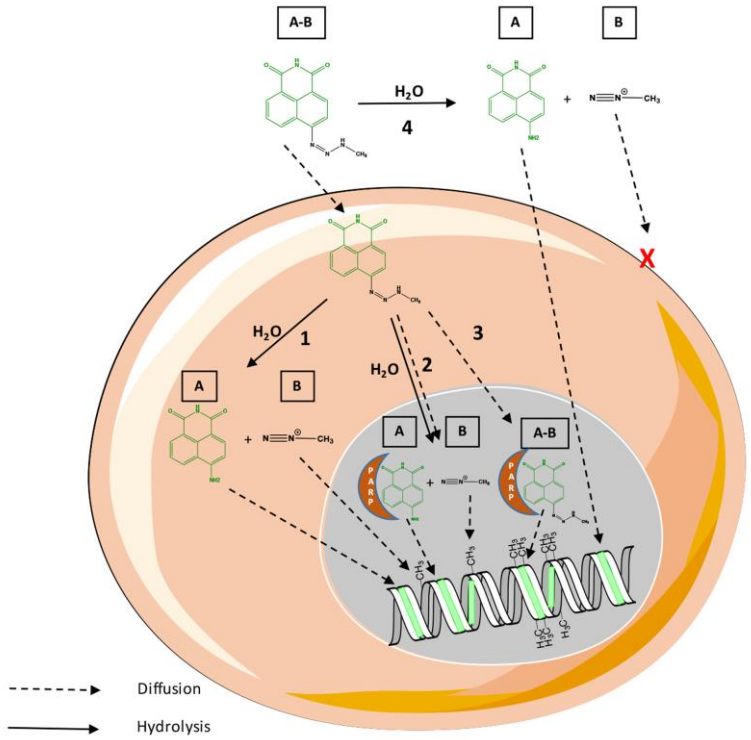

Figure 8. Proposed pathways for the hydrolysis of EG22 and its dual PARP-DNA targeting property. Solid arrows describe hydrolysis and dotted arrows diffusion. EG22 may diffuse in its intact form through the cell membrane to subsequently hydrolyze in the cytoplasm, release ANI and the methyl diazonium species. ANI may then in turn diffuse into the nucleus and either intercalate into the DNA or inhibit PARP. EG22 may also diffuse in its intact form toward the nucleus, intercalate into DNA prior to being converted to ANI and the methyl diazonium species

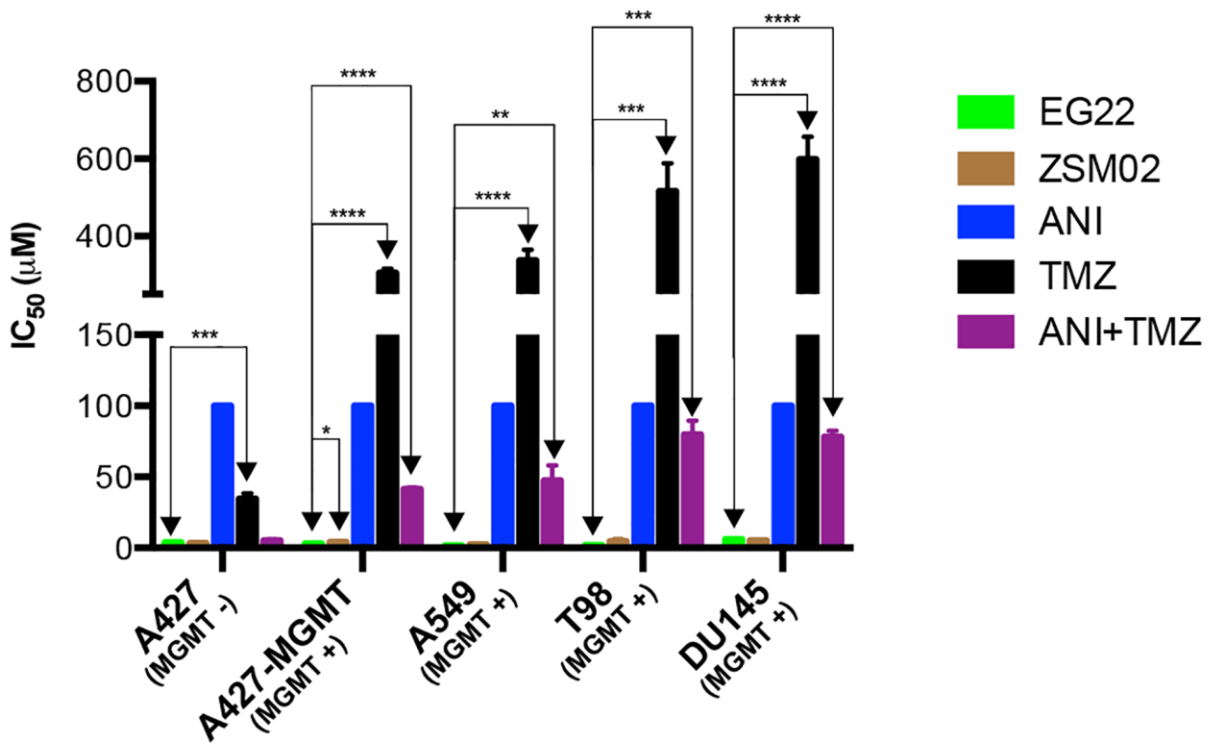

Figure 9. Growth inhibition by EG22 and ZSM02 in a cell panel with varied levels of MGMT. They show similar growth inhibition profile with an increased potency when compared to temozolomide (TMZ), ANI and ANI + TMZ, indicating that their potency is independent of the MGMT status of the cells.

3.4.6 Subcellular localization and mechanism of action

As shown earlier, the hydrolysis of EG22 leads to the release of ANI, an agent that fluoresces in the green and is also known to be able to intercalate into DNA [30]. Thus, its subcellular distribution was analyzed by fluorescence microscopy. Interestingly, as shown in Fig. 10, the green fluorescence was primarily localized in the nucleus, which is consistent with the fact that ANI can intercalate into DNA. This allowed us to propose a mechanism whereby, as depicted in Fig. 8, the intact molecule may be primarily localized in the nucleus where it generates ANI and its alkylating

species in the nucleus, an event that may account for its ability to induce anomalously high levels of DNA lesions when compared with TMZ.

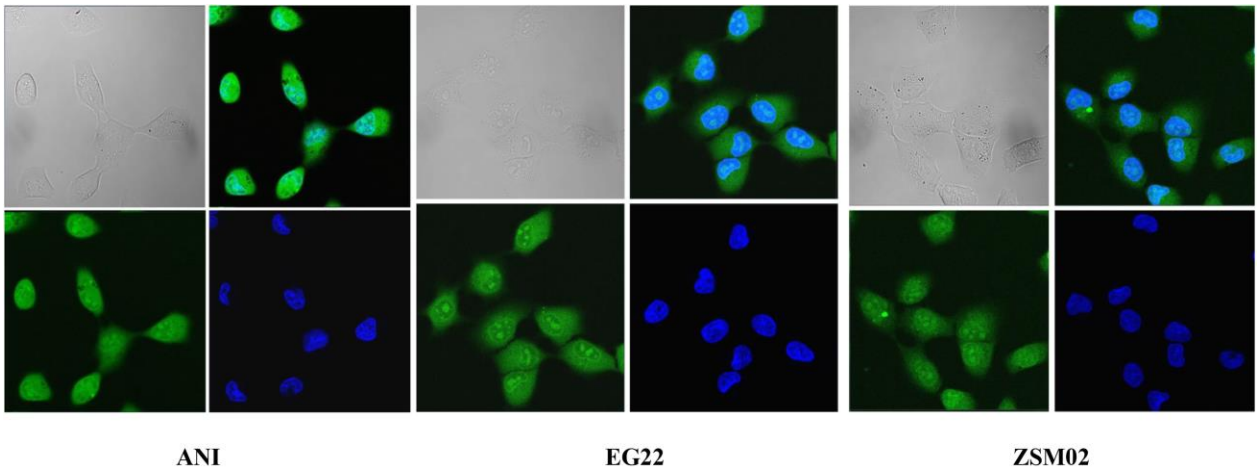

Figure 8. Subcellular distribution of ANI, EG22 and ZSM02 after a 2 h exposure. Following drug treatment, cells were washed with PBS, a drop of DAPI (NucBlue® Live ReadyProbes® Reagent, ThermoFicher Scientific) was added and 3-D images were taken with the appropriate filter. Nuclear localization of the drugs was confirmed by DAPI counterstaining.

3.4.7 Stabilization of EG22 and growth inhibitory profile of the resulting combi-molecule

Although EG22 has been shown to generate anomalously high levels of DNA damage as compared with TMZ, its rate of hydrolysis was considered to be too rapid under physiological conditions ($t_{1/2}$ = 9.76 min) (Fig. 4). Therefore, we sought to stabilize it by acetylating the N3 of the triazene chain. As mentioned earlier, the stable form of EG22, known as ZSM02, has been synthesized and then analyzed by ^{1}H, ^{13}C, ^{15}N NMR and mass spectrometry. Detailed NMR characterization of ZSM02 was reported elsewhere [25]. The potency profile of ZSM02 was studied in comparison with EG22. Although, it did not show selectivity for BRCA1/2 cells when its growth inhibitory potency was compared in the VC8/V79 pair of cell lines, in an isogenic context where VC8 is compared with its BRCA wild type transfectant, ZSM02 showed 3-fold selectivity for the mutant.

Importantly, its potency in a panel of MGMT positive and MGMT negative cell lines paralleled that of EG22 (Fig. 7).

The kinetics of degradation of the stabilized molecule ZSM02 into ANI and methyldiazonium ion was studied and showed a slow release of the active species with a half-life greater than 24 h (Fig. 4) as opposed to the fast decomposition of the prototype molecule EG22. Thus, we have successfully stabilized EG22 by forming ZSM02.

3.5 Discussion

The clinical potency of TMZ is significantly affected by the expression of several DNA repair enzymes, mainly MGMT [19, 31]. Attempts to overcome resistance to TMZ led to several approaches including direct inhibition of MGMT, blockade of abasic sites, PARP inhibition etc. [23, 32, 33, 34, 35, 36, 37, 38]. Alternative targets to sensitize cells to TMZ such as the epidermal growth factor receptor (EGFR) have been investigated by our laboratory [11, 39, 40, 41, 42, 43]. This has led to a novel tumour targeting strategy termed "combi-targeting" according to which molecules designed to block the action of their target and to be hydrolyzed into one inhibitor of the target + another bioactive species should induce strong potency in tumours expressing the target. Combi-molecules designed according to the combi-targeting concept are termed the type I (i.e. they are capable of releasing an inhibitor of EGFR + a DNA damaging species upon hydrolysis) or type II (i.e. they do not require hydrolysis to exhibit their binary EGFR/DNA targeting potency) (Fig. 1) [11, 12, 39, 40, 41, 42, 43, 44, 45, 46, 47]. Here, we sought to apply the type I model to the design of molecules capable of inducing a tandem PARP inhibition and DNA damage. Thus, a molecule (EG22) was synthesized that contained a PARP targeting moiety (ANI)

and a methyl triazene tail designed to be hydrolyzed to a methyl diazonium species targeted to DNA (Fig. 2).

EG22 was not only capable of inducing about 7-fold greater potency against VC8 when compared with its V79 counterpart, but also displayed significant selectivity toward BRCA1/2 deficiency in the isogenic VC8/VC8-BRCA pair of cell lines. Importantly, it was generally more potent than TMZ against both BRCA mutants and wild type cells and when tested against VC8 transfected and its non transfected counterpart, indicating that it is synthetic lethality selective. It is to be noted that its superior potency when compared with BRCA1/2 wild-type and mutated cells is an advantage in tumours that express BRCA1/2 heterogeneously.

A PARP assay was performed to determine the PARP inhibitory potency of EG22 and an alkaline comet assay to demonstrate its DNA damaging properties. The strong PARP inhibitory potency of EG22 is consistent with the type I model of combi-molecules (Fig. 1), which are designed to release an intact inhibitor of the target upon hydrolysis. The rapid conversion of EG22 to ANI in cell culture medium suggests that the latter may be a major contributor to the PARP activity of EG22. Through the alkaline comet assay, the second arm of EG22 shows a dose-dependent DNA damage in both VC8 and V79 after 2 h and the levels of damage were significantly higher than those induced by TMZ and ANI + TMZ. We believe that the anomalously high DNA damage observed may be due to the primary intercalation of EG22 as an intact structure in DNA [27], where it releases its DNA damaging species. This may lead to a localized release of the DNA damaging species and enhancement of DNA damage in the cells. As depicted in Fig. 8, we propose that EG22 can degrade in the extracellular compartment, leading to ANI (path 4), which is capable of diffusing into the cells whereas the methyl diazonium is too unstable to penetrate the cells.

EG22 can also enter the cells as an intact structure (paths 1 and 2) and decompose therein into ANI and the methyl diazonium species that are capable of reaching the nucleus. EG22 may intercalate into the DNA and release both ANI and methyl diazonium in situ (path 3). In the nuclear compartment, both intact EG22 and ANI can bind to and inhibit PARP. The proposed paths for the hydrolysis of EG22 and diffusion of the resulting by-products that appear to concentrate the DNA-targeting and damaging species in the nucleus, are consistent with the observed nuclear localization of the green fluorescence associated with ANI and the anomalously strong DNA damaging potential of EG22.

EG22 is designed to induce the same types of DNA adducts as TMZ, which is inactive against tumours expressing MGMT [14, 20], the sole human enzyme capable of repairing the O6-methylguanine adduct. It should be noted that despite its significant cytotoxicity, O6-methylguanine only accounts for 7% of base adducts induced by TMZ. N7-methylguanine and N3-methyladenine account for 70 and 10% respectively. The latter type of adducts are repaired by the base excision repair machinery [18]. EG22 being designed to induce the same types of lesions as TMZ and able to inflict significantly high levels of DNA damage to the cells while being a potent PARP inhibitor, we sought to determine whether its potency could be superior to that of TMZ in MGMT-expressing cells. Indeed, its 100–300-fold stronger potency in the panel of cells suggests that it is acting by a different mechanism of action when compared with TMZ. Perhaps, tandem blockade of PARP and induction of DNA damage allow to bypass the MGMT-mediated resistance. The levels of potency of EG22 were consistently similar throughout the cell panel whether the cells were BCRA1/2 wild type or mutant and MGMT+ or MGMT-. The ability of a PARP inhibitor to potentiate TMZ in tumour cells has already been reported [23, 35, 36]. However, to our knowledge this is the first report of a small 1,2,3-triazene-containing type I combi-

molecules (MW = 296) capable of behaving like a PARP inhibitor and a DNA alkylating agent and more importantly with growth inhibitory potency stronger than that of a combination of two agents: a PARP inhibitor and a DNA damaging agent of the same structural class. Further attempt to enhance the druggability of the approach led the stabilization of EG22 by acetylating its N3-position to give ZSM02, which slowly released ANI and exhibited strong potency against MGMT cells. It showed less BRCA2 mutant selectivity than EG22, which is perhaps due to its ability to induce sustained release of the DNA damaging species concomitantly with ANI. This mechanism may depress the repair capacity of the wild type cells, thereby reducing the difference in potency when compared with the mutant. Nevertheless, the strong potency of ZSM02 that parallels that of EG22 in the MGMT-expressing cell panel warrants further investigation. Further studies are ongoing to assess its ability to behave as a true masked form of EG22 and to demonstrate its efficacy in vivo.

3.6 Conclusion

In summary, EG22, our combi-molecule targeting PARP and damaging DNA, is the first prototype combining a PARP inhibitor (i.e. ANI) with an N-methyl-1,2,3-triazene as in TMZ and with a MW < 300. It is the first combi-molecule capable of releasing an aromatic amine preferentially localized in the nucleus, as opposed to the perinuclear localization that is typical of aminoquinazolines derived from the hydrolysis of EGFR-targeted combi-molecules reported by our laboratory [47]. Its ability to penetrate the cells and perhaps the nucleus where it may intercalate into DNA leads to an in situ generation of the DNA damaging species. This may account for its ability to generate anomalously high levels of DNA damage. The current work features a new type of DNA damaging agent with enhanced potency against BRCA1/2 mutants and MGMT-proficient tumour cells. Furthermore, its potency against BRCA1/2 wild type

expressing tumours warrants strong activity against tumors in the advanced stages where BRCA1/2 becomes largely heterogeneous. Also, the strong potency of the approach against MGMT-proficient tumour cells indicates that type I agents like EG22 may be developed as a potential alternative to TMZ in advanced tumours characterized by MGMT expression.

List of abbreviations

DNA: Deoxyribonucleic acid

BRCA1/2: Breast Cancer gene 1 or 2

PARP: Poly(ADP-ribose) polymerase

TMZ: Temozolomide

MGMT: O6-Methylguanine Methyltransferase

ANI: 4-amino-1,8-naphthalimide

AIC: 5-aminoimidazole-4-carboxamide

DMEM: Dulbecco's Modified Eagle Medium

TCA: Trichloroacetic Acid

SRB: Sulforhodamine B

PBS: Phosphate-Buffered Saline

FBS: Fetal Bovine Serum

EGFR: Epidermal Growth Factor Receptor

DECLARATIONS

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by CIHR (MOP-130363). Z.S.M. doctoral work was supported by the Fonds Quebecois de Recherche sur la Sante (FQRS) Doctoral Research Award.

Authors' contributions

ZSM participated in the design of the study, carried out the chemical synthesis and purification of ZSM02, molecular and cell studies, and drafted the manuscript. EG carried out the chemical synthesis of EG22 and helped with the references. BJC conceived the study, participated in its design and coordination, and helped draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We wish to thank the McGill NMR Lab and the McGill University Health Center Drug Discovery platform for NMR access.

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Connecting text 2

In the previous chapter, we undertook the biological evaluation of compounds with DNA and PARP arm. Such type of molecules, like PARP inhibitors, were designed to be selective in BRCA1/2 mutant tumours. The results presented previously showed that in BRCA1/2 mutants, the DNA damaging function added in the PARP inhibitor, significantly enhanced the cytotoxicity of the PARP inhibitor. However, despite the success of PARP inhibitors in BRCA1/2 mutants only a small subset of patients can potentially benefit from the approach. Furthermore, despite the potency of the dual PARP-DNA targeting agents, their activity was not biomarker specific. Therefore, we sought to develop a strategy to enhance the selectivity of PARP targeting combi-molecules. In order to expand the use of PARP inhibitors, their targeting to specific biomarkers appears to be a rational strategy.

The epidermal growth factor receptor (EGFR) is one of the most validated cancer target of modern time. It is deregulated in a wide variety of tumours of epithelial origin including: breast, colon, prostate, brain, ovarian, etc. As outlined in our introduction section, several anti-EGFR strategies have been proven to be effective as targeted therapy in the clinical management of solid tumours. Therefore, we initiated a strategy whereby a PARP inhibitor could be target to EGFR-expressing tumours. The design of our strategy coincide with studies that describe high synergy between an EGFR and a PARP inhibitor. The authors suggest that this effect might be due to contextual synthetic lethality between EGFR and PARP which involves the transport of EGFR and PARP transport in the cell. Having established a strong rational for developing EGFR-PARP targeted molecules, we embarqued on a synthetic campaign to achieve the first ever EGFR-PARP targeted molecules. Herein the next chapter, we descried our approach, which consisted of creating molecules in which the PARP moiety is separated from the EGFR one by basic spacer. The nature

of this spacers was based on previous structure activity relationship developed previously in our laboratory. We selected on molecule out of the series which was analyzed for its potency in a wide panel of cell line. Here we described the potency and mechanism of action of the resulting molecules.

CHAPTER 4: DUAL TARGETING OF EGFR AND PARP: A NOVEL SINGLE MOLECULE STRATEGY TO EXPAND THE SELECTIVE POTENCY OF PARP INHIBITION BEYOND BRCA1/2 MUTATION

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4.1 Abstract

Poly(ADP-ribose) polymerase (PARP) inhibitors have emerged as new therapeutic modalities for patients with BRCA1/2 mutations. However, the therapeutic benefit of PARP inhibitors is limited to BRCA1/2 mutations, which accounts for only 5-10% of all hereditary mutations. Despite the remarkable benefit of PARP inhibitors in the clinic, scant strategies exist that seek to expand their benefit beyond the BRCA1/2 mutated tumours. Here we surmised that if PARP inhibitors could be targeted to specific markers in the tumours, their use could be expanded beyond BRCA1/2 mutations. Thus, we engineered olaparib, a clinical PARP inhibitor to target the epidermal growth factor receptor (EGFR), which is overexpressed in a large number of solid tumours. Structural modification of olaparib as a PARP inhibitor warhead and the quinazoline moiety for targeting EGFR led to a series of molecules termed "combi-molecules" with various degrees of dual PARP-EGFR targeting potencies. Studies with the most balanced agent ZSMR06 showed that: (a) it is capable of inducing a dose-dependent inhibition of PARP in isolated enzyme assay and strong DNA PARP trapping potency, (b) it induced a dose-dependent inhibition of EGFR phosphorylation in an isolated kinase and in whole cell assays and this translated into inhibition of downstream signaling of EGFR, (c) in an isogenic cell panel, it was selectively potent against BRCA2 mutant cells and EGFR transfectants, (d) its growth inhibitory potency was stronger than that of olaparib or gefitinib alone and their corresponding equimolar combinations, (e) subcellular distribution analysis showed that it was abundantly localized in the perinuclear region. Studies on the mechanism underlying the strong potency of ZSMR06 revealed that it could also down-regulate BRCA1 and BAP1, a ubiquitin hydrolase that binds to BRCA1. It induced γH2AX foci, with the highest levels detected in the exquisitely sensitive breast cancer cell line MDA-MB-468. This strong potency is believed to be associated with its PARP DNA trapping property in

combination with downregulation of key proteins in homologous recombination. Thus, this study represents the first proof-of-concept of a new targeted strategy to efficiently expand the targeting of PARP inhibitors beyond BRCA1/2 mutations.

4.2 Introduction

Poly(ADP-ribose) polymerase (PARP) is a nuclear protein involved in the maintenance of genomic stability, transcriptional regulation, energy metabolism, DNA methylation, and cell death [1]. When PARP is activated, it catalyzes the formation of a poly-ADP ribose chain of varying lengths and complexity and attaches this chain to itself and other acceptor proteins. This event is one of the first triggered by DNA damage [e.g. single strand breaks (SSBs) and double strand breaks (DSBs)][2]. PARP binds to SSBs and recruit other proteins of the base excision repair machinery to the site of damage. It has been shown that PARP inhibition induces synthetic lethality in BRCA1/2 mutated tumours [3-6]. This arises in tumours with BRCA1/2 mutations in which, PARP-dependent DNA repair is the only alternative to BRCA1/2 deficiency. Thus, blockade of PARP leads to accumulation of DNA damage, and ultimately cell death. Accordingly, over the past decade, PARP inhibition has been actively pursued as a novel approach for the selective therapy of tumours with BRCA1/2 mutations. The therapeutic benefits of PARP inhibitors have now been proven in the clinic against BRCA1/2 mutant breast, ovarian and prostate cancers [7-9]. Disappointingly, resistance to PARP inhibitors has been observed in the clinic. Importantly, the mechanism of resistance is believed to be based on genetic reversion that corrects the original BRCA1- or 2-inactivating mutation [10, 11]. The therapeutic benefit of PARP inhibitors is hitherto limited to BRCA1/2 mutations, which only accounts for 5–10% of all cancers with hereditary mutations in the homologous recombination pathway [12]. Therefore, new strategies are not only required to enhance the potency of PARP inhibitors but also to expand their use beyond BRCA mutant cancers.

Recently, our group utilized a novel strategy developed by our laboratory termed combi-targeting, to design single molecules termed “combi-molecules” capable of simultaneously inducing DNA damage and blocking PARP [13, 14]. The combi-targeting concept postulates that designing “combi-molecules” to block functions or pathways that act cooperatively will lead to agents with superior potency when compared to single agents targeted to each function and their corresponding 2-drug combinations. We have now shown that two types of combi-molecules can be designed with the combi-targeting approach: type I and II. As depicted in figure 1, type I combi-molecules require hydrolysis to induce the combi-targeting properties [15], whereas type II combi-molecules can hit both targets with no requirement for hydrolytic cleavage [16]. Recently, we reported the synthesis of both type I and II PARP-DNA targeting combi-molecules [14]. While these molecules could enhance potency when compared with PARP inhibitors of the same class in BRCA1/2 mutant cells, they also showed significant potency in BRCA1/2 wild type cells and we suggested their potential use at a stage when BRCA1/2 are heterogeneously expressed [13, 14]. The observed potency of the combi-molecules in BRCA1/2 wild type cells is consistent with the fact that PARP inhibitors, have been shown to potentiate the action of the DNA damaging agent in non-synthetically lethal contexts, indicating that their use could be expanded beyond BRCA mutant cancers [17, 18]. Thus, we surmised that perhaps targeting the PARP inhibitors to other cellular dysfunction could lead to non BRCA-mediated selectivity.

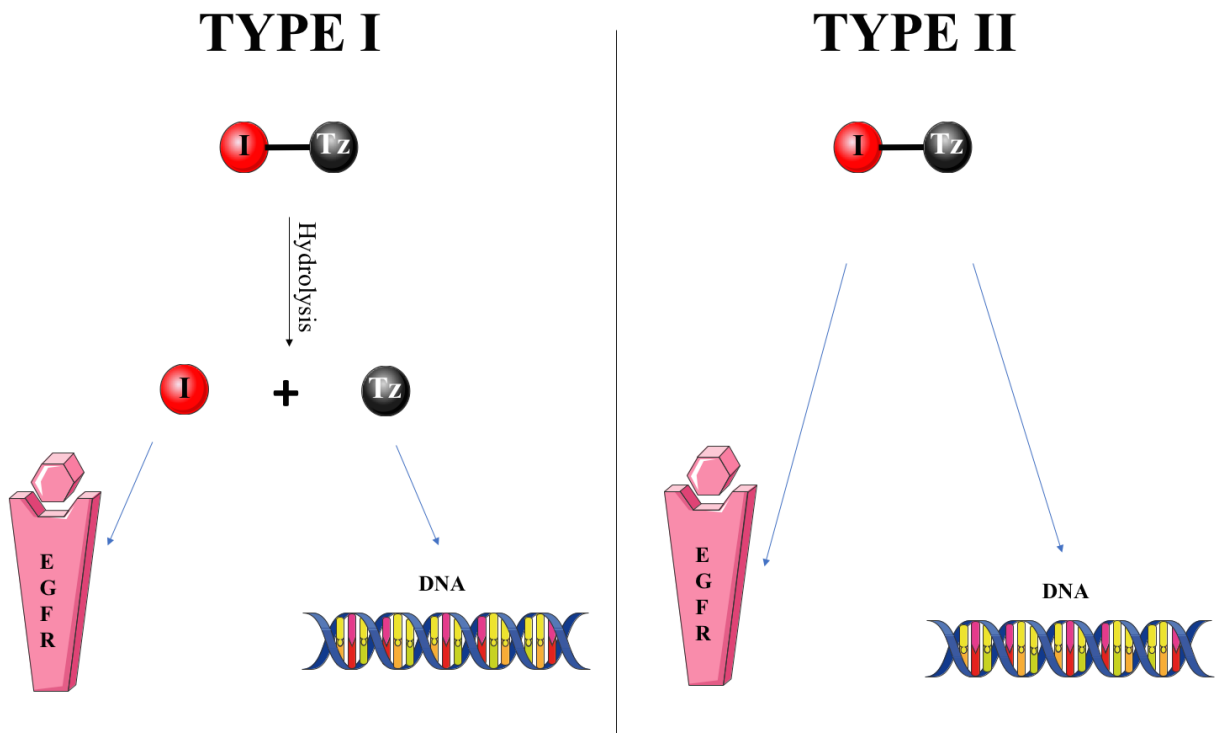


Figure 1. Schematic representation of the principle of type I and type II targeting by combi-molecules. Upon entering the cells, type I combi-molecules bind and inhibit their target as intact molecules. The molecule further releases an inhibitor 'A' and a DNA damaging agent 'B' upon hydrolysis. Type II combi-molecules enter the cells to inhibit their target and damage DNA without hydrolysis.

While several combination modalities have been reported for PARP inhibitors, the concept of targeted PARP inhibitor has not yet been explored. Here using our novel combi-targeting approach, we report on the design of PARP inhibitors targeted to EGFR, a tyrosine kinase receptor overexpressed in several solid tumours, including lung, breast, brain, colon, ovarian, head and neck etc [19]. Overexpression of EGFR is associated with aggressive proliferation and increased invasiveness, migration and survival [20]. EGFR is a 170-kd receptor tyrosine kinase, which upon binding of its cognate ligands (e.g. EGF, TGF□), and following dimerization and auto-

phosphorylation, triggers a cascade of downstream signaling events that translate into activation of growth through the MAPK pathway and antiapoptotic signaling through the PI3K-AKT pathways [21, 22]. Several EGFR-DNA targeting combi-molecules of the type I and II were designed by our group on the premise that blockade of EGFR leads to downregulation of the anti-apoptotic PI3K and DNA repair in EGFR-overexpressing tumour cells [23].

Recently, reports on the relationship between EGFR, PARP, BRCA have begun to emerge. Li et al. [2] reported that PARP inhibition suppressed the growth of EGFR mutant cancer cells by targeting the nuclear localization of pyruvate kinase M2 (PKM2), a Warburg effect-associated enzyme. EGF stimulation is known to induce a translocation of PKM2 to the nucleus where its retention is controlled by PARP-mediated poly-ADP ribosylation. Thus, inhibition of PARP ultimately leads to suppression of its nuclear localization and cell death. Kumaraswamy et al. [24] showed that EGFR expression correlates negatively with that of BRCA1. Likewise, Burga et al. [25] reported that loss of BRCA1 leads to increase in EGFR. Nowsheen et al. [12] observed a synergistic interaction between ABT888, an extremely potent PARP inhibitor and lapatinib, a dual EGFR/HER2 inhibitor. The mechanism underlying such a synergy was believed to be the ability of lapatinib to inhibit the translocation of EGFR and BRCA1 into the nucleus where they play a significant role in homologous recombination repair, thereby leading to accumulation of DNA damage and cell death. Nowsheen et al. [12] referred to this mechanism as “contextual synthetic lethality” between EGFR and PARP. All the above observations lend support to the design of molecules with dual EGFR and PARP targeting potential. To achieve this goal, here we report on the synthesis of novel PARP-EGFR targeting combi-molecules based on structural modifications of olaparib, the first PARP inhibitor approved for clinical use by the FDA [26], and conjugation to an anilinoquinazoline ring system targeted to EGFR via a short linker. The structure of the

EGFR inhibitor moiety straddled that of gefitinib, a clinical inhibitor of EGFR [27]. The design was based on the requirement of the piperazinocarbonyl-fluorobenzomethyl-phthalazinone moiety of olaparib for binding to PARP [28] and the tolerance of the 7-position of the quinazoline class of EGFR inhibitors for bulky substituents [29]. The length of the linker was designed to keep each pharmacophore away from their binding pocket in PARP and EGFR. The analysis of the dual function of the new molecules was performed in a panel of isogenic and established cell lines with varied levels of expression of EGFR. The structure with the strongest EGFR and PARP inhibitory potency, ZSMR06 was selected for further studies in the panel of triple negative breast cancer cells studied by Nowsheen et al. [12]. Molecular analysis for elucidating the mechanism of action of ZRMS06 were focused on the triple negative breast cancer cells, which express wild type BRCA1/2.

4.3 Methods

4.3.1 Chemistry

^{1}H NMR spectra were obtained on a Bruker Ascend^{TM} NMR 400MHz spectrometer. Chemical shifts are given as δ values in parts per million (ppm) and referenced to the residual solvent proton peak. Mass spectrometry was performed at the Drug Discovery Platform at Glen MUHC facility, McGill University, Montreal, Quebec, Canada. Mass spectra were obtained on a Bruker Amazon SL spectrometer using electrospray ionization (ESI). Data are reported as m/z (intensity relative to base peak = 100). All chemicals were purchased from Sigma-Aldrich. ZR2002 and RB10 were synthesized in our lab. N-(3-Chloro-4-fluorophenyl)-6-(3-chloropropoxy)-7-methoxyquinazolin-4-amine and 4-(4-Fluoro-3-(piperazine-1-carbonyl)benzyl)phthalazin-1(2H)-one was purchased from Ark Pharm, Inc. (Arlington Heights, IL, USA). 2-fluoro-5-((4-oxo-3,4-dihydrophthalazin-1-yl)Methyl)benzoic acid was purchased from AstaTech, Inc. (Bristol, PA, USA).

ZSMR02

Compound MR52

To a solution of RB10 (50mg, $1.59x10^{-1}$mM) in dry DMF (1mL) was added 1,1'-carbonyldiimidazole (25.7mg, $1.59x10^{-1}$mM). The mixture was stirred under argon for 1 h at RT. Thereafter, the solution was added dropwise to a solution of piperazine (137mg, 1.59mM) in dry DMF (2 mL). The mixture was stirred under argon overnight at RT. The reaction mixture was evaporated to give a crude product, which was purified by preparative TLC (silica plate, CH_2Cl_2/MeOH 80/20) to give pure compound MR52 (20mg, 40%). ESIMS m/z 427.03 (MH^+). ^1H NMR (400 MHz, DMSO-d_6) δ ppm 9.80 (s, 1H, NH), 8.84 (s, 1H, NH), 8.54 (s, 1H, ArH), 8.49 (d, 1H, J = 2.1 Hz, ArH), 8.20 (t, 1H, J = 1.9 Hz, ArH), 7.88 (d, 1H, J = 8.0 Hz, ArH), 7.83 (dd, 1H, J = 9.0 Hz, J = 2.2 Hz, ArH), 7.72 (d, 1H, J = 8.9 Hz, ArH), 7.33 (t, 1H, J = 8.0 Hz, ArH), 7.27 (d, 1H, J = 7.9 Hz, ArH), 3.43 (t, 4H, J = 5.1 Hz, 2 X $HNCH_2CH_2N$), 2.74 (t, 4H, J = 4.9 Hz, 2 X NCH_2CH_2NH).

Compound ZSMR02

ZSMR02 was synthesized as described in Scheme 1. To solution of 2-fluoro-5-((4-oxo-3,4-dihydrophthalazin-1-yl)Methyl)benzoic acid (7.7mg, $0.026x10^{-2}$mM), HOBt (4.2mg, $3.1x10^{-2}$mM) and DMAP (0.32mg, 0.002mM) in dry DMF, was added EDCI (4.8mg, 0.03mM). The reaction mixture was stirred for 30 min at RT. Thereafter, a solution of MR52 (11mg, $2.58x10^{-2}$mM) was added dropwise. The reaction mixture was stirred over night at RT and evaporated to give ZSMR02, as a crude product, which was purified by preparative TLC (silica plate, CH_2Cl_2/MeOH 92/08). ZSMR02 was obtained as a pure powder (11mg, 100%). ESIMS m/z

707.04 (MH^+). ^1H NMR (400 MHz, DMSO-d_6) δ ppm 12.62 (s, 1H, NH), 9.82 (s, 1H, NH), 9.01 (s, 1H, NH), 8.55 (s, 1H, ArH), 8.48 (d, 1H, J = 2.0 Hz, ArH), 8.27 (dd, 1H, J = 7.8 Hz, J = 1.2 Hz, ArH), 8.20 (t, 1H, J = 1.9 Hz, ArH), 7.98 (d, 1H, J = 8.0 Hz, ArH), 7.93 – 7.80 (m, 4H, ArH), 7.74 (d, 1H, J = 8.9 Hz, ArH), 7.47 (m, 1H, ArH), 7.39 (dd, 1H, J = 6.4 Hz, J = 2.1 Hz, ArH). 7.34 (t, 1H, J = 8.1 Hz, ArH), 7.26 (m, 2H, ArH), 4.35 (s, 2H, $ArCH_2Ar$), 3.70 (s, 2H, NCH_2CH_2N), 3.61 (t, 2H, J = 4.3 Hz, NCH_2CH_2N), 3.45 (t, 2H, J = 4.3 Hz, NCH_2CH_2N), 3.26 (s, 2H, NCH_2CH_2N).

ZSMR03

Compound MR10

To a solution of N-(3-Chloro-4-fluorophenyl)-6-(3-chloropropoxy)-7-methoxyquinazolin-4-amine (100mg, $2.52x10^{-1}$mM) in dry DMF (2mL) was added potassium iodide (2 equivalents). The reaction mixture was stirred for 24 h at RT. Thereafter, the solution was added dropwise to a solution of piperazine (217.4mg, $25.2x10^{-1}$mM) with triethylamine (1.5 equivalent) in dry DMF (5mL). The reaction mixture was stirred for 3 days at 40ºC and evaporated to give MR10, as a crude product, which was purified by preparative TLC (silica plate, CH_2Cl_2/MeOH 80/20). MR10 was obtained as a pure white powder (40mg, 40%). ESIMS m/z 446.17 (MH^+). ^1H NMR (400 MHz, DMSO-d_6) δ ppm 9.57 (s, 1H, NH), 8.50 (s, 1H, ArH), 8.11 (dd, 1H, J = 6.9 Hz, J = 2.7 Hz, ArH), 7.81 (s, 1H, ArH), 7.79 (m, 1H, ArH), 7.46 (t, 1H, J = 9.3 Hz, ArH), 7.22 (s, 1H, ArH), 4.18 (t, 2H, J = 6.3 Hz, OCH_2CH_2), 3.94 (s, 3H, OCH_3), 3.06 (t, 4H, J = 4.8 Hz, 2 X $HNCH_2CH_2N$), 2.56 (m, 6H, 2 X NCH_2CH_2NH & NCH_2CH_2), 2.00 (quint, 2H, J = 6.7 Hz, $OCH_2CH_2CH_2N$).

Compound ZSMR03

ZSMR03 was synthesized as described in Scheme 2. To solution of 2-fluoro-5-((4-oxo-3,4-dihydrophthalazin-1-yl)Methyl)benzoic acid (21mg, $7.05x10^{-2}$mM), HOBt (10.9mg, $8.07x10^{-2}$mM) and DMAP (0.82mg, $6.73x10^{-3}$mM) in dry DMF, was added EDCI (12.6mg, $8.07x10^{-2}$mM). The reaction mixture was stirred for 30 min at RT. Thereafter, a solution of MR10 (30mg, $7.05x10^{-2}$mM) was added dropwise. The reaction mixture was stirred over night at RT and evaporated to give ZSMR03, as a crude product, which was purified by preparative TLC (silica plate, CH_2Cl_2/MeOH 92/08). ZSMR03 was obtained as a pure powder (30mg, 100%). ESIMS m/z 724.35(MH^-). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 12.61 (s, 1H, NH), 9.58 (s, 1H, NH), 8.50 (s, 1H, ArH), 8.26 (dd, 1H, J = 7.9 Hz, J = 1.0 Hz, ArH), 8.11 (dd, 1H, J = 6.9 Hz, J = 2.6 Hz, ArH), 7.97 (d, 1H, J = 7.9 Hz, ArH), 7.89 (dt, 1H, J = 7.2 Hz, J = 1.2, ArH), 7.85 – 7.76 (m, 3H, ArH), 7.45 (t, 1H, J = 9.1 Hz, ArH), 7.43 (m, 1H, ArH), 7.31 (dd, 1H, J = 6.6 Hz, J = 2.2 Hz, ArH), 7.22 (t, 1H, J = 9.1 Hz, ArH). 7.21 (s, 1H, ArH), 4.32 (s, 2H, $ArCH_2Ar$), 4.18 (t, 2H, J = 6.3 Hz, OCH_2CH_2), 3.94 (s, 3H, OCH_3), 3.62 (s, 2H, NCH_2CH_2N), 3.15 (s, 2H, NCH_2CH_2N), 2.45 (s, 2H, NCH_2CH_2N), 2.29 (s, 2H, NCH_2CH_2N), 2.00 (quint, 2H, J = 6.8 Hz, $OCH_2CH_2CH_2N$).

ZSMR04

Compound ZSM04

To solution of 2-fluoro-5-((4-oxo-3,4-dihydrophthalazin-1-yl)Methyl)benzoic acid, (200mg, $6.7x10^{-1}$mM), HOBt (109mg, $8.0x10^{-1}$mM) and DMAP (8.2mg, $6.7x10^{-2}$mM) in dry DMF (4mL), was added EDCI (142 µL, $8.0x10^{-1}$mM). The reaction mixture was stirred for 30 min at RT. Thereafter, a solution of *tert*-butyl piperazine-1-carboxylate (125mg, $6.7x10^{-1}$mM) was added dropwise. The reaction mixture was stirred over night at RT and evaporated to give ZSM04, as a crude product, which was purified by preparative TLC (silica plate, CH_2Cl_2/MeOH 90/10). ZSM04

was obtained as a pure powder (231mg, 74%). ^{1H} NMR (400 MHz, DMSO-d_6) δ ppm 12.58 (s, 1H, NH), 8.26 (d, 1H, J = 7.8 Hz, ArH), 7.96 (d, 1H, J = 8.1 Hz, ArH), 7.90 (t, 1H, J = 7.1 Hz, ArH), 7.83 (t, 1H, J = 7.7 Hz, ArH), 7.44 (m, 1H, ArH), 7.37 (s, 1H, ArH), 7.24 (t, 1H, J = 8.9 Hz, ArH), 4.33 (s, 2H, $ArCH_2Ar$), 4.03 (d, 2H, J = 26.7 Hz, $OCCH_2N$), 3.68 – 3.34 (m, 6H, 3 X NCH_2CH_2N), 3.17 (s, 2H, NCH_2CH_2N). 2.77 (d, 3H, J = 13.1 Hz, NCH_3), 1.36 (d, 9H, J = 26.3 Hz, $C(CH_3)_3$).

Compound ZSM13

To solution of 4-(4-Fluoro-3-(piperazine-1-carbonyl)benzyl)phthalazin-1(2H)-one, (70mg, $1.9x10^{-1}$mM), HOBt (31mg, $2.3x10^{-1}$mM) and DMAP (2.3mg, $1.9x10^{-2}$mM) in dry DMF (2 mL), was added EDCI (40.6 µL, $2.3x10^{-1}$mM). The reaction mixture was stirred for 30 min at RT. Thereafter, a solution of N-(tert-butoxycarbonyl)-N-methylglycine (36.1mg, $1.9x10^{-1}$mM) was added dropwise. The reaction mixture was stirred over night at RT and evaporated to give ZSM13, as a crude product, which was purified by preparative TLC (silica plate, CH_2Cl_2/MeOH 90/10). ZSM13 was obtained as a pure powder (83mg, 81%). ^{1}H NMR (400 MHz, DMSO-d6) δ ppm 12.58 (s, 1H, NH), 8.26 (d, 1H, J = 7.8 Hz, ArH), 7.96 (d, 1H, J = 8.1 Hz, ArH), 7.90 (t, 1H, J = 7.1 Hz, ArH), 7.83 (t, 1H, J = 7.7 Hz, ArH), 7.44 (m, 1H, ArH), 7.37 (s, 1H, ArH), 7.24 (t, 1H, J = 8.9 Hz, ArH), 4.33 (s, 2H, ArCH2Ar), 4.03 (d, 2H, J = 26.7 Hz, OCCH2N), 3.68 – 3.34 (m, 6H, 3 X NCH2CH2N), 3.17 (s, 2H, NCH2CH2N). 2.77 (d, 3H, J = 13.1 Hz, NCH3), 1.36 (d, 9H, J = 26.3 Hz, C(CH3)3).

Compound ZSM14

A solution of ZSM13 (75mg, $1.4x10^{-1}$mM) in HCl (5 mL) and ethanol (2.5 mL) was stirred for 3 h at RT. Thereafter, the solution was basified to a pH of 10 using KOH and extracted using DCM (3 x 25 mL). The organic layers were conserved, dried over Na_2SO_4 and evaporated. ZSM14 was obtained as a pure powder (61.0mg, 100%). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 12.58 (s, 1H, NH), 8.26 (dd, 1H, J = 7.9 Hz, J = 1.0 Hz, ArH), 7.96 (d, 1H, J = 8.1 Hz, ArH), 7.90 (t, 1H, J = 7.6 Hz, ArH), 7.83 (dt, 1H, J = 7.6 Hz, J = 1.1 Hz, ArH), 7.44 (m, 1H, ArH), 7.36 (s, 1H, ArH), 7.23 (t, 1H, J = 8.9 Hz, ArH), 4.33 (s, 2H, $ArCH_2Ar$), 3.66 – 3.34 (m, 6H, 3 X NCH_2CH_2N), 3.17 (s, 2H, NCH_2CH_2N). 2.26 (s, 3H, NCH_3).

Compound ZSMR04

ZSMR04 was synthesized as described in Scheme 3. To a solution of ZR2002 (30mg, $7.9x10^{-2}$mM) in dry DMF (1 mL) was added potassium iodide (2 equivalents). The reaction mixture was stirred for 24 h at RT. Thereafter, the solution was added dropwise to a solution of ZSM14 (34.7mg, $7.9x10^{-2}$mM) with triethylamine (1.5 equivalent) in dry DMF (1mL). The reaction mixture was stirred for 5 days at 40ºC and evaporated to give ZSMR04, as a crude product, which was purified by preparative TLC (silica plate, CH_2Cl_2/MeOH 90/10). ZSMR04 was obtained as a pure powder (7.5mg, 25%). ESIMS m/z 776.31 (MH^-). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 12.59 (s, 1H, NH), 9.35 (s, 1H, NH), 8.37 (s, 1H, ArH), 8.25 (dd, 1H, J = 7.8 Hz, J = 0.9 Hz, ArH), 8.17 (s, 1H, ArH), 7.97 – 7.77 (m, 4H, ArH), 7.57 – 7.38 (m, 2H, ArH), 7.37 – 7.11 (m, 6H, ArH), 6.11 (d, 1H, J = 18.4 Hz, $ArNHCH_2$), 4.32 (s, 2H, $ArCH_2Ar$), 3.65 – 3.38 (m, 6H, 3 X NCH_2CH_2N). 3.12 (s, 2H, NCH_2CH_2N), 2.71 (s, 2H, NCH_2CH_2NH), 2.31 (d, 3H, J = 9.0 Hz, NCH_3).

ZSMR06

Compound MR65

To a solution of ZR2002 (100mg, $2.65x10^{-1}$mM) in dry DMF (2 mL) was added potassium iodide (2 equivalents). The reaction mixture was stirred for 24 h at RT. Thereafter, the solution was added dropwise to a solution of piperazine (228mg, $26.5x10^{-1}$mM) with triethylamine (1.5 equivalent) in dry DMF (5mL). The reaction mixture was stirred for 5 days at 40ºC and evaporated to give a crude product MR65, which was purified by preparative TLC (silica plate, CH_2Cl_2/MeOH 70/30). MR65 was obtained as a pure yellow powder (80mg, 80%). ESIMS m/z 427.17 (MH^+).^{1H} NMR (300 MHz, DMSO-d_6) δ ppm 9.38 (s, 1H, NH), 8.37 (s, 1H, ArH), 8.16 (t, 1H, J = 1.8 Hz, ArH), 7.90 (d, 1H, J = 7.9 Hz, ArH), 7.54 (d, 1H, J = 9.1 Hz, ArH), 7.37 – 7.17 (m, 4H, ArH), 6.04 (t, 1H, J = 5.3 Hz, $ArNHCH_2$), 3.02 (t, 4H, J = 4.9 Hz, 2 X $HNCH_2CH_2N$), 2.65 (t, 2H, J = 6.3 Hz, NCH_2CH_2NHAr), 2.61 (s, 4H, 2 X NCH_2CH_2NH).

Compound MR72

A solution of MR65 (30mg, $7.02x10^{-2}$mM) in dry pyridine (1 mL) was frozen with liquid nitrogen and 4-nitrophenyl chloroformate (14.2mg, $7.02x10^{-2}$mM) was added. The mixture was stirred under argon for 2 h at 0ºC and the reaction was further allowed to reach RT. Thereafter, the solvent was evaporated to give a crude product MR72, which was purified by preparative TLC (silica plate, CH_2Cl_2/MeOH 90/10) to give pure compound MR72 (27 mg, 90%). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 9.40 (s, 1H, NH), 8.38 (s, 1H, ArH), 8.28 (d, 2H, J = 9.2 Hz, ArH), 8.18 (t, 1H, J = 2.0 Hz, ArH), 7.92 (qd, 1H, J = 8.0 Hz, J = 1.0 Hz, ArH), 7.55 (d, 1H, J = 9.0 Hz, ArH), 7.44 (d, 2H, J = 9.2 Hz, ArH), 7.34 (m, 2H, ArH), 7.27 (qd, 1H, J = 7.9 Hz, J = 0.9 Hz, ArH), 7.23 (d, 1H, J = 2.2 Hz, ArH), 6.12 (t, 1H, J = 5.1 Hz, $ArNHCH_2$), 3.65 (s, 2H, $OCNCH_2CH_2N$), 3.50 (s,

2H, $OCNCH_2CH_2N$), 3.41 (q, 2H, J = 5.8 Hz, $HNCH_2CH_2N$), 2.70 (t, 2H, J = 6.6 Hz, NCH_2CH_2NH), 2.58 (s, 4H, 2 X NCH_2CH_2NCO).

Compound MR75

A solution of MR72 (25mg, $4.22x10^{-2}$mM) in dry DMF (1 mL) was added dropwise to a piperazine solution (36.4mg, $42.2x10^{-2}$mM) in dry DMF (2mL). The mixture was stirred under argon for 5 h at 40ºC. The reaction mixture was evaporated to give a crude product, which was purified by preparative TLC (silica plate, CH_2Cl_2/MeOH 80/20) to give pure compound MR75 (10mg, 40%). ESIMS m/z 539.15 (MH^+).

Compound ZSMR06

ZSMR06 was synthesized as described in Scheme 4. To solution of 2-fluoro-5-((4-oxo-3,4-dihydrophthalazin-1-yl)Methyl)benzoic acid (5.5mg, $1.85x10^{-2}$mM), HOBt (3.0mg, $2.22x10^{-2}$mM) and DMAP (0.22mg, $1.85x10^{-3}$mM) in dry DMF, was added EDCI (3.45mg, $2.22x10^{-2}$mM). The reaction mixture was stirred for 30 min at RT. Thereafter, a solution of MR75 (10mg, $1.85x10^{-2}$mM) was added dropwise. The reaction mixture was stirred over night at RT and evaporated to give ZSMR06, as a crude product, which was purified by preparative TLC (silica plate, CH_2Cl_2/MeOH 92/08). ZSMR06 was obtained as a pure powder (9mg, 90%). ESIMS m/z 819.27(MH^+). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 12.61 (s, 1H, NH), 9.40 (s, 1H, NH), 8.37 (s, 1H, ArH), 8.26 (dd, 1H, J = 7.8 Hz, J = 1.0 Hz, ArH), 8.16 (t, 1H, J = 1.7 Hz, ArH), 7.97 (d, 1H, J = 7.9 Hz, ArH), 7.89 (m, 2H, ArH), 7.83 (t, 1H, J = 7.5 Hz, ArH), 7.54 (d, 1H, J = 9.0 Hz, ArH), 7.44 (m, 1H, ArH), 7.34 (m, 3H, ArH), 7.28 – 7.20 (m, 3H, ArH) 6.09 (t, 1H, J = 4.9 Hz, $ArNHCH_2$), 4.33 (s, 2H, $ArCH_2Ar$), 3.66 (s, 2H, NCH_2CH_2N), 3.31 (q, 2H, J = 5.5 Hz,

$NHCH_2CH_2N$), 3.24 – 3.13 (m, 8H, 4 X NCH_2CH_2N), 3.05 (s, 2H, NCH_2CH_2N), 2.64 (t, 2H, J = 6.6 Hz, NCH_2CH_2NH), 2.47 (s, 4H, 2 X NCH_2CH_2N).

4.3.2 Cell culture

VC8 and VC8-BRCA Chinese Hamster Lung cells were generously provided by Dr. Bernd Kaina (Institute of Toxicology, Mainz, Germany). NIH 3T3, NIH 3T3-EGFR mouse fibroblast cell lines and HCT116 colon cancer cell lines were kindly given by Dr. Alaoui-Jamali (Segal Cancer centre and Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, Canada). MDA-MD468 breast cancer, DU145 prostate cancer, A2058 melanoma and A549 lung cancer cell lines were bought from ATCC. MDA-MB453 and MDA-MB231 were generously given by Dr. Ali (the Research Institute of the McGill University Health Centre, Montreal, Canada). T98 glioblastoma cell lines were kindly given by Dr. Sabri (the Research Institute of the McGill University Health Centre, Montreal, Canada). A375 melanoma cell line was kindly provided by Dr. Richard Kremer (Department of Medicine, McGill University Health Centre, McGill University, Montreal, Canada). A549 EGFR-GFP was bought from Sigma Aldrich Canada. All cell lines were maintained in DMEM media from Wisent Bio Products. Media preparation was supplemented with 10% Fetal Bovine Serum (FBS), 12 mL HEPES, 5 mL L-glutamine, 500 □l of gentamicin sulfate, 250 □L of fungisome, and 170 □L of ciprofloxacin. All the bio-products used in the preparation of the media were purchased from Wisent Inc. The cells were grown in Corning cell cultured treated polystyrene flasks, which are placed in an incubator with a stable temperature of 37°C and CO_2 level of 5%. The media of each flask was changed when necessary and cell passaging was done between 85 and 95 % confluence.

4.3.3 In Vitro Growth Inhibition Assay

Cells were plated in 96-well plates (Corning Inc.) at 500-10,000 cells/well in 100 μL medium/well. They were allowed to attach for 24 h and then treated with a wide range of drug concentrations (0.003125 μM to 100 μM). The treatment was designed to be in triplicate for 5 days in the incubator. Following the drug treatment, the cells were fixed with 50 μL per well of cold TCA (50%) for 2 hours at 4 °C. Then, using flow of water, the wells were rinsed four times, dried well as much as possible after last rinse and stained with 50 mL sulforhodamine B (SRB) (0.4 g/100 mL) for at least 1 hour at room temperature. Subsequently, the SRB was rinsed with 1% acetic acid, and allowed to air-dry overnight. Finally, the dye was solubilized in 200 mL of Tris base (10 mM, pH10-10.5) for 2-5 minutes on a shaking platform. Absorbance readings of the solubilized dye were recorded on a ELx808 microplate reader at an optical density of 492 nm. The results were analyzed using GraphPad Prism software to derive a dose-response curve and the IC_{50}. Each experiment was carried out at least 3 times, in triplicate.

4.3.4 In Vitro PARP enzyme assay

The Trevigen HT Universal Colorimetric PARP assay kit with histone-coated strip well was used as per protocol provided by the vendor. Briefly, 50 μl per well of 1X PARP buffer was added to the strip well to rehydrate the histones and the plate was subsequently incubated at room temperature for 30 minutes. The solution was aspirated and replaced with a dose range of ZSMR02, ZSMR03, ZSMR04, ZSMR06 and olaparib (10^{-6} to 10 μM) in duplicate. PARP enzyme (0.5 Unit/well) and a PARP cocktail was then added to the appropriate wells containing inhibitor. A negative control was prepared without PARP to determine the background absorbance, and a positive control without the inhibitor for a 100% reference point. After 60 minutes incubation time, the strip wells were washed twice with 1X PBS + 0.1% Triton X-100 (200 μl/well) followed by

two washes with 1X PBS. Diluted Strep-HRP was then added after the washing and incubated for 60 minutes. Finally, a pre-warmed TACS-Sapphire colorimetric substrate was added to each well, in the dark, for 15 minutes at room temperature, after which the reactions were stopped by adding 0.2M HCl. Optical densities at 450 nm were recorded on ELx808 microplate reader. The results were analyzed using GraphPad Prism software to derive a dose-response curve and the IC_{50} values. The PARP assay was performed twice, in triplicate.

4.3.5 PARP trapping

All steps were carried out as published procedures by Murai *et al* [30] using human leukemic lymphoblast CCRF-CEM cells). In short, cells were collected, lysed, mixed and incubated at 4C for 30 minutes after being treated with olaparib or ZSMR06 for 30 minutes. After centrifugation at 15,000 x g at 4C for 10 minutes, the supernatants were collected and nuclear soluble and chromatin-bound fraction were separated. Immunoblotting was then carried out using standard procedures and Densitometric analyses done using Image J software (NIH).

4.3.6 In Vitro EGFR enzyme assay

96-well plates from Nunc Maxisorp were incubated overnight at 37ºC with poly(L-glutamic acid-L-tyrosine, 4:1) (PGT) in PBS. The kinase reaction was performed by using 13ng/well EGFR (SignalChem Lifesciences, Corp., Richmond, BC, Canada). Each well was treated with a dose range of ZSMR02, ZSMR03, ZSMR04, and ZSMR06 (10^{-4} to 10 μM) in duplicate. Phosphorylation was then initiated by the addition of ATP. The reaction was terminated by removal of reaction mixture, and detection of the phosphorylated substrate by HRP-conjugated anti-phosphotyrosine antibody (Santa Cruz Biotechnology, Dallas, TX, USA). The signals were developed by the addition of 3,3′,5,5′-tetramethylbenzidine peroxidase substrate (KPL, Gaithersburg, MD, USA). The colorimetric reaction was stopped by adding equal volume of

H_2SO_4 (0.09M) to each well. The plates were read at 450nm using a ELx808 microplate reader. The results were analyzed using GraphPad Prism software to derive a dose-response curve and the IC_{50} values.

4.3.7 Western blotting

Cells were plated in 6-well plates and allowed to attach overnight. The cells were rinsed twice with PBS 24 h later and starved overnight on addition of serum-free media. Thereafter, they were treated with different doses of ZSMR02, ZSMR03, ZSMR04, and ZSMR06 for 24 h, washed with PBS (twice) and stimulated with 20 ng/ml EGF for 30 min at 37°C. Cells were then washed, detached by scraping in cold RIPA lysis and extraction buffer (ThermoFisher Scientific, Waltham, MA, USA). Lysates were kept on ice for 30 min and collected by centrifugation at 13,000 rpm for 15 min at 4°C. The concentration of protein was determined using the Bio-Rad protein assay kit (Bio-Rad laboratories, Hercules, CA, USA). Equal amounts of proteins were loaded, resolved on 10% SDS-PAGE and thereafter transferred to a polyvinylidenedifluoride (PVDF) membrane (Milipore, Bedford, MA, USA). Membranes were blocked with 5% milk in TBST (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween 20) overnight at 4°C followed by incubation with phosphotyrosine antibodies such as phospho-EGFR Y1068 (Cell Signaling Technology, Danvers, MA, USA) phospho-Erk1/2 (Cell Signaling Technology, Danvers, MA, USA) and phospho-Akt (Cell Signaling Technology, Danvers, MA, USA) in 5% milk, at 4°C overnight. The membranes were washed with TBST and incubated with respective secondary antibodies for 1h and 30 min at RT in 5% blocking solution. After incubation with antibodies against phosphotyrosines, the membranes were stripped using the Re-Blot Plus Strong buffer (EMD Millipore, Billerica, MA, USA) and probed for total EGFR (Santa Cruz Biotechnology, Dallas, TX, USA), total Erk1/2 (Cell Signaling Technology, Danvers, MA, USA), total Akt (Cell Signaling Technology, Danvers, MA,

USA) and total PAR (Milipore, Bedford, MA, USA) antibodies along with β-actin (Cell Signaling Technology, Danvers, MA, USA) antibody. Immunoblot bands were visualized using ECL kit and enhanced chemiluminescence system (ThermoFisher Scientific, Waltham, MA, USA).

Western blotting for DNA repair proteins was performed essentially as described [31, 32]. In brief, following incubation with the vehicle DMSO or one of the pharmacological compounds, cells were rinsed twice with PBS and stored at -70°C. Crude extracts were prepared by solubilizing proteins in gel sample buffer, pH 8.0, containing protease inhibitors (Roche), 20 mM β-glycerophosphate, 1 mM NaN_3, and 2.5 mM NaF. Samples were incubated for 15 min at 95°C and vortexed 3min with glass beads to shear DNA. After centrifugation (5 min, 13,000 rpm, microfuge), proteins were TCA-precipitated and resuspended in 2x final sample buffer pH 8.0 containing the inhibitors listed above. Aliquots were separated on SDS-PA gels, blotted to nitrocellulose and probed with the antibody of interest, BAP-1, BRCA1, BARD1 (all purchased from Santa Cruz Biotechnology, Dallas, TX, USA), and β-actin (Chemicon, Milipore, Bedford, MA, USA). HRP-conjugated secondary antibodies (Jackson) were raised in donkeys and pre-adsorbed against multiple species. ECL substrates were from LI-COR (Lincoln, NE, USA).

4.3.8 Indirect immunofluorescence and confocal microscopy

All steps were carried out at room temperature following published procedures [33, 34]. Primary antibodies for PARP1 (Santa Cruz Biotechnology, Dallas, TX, USA), γH2AX (Milipore, Bedford, MA, USA) were used to evaluate DNA damage and the nuclear stain DAPI (Sigma Aldrich Canada) was used to confirm the localization of the proteins detected by the antibodies. Images were acquired using the multi-track mode with a LSM780 microscope. Appropriate filter settings were chosen to minimize cross-talk between the channels. Images were processed in Photoshop 8.0.

4.3.9 Confocal microscopy imaging

A549 EGFR-GFP cells in which the EGFR gene has been endogenously tagged with Green Fluorescent Protein (GFP) (commercially available from Sigma-Aldrich) was plated at 60-70% confluence in six-well plates, allowed to adhere overnight, and treated with 25 μM ZSM06 and RB10 for 2 h. After treatment, cells were washed with PBS and 3-D images were taken with the appropriate filter. Only the images corresponding to the equatorial plan of the cells were used to visualize cellular distribution.

4.3.10 Molecular modelling

Molecular modeling was performed with the MOE software package using PARP and EGFR corresponding inhibitors bound to the corresponding protein structure available at the Protein Database (PDB) as templates.

4.3.11 Statistical analysis

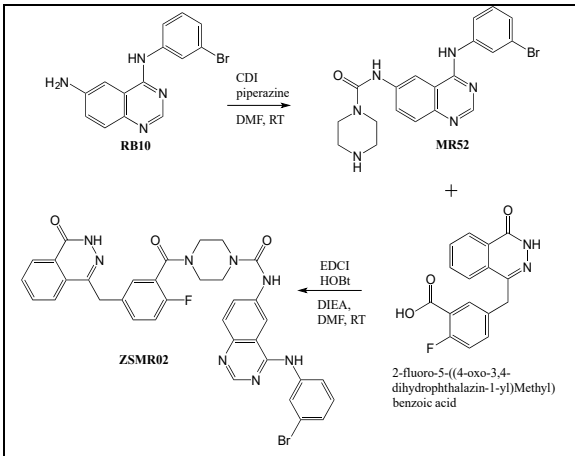
Data were analyzed with Student's two-tailed *t*-test or one-way ANOVA, using GraphPad Prism 5.0 software (GraphPad Prism, San Diego, CA). $P < 0.05$ was defined as statistically significant.

4.4 RESULTS

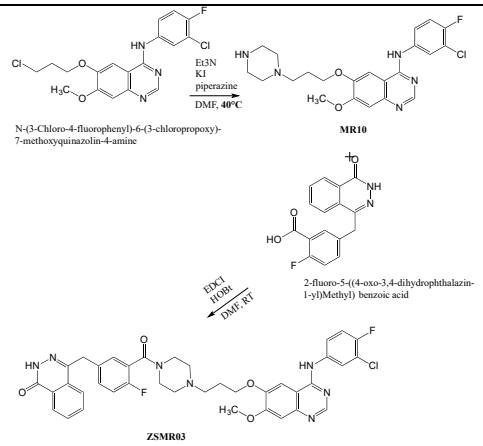
4.4.1 Chemistry

The design and synthesis of the PARP-EGFR combi-molecules were performed according to the type II principle, with a non-hydrolysable linker bridging the two targeted warheads. As depicted in figure 1, type I combi-molecules are designed to be hydrolyzed to release the two targeted species, whereas type II possess two warheads linked by a non-hydrolyzable spacer. The synthesis of the different type II combi-molecules proceeded by forming the amine tagged to the quinazoline moiety and using peptide coupling to link the two moieties. The first molecules synthesized was

ZSMR02, a combi-molecule with no linker, the second without a piperazine carboxamide ZSMR03, and the others ZSMR04, 06 containing like olaparib a carboxamide moiety, which has been shown to accept a hydrogen bond in the binding site of PARP [28]. As depicted in Scheme 1, the synthesis of ZSMR02 started with the coupling of RB10 with piperazine in DMF to give MR52, which was coupled with 2-fluoro-5-(4-oxo-3,4-dihydrophalazin-1-yl)methyl) benzoic acid (FDMB) to give the desired product. In order to enhance EGFR binding potency, the quinazoline moiety was replaced with a moiety whose structure straddle that of gefitinib. Treatment of N-(3-



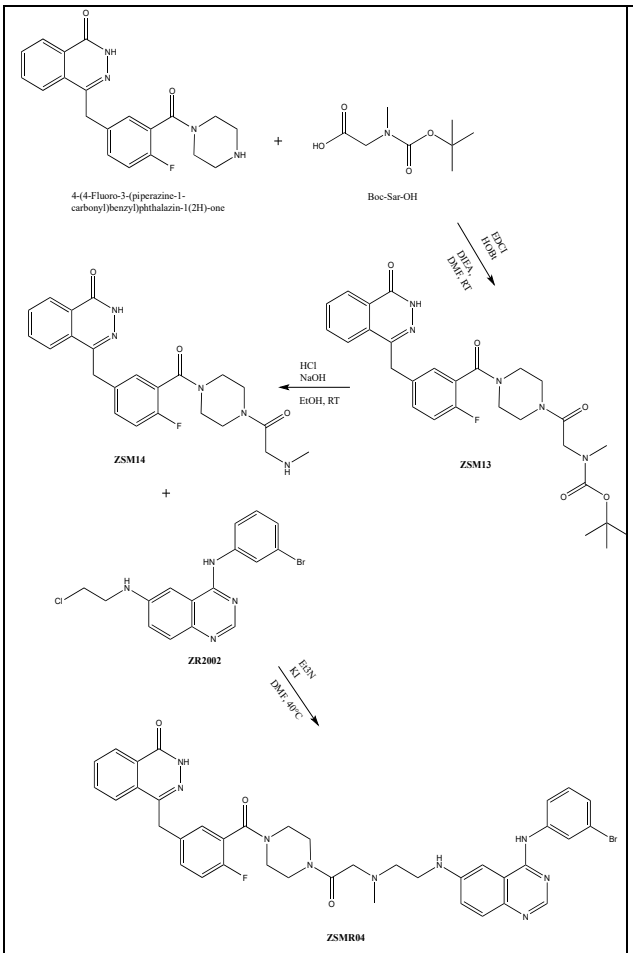
Scheme 1. Synthesis of ZSMR02



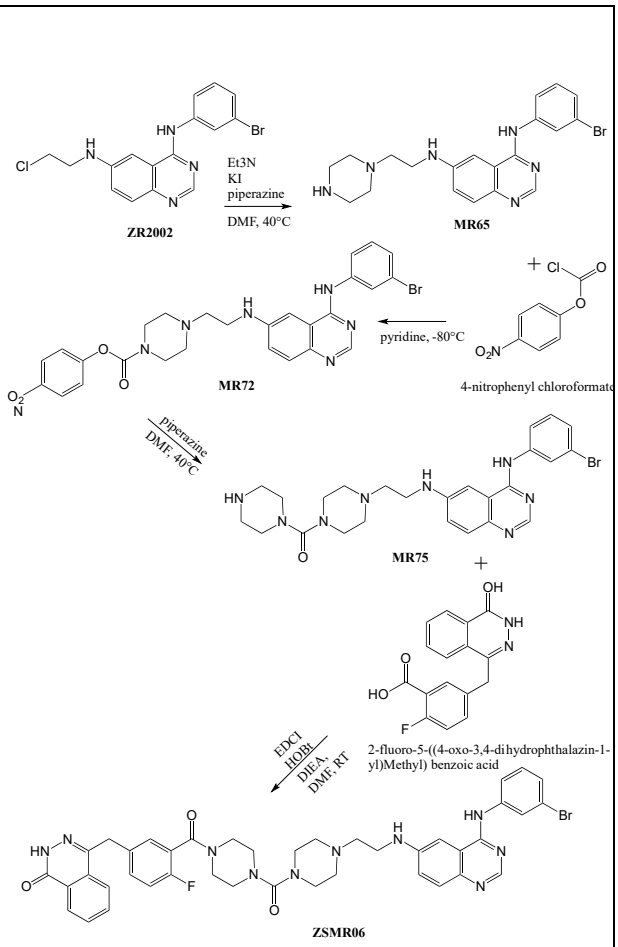
Scheme 2. Synthesis of ZSMR03

chloro-4-fluorophenyl)-6-(3-chloropropoxy)-7-methoxyquinazolin-4-amine with piperazine gave MR10, which was coupled with FDMB to give ZSMR03 as in Scheme 2. Extension of linker length was achieved using ZR2002, a chloroethylaminoquinazoline previously published by our laboratory [35] to alkylate ZSM14 obtained as outlined in Scheme 3, through coupling of FDMB with Boc-Sar-OH and deprotection of the BOC-protected ZSM11. The synthesis of ZRMS06 proceeded as depicted in Scheme 4, starting with the mono-alkylation of ZR2002 to give MR65,

which was coupled with 4-nitrophenyl chloroformate to afford MR72. Treatment with piperazine led to the urea MR75, which was coupled with FDMB to give ZSMR06 with an extended spacer.



Scheme 3. Synthesis of ZSMR04



Scheme 4. Synthesis of ZSMR06

4.4.2 PARP inhibitory potency

(a) In vitro assay

The PARP inhibitory potency of the combi-molecules was examined in a colorimetric PARP assay in histone-coated strip wells. All compounds were capable of inducing a dose-dependent inhibition of PARP with IC_{50} values in the 15-45 nM range. ZSM03, 04, 06 showed identical PARP

inhibitory potency, indicating that the presence of the piperazine carboxamido group did not play a significant role in their potency. The most potent agent with the closest IC_{50} value when compared with olaparib was ZSM02, which contained the carboxamido group but deprived of a liker (Fig 2).

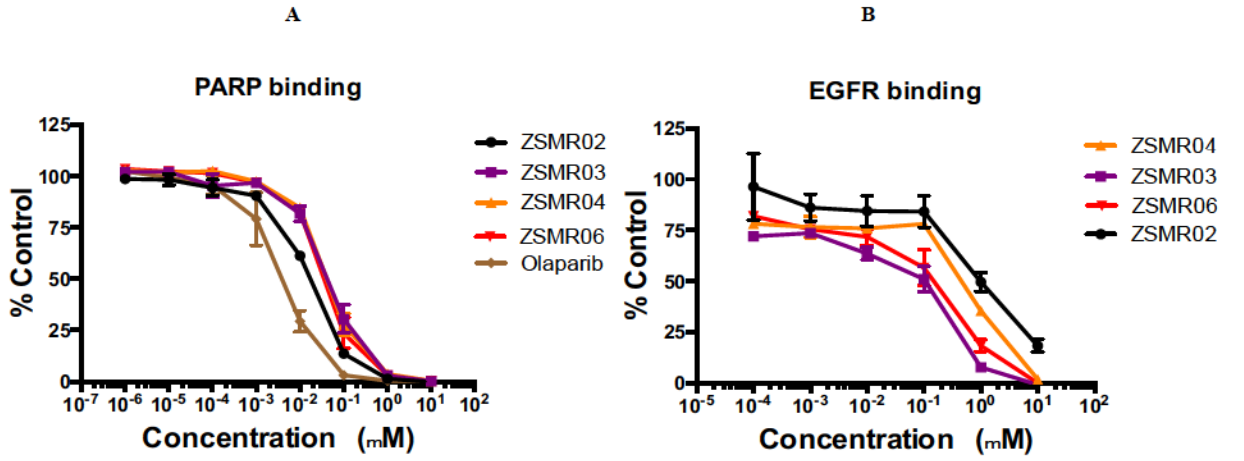


Figure 2. Dual EGFR-PARP targeting by the ZSMR series. (A) Inhibition of PARP activity by the ZSMR series using the Trevigen PARP assay kit, as described in Material and Methods, (B) Dose response curves obtained from a modified ELISA using the phosphorylation of polyglutamate-tyrosine by commercially available purified EGFR and detection with an anti-phosphotyrosine antibody.

(b) Whole cell PAR assay

A western blot detection of poly-ADP ribose chain was performed in whole cells. While the assay did not permit a clear distinction between the potencies of individual compounds, it confirmed that all the combi-molecules could strongly inhibit the formation of poly-ADP ribose chain in the cells, which is consistent with the strong PARP inhibitory potency observed in the enzyme assay (Fig. 3).

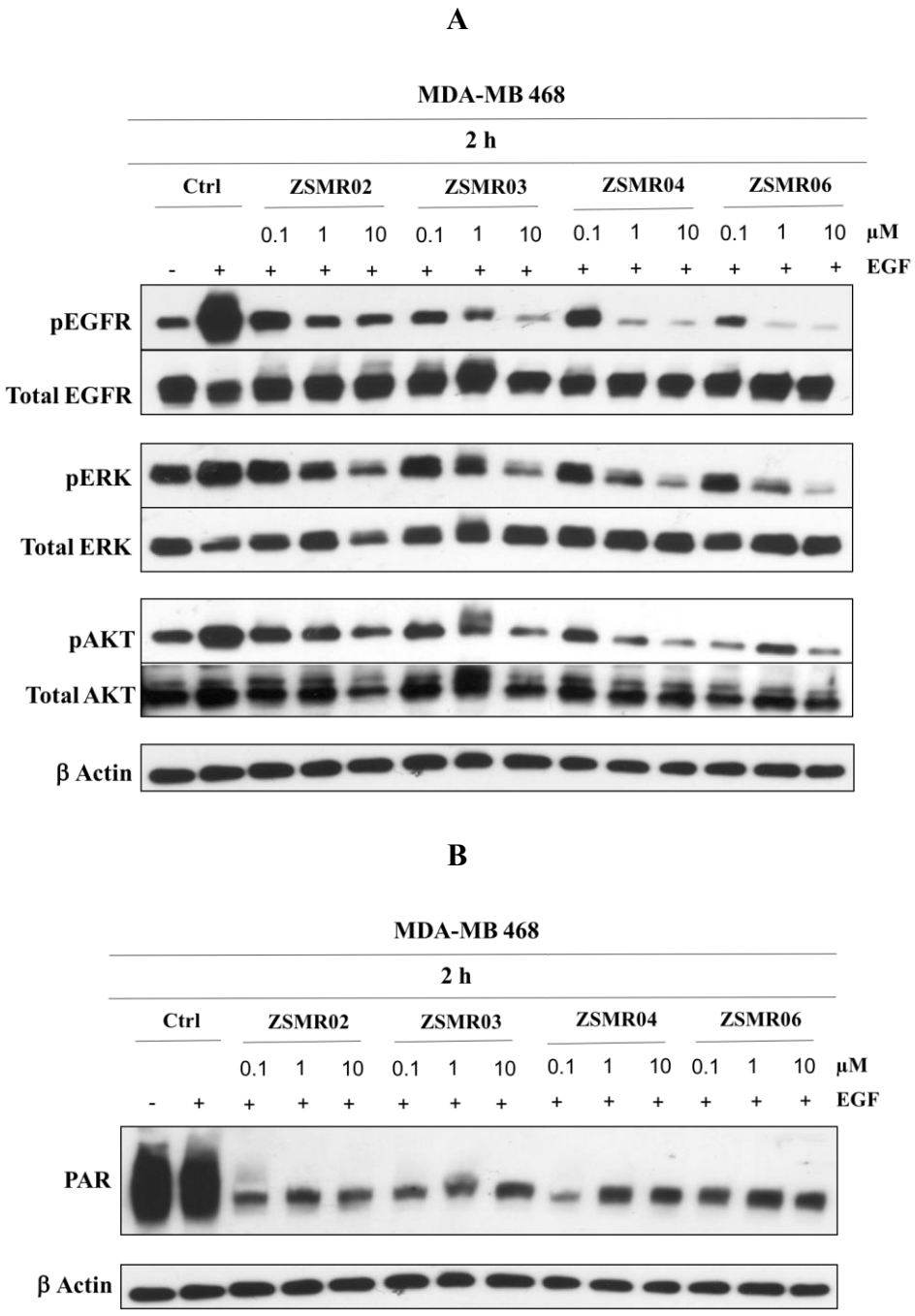


Figure 3. Dual targeting of PARP-EGFR in whole cells. (A) Inhibition of phosphorylation of EGFR and downstream signaling proteins Erk and Akt. (B) Inhibition of PARP activity as determined by the detection of poly-ADP ribose chain (PAR) formation using an anti-PAR antibody.

(c) PARP trapping

PARP trapping assay showed a dose dependent increased in chromatin bound PARP, indicating that ZSMR06 is capable of inducing PARP DNA trapping. As expected, the level of PARP trapping was similar to that of olaparib.

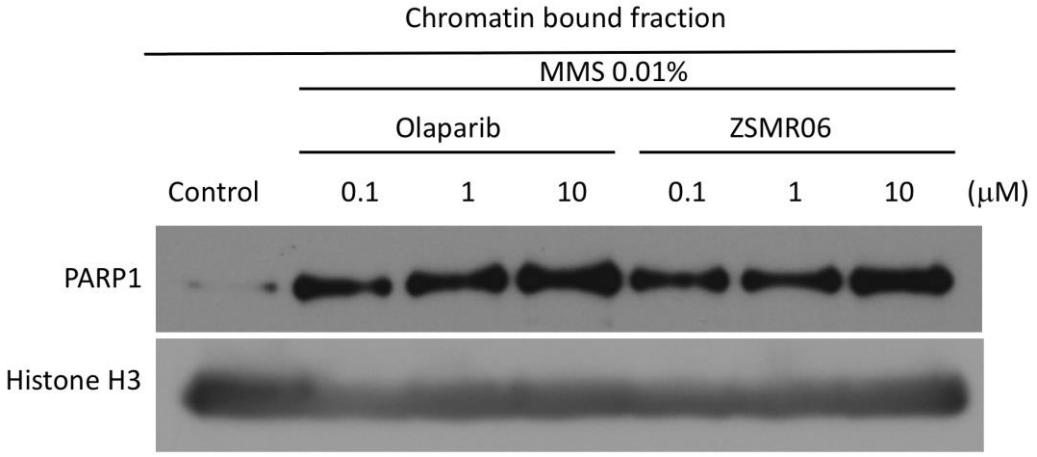


Figure 4. Dose dependent formation of PARP-DNA trapping by olaparib and ZSMR06 in human leukemic lymphoblast CCRF-CEM cells.

4.4.3 EGFR inhibitory potency

(a) Kinase assay

The ability of each agent to inhibit EGFR tyrosine kinase activity was tested in a modified ELISA. All combi-molecules induced a dose-dependent inhibition of EGFR, with IC_{50} values in the 22-860 nM range. ZSM02 that showed the strongest PARP inhibitory activity, was the weakest inhibitor of the series and ZSMR03 or 06 the strongest EGFR inhibitors with IC_{50} in the nanomolar range (Fig. 3).

(b) EGFR phosphorylation and downstream signaling

Inhibition of EGF-induced EGFR phosphorylation was studied in the MDA-MB468 breast cancer cell line. All compounds induced a dose dependent inhibition of EGFR phosphorylation, the most potent being ZSMR04 and 06. Approximately 100% inhibition was seen at 1 μM and this translated into inhibition of phospho-Erk1/2 and phospho-AKT (Fig. 3).

4.4.4 Growth inhibitory profiles

The combi-molecules being dual EGFR-PARP targeting molecules, we compared their potency with that of olaparib in two isogenic pairs of cell lines: VC8 (BRCA2 mutant) and VC8-BRCA2 wild type transfectant or NIH3T3 wild type and NIH3T3-EGFR (transfected with EGFR). Like olaparib, ZSMR02, 03, 04 and 06 showed selective potency against the EGFR transfectant. Likewise, they selectively killed the VC8 (BRCA2 mutant) cells, which is consistent with their strong PARP inhibitory potency (Fig. 5). Interestingly, olaparib despite being a single PARP targeting agent showed selective potency against the EGFR transfectant although its potency against the latter cells was considerably weaker when compared with the combi-molecules (Table 1).

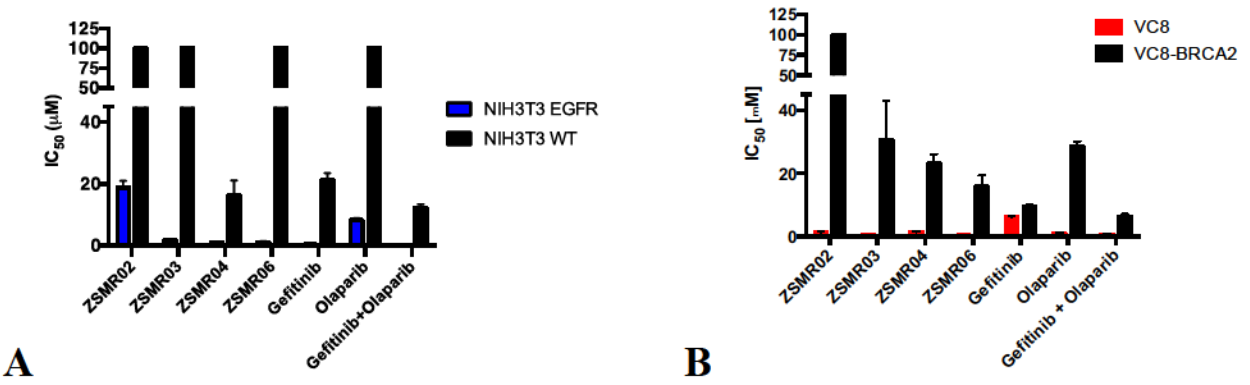


Figure 5. Growth inhibition profile, in isogenic models, comparing the selectivity of individual treatments for two clinical biomarkers: BRCA1/2 and EGFR. **A**, ZSMR02, 03, 04 and 06, similar to gefitinib, the clinical EGFR inhibitor, showed selective potency against the EGFR-transfected cell line, NIH3T3. **B**, ZSMR02, 03, 04 and 06, similar to olaparib, a clinical PARP inhibitor, showed selective potency against the BRCA2 mutant cell line, VC8.

Table 1. Potency of ZSMR combi-molecules on EGFR-proficient and deficient cell lines (IC_{50} μM)

	VC8	VC8-BRCA	Fold selectivity	NIH3T3 WT	NIH3T3 EGFR	Fold selectivity	MDA-MB453	MDA-MB231	MDA-MB468
EGFR status	-	-		-	+++		+	++	++++
ZSMR02	1.37	>100	>50*	>100	19 ± 2.2	>50*	N/A	N/A	N/A
ZSMR03	0.6	70.4	117	>100	1.2 ± 0.03	>50*	4.3 ± 0.31	3 .6 ± 0.31	0.14 ± 0.02
ZSMR04	0.8	27.9	35	16 ± 4.9	1.8 ± 0.27	8.8	5.6 ± 0.56	5.7 ± 0.46	0.37 ± 0.07
ZSMR06	0.55	14.7	27	>100	1.2 ± 0.28	>50*	>100	3.8 ± 0.56	0.031 ± 0.006
Gefitinib+Olaparib	0.95	7.6	8	12 ± 1.1	0.28 ± 0.03	42	5.3 ± 0.89	15 ± 0.60	1.9 ± 0.19
Gefitinib	6.2	10.4	1.7	21 ± 2.3	0.41 ± 0.08	51	7.3 ± 0.88	21 ± 1.2	9.7 ± 0.42
Olaparib	0.97	28.1	29	>100	8.3 ± 0.57	>50*	52 ± 10	79 ± 14.1	4.0 ± 0.29

*Where IC_{50} is determined to exceed 100 μM, the fold selectivity number is set to greater than 50

Further analysis in three established triple negative breast cancer cells with moderate sensitivity to gefitinib showed that the combi-molecules were extremely potent with activities superior to that of olaparib or gefitinib alone and their corresponding equimolar combination (i.e. gefitinib + olaparib) in MDA-MB231 and MDA-MB468. ZSMR06 appeared as the most potent agent, with IC_{50} against MDA-MB468 in the nanomolar range. Their potency appears to increase with the EGFR levels in these cells (MDA-MB468 > MDA-MB231 > MDA-MB453). ZSMR06 induced its strongest potency against MDA-MB-468, which is known to express extremely high levels of EGFR (Fig. 6A and 6B). Similarly, ZSMR06 showed superior potency when compared with the combination of gefitinib + olaparib in a panel lung, prostate, brain, colon, breast cancers and melanoma (Fig. 6C).

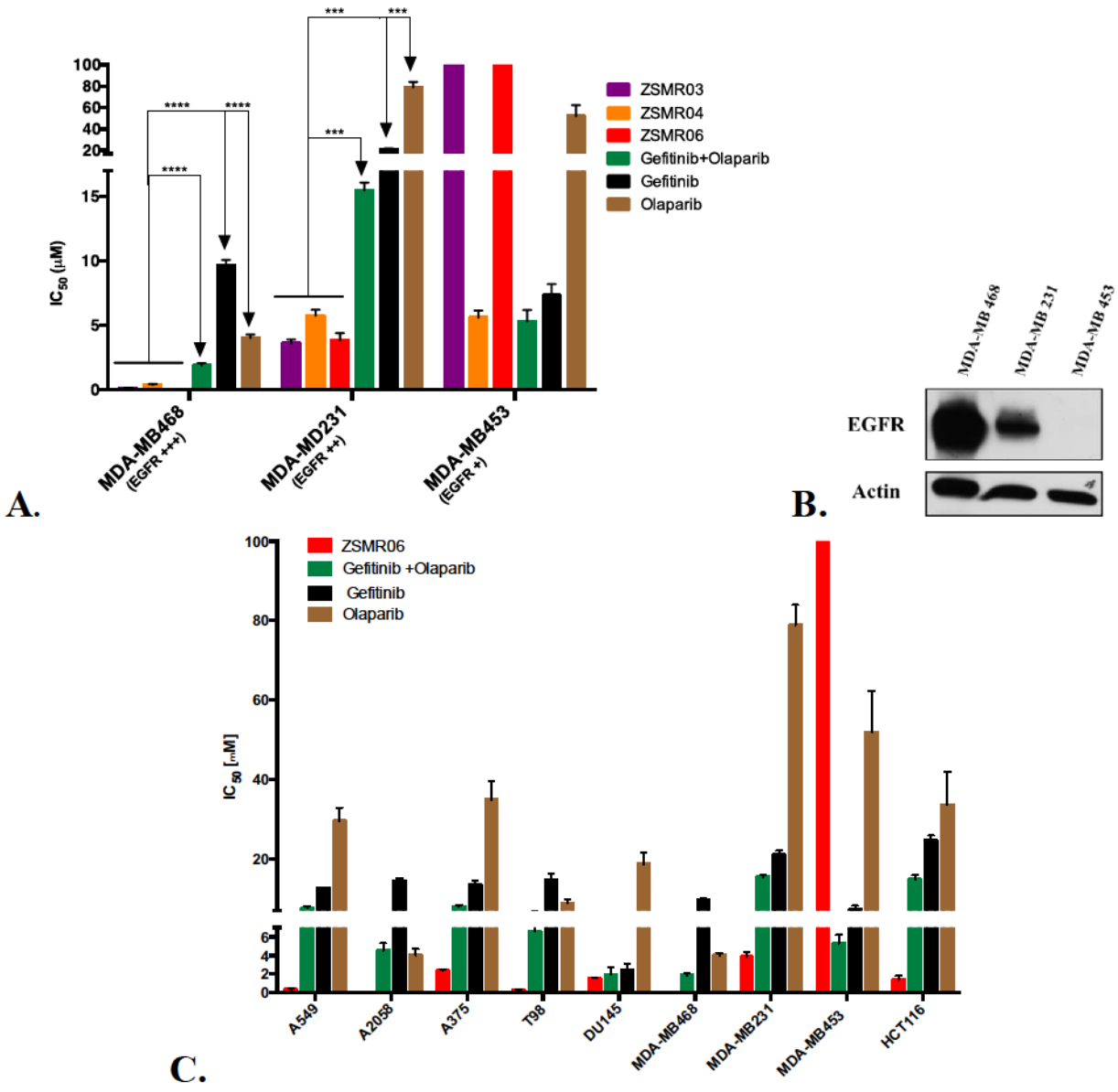
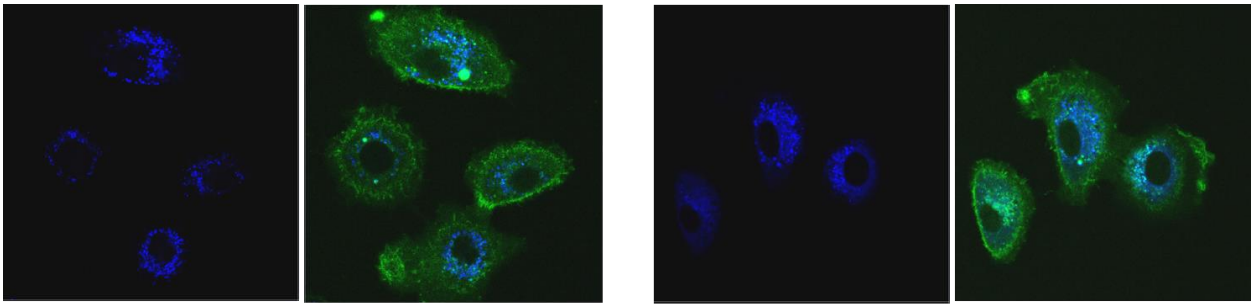


Figure 6. A. Growth inhibition as shown by IC_{50}s, where ZSMR06 shows high potency in growth inhibition when compared to gefitinib, olaparib and gefitinib+olaparib in a panel of EGFR positive cell lines. **B.** Western blot analysis showing EGFR levels of the three triple negative breast cancer cell lines. **C.** Strong inhibitory potency of ZSMR06 in comparison to the combination of gefitinib and olaparib in a panel of solid tumours expressing EGFR.

4.4.5 Fluorescence imaging and modulation of nuclear protein expression

(a) subcellular distribution

ZSMR06 being fluorescent molecule, we compared its subcellular distribution with that of RB10, a small molecular weight and potent inhibitor of EGFR [36]. The results showed that the blue fluorescence associated with ZSMR06 molecule was like for RB10 primarily localized in the perinuclear region (Fig. 7).



ZSMR06 **RB10**

Figure 7. Subcellular distribution of ZSMR06 and RB10, an experimental EGFR inhibitor, in the lung cancer cell line, A549, transfected with GFP-labeled EGFR (green). Both drugs colocalize with EGFR in the perinuclear region after 2h of treatment.

(c) Immunofluorescence of PARP1 and γH2AX

Immunofluorescence staining of PARP1 in the triple negative breast cancer cell line showed that it was well localized within the nucleus regardless of treatment conditions and cell line. Staining with an anti-γH2AX antibody revealed the presence of γH2AX foci in ZSMR06-treated cells (1 μM). Amongst the three cell lines, the levels were most abundant in the MDA-MB-468 cells.

(d) Modulation of BAP1, BARD1 and BRCA1

BAP1, BARD1 and BRCA1 levels were analyzed 16h following cell exposure to ZSMR06 and gefitinib at both 1 μM. ZSMR06 could down-regulate BAP1 (Fig. 9), a ubiquitin hydrolase that binds to BRCA1. It also down-regulated BRCA1 and while concomitantly inducing γH2AX foci (Fig. 8), indicating that the mechanism of action of this compound may be based on its ability to enhance vulnerabilities in these cells.

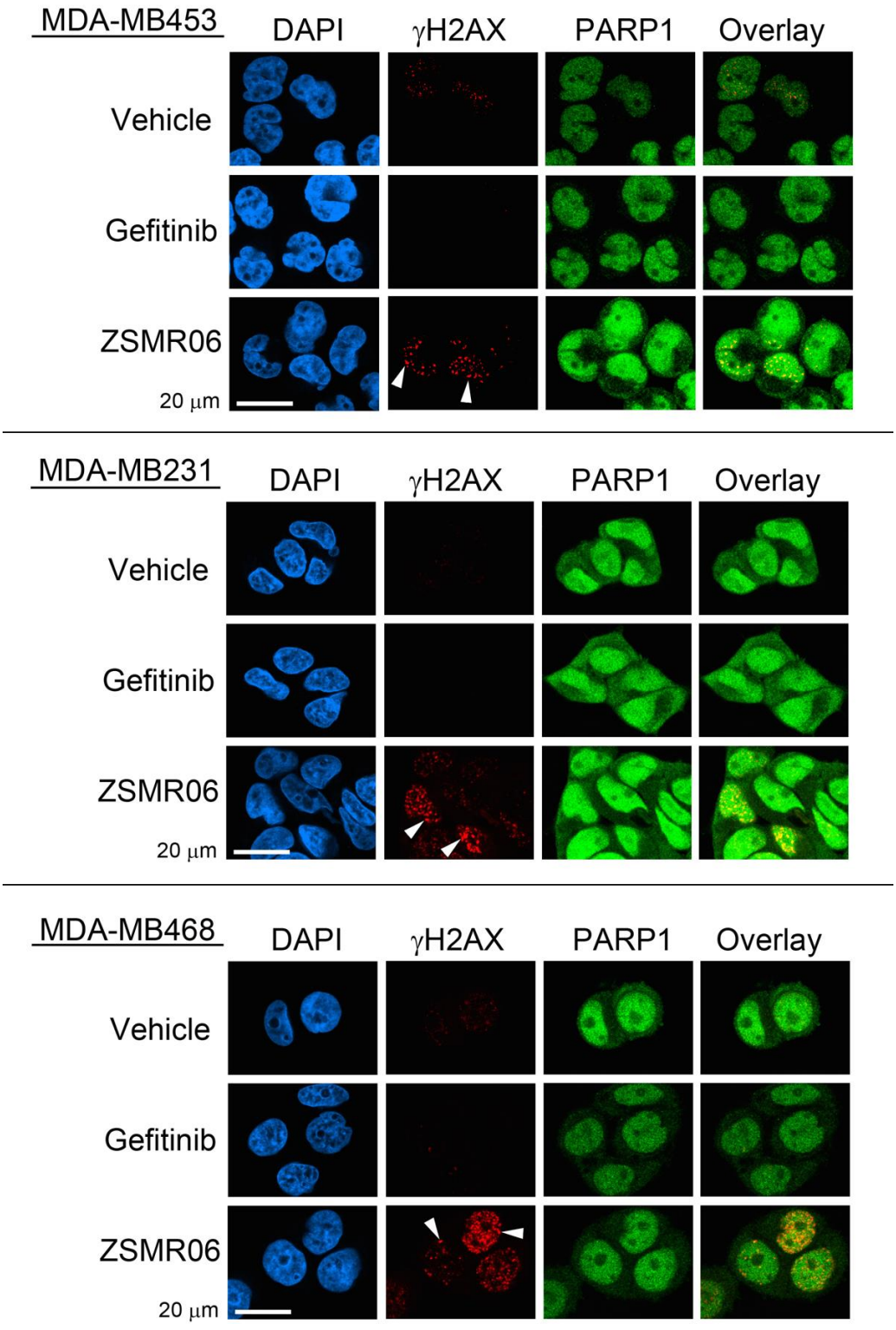


Figure 8. Immunofluorescence staining of PARP1 and γH2AX using DAPI as nuclear counterstaining agent. Formation of γH2AX foci was detetected in the cells treated with ZSMR06.

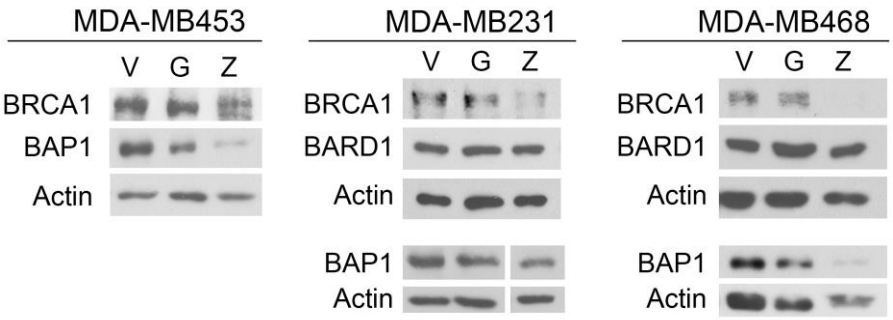


Figure 9. Down-regulation of BAP1 and BRCA1 induced by ZSMR06 in 3 triple-negative breast cancer cells. Complete down-regulation was seen in MDA-MB-468 the most sensitive cell line to ZSMR06 (growth inhibition IC_{50}, 31 nM). V, vehicle; G, Gefitinib; Z, ZSMR06

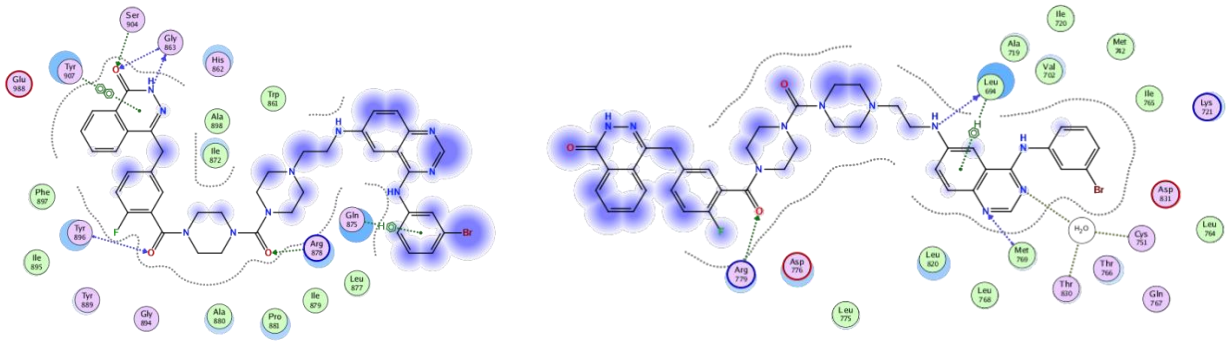
4.5 Discussion

EGFR is one of the most studied cancer targets of the past decade. Its overexpression in many solid tumours including breast, ovarian, prostate, brain, colon, etc. raised the prospect of a broad spectrum of application for anti-EGFR therapy. However, while several EGFR inhibitors and a few anti-EGFR antibodies were approved for clinical use, only a subset of patients with the specific mutations (i.e. L858R) can benefit from treatment with EGFR inhibitors in lung cancer [37, 38] and anti-EGFR antibodies are used in the treatment of a small group of advanced colon cancer patients whose tumours do not carry *ras* mutations [39]. Likewise, given the widespread use of cytotoxic therapy in the clinic, the demonstration that PARP inhibitors could sensitize resistant tumours to DNA damaging agents [18] and also induce selective cytotoxicity against BRCA mutant tumours held great promise [5, 6]. However, olaparib the first PARP inhibitor approved by the FDA, received a limited approval for the treatment of ovarian cancer with germline BRCA1/2 mutations [26], thereby limiting its use to a small percentage of cancer patients. Interestingly, studies establishing a relationship between EGFR, PARP, BRCA have now raised the prospect of expanding the use of PARP inhibitors to a broad spectrum of EGFR-expressing tumours [12, 24,

25]. Indeed, Nowsheen *et al.* [12, 40], reported that the combination of EGFR inhibition, either using the small molecules inhibitor lapatinib or the antibody cetuximab, and the PARP inhibitor ABT888, showed an increase in cytotoxicity. This is associated with the ability of EGFR inhibition to downregulate DNA double strand repair, while PARP inhibition was further enhancing DNA repair deficiency. Here we successfully optimized dual EGFR-PARP targeting molecules, giving rise to ZSMR06, the first single molecule with balanced EGFR-PARP targeting ever reported. Importantly, the combi-molecule consistently showed superior potency when compared with combinations of single molecules in many cell lines.

The ZSMR series of combi-molecules were designed according to a type II approach, indicating that they do not require hydrolysis to release their bioactive species [16]. Thus, optimization for balanced targeting of the intact structure was required. ZSMR02, the first molecule synthesized, showed strong PARP inhibitory potency but little effect on EGFR phosphorylation. Inserting a basic linker between the PARP and EGFR targeting warheads led to agents (e.g. ZSMR03-06) with more balanced targeting properties, perhaps due to the ability of the linker to shift each targeting moiety away from its cognate binding pocket. Indeed, as depicted in figure 10, molecular modelling showed that the olaparib moiety is pointed away from the ATP binding site of EGFR and a similar orientation is observed for the quinazoline moiety when the olaparib warhead is bound to PARP. ZSMR06 was able to achieve nanomolar potency against PARP and nanomolar IC_{50} value against EGFR in *in vitro* assays and in whole cells. Despite its size (MW>500), ZSMR06 and analogues could penetrate the cells and block EGFR autophosphorylation and PAR formation. The ability of ZSMR06 to down-regulate signaling downstream of EGFR signaling (e.g. phospho-Erk1/2, phospho-AKT) and to block PAR formation in the cells are strong evidence of its dual EGFR-PARP targeting properties.

Importantly, isogenic cell selectivity study was required to verify whether the observed target modulation could lead to selective growth inhibition. Indeed, the combi-molecules showed significant selectivity for the NIH3T3-EGFR transfectant and for the VC8 (BRCA2 mutant) cells in an isogenic context. It is also important to note that olaparib, a single targeted agent deprived of EGFR targeting property, showed selectivity for the NIH3T3-EGFR transfectant. This can be due to the reported negative correlation between EGFR and BRCA1 levels [24, 25]. It is to be noted that the potency of the combi-molecules was significantly superior to that of olaparib in the EGFR transfectant and also increased with the EGFR content of the established cell lines. MDA-MB-453, the only cell line found resistant to ZSMR06 is known to exhibit low expression of EGFR [41, 42]. In an extended panel of cell lines including lung, melanoma and colon cancer cell types, the growth inhibitory potency of ZSMR06 was significantly superior to that of the combination of olaparib+gefitinib, indicating an exquisitely strong potency of the single molecule. Although the exact implication of the perinuclear distribution of the combi-molecule in its potency remains to be elucidated, its superior activity may be functionally associated, as previously proposed by Nowsheen *et al.* [12], with interplays between EGFR, BRCA1 and PARP in these cells. To verify this hypothesis, we focused our molecular analyses on the balanced EGFR-PARP targeting combi-molecule ZSMR06 in the panel of triple negative breast cancer cells. Indeed, in addition to being able to down-regulate EGFR downstream signaling, it could induce nuclear γH2AX foci and down-regulation of BRCA1 in these cells.



Binding to PARP-1 Binding to EGFR

Figure 10. Mode of binding of ZSMR06 to the ATP site of EGFR and to the NAD binding site of PARP1.

Interestingly, while the levels of BARD1 [43], a BRCA1-associated protein in the cells remained constant, ZSMR06 down-regulated BAP1, which is a ubiquitin hydrolase responsible for deubiquitination of BRCA1 [44]. This may favour sustained ubiquitination and subsequent degradation of BRCA1 [45], while ZSMR06, perhaps due to its strong PARP-DNA trapping potency, is inducing DNA damage in the cells as evidenced by the formation of γH2AX foci [46]. This vulnerability may be further exacerbated by the proven ability of EGFR inhibition to down-regulate HR and NHEJ [47, 48] in these cell lines. Although the significance of the perinuclear distribution of the combi-molecule remains to be elucidated, strong inhibition of EGFR phosphorylation may prevent its intranuclear translocation where it is known to play a role in NHEJ [48, 49]. Therefore, the combi-molecule may induce an array of events including down-regulations of the MAPK and AKT pathways through blockade of EGFR, inhibition or down-regulation of DNA repair proteins and induction of DNA damage. This array of events may synergize to promote growth inhibition and death in cells overexpressing EGFR.

Conclusion

In summary, we described herein the design and synthesis of the first PARP inhibitors targeted to EGFR, confirmed their dual targeting potency in enzyme assays and demonstrated their ability to modulate their intracellular targets. Studies with the most balanced combi-molecule ZSMR06, demonstrated its ability to induce selective growth inhibition in both BRCA2 and EGFR-expressing isogenic cells and to inhibit or downregulate DNA repair proteins. To our knowledge, ZSMR06 is the first pharmacological modulator of BRCA1 through the downregulation of BAP1. This unique mechanism of action would allow the possible use of this PARP inhibitor, ZSMR06, beyond BRCA1/2 mutations. It is to be noted that while PARP inhibitors are primarily studied in the context of sensitizing tumours to DNA damage, the optimized combi-molecule herein exert exquisitely strong growth inhibitory potency alone, without a cytotoxic functionality. Further studies are required to demonstrate whether they can sensitize cells to DNA damaging agents in an EGFR-dependent fashion.

4.6 References

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4.7 Supplementary material

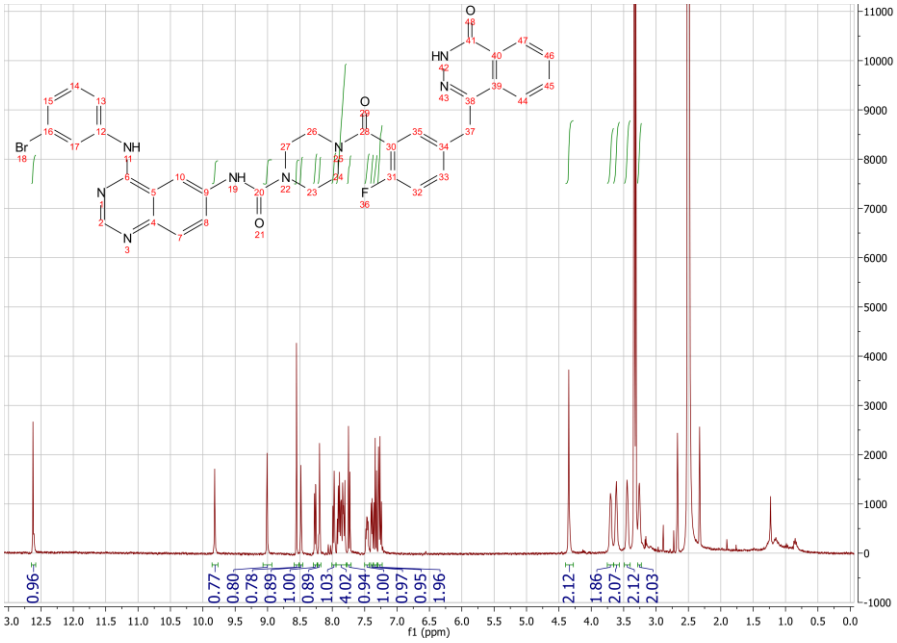

Figure S1. 1H NMR spectrum of ZSMR02

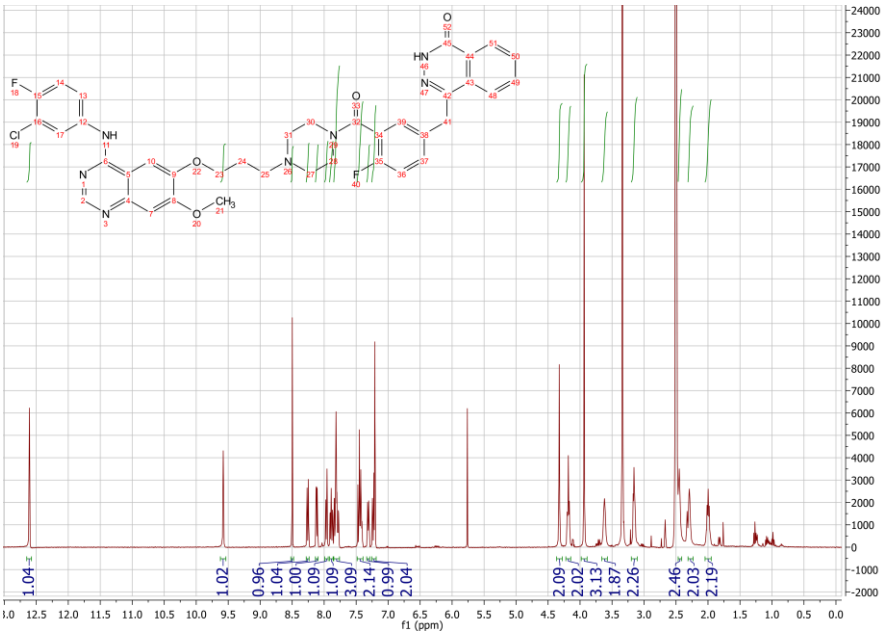

Figure S2. 1H NMR spectrum of ZSMR03

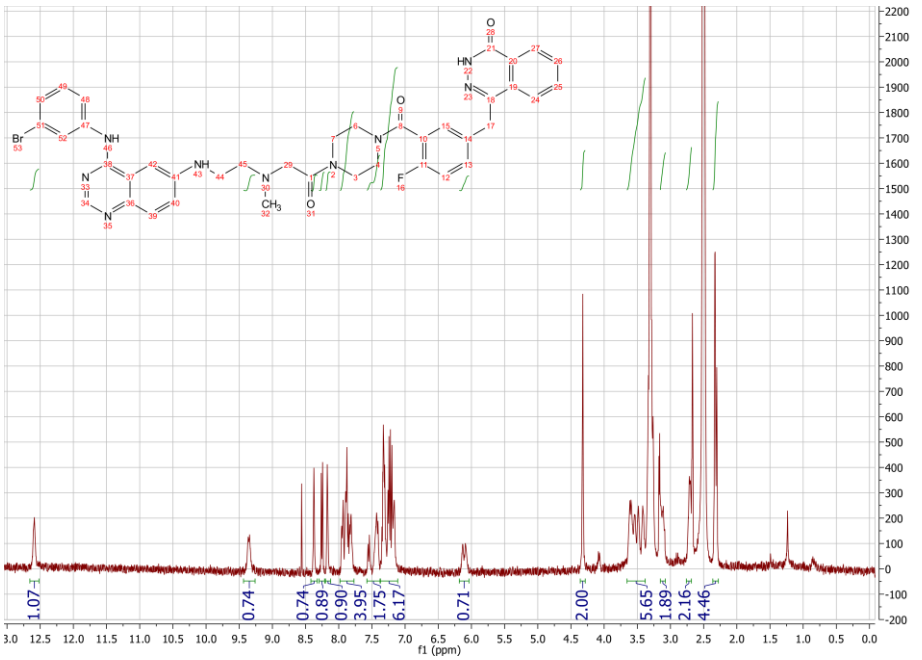

Figure S3. 1H NMR spectrum of ZSMR04

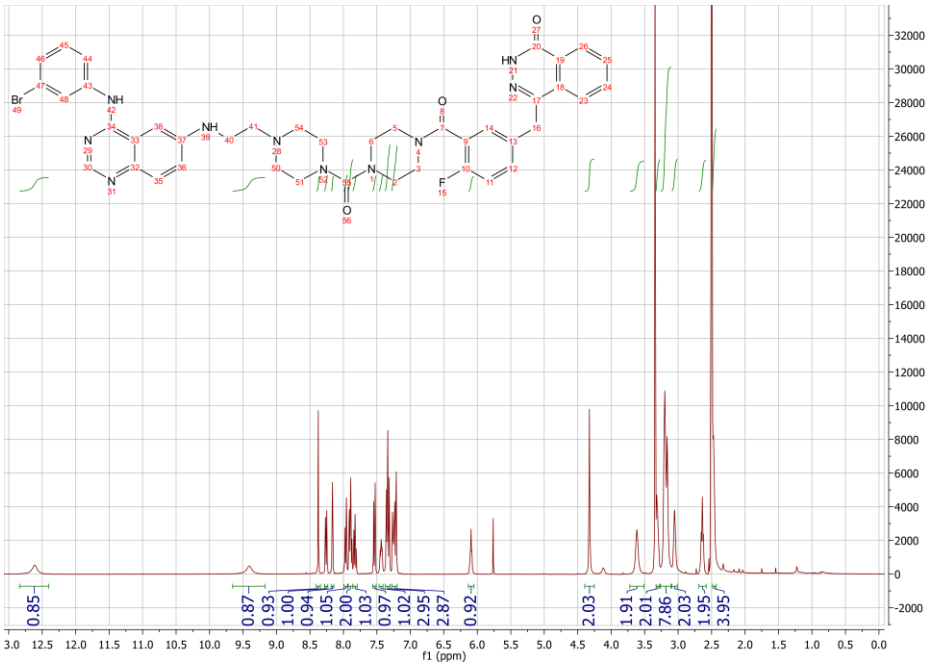

Figure S4. 1H NMR spectrum of ZSMR06

CHAPTER 5: DISCUSSION AND CONTRIBUTIONS TO KNOWLEDGE

In the advanced stages, many cancers are characterized by the overexpression of receptor, redundant or compensatory signaling pathways [1], resistance to apoptosis and expression of DNA repair enzymes that confer resistance to chemotherapy [2, 3]. The multiplicity of targets in the tumours in the advanced stages represents a tremendous challenge to anticancer drug design. For the past decades, the traditional approach to drug design has been centered on a "one-drug-one-target approach''. According to this approach, drugs were designed to be selective for the target believed to be involved in the etiology of the disease. The high attrition rates in single targeted compounds have triggered a paradigm shift in cancer drug design, leading to a novel approach termed ''polypharmacology'' according to which molecules designed to target multiple pathways are more effective anticancer agents than single targeted ones [4]. Despite the success of multitargeted molecules, the rational design of an agent to block two or more targets remain a daunting challenge. This is due to the intolerance of the structure-activity relationship for substituent alterations. Minor structural changes in one targeting moiety can lead to a loss of binding potency of one or the other. Furthermore, dual targeting sometimes requires modifications that can increase the degree of bulkiness of the molecule, thereby affecting its cellular penetration [5]. Accordingly, the challenge of this thesis was to demonstrate the feasibility of molecules with targets as divergent as PARP and DNA or PARP and EGFR, that can synergistically kill tumour cells through simultaneous blockade or damage of the two targets. Additionally, we also sought to achieve this goal by maintaining these molecules in a range that does not affect their cellular penetration.

DNA repair is a major mechanism of resistance to genotoxic chemotherapy. PARP is one such DNA repair protein that is involved in the repair of damage induced by the clinical drug temozolomide. PARP activity in the BER process is directed at the repair of N7-alkylguanine and

N3-methyladenine, two types of lesions that are efficiently repaired by this pathway [6]. Thus, we surmised that a dual targeted molecule design to block PARP and to damage DNA would be a highly effective agent. Here we demonstrated the feasibility of such a type of molecule, with all the desired characteristics:

1) ability to damage DNA,

2) ability to inhibit PARP,

3) ability to localize into the nucleus.

The first attempt to make the proof of concept of a PARP-DNA targeted molecule led to the design of the first prototype termed ‘’EG22’’ contained a naphthalimide moiety and a triazene tail. Unfortunately, this particular molecule was a short-lived species [7]. Despite the ability of this molecule to damage DNA and block PARP, its suitability as a probe was compromised by its short life. Thus, methods for stabilizing this species were urgently needed. This has become one of the first challenges presented to this thesis. In addition to its instability, EG22 also presented the disadvantage of containing an N3 methyl group that is poorly reactive. Therefore, functionalization of N3 was a risky endeavour. Previous work in the literature showed that the 1,2,3 triazene moiety was an extremely unstable linkage highly temperature- and acid-sensitive that could only be stabilized by reducing electron density on the N3 nitrogen [8, 9]. Thus, we attempted to acetylate the N3 of EG22 in order to reduce electron density at N3, thereby stabilizing the 1,2,3 triazene linkage.

A significant body of evidence has accumulated to suggest that in solution, triazenes exist as two tautomeric forms: one termed conjugated and the other non-conjugated. The conjugated form is known to be more stable than its non-conjugated counterpart [10]. However, we expected that due to the reactivity of the acetyl chloride, both of them could be trapped, although in a different ratio,

thereby leading to two different products. Interestingly, the reaction led to one major product and due to the possibilities described earlier, an unequivocal characterization of this molecule was required. Since we also expected products resulting from nitrogen loss, we thought that the most relevant strategy would be ^{15}N labeling of the central nitrogen N2. This would be achieved through substitution of sodium nitrite in the reaction for its commercially available ^{15}N-labeled form [11]. We discovered that despite the instability of the open chain triazene substrate (EG22) and warming the reaction to room temperature, the resulting product did incorporate ^{15}N. The chemical shift of the latter was in the range of previously reported values for such type of compounds. The structure identification was further investigated by modern NMR sequences such as: HSQC and HMBC, that presented the advantage of leading to the detection of all four nitrogens in the structure with chemical shift values matching those of the literature [12]. Furthermore, the 3D configuration of the molecule was solved by x-ray crystallography. It should be noted herein that crystallization of ZSM02 was a daunting challenge. Optimization of the conditions led to a biphasic method using DMF and hexane, which led to small needles. X-ray crystallography showed co-crystallization with one DMF molecule and hydrogen bonding between the two structures at the amidic NH of the two structures. The N3 acetyl 1,2,3-triazene moiety was coplanar with the naphthalimide backbone indicating a partial double bond character of the N2 and N3 despite the cross-conjugation with the carbonyl acetyl group. Overall the study conclusively demonstrated that despite the instability of EG22, we have discovered a reaction condition to successfully acetylate its poorly reactive N3, leading to a structure unequivocally confirmed to be ZSM02.

Importantly, ZSM02 has proven to be extremely potent in cells expressing MGMT, a clinical biomarker of resistance to temozolomide. ZSM02 was significantly more potent than the single molecules, and its corresponding two-drug combinations (ANI+temozolomide). This is the first

time such levels of differential potency were observed when a single dual targeted molecule was compared to its two-drug counterpart in the field of combi-molecules. Confocal microscopy allowed not only to confirm that the molecule was able to diffuse into the cells but also to be localized into the nucleus. This observation allowed us to propose a mechanism for the strong superiority of the combi-molecule over the two-drug combination, which is depicted in figure 1. The nuclear localization of ZSM02 is in agreement with studies by Banerjee *et al* [13] who showed the ability of PARP-4-ANI to intercalate into the DNA. Indeed, the model below (Figure 1), illustrates the mechanism underlying the superior potency of the molecule when compared to the combination of the two individual drugs. The mechanism is primarily based on the fact that the localization of the molecule into the nucleus can lead to a ‘’bystander like effect’’ whereby the nucleus-localized combi-molecule releases the methyldiazonium species *in situ*, thereby concentrating the damage into the nucleus. Indeed, anomalously high levels of DNA damage were observed when cells were exposed to the combi-molecule. This has led to the discovery of perhaps a novel strategy to overcome the intrinsic resistance of tumour cells to DNA methylating drugs. The principle should be that: for a combi-molecule to show significantly higher activity than its two-drug combination counterpart, it must aid in localizing the action of its two arms into cellular compartments where the primary targets can be reached. Indeed, our result is in agreement with previous work published on the principle of combi-targeting led to the conclusion that perinuclear localization of combi-molecules, where they can bind to their EGFR target and subsequently diffuse to the nucleus, was the basis of their mechanism of action, especially when compared with their single drug counterpart [14, 15]. Here, the fact that the PARP targeting element of the molecule was fluorescent, allowed us to clearly map its nuclear localization, where both its DNA damaging and PARP inhibitory components exerts their biological activity.

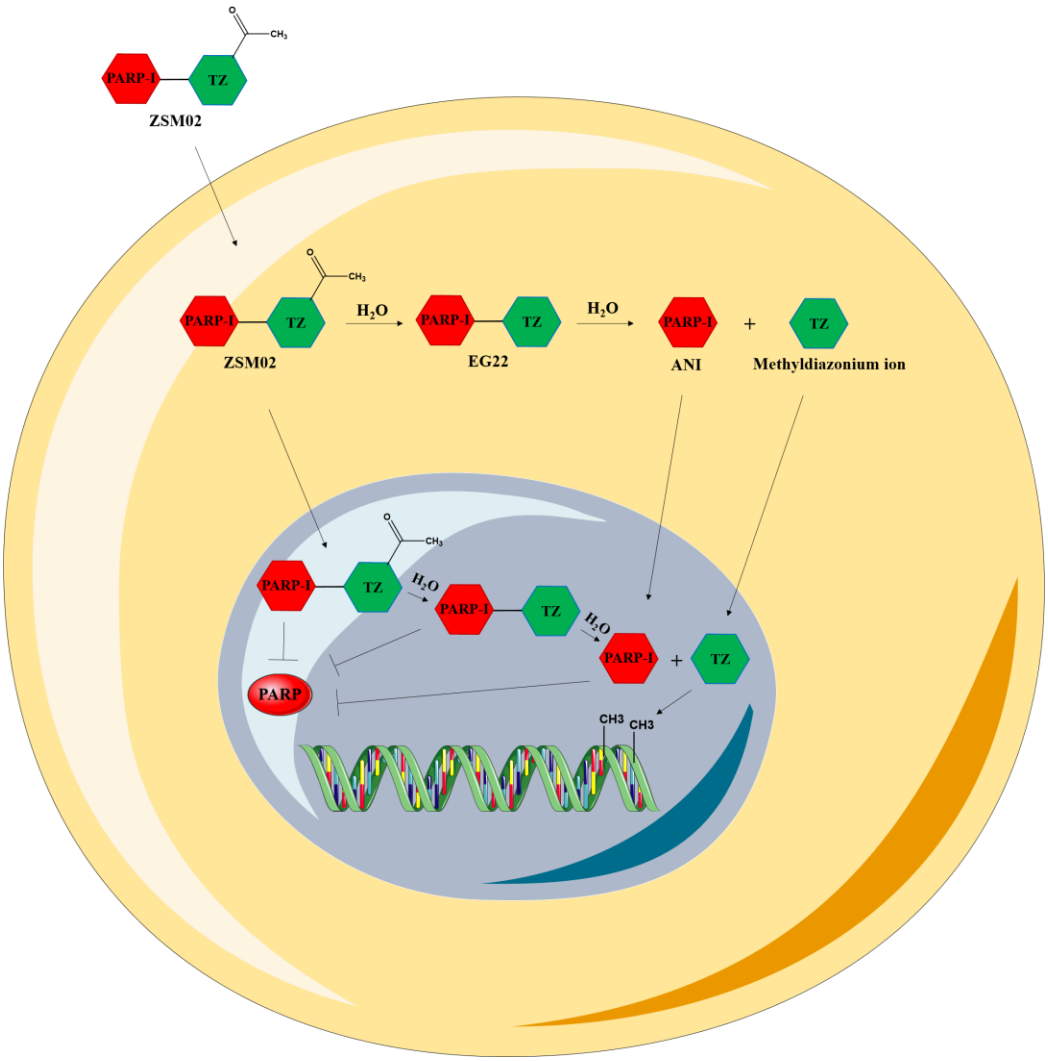

Figure 1. The proposed mechanism of action of ZSM02. ZSM02, the prodrug of EG22, is a combi-molecule with a PARP inhibitory warhead and DNA damaging species. In its intact form, it diffuses through the membrane to subsequently hydrolyze in the cytoplasm and the nucleus, to give EG22, and the PARP inhibitor (PARP-I) and the DNA-damaging agent (Tz). PARP-I may then in turn diffuse into the nucleus and either intercalate into the DNA or inhibit PARP. ZSM02 may also diffuse in its intact form toward the nucleus, intercalate into DNA prior to being converted to EG22, PARP-I, and Tz.

While dual-targeting of PARP and DNA has proven highly effective in vitro, concerns about the toxicity of this approach was a major deterrent in its further development. Indeed, in light of clinical trials demonstrating the toxicities of combinations of PARP inhibitors and DNA damaging agents [16-20], we thought of conferring a targeting element to the approach. Thus, we decided to expand our strategy to the targeting of PARP inhibitors to EGFR, a receptor that is overexpressed in wide range of tumour types including, breast, ovarian, lung, prostate, etc [21]. Work under this strategy led to the discovery of an optimized structure with balanced PARP-EGFR targeting potency, ZSMR06. Both in vitro assay and molecular modeling confirmed the ability of the two moieties of the combi-molecule to bind to their individual targets (i.e. EGFR ATP pocket and PARP NAD+ binding pocket) suggesting that the molecule was optimized to achieve a type of linker between the two moieties that did not hinder their individual potency. The optimized dual potency translated into unique biological properties: a) the first single molecule capable of inducing tandem PARP and EGFR inhibition, leading to superior potency compared to the combination of clinical inhibitors, b) the first single molecule to induce selective targeting of BRCA2 mutation and EGFR overexpression in an isogenic context, c) the first single molecule observed to significantly downregulate BRCA1, and simultaneously inhibiting PARP. Studies on our optimized combi-molecule ZSMR06 are consistent with a possible synthetic lethality between EGFR and PARP inhibition. Indeed, we believe that the mechanism underlying the strong potency of ZSMR06 is its ability to block PARP, EGFR and modulate BRCA1 levels through the downregulation of BAP1, a ubiquitin hydrolase that binds to BRCA1. ZSRM06 induced highly significant levels of downregulation of BRCA1, indicating that the combi-molecule on its own is capable of modulating two DNA repair proteins (e.g. PARP and BRCA1), in addition to robustly block EGFR mediated signaling. We believe that this pleiotropic effect culminated into significant

growth inhibition potency in tumour cells with not only BRCA2 mutation, but also in BRCA1/2 WT, and distorted expression of EGFR. Therefore, this is the first evidence of a novel strategy that can expand the use of PARP inhibitor beyond BRCA1/2 mutation.

Overall, this thesis has contributed to the demonstration of the feasibility of combi-molecules targeted to DNA and PARP, with selective potency for BRCA2 mutant cells. These novel PARP targeted molecules overcame resistance to temozolomide in MGMT expressing cells. The latter molecules showed superior activity when compared to classical combinations, and this is believed to be due to their ability to localize into the nucleus and release their DNA damaging species *in situ*. Importantly, we demonstrated the feasibility of the first class of molecules capable of targeting a PARP inhibitor to EGFR expressing cells. Work with the latter molecules is consistent with a possible synthetic lethality between EGFR and PARP inhibition.

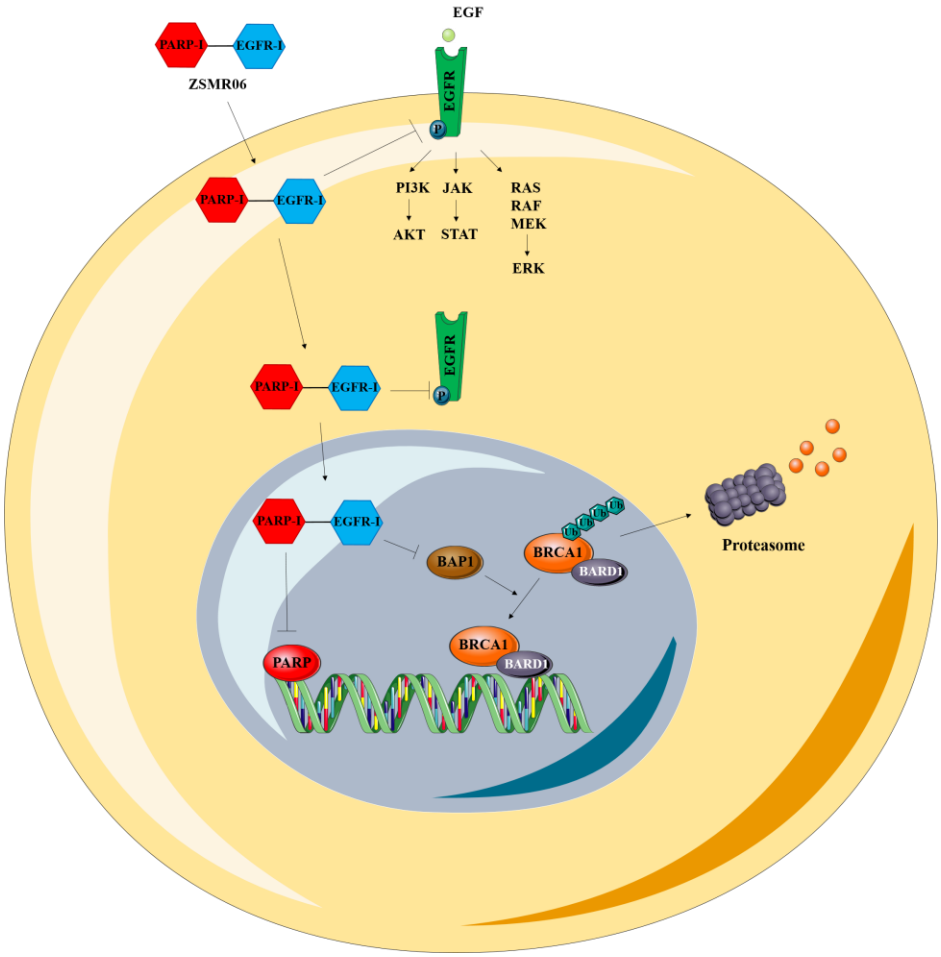

Figure 2. The proposed mechanism of action of ZSMR06. ZSMR06 is a combi-molecule with a PARP inhibitory warhead and EGFR targeting arm. As a stable molecule, ZSMR06 is able to act on both biological targets without the need for being hydrolyzed. It diffuses through the membrane to the cytoplasm, where it can inhibit EGFR through its EGFR inhibitory arm (EGFR-I). The molecule may then further diffuse into the nucleus and inhibit PARP through its PARP inhibitory warhead (PARP-I). ZSMR06 is also able to induce downregulation of the ubiquitin hydrolase, BAP1, which prevents the deubiquitination of BRCA1 and its subsequent degradation. The downregulation of BRCA1 is believed to induce a contextual synthetic lethality with the PARP inhibition of the molecule.

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