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## Identification of DNA repair factors which show a synthetic genetic interaction with MRE11 inhibition

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*Award date:*
2024

*Awarding institution:*
Bangor University

MScRes Molecular Medicine

Bangor University, School of Medical and Health Sciences

# Identification of DNA repair factors which show a synthetic genetic interaction with MRE11 inhibition

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I hereby declare that this thesis is the results of my own investigations, except where otherwise stated. All other sources are acknowledged by bibliographic references. This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless, as agreed by the University, for approved dual awards.

## Abstract

MRE11 is involved in many pathways of DNA damage recognition and repair, and in the overall maintenance of genomic stability. Synthetic lethality is an anticancer therapy approach that exploits the inherent differences between cancerous and non-cancerous cells, and one such strategy involves the targeting to DNA repair proteins. Cancers are commonly deficient in DNA damage recognition and repair pathways, due to increased mutation rates providing more opportunity for chance selective advantage to occur. This, however, results in an increased reliance on alternative repair pathways which can then be targeted by specific small molecule inhibitors to result in synthetic lethality. MRE11 is one such repair factor that has been identified as a potential anticancer target, with its inhibition previously having been shown to sensitise cells to the cancer drugs camptothecin and gemcitabine. Moreover, novel MRE11 inhibitors with improved pharmacological properties compared to those currently available are currently in development. This project screens a variety of selected inhibitors of DNA repair proteins for synthetic interaction with MRE11 inhibition, with the aim of identifying synthetic lethal interactions that could be exploited by novel MRE11 inhibitors clinically. It is shown that synthetic lethal interactions were present for PARP, FEN1 and ATR with MRE11 exonuclease in DT40 *MRE11*$^{+/-}$ cells, with this synthetic lethality also observed for PARP and FEN1 in TK6$^{WT/-}$. However, MRE11 exonuclease inhibition seems to rescue the effects of DNA2 inhibition in DT40 *MRE11*$^{+/-}$, and RAD51 inhibition in TK6$^{WT/-}$, while no effects were shown with the addition of MRE11 exonuclease inhibition for DNA-PK, WRN and ATM in DT40 *MRE11*$^{+/-}$. As MRE11 has been shown to have the ability to remove protein-DNA blocks, such as topoisomerase complexes, from the DNA, it was thought that PARP-trapping inhibitors might work synergistically with MRE11 inhibition, and so inhibitors with different PARP-trapping capabilities were tested. Some evidence of further synthetic genetic interaction between potent PARP-trapper Talazoparib and mirin was observed in TK6$^{WT/-}$, but overall this line of study was inconclusive and requires further investigation. This project provides evidence for synthetic genetic interactions between a variety of DNA repair factors and MRE11 inhibition, that can be used in further investigation with novel MRE11 inhibitors for use in personalised treatment of cancers expressing defects in these DNA repair pathways.

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# 1 Introduction and Literature Review

## 1.1 Cancer and DNA Damage

DNA repair mechanisms and cancer are intrinsically linked. The acquisition of the six hallmarks of cancer, as described by Hanahan and Weinberg (2000), depends on two enabling characteristics, as described by Hanahan and Weinberg (2011): genome instability and mutation, and tumour-promoting inflammation. Cancer development and progression can be viewed as a series of clonal expansions resulting from a mutation or epigenetic change that drives cell division resulting in a selective advantage and eventual out-competition of other non-cancerous cells within the local environment. In normal cells, mechanisms to detect and repair DNA damage that can result in DNA mutation keep mutation rates low, however cancer cells are often characterised by rapid division and increased mutation rates. This is often achieved by compromising the DNA damage recognition mechanisms that would otherwise force the cell into senescence or apoptosis upon recognition of damage (Salk, Fox and Loeb, 2010). In this way, it is beneficial for cancer cells to contain defects in DNA repair pathways, as it allows greater accumulation of mutations that could confer further selective advantage, and this increased genomic instability can result in even further deficiencies in DNA maintenance and repair. While these deficiencies can promote carcinogenesis, they also represent potential targets, exploitable in the treatment of cancer.

Ideal cancer treatments involve the specific targeting of cancerous cells, while sparing non-cancerous cells to limit toxicity to the patient. The synthetic lethality (SL) approach has a been popular line of investigation for novel cancer therapies and is defined by non-lethal mutations (or alterations to expression or activity such as by gene knockout or inhibitors) in different genes resulting in lethality when combined (Nijman, 2011; Guo *et al.*, 2020). In cancer treatment, SL strategies involve the exploitation of cancer-specific defects or deficiencies, such as in DNA repair, to target gene products essential for the cancer cells survival, while leaving normal cells unharmed due to intact redundant pathways (SL is summarised visually in Figure 1; Gavande et al., 2016; Guo et al., 2020). For example, a major breakthrough in cancer treatment, and in the SL approach, was the use of PARP inhibitors in BRCA-deficient ovarian cancer (Gavande *et al.*, 2016). As previously discussed, cancer cells are characterised by their genomic instability caused by defects in DNA damage recognition and repair genes. BRCA1 and 2 play an integral role in the repair of double-strand DNA breaks, while PARP is more known for its role in single-strand break repair. By inhibiting PARP, single-strand breaks accumulate in the DNA, and when left unrepaired are converted to double-strand breaks during DNA replication, however these are also not repairable in BRCA-deficient cancer cells. Therefore, the combination of these two deficiencies proves synthetically lethal to cancer cells but not to BRCA-proficient cells, by exploiting their inherent differences from non-cancer cells.

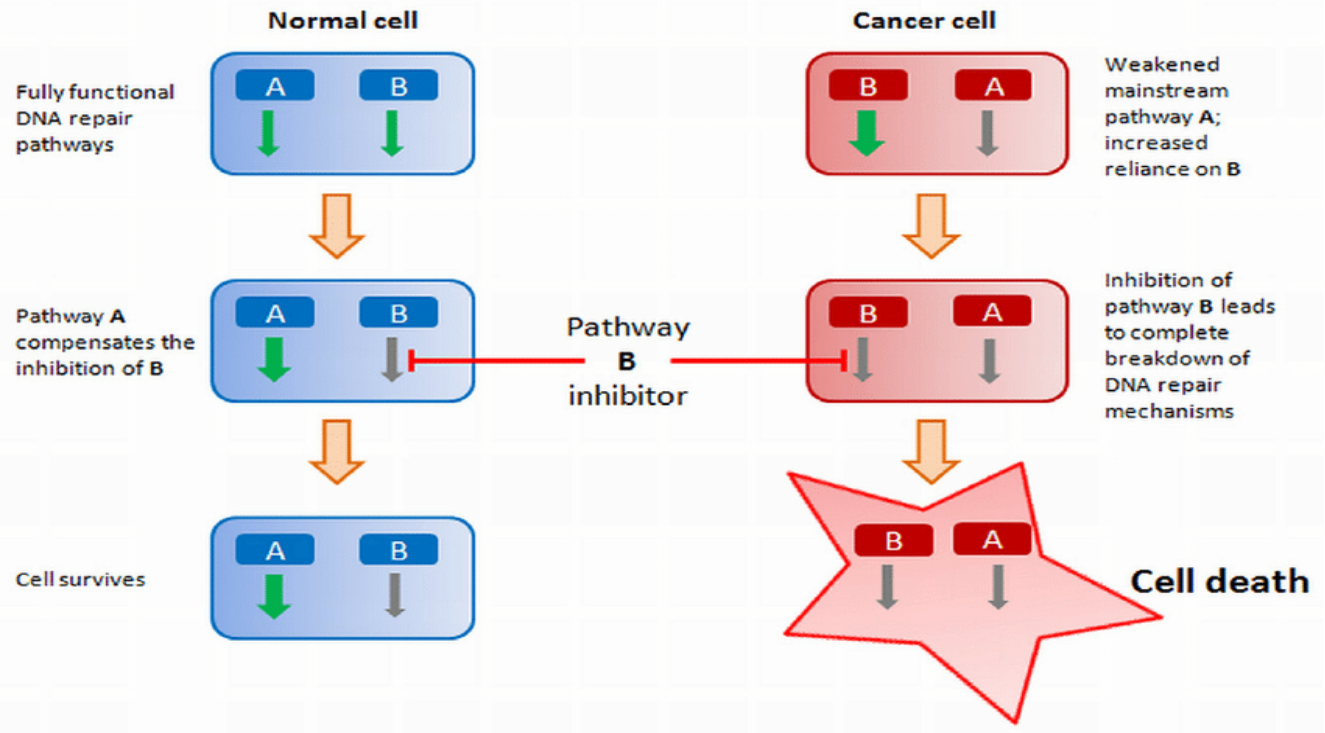


**Figure 1. Synthetic lethality-based approach to cancer therapy, targeting DNA repair pathways.** Diagram from Liu et al. (2014).

Furthermore, the inherent defects in DNA repair present in cancer cells make them sensitive to DNA-damaging agents. Gemcitabine (2′,2′-difluoro-2′-deoxycytidine; dFdC) is a pyrimidine analogue that becomes incorporated into the DNA during replication at the elongating end of nascent DNA. This prevents the progression of DNA polymerase, leading to strand termination and activation of cell cycle checkpoints (Galmarini, Mackey and Dumontet, 2002). The termination of DNA replication, or stalling of the replisome upon encountering a nucleoside analogue, results in the activation of DNA repair mechanisms, which can induce cell cycle arrest or even cell death in response to the obstacle to normal cell cycle progression (Ewald, Sampath and Plunkett, 2008). However, the activation of DNA repair mechanisms can also aid in resistance to this form of treatment. Another form of DNA-damaging cancer treatment is topoisomerase poisons such as camptothecin and etoposide. Topoisomerases mitigate against torsional stress within DNA, and catalyse the cleavage of phosphodiester bonds, bind DNA ends to protect them, and aid in the re-ligation of topoisomerase-created breaks (Sun *et al.*, 2020). Topoisomerase-DNA covalent complexes (TOPcc) are required for the proper function of topoisomerases, however failure to re-ligate and repair these breaks results in lethal DNA-protein crosslinks. Topoisomerase poisons bind TOPcc and trap topoisomerases to the DNA, disrupting replication and leading to polymerase and replication fork collisions that greatly impair genome stability and often result in cell cycle arrest or cell death (Froelich-Ammon and Osheroff, 1995).

These observations highlight how DNA repair mechanisms, the induction of DNA damage, and cancer are intimately linked. Increased mutation rates are promoted in cancerous cells by deficiencies in DNA repair pathways, which can result in additional DNA repair defects which may further aid or hinder cancer progression. Synthetic lethality approaches to cancer treatment exploit these defects to selectively target cancer cells over non-cancer cells through inhibition of redundant repair pathways, and DNA-damaging agents can prove particularly lethal to cancer cells with DNA repair defects. However, while deficiencies in DNA repair pathways can be exploited for the treatment of cancer, intact or even overactive repair pathways can work against common cancer therapies by preventing or reversing damage caused to the DNA, resulting in treatment resistance.

## 1.2 An Overview of DNA Repair Pathways and Proteins

### 1.2.1 DNA Damage Recognition and Response

DNA damage events naturally occur daily within the human genome, ranging from simple base modifications and mismatches, to DNA-protein adducts, inter- and intra-strand crosslinks, and DNA single-strand breaks (SSBs) and double-strand breaks (DSBs). Cells are frequently subjected to stress-induced DNA damage, but have evolved several different, often redundant, repair pathways and cell cycle checkpoints vital for the life of individual cells to whole organisms. However, under excessive stress, or in the case of defects in certain or multiple repair pathways and checkpoints, accumulation of damage to the DNA leads to cell death in order to prevent carcinogenesis.

The DNA damage response (DDR) is initiated by recognition of the specific type of DNA damage present, and this determines repair pathway taken. Defects in DDR leads to genomic instability and predisposes the individual to cancer (Dupré *et al.*, 2008). The initial response involves the phosphorylation of one of three phosphatidylinositol-3 kinase-related kinases (PIKKs): Ataxia-telangiectasia mutated (ATM), Ataxia telangiectasia and Rad3-related (ATR) or DNA-dependent protein kinase (DNA-PK). The PIKK that is activated is dependent on the type of DNA damage and helps direct which repair pathway is required (visual summary in *Figure 2*). In the presence of DSBs, ATM dissociates into phosphorylated monomers, resulting in the phosphorylation of several of its substrates, such as p53, Chk2, Nbs1 and BRCA1 to coordinate cell cycle arrest and repair of DNA damage (Hickson *et al.*, 2004; Dupré *et al.*, 2008). ATM and

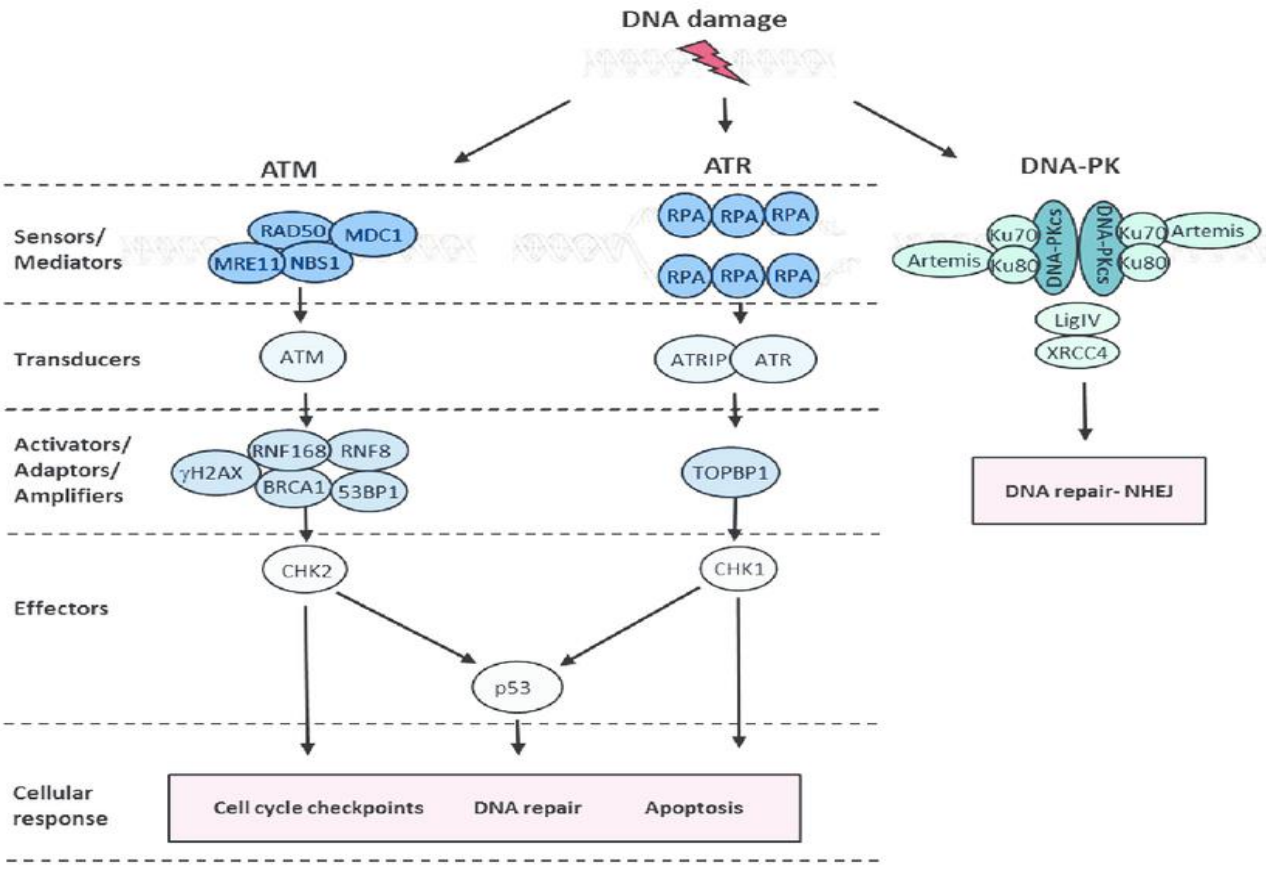


**Figure 2. An overview of the ATM, ATR, and DNA-PK signalling pathways in the DNA damage response (DDR).** Depending on the type of DNA damage, a different PIKK is activated and recruits a series of downstream proteins to initiate the appropriate response.
Diagram from Ryan, Hollingworth and Grand (2016).

its many substrates act as parts of cell cycle checkpoints, controlling G1/S-, S-phase-, and G2/M-arrest (Lavin and Kozlov, 2007; Jin and D.-Y. Oh, 2019). Mutation of ATM is shown to occur in the human autosomal recessive disorder ataxia-telangiectasia (A-T) and shows cell failure to initiate cell cycle arrest after DSB induction, alongside hypersensitivity to ionising radiation (IR) (Hickson *et al.*, 2004). Many proteins interact with ATM in the recognition and repair of DSBs, a major one being the Mre11-Rad50-Nbs1 (MRN) complex. In fact, there is mounting evidence that MRN can initially be recruited to DSB sites and activate ATM, which then in turn phosphorylates the MRN complex and other ATM substrates, with DNA-damage induced MRE11 UFMylation needed for MRN complex formation and activation of ATM (Jin and D.-Y. Oh, 2019). However, while the MRN complex is needed for the efficient activation of ATM, ATM has been shown to be active in the absence of Nbs1 and in environments with excess DNA ends which seems to allow a bypass of MRN-dependent monomerization of ATM (Lavin and Kozlov, 2007). Upon activation of ATM, it can phosphorylate p53, a central part of the G1/S checkpoint, which leads to the inhibition of cell cycle progression from G1 into S-phase, or results in the induction of apoptosis (Lavin and Kozlov, 2007). In fact, ATM deficiency is a common feature in many cancers, with relatively low ATM expression observed in 21.4-63.9% of gastric cancers, and 31% of breast cancers, which fits with the common cancer characteristic of

dysregulated replication and cell-death mechanisms (Jin and D.-Y. Oh, 2019). Conversely, the activation of ATM signalling is a mechanism of chemo- and radiotherapy resistance, and the inhibition of ATM activity has been investigated in the context of improving patient response to anti-cancer therapies across many tumour types. Caffeine and wortmannin are two early compounds found to inhibit ATM and sensitise cells to IR, however their lack of specificity, low potency and high toxicity restricted further development (Gavande *et al.*, 2016). The first selective ATM inhibitor was KU-55933, an ATP-competitive inhibitor which showed high selectivity over other PIKKs and sensitised tumour cells to IR and drugs such as camptothecin, and, in the presence of DSBs, KU-55933 significantly blocks HR repair signals, and causes the loss of IR-induced cell cycle arrest (Hickson *et al.*, 2004; Jin and D.-Y. Oh, 2019).

In contrast to ATM, ATR is activated by structures such as ssDNA, SSBs and junctions present between ss- and dsDNA. In eukaryotes, ssDNA is first detected by replication protein A (RPA), which coats the ssDNA and invites the binding of ATR-interacting protein (ATRIP) in turn enabling the ATR-ATRIP complex to localise at sites of DNA damage (Shiotani and Zou, 2009). To initiate DNA-damage checkpoint responses, ATR phosphorylates a range of substrates, the best-characterised one being checkpoint kinase 1 (Chk1), which in response to DNA damage is phosphorylated by ATR on multiple residues, stimulating its kinase activity and release from chromatin. Activated Chk1 then phosphorylates many substrates involved in activation of cell cycle arrest, DNA repair, and replication fork stabilisation (Shiotani and Zou, 2009). Inhibition of ATR activity results in accelerated progression of DNA-damaged cells from G2 to M-phase, resulting in disrupted DNA repair and mitotic catastrophe (Su *et al.*, 2023). This has been shown to sensitise tumours to DNA damaging agents, as inhibiting ATR increases replication stress and therefore results in an accumulation of DSBs at sites of DNA damage. VE-821 is a potent and selective ATR inhibitor and shows single-agent activity in radiotherapy-resistant tumours and has been shown to inhibit radiation- and gemcitabine-induced phosphorylation of Chk1, inhibiting G2/M arrest and increasing DNA damage resulting in cancer cell death (Prevo *et al.*, 2012; Gavande *et al.*, 2016). Berzoserib (M6620) was developed based on ATR inhibitor VE-821, and so far has been the first ATR inhibitor to enter clinical trials, with 68% of small cell lung cancer patients treated with berzosertib and topotecan showing tumour regression (Su *et al.*, 2023). While ATR can respond directly to issues with DNA replication, its activation at DSBs is dependent on ATM. As previously discussed, a DSB is recognised by MRN which stimulates ATM activity, which can be activated throughout the cell cycle, while ATR is activated mostly during S- and G2-phase (Shiotani and Zou, 2009). The function of ATM and ATR activation at DSBs is mostly to do with DNA end resection, and it's likely the accumulation of ssDNA during resection that stimulates ATR activation (Shiotani and Zou, 2009; Jin and Oh, 2019).

1.2.2 DNA Single-Strand Break (SSB) Repair

The presence of an SSB recruits many DDR sensor proteins, as discussed, including RPA, ATR, Chk1 and poly(ADP-ribose) polymerases (PARPs), to activate their substrate molecules and initiate repair or cell death, as persistence of SSBs results in their conversion to more lethal DSBs during replication (Jin and Oh, 2019). The major pathways involved in the repair of SSBs are base excision repair (BER) and nucleotide excision repair (NER), as outlined in *Figure 3*. PARPs are a family of enzymes that catalyse the poly(ADP-ribosyl)ation (PARylation) of proteins to modify their structural properties, using $NAD^+$ as a substrate (Yelamos *et al.*, 2011). PARP1 and 2 activities have been shown to be stimulated by breaks in the DNA and share a dual role in sensing and transducing signals of DNA damage.

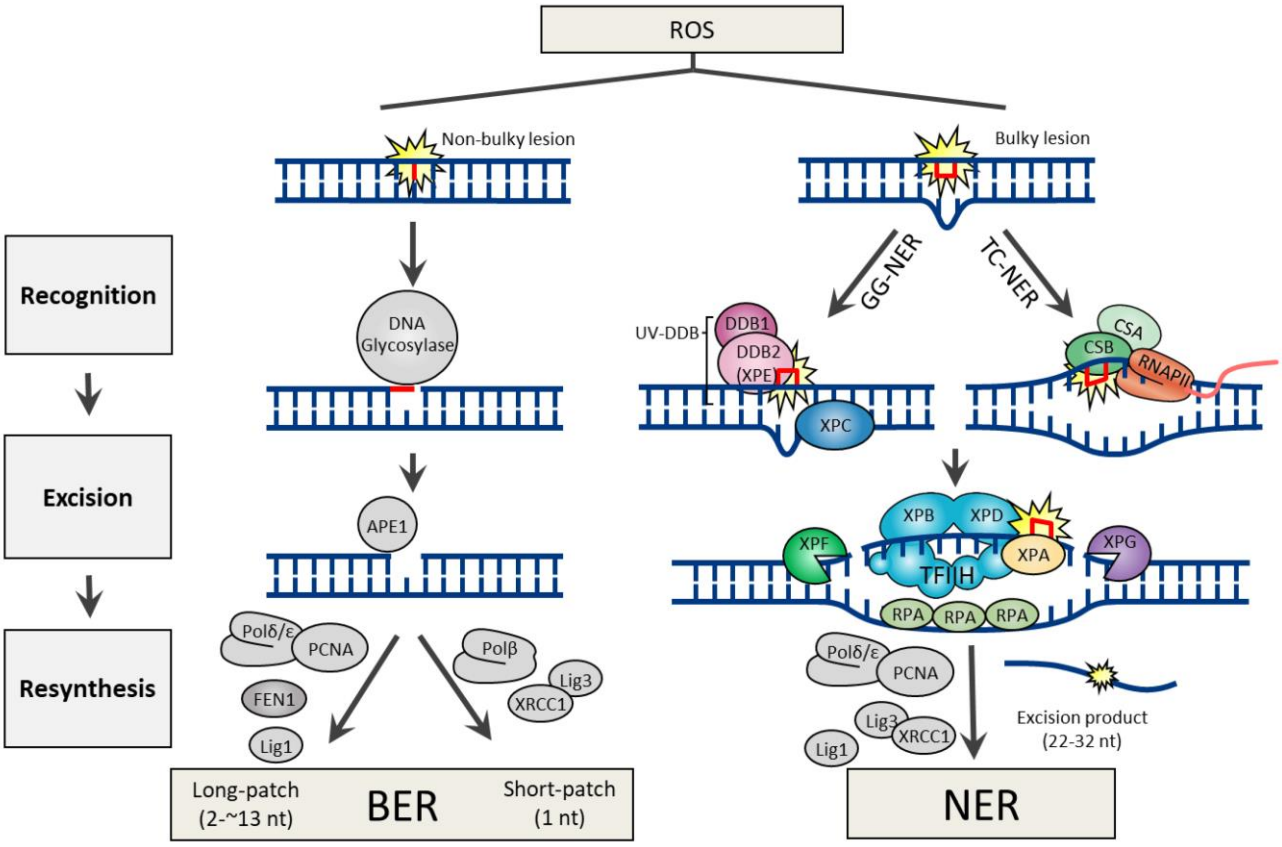


**Figure 3. Pathways and proteins involved in the repair of DNA single-strand breaks (SSBs).** Depending on the type of lesion, repair of SSBs can be carried out by base excision repair (BER) or nucleotide excision repair (NER).
Diagram by Lee and Kang (2019).

In BER, various DNA glycosylases recognise the presence of damaged bases and catalyses base removal resulting in an abasic site. This recruits apurinic/apyrimidinic endonuclease 1 (APE1) to create a nick in the phosphodiester bond 5' to the abasic site (Abbotts and Wilson, 2017). At laser-induced DNA damage sites, PARP1 is shown to accumulate rapidly, with PARP2 accumulation seemingly dependent on PARP1 (Yelamos *et al.*, 2011) Both interact with XRCC1 – a scaffold protein known to stimulate most BER factors – and recruit by flap endonuclease-1

(FEN1) to damage sites (Yelamos *et al.*, 2011; Yang, Hu and Guo, 2022). After recognition and BER activation, repair can be completed by either long-patch (LP-BER) or short-patch (SP-BER) BER. LP-BER involves the excision of at least two nucleotides with displacement synthesis catalysed by Pol β resulting in a flap cut by FEN1 activity prior to ligation of the gap (Gavande *et al.*, 2016). As longer ssDNA flaps tend to become coated in RPA, and this can inhibit FEN1-mediated cleavage, DNA2 (DNA replication helicase/nuclease 2) is recruited to cut the flap length to a size suitable for FEN1 processing (Pawłowska, Szczepanska and Blasiak, 2017). SP-BER excises just one nucleotide and the 5' lyase activity of Pol β cleaves the 5' deoxyribose-phosphate (dRP) and adds a single base before the ligation step, which involves DNA ligase I or DNA ligase III in complex with XRCC1 (Gavande *et al.*, 2016).

A crucial protein in BER is the aforementioned FEN1, which is also essential for RNA primer removal in the processing of Okazaki fragments during DNA replication, and in the removal of flap structures formed in LP-BER (Zheng *et al.*, 2011). FEN1 deficiency is characterised by defects in DNA replication and cell proliferation failure, while overexpression of FEN1 has been linked to drug resistance in many cancers (Yang, Hu and Guo, 2022). FEN1 inhibitors have been shown to be effective in treating HR-defective tumours and increased sensitivity to chemotherapeutic agents (He *et al.*, 2018; Guo *et al.*, 2020; Yang, Hu and Guo, 2022). WRN is another DDR protein, though more known for roles in DSB repair, that functions in BER through it's ATP-dependent helicase activities (Ahn *et al.*, 2004). WRN has been shown to interact with Pol β to stimulate strand displacement, and is able to unwind many SSB BER intermediates (Ahn *et al.*, 2004).

Another pathway in the repair of SSBs in humans is NER, which is involved in the repair of bulky DNA adduct damage (Woods and Turchi, 2013). Identification of bulky adduct damage can occur via sensor proteins that recognise distortions and chemical modifications, such as by PARP, or recognition is transcription-coupled (Yelamos *et al.*, 2011; Gavande *et al.*, 2016). Recognition triggers the localisation of specific endonucleases such as XPG and ERCC1/XPF to hydrolyse phosphodiester bonds 3' and 5' to the adduct, respectively, to be followed by resynthesis and ligation to result in faithfully repaired DNA (Gavande *et al.*, 2016).

### 1.2.3 DNA Double-Strand Break (DSB) Repair: Non-Homologous End-Joining (NHEJ)

While PARP and ATR signalling, and the BER and NER repair pathways, focus on processing SSBs, DSBs are considered more lethal to the cell and, upon recognition, are funnelled into repair via non-homologous end-joining (NHEJ) or homologous recombination (HR).

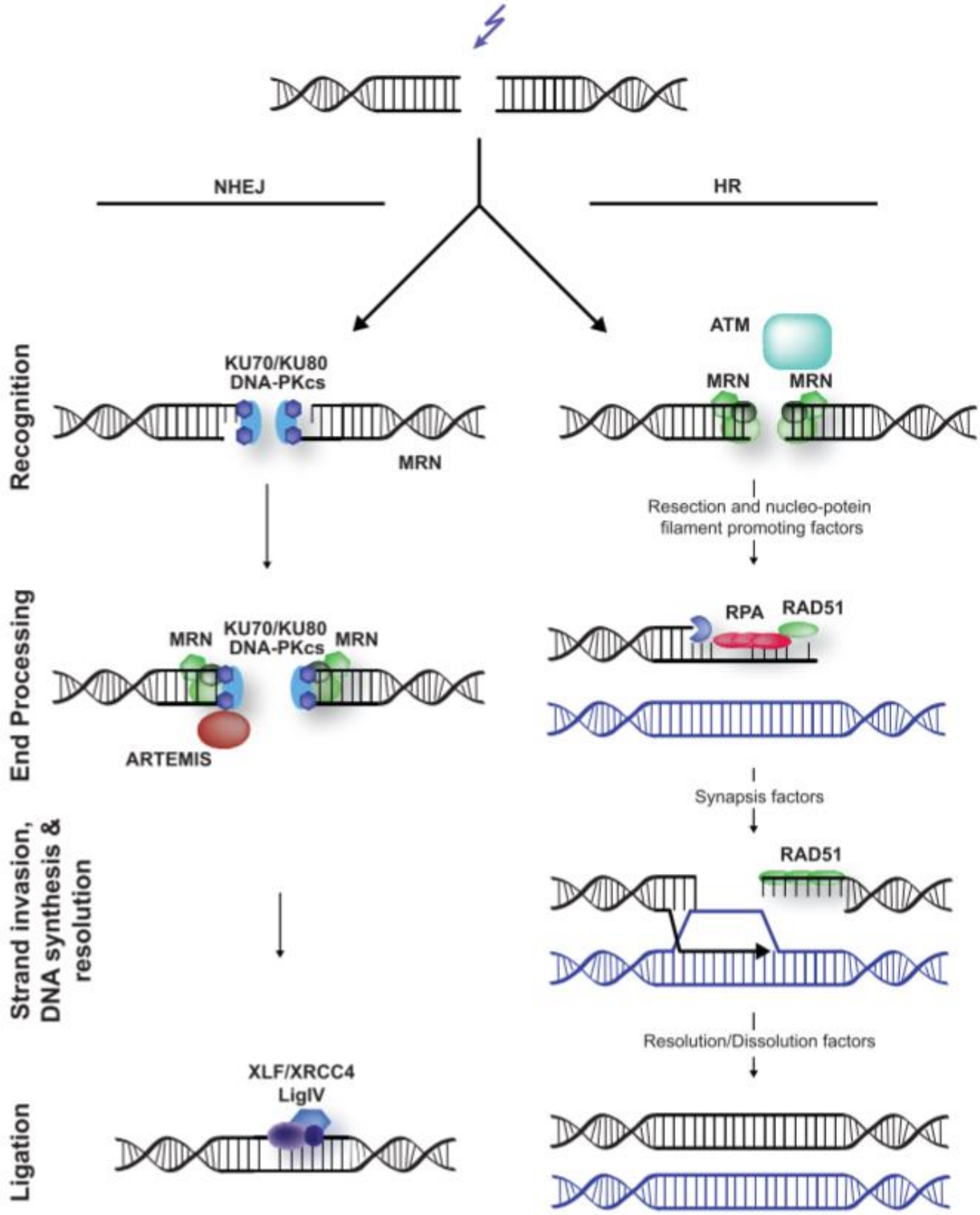


**Figure 4. Pathways and proteins involved in DNA double-strand break (DSB) repair.**
Depending on a range of factors, repair of DSBs can be carried out by non-homologous end joining (NHEJ) or homologous repair (HR).
Diagram by Lans, Marteijn and Vermeulen (2012).

NHEJ is active throughout the cell cycle and is the dominant pathway choice for repair of DSBs in higher eukaryotes (Jeggo and Lavin, 2009). NHEJ can be summarised by four steps, starting with recognition of DNA termini by the recruitment of the Ku (Ku70 and Ku80) complex to DSBs. DNA-bound Ku recruits DNA-PKcs to form a heterotrimeric complex with kinase activity activated by being bound to DNA termini, and bridges DNA ends via the formation of the synaptic complex. DNA-PK then further coordinates repair processes by autophosphorylation and phosphorylation of other targets to initiate DNA end processing by nucleases such as Artemis, and polymerases PNK and TDP. The final step is ligation of the processed DNA ends of the DSB by the ligase IV/XRCC4/XLF complex (Jeggo and Lavin, 2009; Gavande *et al.*, 2016).

DNA-PK is central to the NHEJ response to DSBs, and plays key roles in VDJ recombination, apoptosis activation, and in telomere maintenance, all associated with DNA breaks (Goodwin and Knudsen, 2014). DNA-PK is a nuclear serine/threonine kinase activated by dsDNA ends and consists of a catalytic subunit (DNA-PKcs) and DNA-end binding component Ku. In NHEJ, DNA-PKcs tethers DNA termini in a synaptic complex of two DNA-PKcs molecules to facilitate rejoining (Burma and Chen, 2004). DNA-PK also functions in recruiting DNA ligase IV-XRCC4 complex for end rejoining, with DNA-PK shown to phosphorylate XRCC4 *in vitro* (Burma and Chen, 2004). The autophosphorylation of DNA-PKcs is thought to be an early and integral step in DSB repair by NHEJ, however decreases in phosphorylation have been shown in A-T cells, suggesting a role of ATM in DNA-PKcs phosphorylation (Chan *et al.*, 2002). DNA-PK activity has been related to a decreased in patient response to DNA-damaging agents, and to treatment resistance in cancer, as well as correlated with poorer prognosis in many types of tumours (Goodwin and Knudsen, 2014). This has made it an attractive target for the development of small molecule inhibitors, specifically targeting the ATP-binding site of the kinase domain (Gavande *et al.*, 2016). Early inhibitors include wortmannin and LY294002, which conferred potent radiosensitisation but were impractical for clinical applications. However, LY294002 went on to be used as a lead compound in further modification to eventually develop NU744, which displays high selectivity for DNA-PK over other PIKKs, sensitisation to IR and etoposide in colon cancer cells, and increased HR activity (Leahy *et al.*, 2004; Zhao *et al.*, 2006; Davidson *et al.*, 2013).

WRN has also been shown to play a major role in NHEJ, specifically in the choice between classical/canonical (c-) and alternative (alt-) NHEJ pathways. C-NHEJ fuses DNA ends without any sequence homology and is dependent on Ku, whereas alt-NHEJ links DNA ends showing microhomologies, with the aid of MRE11 and is Ku-independent (Reginato and Cejka, 2020). WRN is a RECQ-like helicase with both 3’-5’ exonuclease and helicase activities, which, when mutated in Werner syndrome (WS), results in deletions developing at non-homologous DNA ends (Chen *et al.*, 2003). People with WS show premature ageing and a predisposition to

cancer and cardiovascular disease. The extensive deletion observed at non-homologous ends in WS cells suggests WRN helicase may unwind DSBs in search for local homology and WRN helicase unwinds while the exonuclease activity may trim DNA ends in preparation for ligase IV and XRCC4 (Chen *et al.*, 2003). Furthermore, when WRN is recruited to DSBs, associates with Ku to promote c-NHEJ by helicase and exonuclease activities, and by preventing the localisation of MRE11 and CtIP and protecting DNA ends from resection and alt-NHEJ (Franchitto and Pichierri, 2004; Shamanna *et al.*, 2016). This role of WRN in NHEJ pathway choice is supported by WRN-deficient, and WS, cells being prone to the more mutagenic alt-NHEJ pathway (Shamanna *et al.*, 2016). Moreover, inhibition of WRN helicase by NSC617145 results in increased accumulation of DNA-PKcs and rad51 foci, implying HR activation, and further suggesting a role for WRN in activation of NHEJ (Aggarwal *et al.*, 2013). NSC617145 negatively affected cell proliferation and induced DNA damage, as well as showing synergistic effects with mitomycin C to induce DSBs (Aggarwal *et al.*, 2013).

While NHEJ is a fast method of DSB repair and is active in all stages of the cell cycle, it is also error-prone due to the lack of reliance on homology leading to less faithful repair. Over-reliance on NHEJ can result in an accumulation of mutations, eventually leading to cell death. HR, on the other hand, uses a homologous template for faithful repair, and is most active in S- and G2-phase due to the presence of sister chromatids.

### 1.2.4 DNA Double-Strand Break (DSB) Repair: Homologous Recombination (HR)

HR is a major pathway for the accurate repair of DSBs and inter- and intra-strand crosslinks, as well as playing a critical part in restarting stalled replication forks and ensuring proper chromosome segregation in meiosis (Gavande *et al.*, 2016). HR is favoured over NHEJ in the repair of DSBs when ‘dirty’ ends, or just the one end, is present, such as with replication-associated DSBs (Bonilla *et al.*, 2020). Upon DSB recognition, and if not prevented by NHEJ proteins such as WRN, the MRN complex with CtIP binds the DNA ends of the DSB and exposes 3’ ssDNA overhangs through short-range resection (pathway summarised in *Figure 4*). Long-range end resection then takes place through the action of EXO1 or DNA2 with Bloom syndrome protein (BLM). The exposed ssDNA gets coated in RPA to prevent further degradation and formation of secondary structures until Rad51 replaces RPA to assemble the nucleoprotein filament to begin the homology search (Bonilla *et al.*, 2020). Invasion of Rad51-ssDNA into homologous dsDNA leads to heteroduplex DNA and formation of a Holliday junction (Gavande *et al.*, 2016). The dissociation of Rad51 allows the start of the synthesis step of HR, and the final ligation of DNA ends.

The RAD51 protein is key to HR repair of DSBs and is evolutionarily conserved from bacteriophages to mammals (Huang *et al.*, 2011). There is significant evidence for Rad51’s role in cancer and its treatment, from germline mutations in Rad51 paralogs leading to increased

susceptibility to epithelial ovarian cancer, increased RAD51 expression in almost all cancers, and many cancer cell lines showing increases in Rad51 foci independent of induced DNA damage, consistent with increased replication stress seen in rapidly dividing cancer cells (Klein, 2008; Mathews *et al.*, 2011; Song *et al.*, 2015). Furthermore, lower RAD51 expression, or its inhibition, has been shown to sensitise cancer cells to cisplatin or IR treatment, while overexpression results in hyperrecombination which promotes cancer progression and confers DNA-damaging agent resistance (Liu *et al.*, 2011; Bonilla *et al.*, 2020). This makes RAD51 an attractive target for cancer therapies, with main strategies involving the exploitation of RAD51 overexpression in cancer by locking RAD51 in active formation and further promoting the formation of toxic RAD51 complexes, or by inhibition of the DNA strand exchange activity of RAD51 by preventing binding to ssDNA (Gavande *et al.*, 2016). The small molecule RAD51 inhibitor B02 has been found to specifically inhibit RAD51 binding to DNA, and therefore inhibits HR and increases sensitivity to DNA damage such as by IR or etoposide (Alagpulinsa, Ayyadevara and Shmookler Reis, 2014). B02 impacts RAD51's ability to form the nucleoprotein filament by targeting its protein-DNA interactions, and also the binding of dsDNA to RAD51 during the homology search (Huang *et al.*, 2012).

Break-induced replication (BIR) is a pathway employed during repair by HR to repair one-ended DSBs, such as at stalled replication forks and in the process of telomere lengthening, often though the utilisation of microhomologies (Malkova and Ira, 2013). BIR can be both RAD51-dependent and -independent, and while this pathway aids in the repair of lethal DSBs, it has also been shown to be a source of many genomic rearrangements and play a role of the promotion of cancer progression (Malkova, 2018).

### 1.2.5 More than Just Repair: DDR Proteins at Stalled Replication Forks and Telomeres

The genome is at its most vulnerable during DNA replication, with many structures or lesions with the capability to prevent replication fork progression, leading to stalled forks prone to deleterious breakage or collapse. Cells therefore rely on cell cycle checkpoints during replication to prevent cell cycle progression and origin firing in the event of stalling. ATR and Chk1 are essential to these mechanisms, and loss of these has been shown to result in replication fork collapse and chromosomal rearrangements, increasing cancer predisposition. The progression of the replication fork is halted in order to deal with the offending barrier, which can involve repair of lesions, removal of interfering structures, or bypass to allow for post-replication repair. Stalled forks can lead to deleterious effects through the accumulation of large sections of ssDNA more easily targeted by nucleases and development into DSBs, if not properly protected. Therefore, in response to fork stalling, the intra-S-phase/replication checkpoint is activated, with ATR recruited to the ssDNA by RPA binding at stalled replication forks to phosphorylate downstream effectors such as Chk1. The activation of these checkpoint kinases blocks progression of the cell cycle, prevents further origin firing, and stabilises the

stalled fork to allow eventual repair and restart (Paulsen and Cimprich, 2007). ATR aids in the stabilisation of the replisome components to prevent dissociation, and also prevents recombination through inhibition of nucleases cleaving the stalled fork structure and downregulating HR components while upregulating helicase activity to help fork restart (Paulsen and Cimprich, 2007). Rad51 also plays a similar role at stalled forks, by protecting the DNA ends from uncontrolled degradation. Nuclease activity, such as by DNA2 and MRE11, is needed for HR-mediated fork restart, however uncontrolled nuclease degradation can result in over-resection of the stalled fork and have deleterious effects (Zheng *et al.*, 2020). Rad51 therefore, while also promoting the action of DNA2 and MRE11 to aid restart, also protects the fork, alongside CtIP, from excessive degradation through nuclease action.

In some cases, when a stalled fork cannot be rescued, a restart can occur directly though helicases such as RECQ1, or via DNA2-WRN-mediated resection to produce 3' ssDNA overhangs to initiate HR-mediated fork restart by activating Rad51 filament formation and invasion of homologous dsDNA (Costanzo, 2011; Pawłowska, Szczepanska and Blasiak, 2017; Bonilla *et al.*, 2020). C5 is a compound that has been shown to inhibit the nuclease, DNA dependent ATPase, helicase and DNA binding activities of DNA2, and confers toxicity through suppressing replication-coupled DSB end resection and replication fork restart (Liu *et al.*, 2016). It is also shown to inhibit the over-resection of nascent DNA in cells deficient in fork protection, adding further evidence to DNA2's role at stalled forks (Liu *et al.*, 2016; Zheng *et al.*, 2020).

PARP1 and 2 also play a role in detecting stalled replication forks and recruit Mre11 for the DNA end processing and ssDNA gap enlargement needed in HR, and this is supported through observations of HR inhibition and suppressed Rad51 expression when PARP is disrupted (Hashimoto *et al.*, 2010; Yelamos *et al.*, 2011). FEN1 is also implicated in the rescue of stalled replication forks, where it forms a complex with WRN and MRE11 in response to the damage that accumulates at these structures (Franchitto and Pichierri, 2004). FEN1 endonuclease activity is important in the cleavage of regressed forks to resolve the resulting 'chicken foot' structure, and also in targeting the ssDNA to create DSBs in order to initiate BIR when needed (Zheng *et al.*, 2011). Inhibition or depletion of replication fork regulators such as FEN1 has been shown to result in PARP1 accumulation and an increase in XRCC1-mediated (SSB) processing over HR-mediated restart (Rose *et al.*, 2020). This exemplifies how DDR proteins, from across both SSB and DSB repair pathways, interact and are intricately involved in the response to stalled replication forks. This occurs similarly in the maintenance of telomeres.

Telomeres are nucleoprotein structures that cap the ends of chromosomes in eukaryotes to provide protection from degradation. In mammals, telomeres consist of a series of TTAGGG repeats that end in a 3' G-rich ss-overhang that is prone to folding into a structure termed the t-loop, which may play a role in protecting the DNA ends from being recognised as DSBs and activating DNA damage checkpoints (Multani and Chang, 2007). However, the G-rich ssDNA at

telomeres can also result in the formation of guanine quartets (G4s) or tape-like G-ribbons which can fold to form 4-stranded DNA which can inhibit replication (Pawłowska, Szczepanska and Blasiak, 2017). These structures are resistant to most replication-related helicases and nucleases, however DNA2 has been shown to bind these structures with high affinity, further evidenced by DNA2-depletion resulting in an accumulation of cells at the G2/M checkpoint, suggesting chromosomal damage leading to genome instability and cell cycle arrest (Pawłowska, Szczepanska and Blasiak, 2017). FEN1 also plays a role at G-rich telomeres, with FEN1 deficiency being characterised by minisatellite expansion or contractions, and mutations affecting telomere stability through the promotion of telomere fusions, and, in cancer cells, can induce telomere shortening (Zheng *et al.*, 2011).

There is further evidence of PARP2's involvement in telomere maintenance, via its interaction with telomeric repeat binding factor 2 (TRF2). TRF2 is integral to the protection of telomeres through its ability to affect DNA damage signalling and interact with repair proteins. PARP2 regulates TRF2 through its DNA-binding activity and acts to open the t-loop in response to DNA damage and allow access of DDR proteins (Yelamos *et al.*, 2011). For example, PARP2$^{-/-}$ mouse embryonic fibroblasts, while showing unperturbed telomere length and capping, do show increased chromosome ends lacking any detectable TTAG repeats, suggesting a role for PARP2 in regulating TRF2 as a central part of maintaining telomeres and genome stability (Gomez *et al.*, 2006). PARP1 has also been shown to interact with TRF2 in the presence of ssDNA damage induced by G4 structures, likely to initiate the DDR, and this localisation is shown to be inhibited by PARP inhibitor treatment (Salvati *et al.*, 2010). TRF2, as part of its functions in regulating DNA damage signalling at telomeres, interacts with both ATM and the MRN complex, showing to be a specific inhibitor of ATM kinase activity on telomeric DNA, and potentially protects from MRE11 degradation also (Lavin and Kozlov, 2007). Finally, DNA-PKcs and Ku have been implicated in masking chromosome ends in order to prevent recognition as DSBs by DDR machinery, and in the absence of functional telomerase, DNA-PKcs deficiency specifically resulted in enhanced telomere degradation (Burma and Chen, 2004).

Slijepcevic (2006) proposes an "integrative model" describing the role of DDR proteins at telomeres, further backed up by the observation of TRF2 not being confined to telomeres, and instead shown to localised to sites of DNA damage independently of other DDR proteins (Bradshaw, Stavropoulos and Meyn, 2005). Slijepcevic describes how at least 14 mammalian DDR proteins have been implicated in telomere maintenance, with cells defective in DSB factors, such as RAD51, MRE11, Ku and DNA-PKcs, also showing a loss of telomeric capping functions. The similarities in replication-inhibitory structures, lesions and damage events makes these DDR proteins qualified for function both at telomeres and the genome at large. It is more than likely this "integrative model" could be extended to include the processing of stalled

replication forks too, seeing as many of these proteins are implicated in all three environments, playing similar roles.

### 1.3 The MRN Complex: Role in Cancer Development and Treatment Resistance

MRE11, and the MRN complex as a whole, is highly conserved, with Mre11 nuclease activity specifically essential for mammalian cell survival (Costanzo, 2011). Depletion of Mre11 in *Xenopus* egg extracts leads to accumulation of replication-dependent lethal DSBs, and cells containing Mre11 nuclease-dead alleles show chromosomes developing structural abnormalities, defects in the repair of IR-induced DNA damage, and sensitivity to replication fork stalling agents (Trenz *et al.*, 2006; Buis *et al.*, 2008). Mutations within the MRN complex, specifically in MRE11 and NBS1, result in diseases such as Nijmegen breakage syndrome and ataxia telangiectasia-like disorder (ATLD), which are known to predispose individuals to cancer (Williams, Williams and Tainer, 2007).

The MRN complex is unique in that it participates in most, if not all, pathways of the DDR, and is among the first factors to be recruited to DNA breaks. The MRN complex consists of the nuclease MRE11, ATPase RAD50, and structural protein NBS1 (Reginato and Cejka, 2020). Its role in checkpoint signalling involves its rapid localisation to DSBs and activation of ATM kinase activity which phosphorylates further DDR proteins, and MRN itself. Phosphorylation by ATM of CtIP (a DDR protein closely associated with MRE11 and its roles in HR) is needed for the first step of DNA end resection, and phosphorylation of WRN helicase promotes rapid recovery from replication stresses (Regal *et al.*, 2013). Initial short-range resection is undertaken by MRN and its co-factor CtIP, and also has the capability to process DNA ends containing secondary structures and protein blocks. Downstream of this long-range end resection is performed by EXO1 and DNA2, and even these require the MRN complex in a structural role to promote this pathway (Reginato and Cejka, 2020) In both HR and NHEJ, processing of ‘dirty’ DNA ends, such as those with topoisomerase adducts, requires either direct Mre11 nuclease activity, or promotion by the MRN complex to process (Shibata *et al.*, 2014) Furthermore, the signalling cascade that is initiated by MRNs initial activation of ATM can trigger changes in chromatin conformation that allows the recruitment of repair factors to the site, regardless of the pathway being undertaken (Reginato and Cejka, 2020).

Due to MRE11s central role in the signalling and repair of DNA damage, it is intuitive that alterations to its functions can lead to the development of cancerous cells, and defects have been shown to sensitise cells to treatment, however it has also been implicated in the resistance to several common cancer therapies (*Figure 5*). In *Schizosaccharomyces pombe*, Rad32 (Mre11) nuclease activity plays a role in the removal of TOPcc from DNA ends, along with Ctp1 (CtIP homolog), after treatment with topoisomerase poisons such as Camptothecin and etoposide (Figure 5A; Hartsuiker, Neale and Carr, 2009). The removal of TOPcc after

treatment with these cancer drugs can confer resistance to topoisomerase poisons, as the cancer-targeting DNA damage is being prevented. A similar role for MRE11 is seen in the resistance to nucleoside analogues, where it's been shown that following gemcitabine (dFdC) treatment, MRE11 3' to 5' exonuclease activity is involved in the removal of nucleoside analogues from nascent DNA ends during replication, with inhibition of MRE11 exonuclease showing sensitisation to dFdC and an increased of dFdC in genomic DNA (Figure 5B&C; Boeckemeier et al., 2020).

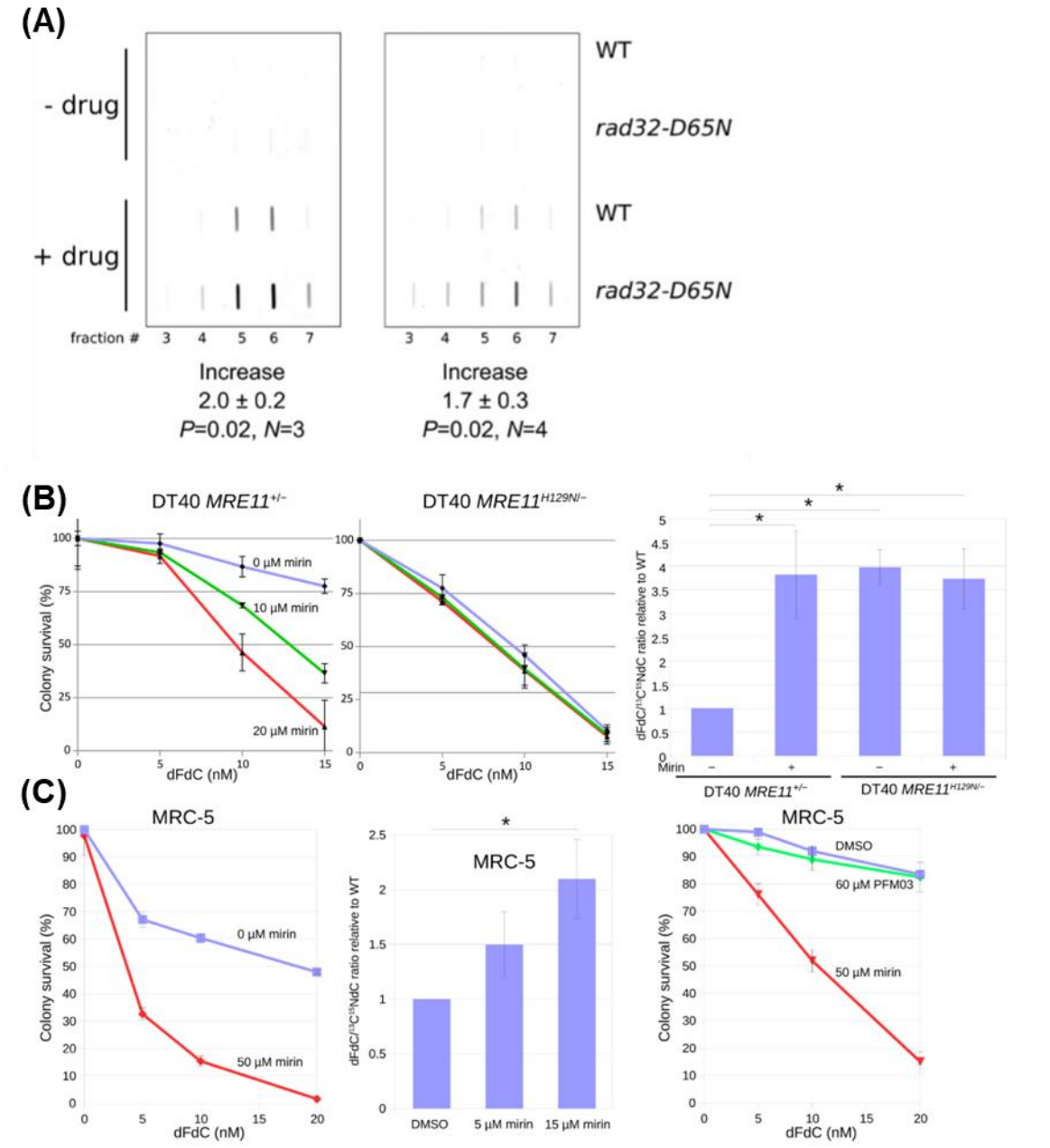


**Figure 5. Mutation or inhibition of Mre11 nuclease activity shows sensitisation to topoisomerase poison camptothecin, and nucleoside analogue gemcitabine (dFdC), suggesting a role for Mre11 in resistance to these cancer treatments.**
**(A)** Hartsuiker, Neale and Carr (2009) describe a role for Mre11 nuclease activity in the removal of trapped topoisomerases from the DNA, by showing covalently bound topoisomerase levels to be increased in the nuclease-dead mutant compared to the wild-type (WT) when treated with Top2 inhibitor TOP-53 (left) or Top1 inhibitor camptothecin (right). **(B & C)** Boeckemeier et al. (2020) suggest Mre11 exonuclease activity is needed for resistance to dFdC, via its removal from the DNA. These findings show sensitisation to dFdC in **(B)** chicken DT40 cells with Mre11 exonuclease inhibition by mirin and in mutant Mre11$^{H129N/-}$ (nuclease-dead), and in **(C)** human MRC-5 with mirin, but no sensitivity observed with Mre11 endonuclease inhibitor PFM03.

With its various roles in the development, progression, and resistance to treatment of cancer, the MRN complex, specifically MRE11, has become an attractive target for inhibition in cancer therapies. Dupré et al. (2008), using forward chemical genetic screening, identified mirin (Z-5-

(4-hydroxybenzylidene)-2-imino-1,3-thiazolidin-4-one) as an MRN inhibitor (*Figure 6*). They showed it to inhibit MRN-dependent activation of ATM, while not having a direct effect on ATM kinase activity, prevent G2/M checkpoint activation and HR, and to inhibit Mre11 3' to 5' exonuclease activity. Importantly, mirin was not found to affect the ability of Mre11 to interact with its MRN partners Rad50 and Nbs1, or ability to bind damaged DNA. Phosphorylation of Chk2, and the repair of DSBs by HR induced by IR, was reduced after treatment with mirin, and therefore cells were sensitised to radiation therapy (Piscitello *et al.*, 2018). However, it was discussed by Dupré et al. (2008) that mirin is a relatively weak inhibitor of Mre11, and its specificity to Mre11 as a sole target wasn't confirmed.

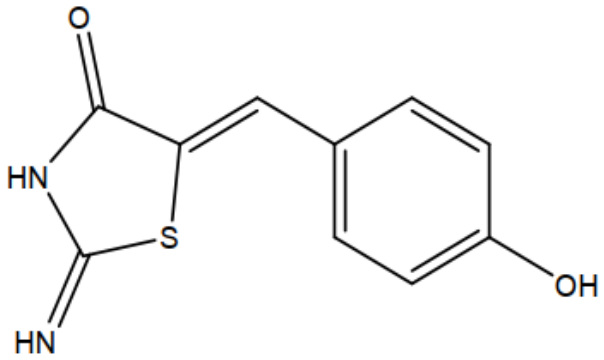


**Figure 6. Structure of mirin, an Mre11 3'-5' exonuclease inhibitor, identified by** Dupré et al. (2008)**.**

Three derivatives of mirin were developed (PFM01, PFM03 and PFM39) with only PFM39 shown to specifically inhibit MRE11 exonuclease, while the others specifically inhibited MRE11 endonuclease activity, though all three inhibited MRE11-associated DSB end resection (Shibata *et al.*, 2014). However, discovering other small molecule inhibitors of Mre11, or specifically Mre11 exonuclease activity, has been difficult (Shin *et al.*, 2007; Kobayashi *et al.*, 2010; Wang *et al.*, 2021).

## 1.4 Project Rationale and Aims

MRE11 has been shown to have great potential as a target in cancer treatment, with its roles in the initial development of cancer, and in resistance to current cancer treatments. The exploitation of synthetic lethal interactions is also an emerging strategy in the development of cancer therapies, with the targeting of DNA repair pathways being a key focus.

The aim of this project is, initially, to replicate and verify previous findings (*Figure 7*) utilising the Opentrons pipetting robot and new protocols, before using this same high-throughput drug screening process to test a variety of DNA repair inhibitors in combination with MRE11 exonuclease inhibitor mirin, to investigate potential synthetic lethal interactions that could be used to inform the development of more personalised cancer treatments. An ongoing project with the lab group is to identify novel MRE11 inhibitors with improved properties in comparison to mirin. Therefore the aim of this project is to identify novel and clinically relevant synthetic interactions between DNA repair pathways that could be exploited with these novel MRE11 inhibitors.

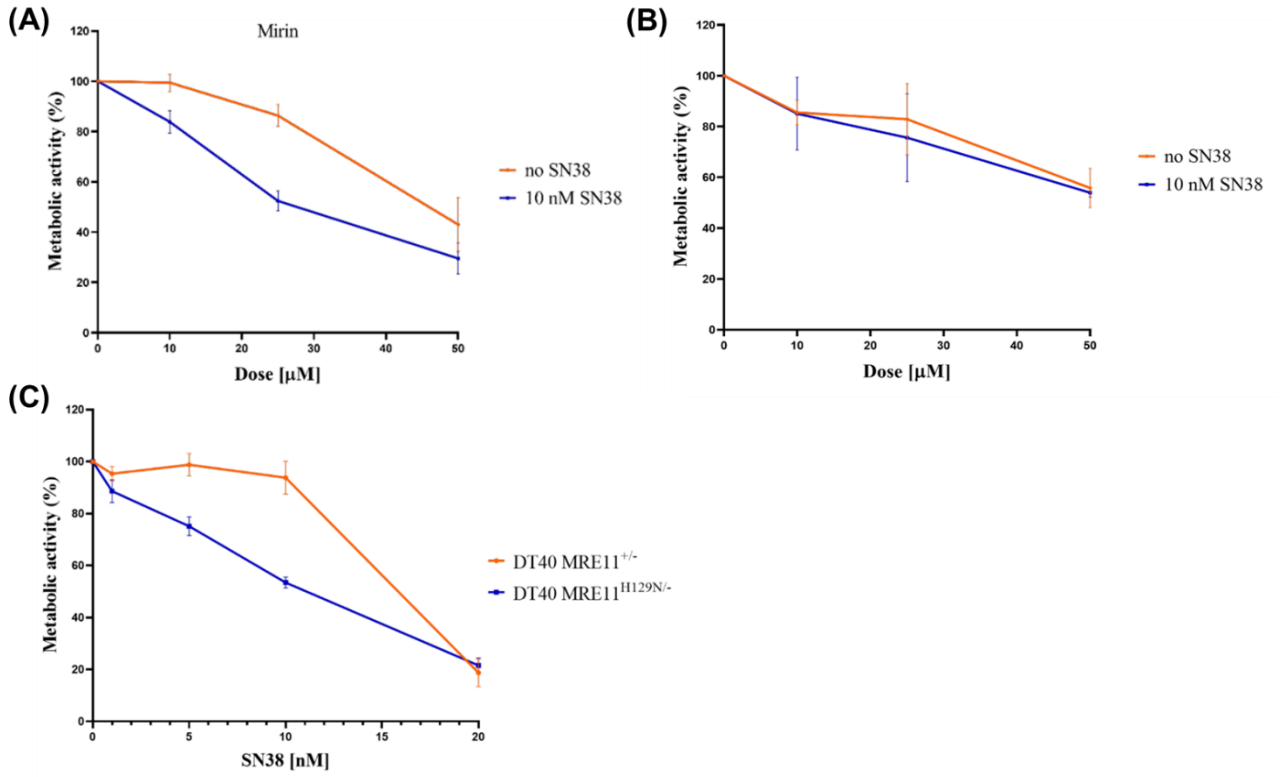


**Figure 7. Previous data in DT40 cells showing interactions of MRE11 with topoisomerase poison SN38 (camptothecin derivative).**
Increasing doses of MRE11 exonuclease inhibitor mirin with and without the addition of 10nM SN38 in **(A)** DT40 *MRE11*$^{+/-}$ cells and **(B)** DT40 *MRE11*$^{H129N/-}$ (nuclease-dead) cells. **(C)** Comparison of DT40 *MRE11*$^{+/-}$ and *MRE11*$^{H129N/-}$ at increasing SN38 doses.
Data from unpublished PhD thesis by Salerno (2020) at Bangor University.

# 2 Materials and Methods

## 2.1 Cell Culturing and Maintenance

Details on cell lines used can be found in *Table 1*.

DT40 are chicken B-lymphocytes cells derived from an avian leukosis virus induced lymphoma. They were cultivated in RPMI 1640 medium (Gibco) supplemented to contain 10% foetal-bovine serum (FBS Supreme, PAN Biotech), 1% chicken serum (PAN Biotech), 1% L-Glutamine (Sigma-Aldrich), and 1% Penicillin-Streptomycin (Gibco). DT40 were incubated at 39°C with 5% $CO_2$ and approximately 95% humidity, as suggested by Ridpath et al. (2011). Cells were sub-cultured and maintained at a density between $5x10^4$ and $7x10^5$ to ensure cells remain in log phase for experimental use.

TK6 human lymphoblast cells were cultivated in RPMI 1640 medium with 10% donor horse serum (PAN Biotech), 1% L-Glutamine and 1% Penicillin-Streptomycin. Cells were incubated at 37°C with 5% $CO_2$ and approximately 95% humidity and maintained at a density between $1x10^5$ and $1x10^6$.

Cells were counted using an automated Bio-Rad TC20 cell counter, and manually using a hemocytometer, after staining with trypan blue (ThermoFisher Scientific). Cells were kept in culture for a maximum of 5 weeks, and frozen cell stocks, of approximately $3x10^6$ cells/mL in FBS and 10% DMSO, were kept in -80°C freezers and liquid nitrogen for renewing cell-line cultures.

| Cell Lines | Organism | Tissue/Cell type | Origin |
|---|---|---|---|
| DT40 *MRE11*$^{+/-}$ | *G.gallus* | Lymphocyte | Prof. Takeda, Kyoto University |
| DT40 *MRE11*$^{H129N/-}$ | *G.gallus* | Lymphocyte | Dr. Vernon, Bangor University |
| TK6$^{WT/-}$ | *H.sapien* | Lymphoblast | Dr. Robinson, Bangor University |

**Table 1. Cell lines used in this research project.** The DT40 *MRE11*$^{+/-}$ cell-line contains one of the two wild-type (WT) MRE11 alleles knocked out, and is the parent of the DT40 *MRE11*$^{H129N/-}$ which is considered an MRE11 'nuclease-dead' cell-line containing a mutation within the *MRE11* nuclease domain.

## 2.2 Compound Preparation

All compounds used for cell survival assays were made up into 10mM stocks in 100% DMSO from powder as purchased from suppliers. Depending on the working concentrations required for each compound, further stock solutions were made for 1mM, 100µM and 1µM in cell culture media, and kept frozen. Working stocks of compounds to be used in cell survival assays were made up in relevant cell culture media, and from stock solutions that ensured final DMSO

content was at or below 1%. These were batch-made to ensure consistency between repeat assays.

For RADAR assays, further stocks were again made from 10mM stocks in 100% DMSO, and diluted to required working concentrations in DMSO. As only 1µL of each compound was added to 1mL of cells for sample preparation, this meant DMSO content was still kept around 1%.

| Compound | Manufacturer | 100% DMSO Stocks | Stocks in cell media |
|---|---|---|---|
| Mirin | Sigma-Aldrich | 25.6mM, 10mM, 5mM | |
| SN-38 | Sigma-Aldrich | 10mM, 100µM, 10µM, 1µM | 1mM, 100µM, 2µM |
| AZD2461 | Sigma-Aldrich | 10mM, 500µM | 1mM |
| BMN-673 | MedChem Express | 10mM, 6µM | 1mM |
| BSI-201 | LKT Labs | 10mM, 2mM | 1mM |
| VE-821 | Sigma-Aldrich | 10mM | 1mM |
| B02 | Sigma-Aldrich | 10mM | 1mM |
| LNT1 | Sigma-Aldrich | 10mM | 1mM |
| C5 | MedChem Express | 10mM | 1mM |
| NSC617145 | Tocris | 10mM | 1mM |
| NU7441 | Tocris | 10mM | 1mM |
| KU55933 | Sigma-Aldrich | 10mM | 1mM |
| XTT sodium salt | Discovery Fine Chemicals | N/A | N/A |

## 2.3 Cell Survival XTT Assays

XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)−5-[(phenylamino) carbonyl]−2H-tetrazolium hydroxide) is converted by metabolically active cells into a water-soluble formazan dye with absorbance at 450nm. XTT stocks were prepared with 100 mg XTT sodium salt (Discovery Fine Chemicals), 2mg phenazine methosulphate (PMS; ThermoFisher Scientific) and 660µL DMSO, from which a working solution was created with a volume of 9.1µL XTT stock in 2.5mL supplemented RPMI media. The protocol used and described is adapted from Ridpath et al. (2011)

Drug plate production was automated using the Opentrons O2 pipetting robot. A diagram demonstrating the layout of the drug plates is shown below in *Figure 8*. The drug plates consisted of a serial dilution of the 'test drug' (i.e. specific DNA repair inhibitor being tested) and alternating addition of a 'poison' compound, typically mirin (Sigma-Aldrich). All wells contain a total volume of 200µL after cell addition.

50µL of cells are added to each necessary well, with DT40 *MRE11*$^{+/-}$ cells diluted to 100,000 cells/mL while DT40 *MRE11*$^{H129N/-}$ and TK6 were diluted to a density of 120,000 cells/mL. Plates

were then incubated at conditions specific to the cell line (previously described) for 48 hours, aiming for approximately 1,000,000 cells/mL within the wells for optimal absorbance readings. 80µL of XTT working solution was added to each well along with resuspension of the cells. After a 3-hour incubation, absorbance was read at 450nm using an ELx800$^{TM}$ Absorbance Microplate Reader and Gen5 software and the results were saved in Microsoft Excel and processed using Prism Graph-Pad.

Sample absorbance readings were corrected for the absorbance of media and drug alone, and an average of the 3 technical repeats within the plates was taken. Estimated cell survival was calculated by comparing treated wells to untreated control wells, and expressed as a relative percentage. Data are reported as means ± standard deviation of triplicate biological samples.

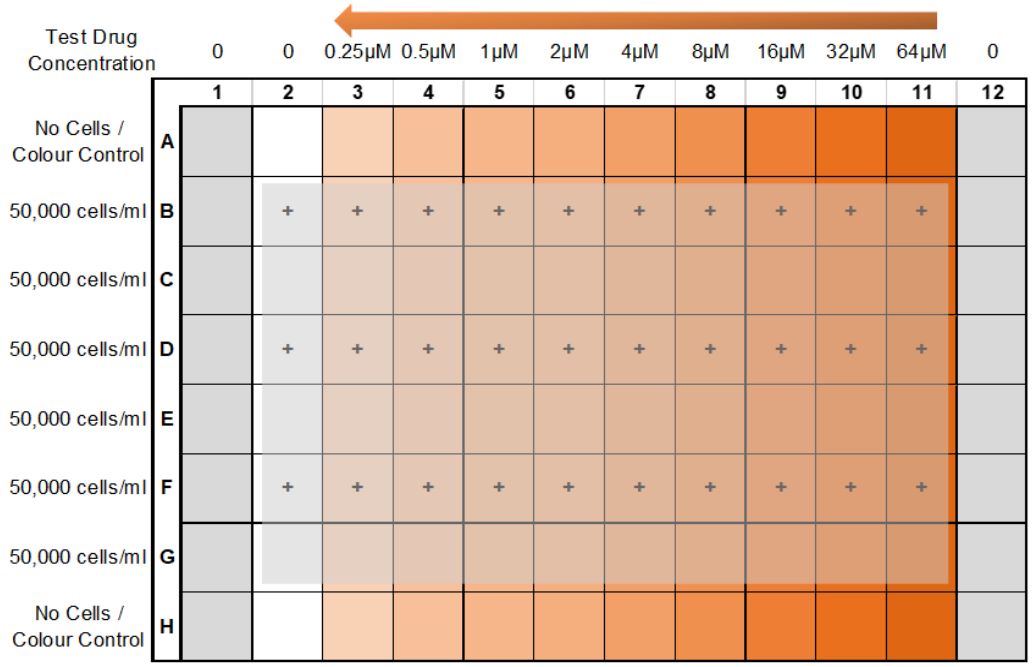


**Figure 8. Layout of the 96-well plates used to produce the drug plates for XTT assays**. '+' indicates the addition of the 'poison' compound, while remaining wells are supplemented with RPMI media to equalise the volumes. Columns 1 and 12 are considered blanks, containing only RPMI media. A serial dilution of the test drug is performed with the highest concentration being at column 11. Test drug concentrations shown are examples only and don't apply to all assays. Column 2 contains no test drug and is used as control wells. 100,000 cells/mL are added to each well indicated in the gray box, which is diluted to 50,000 cells/mL with drug and media addition. Rows A and H contain no cells and are used as colour control wells to account for any coloured compounds affecting absorbance readings.

## 2.4 RADAR Assays

RADAR (rapid approach to DNA adduct recovery) is a method designed to quantitatively detect topoisomerase-covalent complexes (TOPcc) using samples of isolated DNA from treated cells loaded on a slot blot. The protocol used and described below is adapted from Kiianitsa and Maizels (2013).

2.4.1 DPCC Isolation

Each sample consisted of 1mL of approximately $1x10^6$ DT40 $Mre11^{+/-}$ cells treated with the required drugs, as shown in *Figure 9*, incubated in a 24-well plate at cell culture conditions (described previously) for 1 hour.

Samples were then transferred to 1.5mL Eppendorf tubes, culture medium aspirated, and 1mL of lysis reagent (or DNAzol™, Invitrogen) was added and incubated at 65°C for 15 minutes. Lysis reagent consisted of 6M guanidinium isothiocyanate (GTC), 10mM Tris-HCL (pH6.5), 20mM EDTA, 4% Triton x100 and 1% Sarkosyl.

DNA-protein covalent complexes (DPCC) were precipitated with addition of 0.5mL 100% ethanol at -20°C for 5 minutes, followed by centrifugation at maximum speed for 15 minutes, and aspiration of the supernatant. The pellet was washed twice in 1mL 75% ethanol (centrifugation at maximum speed for 10 minutes followed by aspiration of ethanol). Pellet was briefly left to dry to allow evaporation of ethanol, before dissolving in 200µL 8mM NaOH and kept overnight at -20°C.

| | | DMSO | Mirin (5mM) | AZD2461 (500µM) | Mirin & AZD2461 | |
|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** |
| DMSO | **A** | / | DMSO Control | Mirin Control | AZD2461 Control | Mirin & AZD2461 Control | / |
| 0.1µM SN-38 | **B** | / | 0.1µM SN-38 Control | Mirin & 0.1µM SN-38 | AZD2461 & 0.1µM SN-38 | Mirin, AZD2461 & 0.1µM SN-38 | / |
| 1µM SN-38 | **C** | / | 1µM SN-38 Control | Mirin & 1µM SN-38 | AZD2461 & 1µM SN-38 | Mirin, AZD2461 & 1µM SN-38 | / |
| 10µM SN-38 | **D** | / | 10µM SN-38 Control | Mirin & 10µM SN-38 | AZD2461 & 10µM SN-38 | Mirin, AZD2461 & 10µM SN-38 | / |

**Figure 9. Layout of the 24-well plates used for incubating RADAR samples.** Each well consisted of $1x10^6$ cells in 1mL culture medium and a total of 30µL of treatment compounds (10µL of each, and total made up to 30µL with DMSO where needed). Compounds include; Mre11 exonuclease inhibitor Mirin (Sigma-Aldrich), PARP inhibitor AZD2461 (Sigma-Aldrich) and topoisomerase poison SN-38 (7-ethyl-10-hydroxycamptothecin; Sigma-Aldrich).

Samples were then thawed and placed in a Thriller shaking incubator at 65°C, 700rpm for 30 minutes before centrifugation and extraction of 150µL of supernatant (dissolved DPCC) and disposal of the debris pellet. DNA was quantified using the Qubit™ High Sensitivity dsDNA Quantification Assay Kits (Invitrogen).

### 2.4.2 DPCC Immunodetection

Samples were diluted to the required concentration in 8mM NaOH. The target concentration was 200ng of DNA in 200µL. Due to variations in DNA quantity within and between sample sets this wasn't always achieved, however samples were diluted to equal concentrations within sample sets, normalised to the lowest concentration.

Samples were loaded onto a PVDF membrane (Amersham) using a vacuum slot-blot manifold and then blocked in 0.5% milk powder dissolved in Tris-buffered saline (TBS; 10mM Tris, 150mM NaCl, pH 7.6) containing 0.1% Tween 20 (TBST) for 1 hour. Primary antibodies (mouse monoclonal anti-TOPO I-DNA covalent complexes, clone 1.1A, M-MABE1084) were added at 1:1,000 dilution and incubated overnight at 4°C on a rocker. The membrane was then washed 3 times for 10 minutes with TBST before being incubated with secondary antibodies (rabbit anti-mouse IgG [H + L], HRP; Invitrogen) at 1:2,000 dilution for 1 hour at room temperature. After 3 more 10 minute washes with TBST, 5 mL enhanced chemiluminescence (ECL; Thermo Scientific) solution was added and the membrane was imaged using a Bio-Rad ChemiDoc Imager and quantified and presented with use of Bio-Rad ImageLab.

# 3 Results

## 3.1 Optimisation of Cell Survival Assays with Opentrons Pipetting Robot

Before drug screening could begin, the optimal conditions for cell growth within the 96-well plates needed to be determined. These tests were performed for all cell-lines used. *Figure 10* shows some of the experiments that informed the final conditions used for the drug screening plates in DT40 *MRE11*$^{+/-}$ cells.

Serial dilutions of the cells were performed in 96-well plates using the Opentrons Pipetting Robot – starting at a cell density of 1x10$^6$ cells/mL and halved for each dilution down to just under 2x10$^3$ cells/mL, giving a total of 10 different seeding densities, each with 6 replicates. The highest absorbance value was shown to be when cells seeded at 1.25x10$^5$ are incubated for 48 hours before XTT addition, giving an average OD of 1.685 at 450nm.This was closely followed by cells seeded at 5x10$^5$, and 1x10$^6$ incubated for 24-hours (average ODs 1.443 and 1.418 respectively). This was further informed by the data shown in *Figure 10.B* showing the highest final cell density was reached after 48-hours incubating cells seeded at 2.5x10$^5$, reaching just over 1x10$^6$ cells/mL, though higher cell density doesn't seem to reflect a higher absorbance value, which is measuring cell metabolism. These values also don't suggest the cells are dividing at the expected rate (every 8 hours for DT40) and assays informed by this data were showing inconsistent cell growth and viability.

This led to a change from FBS 'Good' to FBS 'Supreme'. As a change in cell growth media had been made, and differences in cell growth had been observed in routine tissue culture, the serial dilution experiment was repeated, shown in *Figure 10.C*. While absorbance was again observed at 1.25x10$^5$ cells/mL seeded after 48 hours, it was shown that slight differences in seeding densities had a less dramatic impact as they did using FBS 'Good' (*Figure 10.A*), and represented a more stable and reliable growth curve of the cells in these conditions. This is again demonstrated in *Figure 10.D* where much higher final cell densities are reached compared to *Figure 10.*B, within the same time frame, with live cell density peaking at just below 3x10$^6$ cells/mL, compared to 1x10$^6$ cells/mL previously, suggesting a more nutrient rich environment is allowing the cells to remain viable and metabolise for longer and at greater densities.

Based on these results, and further tests analysing the DT40 *MRE11*$^{+/-}$ growth curve and 'cliff-edge' of cell viability at higher cell densities, a concentration of 1x10$^5$ cells/mL (which results in a final concentration of 5x10$^4$ cells/mL in final 96-well plate) was chosen, and cells were incubated with inhibitors for 48 hours before XTT addition. Similar tests were performed for DT40 *MRE11*$^{H129N/-}$ and TK6 WT$^{/-}$ where a seeding density of 1.2x10$^5$ and 1.6x10$^5$ cells/mL, respectively, incubated over 48 hours, were chosen. These differences in seeding densities reflect the differences in replication times of the different mutants and cell lines. When observed

under the microscope, these conditions resulted in near- or full cell confluency within control wells, and resulted in less variable growth and absorbance values, as further discussed below.

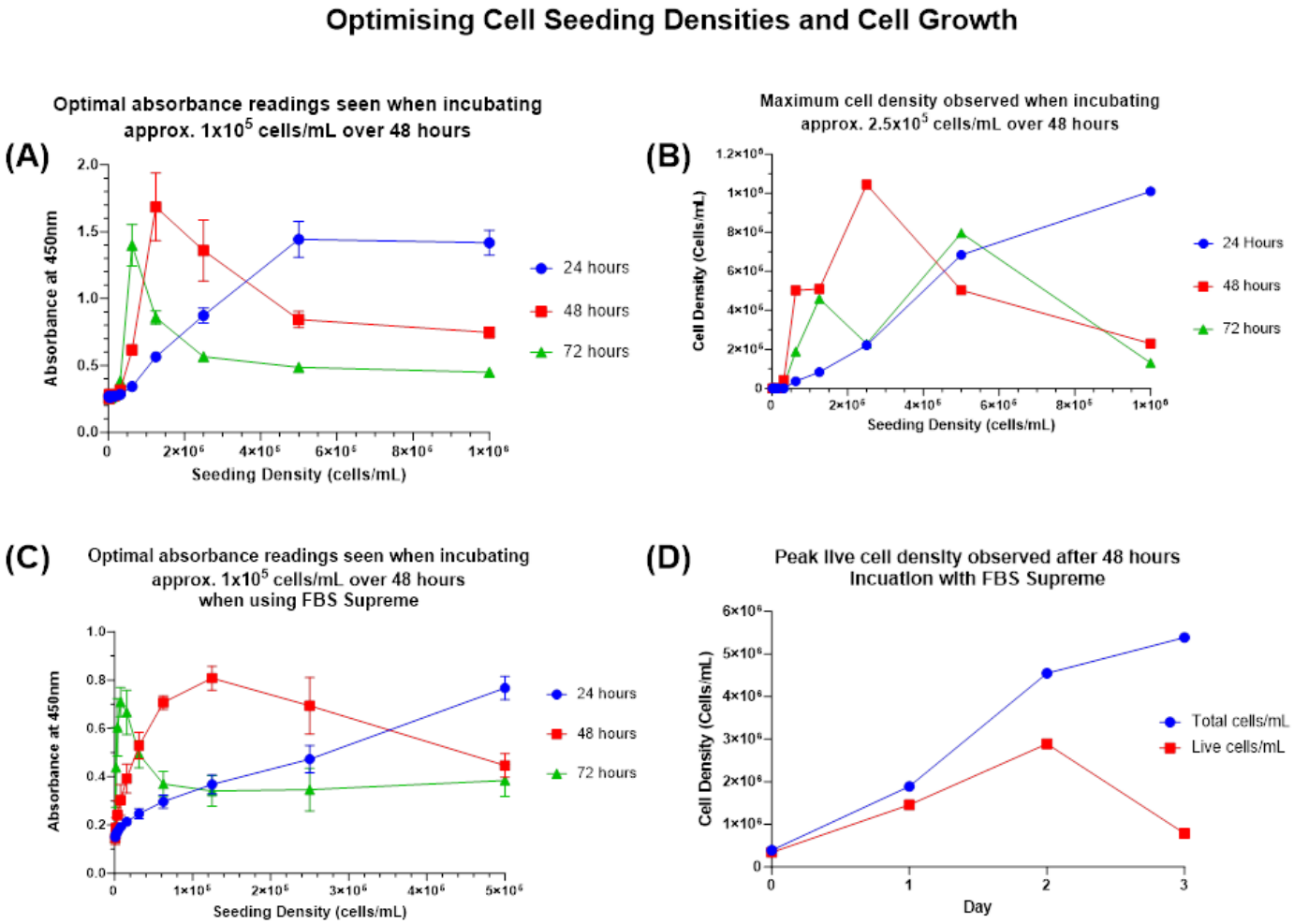


**Figure 10. Serial dilutions of DT40 *MRE11+/-* cells were performed and incubated for 24, 48, and 72 hours to determine optimal conditions for cell survival assays.**
**(A)** Serial dilutions (100µL) were performed in 96-well plates simultaneously and absorbance was read at different time points to determine optimal seeding density and incubation time based on peak XTT absorbance at 450nm. **(B)** A sample from each dilution in **A** was taken after the allotted time and cell density determined based on average live cell number counted using a hemocytometer and light microscope after staining with trypan blue. **(C)** Experiment replicated with cells grown in FBS Supreme, rather than FBS Good. **(D)** Cell counts derived from cell grown in FBS Supreme over 3 days.

It's also important to note that, while comparatively lower absorbance values are shown in *Figure 10.C* compared to *10.A* (with the switch to FBS Supreme), this is not representative of future assays as these initial experiments were performed at a time when absorbance readings

were not ideal but was later remedied by optimisation described below. In general, the use of FBS Supreme at the seeding density and incubation time determined by *Figure 10.C* resulted in higher and more consistent absorbance values, and more reliable cell growth than before.

Next, the reproducibility of the survival assays themselves needed to be addressed. As shown in *Figure 11.A* and *11.B*, there was significant variability between assays (comparing the blue lines), as indicated by the large error bars and differences in absorbances at similar concentrations. Mirin dilutions with and without the addition of SN-38 were run at this stage of optimisation as the expected results were known and so were utilised as control assays, in addition to aiming to replicate previous students' results (see *Figure 7* in *1.4 Project Rationale and Aims*) before proceeding forward. One of the earliest changes made was in controlling the temperature of the cells within the 96-well plates during pipetting. This was achieved through the addition of a temperature module to the Opentrons pipetting robot, minimising the time cells and plates spent in the robot by optimising the protocol, and by thoroughly ensuring cell medium and drug plates were warmed to the appropriate temperature before the addition of cells. The change from FBS 'Good' to FBS 'Supreme' also made a huge impact on cell stability, as previously discussed. The addition of resuspension of cell samples before being added to the drug plates, and within the plates also aided in reducing assay variability, shown in *Figure 11.*C. However, more variability than desired was still present, and the expected results were not being reproduced.

A major step forward was in the observation and control of the cell growth curve. Initially, a culture of cells showing >90% viability was considered suitable for experimental use, but due to large inconsistencies across experiments this was re-examined and decided that cell density was just as, if not more, important to consider. For DT40 *MRE11*$^{+/-}$ cells, for example, a 'cliff-edge' phenomenon was observed where once reaching a certain density (approx. $7x10^5$ cells/mL), the log-phase of exponential cell growth rapidly declined, and if experimental samples were taken of cells at or just before this point, there was significant delay of cell growth within the plates. So, while at the point of sample taking the cell viability was ideal, regardless of what density they were diluted to, if the cells were at or close to this 'cliff-edge' they would experience a rapid decline in growth rate resulting in final cell densities after 48-hours incubation being significantly less than expected, and contributing to the inconsistent results between assays (as cells of similar viability, but not cell density, were being sampled). After this connection was made, it was ensured that experimental samples were taken from cell cultures ≤$5x10^5$ cells/mL to ensure decent leeway before the end of log-phase. This resulted in significantly improved repeatability of the assays, and higher absorbance values due to improved cell number and viability.

The effect of pipetting speeds within the Opentrons Pipetting Robot on cell viability was also considered, however the differences observed between the default (94µL/s), medium (62µL/s),

and slow (31µL/s) pipetting speeds were considered minimal or non-existent, so this was not altered moving forward.

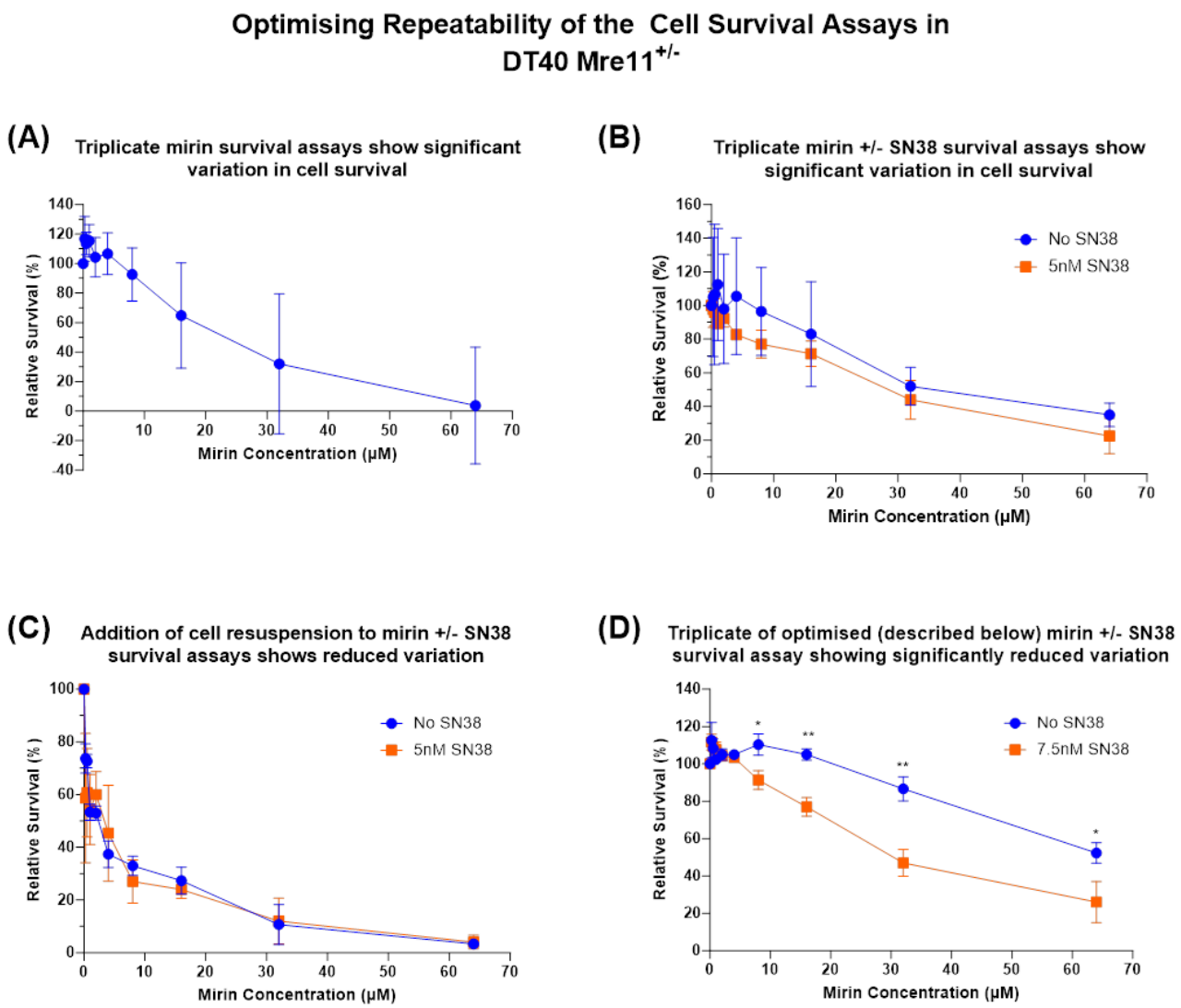


**Figure 11. High variability was observed across technical and biological triplicate assays which was significantly reduced after a period of optimisation of cell growth and Opentrons protocols to eventually allow effective and reliable drug screening.**
Three identical plates were created simultaneously using the Opentrons Pipetting Robot consisting of a **(A)** mirin serial dilution and **(B)** additions of a single concentration (5nM) of SN-38. **(C)** Showing the effects of the addition of cell resuspension to the Opentrons protocols, and **(D)** a further significant reduction in biological variability and a demonstration of the expected result through several optimisation steps.

Asterix (*) represent level of significant difference, as determined by t-test, between 'No SN38' and '7.5nM SN38' at that mirin concentration (n=3), where * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. Each data point represents an average of 3 (6 for **A**) technical repeats for each assay, which were biologically repeated in triplicate to give an average percentage survival plotted here, with deviation from the mean represented by error bars.

To keep laboratory waste to a minimum, reusable reagent reservoirs were utilised within the Opentrons Pipetting Robot for holding culture media, diluted inhibitors and XTT, and cells for use in the drug screening assays. These reservoirs were rinsed after use, run through a laboratory-dishwasher, and autoclaved (sealed) before reuse. Manual assays were run to replicate the same protocol as the pipetting robot (and in tissue culture conditions) to test for any differences in variability and cell growth, and this showed significant improvement in final cell density of the assays (*Figure 12.B*), seen by the reduction in error bars compared to when using the reusable reservoirs. As different reservoirs are used to run assays manually, disposable (one-use) reservoirs (INTEGRA and ChannelMate, StarLab) were trialled in the pipetting robot, again showing significant improvement to cell density, and reduced variability (error bars) between assays (*Figure 12.A*).

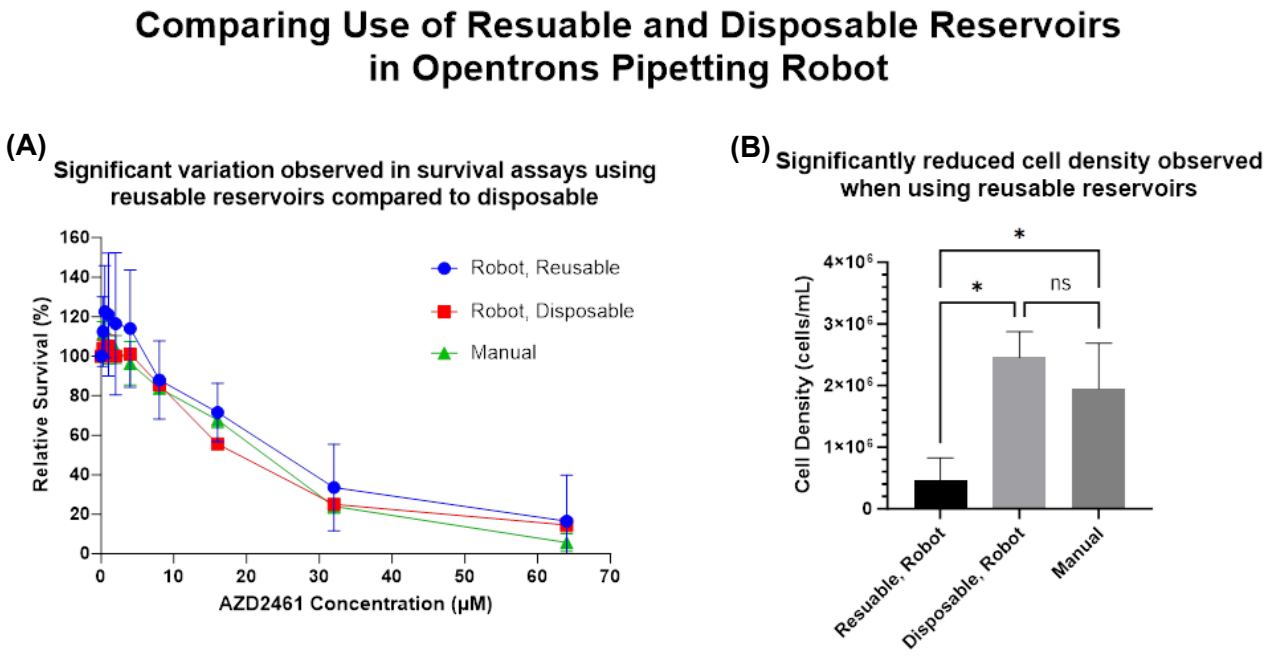


**Figure 12. A switch from reusable to disposable reagent reservoirs was made, resulting in significantly reduced variability, and improved cell growth, viability, and absorbance readings.**
Asterix (*) represent level of significant difference, as determined by one-way ANOVA (n=3), where * $p \leq 0.05$ and ns denotes no significant difference.

Overall, these optimisation tests, changes and general improvement in tissue culture practices eventually resulted in a reliable, reproducible, and semi-automated drug screening assay that would allow the project to move forward. A description of the final assay and cell conditions can be found in *Materials and Methods*. Using the optimised assay, a successful triplicate biological repeat of the mirin-SN-38 assay was achieved, shown in *Figure 11.D*, showing significant differences between the effects of mirin alone and in combination with 7.5nM SN-38, as well as massively reduced variability between both technical and biological repeats represented by

minimal error bars. These successful control assays signalled the go-ahead for the screening of DNA repair inhibitors in combination with mirin to begin.

### 3.2 Screening DNA Repair Inhibitors in Combination with Mirin in DT40 *MRE11*$^{+/-}$ Cells

To test for potential synthetic genetic interactions between the MRE11 exonuclease inhibitor mirin and various selected DNA repair inhibitors, inhibitors were first tested in DT40 *MRE11*$^{+/-}$ cells as a simple serial dilution to determine appropriate concentration ranges for each. Following this, each inhibitor was screened using serial dilution (within the optimised concentration range) with and without the addition of 20μM mirin. This mirin concentration was also determined using serial dilution and was chosen based on approximately 80% cell survival at this concentration, to allow for additional lethality in combination with other inhibitors to be observed. Relative cell survival was calculated based on the control/'no-drug' wells being representative of 100% cell survival, with increasing inhibitor concentration having cell survival represented as a percentage of this. It is important to note, however, that in the mirin combination assays 20μM was included in control wells, meaning, while represented at 100% survival still, cell survival was actually around 80% of that seen in absence of mirin. Results are visualised this way in order to better observe potential synthetic lethal interactions, rather than just the additive effect of two inhibitors on cell survival. Results of the DNA repair inhibitors in combination with mirin assays in DT40 *MRE11*$^{+/-}$ cells are shown in *Figure 13*. Inhibitors were initially screened in this mutant cell line due to its ease of use, and due to also having access to a 'nuclease-dead' mutant (DT40 *MRE11*$^{H129N/-}$) to confirm the effects of mirin are due to inhibition of MRE11 3'-5' exonuclease activity.

Inhibition of PARP 1&2 activity by Olaparib-analogue AZD2461 showed steady and significant effects on DT40 Mre11$^{+/-}$ cell survival at increasing concentrations. Cells were significantly sensitised to PARP inhibition with the addition of inhibiting Mre11 exonuclease activity by mirin (*Figure 13.A*). While differences in relative cell survival with and without mirin are observed at all concentrations tested, they were only considered statistically significant from 0.25-16μM AZD2461 ($p \leq 0.01$), likely higher concentrations significantly compromising cell survival and so any additional cell death makes a less significant difference in absorbance values, and therefore calculated relative survival.

# DT40 Mre11$^{+/-}$ Cell Survival with DNA Repair Inhibitors and Mirin

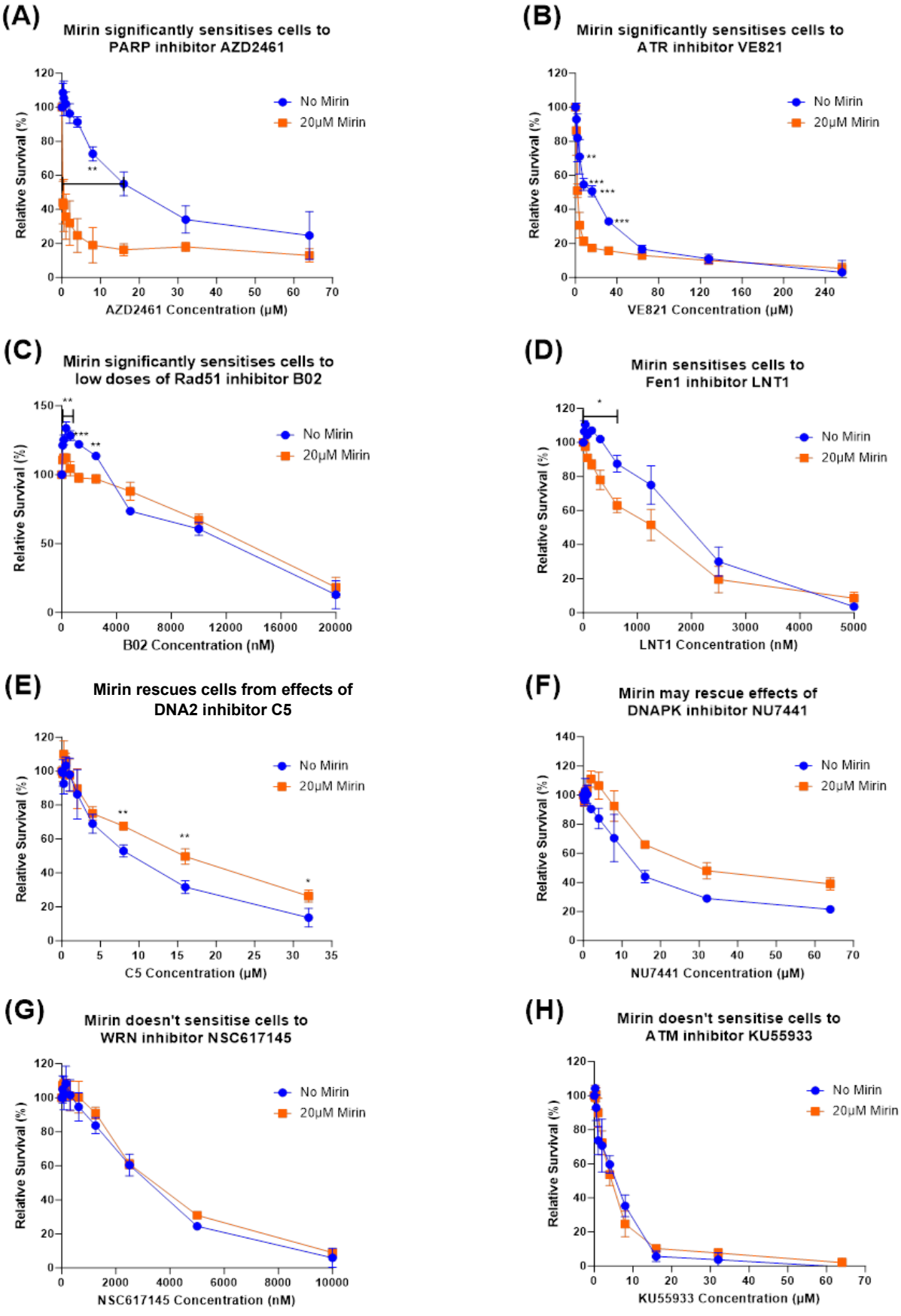

**Figure 13. Screening a variety of DNA repair inhibitors in combination with MRE11 exonuclease inhibitor, mirin (20μM), in DT40 *MRE11*$^{+/-}$ cells to determine any synergistic or synthetical genetic effects.**
All inhibitors were tested using serial dilution (9 different concentrations, halved each time). A 0μM/control condition was included and used to determine relative cell survival at subsequent concentrations based on 0μM representing 100% survival. It is important to note, mirin was included in these control wells meaning for the 20μM Mirin condition, what is considered 100% survival is actually approximately 80%, but is represented this way to better visualise synthetic lethality rather than just additive effects of another inhibitor. **(A)** PARP inhibition by AZD2461 (Sigma-Aldrich), at a concentration ranging from 0.25-64μM. **(B)** ATR inhibition by VE-821 (Sigma-Aldrich), ranging from 1-256μM. **(C)** RAD51 inhibitor B02 (Sigma-Aldrich) concentrations from 78nM- 20μM. **(D)** FEN1 inhibitor LNT1 (Sigma-Aldrich) concentrations ranging from 19.5nM-5μM. **(E)** DNA2 inhibitor C5 (MedChem Express) used at 0.125-32μM. **(F)** DNA-PK inhibitor NU7441 (Tocris) used at 0.25-64μM. **(G)** WRN inhibitor NSC617145 (Tocris), used at 10μM-39nM. **(H)** ATM inhibitor KU55933 (Sigma-Aldrich), used at 64-0.25μM.

Asterix (*) represent level of significant difference, as determined by t-test, between 'No Mirin' and '20μM Mirin' at that inhibitor concentration (n=3, except for **F** where n=2), where * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. Where several consecutive points show the same level of significance, or points are too clustered to differentiate, the group of points being referred to is indicated with a capped horizontal line. No asterix (*) suggests a non-significant difference.
Each data point represents an average of 3 technical repeats for each assay, which were biologically repeated in triplicate to give an average percentage survival plotted here, with deviation from the mean represented by error bars, with the exception of **F** where only 2 biological repeats are represented. Where error bars are not visible, standard deviation is too small to visualise on this figure.

Similarly, the addition of mirin to cells treated with VE-821, an ATR inhibitor, also significantly sensitise cells (*13.B*), particularly at VE-821 concentrations 4μM ($p \leq 0.01$) and 8-32μM ($p \leq 0.001$), after which relative cell survival falls to near 0% and differences are no longer observed.

Interestingly, treatment with RAD51 inhibitor B02 increased DT40 *MRE11*$^{+/-}$ cell survival at lower doses (≤2,500nM), relative to control condition containing no inhibitors (considered 100%), but the addition of mirin at these B02 concentrations significantly reduces cell survival (*Figure 13.C;* 78-625nM $p \leq 0.01$, 1250nM $p \leq 0.001$, 2500nM $p \leq 0.01$). Concentrations at or above 5000nM (5μM) B02 seem to no longer be beneficial to the cells, and relative survival drops significantly, and the addition of 20μM mirin no longer shows a difference in terms of cell survival.

Treatment with mirin alongside FEN1 inhibitor LNT1 also seems to somewhat sensitise cells (*13.D*), though to a lesser degree than observed in other inhibitors, with differences considered

significant only at 39-625nM to $p \leq 0.05$, after which higher degrees of variation seem to limit observations of significant differences.

On the other hand, inhibition of Mre11 exonuclease activity by mirin seems to significantly rescue the effects of higher doses of C5, a DNA2 inhibitor (*Figure 13.E;* 8-16μM $p \leq 0.01$, 32μM $p \leq 0.05$). A similar effect of mirin is seen alongside DNA-PK inhibitor NU7441 (*13.F*), where some rescue of cell survival appears to occur at almost all concentrations, however the differences in relative survival between the absence and presence of mirin did not turn out to be significant to $p \leq 0.05$, potentially due to only two biological repeats being represented in the data rather than the optimal three.

For WRN inhibitor NSC617145 (*13.G*) and ATM inhibitor KU55933 (*13.H*) no differences in cell survival in the absence and presence of mirin were observed ($p \geq 0.05$).

### 3.3 Testing DNA Repair Inhibitors in DT40 Mre11 Nuclease-Dead Mutants

Initially the DNA repair inhibitors were screened by comparing effects on survival of DT40 *MRE11*$^{+/-}$ and *MRE11*$^{H129N/-}$ (Mre11 nuclease-dead) mutants due to issues with the original assay, however it was also a useful tool to test if the effects induced by mirin was MRE11 nuclease-dependent, as mirin is thought to be a specific inhibitor of MRE11's 3'-5' exonuclease activity. As shown in *Figure 14*, three DNA repair inhibitors were screened this way initially; PARP inhibitor AZD2461, RAD51 inhibitor B02, and ATR inhibitor VE-821, as at the time these were showing the most intriguing effects in the *MRE11*$^{+/-}$ mutant in combination with mirin. However, the results from these screens, performed in triplicate, were unexpected. The rationale to begin with was that using an MRE11 nuclease-dead mutant would be equivalent to inhibiting MRE11 activity with mirin, and so similar effects to those shown in *Figure 13* were expected. This was not the case, and for all three inhibitors the nuclease-dead *MRE11*$^{H129N/-}$ mutant showed significantly less sensitivity than *MRE11*$^{+/-}$.

From this, it was thought either some MRE11 activity, but not nuclease, was needed for sensitivity to these DNA inhibitors to occur, or the DT40 *MRE11*$^{H129N/-}$ mutant cell-line was no longer expressing the expected phenotype due to repeated culturing and freezing cycles potentially introducing unknown and confounding mutations, which is more likely to occur in mutants deficient in DNA repair, such as *MRE11*$^{H129N/-}$. To test this, the effects of mirin and SN-38 on the *MRE11*$^{H129N/-}$ mutant, compared to *MRE11*$^{+/-}$, were investigated as shown in *Figure 15*.

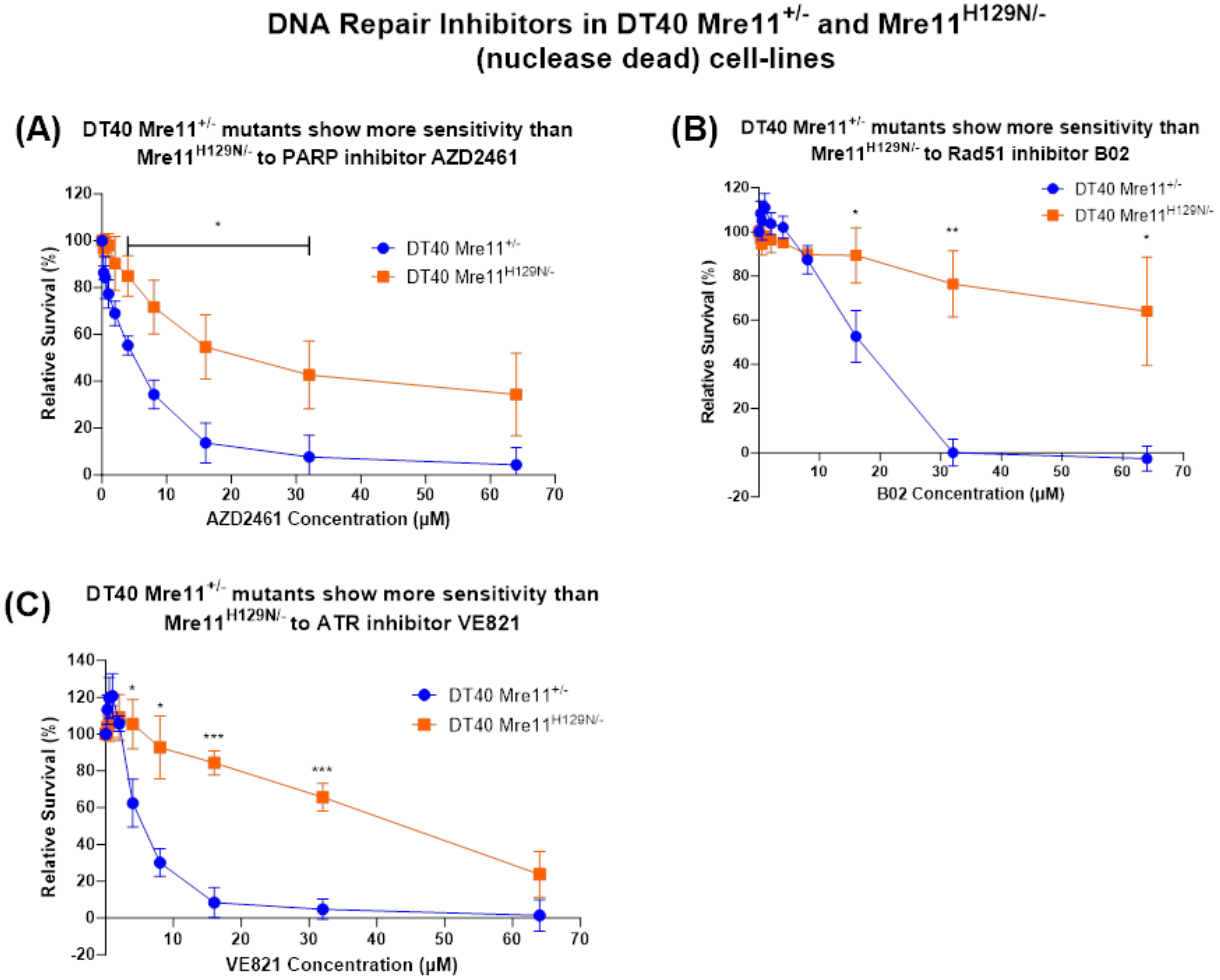


**Figure 14. Screening DNA repair inhibitors in MRE11 'nuclease-dead' mutants (DT40 *MRE11$^{H129N/-}$*) and comparing the effects seen in corresponding *MRE11$^{+/-}$* DT40 mutant to confirm result in *MRE11$^{+/-}$* using mirin.**

All inhibitors were tested using serial dilution (9 different concentrations, halved each time). A 0μM/control condition was included and used to determine relative cell survival at subsequent concentrations based on 0μM representing 100% survival. Two identical plates containing serial dilutions of each inhibitor were made, each plate having a different cell line added and incubated for 48-hours. **(A)** PARP inhibition by AZD2461 **(B)** RAD51 inhibition by **(C)** ATR inhibition by VE-821.

Asterix (*) represent level of significant difference, as determined by t-test, between '*MRE11$^{+/-}$*' and '*MRE11$^{H129N/-}$*' at that concentration (n=3), where * p ≤ 0.05, ** p ≤0.01 and *** p ≤0.001. Where several consecutive points show the same level of significance, or points are too clustered to differentiate, the group of points being referred to is indicated with a capped horizontal line. No asterix (*) suggests a non-significant difference.

Each data point represents an average of 6 technical repeats for each assay, which were biologically repeated in triplicate to give an average percentage survival plotted here, with deviation from the mean represented by error bars.

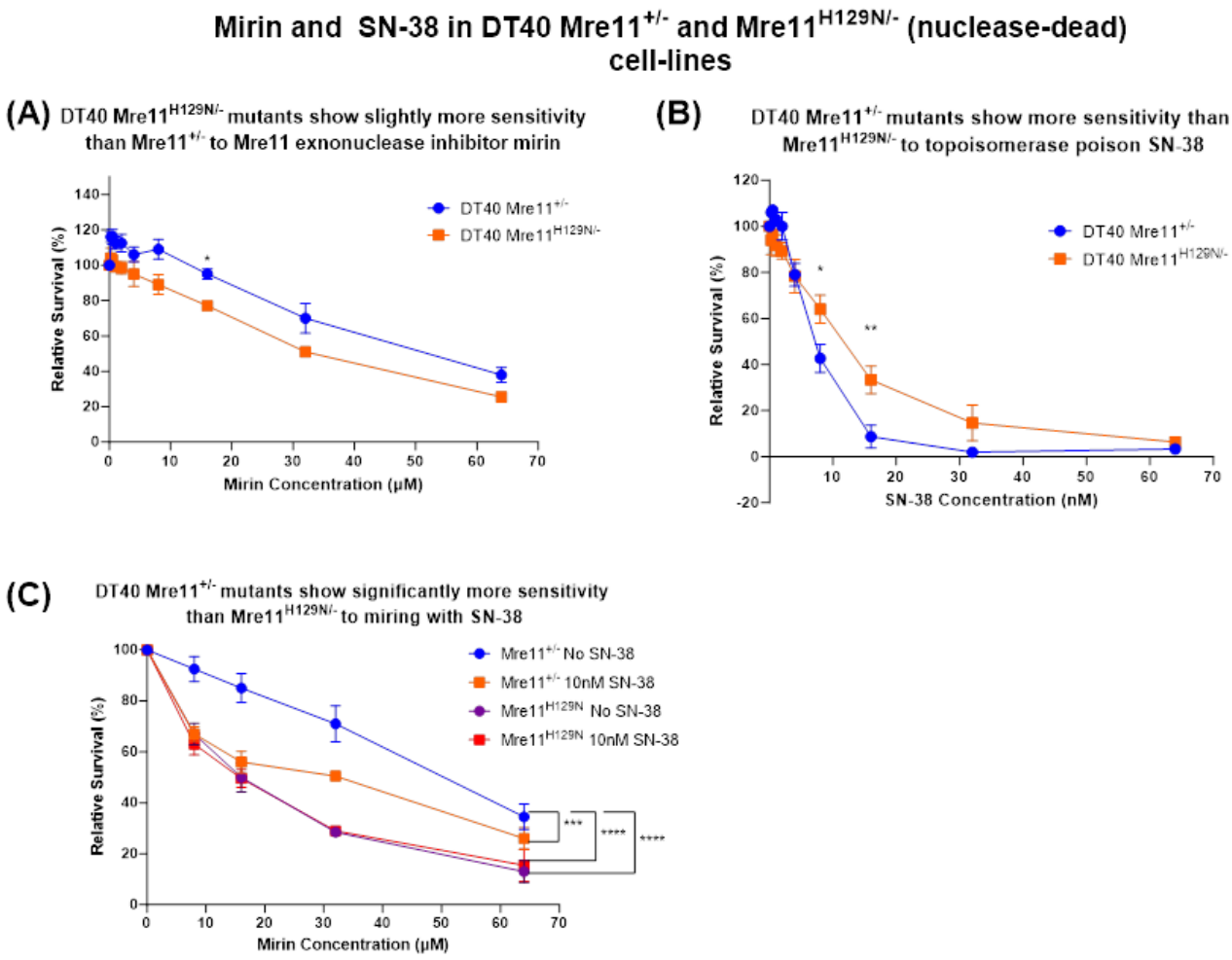


**Figure 15. Testing DT40 *MRE11$^{H129N/-}$* mutant in comparison to *MRE11$^{+/-}$* for sensitivity to mirin and SN-38 to determine if expected phenotypes are still being expressed.**
Comparing effects on DT40 MRE11$^{+/-}$ and MRE11$^{H129N/-}$ survival of **(A)** inhibition of MRE11 exonuclease activity by mirin **(B)** topoisomerase poison SN-38 and **(C)** both mirin and SN-38 in combination.

Asterix (*) represent level of significant difference between 'MRE11+/-' and 'MRE11$^{H129N/-}$' , determined by t-test, and two-way ANOVA for comparisons in **C**, (n=3), where * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$ and **** $p \leq 0.0001$. Where several consecutive points show the same level of significance, or points are too clustered to differentiate, the group of points being referred to is indicated with a capped horizontal line. No asterix (*) suggests a non-significant difference.
Each data point represents an average of 6 technical repeats for each assay (except for **C** which has 3), which were biologically repeated in triplicate to give an average percentage survival plotted here, with deviation from the mean represented by error bars.

The inhibition of MRE11 exonuclease activity by mirin would be expected to have no impact on cell survival in the MRE11 nuclease-dead DT40 *Mre11$^{H129N/-}$* mutant, however, as shown in *Figure 15.A*, *Mre11$^{H129N/-}$* cells show slightly increased sensitivity to mirin compared to *Mre11$^{+/-}$* cells, with a difference considered significant at 16μM to $p \leq 0.05$. This alone suggests either off-target effects of mirin, not specific to MRE11 nuclease activity which has already been knocked out in the nuclease-dead mutant, or MRE11 nuclease activity is no longer deactivated in the *Mre11$^{H129N/-}$* mutant due to further mutation or contamination from other cell-lines. Furthermore, from previous students' results (*Figure 7*) and other studies, the MRE11 nuclease-dead mutant would be expected to show significant sensitivity to the topoisomerase poison SN-

38. However, here *Mre11*$^{+/-}$ shows more sensitivity than *Mre11*$^{H129N/-}$, and at certain SN-38 concentrations this difference is significant to $p \leq 0.05$ and 0.01 (*15.B*). Shown in *15.C*, the same control assay as shown in *Figure 11.D* was performed in both the DT40 *Mre11*$^{+/-}$ and *Mre11*$^{H129N/-}$ alongside each other. The nuclease-dead mutant, here, is still showing effects on cell survival from MRE11 exonuclease inhibition by mirin, but additionally is not sensitised at all by the addition of SN-38. *Mre11*$^{+/-}$, on the other hand, does show the expected results, with a significant difference between the 'No SN-38' and '10nM SN-38 to $p \leq 0.001$, suggesting the issue is not within the assay itself.

Due to the potential for mutation within the DT40 *Mre11*$^{H129N/-}$ cell-line, and time limitations meant that further investigation into this could not be carried out within the scope of the project. Therefore, further testing with the nuclease-dead mutant was stopped.

### 3.4 Testing Promising DNA Repair Inhibitors in Combination with Mirin in TK6$^{WT/-}$ Cells

Following on from the screening of a range of DNA repair inhibitors in DT40 *Mre11*$^{+/-}$ in combination with MRE11 exonuclease inhibition by mirin (*Figure 13*), those showing potential for synthetic lethality or otherwise interesting interactions were selected and further screened in the human cell line TK6$^{WT/-}$. Accounting for increased sensitivity of TK6 to mirin, a lowered dose of 10μM was used to result in a comparable approximate 80% cell survival with mirin treatment alone. Results from these screenings in TK6$^{WT/-}$ are shown in *Figure 16*.

TK6$^{WT/-}$ cells are sensitised to PARP inhibitor AZD2461 (Olaparib analogue) when combined with Mre11 exonuclease inhibitor mirin (*Figure 16.A*), with more significant differences observed in relative cell survival with and without mirin seen at lower AZD2461 doses, likely as it increases cell survival drops below a threshold where additional lethality has less effects on absorbance values and therefore cannot show a significant difference. Interestingly, the extent to which mirin sensitises cells to PARP inhibition seems to be less potent in TK6 compared to DT40 *Mre11*$^{+/-}$, which can be seen in *Figure 13.A* for comparison.

On the other hand, a significant rescue effect is seen when MRE1 exonuclease activity is inhibited alongside RAD51 inhibition by B02 (*16.B*). This difference in relative survival is significant at almost all concentrations of B02 and remains relatively consistent also. In DT40 *Mre11*$^{+/-}$ (*Figure 13.C*) inhibition of RAD51 alone seemed to be beneficial to the cells at lower concentrations, shown by an initial increase in cell survival apparently mitigated against

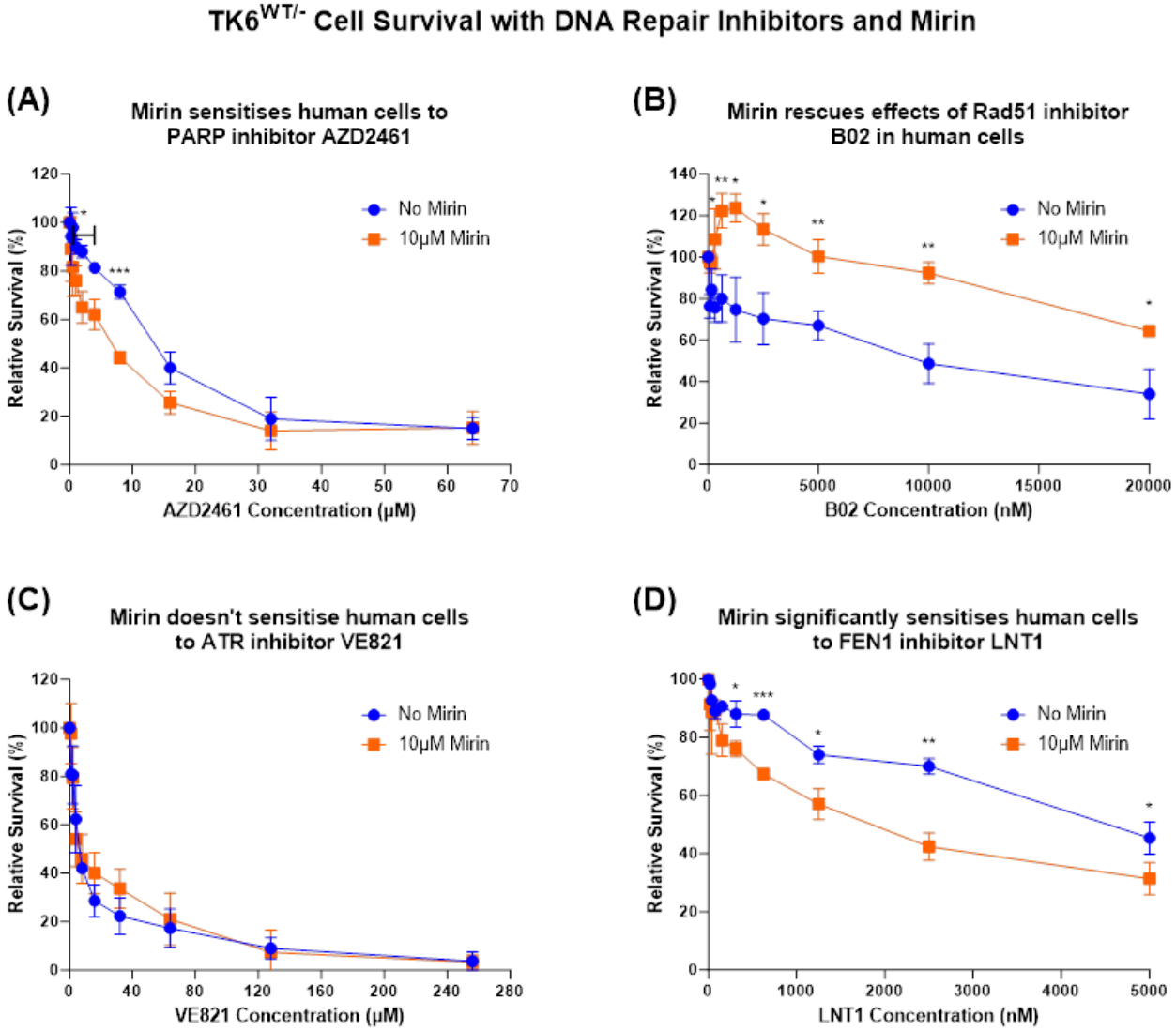


**Figure 16. Screening promising DNA repair inhibitors in human TK6$^{Wt/-}$ in combination with mirin (10μM), to investigate whether synergistic of synthetic lethal effects seen previously in DT40 translate to human cells.**

All inhibitors were tested in the same manner as described for DT40 *Mre11*$^{+/-}$ in *Figure 13* but using 10μM mirin due to increased sensitivity of TK6 $^{WT/-}$. **(A)** PARP inhibition by AZD2461**(B)** RAD51 inhibition by **(C)** ATR inhibition by VE-821 **(D)** FEN1 inhibition by LNT1.

Asterix (*) represent level of significant difference, as determined by t-test, between 'No Mirin' and '10μM Mirin' at that inhibitor concentration (n=3), where * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. Where several consecutive points show the same level of significance, or points are too clustered to differentiate, the group of points being referred to is indicated with a capped horizontal line. No asterix (*) suggests a non-significant difference.
Each data point represents an average of 3 technical repeats for each assay, which were biologically repeated in triplicate to give an average percentage survival plotted here, with deviation from the mean represented by error bars. Where error bars are not visible, standard deviation is too small to visualise on this figure.

by MRE11 inhibition, before becoming progressively more lethal at higher concentrations with no observable effects with the addition of mirin. This is contrasted here in *Figure 16.B* where no increase in relative survival is observed with B02 treatment alone, however when combined with mirin a significant increase in cell survival is seen relative to the control conditions, suggesting a combined effect of RAD51 and MRE11 inhibition actually benefitting cell survival. This remains

consistent over increased B02 concentrations, with relative survival gradually decreasing but the effects of B02 consistently being mitigated by mirin by at least 30%, and continuing to show a significant difference in relative cell survival.

ATR inhibitor VE-821, however, shows no additional sensitisation of TK6 cells when combined with mirin *(16.C*), with no differences in relative survival shown. This is in contrast to the effects seen in DT40 *Mre11*$^{+/-}$ where, especially at lower concentrations before survival is significantly compromised, mirin resulted in significant sensitisation to ATR inhibition by VE-821 (*Figure 13.B*).

FEN1 inhibitor LNT1, however, while showing some sensitisation when combined with mirin in DT40 *Mre11*$^{+/-}$ (*Figure 13.D*), shows even more significant differences in relative cell survival in TK6 when comparing effects seen with and without mirin *(16.D*). These differences are significant at almost all LNT1 concentrations tested. While LNT1 resulted in almost 100% lethality in the *Mre11*$^{+/-}$ mutants, TK6$^{WT/-}$ seem to be less sensitive to FEN1 inhibition by LNT1 on it's own, with only approximately 50% lethality at the same concentration (5000nM/5μM). However lethality is greatly increased when introducing MRE1 exonuclease inhibition simultaneously, with at least 20% difference in mean survival observed consistently at and above 625nM LNT1. From the scope of the graph presented in *Figure 16.D*, it appears the significant effect of mirin may decrease with further increase in LNT1 concentration, which may more closely reflect the effect seen in *Mre11*$^{+/-}$ where sensitivity to LNT1 in higher.

### 3.5 Further Investigating Potential for MRE11 Inhibition to Sensitise Cells to PARP Inhibitors

As a result of PARP inhibitor AZD2461 in combination with mirin showing the largest and most consistent significant difference in cell survival in DT40 *Mre11*$^{+/-}$ (*Figure 13.A*) and similar effects also observed in human TK6$^{WT/-}$ (*Figure 16.A*), this was further investigated by sourcing other PARP inhibitors that supposedly varied in their mechanism of inhibiting PARP activity. It is important to note that at the time of this line of investigation beginning, not all results from other inhibitors had been obtained, and so at the time AZD2461 was the only inhibitor showing intriguing results warranting further investigation. Since then, other inhibitors, such as those targeting ATR, FEN1 and DNA2, would also be interesting to test similarly, but time limitations prevented this.

The effects of mirin in sensitising DT40 *Mre11*$^{+/-}$ cells to PARP inhibition by AZD2461 is shown again in *Figure 17.A* for ease of comparison, and it can be seen here that PARP inhibitors Talazoparib (BM-673; *17.B*) and Iniparib (BSI-201; *17.C*) do not show increased sensitisation of cells in combination with MRE11 exonuclease inhibition by mirin. This was an interesting discovery as all three inhibitors act to specifically inhibit PARP activity and are shown here to impact cell survival presumably through PARP inhibition, however their differences in mechanism of action may be where MRE11-dependent sensitisation resides.

Further testing in TK6$^{WT/-}$ *(Figure 18*) interestingly showed that, in this human cell-line, MRE11 exonuclease inhibition did confer sensitivity to PARP inhibition by Talazoparib (*18.B*), with significant differences observed when comparing relative cell survival with and without the addition of mirin. This was an intriguing finding as no such differences were observed in DT40 *Mre11*$^{+/-}$*.* TK6 does seem to be slightly less sensitive to Talazoparib treatment overall, however not by much, though this might be affecting the ability to observe synergistic effects.

Again, MRE11 inhibition in combination with PARP inhibition by Iniparib (*18.C*) does not show any increased sensitivity in TK6$^{WT/-}$, similarly to its effects in DT40 *Mre11*$^{+/-}$*.*

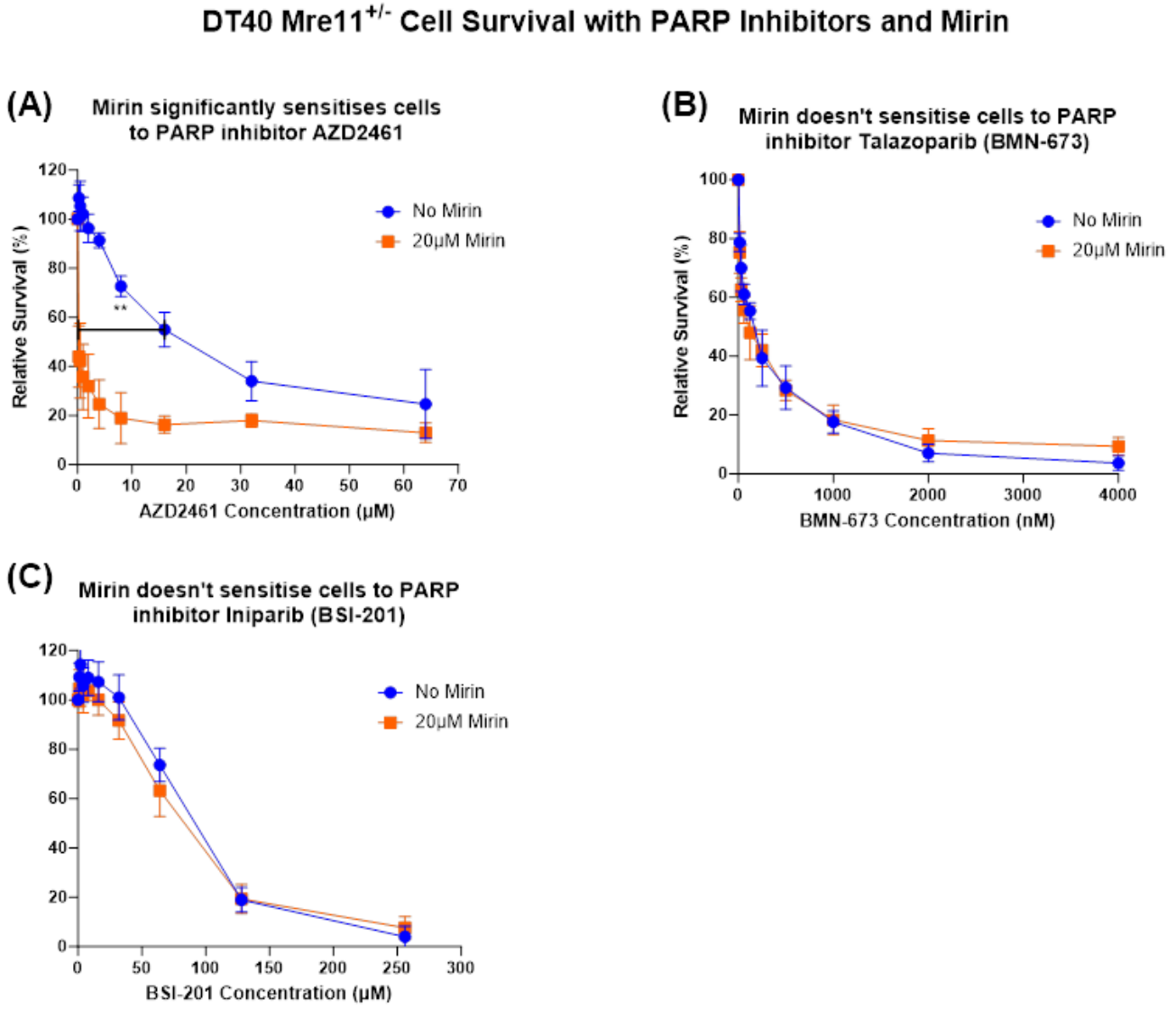


**Figure 17. Testing two additional PARP inhibitors with different modes of action in DT40 *Mre11*$^{+/-}$ cells, in combination with MRE11 exonuclease inhibitor mirin to test for any differences in sensitisation effects.**

All inhibitors were tested in the same manner as described in *Figure 13* with a serial dilution of PARP inhibitor with and without the addition of 20μM mirin. PARP inhibition by **(A)** AZD2461 **(B)** Talazoparib (BMN-673; MedChem Express) and **(C)** Iniparib (BSI-201; LKT Labs)

Asterix (*) represent level of significant difference, as determined by t-test, between 'No Mirin' and '10μM Mirin' at that inhibitor concentration (n=3), where * $p \leq 0.05$, and ** $p \leq 0.01$. Where several consecutive points show the same level of significance, or points are too clustered to differentiate, the group of points being referred to is indicated with a capped horizontal line. No asterix (*) suggests no significant difference.

Each data point represents an average of 3 technical repeats for each assay, which were biologically repeated in triplicate to give an average percentage survival plotted here, with deviation from the mean represented by error bars. Where error bars are not visible, standard deviation is too small to visualise on this figure.

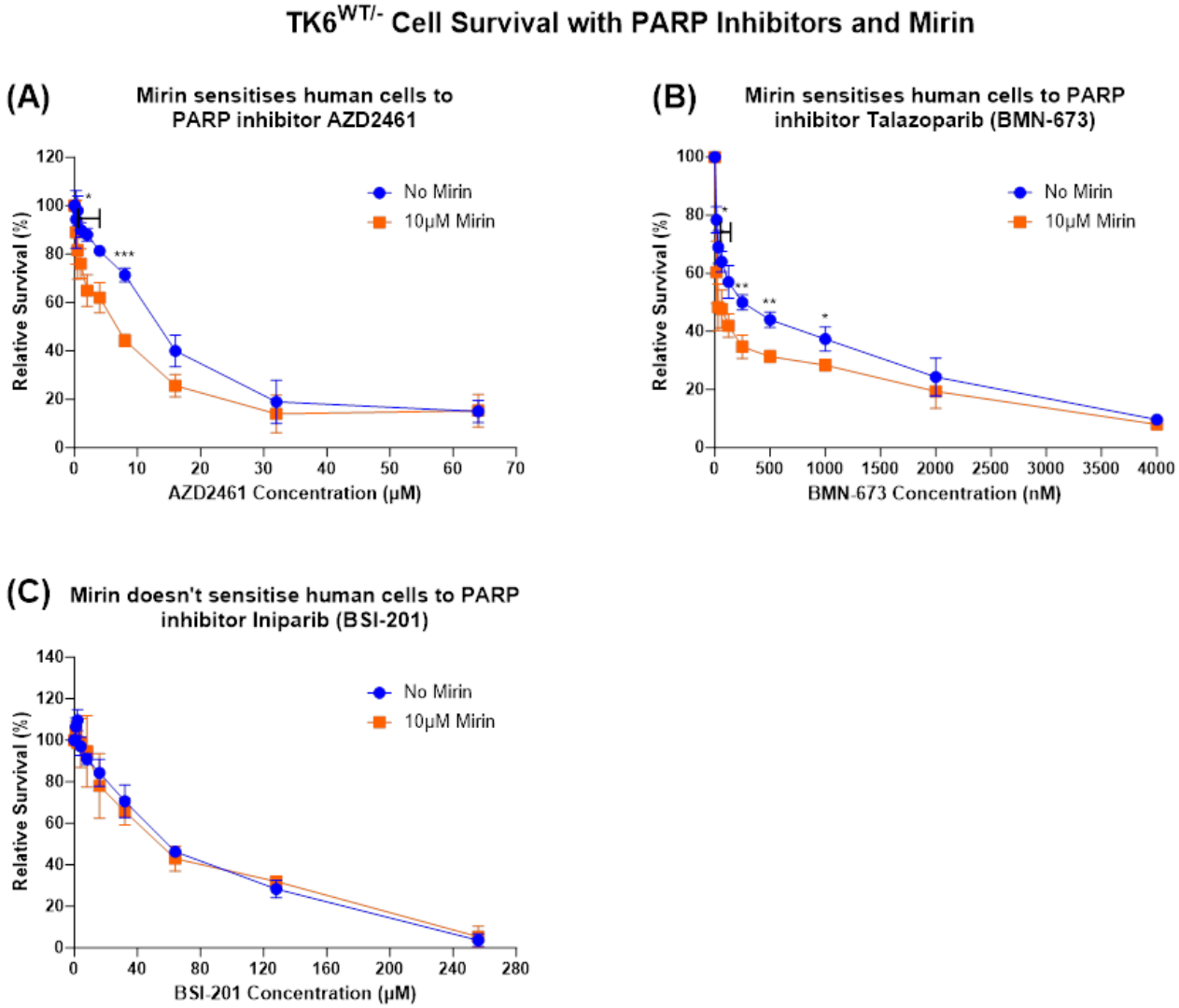


**Figure 18. Testing two additional PARP inhibitors with different modes of action in TK6$^{WT/-}$ cells, in combination with MRE11 exonuclease inhibitor mirin to test for any differences in sensitisation effects in human cells.**

All inhibitors were tested in the same manner as described in *Figure 13* with a serial dilution of PARP inhibitor with and without the addition of 10μM mirin to account for TK6 sensitivity to mirin. PARP inhibition by **(A)** AZD2461 **(B)** Talazoparib (BM-673) **(C)** Iniparib (BSI-201).

Asterix (*) represent level of significant difference, as determined by t-test, between 'No Mirin' and '10μM Mirin' at that inhibitor concentration (n=3), where * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$. Where several consecutive points show the same level of significance, or points are too clustered to differentiate, the group of points being referred to is indicated with a capped horizontal line. No asterix (*) suggests no significant difference.

Each data point represents an average of 3 technical repeats for each assay, which were biologically repeated in triplicate to give an average percentage survival plotted here, with deviation from the mean represented by error bars. Where error bars are not visible, standard deviation is too small to visualise on this figure.

3.6 Effects of simultaneous MRE11 & PARP inhibition on treatment with topoisomerase poisons

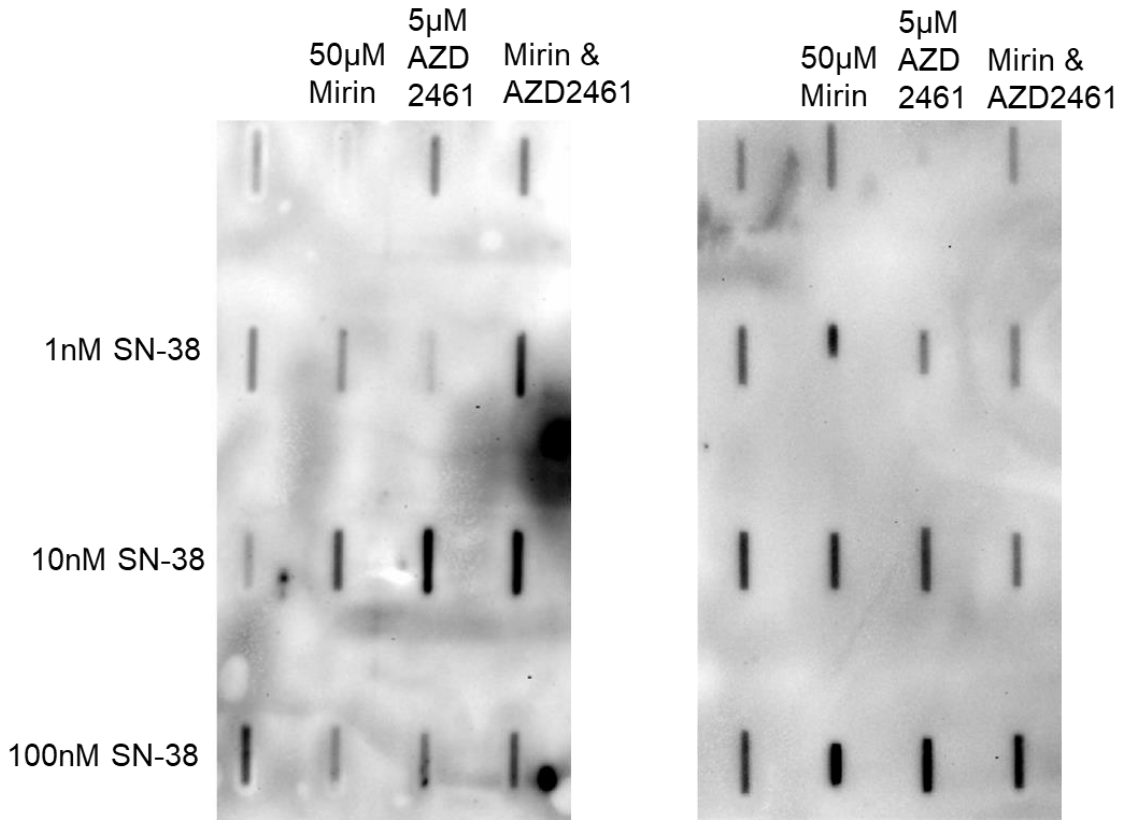


**Figure 19. RADAR images showing the immunodetection of topoisomerase-DNA covalent complexes (TOPcc) present in samples derived from DT40 *MRE11*$^{+/-}$ cells treated with the combination of MRE11 exonuclease inhibitor mirin, PARP inhibitor AZD2461, and topoisomerase poison SN-38.**

With the known role of PARP in the signalling and response to DNA damage, especially in response to bulky DNA adducts, the combination of mirin, AZD2461 and SN-38 was tested on DT40 *MRE11*$^{+/-}$ cells, as shown in *Figure 19*.

Most RADAR assays were unsucessful, and those presented were not sufficient to quantify the increases in TOPcc potentially observed. Some increase in TOPcc is observed with increasing SN-38 concentrations, and the first image shows a potential for the combination of AZD2461 and mirin to also increase TOPcc, but the same cannot be said for the second image, and time contraints prevented further investigation of this.

# 4 Discussion

## 4.1 MRE11 Exonuclease Inhibition by Mirin Increases Sensitivity to Topoisomerase Poison SN-38

The initial part of this project was to optimise the drug screening assay used in this project and replicate previous students' results using the altered assay for use in the Opentrons pipetting robot to ensure the assay was working as intended, and that each result was reproducible. *Figure 7.A* (in *1.4 Project Rationale and Aims*) shows the effects of mirin and SN-38 on DT40 $Mre11^{+/-}$ cells, using a manual version of the current assay, and performed with a lower cell concentration but over three days rather than two, by a previous student in the lab. *Figure 11.D* (in *3.1 Optimisation of Cell Survival Assays with Opentrons Pipetting Robot*) shows the same, using the Opentrons pipetting robot and implementing a series of changes to optimise the assay, as outlined previously in *Results* and also *Materials and Methods*. Both graphs show very similar and comparable results, showing the sensitisation of the cells to SN-38 by inhibiting MRE11 exonuclease activity with mirin, and this being a reproducible result using the optimised assay allowed the project to move forward into testing the different DNA repair inhibitors alongside mirin.

However, there were issues in reproducing previously observed results in the nuclease-dead cell-line (DT40 $MRE11^{H129N/-}$). *Figure 7.B* shows that previously there had been no significant differences in the sensitivity to SN-38 in the nuclease-dead cell-line with the inhibition of MRE11 by mirin, which is understandable as the cell-line is already deficient in MRE11 nuclease activity, so inhibiting its exonuclease activity shouldn't confer any additional sensitivity. Furthermore, *7.C* shows that $MRE11^{H129N/-}$ is significantly more sensitive than $MRE11^{+/-}$ to SN-38 treatment alone, due to the lack of functional MRE11 nuclease activity. These effects were not observed in this project, however. *Figure 15.B* shows $MRE11^{+/-}$ to be more sensitive to SN-38 than $MRE11^{H129N/-}$. While no significant differences were observed between the nuclease-dead cells treated with mirin and SN-38 vs. SN-38 alone in *15.D*, suggesting the cells are still MRE11 nuclease-dead but mutations outside of MRE11 are conferring SN-38 resistance, a sensitivity to mirin was observed in the results shown in *7.B*.

This led to the reliability of the nature of the nuclease-dead cell-line to be brought into question, as despite multiple repeats and attempts at improving the assay, $MRE11^{H129N/-}$ did not seem to show the same sensitivity to SN-38 compared to the $MRE11^{+/-}$ cell-line that was seen before, and it was showing sensitivity to mirin that wasn't previously observed. It is possible that the cell-line became contaminated with DT40 wild-type or other cell-lines in use before the start of this project. Alternatively, repeated waking-and-freezing cycles of the cell stocks over a long period of time, and the nature of the MRE11 mutation causing repair defects, may have resulted

in accumulation of mutations that altered the phenotype, or reversed the *MRE11*$^{H129N}$ mutation, resulting in the different results observed.

Cell stocks were kept frozen at -80˚C and/or in liquid nitrogen (described in more detail in *2.1 Cell Culturing and Maintenance*) and to test for potential contamination of the nuclease-dead stocks in use, older stocks were awoken from a range of batches as far back as 2018, with the hopes of including stocks that were potentially in use at the time the previous students' results were generated. However similar effects were observed across all stocks tested, and due to time constraints, this line of investigation was dropped for the time-being. To reliably determine the current nature of the DT40 *MRE11*$^{H129N/-}$ cell-line kept in frozen stocks, samples would need to be sent off for sequencing and this was not within the scope of the current project. Therefore, while some of the inhibitors were screened in the nuclease-dead cell-line, this was no longer pursued as the results could not be relied upon as the nature of the cell-line could not be confidently confirmed. However, once done so, it would be intriguing to test these DNA repair inhibitors in the nuclease-dead cell-line to determine whether any effects observed in combination with mirin were endonuclease-dependent, as this could aid in furthering the understanding of these interactions.

Due to the previously shown effects of MRE11 removing bound topoisomerase from DNA, such as by Hartsuiker, Neale and Carr (2009), and the potential for PARP to affect the detection and signalling of bulky DNA adducts (Gavande *et al.*, 2016), RADAR assays were performed to test for interaction between the two, as shown in *Figure 19.* However, due to time constraints and issues in optimsing this assay, the effects of the combined inhibition of MRE11 exonuclease and PARP on the accumulation of TOPcc with SN-38 treatment was inconclusive, and requires further investigation.

## 4.2 Simultaneous Inhibition of PARP and MRE11 Exonuclease Activities Results in Synthetic Lethality

PARP has long been an attractive target for selective targeting of HR-deficient cancers, with the rationale behind this is the concept of synthetic lethality. Inhibition of PARP would lead to an increase in SSBs, due to PARPs integral role in the recognition and signalling for SSB repair, and the persistence of unrepaired SSBs results in conversion to DSBs during DNA replication (Gavande *et al.*, 2016). In BRCAmut, HR-deficient, or HR-inhibited, cancer cells, the repair of these DSBs would be defective, leading to lethality. It has been shown that in HR-defective cells, PARP inhibition promotes repair by NHEJ, and an intact NHEJ and 53BP1 (p53-binding protein-1) pathway is needed for synthetic lethality to occur (Javle and Curtin, 2011; Patel, Sarkaria and Kaufmann, 2011). Therefore it was hypothesised that inhibiting MRE11 exonuclease activity, a crucial protein in the detection and repair of DSBs by HR, could confer similar synthetic lethality when also inhibiting PARP.

Here, it is shown that inhibition of the exonuclease activities of MRE11 using the small molecule inhibitor mirin, sensitises both chicken DT40 Mre11$^{+/-}$ and human TK6$^{WT/-}$ cell-lines to PARP inhibition by Olaparib analog AZD2461 (see *Figures 13.A & 16.A*). These findings are backed up by previous research on PARP inhibition in HR-defective backgrounds, as previously described. It is important to note that the DT40 cell line is derived from chicken lymphoma cells, and are reported to lack functional p53, while TK6 are derived from human lymphoblast expressing wild-type p53 (Skopek *et al.*, 1978; Little *et al.*, 1995; Ridpath *et al.*, 2011). As synthetic lethality with PARP inhibition in HR-defective backgrounds is reliant on intact 53BP1 pathways, the lack of functional p53 in DT40 may have an effect on this. However, a very clear additive/synthetic lethal effect is still observed in the DT40 mutants, and studies have shown that while 53BP1 is mainly characterised by its interaction with p53, this is largely downstream of many DDR pathways and involved in the activation of apoptosis, and in-fact many of 53BP1's roles in DNA repair by NHEJ are p53-independent (Mirza-Aghazadeh-Attari *et al.*, 2019). DT40 is also a cancer cell-line, whereas TK6 is not, and so differences observed between the two may also be influenced by cancer characteristics such as increased proliferation, reliance on certain DDR pathways *etc.*.

Surprisingly, the MRE11 nuclease-dead DT40 mutant (DT40 *MRE11$^{H129N/-}$)* showed significantly less sensitivity to AZD2461 than the *MRE11$^{+/-}$* (*Figure 14.A*). It was expected here that comparing the *MRE11$^{+/-}$* and *MRE11$^{H129N/-}$*DT40 cell-lines for sensitivity to AZD2461 would be similar to comparing *MRE11$^{+/-}$* with and without the addition of mirin, as in *Figure 13*. Therefore a similar response was expected to be observed, with the mutation of MRE11 nuclease activity showing a similar effect to inhibition by mirin. However, the opposite was observed, and while the reliability of the *MRE11$^{H129N/-}$* genotype/phenotype has been questioned, and discussed previously, alternatively it may suggest a role for MRE11's endonuclease activities in conferring synthetic lethality with PARP inhibition. Mirin is shown to inhibit MRE11 exonuclease activity, whereas the nuclease-dead mutant should have both MRE11 exo- and endonuclease activity knocked out. MRE11 endonuclease activity is what initiates end resection in HR, and is followed by bidirectional resection by MRE11 exonuclease to finally commit the cell to DSB repair by HR (Shibata *et al.*, 2014). Furthermore, it has been shown that inhibition of MRE11 endonuclease prevents repair by HR and instead promotes NHEJ, while exonuclease inhibition results in more general repair defects, and that complete silencing of MRE11 reduces NHEJ efficiency (Rass *et al.*, 2009; Shibata *et al.*, 2014, 2017). This could therefore explain why a lack of any functional MRE11 nuclease activities results in less sensitivity to AZD2461, as an intact NHEJ pathway is needed for PARP inhibition to confer synthetic lethality. In NHEJ, other exonucleases, such as WRN, are commonly utilised over MRE11, therefore mirin does not leave NHEJ significantly compromised by inhibiting MRE11 exonuclease.

4.2.1 Role of the Mechanism of PARP Inhibition on Synthetic Lethal Interactions with Mirin

The rationale behind investigating the effects of different mechanisms of PARP inhibition was the potential for MRE11 to have a role in the removal of trapped PARP from the DNA, in a similar fashion to how MRE11 has been shown to remove TOPcc from the DNA after treatment with topoisomerase poisons (Hartsuiker, Neale and Carr, 2009). With the addition of MRE11 exonuclease inhibitor mirin resulting in significant sensitisation of both DT40 *MRE11*$^{+/-}$ and TK6$^{WT/-}$ cells to PARP inhibition by AZD2461, a potential explanation for this was mirin preventing MRE11 from being able to confer a level of resistance to PARP inhibition by removing trapped PARP, but with some cytotoxic effects still observed without mirin due to the catalytic inhibition also taking place. Therefore Talazoparib (a potent PARP-trapper) and Iniparib (known to have little-to-no PARP-trapping ability) were chosen to help test this theory.

PARP inhibitors were initially designed to block PARP's catalytic activity in the synthesis of pADPr chains, and were created to resemble its by-product nicotinamide (Javle and Curtin, 2011). Most PARP inhibitors compete with PARP's substrate $NAD^+$ for access to the enzymatically active site and prevent the modification and recruitment of DDR proteins, and release of PARP itself from the DNA, by inhibiting pADPr chain formation (Gavande *et al.*, 2016). Olaparib (AZD2281) was the first PARP inhibitor to be granted FDA approval in 2014, as a monotherapy in BRCAmut ovarian cancer (Gavande *et al.*, 2016). However, resistance to Olaparib was shown to be due to overexpression of P-glycoprotein, resulting in the development of AZD2461 to overcome this, and was found to be better tolerated in mice (O'Connor *et al.*, 2016; Reilly *et al.*, 2019). It was shown that, after treatment, PARP was trapped to the DNA by hydrogen bonding at unrepaired SSBs, resulting in replication fork stalling at these protein-DNA adducts, and generation of lethal DSBs (O'Connor *et al.*, 2016). These DSBs would usually be repaired by HR but in HR-defective backgrounds (such as BRCAmut), the more error-prone NHEJ pathway is utilised resulting in mutation accumulation. The prevention of PARPs release from SSBs is referred to as PARP-trapping. Different PARP inhibitors vary in their PARP-trapping abilities, though the extent to which they trap PARP does not correlate with their general cytotoxicity, and their inhibitory effects also rely on their catalytic inhibitory properties also (Murai *et al.*, 2012; Rose *et al.*, 2020). Olaparib, and analog AZD2461, are considered to be relatively weak PARP-trappers, though are shown to confer some cytotoxicity this way, but instead rely mostly on catalytic inhibition of pADPr synthesis by outcompeting $NAD^+$ (Murai *et al.*, 2012). Talazoparib (BMN-673), however, is considered to be a highly potent PARP-trapper, shown to be nearly 100-fold more effective at PARP-trapping than Olaparib (Murai *et al.*, 2014; Giudice *et al.*, 2022). Iniparib (BSI-201), on the other hand, rather than competing with $NAD^+$ to confer catalytic inhibition, is thought to remove zinc from the zinc-finger domains of PARP1, preventing its activation in response to DNA damage and is not known to display any PARP-trapping ability (Mendeleyev *et al.*, 1995; Javle and Curtin, 2011).

However, in DT40 Mre11$^{+/-}$, neither Talazoparib nor Iniparib showed increased sensitivity with MRE11 inhibited by mirin (*Figure 17*). TK6$^{WT/-}$, however, were significantly sensitised to Talazoparib by mirin, while Iniparib again was not (*Figure 18*). Based on this alone, it's difficult to say whether MRE11 may play a role in removing trapped PARP, as both PARP-trappers (AZD2461 and Talazoparib) show increased lethality with mirin in TK6$^{WT/-}$ while Iniparib (non PARP-trapping) does not, however the same is not seen in DT40 *MRE11*$^{+/-}$. It would be interesting to compare the effects of the three PARP inhibitors in DT40 *MRE1*$^{H129N/-}$, to see if endonuclease activity is at all involved, but as previously explained the phenotype of the nuclease-mutant could not be confirmed within the timeframe of the project.

To further this line of investigation, RADAR assays were considered and samples from DT40 Mre11$^{+/-}$ cells treated with the three PARP inhibitors, with and without mirin, were prepared to be loaded onto a membrane with PARP antibodies, however time limitations meant this was unable to be completed. The idea here was that if MRE11 exonuclease activity were to have a role in the removal of trapped PARP after PARP inhibitor treatment, higher concentrations of PARP would be detected by anti-PARP antibodies in samples with Talazoparib and mirin, followed by AZD2461 and mirin, compared to the PARP inhibitors alone, mirin alone, or any samples treated with Iniparib. It was also noted, however, that due to PARP being bound to DNA via hydrogen bonding, not covalent bonds, detection with antibodies in a RADAR assay might not be suitable, and due to time constraints this was not made a priority (Murai *et al.*, 2014; Gavande *et al.*, 2016).

Overall, PARP inhibitors have been well studied and documented in furthering synthetic lethality strategies for cancer treatment, with much evidence suggesting synthetic lethality being conferred by PARP inhibition in HR-deficient backgrounds. This is shown here by the inhibition of key HR protein MRE11 by mirin showing significant sensitising effects in both chicken DT40 Mre11$^{+/-}$ and human TK6$^{WT/-}$ to PARP inhibition by Olaparib analog AZD2461. Investigation into MRE11 playing a role in the removal of trapped PARP was inconclusive, with sensitisation by mirin seen with potent PARP-trapper Talazoparib, while none observed in catalytic PARP inhibitor Iniparib, but this was not consistent across both cell lines, and further investigation if needed to confirm this theory. While PARP mutations are not as commonly found in cancer as with other DDR proteins, such as RAD51, PARP inhibition has been shown to be beneficial in HR-deficient cancers. Mirin, or other similar novel MRE11 inhibitors, could be utilised to confer some, or enhance previously existing, HR-deficiency in cancer treatment by PARP inhibitors.

## 4.3 Simultaneous Inhibition of FEN1 and MRE11 Exonuclease Activities Results in Synthetic Lethality

FEN1 is a flap endonuclease crucial to the processing of Okazaki fragments during DNA replication, the removal of DNA flaps in BER, and in the rescue of stalled replication forks. It has

been previously proposed that, similar to PARP inhibitors, cancers with HR defects would be sensitive to loss or inhibition of FEN1 (Guo *et al.*, 2020). Mutant yeast strains deficient for *Rad27* (yeast FEN1 homolog) show increased dependence on HR, and in fact it was not possible to generate viable double mutants of *rad27* and *mre11* (Symington, 1998). Synthetic lethal interactions have also been described in human colorectal and gastric cancer cell lines with microsatellite instability between FEN1 inhibition and the already mutated MRE11 (Ward, McHugh and Durant, 2017). The same study showed Rad51 foci to accumulate when FEN1 was inhibited, and the induced toxicity was increased in HR-disrupted cells, similar to what is described for PARP inhibitors though interestingly in this case sensitivity to PARP or other DDR protein inhibition was not observed (Ward, McHugh and Durant, 2017). It has also previously been shown that, alongside MRE11 depletion sensitising cells to FEN1 inhibition, as does MRE11 inhibition by mirin (van Pel *et al.*, 2013).

Here it is shown that some sensitivity is shown to FEN1 inhibition by LNT1 in DT40 Mre11$^{+/-}$ cells with the addition of mirin (*Figure 13.D*), but this sensitivity is significantly more pronounced in TK6$^{WT/-}$ (*Figure 16.D*). Interestingly, it is noted by Asagoshi et al. (2010) that FEN1 is seemingly nonessential in the chicken DT40 cell-line, but is much more integral to DDR in human cells. This could explain the differences seen here between the DT40 and TK6 cell-lines, where effects of FEN1 inhibition are still observed in the Mre11$^{+/-}$ and possibly affected by the presence of a single copy of functional MRE11, compared to TK6 where a significant differences in relative cell survival is seen when MRE11 exonuclease activities are inhibited alongside FEN1.

Limited information is available for LNT1 and details on the mechanism of FEN1 inhibition could not be elucidated. Other FEN1 inhibitors with differing modes of inhibition would be interesting to test, such as C8 used by Guo et al. (2020) which is thought to inhibit endonuclease activity by obstructing metal ions vital for catalysis (Tumey *et al.*, 2005).

It's likely the reason for synthetic lethal interaction between MRE11 and FEN1 is similar to the rationale behind PARP-MRE11 synthetic lethality. FEN1 is crucial to a major pathway of SSB repair and is also involved in other activities that when compromised can induce genome instability through the accumulation of SSBs (processing of Okazaki fragments, rescue of stalled replication forks and telomere maintenance; Zheng et al., 2011). The unrepaired SSBs are converted to DSBs, and in cancer cells deficient for MRE11, or when MRE11 activity is inhibited by mirin, the repair of these DSBs is impaired resulting in synthetic lethality.

### 4.4 Inhibition of MRE11 Exonuclease Activities Sensitises DT40 Mre11$^{+/-}$ Cells, but not Human TK6$^{WT/-}$, to ATR Inhibition

ATR can respond to a wide range of DNA damage but is primarily activated by RPA-coated ssDNA and associated with the signalling of SSBs but generally does not directly partake in

DNA repair (Shiotani and Zou, 2009). The MRN complex is involved in the ATR signalling pathway both upstream and downstream of ATR activation. It has been shown that phosphorylation of ATR substrates is decreased in MRE11-deficient cells after treatment with lower doses of UV or HU, but MRN is however dispensable in higher dose treatment (Olson *et al.*, 2007). Furthermore, studies have suggested ATR inhibition may be particularly effective in HR-deficient cancer cells, and confer lethality through mainly replication stress-induced DNA damage (Toledo et al., 2011; Min et al., 2017). Therefore it was hypothesised that a synthetic lethal interaction would be observed when simultaneously inhibiting MRE11 exonuclease activity, and ATR. Although, it was also found that ATR inhibition, at least by inhibitor AZD6738, downregulated other DDR proteins including MRE11, which may limit the usefulness of simultaneously inhibiting MRE11 exonuclease and ATR (Min *et al.*, 2017).

DT40 Mre11$^{+/-}$ cells showed significantly increased sensitisation to ATR inhibition by VE-821 with the addition of mirin (*Figure 13.B*) however the same was not observed in human TK6$^{WT/-}$ (*Figure 16.C*). This may be due to the effectiveness of ATR inhibitors in HR-deficient cancers being dependent on p53 or ERCC1 deficiency and so the addition of a lack of functional p53 in DT40 on top of MRE11 inhibition may explain the differences in sensitivity (Ridpath *et al.*, 2011; Toledo *et al.*, 2011). Furthermore, ATR is a responder primarily to replication stress, though can be activated by other DDR proteins to take part in DNA repair, and as previously mentioned the cytotoxicity of ATR inhibition seems to come from DNA damage induced by replication stress (Min *et al.*, 2017). Cancer cells undergo much more replication stress than non-cancerous cells due to rapid reproduction and mutations of checkpoint and maintenance pathways. The chicken lymphoma DT40 cell-line may experience a greater degree of replication stress than the non-cancer TK6 cell-line, and so therefore is more reliant on a combination of ATR and MRE11 for maintenance of genomic stability. This could be exploited clinically as it is common for cancers to be deficient for p53, and the greater reliance on replication stress in ATR-deficient cancers could mean that treatment with novel MRE11 inhibitors could prove beneficial.

It has been shown previously that inhibition of ATR can increase MRE11- and EXO1-mediated degradation of nascent DNA at PrimPol-induced ssDNA gaps, suggesting a role for ATR in preventing MRE11 exonuclease activity in this context (Leung *et al.*, 2023). ATR also plays a role in the stabilisation of replication forks, as part of responding to replication stress, by stabilising the replisome to prevent it from dissociation (Paulsen and Cimprich, 2007). In the absence of ATR, stalled replication forks are shown to collapse and result in deleterious structures. Nucleases involved in cleaving stalled fork structures, and proteins involved in carrying out HR, are suppressed by ATR at these stalled replication forks, and instead helicase activities that promote fork reversal are promoted (Paulsen and Cimprich, 2007). Therefore a complicated interaction between ATR and MRE11 inhibition may be taking place. On one hand DNA end-resection factors are needed for ATR activation at DSBs and ATR inhibition is

suggested to increase replication stress-induced damage in HR-deficient cells. But, on the other hand, ATR also suppresses HR factors at stalled replication forks, and has specifically been shown to play a role in preventing MRE11-mediated degradation at ssDNA gaps. The former would result in synthetic lethality with simultaneous inhibition of MRE11 and ATR, while the latter would suggest that inhibiting MRE11 may rescue deleterious effects of excess DNA degradation occurring in the absence of ATR. In the DT40 cancer cell-line, the increased presence of replication stress may be overshadowing any benefits of simultaneous inhibition, along with the added lack of functional p53 that would be allowing cells to continue past checkpoints into mitosis and result in cell death that way. Whereas in the non-cancerous TK6 cell-line, a combination of the synthetic lethal and beneficial effects may be almost 'cancelling out' any observable effects.

Showing initial promising results in DT40 *MRE11*$^{+/-}$ using simultaneous ATR and MRE11 exonuclease inhibition, VE-821 was also tested in the DT40 *MRE11*$^{H129N/-}$ nuclease-dead mutant (*Figure 14.C*). Again, the DT40 *MRE11*$^{+/-}$ showed significantly more sensitivity to ATR inhibition by VE-821 than the nuclease-dead mutant. As previously discussed, this may be a result of phenotype-altering mutations in DT40 *MRE11*$^{H129N//-}$, or it may suggest that MRE11 endonuclease activity is in some way involved in the synthetic lethal interactions between ATR and MRE11, although there is no clear evidence-based explanation for this.

## 4.5 RAD51 Inhibition may be Beneficial at Low levels in DT40 Mre11$^{+/-}$ Cancer Cells, but not in Human TK6$^{WT/-}$, Which are Rescued from Lethal RAD51 Inhibition by MRE11 Exonuclease Inhibition

Rad51 is crucial for DSB repair by HR, as it is the protein that facilitates the search and subsequent strand invasion of homologous sequences to allow accurate repair. Rad51 also works closely with MRE11, and the MRN complex as a whole, both in HR and in HR-independent roles at stalled replication forks (Hashimoto *et al.*, 2010). Studies such as Gu et al. (2022) propose and demonstrate potential synthetic lethality in inhibiting Rad51 alongside other DDR proteins, but mostly focus on the damage-signalling kinases such as ATM, ATR and DNA-PK.

With Rad51 inhibitor B02, DT40 *MRE11*$^{+/-}$ showed increased cell survival compared to control conditions at lower doses of B02 alone (≥4μM), after which survival goes back to baseline and so differences are observed with the addition of mirin (*Figure 13.C*). In DT40 *MRE11*$^{H129N/-}$, similarly to effects seen with PARP and ATR inhibitors, significantly less sensitisation was seen compared to that in the *MRE11*$^{+/-}$ (*Figure 14.B*). Although, at lower doses of B02, as before, show some increase to survival of DT40 *MRE11*$^{+/-}$ cells, but not significantly so. It would be interesting to observe if a greater effect can be seen if these doses were expanded upon and included even lower doses as shown in *Figure 13.C*. Rad51 overexpression is commonly

observed in cancer cells, and has been shown to result in hyperrecombination that can confer resistance to some cancer therapies, and aid in cancer cell survival and proliferation (Bonilla *et al.*, 2020). The increase in Rad51 expression and foci observed in many cancers is a result of increased transcription of RAD51, not due to gene amplification, likely due to the increased replication stress of rapidly dividing cells, however a very tight control of RAD51 expression, and HR, is needed to maintain genomic stability (Gavande *et al.*, 2016). RAD51 has been shown to promote the formation of RNA-DNA hybrids that can induce chromosomal instability, *in vivo* (Wahba, Gore and Koshland, 2013). Furthermore, in multiple myeloma Rad51 overexpression resulted in increased HR activity, as expected, but RAD51 inhibition by siRNA resulted in a decrease in mutation acquisition (Shammas *et al.*, 2009). In fact, previous studies have attempted to exploit the overexpression of RAD51 in cancer cells. It has been shown that overexpressed RAD51 can lead to accumulation of genotoxic RAD51 complexes at undamaged DNA sites, and further stimulating RAD51 binding to DNA can induce cancer cell death, showing that despite RAD51's many beneficial roles, slight dysregulation of expression in either direction can be lethal (Mason *et al.*, 2014). Cancer cells are known to benefit from a certain level of dysregulated DNA repair and increased cell proliferation, as this allows for faster generation of genomic changes that may confer beneficial phenotypes. These findings suggest RAD51 is one such target whose expression is commonly altered to allow for higher rates of HR in the hopes of aiding disease progression. As DT40 is a cancer cell line, it likely undergoes higher amounts of replication stress and mutation, potentially through the action of overexpressed RAD51. Assays were performed over 48 hours, containing approximately 6 replication cycles, and so while this is not a time period over which significant mutation and clonal expansion could take place, the presence of hyperactive recombination could be a hinderance to cells that by chance generate deleterious mutations. Therefore, lower doses of RAD51 inhibitor B02 may have increased cell survival from the control baseline by reducing hyperrecombination, and limiting the number of deleterious mutations resulting in cell death. While increasing doses of B02 cross a threshold from keeping hyperrecombination under control, to inhibiting the accurate repair of DNA damage, and so is no longer beneficial to cell survival.

When tested in $TK6^{WT/-}$, inhibition of MRE11 exonuclease activity by mirin seemed to significantly rescue the effects of RAD51 inhibition by B02 (*Figure 16.B*). Again, at lower doses of B02, cell survival increases above the defined "100%" based on a no-drug control, but this time this effect is only observed in cells treated with both B02 and mirin. Consistently across the concentration range tested, the inhibition of MRE11 exonuclease by mirin shows a rescue from the effects of RAD51 inhibition by B02, and to a significant degree. MRE11-mediated resection of nascent DNA ends is needed for the restart of stalled replication forks in order to increase the ssDNA gaps and template available for RAD51-mediated strand-switching and invasion (Hashimoto *et al.*, 2010). However, this process needs to be tightly controlled, as over-resection of DNA ends can be deleterious. At stalled replication forks, unprotected arms act as a signal

and entry point for CtIP-MRE11 with EXO1, or DNA2, mediated degradation (Bonilla *et al.*, 2020). RAD51 has been shown to play a role in protecting nascent DNA from MRE11-mediated degradation and resection during DNA replication and at stalled forks (Costanzo, 2011). This is supported by the accumulation of hyperresected DSBs in RAD51-deficient yeast cells, and has been shown to be, at least in part, a result of MRE11 exonuclease activity through the use of mirin (Hashimoto *et al.*, 2010). Therefore, the results seen here in TK6 may be the effect of Rad51 inhibition by B02 leading to unimpeded DNA end resection by MRE11, proving lethal to the cells. So, when B02 is combined with MRE11 exonuclease inhibitor mirin, the deleterious over-resection of DNA ends in the absence of RAD51 is prevented, and so a rescue effect is observed.

It is important to note, in the processing of results, the control “100% cell survival” condition was based on the absence of the inhibitor being tested – so the control for cells not treated with mirin contained neither B02 nor mirin, while the control for cells treated with mirin contained 10μM mirin (for $TK6^{WT/-}$) but no B02. Therefore, an initial increase in cell survival seen when B02 and mirin are combined could be explained by cell survival being calculated relative to its control. The “100% survival” control here contains mirin at a concentration intended to result in approximately 20% cell lethality, while subsequent data points contain both B02 and mirin, and experiencing the rescue effect of these combined, and so therefore are presenting as higher than 100% cell survival.

It is interesting that drastically different effects of combining MRE11 and RAD51 inhibition are observed between the two cell lines. As mentioned previously, DT40 are a cancer cell line derived from chicken lymphoma, compared to TK6, while immortalised, is a non-cancerous human cell line. The differences observed here between the two may be explained by the increased replication stress, and potential overexpression of RAD51 to compensate in DT40, as a cancer cell line. Inhibiting both MRE11 and RAD51 is essentially only targeting HR, and so it would make sense for this to not necessarily result in any synthetic lethality in an otherwise DNA repair-proficient cell-line as alternative pathways are available. Ignoring the initial increase in DT40 $MRE11^{+/-}$ cell survival at lower B02 doses, no differences are seen in cell survival in the presence or absence of mirin. DT40 is a rapidly proliferating cancer cell-line, and potentially exhibiting an overexpression of RAD51 as previously hypothesised, and also has been shown to lack functional p53, which is activated in response to DNA damage to initiate cell cycle arrest to allow for either DNA repair or apoptosis (Ridpath *et al.*, 2011; Mijit *et al.*, 2020). TK6, on the other hand, is a non-cancer cell-line, and contains functional p53, so the deleterious effects of MRE11-mediated hyper-resection in the absence of RAD51 may be activating checkpoints leading to cell cycle arrest or cell death in TK6, where this checkpoint may be passed in DT40. However the effects of immortalising the TK6 cell line should not be disregarded, and may in fact be experiencing some degree of replication stress. The assay taking place over just the two

days may prevent the potential long-term effects of this on DT40 from being observed, and so a different assay may be recommended to pursue this further, such as by observing more long-term survival with cell subculturing and consistent inhibitor dosing, or sending samples for DNA sequencing to determine if accumulation of mutations is taking place.

## 4.6 Inhibition of MRE11 Exonuclease Activities Rescues DT40 Mre11$^{+/-}$ Cells from Effects of DNA2 Inhibition

DNA2 is a helicase and nuclease with crucial roles in responding to DNA replication stress through the processing of Okazaki fragments, DNA end resection in HR, and in the stabilisation of stalled replication forks (Liu *et al.*, 2016; Pawłowska, Szczepanska and Blasiak, 2017; Zheng *et al.*, 2020). The small molecule inhibitor C5 has been shown to bind DNA2's helicase domain and successfully inhibit both its nuclease and helicase activities, resulting in reduction of both DNA end resection and restart of stalled forks, and sensitising cancer cells to chemotherapeutic agents (Liu *et al.*, 2016). DNA2 works very closely alongside MRE11, which functions both upstream and downstream of DNA2's role in end resection. DSB repair by HR is initiated by resection of DNA ends to generate 3' ssDNA promoted by CtIP and MRE11, followed by long-range resection by DNA2 and EXO1, which leads to the inhibition of NHEJ and repair by HR proceeds (Hoa *et al.*, 2015; Bonilla *et al.*, 2020).

Based on the current literature, it was hypothesised that there would either be a synthetic lethal effect, or no additional effects observed when simultaneously inhibiting MRE11 and DNA2. MRE11, activated by CtIP, nicks DNA near the DSB, allowing EXO1 and DNA2-BLM to resect DNA 5' to 3' away from the nick, while MRE11 resects 3' to 5' towards the DNA ends (Ferrari *et al.*, 2015). Recent studies, such as Tatsukawa et al. (2024), describe DNA2 to be the major nuclease involved in long-range resection of DSBs in *Xenopus* extracts, with end resection by DNA2 being dependent on MRE11 activity. They further showed that DNA2 depletion inhibited DSB end resection to a greater extent than MRE11/RAD9-double depletion, further showing a far greater reliance on DNA2 than MRE11 for resection prior to HR. This is supported by Oh and Symington (2018) who show that in the absence of Mre11 nuclease, the resection of DSBs occurs mainly through Sgs1-Dna2 activity (BLM/WRN and DNA2 homologs in *Saccharomyces cerevisiae* / budding yeast), and Paull (2010) discuss Mre11 nuclease-deficient cells are sensitised to IR when Dna2 nuclease is also depleted. Shim et al. (2010a) further explain that, while the Mre11 nuclease activities are not required for efficient loading of Dna2 and Exo1, it is needed for resection to take place in the absence of these resection enzyme. Exo1 and Dna2 have been shown to be unable to resect DNA ends bound with the Ku heterodimer, and Mre11 nuclease activity is needed in order expose ssDNA overhangs needed for HR initiation (Ferrari *et al.*, 2015; Oh and Symington, 2018). MRN has been shown to recruit Exo1 and Dna2 to DSBs with non-catalytic activities, with mutations removing Mre11 nuclease activity conferring mild defects in DSB resection while complete Mre11 knockouts show more severely defective

end resection, and absence of recruited Exo1 and Dna2 (Mimitou and Symington, 2009; Shim *et al.*, 2010b). These defects are then shown to be rescued through the deletion of Ku, suggesting Ku prevents access to DNA ends, and requires Mre11 nuclease activity for its removal (Shim *et al.*, 2010b). However, it is likely that Mre11 endonuclease activity is what is required for removal of Ku, with studies such as Paull (2018) demonstrating initial endonucleolytic excision of 5' strand next to the DSB, followed by 3' to 5' exonucleolytic degradation towards break ends to enlarge and allow penetration of EXO1 and DNA2 for more extensive resection. This shows that while MRE11 and DNA2 share a close relationship and common functions, they are not completely redundant. In absence of Mre11 nuclease activity, DSB resection for efficient repair by HR becomes more reliant on Dna2 and Exo1, largely due to persistent Ku bound to DSB ends preventing further resection and HR factor recruitment. Mre11 has also been shown to be involved in alt-NHEJ, through resecting DNA ends to expose microhomologies (McVey and Lee, 2008). Together with MRE11s roles in NHEJ, and the greater dependence on DNA2 that may occur when inhibiting MRE11 nuclease activity, it's possible that inhibiting both MRE11 and DNA2 could result in synthetic lethality.

In fact, in the absence of DNA2, MRE11 exonuclease activity was deleterious in DT40 *MRE11*$^{+/-}$ cells (*Figure 13.E*), which was an unexpected result. As previously mentioned, it is Mre11 endonuclease activity, rather than exonuclease, that was implicated in the removal of Ku from DSB ends, however this would more likely result in a lack of sensitisation when inhibiting MRE11 exonuclease, rather than a rescue effect. Folly-Kossi et al. (2023) report that DNA2 is often overexpressed in cancers containing mutant p53, and DT40 is a cancer cell-line lacking functional p53 so there is the potential for overexpression of DNA2 in DT40 resulting in over-resection, but that also wouldn't necessarily result in a rescue effect when inhibiting both DNA2 and MRE11.

Weinstock et al. (2006) show that over-resection of DSBs can result in the excessive initiation of HR, resulting in increased toxic DNA intermediates and rearrangements and, paradoxically, dysregulated DSB resection could in fact promote the more error-prone alt-NHEJ pathway. The persistent binding of Mre11 at DSBs can be a barrier to the end resection required for HR, which could aid in alleviating stress in the case of excessive HR initiation, however this can be by-passed by Sgs1-Dna2 resection in some cases (Bernstein *et al.*, 2013). In this context, DNA2 could be allowing the by-pass of MRE11 blocks at DNA ends that are preventing excessive HR, especially if DNA2 is overexpressed in DT40 as hypothesised above, so its inhibition would be preventing this by-pass from occurring. However the addition of MRE11 exonuclease inhibition here still wouldn't result in the observed rescue effect.

Interestingly, Shibata et al. (2014) found that PFM39 (an MRE11 exonuclease inhibitor) rescued repair defects in EXO1/BLM-depleted cells, and it was suggested and experimentally shown that the combined inhibition of these exonuclease activities promoted DSB repair by NHEJ.

Shibata et al. suggest that when either MRE11 exonuclease or EXO1/BLM activities are lost, neither NHEJ or HR can occur due to their requirement for generating ssDNA gaps and it is this extension of ssDNA that prevents repair by NHEJ, but when both types of exonuclease activities are lost, the nick created by MRE11 endonuclease activity is not enlarged at all, and progression to NHEJ is allowed. This, therefore, could explain the result observed here as, while Shibata et al. do not reference DNA2, studies occurring since show it to be the major factor in DSB end resection and is often in complex with BLM (Tatsukawa *et al.*, 2024). DNA2 inhibition by C5 in DT40 *MRE11*$^{+/-}$ cells likely impaired the cells' ability to enlarge the ssDNA gaps but not significantly enough to allow the progression to NHEJ as other exonucleases were still present, but resulted in prevention of both HR and NHEJ due to HR factors being unable to localise at the site. However, when combining this with MRE11 exonuclease inhibition, the extension of the ssDNA gap created by MRE11 endonuclease is completely inhibited and so repair of DNA damage is allowed to occur by NHEJ, resulting in a rescue from lethality. This could be tested in future investigations by treating cells with an NHEJ inhibitor to determine whether this causes the rescue effect to no longer be present. Overall, while an unexpected result, the rescue from the effects of DNA2 inhibition by the addition of inhibiting MRE11 exonuclease activity can be explained by the currently literature.

## 4.7 Potential Rescue of DT40 Mre11$^{+/-}$ Cells from Effects of DNA-PK Inhibition by Inhibition of MRE11 Exonuclease Activities

DNA-PK is a DDR protein involved in the early detection of DSBs, and their repair by NHEJ. It has been shown that HR activity increases in cells defective in DNA-PK, and Ku70 (part of DNA-PK, alongside DNA-PKcs and Ku80) has been proposed as an important component in the choice between DSB repair pathways, with its presence promoting repair by NHEJ and absence allowing recruitment of MRE11 and other HR proteins (Goedecke *et al.*, 1999; Burma and Chen, 2004). Synthetic lethal interactions have also been identified between DNA-PKcs and many proteins involved in repair by HR (Goodwin and Knudsen, 2014). Based on this, it was expected that MRE11 inhibition would lead to sensitisation of DT40 cells to DNA-PK inhibition, due to interference in both major DSB repair pathways.

However, no significant differences were observed when combining NU7441 (DNA-PK inhibitor) with mirin in DT40 Mre11$^{+/-}$ cells in this case (*Figure 13.F*). In general, inhibiting both DNA-PK and MRE11 exonuclease activity does instead show increased cell survival compared to inhibiting DNA-PK alone, suggesting a potential rescue effect, but the differences were not considered significant enough to conclude this. Similarly to the effects seen with Rad51 inhibition, an initial increase in cell survival above 100% is seen at lower NU7441 concentrations when combined with mirin, after which a steady decline is observed, with a consistent difference in cell survival between cells treated with mirin and without.

Deshpande et al. (2020) describe the Ku heterodimer to have a relatively high affinity for DNA ends so, along with the generally high concentrations of DNA-PK in higher eukaryotic cells, DSBs are likely rapidly bound by DNA-PK upon formation. The removal of DNA-PK from DNA ends is facilitated either by Ku being phosphorylated by DNA-PKcs to reduce its binding affinity, or endonuclease activity by MRE11. They also show that MRE11-mediated dsDNA cleavage is promoted by DNA-PK being stably bound to DNA ends which ensures ends undergoing successful end-joining are not cleaved by MRE11, as DNA-PKcs would dissociate from the DNA in the event of successful end-joining. Using the DNA-PK inhibitor NU7441, Deshpande et al. found increased efficiency of MRE11-mediated DNA cleavage, with the DNA-PK inhibitor preventing the kinase activities of DNA-PKcs, and therefore not allowing for autophosphorylation and dissociation from DNA ends. However, it was specifically MRE11 endonuclease activity, tested using endonuclease inhibitors PFM01 and 03, that acts on DNA-PK-bound DNA ends, with exonuclease inhibitor PFM39 showing significantly less sensitisation.

Therefore, DNA-PK inhibition by NU7441 leads to persistent DNA-PK stably bound to DSBs, promoting cleavage by MRE11 endonuclease activity to remove bound DNA-PK and initiate end resection by MRE11 exonuclease activity, creating 3' ssDNA needed for initiating the homology search (Shibata *et al.*, 2017; Deshpande *et al.*, 2020). This could result in a similar effect as seen with inhibiting both RAD51 and MRE11, where inhibiting DNA-PK alone results in an increase in DNA end resection by MRE11, promoting genomic instability, and so the addition of mirin reducing this dysregulated resection and confers a rescue effect. However the results shown in *Figure 13.F* did not show a significant enough difference to conclude a rescue effect.

On the other hand, Tavecchio et al. (2012), also hypothesising that defective DNA-PK would result in more reliance on HR for DSB repair, instead found that IR-induced Rad51 foci were decreased by treatment with NU7441, and they suggest a potential role of DNA-PK kinase activity in HR. However, this could instead be due to NU7441 resulting in DNA-PK remaining stably bound to DNA, as described by Deshpande et al. (2020), as Ku does not dissociate from DNA ends and its continued presence instead promotes NHEJ protein recruitment, as discussed by Goedecke et al. (1999). However, if DNA-PK kinase activity were to play a role in HR alongside MRE11, this could aid in explaining the lack of significant differences seen here between survival of cells treated with both mirin and NU7441, and NU7441 alone.

Additionally, both DNA-PKcs and Ku have been implicated in telomere capping, by masking chromosome ends to prevent recognition as DSBs, and it has been shown that DNA-PK deficiency can lead to enhanced telomere degradation (Burma and Chen, 2004). It has also been shown that Mre11 has functions in telomere length maintenance, with MRE11 nuclease activities shown to remove 3' telomeric overhangs and promote chromosome fusions in the absence of TRF2 (Dupré *et al.*, 2008; Deng *et al.*, 2009). Moreover, a recently published study by Audry et al. (2024) shows MRE11 nuclease activity to be directly involved in telomere

degradation when not properly protected. It is possible that DNA-PK inhibition may leave telomeric DNA ends unprotected, and allow for MRE11-mediated degradation. This would explain the slight rescue from effects of DNA-PK inhibition seen when inhibiting MRE11 exonuclease activity, as DNA-PK inhibition alone may be allowing for increased telomeric degradation which is prevented when MRE11 exonuclease is inhibited.

Overall, no statistically significant differences were observed between DT40 *MRE11*$^{+/-}$ cells treated with DNA-PK inhibitor NU7441 alone compared to those treated with both NU7441 and mirin. While there is significant evidence to support a rescue effect, as well as to the contrary with the potential for synthetic lethal interactions to take place, it is perhaps the combination of all these described effects that result in there being no overall significant increase or decrease in sensitisation, or other confounding factors may be influencing results. Alternatively, biological repeats of the assay could result in the observation of a significant difference, and could be further investigated in the future.

## 4.8 Inhibition of MRE11 Exonuclease Activities Does Not Sensitise DT40 Mre11$^{+/-}$ Cells to WRN Inhibition

WRN is a helicase that functions in DSB repair by NHEJ and HR, with both 3'-5' exonuclease and helicase activity, and also likely functions in SSB repair by BER, telomere capping, and in restoring replication forks (Chen *et al.*, 2003; Ahn *et al.*, 2004; Franchitto and Pichierri, 2004; Multani and Chang, 2007). Most notably, WRN is essential in the choice between the c-NHEJ and alt-NHEJ, with WRN-deficient cells shown to be prone to the more mutagenic alt-NHEJ pathway (Shamanna *et al.*, 2016). It was hypothesised that cells would be sensitised to WRN inhibition when combined with inhibiting Mre11 exonuclease activity, based on previous studies suggesting a synergistic interaction between the two. WRN inhibition by NSC617145 has been shown to result in increased RAD51 foci after treatment with mitomycin C, suggesting an increased dependence on HR for DSB repair (Aggarwal *et al.*, 2013). This is likely due to WRN's close ties to functional NHEJ, with characterised interactions with Ku and DNA-PKcs, and extensive deletion of non-homologous DNA ends being observed in WS cells, shown to be rescued by the expression of WT WRN (Chen *et al.*, 2003). Therefore, targeting MRE11 function could prove to be synthetically lethal due to significant interference occurring in both major DSB repair pathways. Furthermore, it has been suggested that WRN plays a role in the repair of SSBs by BER, with interactions with Pol β to stimulate strand displacement synthesis, and WRN helicase activity shown to unwind a range of SSB BER intermediates (Ahn *et al.*, 2004). Similar to the synthetical lethality of inhibiting PARP in HR-deficient cancers, limiting the ability to repair SSBs leads to an accumulation of lethal DSBs, which MRE11 plays an integral role in the repair of. Therefore, a combination of WRN's roles in SSB repair and DSB repair by NHEJ suggested that inhibiting its function alongside inhibiting MRE11 would prove synthetically lethal by

increasing SSBs development into DSBs, and then preventing their repair by both NHEJ and HR.

It was also hypothesised, however, that the opposite effect was also possible, as there was evidence that inhibiting MRE11 exonuclease activity could in fact rescue cells from the effects of WRN inhibition, due to WRNs roles in protecting DNA from nuclease degradation. WRN has been shown to inhibit MRE11 and CtIP recruitment in order to protect telomeres from nuclease degradation (Shamanna *et al.*, 2016). WS is characterised by the rapid onset of cell senescence and premature ageing, which is linked to defects in telomere maintenance caused by WRN-deficiency (Multani and Chang, 2007). Together with MRE11's known ability to degrade telomeres in the absence of proper DNA-end protection, this could suggest that in WRN inhibition could result in increased telomere shortening which could be prevented by inhibiting MRE11. This is further backed up by the absence of WRN exonuclease activities resulting in excessive MRE11- and EXO1-mediated degradation of nascent DNA at stalled replication forks induced by camptothecin treatment, with replication tract length being restored by treating the cells with mirin (Iannascoli *et al.*, 2015). WRN also has non-catalytic functions in preventing DNA degradation by MRE11, such as through association with NBS1 and promoting RAD51 binding which prevents ends from MRE11 resection (Iannascoli *et al.*, 2015). Therefore an alternative hypothesis would have been, in the case that the simultaneous knockout of BER repair of SSBs and DSB repair by NHEJ and HR did not occur when inhibiting WRN and MRE11, that a similar effect as seen in inhibiting RAD51 would occur, where inhibiting MRE11 exonuclease activity rescued the over-resection or degradation potentially seen with WRN inhibition.

However, no significant differences were observed between DT40 Mre11$^{+/-}$ cells treated with both NSC617145 and mirin, compared to those treated with NSC617145 alone (*Figure 13.G*). So MRE11 inhibition neither sensitised cells to, or rescued cells from the effects of WRN inhibition, which was a surprising result. There is evidence suggesting that WRN and MRE11 function together in a range of repair pathways, which in isolation of other evidence would explain there being no additional effects when inhibiting them both. For example, WRN and MRE11 have been shown to form a complex and function together at stalled replication forks, with the absence of either resulting in higher instances of chromosome rearrangements (Franchitto and Pichierri, 2004). Mutations that alter the function of WRN or MRE11 are also shown to increase DNA breaks during replication, and in WS cells these DNA breaks were not further increased by RNAi MRE11 silencing, suggesting WRN and MRE11 acting within the same pathway in this context (Franchitto and Pichierri, 2004). However, this explanation is not in isolation of other evidence, as previously outlined, and therefore cannot completely explain the result seen here.

Aggarwal et al. (2013) report NSC617145 to negatively affect cell proliferation, and confer its genotoxicity through the induction of WRN-dependent DNA damage. While Aggarwal et al. show NSC617145 to inhibit WRN helicase activity dose-dependently, they report there to be no detectable effects on WRN exonuclease activity, which may be contributing to the lack to expected results in this case.

Datta et al. (2021) clearly outline the independent effects of WRN helicase and exonuclease in the context of telomere maintenance and interactions with MRE11. Using DNA fibre experiments, Datta et al. show that loss of inhibition of WRN helicase activity is what compromises replication fork restart in BRCA2-mutated cells, and that WRN exonuclease is not required there. However, they observed both WRN helicase and exonuclease activities aided in rescuing nascent strand degradation in the BRCA-mutated cancer cells, and WRN depletion resulted in enhanced degradation, showing a clear role of WRN in preventing over-degradation at unprotected replication forks. They then saw that inhibition of MRE11 rescued this over-degradation of nascent DNA that results from WRN helicase inhibition, and found reduced nucleolytic cleavage of synthetic reversed replication forks by MRE11 nuclease activities when in the presence of WRN helicase *in vitro*. These findings by Datta et al., especially as they make use of NSC617145 as their WRN inhibitor, do suggest a greater influence of WRN helicase activities in the context of preventing DNA degradation by MRE11, rather than exonuclease activities, and so does not support the line of thinking that NSC617145's lack of effect on WRN exonuclease is the reason behind the unexpected results.

Intriguingly, Datta et al. found that inhibition of WRN using NSC617145 resulted in destabilisation of replication forks to a higher degree than that seen when depleting WRN *in vivo*, suggesting addition effects of NSC617145 than just the inhibition of WRN, or exonuclease activities of WRN played a bigger role than was being observed. They showed NSC617145 was in fact a specific WRN helicase inhibitor, and that it binds WRN and exerts its genotoxicity WRN-dependently, however what was observed was the persistence of WRN bound to chromatin in cells treated with NSC617145. This suggests that the inhibitory effects on WRN are being enacted through trapping WRN to the DNA, similarly to the PARP-trapping mechanism utilised by many PARP inhibitors. Datta et al. suggest that the increase in fork instability after NSC617145 treatment compared to WRN knockouts is likely due to the combination of WRN helicase inhibition and the accumulation of WRN-DNA complexes impeding replication fork progression. They tested this experimentally by showing the slowed replication forks induced by NSC617145 were rescued when depleting WRN. Furthermore, they saw that these NSC617145-induced stalled forks underwent increased MRE11-mediated degradation. While this still implies a rescue effect should be observed when inhibiting MRE11 alongside WRN, the increase in replication stress would result in the majority of DNA breaks induced by WRN inhibition occurring during replication, resulting in a higher likelihood that they'd be repaired by

HR rather than NHEJ, in which case inhibiting MRE11 could prove synthetically lethal. A combination of a rescue and synthetic lethal effect could result in the lack of either observed here. Moreover, the mechanism of WRN inhibition by NSC617145 being to trap it to the DNA could therefore inhibit the non-catalytic activities of WRN also, such as the structural roles it plays in repair by HR, though this doesn't seem to be discussed by Datta et al. (Chen *et al.*, 2003).

Overall, WRN and MRE11 seem to have a complicated relationship, from working within the same pathways, functioning to both activate and inhibit each other, and a range of evidence supports both their inhibition conferring synthetic lethality and also the potential for rescue effects. At lot of the studies discussing the interactions of MRE11 and WRN may be doing so in the context of the addition or existence DNA damage, such as by treating cells with topoisomerase poison camptothecin, or using BRCA2-mutated cancer cell-lines (Iannascoli *et al.*, 2015; Datta *et al.*, 2021). In this project, DNA-damaging agents are not utilised, though this was going to be the next stage of investigation. It would be interesting to further investigate the effects of simultaneous WRN and MRE11 inhibition across different cell-lines, and in the presence of DNA-damage agents such as camptothecin derivative SN-38, and also see if different WRN inhibitors utilising different mechanisms would confer different effects to those observed here.

## 4.9 Inhibition of MRE11 Exonuclease Activities Does Not Sensitise DT40 Mre11$^{+/-}$ Cells to ATM Inhibition

ATM is a key protein in the activation of cell cycle checkpoints in the response to DSBs, ATM deficiency is known to result in ataxia-telangiectasia (A-T), characterised by chromosomal instability and predisposition to cancer (Lavin and Kozlov, 2007; Jin and D. Y. Oh, 2019). Due to the close functional ties between ATM and MRE11, as well as them both functioning in the same pathways, such as the recognition of DSBs and initiation of their repair by HR, it was not hypothesised that a strong, if any, synthetic lethal interaction would be observed with their combined inhibition.

As expected, no significant differences were found between DT40 Mre11$^{+/-}$ cells treated with both mirin and ATM inhibitor KU55933 compared to those treated with KU55933 alone (*Figure 13.H*).

Many studies have shown that proper and efficient activation of ATM in response to DSBs is dependent on functional MRN, with MRE11 and RAD50 shown to recruit ATM to DNA ends, and the complete MRN complex being required to stimulate ATM's kinase activity (Uziel *et al.*, 2003; Lavin and Kozlov, 2007). Furthermore, both NBS1 and MRE11 have been shown to be phosphorylated by ATM in response to DSBs, with this phosphorylation crucial for their roles in the activation of cell cycle checkpoints and allowing for effective DSB repair by HR (Uziel et al.,

2003). KU55933 was the first selective ATM inhibitor discovered, and has been shown to significantly impair HR, observed through the reduction of RAD51 foci in human melanoma cells (Jin and D. Y. Oh, 2019). While KU55933 is unfortunately not approved for use *in vivo* due to its high lipophilicity, analogs such as KU60019 have since been produced that show better pharmacokinetics and have proceeded to clinical trials for kidney cancers (ClinicalTrials.gov ID: NCT03571438;Jin and Oh, 2019).

While no synergistic effects were observed by combining MRE11 and ATM inhibition, this was an expected result, and it backed up by the literature in the field. Furthermore, this result aids in providing further confidence that results obtained with other DDR protein inhibitors, where the literature is less clear on what result is to be expected.

## 5 Conclusions and Future Directions

The aim of this project was to investigate the potential for synthetic lethality, or otherwise interacting effects, of a range of DNA repair proteins with MRE11 exonuclease activity to be exploited by novel MRE11 inhibitors currently in development, and in order to inform potential routes for improving personalised cancer therapy. In this project, synthetic lethal interactions were shown to be present between PARP, FEN1 and ATR with MRE11 exonuclease in both DT40 Mre11$^{+/-}$ cells, with this synthetic lethality also observed for PARP and FEN1 in TK6$^{WT/-}$, but not for ATR. However, MRE11 exonuclease inhibition seems to rescue the effects of DNA2 inhibition in DT40 Mre11$^{+/-}$, and RAD51 inhibition in TK6$^{WT/-}$. No effects were shown with the addition of MRE11 exonuclease inhibition for DNA-PK, WRN and ATM in DT40 Mre11$^{+/-}$.

MRE11 exonuclease inhibition having no addition effect on ATM inhibition was largely an expected result, with ATM's activation being largely dependent on MRE11, and ATM itself in turn playing a role in activating MRE11 activities downstream, so their combined inhibition has little additive effect and leaves alternative pathways available for repair of DNA damage. The lack of effect observed with MRE11 and WRN inhibition together was, however, unexpected. There was potential for inhibiting both MRE11 and WRN to prove synthetically lethal due an increased dependence on HR with WRN inhibition, due to its role in NHEJ and SSB repair by BER. Alternatively MRE11 exonuclease inhibition could have rescued cells from increased telomere degradation in the absence of WRN. Potentially the combination of both the lethal and rescuing effects resulted in the lack of either being observed, though an alternative, or additional, explanation is that the WRN inhibitor NSC617145 has been shown to trap WRN to the DNA, so while this may prevent the helicase and structural functions of WRN, it may still allow for repair of DNA damage by WRNs exonuclease activities. DNA-PK was another that was hypothesised to be synthetically lethal with MRE11, as it was thought that inhibiting both would effectively disrupt both HR and NHEJ repair of DSBs. However, so significant effects were observed, and if anything there was minor rescue of the effects of DNA-PK inhibition by inhibiting MRE11

exonuclease. It is thought that this may be due to the inhibition of DNA-PK's kinase activities also means it cannot autophosphorylate, and therefore cannot dissociate from DNA. DNA-PK being stably bound to DSBs promotes MRE11 endonuclease cleavage, which allows for more extensive end resection by MRE11 and other exonucleases. Furthermore, DNA-PK plays an important role in telomere capping and protection of telomeres from nuclease degradation, so in both contexts the inhibition of DNA-PK could be resulting in over-resection by MRE11, and so inhibiting its exonuclease activities could be conferring some rescue effects, which combined with any potential synthetic lethality could explain the lack of significant effects observed.

A significant rescue effect by MRE11 exonuclease inhibition was observed when inhibiting DNA2. MRE11 and DNA2 are closely associated and share similar functions, however not redundantly. MRE11s roles in NHEJ and the greater dependences on DNA2 observed when MRE11 nuclease in inhibited or depleted suggested the potential for synthetic lethality. However, it may be possible that by partially impairing DSB end resection through the inhibition of DNA2, neither HR nor NHEJ could proceed to repair DNA damage. When MRE11 exonuclease inhibition is included, this completely prevents DSB resection, and allows repair by NHEJ to take place, resulting in a rescuing effect. This could be further investigated in the future with the addition of a NHEJ inhibitor to determine whether the rescue effect persists or not in this context. RAD51 also showed an interesting interaction with MRE11. RAD51 overexpression is a common factor in many cancers, leading to hyperrecombination and the promotion of RNA-DNA hybrids that can increase the mutational burden. The chicken DT40 lymphoma cell-line may be overexpressing RAD51 and so low levels of RAD51 inhibition would prove beneficial to cell survival. This is not the case in the non-cancer cell-line TK6, where a rescue effect is observed, likely due to the lack of RAD51 leading to unimpeded DSB resection by MRE11 exonuclease, so inhibiting this activity alleviated this effect. Similarly, DT40s increased replication stress due to its nature as a cancer cell-line likely makes it more reliant to the mechanisms involved in responding to and resolving replication-associated stress, such as ATR, whereas TK6 would be less dependent on these functions. A sensitisation to ATR inhibition by MRE11 exonuclease inhibition was observed in DT40 $Mre11^{+/-}$, suggesting synthetic lethality, however the same was not observed in $TK6^{WT/-}$. Resection factors are needed for ATR activation to DSBs, and ATR inhibition has been shown to increase replication stress-induced damage in HR-deficient cells, however ATR also plays a role in suppressing HR factors at stalled replication forks, and prevents MRE11-mediated degradation of ssDNA to maintain genomic instability. These complicated interactions between ATR and MRE11 may be the cause of there being not differences observed when inhibiting ATR alone or with MRE11 inhibition in TK6, however in DT40 the increased replication stress and lack of p53 could be resulting in cells containing damaged DNA being allowed passage into mitosis, eventually leading to cell death.

Less complex interactions are reported between FEN1 and MRE11. FEN1 is primarily involved in the repair of SSBs, and previous studies have suggested HR-defective cancers to be sensitive to FEN1 inhibition. This project further showed this, with FEN1 and MRE11 exonuclease inhibition proving synthetically lethal. The increased sensitivity of human TK6 cells to this combined inhibition over DT40 could be due to FEN1 being considered non-essential in chicken DT40 cells, but is found to be essential in the human DDR.

Finally, a major focus of this project was on the combination of MRE11 exonuclease and PARP inhibition, and how the different mechanisms of inhibiting PARP may alter the potential for synthetic lethality. Synthetic lethal interaction of MRE11 and PARP was observed in both DT40 *MRE11*$^{+/-}$ and TK6$^{WT/-}$ when using PARP inhibitor AZD2461. These findings support previous studies and clinical trials in the field, investigating the synthetic lethality of PARP inhibition in HR-defective backgrounds. However, the DT40 *MRE1*$^{H129N/-}$ nuclease-dead mutant did not show additional sensitivity, suggesting a potential role of MRE11 endonuclease activity and NHEJ in conferring synthetic lethality with PARP inhibition, however this was inconclusive. Based on the highly significant sensitisation to PARP inhibition observed with MRE11 exonuclease inhibition, and previous studies demonstrating MRE11's ability to remove blockages from the DNA, such as topoisomerases and nucleoside analogs, it was hypothesised that MRE11 may have the ability to remove trapped PARP, therefore proving synthetically lethal with their combined inhibition. Talazoparib, a significantly more potent PARP-trapper than Olaparib analog AZD2461, showed increased sensitivity with the addition of MRE11 exonuclease inhibition in TK6$^{WT/-}$, while Iniparib, which is not known for any PARP-trapping abilities, showed no sensitisation with MRE11 exonuclease inhibition in either cell-line tested. Unfortunately, DT40 *MRE11*$^{+/-}$ was not sensitised to Talazoparib by MRE11 inhibition, otherwise there would be strong case for MRE11 exonuclease in removing trapped PARP from DNA.

Beside further investigation already mentioned, inhibitors shown in this project to confer sensitisation or rescue by MRE11 exonuclease inhibition could be further tested in other human cell-lines, specifically a range of cancer cell-lines. The human cell-line utilised in this project was not derived from cancer, and so does not contain many of the characteristics that might influence synthetic genetic interactions between the DDR proteins tested. Therefore, to further determine their usefulness in combination with novel MRE11 inhibitors clinically, the effects in cancerous, or even HR-defective, contexts would be needed. Additionally, as partially investigated with PARP inhibitors, inhibitors of these DDR proteins with differing mechanisms of inhibition could be tested in a similar way, in order to determine if any synthetic genetic interactions are due to inhibition of the protein itself, of influenced by the mechanism of inhibition. Particularly with MRE11's previously displayed roles in the removal of trapped proteins from the DNA, inhibitors that utilise a trapping-mechanism may interact differently with MRE11 exonuclease inhibition than those that confer inhibition differently. Furthermore, the use

of the DT40 *MRE11*$^{H129N/-}$ nuclease-dead cell-line, or alternatively MRE11 endonuclease inhibitors, to determine if observed effects require MRE11 endonuclease activity or not, would be an interesting line of further investigation. The novel MRE11 inhibitors currently in development could also be tested alongside the DDR inhibitors tested here, as their mechanism of inhibiting MRE11 could differ to that of mirin, and contain more clinically relevant pharmacokinetics.

Overall, the findings in this project provide evidence of synthetic genetic interaction between MRE11 exonuclease and a range of DNA repair proteins that could help inform more effective personalised cancer therapy, through the use of novel MRE11 inhibitors in cancers expressing defects in these DNA repair proteins.

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