DESIGN, SYNTHESIS AND BIOLOGICAL ACTIVITY OF NOVEL MOLECULES DESIGNED TO TARGET PARP AND DNA

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This thesis is dedicated to anyone and everyone who must face the daunting challenge of bringing to the laboratory an indomitable spirit and passion for research while simultaneously reserving understanding, tranquility and love towards your wife and family. The road towards your goal might be steep, but the view once you have arrived at your destination makes the journey all the more rewarding.

TABLE OF CONTENTS

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ABSTRACT

According to the most recent Canadian statistics, the expected likelihood of women developing either breast or ovarian cancer throughout their lifetime is 26.1% and 2.9% respectively. Furthermore, breast cancer in women is the most prominent in the list of expected cancer development and its percentage is approximately twofold that of second place: lung cancer. Common to both men and women, pancreatic cancer has a 2.4% and 2.5% incidence of development respectively. In the previous decade, a commonality in these cancers has been discovered. in that a mutation in DNA repair proteins known as *BRCA1* and *2* renders these gene products non-functional. A DNA repair protein, known as poly (ADP-Ribose) polymerase (PARP), then becomes the only means by which mutated cells with defective *BRCA* gene products can repair DNA. As a result, if PARP is inhibited within mutated cells with defective *BRCA* gene products, they become incapable of repairing DNA lesions and ultimately undergo cell death. This translated into a novel strategy for the selective therapy of tumours with cancer susceptibility gene termed "synthetic lethality". Despite the successful proof-of-concept for synthetic lethality in the clinic, acquired resistance have been reported. In order to enhance the potency of the approach, we sought to synthesize PARP inhibitors capable of not only blocking PARP function but also alkylating DNA. Thus, we identified EG40, a PARP inhibitor carrying a chloroethyltriazolinium moiety. Using the sulphorhodamine B (SRB) assays, we showed that EG40 is 2.5-fold more potent than its PARP inhibitor counterpart, PARP-4-ANI, and displays 25-fold selectivity for the *BRCA2* mutant in an isogenic pair of cell lines. We assessed the binary targeting ability of EG40 using the comet assay to measure the extent of DNA damage, as well as using a PARP assay to measure the extent of PARP inhibition. EG40 inhibits PARP 20-fold more weakly when compared to the naked PARP-4-ANI scaffold and induces significant DNA damage against VC8 mutant cells. In

conclusion, we have developed a drug that acts more effectively in terms of potency while maintaining selectivity for its counterpart. Furthermore this drug is effective in inhibiting PARP and causing DNA damage. This gives prima facie evidence that a single molecule termed combi-molecule with dual PARP-DNA targeting function can be highly effective in tumors containing *BRCA1* or *2* mutations.

RÉSUMÉ

Selon les plus récentes statistiques canadiennes, la probabilité qu'une femme développe un cancer du sein ou de l'ovaire au cour de sa vie est de 26,1% et de 2,9% respectivement. En outre, la probabilité de cancer du sein chez les femmes est deux fois plus élevée que celui de poumon. Au cours de la décennie précédente, un point commun entre ces cancers a été découvert: il s'agit d'une mutation dans les protéines de réparation d'ADN appelés *BRCA1* et *BRCA2* qui les rend afonctionnels. Dans ces cancers, une protéine de réparation d'ADN, connu sous le nom de poly(ADP-ribose) polymérase (PARP), est le seul moyen par lequel les cellules mutées peuvent réparer l'ADN. Par conséquent, si la PARP est inhibée dans les cellules mutées, celles-ci deviennent incapables de réparer les lésions de l'ADN et meurent. Ainsi, l'inhibition de PARP est devenue une nouvelle stratégie pour le traitement sélectif des tumeurs avec gène de susceptibilité au cancer, appelées: "létalité synthétique". Malgré le succès de la preuve de concept de létalité synthétique en clinique, des cas de résistance aux inhibiteurs de PARP ont été rapportés. Afin d'améliorer la puissance de l'approche, l'objectif de cette thèse a été de concevoir les inhibiteurs de PARP capables non seulement de bloquer la fonction de PARP, mais aussi d'endommager l'ADN. Le travail effectué dans le cadre de cette thèse nous a permis de découvrir une molécule dénommée EG40, un phtalimide couplé a un groupement chloroethyltriazolinium. En utilisant un essai appelé la sulforhodamine B (SRB), nous avons montré que EG40 est 2,5 fois plus puissant qu'un inhibiteur de PARP dénommé PARP-ANI et démontre une sélectivité d'inhibition de croissance de 25 fois pour le mutant *BRCA2* dans une paire isogénique de lignées cellulaires. Nous avons employé le test des comètes pour démontrer la capacité de EG40 à endommager l'ADN et celui d'inhibition de PARP pour mesurer sa capacité à inhiber la fonction de ce dernier. La capacité d'EG40 à inhiber PARP a été de 20 fois plus faible que celle d'ANI,

mais les résultats du tests de comètes prouvent qu'elle est capable d'induire des dommages significatifs dans l'ADN des cellules. En conclusion, nous avons développé un médicament potentiel qui agit plus efficacement en termes d'induction de la mort des cellules tumorales *in vitro* qu'un inhibiteur standard de PARP, tout en maintenant une sélectivité pour les cellules exprimant une mutation dans le gène de *BRCA1* ou *BRCA2*. En outre, ce médicament potentiel est efficace dans l'inhibition de la PARP et est capable d'induire des lésions dans l'ADN. Ainsi, nous avons obtenu les premières évidences de la faisabilité d'un molécule unique appelée "combi-molécule" capable d'exercer la double fonction de ciblage de PARP et d'endommagement de l'ADN, tout en gardant un effet sélectif et prononcé contre les tumeurs exprimant des genes *BRCA1* ou *BRCA2*.

LIST OF ABBREVIATIONS

BER = Base Excision Repair

BLM = Bloom Syndrome Helicase

BRCA1/2 = Breast Cancer Susceptibility Gene ½

CtIP = C-terminal Interacting Protein

DMEM = Dulbecco's Modified Eagle Medium

DMSO = Dimethyl Sulfoxide

DNA2 =

DSB = Double Strand Break

EGFR = Epidermal Growth Factor Receptor

GGR = Global Genomic Repair

HR = Homologous Repair

HSQC = Heteronuclear Single Quantum Coherence

MMR = Mismatch Repair

NAD = Nicotine Adenine Dinucleotide

NER = Nucleotide Excision Repair

NHEJ = Non-Homologous End Joining

NMR = Nuclear Magnetic Resonance

PAR = Poly (ADP-Ribose)

PARP = Poly (ADP-Ribose) Polymerase

PARP-4-ANI = PARP-4-amino-1,8-napthalimide

PARP-5-AIQ = PARP-5-amino-isoquinolinone

PTEN = Phosphatase and Tensin Protein

PBS = Phosphate Buffered Saline

ROS = Reactive Oxygen Species

SDSA = Synthesis Dependent Strand Annealing

SRB = Sulforhodamine B

SSB = Single Strand Break

ssDNA = Single Stranded Deoxyribonucleic Acid

TCA = Trichloroacetic Acid

TCR = Transition Coupled Repair

TEM = Temozolomide

XRCC1 = X-ray cross complementation protein 1

LIST OF FIGURES

Chapter 1: Background and Introduction

Chapter 1: BACKGROUND AND INTRODUCTION

1.1) FOREWORD

This thesis is presented in manuscript form, in partial agreement with the terms listed by the Faculty of Medicine, division of Pharmacology and Therapeutics at McGill University.

1.2) PREFACE

A variety of somatic and germline mutations in healthy cells are what give rise to carcinogenesis. In the latter case, mutated tumour suppressor genes have been shown to be hereditary and lead offspring to an increased cancer risk[1]. The *BRCA1* and *2* genes belong to this category in which individuals have an increased risk for breast, ovarian, pancreatic and prostate cancers[2]. In addition to a larger likelihood of cancer comes deficiencies in vital DNA repair pathways. When one DNA repair pathway is hindered due to a genetic mutation, alternative pathways attempt to compensate. In the case of *BRCA1* and *2* mutations, the compensatory pathway is base excision repair (BER)[3] and the cells become highly dependent on this pathway. Should this compensatory pathway become blocked at a key regulator, the oncogenic cell loses its two most important methods for the repair of DNA lesions. Genomic instability ensues due to a build-up of unrepaired lesions which ultimately triggers cell death[4]. When tumours show signs of *BRCA1* or *2* mutation, the possibility for the cellular condition of "synthetic lethality" becomes apparent. Currently, the synthetic lethality strategy in this context is to pharmacologically inhibit a key player in BER, poly(ADP-Ribose) polymerase (PARP), in a tumour with *BRCA1* or *2* mutation[5,6]. With the main and alternative DNA repair pathways dysfunctional, the cell dies. However, cells will only die in the event of a high levels of DNA damage. Upon addition of a cytotoxic moiety, DNA damage can occur at a faster rate than spontaneous DNA damage[7].

Therefore, it becomes apparent to test the effectiveness of the addition of a PARP inhibitor and a DNA alkylator[7]. The goal of this thesis is to determine the potency and usefulness of PARP and DNA targeted molecules in tumours with *BRCA1* or *2* mutation. Prior to describing our approach in this thesis, a description of principles associated with the role of *BRCA1* and *2*, PARP involvement in BER and synthetic lethality are given below. A short review of the principle of combi-targeting is included prior to the description of the objectives of the thesis.

1.3) GENERAL CARCINOGENESIS

1.3.1) PARP Mediated DNA Repair

Poly(ADP-Ribose) polymerase (PARP) is a ubiquitous protein, which is responsible for the repairs of DNA single strand breaks (SSB) through a process known as the base excision repair (BER) pathway[8] (Figure 1). This pathway in particular is responsible for the repair of small lesions in the DNA helix as well as the removal of incorrect DNA base pairs[3]. The process for BER is relatively straightforward. The first step involves the removal of the incorrect base pair. This involves DNA glycosylases than can hydrolyze the glycosidic bonds. Typical glycosylases that act this way include N-methylpurine DNA glycosylase, uracil DNA glycosylase, thymine DNA glycosylase and several others[9]. The second step involves an endonuclease that further cleaves the DNA strand to yield an apurinic site that contains a 3'-hydroxyl group on one end and on the opposite end a 5'-deoxyribose-5-phosphate[8]. The main group of endonucleases recruited by BER is apurinic/apyrimidinic endonuclease 1 series. At this stage in the process, short chain BER or long chain BER can occur. In the case of short chain BER, DNA polymerase β is responsible for synthesizing the appropriate sequence complementary to the undamaged base[10]. Then a complex of ligases, consisting of DNA ligase 3 and X-ray cross-complementation protein 1 (XRCC1) are responsible for ligating this newly synthesized strand to the DNA[11]. Long chain BER is similar on

concept with the sole exception that the proteins implicated are different. In the case of the DNA synthesis step, DNA polymerase δ or ε, as well as DNA polymerase β itself, can be responsible for the synthesis of the long chain DNA strand that is 2 to 12 nucleotides long[12]. Structure specific flap endonuclease 1 removes what is unnecessary from the nucleotide sequence and DNA ligase 1 is then implicated in attaching this nucleotide sequence to the DNA[13]. Regardless of short or long chain BER, as soon as the DNA ligase has attached the new sequence of DNA to the strand, the pathway is complete and the DNA successfully repaired.

PARP is an accessory protein that has the ability to recruit XRCC1 and DNA polymerase β to the site of a single strand break[14]. PARP1 in particular is known to have a very high affinity for SSB and acts as a sensory enzyme[15]. Once PARP binds to the location of the DNA strand break, using its two zinc fingers located within the DNA binding domain[16], it either homo- or heterodimerizes with PARP-1 or PARP-2 to allow for the polymerization of nicotinamide adenine dinucleotide (NAD) to form a long poly(ADP ribose) (PAR) chain[17]. Subsequent downstream proteins, such as the aforementioned XRCC1, as well as DNA polymerase β, are then utilized to continue the BER pathway[14]. Once these additional proteins are recruited and PARP has been activated, it can unbind from the SSB and allow the other players in the BER pathway to finish repairing the lesion. Interestingly, it was discovered that PARP is solely implicated in the short chain BER pathway and is not a player in the long chain pathway[18]. Furthermore, an inhibition of this PARP accessory proteins leads to hypersensitivity towards alkylating agents[19], giving possible implications for PARP to be targeted to render tumours more sensitive towards chemotherapy. Therefore, outside of the BER pathway, PARP also appears to have a general role in protection from endogenous and exogenous alkylation potential[15]. Evidently, PARP plays a crucial role in the genomic stability of cells.

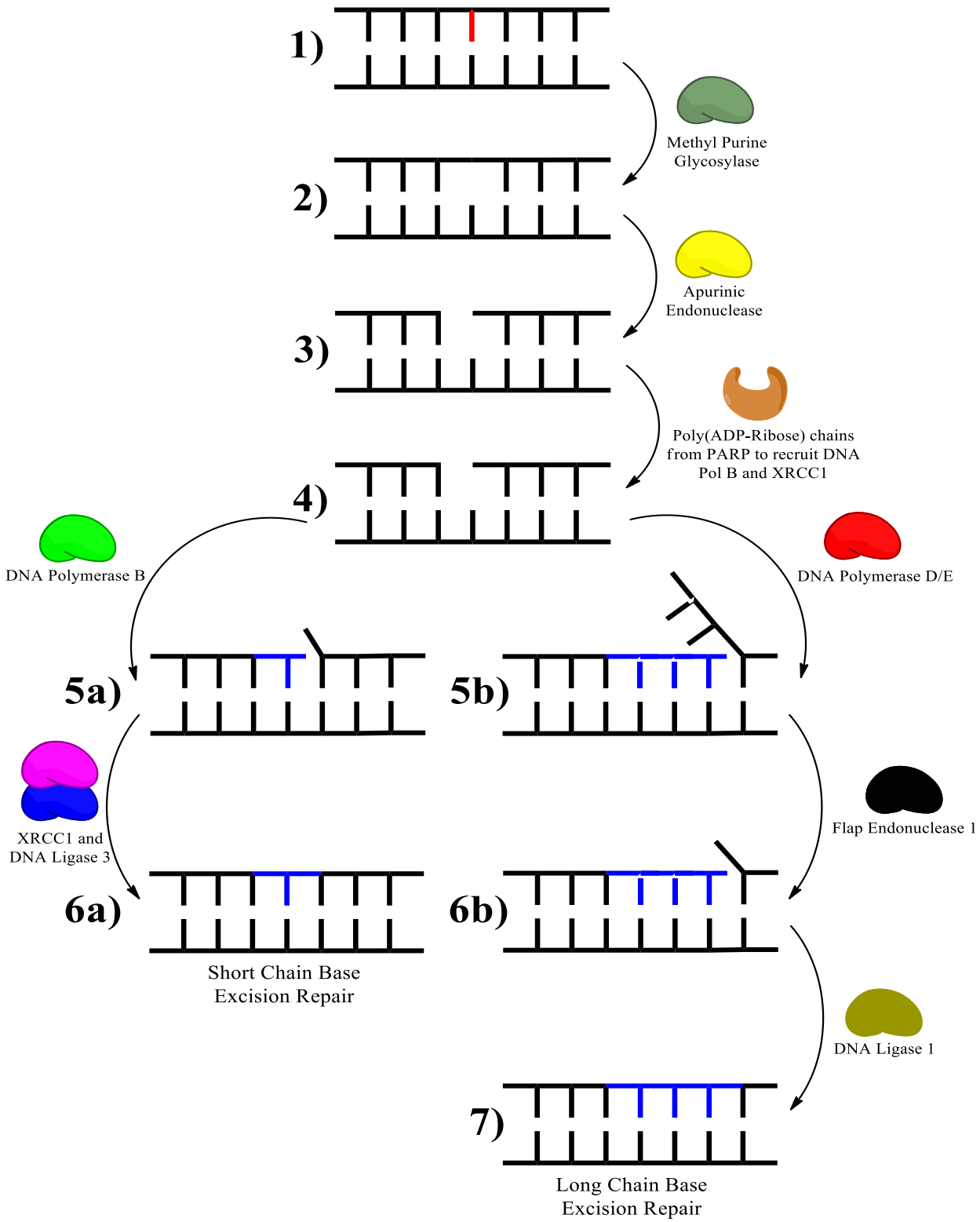

Figure 1: Mechanism of base excision repair (BER) for short chain (left) and long chain (right) pathways.

If this type of lesion, be it from a mismatched base or from an alkylator, remains unrepaired, a double strand break will ensue. Double strand breaks (DSB) are known to be much more cytotoxic by comparison to a SSB[20]. Once the DSB has occurred, PARP may no longer play a role in its repair. Instead, the process of homologous recombination or non-homologous end joining will become implicated in the repair of the DSB[21].

1.3.2) *BRCA* Mediated DNA Repair

The gene products of *BRCA1* and *2* are extremely important for the process of homologous recombination (HR) (Figure 2), a mechanism directed at the repair of DNA double strand breaks, interstrand crosslinks and lesions occurring at the replication forks for DNA synthesis[22]. Double strand breaks can be caused by numerous events, such as radiation, endonucleases, replication fork DNA transcription errors due to unrepaired SSBs and reactive oxygen species (ROS)[23]. Such products include BARD1 on the *BRCA1* gene and RAD51 on the *BRCA2* gene[24]. The HR pathway is complex, with several different products possible, such as the gene conversion product, the crossover product, the gene conversion combined with crossover product, and less commonly, the non-crossover product[25]. In addition, the process of HR requires several steps, commonly occurs in either the S or G2 phase of the cell cycle where the process of HR is upregulated and many implicated proteins[26]. Of these proteins, there are two categories: the first correspond to those that function as enzymes, responsible for the synthesizing the DNA repair components and the second are proteins that assist in and the regulation of the enzymatic proteins[23]. In particular, HR is highly important for the prevention of cancer onset due to its near perfect DNA repair functionality. This process also serves as a metaphorical beacon to signal when a cell has too much damage and should no longer be replicated. This allows for the genomic stability of the cell to remain intact. However, there are four potential mechanisms that could threaten this stability: 1) an incorrect repair of DNA

damage causing mutations, 2) errors in DNA replication, 3) errors in chromosomal distribution in mitosis and 4) failures to detect serious DNA lesions at cell cycle checkpoints[23].

When a double strand break occurs, an MRN complex which consists of MRE11, RAD50 and NBS1, as well as several other proteins are localized to the site of DNA damage. This MRN complex is responsible for the recruitment of *BRCA1* and C-terminal interacting protein (CtIP)[27]. The latter form a complex responsible for the resection of 5' DNA strand to expose the 3'-hydroxyl terminus[28], which yields single stranded DNA (ssDNA). A second complex comprising of bloom syndrome helicase (BLM) and DNA2 endonuclease becomes implicated in the resection of the 3' DNA strand[29]. Once strands are resected, numerous proteins such as *BRCA1*, PALB2 and *BRCA2* are responsible for the recruitment of RAD51[30]. This forms a RAD51 filament is responsible for the localization of homologous templates and DNA strand exchanges[23]. Once the appropriate template is found, there is invasion of the 3' hydroxyl end to the adjacent sister chromatid, whereupon DNA polymerase uses the chromatid template to make an identical copy of the required DNA strand. The synthesized strands are ligated to the original sequence by DNA ligase. In mammals, it is assumed that the BRC repeats in *BRCA2* are responsible for re-establishing the double stranded helix of DNA at this particular point[31]. Two end products become possible now: the synthesis-dependent strand annealing product (SDSA) or the cross over product[32]. The former involves the return of the invading ssDNA to its original chromatid (no DNA crossover) once DNA synthesis is completed and anneals it to the 5' end. It is this newly formed template that is used to repair the other strand on the same chromatid, resulting in the full repair of the DSB. In the latter case, sister chromatids share DNA strands with each other and create DNA overlaps at two points. These overlap points in the latter case are called Holliday junctions[33]. These junctions can be cleaved in two distinct ways, either both in a similar fashion (both horizontally or both

vertically) or a horizontal and a vertical cleavage, giving either a non-crossover product or a crossover product respectively. Once the Holliday junctions are cleaved using an endonuclease and subsequently ligated using DNA ligase, the DNA is fully repaired[34]. It is also worth mentioning that in the event of base pair mismatches, the mismatch repair (MMR) pathway may be used to ensure that the HR pathway remains error free[35].

HR also plays a crucial role in the repair of interstrand crosslinks, in conjunction with the nucleotide excision repair (NER) pathway, another process known in the repair of SSB (Figure 3). These interstrand crosslinks are detrimental to genomic stability wherein they cause several mutations during DNA transcription and replication[36]. When DNA incurs an interstrand crosslink, either from chemotherapy or other environmental carcinogens, the NER pathway becomes activated. There are two main sensors that determine when NER should be activated. The first is the sub-pathway of global genomic repair (GGR), responsible for using an XPC/hHR23A/B protein complex to determine the location of DNA damage and the other is transcription coupled repair (TCR), which involve the use of CSA and CSB sensory proteins to recruit the TFIIH complex and begin the nucleotide excision process so RNA polymerase II can continue to transcribe the DNA[37]. Naturally, the subpathway used is dependent on the current ongoing processes in the cell, TCR being used during DNA transcription and GGR under general purposes[38]. Regardless of the sensory protein complex used, the recruited TFIIH acts to unwind the DNA, breaking the hydrogen bonds between base pairs creating what is known to be a bubble structure[39]. Without a ssDNA filament to work with, the NER pathway will be unable to properly excise the damaged portion of DNA. Once unwound, the first excision in the damaged DNA strand occurs approximately 6 bases after the crosslink by the XPG endonuclease and the second incision is done thereafter approximately 22 bases before the DNA damage by the ERCC1/XPF

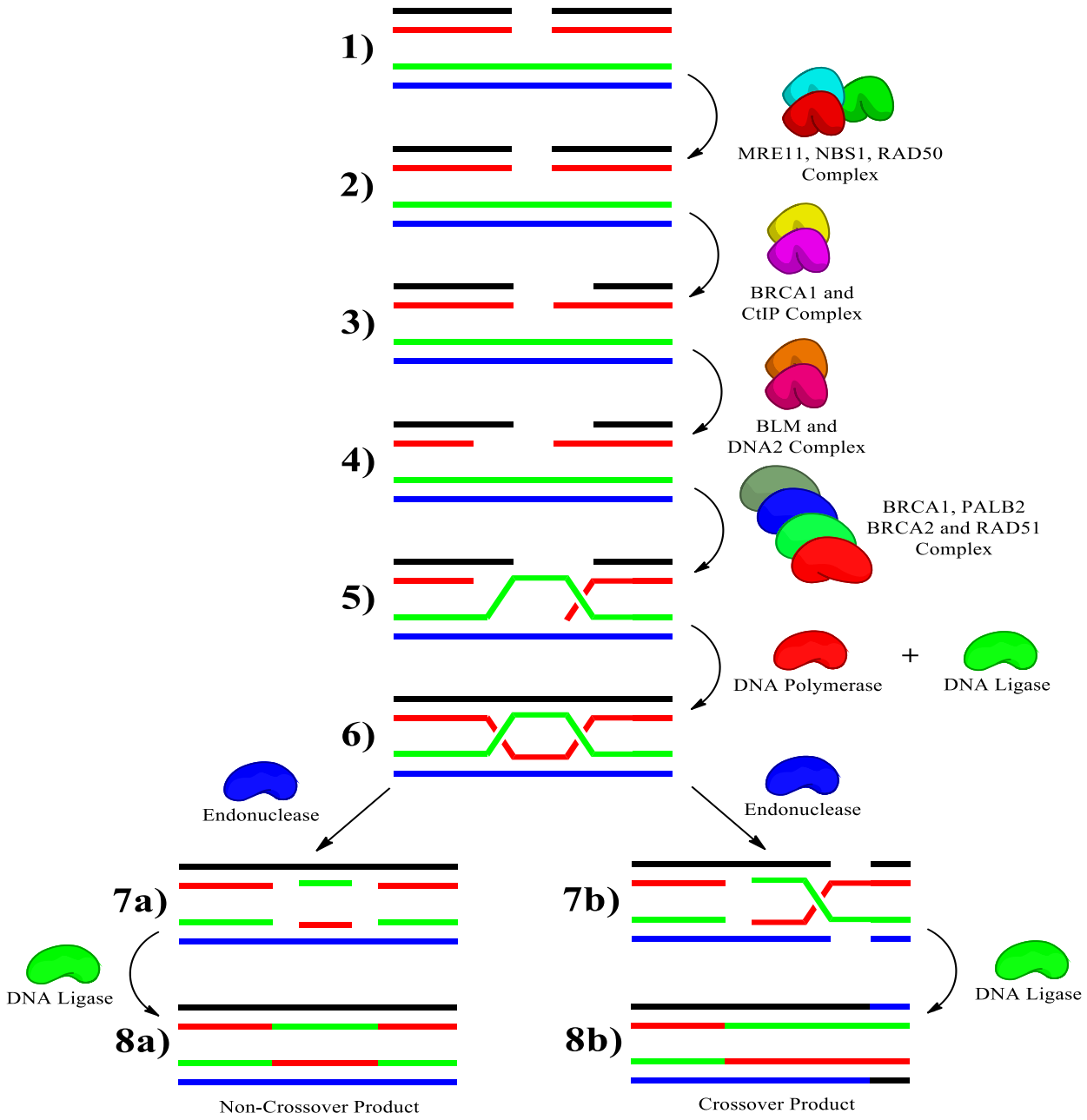

Figure 2: Mechanism of homologous recombination for non-crossover (left) and crossover (right) products.

endonuclease complex[37]. At this point, the strand of nucleotides is free to move, bound only by its interstrand crosslink. The sister chromatid is then located, used as a template and traditional HR processes as described above ensue to result in the repair of the cross-linked DNA[40]. The only

difference occurs when the Holliday junctions are formed that the aforementioned NER endonucleases are responsible for the excision of the second portion of cross-linked DNA, completely removing it from the structure[23].

Replication forks are essential for cells undergoing mitosis, allowing for the effective transcription of DNA for both resulting cells[41]. Briefly stated, a helicase known as Mcm2-7 is responsible for the unwinding of the DNA and a topoisomerase is responsible for the removal of hydrogen bonding interactions between the strands[42]. What results is a two ssDNA tails, the first being the leading strand and the second being the lagging strand. Two individual DNA polymerases (Pol α and Pol δ) begin synthesizing in the 5' to 3' direction the complementary DNA sequence from the leading or lagging tail template[43]. Remarkably, the leading tail side has the polymerase synthesizing its complementary DNA strand in the same direction as the fork movement. In the case of the lagging tail, a different mechanism is employed. An RNA primase synthesizes a short RNA primer to serve as a signal for the DNA polymerase to begin synthesizing the complementary DNA strand for the lagging tail. The polymerase continues to produce DNA until it reaches the preceding RNA primer, which signals the polymerase to stop its synthesis. It then detaches itself from the lagging strand and forms a segment of DNA known as an Okazaki fragment[44]. This process repeats itself numerous times to synthesize complementary DNA for the lagging tail in the direction opposing the flow of the replication fork[42]. This is the process that occurs in a typical replication fork, however, in the event there is a lesion to a base along the DNA strand, a stall in the replication fork will occur. Furthermore, if this error is not repaired, or if the replication fork is not able to continue, a double strand break may ensue and result in the death of the cell[45].

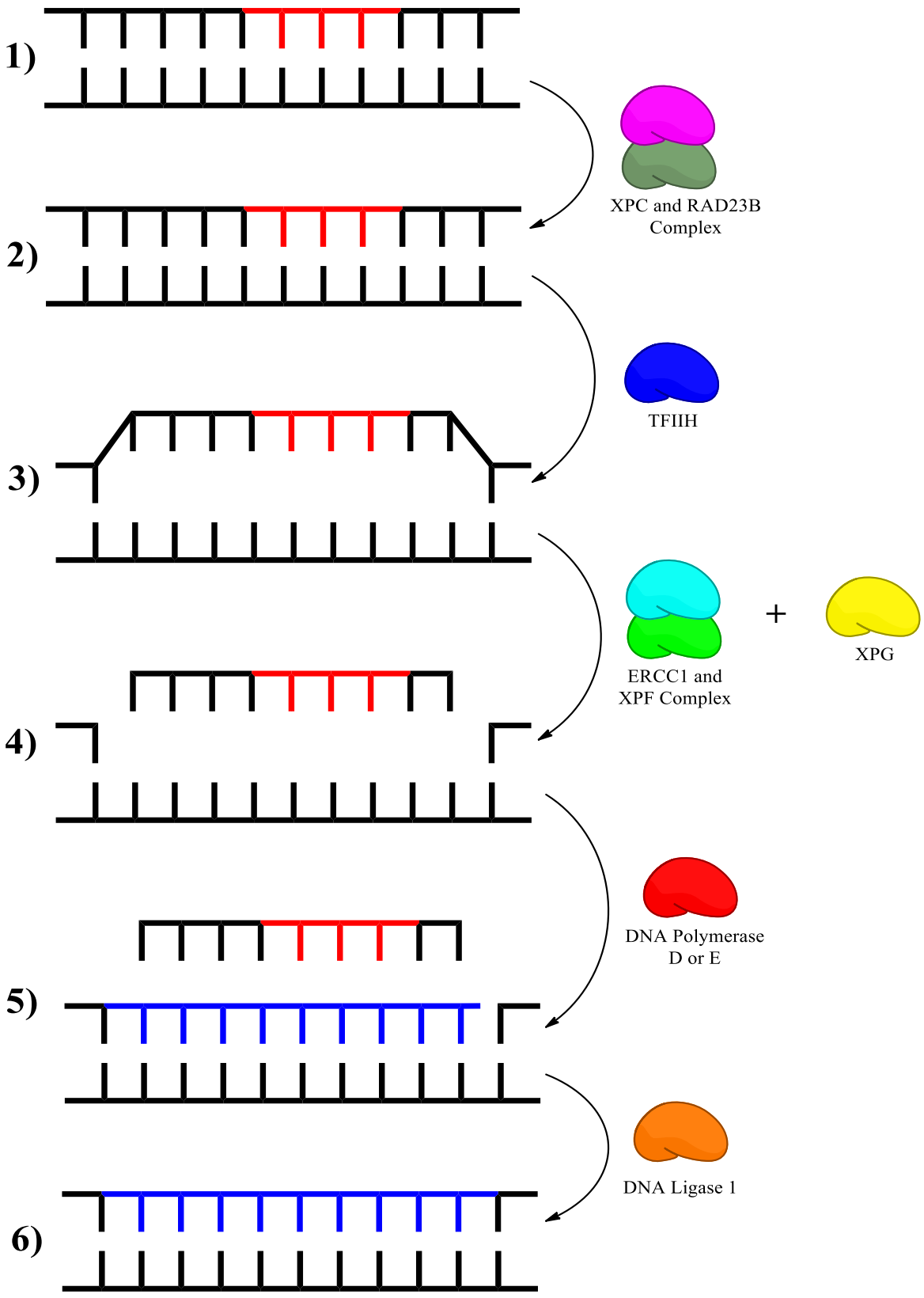

Figure 3: Mechanism of nucleotide excision repair.

A lesion can either include damage to one of the DNA bases or a SSB altogether. In the case of damage to the DNA base, there is the possibility of two methods with which to repair the DNA, either through replication fork regression or strand invasion, as observed in the HR pathway[23]. Synthesis on the undamaged pair of ssDNA will continue until both the leading and lagging strands are both halted. Evidently, the HR pathway is extremely crucial for the maintenance of genomic stability. For any of these aforementioned processes to occur, *BRCA* gene products, as well as numerous other proteins must be synthesized[46]. In the case of dysfunctional *BRCA* gene products, either on *BRCA1* or *2*, HR will not work. Rather, the double strand break repair pathway of non-homologous end-joining (NHEJ) will take over. This form of DNA repair, which uses DNA ligases to arbitrarily re-attach the DNA strands, often involves insertion and deletion events, which causes it to be error prone and lead to genomic instability[16].

1.3.3) Concept of Synthetic Lethality

The concept of synthetic lethality has been growing in popularity ever since it was discovered. As aforementioned, in the case of *BRCA1* and *2* tumours, only NHEJ is available to repair the DSB and the single strand DNA repair pathways are unaffected. This is an error prone pathway and has the potential to lead to genomic instability[47]. It can be said that both the *BRCA* gene product(s) and PARP are two redundant proteins that are present within the cell. However, in the case of some cancers, there are some gene products that are mutated, such as *BRCA1* or *2* in the case of breast, ovarian, pancreas and prostate cancers[2]. Therefore, the remaining gene product, PARP in this case, is able to take over and repair the DNA damage incurred by the cells. In the case of cells with functional *BRCA* gene products, they can repair the double strand break that ensues from an unrepaired single strand break. Through the use of pharmacological inhibition of PARP in the presence of a *BRCA* mutated tumour, two major DNA repair pathways no longer

function properly[48]. Therefore, in *BRCA1* or *2* mutated tumours, when PARP is inhibited pharmacologically, both products are unable to exert their effects on DNA repair. As a result, the cell accumulates lesions and ultimately dies. This form of combination is known as synthetic lethality and is currently being used in the clinic in *BRCA* mutated advanced ovarian cancers[49]. Although the first approved inhibitor for the treatment of ovarian cancer is olaparib, there are many other PARP inhibitors currently in clinical trials. Such examples include veliparib, iniparib and ABT-888[50]. The remarkable fact regarding this synthetic lethality scheme is that it is selective for the tumour cells exclusively. Oncogenic cells with *BRCA* mutation and PARP inhibition lose all ability to repair their DNA lesions and undergo cell death, whereas healthy cells with functional *BRCA* gene products are able to take over in the event of PARP inhibition[51].

Once such example that also implicates PARP is the synthetically lethal combination of PARP inhibition in the context of Phosphatase and Tensin Homolog (*PTEN*) gene mutated tumours[52]. *PTEN* acts similarly to *BRCA1* or *2* in that it is responsible for some components in DNA repair pathways. In particular, *PTEN* is a tumour suppressor gene, acting as a regulator of proliferation and pro-apoptotic pathways as well as in genomic stability through DNA repair[53]. Due to the latter function of the *PTEN* protein, the pharmacological inhibition of PARP in tumours with mutated *PTEN* leads to selective oncogenic cell death. Another, yet fairly novel, synthetic lethality approach involves targeting EZH2 methyltransferease in the presence of *ARID1A* gene mutated cancers[54]. The *ARID1A* gene product acts as a chromatin remodeler and EZH2 activity is responsible for silencing gene expression. The novelty of this strategy arises from the fact that no DNA repair pathways are targeted and that it is a relationship between epigenetic events. The synthetically lethal combination arises from the fact that in *ARID1A* mutated tumours, EZH2 is

upregulated and becomes a vital component in cell viability[54]. The EZH2 addicted tumour logically would undergo cell death in the event this silencer is inhibited pharmacologically.

1.3.4) Applications to Cancer

This synthetically lethal strategy allows for natural cell death. It takes advantage of spontaneous DNA damage to kill oncogenic cells (Figure 4).

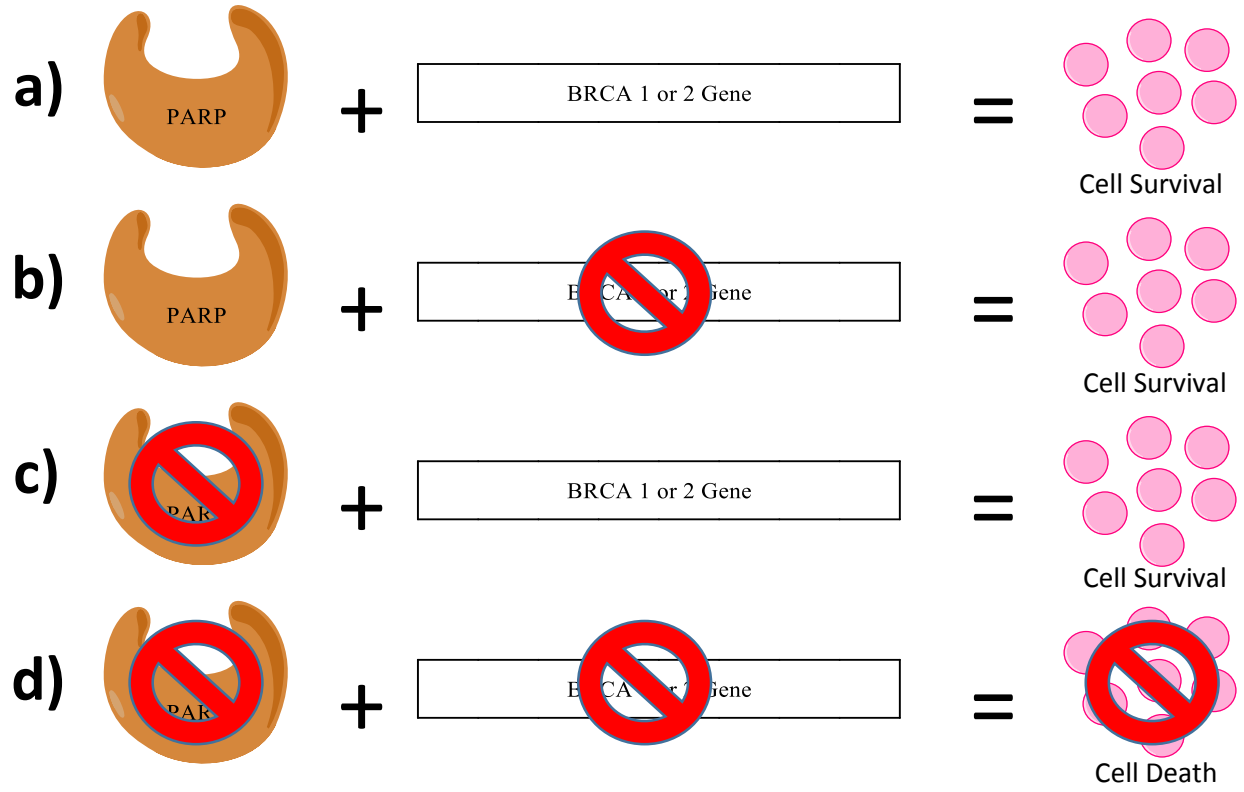

Figure 4: Concept of synthetic lethality.

There have been some concerns about possibilities of resistance to this synthetically lethal scheme. Regretfully, as this is a fairly novel discovery, the mechanism of resistance is still yet to be elucidated[16]. Once resistance is expressed, the synthetic lethality scheme of PARP inhibition on *BRCA* mutated tumours is no longer effective and conventional treatment follows thereafter. It is for this reason that clinical trials with PARP inhibitors are not just testing the efficacy of the inhibitor alone. Rather, they are also testing them in combination with DNA damaging agents,

such as temozolomide, gemcitabine, platinum salts, etc.[50]. The goal of this treatment is to use PARP inhibition as a chemosensitizer to cytotoxic agents.

1.4) ALKYLATORS AND TRIAZENES

As many pharmaceutical companies have concluded, a possible manner to accelerate tumour killing kinetics is to add a clinical agent that is also able to damage DNA[55]. Although the DNA damage would not be selective (as is the case with conventional chemotherapy) healthy cells with functional *BRCA* gene products would be able to repair the lesions caused by the alkylating agent even with PARP inhibition. Conversely, the tumour with mutated *BRCA* and PARP inhibition will not be able to repair the DNA damage induced by the alkylator. With regards to current small molecule alkylating agents, such as temozolomide (TEM) or chlorambucil, additional amounts of DNA damage would be able to be administered in this synthetic lethality approach. Temozolomide is a clinical alkylating agent that under physiological conditions releases a methyl diazonium ion and an inactive metabolite[56]. The methyl diazonium being a highly unstable species, it is susceptible to nucleophilic attacks from specific atoms of the DNA base pairs. The diazonium ion is able to methylate the N7- and N3-position of adenine and the O6-position of guanine, ultimately leading to single strand breaks[57]. In the event that methylated DNA is not repaired, subsequent cell divisions would cause genomic instability and ultimately lead to cell death. Chlorambucil also acts as an alkylating agent but induces DNA interstrand cross links[58]. The effect of cross-linking DNA results in double strand breaks leading to significant genomic instability. Thus, in cells with *BRCA1* or *2* mutation, PARP inhibition will leave the cells without alternative mechanism of DNA repair, thereby leading to cell death.

1.5) COMBI-TARGETING CONCEPT

The combi-targeting concept has been initiated within our laboratory[59,60]. It consists of designing molecules termed combi-molecules to act on two specific targets (e.g. DNA and tyrosine receptor kinase). Furthermore, combi-molecules can be categorized into three classes. First are the type I combi-molecules, which are designed to hydrolyze under physiological conditions to yield two active moieties. Common examples of this type of combi-molecule include epidermal growth factor receptor (EGFR) inhibitors coupled to alkylating agents[61,62,63]. Second are the type II combi-molecules, which do not require dissociation to exert their dual targeting activities. Common examples from our laboratory include JDD36[64]. The third type of combi-molecule is type III, which was recently described in our laboratory[65]. They are a hybrid class of combi-molecules, able to combine the stability granted from a type II combi-molecule with the simultaneous dual action typically gained from hydrolysis of the type I combi-molecules.

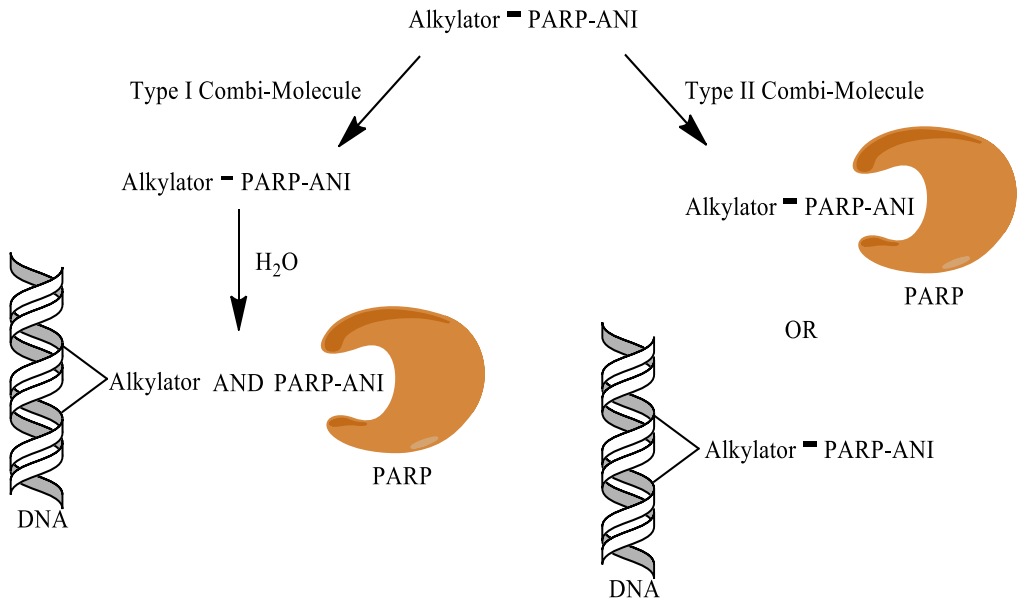

Figure 5: The combi-targeting concept: difference between type I and type II combi-molecules.

Here, we synthesized a wide panel of type I and type II molecules designed to target PARP and DNA. Combi-molecules were designed to carry three different types of alkylating agents using two PARP inhibitory scaffolds. The alkylating species tethered to the PARP inhibitor arm include a monomethyltriazene, a dimethyltriazene and a bis(2-chloroethyl)triazene. The mechanism of action of the PARP-monomethyltriazene is comparable to temozolomide. Both types of compounds release the methyl diazonium ion, which is known to methylate DNA[57]. Our purpose was to enhance the potency of PARP inhibitors by adding an additional DNA damaging component to their aromatic scaffold.

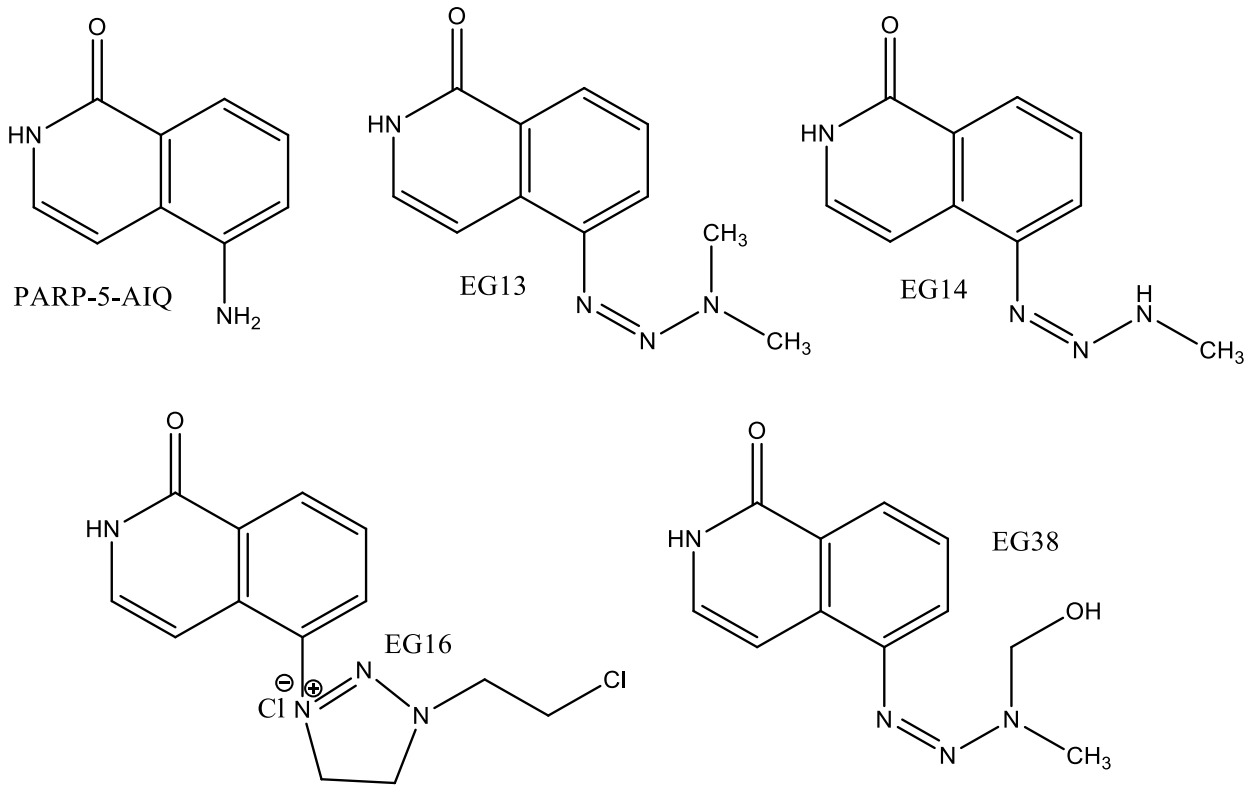

Figure 6: PARP-5-AIQ Series Molecules

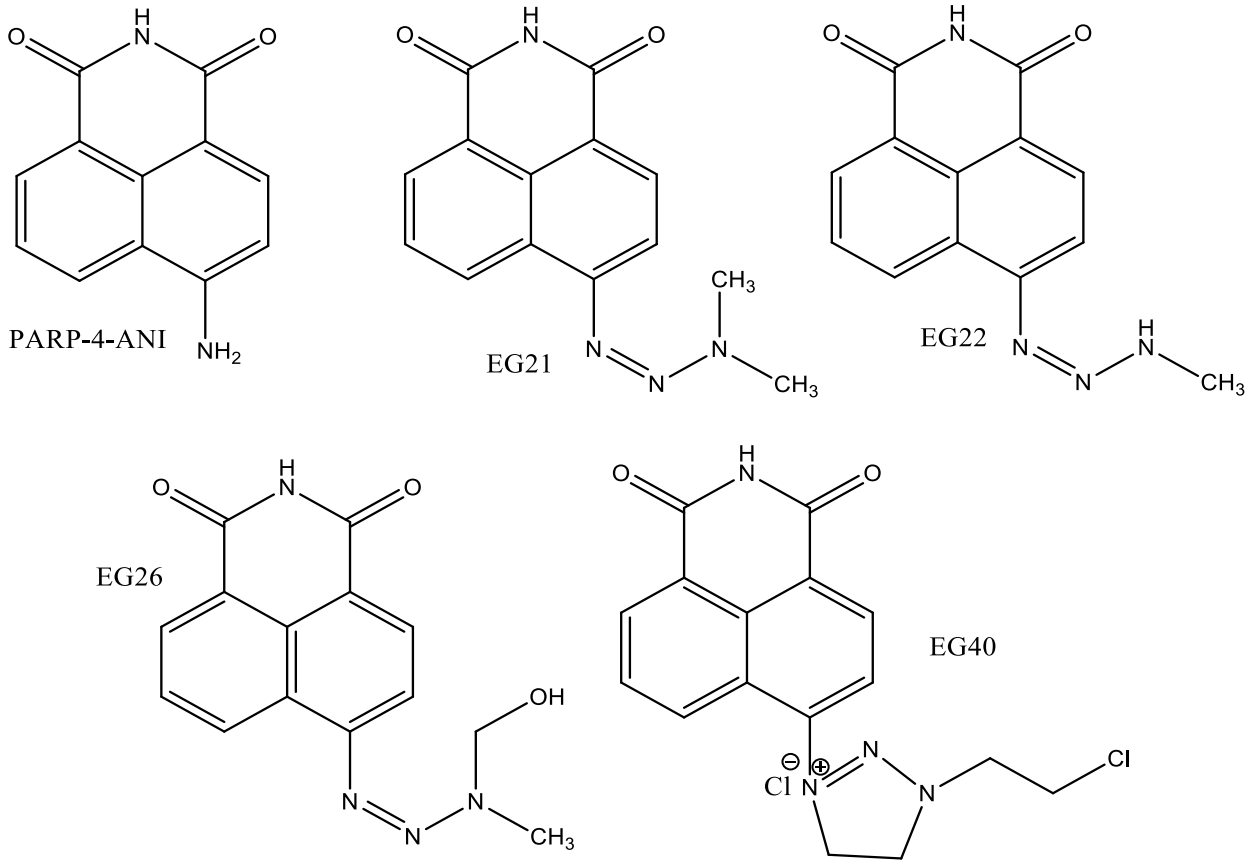

Figure 7: PARP-4-ANI Series Molecules

1.6) RESEARCH OBJECTIVES

1.6.1) Statement of Purpose

Breast and ovarian are amongst the most common cancers in women. A subset of patients carry tumours with *BRCA* mutations that are associated with severe DNA repair deficiency. In these tumours, the only alternative DNA repair is the BER pathway. Pharmacological inhibition of PARP leads to cell death in these tumours. Therefore, PARP targeting has become a selective cytotoxic therapy. However, despite the recent approval of the first PARP inhibitors for the clinical management of ovarian cancer in conjunction with a diagnostic kit to screen for *BRCA* status,

several limitations have been reported for the approach during clinical development, including disappointing overall survival, resistance associated with re-activation of *BRCA1* or *2* and multidrug resistance mechanisms. The goal of this thesis is to make the proof-of-concept of a novel approach that consists of single molecules termed "combi-molecules" to behave as both a PARP inhibitor and a DNA damaging agent. This concept was based on the hypothesis that inflicting further DNA damage to *BRCA1* or *2* deficient cells while PARP is inhibited will enhance cell killing. This may well represent a novel approach to prolong survival with the synthetic lethality concept.

1.6.2) Contribution of Authors

I carried out all experimental work, with the sole exception of replicates on the standard PARP-4-ANI. This was performed by Zhor Senhaji Mouhri, a PhD student in our laboratory. I alone was responsible for the synthesis, isolation and characterization of combi-molecules EG13, EG14, EG16, EG21, EG22, EG26, EG38 and EG40.. I was also responsible for the sulforhodamine B assays done to assess the potency and selectivity of each synthesized molecule, as well as the sulforhodamine B assays to compare the potency of the combi-molecule EG40 to that of known DNA targeting analogs. I performed the PARP and the comet assay on EG40Lastly, it was Chris Williams who prepared the molecular modelling figures.

Chapter 2: DESIGN, SYNTHESIS AND BIOLOGICAL ACTIVITY OF NOVEL MOLECULES DESIGNED TO TARGET PARP AND DNA

CHAPTER 2

DESIGN, SYNTHESIS AND BIOLOGICAL ACTIVITY OF NOVEL MOLECULES DESIGNED TO TARGET PARP AND DNA

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2.1) ABSTRACT

In order to enhance the cytotoxic potential of poly(ADP-ribose) polymerase (PARP) inhibitors in *BRCA1* or *2* deficient tumours, we designed a series of molecules containing a 1,2,3-triazene moiety tethered to a PARP targeting scaffold. A cell-based selectivity assay involving a *BRCA2*-deficient Chinese hamster cell line and its corresponding *BRCA2* wild type transfectant, was used to predict the PARP targeting potential of the latter agents. The results showed that adding a DNA damaging function to the PARP inhibitors decreased but did not abrogate the selective targeting of the *BRCA2*-deficient cells. The DNA damaging moiety augmented the potency in *BRCA2* deficient cells by 2-20 fold. The most selective dual PARP-DNA targeting agent EG40 was found to possess dual DNA and PARP targeting properties.

2.2) INTRODUCTION

The past five years have seen significant development in the field of DNA repair inhibitors[1]. In this context, a cellular condition termed "synthetic lethality" is being targeted for selective chemotherapeutic intervention against solid tumours of the breast, ovary and pancreas. Synthetic lethality arises when one of two genes "A" or "B" is mutated and the functions of a non-mutated gene "A" or "B" are required to rescue the cells from the dysfunction of the mutated one[2,3]. Therefore, in cells in which gene "A" is mutated, blockade or dysfunction of gene product "B", leads to cell death. As an example, one such occurrence is the mutation of the *BRCA1* or *2* genes[4], which are tumour suppressor genes involved in homologous recombination repair[5]. In the context of homologous recombination repair, *BRCA1* and *2* form a complex with PALB2[6] and RAD51 that relocates to the site of damage[7]. Mutation of *BRCA1* and *2* leads to loss of CtIP and PALB2 recruitment and RAD51 activation respectively[8,9]. This subsequently leads to loss of DNA repair function. An alternative to this deficiency is the expression of the *PARP* gene product commonly referred to as poly(ADP-ribose) polymerase (PARP)[10]. The latter is responsible for the recruitment of DNA polymerase and XRCC1 via poly(ADP-ribose) chains, leading to a base excision repair (BER) mechanism[11]. Activation of BER can compensate for the loss of homologous recombination repair functions. Since this is the only alternative for rescuing the cells in *BRCA1* or *2* mutant cells, blockade of PARP creates a synthetic lethality condition that ultimately leads to cell death[12]. This confers to PARP inhibitors the unique ability to selectively induce cell death in *BRCA1* or *2* mutated tumours[13].

The first generations of PARP inhibitors, including nicotinamide, 3-aminobenzamide and 2-methylquinazolin-4-[3H]-one were rather weak, with IC_{50} for PARP inhibition ranging from 120 μM to 5 μM[14,15]. While the later generations of PARP inhibitors (e.g. PARP-5-

aminoisoquinolinone and PARP-4-amino-1,8-naphthalimide), were more potent[15], it was not until 2014 that the first PARP inhibitor, olaparib, was approved for the treatment of ovarian cancer[16]. Despite being considered to be a selective cancer targeting approach, the overall survival obtained with PARP inhibitors as single agents in patients with *BRCA1* or *2* mutations has been disappointing[17]. Furthermore, mechanisms of resistance to the synthetic lethality condition brought on by PARP inhibition in *BRCA1* and *2* mutated tumours have emerged *in vivo*[18]. Some observed mechanisms include: reactivation of *BRCA1* and *2*[19], spontaneous mutation of the *TP53BP1* gene and p-glycoprotein 1 upregulation[20]. Thus, strategies directed at augmenting the potency of PARP inhibitors are under evaluation[21]. To enhance therapeutic potency, combinations of PARP inhibitors with DNA damaging agents are currently being investigated in several clinical trials to overcome drug resistance[22-24].

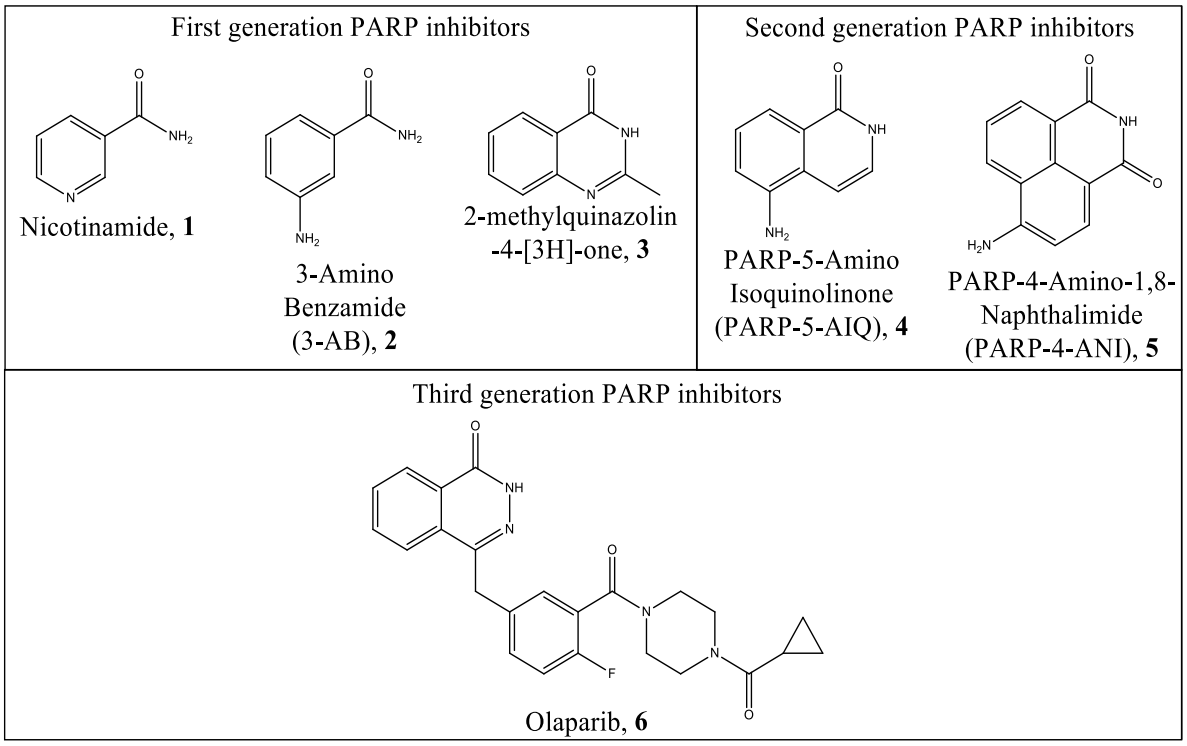
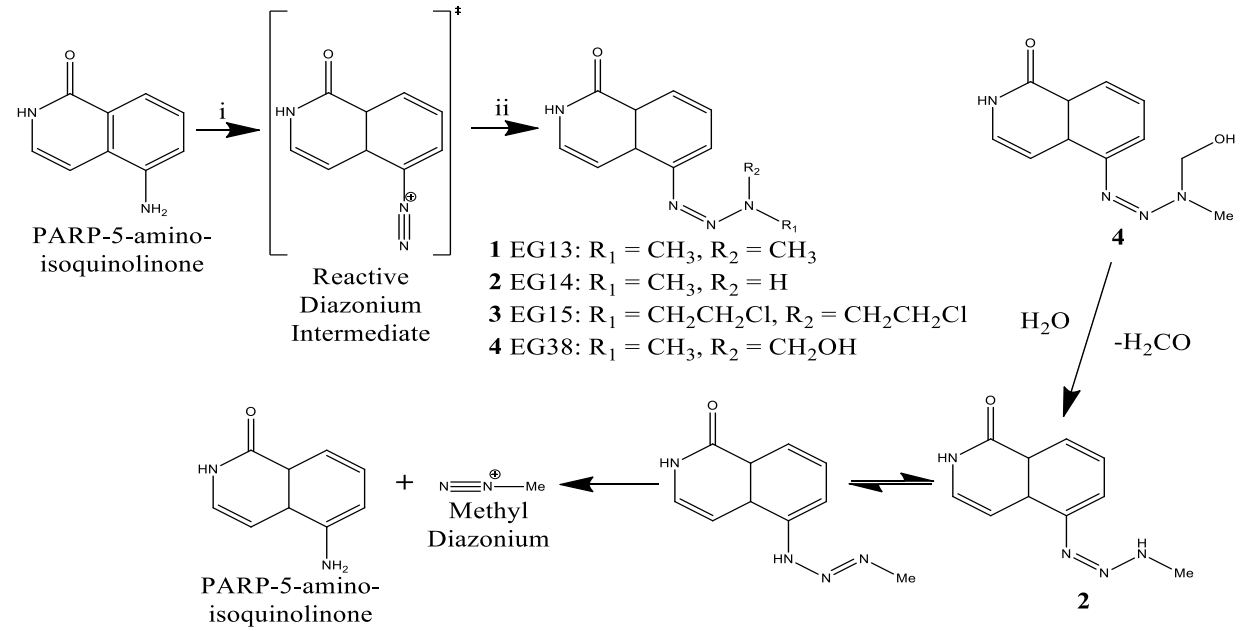


Figure 8: Examples of first, second and third generation PARP inhibitors.

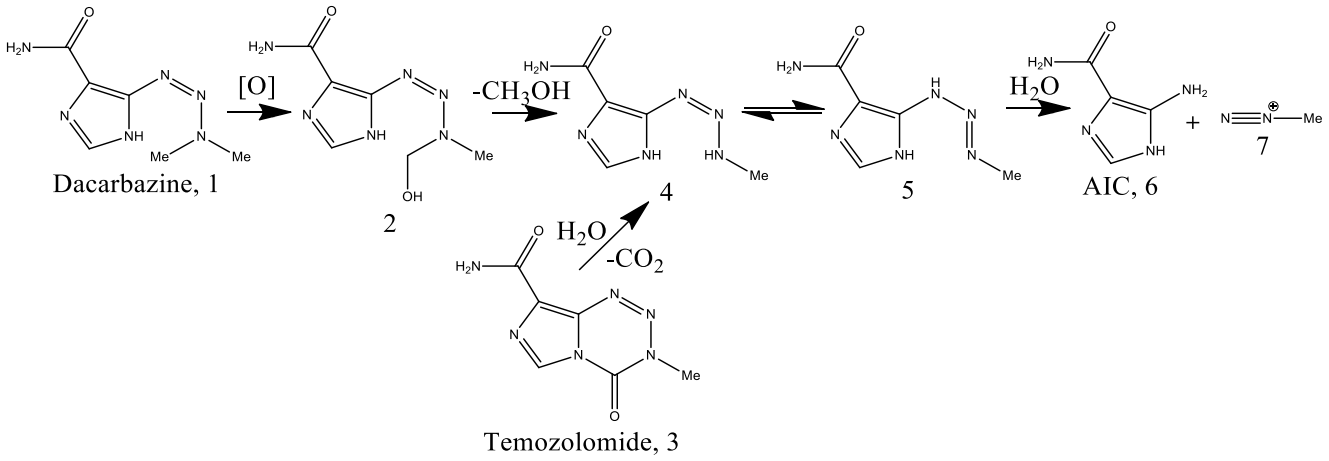
Recently, to circumvent problems associated with classical combinations of cytotoxic drugs, we developed a novel approach, termed "combi-targeting", that sought to design compounds termed "combi-molecules'' to not only possess DNA damaging properties, but also kinase targeting functions[25,26]. This approach was designed to selectively enhance the cytotoxic potential of the DNA damaging arm in tumours with disordered kinase-mediated signaling[27]. Here, given that synthetic lethality is a selective tumour targeting approach, we sought to design new molecules with DNA damaging properties that are targeted to the DNA repair protein PARP. The purpose of synthesizing such "combi-molecules" is to not only induce strong DNA damage in these cells but also deprive them from BER, the only alternative DNA repair mechanisms in *BRCA1* and *2* deficient cells. To achieve this goal, our strategy was to tether a DNA damaging moiety to a PARP targeting scaffold, while keeping the molecular size small enough to avoid problems associated with cell penetration or drug efflux mechanisms[28]. We first identified the isoquinolinone scaffold as the most suitable structure for our approach and appended a 1,2,3-triazene linkage to the 5-position of the ring, leading to structures **1-4** (Scheme 1). As depicted in Scheme 1, the triazene tail was designed to release the DNA alkylating methyl diazonium species and restore the intact PARP inhibitor upon hydrolysis.

The design of this series was based upon the mechanism of action of the lead clinical DNA alkylating agent termed temozolomide **3** (Scheme 2), a prodrug, which requires hydrolysis to generate the monoalkyltriazene **4**[29]. The latter is known to undergo further hydrolysis to give rise to amine **6** and the DNA methyl diazonium alkylating species **7**[30]. Based upon the latter model, structures **2** and **4** were expected to regenerate their parent aminoisoquinolinone (PARP inhibitor) and the DNA damaging methyl diazonium species (Scheme 1). We also designed structures **1** and **3**, based upon the mechanism of action of dacarbazine (see **1**, Scheme 2), which requires metabolic

activation to generate monoalkyltriazene **4**[31]. Structure **3** containing a bis-chloroethyl triazene moiety cyclized at room temperature to give the triazolinium EG15. It is now know that bis-chloroethyl triazenes spontaneously cyclize to a monochlorethyltriazolinium ion that retains DNA damaging capacity[32,33].



Scheme 1: Synthesis of PARP-5-AIQ series. Reagents and conditions: i: NaNO2, H+ at -5^{0}C. ii: Corresponding Amine at -5^{0}C.



Scheme 2: Generation of methyl diazonium from prodrugs dacarbazine and temozolomide.

2.3) RESULTS

The synthesis of the PARP-5-AIQ combi-molecule series proceeded according to Scheme 1. Briefly, PARP-5-AIQ was diazotized *in situ* and monomethylamine, dimethylamine, or bis(2-chloroethyl)amine were added. This was followed by the neutralization of the mixture to provide the corresponding mono- and dialkyltriazenes **1-4**. For the synthesis of the corresponding methylol, a mixture of methylamine and formaldehyde was added to the diazonium salt.

In order to determine the potential PARP targeting properties of the newly synthesized compounds, a cell-based assay was used. It consisted of a pair of cell lines originating from Chinese hamster lung cancer cells that included VC8 cells with *BRCA2* mutation and its *BRCA2* wild type transfectant. Selective targeting of the *BRCA2* mutant would indirectly indicate potential PARP targeting properties. The results showed that the lead PARP-5-AIQ inhibitor was virtually inactive in growth inhibition assay. Accordingly, no selectivity was observed (Figure 2). Nevertheless, selectivity was observed for the DNA alkylating combi-molecules **2-4**, which was attributed to their ability to damage DNA. These molecules being designed to regenerate PARP-5-aminoisoquinolinone, which was found to be inactive, we consequently concluded that any effect observed with **2-4** was due to their DNA alkylating potential. Therefore, keeping the design strategy intact, we sought for other amine containing PARP inhibitors. We found that the most potent of the many aromatic amines described in the literature was PARP-4-amino-1,8-naphthalimide (Scheme 3). Thus, we applied our strategy to a structural modification of the latter and this led to compounds **5-8**.

Using the cell-based assay, the selectivity of the resulting compounds were analyzed. The results showed that the lead PARP-4-amino-1,8-naphthalimide was 77-fold selective for the VC8 mutant (Figure 3), which suggested that this scaffold was perhaps targeted to PARP and hence

considered adequate for our targeting model. As shown in Table 1, adding a DNA damaging moiety to the PARP-4-ANI, decreased selectivity. However, as per our hypothesis, structures **2-4** and **6-8** showed that adding a cytotoxic moiety to the PARP targeting scaffold led to enhanced potency in the *BRCA2* mutant. This indicates that indeed, although PARP inhibitors are known to induce significant DNA damage in cells with *BRCA1 or 2*, addition of further damage can enhance their cytotoxic activity.

Pooling all of the selectivity data observed for all the compounds in the study, we obtained an average selectivity graph that showed EG40 to be the most selective combi-molecule of the panel (Figures 2 and 4). We therefore proceeded to investigate its dual targeting potential at the molecular level.

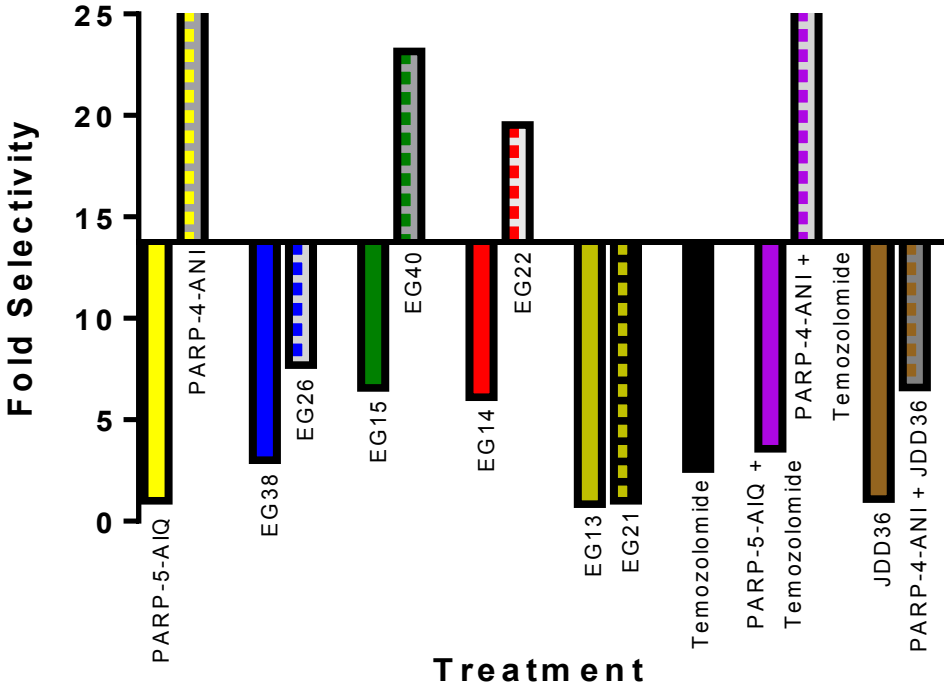
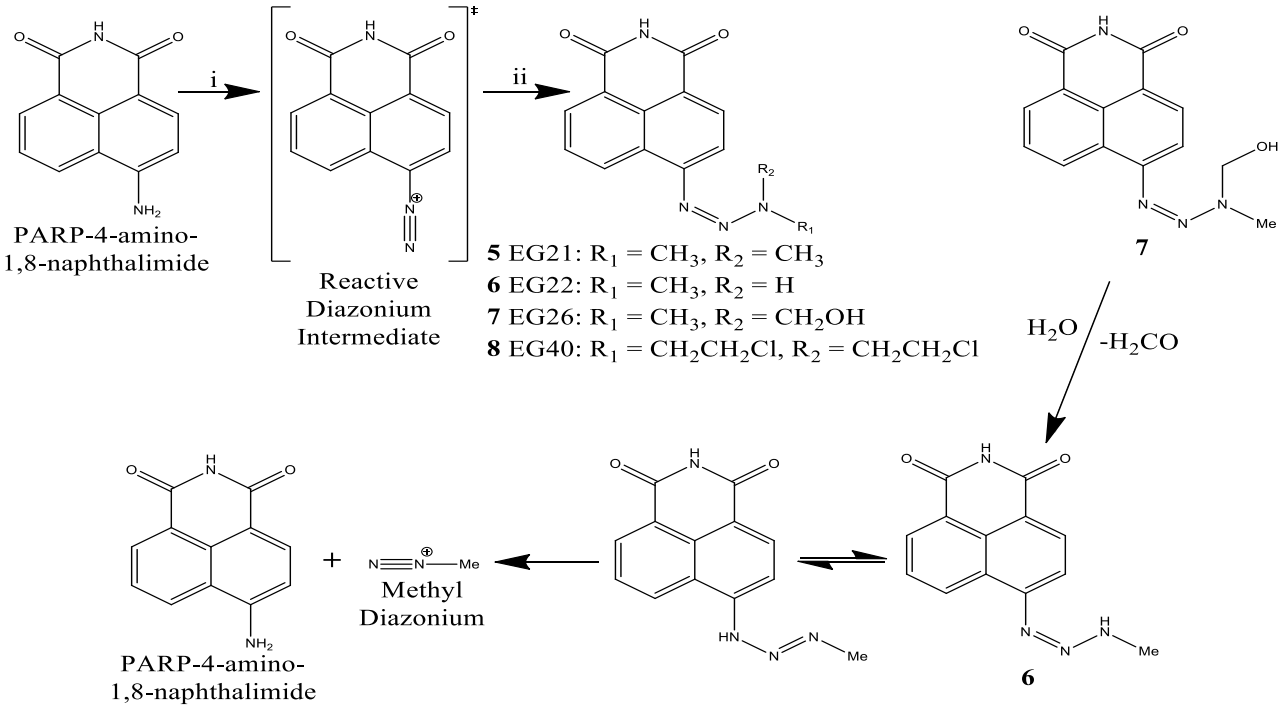


Figure 9: Pooled fold selectivity data of EG series between isogenic pair VC8 and VC8-*BRCA*.



Scheme 3: Synthesis of PARP-4-ANI Series. Reagents and conditions: i: NaNO2, H+ at -5^{0}C. ii: Corresponding Amine at -5^{0}C.

Furthermore, EG40 presents a unique chloroethyltriazolinium DNA damaging moiety, resulting from the cyclization of one of the chloroethyl groups. As outlined previously, we attributed the selectivity of all the triazolinium systems shown in Table 1 to their ability to damage DNA. However the contribution of the PARP targeted moiety to PARP inhibition remained to be demonstrated. Although the uniquely high selectivity of EG40 was an indirect evidence of PARP targeting, we sought to dissect the properties of its two targeting arms, using a PARP assay for PARP inhibition and the comet assay for analyzing its DNA damaging properties.

The results showed that our combi-molecule **8** induced a dose dependent inhibition of PARP with an IC_{50} of 1.7μM (Figure 4), which is in the same range as its IC_{50} for growth inhibition in the VC8 *BRCA2* mutant cells. This indicated that perhaps the PARP inhibitory potency of EG40 contributes to its growth inhibitory potency. Having confirmed that EG40 is a potent PARP

inhibitor, we sought to determine whether it could induce DNA strand breaks using the comet assay. The results showed that EG40 could induce high levels of DNA damage in both VC8 *BRCA2* mutant cells (Figure 5). For comparison, the extent of DNA damage induced by an analogous compound, JDD36[34], carrying the chloroethyltriazolinium function was also analyzed. Both compounds induced high levels of DNA damage, regardless of their aromatic warhead.

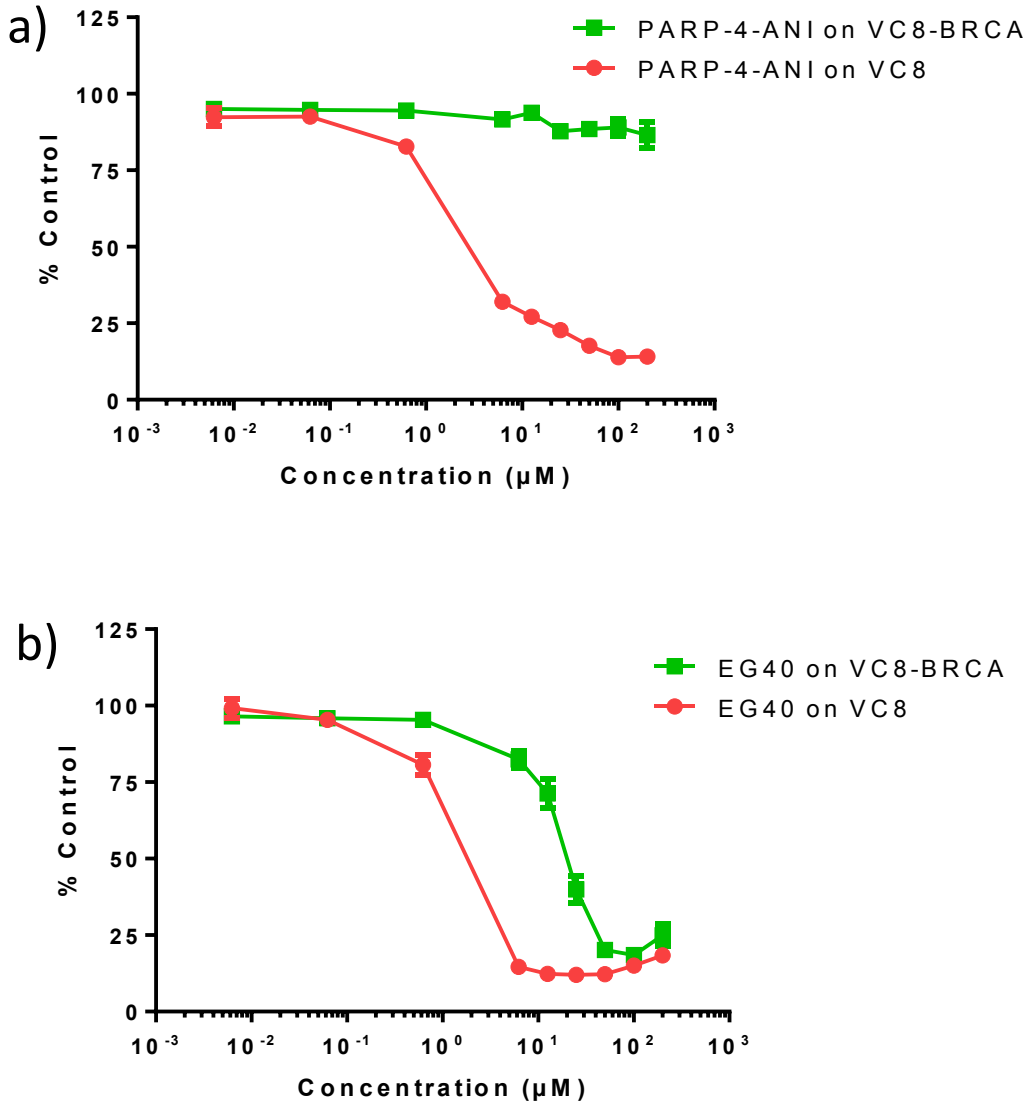
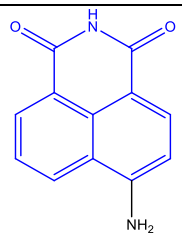
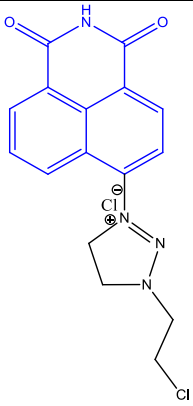
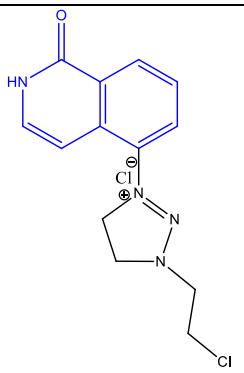
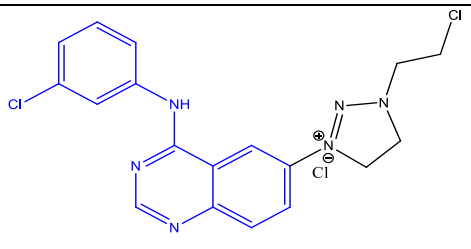


Figure 10: Selective VC8 mutant targeting by a) PARP-4-ANI and b) EG40. Data are means and SEM from 4 different experiments.

Table 1: Addition of a cytotoxic moiety increases the potency on the VC8 mutant.

Treatment	Structure	IC_{50} on VC8 (μM)
PARP-4-ANI		2.59
EG40		1.00
EG15		23.92
JDD36		15.17

Therefore, we believe that the DNA damage potential can be attributed to the chloroethyltriazolinium DNA damaging function and not to DNA intercalation. In corroboration, fluorescence microscopy did not show preferential nuclear localization of EG40 (Figure 6).

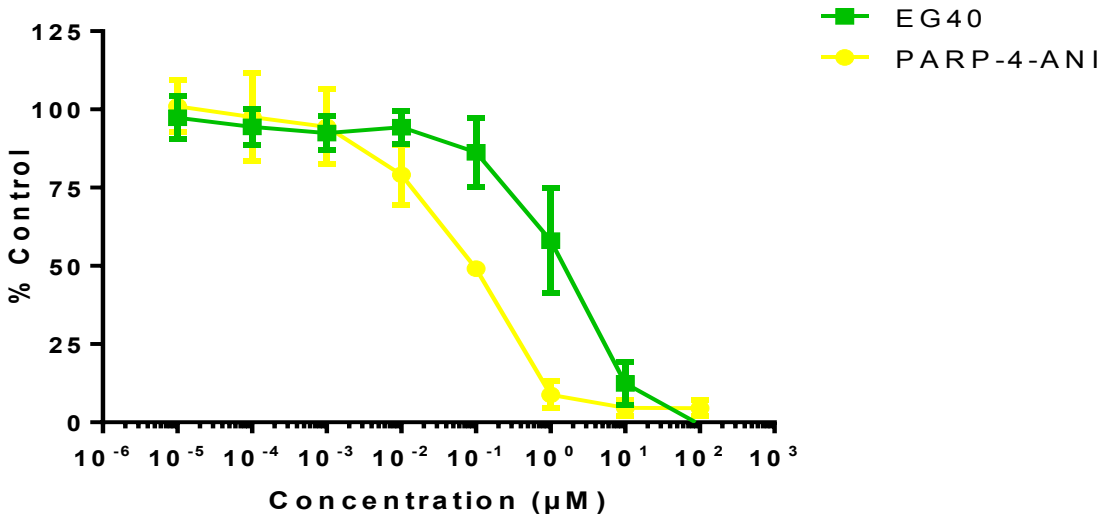


Figure 11: Dose dependent inhibition of EG40 in a PARP assay. Its parental PARP-4-ANI, a known PARP inhibitor, was used as a reference. Data are means and SEM from 2 different experiments.

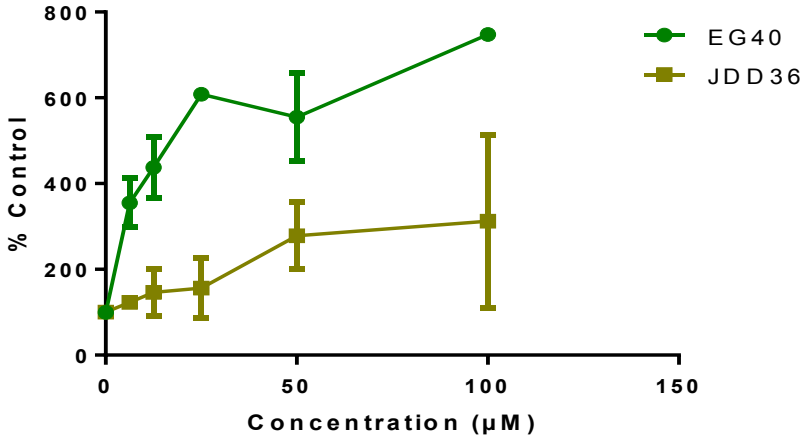
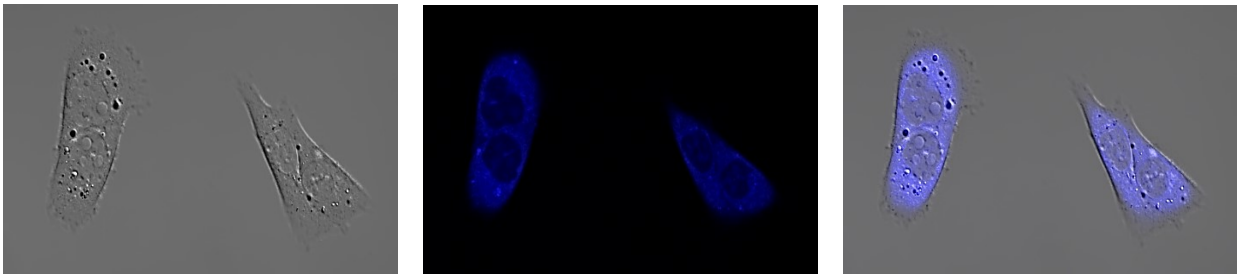


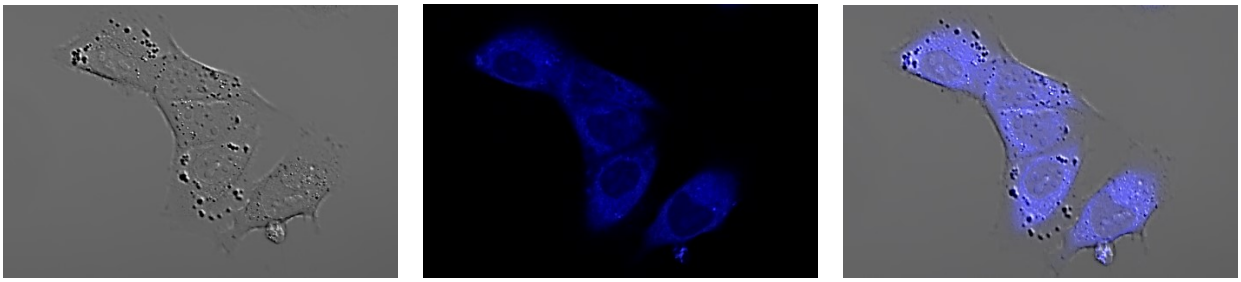
Figure 12: DNA damage induced by EG40 in VC8 mutant cells in comparison with JDD36. Data are means and SEM from 2 different experiments.

a)



VC8-*BRCA* Bright Field 25µM EG40	VC8-*BRCA* DAPI Filter 25µM EG40	VC8-*BRCA* Merge 25µM EG40

b)



VC8 Bright Field 25µM EG40	VC8 DAPI Filter 25µM EG40	VC8 Merge 25µM EG40

Figure 13: Subcellular distribution of EG40 in a) VC8 *BRCA* transfectant cells and b) VC8 mutant cells.

2.4) DISCUSSION

The current study was designed to establish the feasibility of molecules with dual PARP and DNA targeting properties. The requirement for grafting a DNA alkylating portion to the molecule led to a novel class of aromatic amine containing PARP inhibitors. While the latter amines have not reached the clinical setting, they have been reported to have potent PARP inhibitory potency[15,34]. However, in our cell-based assay, our aminoisoquinolinone and its derived combi-molecules did not show any significant growth inhibitory potency against the *BRCA2* mutant. Importantly, the lead aminoisoquinolinone did not show any selectivity for the *BRCA2* mutant form. Thus, we surmised that the 2-7-fold selectivity observed for these isoquinolinone combi-molecules could be attributed solely to their DNA damaging potential.

Thus, in order to achieve our proof-of-concept, a lead aromatic amine-containing PARP inhibitor with *BRCA2* selectivity was needed. Indeed, the aminonaphthalimide system showed significantly stronger potency against the *BRCA2* mutant in our cell-based selectivity assay, indicating that is has met the *sine qua non* for further design of combi-molecules. Our results indicate that adding a DNA damaging moiety to a PARP inhibitor scaffold decreases but does not abrogate its *BRCA2* selectivity. Indeed, PARP-4-ANI was 77-fold selective for the mutant, while the selectivity of the combi-molecule **8** was 25-fold, indicating that it retained significant selectivity for the *BRCA2* mutant. The decrease in selectivity may be due to the fact that inhibition of PARP in the *BRCA2* transfected VC8 cells enhances the potency of the DNA damaging agent in the latter cells, thereby leading to a decrease in the wild type/mutant IC_{50} ratios. Nevertheless, the observed 25-fold selectivity can be considered to be a significant retention of selective *BRCA2* mutant cell targeting potency.

Importantly, as per our primary objective, we have successfully achieved a significant enhancement in potency against the *BRCA2* mutant. Indeed, despite the great sensitivity of VC8 cells to naked PARP inhibitors, our dual targeting combi-molecules could induce 2-20-fold stronger potency than PARP 4-ANI. We believe that this enhanced potency may be attributed to its ability to inhibit PARP in the cells. Indeed, we showed that EG40, the most selective compound, could induce a dose-dependent inhibition of PARP. Further analysis by molecular modelling demonstrated that despite the steric hindrance conferred by the triazolinium group, EG40 could be docked into the binding pocket with possible hydrogen bonding interaction through its imide moiety with the NAD binding pocket. However, the triazolinium group would produce large clashes with the Lys903 and Glu998, which may explain its significantly weaker inhibition activity when compared with PARP 4-ANI (see Figure 7).

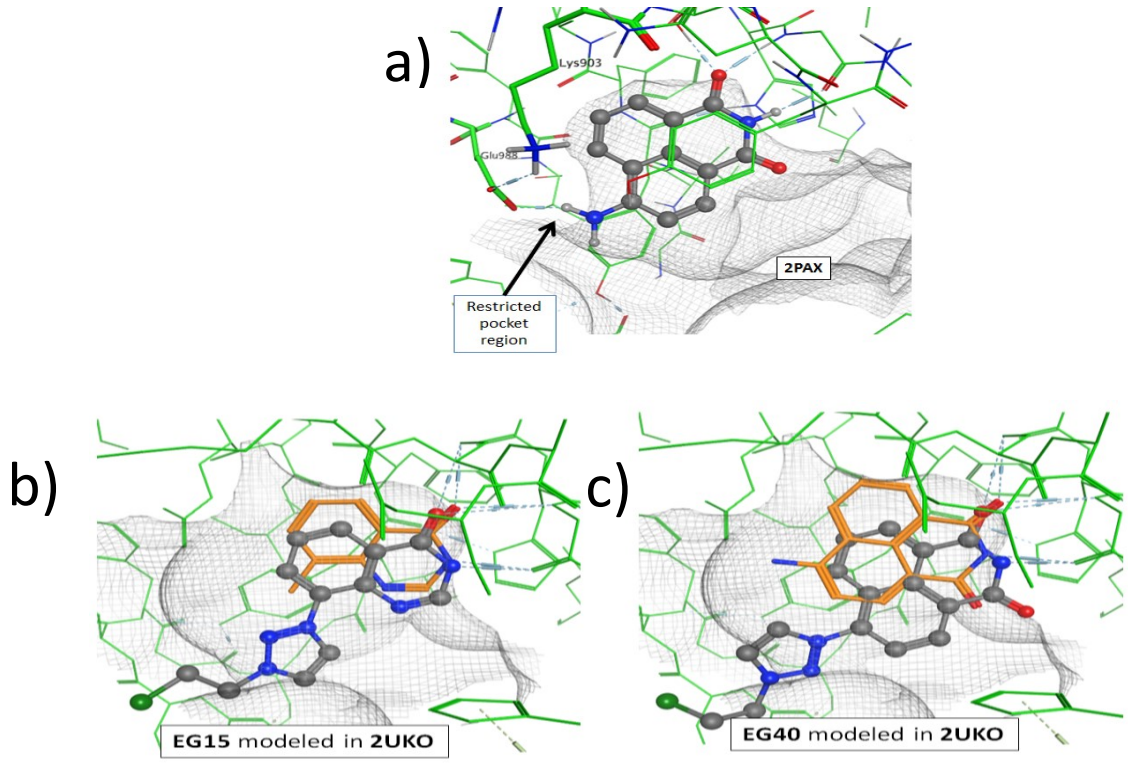


Figure 14: The naked PARP-4-ANI inhibitor (a), EG15 (b) and EG40 (c) in the NAD binding pocket

In this study, it should be mentioned that in contrast to the aminoisoquinolinone, the naphthalimide moiety has been reported to intercalate into the DNA[35]. Our data involving the comparison of other triazolinium compounds suggests that the DNA damaging property of the molecule is solely due to the chloroethyltriazolinium moiety. Fluorescence microscopy analysis did not show preferential distribution in the nucleus, a subcellular distribution profile that is typical of DNA intercalating agent. Indeed, chloroethyltriazolinium compounds, regardless of their aromatic moiety [e.g. imidazole[36-38], quinazoline[33], isoquinolinone, naphthalimide] induced significant levels of DNA damage in these cells as revealed by the comet assay.

In summary, we have successfully synthesized molecules targeting PARP and DNA, demonstrated their ability to enhance potency in the *BRCA2* mutant, as well as superior activity when compared with individual combinations of PARP inhibitors and DNA damaging agents. Further studies are required to demonstrate the *in vivo* potency of the approach.

ACKNOWLEDGEMENT

We thank the Canadian Institute of Health Research (CIHR) for financial support, Dr. Bernd Kaina for the isogenic pair of cells and Dr. Christopher Williams for the molecular modeling.

2.5) MATERIALS AND METHODS*

*For publication, this will be sent as supplemental material.

2.5.1) Chemistry

EG13: The bismethyl triazene compound, EG13, was synthesized as described in Scheme 1: PARP-5-amino-isoquinolinone (50mg, 0.312mmol) was dissolved in 50%/50% solution of water/hydrochloric acid (2.5mL/2.5mL) in the dark. The mixture was cooled to -5^{0}C. A solution of sodium nitrite in water (2eq in 1mL) was added dropwise. After 15 min at -5^{0}C, dimethylamine (6eq) was added slowly dropwise directly. After 30 min at -5^{0}C, the mixture was added dropwise to a saturated solution of sodium bicarbonate and left to precipitate for an hour. Filtration of the mixture yielded a light-brown solid (54mg, 80%). ^{1}H NMR (300MHz, DMSO-d_6) δ ppm 11.24 (br d, 1H, *J = 3.9Hz*, NHCH), 7.95 (d, 1H, *J = 7.5Hz*, ArH), 7.58 (dd, 1H, *J = 7.8Hz, 1.2Hz*, ArH), 7.36 (t, 1H, *J = 8.1Hz*, ArH), 7.13 (m, 1H, ArH), 7.04 (d, 1H, *J = 6.9Hz*, ArH), 3.53 (br s, 3H, NCH_3), 3.22 (br s, 3H, NCH_3). ^{13}C NMR (400MHz, DMSO-d_6): δ ppm 162.16, 145.44, 133.13, 128.50, 127.61, 126.79, 123.66, 118.60, 10076, 43.37 and 36.50 . ESI m/z 217 (MH^+).

EG14: The methyl triazene compound, EG14, was synthesized as described in Scheme 1: PARP-5-amino-isoquinolinone (50mg, 0.312mmol) was dissolved in 50%/50% solution of water/hydrochloric acid (2.5mL/2.5mL) in the dark. The mixture was cooled to -5^{0}C. A solution of sodium nitrite in water (2eq in 1mL) was added dropwise. After 15 min at -5^{0}C, methylamine (6eq) was added slowly dropwise directly. After 30 min at -5^{0}C, the mixture was added dropwise to a saturated solution of sodium bicarbonate and left to precipitate for an hour. Filtration of the mixture yielded a dark-brown solid (41mg, 65%). ^{1}H NMR (300MHz, DMSO-d_6) δ 11.26 (br s, 1H, NH), 10.72 (br s, 1H, NH), 7.96 (d, 1H, *J = 8.1Hz*, ArH), 7.57 (d, 1H, *J = 7.5Hz*, ArH), 7.39 (t, 1H, *J = 7.8Hz*, ArH), 7.14 (d, 1H, *J = 7.2Hz*, ArH), 7.04 (d, 1H, *J = 7.5Hz*, ArH), 7.95 (d, 1H, *J = 9Hz*, ArH), 3.08 (br s, 3H, CH_3). ^{13}C NMR (400MHz, DMSO-d_6): δ ppm 162.16, 145.83, 133.26, 128.47, 127.59, 126.81, 123.82, 118.73, 100.80 and 30.97. ESI m/z 203 (MH^+).

EG16: The triazolinium compound, EG16, was synthesized as described in Scheme 1: PARP-5-amino-isoquinolinone (50mg, 0.312mmol) was dissolved in 50%/50% solution of water/hydrochloric acid (2.5mL/2.5mL) in the dark. The mixture was cooled to -5^{0}C. A solution of sodium nitrite in water (2eq in 1mL) was added dropwise. After 15 min at -5^{0}C, a solution of bis (2-chloroethyl) amine hydrochloride in water (6eq in 2mL) was added slowly dropwise directly. After 30 min at -5^{0}C, the mixture was added dropwise to a saturated solution of sodium bicarbonate and left to precipitate for an hour. Filtration of the mixture yielded a dark-brown solid (10mg, 10%). ^{1}H NMR (300MHz, DMSO-*d*$_6$) δ 11.72 (br d, 1H, *J = 5.4Hz*, NH), 8.38 (d, 1H, *J = 8.1Hz*, ArH), 8.00 (dd, 1H, *J = 7.8Hz, 1.2Hz*, ArH), 7.67 (t, 1H, *J = 7.8Hz*, ArH), 7.37 (m, 1H, ArH), 6.77 (d, 1H, *J = 7.5Hz*, ArH), 4.92 (t, 2H, *J = 13.2Hz*, CH_2), 4.69 (t, 2H, *J = 12.9Hz*, CH_2), 4.56 (t, 2H, *J = 5.4Hz*, CH_2), 4.15 (t, 2H, *J = 5.1Hz*, CH_2). ^{13}C NMR (400MHz, DMSO-*d*$_6$): δ ppm 161.29, 132.64, 132.12, 131.79, 129.74, 128.40, 128.15, 126.80, 99.18, 55.96, 54.94, 54.79 and 40.82. ESI m/z 311 (M^-).

EG38: The methylol triazene compound, EG38, was synthesized as described in Scheme 1: PARP-5-amino-isoquinolinone (50mg, 0.312mmol) was dissolved in 50%/50% solution of water/hydrochloric acid (2.5mL/2.5mL) in the dark. The mixture was cooled to -5^{0}C. A solution of sodium nitrite in water (2eq in 1mL) was added dropwise. After 15 min at -5^{0}C, a solution of hydrochloric acid (1eq), formaldehyde (30eq) and methylamine (10eq) was added slowly dropwise directly. After 30 min at -5^{0}C, the mixture was added dropwise to a saturated solution of sodium bicarbonate and left to precipitate for an hour. Filtration of the mixture yielded a light-pink solid (48mg, 66%). ^{1}H NMR (300MHz, DMSO-*d*$_6$) δ 11.15 (vbr s, 1H, NH), 8.04 (dd, 1H, *J = 9.3Hz, 8.4Hz*, ArH), 7.64 (t, 1H, *J = 7.8Hz*, ArH), 7.47 (m, 2H, ArH), 7.12 (dt, 1H, *J = 20.1Hz, 7.2Hz*, ArH), 5.33 (s, 1H, OH), 5.17 (s, 2H, CH_2), 3.23 (s, 3H, CH_3). ^{13}C NMR (400MHz, DMSO-*d*$_6$): δ

ppm 161.17, 145.16, 132.64, 131.60, (2C) 127.19, 124.86, 119.31, 100.95, 78.52 and 33.66. ESI m/z 233 (MH^+).

EG21: The bismethyl triazene compound, EG21, was synthesized as described in Scheme 2: PARP-4-amino-1,8-napthalimide (50mg, 0.236mmol) was dissolved in trifluoroacetic acid (5mL) in the dark. The mixture was cooled to -5^{0}C. A solution of sodium nitrite in water (2eq in 1mL) was added dropwise. After 15 min at -5^{0}C, dimethylamine (6eq) was added slowly dropwise directly. After 30 min at -5^{0}C, the mixture was added dropwise to a saturated solution of sodium bicarbonate and left to precipitate for an hour. Filtration of the mixture yielded a light-brown solid (47mg, 74%). ^{1}H NMR (300MHz, DMSO-*d_6*) δ 11.59 (s, 1H, NH), 8.98 (dd, 1H, *J = 8.4Hz, 0.9Hz*, ArH), 8.45 (dd, 1H, *J = 7.1Hz, 1.2Hz*, ArH), 8.38 (d, 1H, *J = 8.1Hz*, ArH), 7.82 (t, 1H, *J = 7.8Hz,* ArH), 7.70 (d, 1H, *J = 8.1Hz,* ArH), 3.69 (s, 3H, CH_3), 3.43 (s, 3H, CH_3). ^{13}C NMR (400MHz, DMSO-*d_6*): δ ppm 164.87, 164.41, 151.64, 131.72, 130.84, 130.65, 130.53, 127.34, 126.55, 122.82, 118.26, 112.12, 44.21, 37.39. ESI m/z 269 (MH^+).

EG22: The methyl triazene compound, EG22, was synthesized as described in Scheme 2: PARP-4-amino-1,8-napthalimide (50mg, 0.236mmol) was dissolved in trifluoroacetic acid (5mL) in the dark. The mixture was cooled to -5^{0}C. A solution of sodium nitrite in water (2eq in 1mL) was added dropwise. After 15 min at -5^{0}C, methylamine (6eq) was added slowly dropwise directly. After 30 min at -5^{0}C, the mixture was added dropwise to a saturated solution of sodium bicarbonate and left to precipitate for an hour. Filtration of the mixture yielded a dark-brown solid (34mg, 57%). ^{1}H NMR (300MHz, DMSO-*d_6*) δ 11.61 (s, 1H, NH), 11.44 (q, 1H, *J = Hz*, $NHCH_3$), 8.97 (dd, 1H, *J = 8.4Hz, 0.9Hz*, ArH), 8.46 (dd, 1H, *J = 7.2Hz, 1.2Hz*, ArH), 8.39 (d, 1H, *J = 8.1Hz*, ArH), 7.83 (t, 1H, *J = 8.0Hz*, ArH), 3.26 (d, 1H, *J = 4.2Hz*, $NHCH_3$). ^{13}C NMR (400MHz, DMSO-

d6): δ ppm 164.89, 164.42, 152.11, 131.72, 130.86, 130.62, 130.48, 127.57, 127.46, 126.54, 118.43, 112.31 and 31.81. ESI m/z 253.0732 (MH^-).

EG26: The methylol triazene compound, EG26, was synthesized as described in Scheme 2: PARP-4-amino-1,8-napthalimide (50mg, 0.236mmol) was dissolved in trifluoroacetic acid (5mL) in the dark. The mixture was cooled to -5^{0}C. A solution of sodium nitrite in water (2eq in 1mL) was added dropwise. After 15 min at -5^{0}C, a solution of hydrochloric acid (1eq), formaldehyde (30eq) and methylamine (10eq) was added slowly dropwise directly. After 30 min at -5^{0}C, the mixture was added dropwise to a saturated solution of sodium bicarbonate and left to precipitate for an hour. Filtration of the mixture yielded a light-brown solid (25mg, 37%). ^{1}H NMR (300MHz, DMSO-*d6*) δ 11.15 (br s, 1H, NH), 8.97 (dd, 1H, *J = 8.4Hz, 1.2Hz*, ArH), 8.47 (dd, 1H, *J = 7.2Hz, 1.2Hz*, ArH), 8.42 (d, 1H, *J = 8.4Hz*, ArH), 7.86 (t, 1H, *J = 8.0Hz*, ArH), 7.44 (d, 1H, *J = 8.1Hz,* ArH), 6.58 (t, 1H, *J = 7.5Hz,* CH2OH), 5.26 (d, 2H, *J = 6.9Hz*, CH2OH), 3.39 (s, 3H, CH3). ^{13}C NMR (400MHz, DMSO-*d6*): δ ppm 164.82, 164.38, 151.21, 131.57, (2C) 130.71, 130.40, 127.55, 126.85, 122.89, 119.17, 112.84, 79.11 and 34.25. ESI m/z 283 (MH^-).

EG40: The triazolinium compound, EG40, was synthesized as described in Scheme 2: PARP-4-amino-1,8-napthalimide (50mg, 0.236mmol) was dissolved in trifluoroacetic acid (5mL) in the dark. The mixture was cooled to -5^{0}C. A solution of sodium nitrite in water (2eq in 1mL) was added dropwise. After 15 min at -5^{0}C, a solution of bis (2-chloroethyl) amine hydrochloride in water (6eq in 2mL) was added slowly dropwise directly. After 30 min at -5^{0}C, the mixture was added dropwise to a saturated solution of sodium bicarbonate and left to precipitate for an hour. Filtration of the mixture yielded a dark-brown solid (64mg, 74%). ^{1}H NMR (300MHz, DMSO-*d6*) δ 12.01 (br s, 1H, NH), 8.76 (dd, 1H, *J = 8.7Hz, 0.9Hz*, ArH), 8.59 (d, 1H, *J = 8.1Hz,* ArH), 8.58 (dd, 1H, *J = 7.2Hz, 0.9Hz*, ArH), 8.15 (d, 1H, *J = 8.1Hz*, ArH), 8.03 (t, 1H, *J = 8.1Hz*, ArH), 8.76

(dd, 1H, *J = 8.7Hz, 0.9Hz*, ArH), 5.10 (t, 2H, *J = 13.5Hz*, CH_2), 4.79 (t, 2H, *J = 12.9Hz*, CH_2), 4.71 (t, 2H, *J = 5.4Hz*, CH_2), 4.23 (t, 2H, *J = 5.1Hz*, CH_2). ^{13}C NMR (400MHz, DMSO-d_6): δ ppm 164.26, 163.59, 137.41, 131.42, 130.31, (2C) 130.09, 129.20, 124.97, 124.21, 123.73, 121.85, 55.54, 55.43, 55.35 and 40.75. ESI m/z 329.0806 (M^+ without Cl).

Molecular purity was assessed on the basis of ^1H NMR, ^{13}C NMR in tandem with heteronuclear single quantum coherence (HSQC) and mass spectrometry. EG14 and EG22 due to their lack of stability were only able to have approximately 90% purity as determined by ^1H NMR. All other synthesized molecules were in excess of 90% purity. ^1H NMR analyses were performed on a Varian 300MHz spectrometer. ^{13}C NMR as well as the HSQC analyses were performed on a Bruker 400MHz spectrometer. Mass spectrometry analysis for EG22, PARP-4-ANI and EG40 were done on a Thermo Exactive Plus Orbitrap-API high resolution mass spectrometer and the remaining molecules were analyzed via the Bruker AmaZon SL nominal mass spectrometer.

2.5.2) Biology

2.5.2.1) Cell Culture

The three cell lines used include VC8, V79 and VC8 transfected with the *BRCA* gene (VC8-*BRCA*). All cell lines originated from Chinese Hamster lung cancer and consequently were maintained under identical culture conditions. VC8 is *BRCA* deficient whereas V79 and VC8-*BRCA* are *BRCA* proficient. Cells vials that were stored in a -80^{0}C freezer were thawed in a 37^{0}C incubator with 5% CO_2. Thawed cell suspension was then aliquoted into a 15mL centrifuge tube and supplemented with 4mL of DMEM media. The suspension was centrifuged at 1,500rpm for 5 minutes and the supernatant discarded. 10mL of fresh DMEM media was added to the tube and the cell pellet resuspended. The cell suspension was transferred to a T75 flask and incubated at

37^0C with 5% CO_2. Media was exchanged maximum every four days as required via aspiration of old media within the T75 flask and replacement of 10mL of fresh DMEM media. Once cells reached appropriate confluency and required passaging, old media was aspirated and washed with 5mL of PBS. After the PBS wash was aspirated, 2mL of 0.25% Trypsin was aliquoted into the T75 flask and incubated at 37^0C for 1 minute. The action of Trypsin was halted using 5mL of fresh DMEM media and the resulting cell suspension transferred to a 15mL centrifuge tube. The cell suspension was then centrifuged at 1,500rpm for 5 minutes and the supernatant discarded. The cell pellet was then resuspended in 10mL of fresh DMEM media and a fraction was either aliquoted into a new T75 flask, aliquoted for experiments or frozen for storage. In the case of cell freezing, DMSO is added to make 5% DMSO in DMEM and 1mL of the cell suspension is aliquoted into cryovials. The freezing process involves 30 minutes at 4^0C, 2 hours at -20^0C and long term storage at -80^0C. Cell vials were in stasis for at least one month before thawing for cell culture.

2.5.2.2) Drug Treatment

20mM stock solutions of all drugs were prepared in sterile DMSO. Stock solutions were then diluted to the appropriate concentration (dependent on the assay) in DMEM media for growth inhibition assays and comet assays. Exceptions to the specified dug treatment were EG14 and EG22. Due to their unstable nature, drug dilutions were prepared in DMSO and each concentration was further diluted in DMEM media for subsequent treatment to minimize contact with aqueous media. For the PARP assay, proprietary PARP buffer 1X was used to dilute the stock solutions to the necessary concentration.

2.5.2.3) Growth Inhibition Assay

The growth inhibition assay used was the SRB assay. Once T75 flasks reached a confluency of approximately 60 to 100%, media was aspirated and the cells were washed with

5mL of PBS to remove residual media. 2mL of trypsin was used to detach the cells from the flask. Once the cells were detached, 5mL of DMEM was added to the cell suspension and the suspension transferred to a sterile 15mL centrifuge tube. The cell suspension was centrifuged for 1,500rpm for 5 minutes and the supernatant discarded. The cell pellet was resuspended with 10mL of fresh DMEM media. 100µL of cell suspension was mixed with 100µL of tryptan blue. 10µL of the resulting mixture was injected into the hemocytometer for cell counting. Once cell concentration was determined, the number of cells/well (dependent on cell line and day of treatment) were plated in 96 well plates. For V79, cells/well to plate were 1,000 for the first day of treatment and 500 for the second day of treatment. For VC8, cells/well to plate were 10,000 for the first day of treatment and 7,500 for the second day of treatment. For VC8-*BRCA*, cells/well to plate were 5,000 for the first day of treatment and 3,000 for the second day of treatment. The wells at the extremities of the plate were filled with 200µL of sterile PBS to reduce changes in well volume due to evaporation. The cell suspension was diluted according to the cell line and 100µL of cell suspension was plated into the required number of 96 well plates. Plates were stored for either one or two days (dependent on day of treatment) in a 37^0C incubator with 5% CO_2.

Drug treatment was done by diluting the 20mM stock solutions of drug in DMEM media, up to a maximum concentration of 2% DMSO in DMEM media. In the case of unimolecular drug combinations, 40µL of each drug stock solution was diluted in 2mL of pure DMEM media. Subsequent tubes contained 2% DMSO in DMEM media as a diluent to maintain the concentration of DMSO constant throughout treatments. The highest concentration was serially diluted by half for 6 dilution tubes and then by 1/10 for 3 dilution tubes, yielding the following concentration range: 400µM, 200µM, 100µM, 50µM, 25µM, 12.5µM, 1.25µM, 0.125µM, 0.0125µM and control (2% DMSO in DMEM media). 100µL of each drug concentration was aliquoted into each

well in triplicate and the treated cells were incubated for 5 days in a 37^0C incubator with 5% CO_2. After incubation, cells were fixed with 50µL of 50% trichloroacetic acid (TCA) and kept at 4^0C for 2 hours. Plates were then rinsed with tap water 4 times and left to dry overnight. 50µL of 0.4% Sulforhodamine B dye was added to each well and incubated for approximately 30 minutes. Plates were then washed with 1% acetic acid 4 times and allowed to dry overnight.

Prior to spectrometric analysis, 200µL of 10mM Tris base was added to each well and incubated for 5 minutes. The optical density was measured using a plate reader at 490nm.

2.5.2.4) PARP Assay

The Universal Colorimetric PARP assay was used to measure the binding affinity of the PARP inhibitory moiety of EG40 when compared to the original PARP inhibitor. All solutions prepared in this experiment were placed over ice. The PARP assay strips were rehydrated with 50µL of 1X PARP buffer and incubated for 30 minutes. Prepared drug dilutions involved 1 in 10 serial dilutions with a concentration range of 400µM, 40µM, 4µM, 0.4µM, 0.04µM, 0.004µM, 0.0004µM and 0.00004µM diluted in 1X PARP buffer. This concentration range was subjected to both PARP-4-ANI and EG40. The 1X PARP buffer was then discarded and 12.5µL of each drug dilution was added in duplicate in the wells. Add 12.5µL of PARP HSA enzyme in duplicate to each of the wells. Once both are added, incubate the plate for 10 minutes. After incubation, add 25µL of 1X PARP cocktail (mixture of activated DNA, proprietary PARP cocktail solution, diluted in 1X PARP buffer) in duplicate to each of the wells. Positive controls were prepared by only adding PARP HSA enzyme and PARP cocktail whereas the negative control was prepared by only adding inhibitor and PARP cocktail. In both cases, the final volume in these wells totaled 50µL. The plate was then incubated at room temperature for 1 hour. After incubation, the plate was washed twice with 0.1% PBS and twice with PBS. 50µL of 1X Strep-HRP diluted in

proprietary Strep diluent was then added to each of the wells and again incubated at room temperature for 1 hour. The plate was thereafter washed twice with 0.1% PBS and twice with PBS. 50µL of TACS Sapphire colorimetric substrate was then added to each of the wells in the dark and incubated in aluminum foil for 15 minutes at room temperature. The reaction with the substrate was then stopped using 50µL of 0.2N hydrochloric acid. The plate was then incubated for 5 minutes and the optical density read using a plate reader at 450nm.

2.5.2.5) Alkaline Comet Assay

The alkaline comet assay was used to measure the quantity of single and double strand breaks caused by alkylating agents. Passaged cells of VC8 and VC8-*BRCA* were diluted in the appropriate amount of fresh DMEM media. 1mL aliquots of cell suspensions were plated into each well for the required number of 6 well plates. Both cell lines were grown in these 6 well plates until 50-90% confluent.

Once appropriate confluency was reached, drug treated was administered. Drug dilutions were serially diluted by 1 in 2, yielding a concentration range of 100µM, 50µM, 25µM, 12.5µM and 6.25µM, diluted in 0.25% DMSO in DMEM media. A control treatment with only 0.25% of DMSO in DMEM media was also administered. This concentration range was applied to EG40, JDD36 and Chlorambucil. 1mL of each drug dilution was added to each well and incubated at 37^0C with 5% CO_2 for 2 hours. During treatment time, lysis buffers, agarose solution and films were prepared. Lysis buffer A consisted of 2.5M sodium chloride, 100mM of tetra sodium ethylene diamine tetraacetic acid, 10mM of Tris base and 35mM of n-Lauroyl sarcosine. Lysis buffer B was identical to lysis buffer A except for the absence of the n-lauroyl sacrosine. Agarose was prepared in a concentration of 0.75% in distilled water. The films were cut to hold 6 agarose gel chambers per film. The chambers were filled with liquefied agarose gel and pressed onto the hydrophobic

side of the film. Chambers were removed once the gel had polymerized. After treatment time, the cells had their treatment aspirated and washed with PBS. The treated cells then had 0.500mL of trypsin with 0.25% EDTA and were incubated at 37^0C for 1 minute. Once cells had detached from the plate, 1mL of fresh DMEM media was used to halt the trypsin. The cell suspension in each well was transferred into a 1.5mL Eppendorf tube, kept on ice and centrifuged for 3 minutes at 2000rpm at 4^0C. The media was then aspirated from each of the Eppendorf tubes and the cell pellet resuspended with 1mL of PBS. The tubes were again centrifuged for 3 minutes at 2000rpm and again the supernatant discarded. 1mL of PBS was added to resuspend the cell pellet. In a clean 1.5mL Eppendorf tube, 270µL of liquefied agarose and 30µL of cell suspension were mixed and of this, 150µL was loaded into the gel chamber on the film and allowed to polymerize. During polymerization, 44.5mL of lysis buffer A, 5mL of DMSO and 0.5mL of Triton X-100 were placed in a 50mL centrifuge tube. Once polymerization of this mixture was complete, the modified lysis buffer A was carefully poured over the gel in a petri dish and kept in aluminum foil overnight at 4^0C.

Lysis buffer A was then removed the next morning and washed 3 times with distilled water. 50mL of lysis buffer B was then added to the film inside the petri dish, the film covered with aluminum foil again, and incubated in 37^0C with 5% CO_2 for 1 hour. Thereafter, the lysis buffer B was removed and the film washed 3 times with distilled water. 50mL of unwinding buffer, consisting of 300mM of sodium hydroxide, 10mM of tetra sodium ethylene diamine tetraacetic acid and 7mM of 8-hydroxyquinoline in a diluent of 2% DMSO in distilled water, was poured onto the gel in the petri dish and incubated at room temperature in the dark for 30 minutes. Then the gel was placed into an electrophoresis apparatus, submerged with approximately 250mL of unwinding buffer and run under the following conditions: 400mA and 20V for 20 minutes. After

electrophoresis, the gel was then placed into a petri dish containing 50mL of neutralizing buffer (1.0M ammonium acetate) and allowed to incubate in the dark for 30 minutes. The film was then washed with approximately 50mL of 100% ethanol in the dark at room temperature for 2 hours. The ethanol was removed thereafter and the gels allowed to dry overnight.

Once dry, 4µL of CYBR Gold was diluted in 40mL of distilled water, poured over the films in a petri dish and incubated in the dark for 13 minutes. The dye was thereafter removed from the dish and the films were dried overnight. For imaging analysis, the Leica fluorescence microscope was used and the tail moments of the comets were measured using Comet Assay IV software.

REFERENCES

1. Kelley, M.R., Logsdon, D. & Fishel, M.L. Targeting DNA repair pathways for cancer treatment: what's new? *Future Oncology* **10**, 1215+ (2014).
2. Kaelin, W.G. The Concept of Synthetic Lethality in the Context of Anticancer Therapy. *Nat Rev Cancer* **5**, 689-698 (2005).
3. Fang, B. Development of Synthetic Lethality Anticancer Therapeutics. *Journal of Medicinal Chemistry* **57**, 7859-7873 (2014).
4. Rios, J. & Puhalla, S. PARP inhibitors in breast cancer: BRCA and beyond. *Oncology (Williston Park, N.Y.)* **25**, 1014-1025 (2011).
5. Shuen, A., Reis-Filho, J. & Foulkes, W. The Role of BRCA1 and BRCA2 in Anticancer Drug Therapy. in *Advances in DNA Repair in Cancer Therapy*, Vol. 72 (eds. Panasci, L., Aloyz, R. & Alaoui-Jamali, M.) 143-155 (Springer New York, 2013).
6. Al Abo, M.*, et al.* Compensatory Functions and Interdependency of the DNA-Binding Domain of BRCA2 with the BRCA1–PALB2–BRCA2 Complex. *Cancer Research* **74**, 797-807 (2014).
7. Sy, S.M.H., Huen, M.S.Y., Chen, J. & Livingston, D.M. PALB2 Is an Integral Component of the BRCA Complex Required for Homologous Recombination Repair. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 7155-7160 (2009).
8. Zhang, F., Fan, Q., Ren, K. & Andreassen, P.R. PALB2 Functionally Connects the Breast Cancer Susceptibility Proteins BRCA1 and BRCA2. *Molecular Cancer Research* **7**, 1110-1118 (2009).
9. Zhang, J. The role of BRCA1 in homologous recombination repair in response to replication stress: significance in tumorigenesis and cancer therapy. *Cell Biosci* **3**, 1-14 (2013).

10. Li, S.-X., Sjolund, A., Harris, L. & Sweasy, J.B. DNA repair and personalized breast cancer therapy. *Environmental and Molecular Mutagenesis* **51**, 897-908 (2010).
11. Horton, J.K.*, et al.* XRCC1 and DNA polymerase beta in cellular protection against cytotoxic DNA single-strand breaks. *Cell research* **18**, 48-63 (2008).
12. Fong, P.C.*, et al.* Inhibition of Poly(ADP-Ribose) Polymerase in Tumors from BRCA Mutation Carriers. *New England Journal of Medicine* **361**, 123-134 (2009).
13. Chan, S.L. & Mok, T. PARP inhibition in BRCA-mutated breast and ovarian cancers. *Lancet (London, England)* **376**, 211-213 (2010).
14. Uchigata, Y., Yamamoto, H., Kawamura, A. & Okamoto, H. Protection by superoxide dismutase, catalase, and poly(ADP-ribose) synthetase inhibitors against alloxan- and streptozotocin-induced islet DNA strand breaks and against the inhibition of proinsulin synthesis. *Journal of Biological Chemistry* **257**, 6084-6088 (1982).
15. Curtin, N.J. PARP inhibitors for cancer therapy. *Expert Reviews in Molecular Medicine* **7**, 1-20 (2005).
16. American Association for Cancer Research. Olaparib Approved for Advanced Ovarian Cancer. *Cancer Discovery Cancer Discovery* **5**, 218 (2015).
17. Harter, P.*, et al.* Olaparib maintenance therapy in patients with platinum-sensitive relapsed serous ovarian cancer and a BRCA mutation: Overall survival adjusted for post-progression PARP inhibitor therapy. *Gynecologic Oncology* **137, Supplement 1**, 8 (2015).
18. Bouwman, P. & Jonkers, J. Molecular Pathways: How Can BRCA-Mutated Tumors Become Resistant to PARP Inhibitors? *Clinical Cancer Research* **20**, 540-547 (2014).
19. Dhillon, K.K., Swisher, E.M. & Taniguchi, T. Secondary mutations of BRCA1/2 and drug resistance. *Cancer Science* **102**, 663-669 (2011).

20. Sonnenblick, A., de Azambuja, E., Azim Jr, H.A. & Piccart, M. An update on PARP inhibitors-moving to the adjuvant setting. *Nat Rev Clin Oncol* **12**, 27-41 (2015).
21. Clark, C.C., Weitzel, J.N. & O'Connor, T.R. Enhancement of Synthetic Lethality via Combinations of ABT-888, a PARP Inhibitor, and Carboplatin In Vitro and In Vivo Using BRCA1 and BRCA2 Isogenic Models. *Molecular Cancer Therapeutics* **11**, 1948-1958 (2012).
22. Liang, H. & Tan, A. PARP Inhibitors. *Curr Breast Cancer Rep* **3**, 44-54 (2011).
23. Plummer, R.*, et al.* A phase II study of the potent PARP inhibitor, Rucaparib (PF-01367338, AG014699), with temozolomide in patients with metastatic melanoma demonstrating evidence of chemopotentiation. *Cancer chemotherapy and pharmacology* **71**, 1191-1199 (2013).
24. Norris, R.E., Adamson, P.C., Nguyen, V.T. & Fox, E. Preclinical evaluation of the PARP inhibitor, olaparib, in combination with cytotoxic chemotherapy in pediatric solid tumors. *Pediatric Blood & Cancer* **61**, 145-150 (2014).
25. Rachid, Z., Brahimi, F., Katsoulas, A., Teoh, N. & Jean-Claude, B.J. The Combi-Targeting Concept: Chemical Dissection of the Dual Targeting Properties of a Series of "Combi-Triazenes". *Journal of Medicinal Chemistry* **46**, 4313-4321 (2003).
26. Larroque, A.-L.*, et al.* Synthesis of Water Soluble Bis-triazenoquinazolines: An Unusual Predicted Mode of Binding to the Epidermal Growth Factor Receptor Tyrosine Kinase. *CBDD Chemical Biology & Drug Design* **71**, 374-379 (2008).
27. Banerjee, R., Huang, Y., McNamee, J.P., Todorova, M. & Jean-Claude, B.J. The Combi-Targeting Concept: Selective Targeting of the Epidermal Growth Factor Receptor- and Her2-

Expressing Cancer Cells by the Complex Combi-Molecule RB24. *Journal of Pharmacology and Experimental Therapeutics* **334**, 9-20 (2010).

28. Nendza, M. & Müller, M. Screening for low aquatic bioaccumulation (1): Lipinski's 'Rule of 5' and molecular size. *SAR and QSAR in Environmental Research* **21**, 495-512 (2010).
29. Hvizdos, K.M. & Goa, K.L. Temozolomide. *CNS Drugs* **12**, 237-243 (1999).
30. Marchesi, F.*, et al.* Triazene compounds: Mechanism of action and related DNA repair systems. *Pharmacological Research* **56**, 275-287 (2007).
31. Amirmostofian, M.*, et al.* Synthesis and Molecular-cellular Mechanistic Study of Pyridine Derivative of Dacarbazine. *Iranian Journal of Pharmaceutical Research : IJPR* **12**, 255-265 (2013).
32. Costanza, M.E.*, et al.* Results of a randomized study comparing DTIC with TIC mustard in malignant melanoma. *Cancer* **37**, 1654-1659 (1976).
33. Qiu, Q.a.*, et al.* Type II combi-molecules: design and binary targeting properties of the novel triazolinium-containing molecules JDD36 and JDE05. *Anti-Cancer Drugs* **18**, 171-177 (2007).
34. McDonald, M.C., et al. Effects of 5-aminoisoquinolinone, a water-soluble, potent inhibitor of the activity of poly (ADP-ribose) polymerase on the organ injury and dysfunction caused by haemorrhagic shock. BPH British Journal of Pharmacology 130, 843-850 (2000).
35. Li, X.*, et al.* The novel anti-tumor agents of 4-triazol-1,8-naphthalimides: Synthesis, cytotoxicity, DNA intercalation and photocleavage. *European Journal of Medicinal Chemistry* **46**, 1274-1279 (2011).

36. Pittillo, R.F. & Hunt, D.E. 5(or 4)-(3,3-bis(2-chloroethyl)-1-triazeno)imidazole-4(or 5)-carboxamide (NSC-82196): microbiologic evaluation and assay. *Cancer chemotherapy reports. Part 1* **51**, 213-223 (1967).
37. Shealy, Y.F., Krauth, C.A., Holum, L.B. & Fitzgibbon, W.E. Synthesis and properties of the antileukemic agent 5 (or 4)-[3, 3-bis (2-chloroethyl)-1-triazeno] imidazole-4 (or 5)-carboxamide. *Journal of pharmaceutical sciences* **57**, 83-86 (1968).
38. Vogel, C.L.*, et al.* Preliminary clinical trials and clinical pharmacologic studies with 5-(3,3-bis(2-chloroethyl)-1-triazeno)-imidazole-4-carboxamide (NSC-82196) given orally. *Cancer chemotherapy reports. Part 1* **55**, 159-165 (1971).

Chapter 3: Discussion and Contribution to Knowledge

Chapter 3: DISCUSSION AND CONTRIBUTION TO KNOWLEDGE

3.1) DISCUSSION AND CONTRIBUTION TO KNOWLEDGE

Medical treatment of cancer is largely based on cytotoxic therapy involving DNA directed agents[66] (e.g cisplatin, cyclophosphamide, doxorubicin, temozolomide etc.). The major drawbacks of such therapies is their ability to block targets that are common to both normal and tumour cells. In particular, DNA damaging agents such as alkylating, metal complexing agents or radiotherapy, seek to kill the cells by inducing DNA lesions that can trigger apoptosis or other mechanisms of cell death. The toxicities associated with such mechanisms of antitumour action is a major deterrent in their use in the clinical management of solid tumours. More importantly, despite the potency of the DNA damaging agents, many patients fail to respond or develop resistance to such types of therapeutic modalities[67]. The toxicity and poor therapeutic index of these agents in some tumours have been attributed to a lack of tumour selectivity. Recently, the enthusiasm for DNA damage based therapy has been reawakened by the discovery of tumours with a specific cancer susceptibility gene that are uniquely sensitive to DNA damaging agents (e.g. alkylators and radiation). These genes termed *BRCA1* and *2,* which are involved in HR repair, confer DNA repair deficiency to their host tumours, thereby rending them more sensitive to DNA damage[68]. More importantly, these tumour cells express PARP as their sole alterative DNA repair protein. Accordingly, targeting the latter enzyme has revealed a significant vulnerability in these cells that would expose them to rapid DNA damage induced death. Therefore, because blocking PARP and mutation of the *BRCA1* or *2* cooperatively lead to cell death, the two genes responsible for the latter conditions are said to be synthetically lethal. Since the discovery of this principle, many genes whose dysfunction cooperate to lead to cell death have been identified[69]. Here, we have focused on synthetic lethality mediated by PARP inhibition and *BRCA1* and *2* mutations. During

the course of this study, the clinical proof-of-concept of targeting PARP in patients with *BRCA1* and *2* has been validated by the complete and final approval of the first PARP inhibitor by the FDA. This approval was limited, recommended for advanced stage ovarian cancers and specifically restricted to *BRCA1* or *2* mutated patients. Despite the significant enthusiasm for synthetic lethality, a large number of PARP inhibitors in development failed due to poor clinical outcomes[70], which may be associated to their lack of potency. Therefore, we hypothesized that if one agent has the dual ability to not only damage DNA but also to block PARP, the sole alternative mechanism in the cells, it would be a more potent therapy for *BRCA1* and *2* cells than the currently available or investigated agents. We believed that blocking PARP in these cells expressing cancer vulnerability genes while damaging their DNA would lead to enhanced cell-killing in these tumours.

In this thesis, we contributed to the first proof-of-concept that verifies this hypothesis by designing and synthesizing a large number of molecules from different classes, altering both the PARP targeting head and DNA damaging tail. We demonstrated herein that such molecules can damage DNA and inhibit PARP. More importantly, they can retain significant levels of selectivity (25-fold) for *BRCA1* and *2* mutant cells. While the dual PARP targeting properties of these novel dual targeted combi-molecules were not refined in this thesis, using molecular modelling, we have contribute to the rationalization of their PARP inhibitory potency. Indeed, in contrast to a standard PARP inhibitor, PARP-4-ANI, the position of the alkylating group have been shown through calculations to clash with the NAD binding pocket of PARP, suggesting that further design of compounds with refined potency should be directed at placing the DNA damaging group in a less restricted area of NAD binding pocket.

The results presented in this thesis *in toto* give *prima facie* evidence of the strong potency associated with tandem targeting of PARP and DNA using a single molecule. Importantly, while selectivity was decreased when compared with a naked PARP inhibitor, selectivity was not abrogated by the approach. Further studies are required to demonstrate the potency of this novel approach *in vivo*.

3.2) REFERENCES

1. Myers, M.P.*, et al.* The lipid phosphatase activity of PTEN is critical for its tumor supressor function. *Proceedings of the National Academy of Sciences* **95**, 13513-13518 (1998).
2. Mersch, J.*, et al.* Cancers associated with BRCA1 and BRCA2 mutations other than breast and ovarian. *Cancer* **121**, 269-275 (2015).
3. Li, S.-X., Sjolund, A., Harris, L. & Sweasy, J.B. DNA repair and personalized breast cancer therapy. *Environmental and Molecular Mutagenesis* **51**, 897-908 (2010).
4. Kornienko, A., Mathieu, V., Rastogi, S.K., Lefranc, F. & Kiss, R. Therapeutic Agents Triggering Nonapoptotic Cancer Cell Death. *Journal of Medicinal Chemistry* **56**, 4823-4839 (2013).
5. Comen, E.A.M.D. & Robson, M.M.D. Poly(ADP-Ribose) Polymerase Inhibitors in Triple-Negative Breast Cancer. *Cancer Journal January/February* **16**, 48-52 (2010).
6. O'Connor, M.J. Development of the PARP inhibitor olaparib to generate new therapies for cancer patients. *Mutagenesis* **27 (6)**, 799 (2012).
7. Clark, C.C., Weitzel, J.N. & O'Connor, T.R. Enhancement of Synthetic Lethality via Combinations of ABT-888, a PARP Inhibitor, and Carboplatin In Vitro and In Vivo Using BRCA1 and BRCA2 Isogenic Models. *Molecular Cancer Therapeutics* **11**, 1948-1958 (2012).
8. Sung, J.S. & Demple, B. Analysis of Base Excision DNA Repair of the Oxidative Lesion 2-Deoxyribonolactone and the Formation of DNA–Protein Cross-Links. in *Methods in Enzymology*, Vol. Volume 408 48-64 (Academic Press, 2006).
9. Korolev, V.G. Base Excision Repair of DNA: Glycosylases. *Russ J Genet* **41**, 583-592 (2005).

10. Sung, J.-S. & Demple, B. Roles of base excision repair subpathways in correcting oxidized abasic sites in DNA. *FEBS Journal* **273**, 1620-1629 (2006).

11. Hang, B. *Dna Repair, Genetic Instability, And Cancer*, (World Scientific & Imperial College Press, Singapore, SGP, 2007).

12. Ma, W.*, et al.* The Transition of Closely Opposed Lesions to Double-Strand Breaks during Long-Patch Base Excision Repair Is Prevented by the Coordinated Action of DNA Polymerase δ and Rad27/Fen1. *Molecular and Cellular Biology* **29**, 1212-1221 (2009).

13. Ranalli, T.A., Tom, S. & Bambara, R.A. AP Endonuclease 1 Coordinates Flap Endonuclease 1 and DNA Ligase I Activity in Long Patch Base Excision Repair. *Journal of Biological Chemistry* **277**, 41715-41724 (2002).

14. Malanga, M. & Althaus, F.R. The role of poly(ADP-ribose) in the DNA damage signaling network1. *Biochemistry and Cell Biology* **83**, 354-364 (2005).

15. Masaoka, A., Horton, J.K., Beard, W.A. & Wilson, S.H. DNA polymerase β and PARP activities in base excision repair in living cells. *DNA Repair* **8**, 1290-1299 (2009).

16. Javle, M. & Curtin, N.J. The potential for poly (ADP-ribose) polymerase inhibitors in cancer therapy. *Therapeutic Advances in Medical Oncology* **3**, 257-267 (2011).

17. de Murcia, G.*, et al.* Structure and function of poly(ADP-ribose) polymerase. *Mol Cell Biochem* **138**, 15-24 (1994).

18. Godon, C.*, et al.* PARP inhibition vs. PARP-1 silencing: different outcomes in terms of single-strand break repair. *European Journal of Cancer Supplements* **6**, 55 (2008).

19. Horton, J.K. & Wilson, S.H. Hypersensitivity phenotypes associated with genetic and synthetic inhibitor-induced base excision repair deficiency. *DNA Repair* **6**, 530-543 (2007).

20. Tounekti, O., Kenani, A., Foray, N., Orlowski, S. & Mir, L.M. The ratio of single- to double-strand DNA breaks and their absolute values determine cell death pathway. *Br J Cancer* **84**, 1272-1279 (2001).

21. Farlow, A., Meduri, E. & Schlötterer, C. DNA double-strand break repair and the evolution of intron density. *Trends in Genetics* **27**, 1-6 (2011).

22. Zhang, J. The role of BRCA1 in homologous recombination repair in response to replication stress: significance in tumorigenesis and cancer therapy. *Cell Biosci* **3**, 1-14 (2013).

23. Wei, Q.Y. *Dna Repair, Genetic Instability, And Cancer*, (World Scientific & Imperial College Press, Singapore, SGP, 2007).

24. Venkitaraman, A.R. Cancer Susceptibility and the Functions of BRCA1 and BRCA2. *Cell* **108**, 171-182 (2002).

25. Aguilera, A. & Rothstein, R. Molecular genetics of recombination. (2007).

26. Xu, X.*, et al.* The yeast Shu complex utilizes homologous recombination machinery for error-free lesion bypass via physical interaction with a Rad51 paralogue. *PloS one* **8**(2013).

27. Roy, R., Chun, J. & Powell, S.N. BRCA1 and BRCA2: different roles in a common pathway of genome protection. *Nat Rev Cancer* **12**, 68-78 (2012).

28. Hoa, N.N.*, et al.* BRCA1 and CtIP Are Both Required to Recruit Dna2 at Double-Strand Breaks in Homologous Recombination. *PLoS ONE* **10**, e0124495 (2015).

29. Mimitou, E.P. & Symington, L.S. Nucleases and helicases take center stage in homologous recombination. *Trends in biochemical sciences* **34**, 264-272 (2009).

30. Zhang, F., Fan, Q., Ren, K. & Andreassen, P.R. PALB2 Functionally Connects the Breast Cancer Susceptibility Proteins BRCA1 and BRCA2. *Molecular Cancer Research* **7**, 1110-1118 (2009).

31. Sy, S.M.H., Huen, M.S.Y., Chen, J. & Livingston, D.M. PALB2 Is an Integral Component of the BRCA Complex Required for Homologous Recombination Repair. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 7155-7160 (2009).

32. Frankenberg-Schwager, M*., et al.* Single-Strand Annealing, Conservative Homologous Recombination, Nonhomologous DNA End Joining, and the Cell Cycle-Dependent Repair of DNA Double-Strand Breaks Induced by Sparsely or Densely Ionizing Radiation. *rare Radiation Research* **171**, 265-273 (2009).

33. Lushnikov, A.Y., Bogdanov, A. & Lyubchenko, Y.L. DNA recombination: holliday junctions dynamics and branch migration. *The Journal of biological chemistry* **278**, 43130-43134 (2003).

34. Murphy, C.G.M.B.B.B.A.O. & Moynahan, M.E.M.D. BRCA Gene Structure and Function in Tumor Suppression: A Repair-Centric Perspective. *The Cancer Journal* **16**, 39-47 (2010).

35. Sameer, A.S., Nissar, S. & Fatima, K. Mismatch repair pathway. *European Journal of Cancer Prevention European Journal of Cancer Prevention*, 1 (2014).

36. Vasquez, K.M. Targeting and processing of site-specific DNA interstrand crosslinks. *EM Environmental and Molecular Mutagenesis* **51**, 527-539 (2010).

37. Lei, L. *Dna Repair, Genetic Instability, And Cancer*, (World Scientific & Imperial College Press, Singapore, SGP, 2007).

38. Hanawalt, P.C. Subpathways of nucleotide excision repair and their regulation. *Oncogene* **21**, 8949-8956 (2002).

39. You, J.-S., Wang, M. & Lee, S.-H. Biochemical Analysis of the Damage Recognition Process in Nucleotide Excision Repair. *Journal of Biological Chemistry* **278**, 7476-7485 (2003).

40. Sasaki, M.S., Takata, M., Sonoda, E., Tachibana, A. & Takeda, S. Recombination repair pathway in the maintenance of chromosomal integrity against DNA interstrand crosslinks. *Cytogenetic and genome research* **104**, 1-4 (2004).

41. Michel, B., Grompone, G., Florès, M.J. & Bidnenko, V. Multiple pathways process stalled replication forks. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 12783-12788 (2004).

42. Quan Ge, X. & Julian Blow, J. Chapter 1 Conserved Steps in Eukaryotic DNA Replication. in *Molecular Themes in DNA Replication* 1-21 (The Royal Society of Chemistry, 2009).

43. Garg, P. & Burgers, P.M.J. DNA Polymerases that Propagate the Eukaryotic DNA Replication Fork. *Critical Reviews in Biochemistry and Molecular Biology* **40**, 115-128 (2005).

44. Tougu, K. & Marians, K.J. The Interaction between Helicase and Primase Sets the Replication Fork Clock. *Journal of Biological Chemistry* **271**, 21398-21405 (1996).

45. Nahas, S.A. & Gatti, R.A. DNA double strand break repair defects, primary immunodeficiency disorders, and 'radiosensitivity'. *Current opinion in allergy and clinical immunology* **9**, 510-516 (2009).

46. Nagaraju, G., Scully, R. & Replication Fork Repair, P. Minding the gap: The underground functions of BRCA1 and BRCA2 at stalled replication forks. *DNA Repair* **6**, 1018-1031 (2007).

47. Brady, N., Gaymes, T.J., Cheung, M., Mufti, G.J. & Rassool, F.V. Increased error-prone NHEJ activity in myeloid leukemias is associated with DNA damage at sites that recruit key nonhomologous end-joining proteins. *Cancer research* **63**, 1798-1805 (2003).

48. Ellis, P., Stebbing, J. & Tutt, A. PARP inhibitors in BRCA1-/BRCA2-associated and triple-negative breast cancers. *Future Oncology* **6**, 485+ (2010).

49. Deeks, E. Olaparib: First Global Approval. *Drugs* **75**, 231-240 (2015).

50. Glendenning, J. & Tutt, A. PARP inhibitors – current status and the walk towards early breast cancer. *The Breast* **20, Supplement 3**, S12-S19 (2011).

51. Liu, X. PARP inhibition as a prototype for synthetic lethal screens. *Methods Mol Biol* **986**, 123-137 (2013).

52. Brough, R., Frankum, J.R., Costa-Cabral, S., Lord, C.J. & Ashworth, A. Searching for synthetic lethality in cancer. *Current Opinion in Genetics & Development* **21**, 34-41 (2011).

53. Nguyen, S.D. *Tumor Suppressors*, (Nova Science Publishers, Inc., New York, NY, USA, 2011).

54. Bitler, B.G.*, et al.* Synthetic lethality by targeting EZH2 methyltransferase activity in ARID1A-mutated cancers. *Nat Med* **21**, 231-238 (2015).

55. Hashimoto, J.*, et al.* O3-049 * SYNERGISTIC INTERACTION BETWEEM OLAPARIB, A PARP INHIBITOR, AND CYTOTOXIC AGENT IN TRIPLE NEGATIVE BREAST CANCER. *Annals of Oncology Annals of Oncology* **24**, ix59 (2013).

56. Hvizdos, K.M. & Goa, K.L. Temozolomide. *CNS Drugs* **12**, 237-243 (1999).

57. Marchesi, F.*, et al.* Triazene compounds: Mechanism of action and related DNA repair systems. *Pharmacological Research* **56**, 275-287 (2007).

58. Begleiter, A., Mowat, M., Israels, L.G. & Johnston, J.B. Chlorambucil in Chronic Lymphocytic Leukemia: Mechanism of Action. *Leukemia & Lymphoma* **23**, 187-201 (1996).

59. Matheson, S.L., McNamee, J. & Jean-Claude, B.J. Design of a chimeric 3-methyl-1,2,3-triazene with mixed receptor tyrosine kinase and DNA damaging properties: a novel tumor targeting strategy. *The Journal of pharmacology and experimental therapeutics* **296**, 832-840 (2001).

60. Brahimi, F.*, et al.* Inhibition of epidermal growth factor receptor-mediated signaling by "Combi-triazene" BJ2000, a new probe for Combi-Targeting postulates. *The Journal of pharmacology and experimental therapeutics* **303**, 238-246 (2002).

61. Rachid, Z., Brahimi, F., Katsoulas, A., Teoh, N. & Jean-Claude, B.J. The Combi-Targeting Concept: Chemical Dissection of the Dual Targeting Properties of a Series of "Combi-Triazenes". *Journal of Medicinal Chemistry* **46**, 4313-4321 (2003).

62. Domarkas, J.*, et al.* The Combi-Targeting Concept: Synthesis of Stable Nitrosoureas Designed to Inhibit the Epidermal Growth Factor Receptor (EGFR). *Journal of Medicinal Chemistry* **49**, 3544-3552 (2006).

63. Banerjee, R., Huang, Y., McNamee, J.P., Todorova, M. & Jean-Claude, B.J. The Combi-Targeting Concept: Selective Targeting of the Epidermal Growth Factor Receptor- and Her2-Expressing Cancer Cells by the Complex Combi-Molecule RB24. *Journal of Pharmacology and Experimental Therapeutics* **334**, 9-20 (2010).

64. Qiu, Q.a., *et al.* Type II combi-molecules: design and binary targeting properties of the novel triazolinium-containing molecules JDD36 and JDE05. *Anti-Cancer Drugs* **18**, 171-177 (2007).

65. Rao, S., *et al.* Target Modulation by a Kinase Inhibitor Engineered to Induce a Tandem Blockade of the Epidermal Growth Factor Receptor (EGFR) and c-Src: The Concept of Type III Combi-Targeting. *PLoS ONE PLOS ONE* **10**, e0117215 (2015).

66. Li, F., Zhao, C. & Wang, L. Molecular-targeted agents combination therapy for cancer: Developments and potentials. *International Journal of Cancer* **134**, 1257-1269 (2014).

67. Brown, R. & Böger-Brown, U. Cytotoxic drug resistance mechanisms. (1999).

68. Drew, Y. & Calvert, H. The Potential of PARP Inhibitors in Genetic Breast and Ovarian Cancers. *NYAS Annals of the New York Academy of Sciences* **1138**, 136-145 (2008).

69. Bajrami, I., *et al.* Synthetic lethality of PARP and NAMPT inhibition in triple-negative breast cancer cells. *EMBO Mol Med* **4**, 1087-1096 (2012).

70. Harter, P., *et al.* Olaparib maintenance therapy in patients with platinum-sensitive relapsed serous ovarian cancer and a BRCA mutation: Overall survival adjusted for post-progression PARP inhibitor therapy. *Gynecologic Oncology* **137, Supplement 1**, 8 (2015).