

**Identification and characterisation
of novel, natural product
inhibitors of the poly (ADP-ribose)
polymerase (PARP) enzyme for
anti-cancer and other therapeutic
applications**

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Abstract

Interest in the enzyme poly (ADP-ribose) polymerase (PARP) has grown significantly in recent years, especially in relation to the potential to harness for therapeutics development its multiple roles in detecting and repairing DNA damage and in non-oncological scenarios such as its mediation of neuronal death in neurodegeneration. Human cancer cells with a defective homologous recombination pathway due to deleterious mutations in their tumour suppressor genes such as *BRCA1* and *BRCA2*, and which often rely on PARP for their single-strand DNA repair, can be selectively targeted through pharmacological inhibition of PARP, which results in their death through synthetic lethality. Currently, most PARP inhibitors (PARPi) are synthetic and designed with the benzamide motif to mimic the nicotinamide moiety of NAD^+ , the substrate for PARP, but which is also a substrate for some related enzymes.

Only four drugs are currently approved as PARPi and used in the clinic: olaparib, rucaparib, niraparib and talazoparib. They are competitive inhibitors of NAD^+ and, while relatively safe, they have drawbacks, including high cost and the development of resistance to them by cancer cells. It is, therefore, important to identify novel PARPi scaffolds that can generate a new pipeline of PARPi drugs, especially those that are not competitive inhibitors of NAD^+ . As natural products are a proven, veritable source of new drugs, with significant diversity for exploring chemical space, the aim of this PhD project was to identify and characterise new, natural product PARPi.

A cell-based model assay was optimised for screening and characterisation, in which PARP-mediated cell death was induced by the alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), such that an agent with the ability to protect against the PARP1-mediated cell death was considered a PARPi. Morphological changes following the treatments were also captured using brightfield microscopy.

The model assay was validated by using it to assess the relative potencies of olaparib, a clinically approved PARPi, and its fluorescent derivative, PARPi-FL. The results showed that PARPi-FL ($EC_{50} = 55.7$ nM) was less potent than olaparib ($EC_{50} = 23.1$ nM) (as assessed by the MTT assay), which was corroborated by the results of a cell-free (biochemical) assay with IC_{50} values of 17.7 and 5.4 nM, respectively. The efficacies and potencies of the four clinically approved PARPi were also determined. The EC_{50} values (nM) revealed the following rank order of potencies: talazoparib (3.6) > rucaparib (8.4) > niraparib (17.1) > olaparib (23.1), which was consistent with the potency rankings of these PARPi in previous studies, thus validating the assay. When employed in a time-course experiment, the assay uniquely revealed that olaparib and DPQ could continue to significantly protect against PARP1-mediated cell death even when added up to 12 h and 8 h, respectively, after initiation of the MNNG insult.

The assay was utilised to screen a small commercial library of 502 natural compounds, as well as a small, customised set of compounds, for PARP-inhibiting activity. While the few original hits did not sustain activity in re-screening, the work inspired a recourse to 4'-methoxyflavone (4MF) and 3',4'-dimethoxyflavone (DMF), two flavones that have been previously found to inhibit parthanatos. These two

natural products were further validated in the model assay, and their potencies (EC_{50}) were 6.3 and 5.9 μ M, respectively, based on the MTT assay. A cell-free, PARP binding assay also confirmed that 4MF and DMF could directly inhibit the PARP1 enzyme, with IC_{50} values of 2.54 and 2.07 μ M, respectively. A combination of 4MF or DMF with cisplatin or doxorubicin revealed that 4MF (5 and 10 μ M) could sensitise HeLa cells to the effect of cisplatin or doxorubicin.

Structure-activity relationship (SAR) study of 4MF and DMF showed that they had weak antioxidant activity, based on the DPPH free radical-scavenging activity, whereas their hydroxy- derivatives 4'-hydroxyflavone (4HF) and 3',4'-dihydroxyflavone (DHF) exhibited higher and significant antioxidant activity: 55.9% and 77.9% for 4HF and DHF, respectively, compared to 4MF (17.3%) and DMF (15.5%). However, a reactive oxygen species (ROS) assay that measures intracellular levels of ROS revealed that none of 4MF, DMF and 4HF altered hydrogen peroxide-induced intracellular ROS levels in HeLa cells while, on the other hand, DHF significantly decreased the ROS levels ($p < 0.001$). The PARP-inhibitory activities of 4MF, DMF, 4HF and DHF at 20 μ M in a cell-free assay were 21.4%, 17.5%, 35.2% and 24.3%, respectively. In contrast, in the cell-based assay, the EC_{50} values for their effects against MNNG-induced, PARP-mediated parthanatos were 6.3, 5.9, 24.8 and 62.9 μ M, respectively. These findings suggest that 4HF and DHF are stronger antioxidants than 4MF and DMF, and that, conversely, 4MF and DMF are more potent PARP inhibitors than 4HF and DHF. This strongly suggests that a significant antioxidant property does not necessarily correlate directly with a significant PARP-inhibitory property, and vice-versa.

The SAR study revealed the importance of the 3' and 4' positions on the B-ring of the flavone compared to substitution on the A-ring, especially on the C7 position. *In silico* docking simulation indicated that 4MF, DMF, 4HF and DHF could each bind effectively to PARP1 and PARP2 enzymes. Furthermore, the physicochemical properties of these four flavones fulfilled drug-likeness criteria according to the Lipinski's rule of five.

In conclusion, this study has successfully demonstrated that PARPi could be obtained from natural sources and 4MF, DMF, 4HF and DHF, in particular, as flavones, have mild-to-moderate PARP-inhibitory activity and thus represent chemical scaffolds that could inspire the development of novel PARPi for the treatment of cancers and non-oncological conditions, including neurological and neurodegenerative diseases.

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List of Abbreviations

Å	Angstrom
µg	Microgram
µl	Microlitre
µM	Micromolar
4MF	4'-methoxyflavone
6-OHDA	6-hydroxydopamine
AB	AlamarBlue
ABC	ATP-binding cassette
AD	Alzheimer's disease
ADP	Adenosine diphosphate
ADPr	ADP-ribose
AIF	Apoptosis-inducing factor
AMPK	AMP-activated protein kinase
aNHEJ	Alternative nonhomologous end-joining
ANK	Ankyrin domain
ANOVA	Analysis of Variance
ART	ADP-ribosyltransferase
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia and Rad3-related protein
Aβ	Amyloid-β
BER	Base excision repair
BRCA1	Breast cancer type 1 susceptibility protein
BRCA2	Breast cancer type 2 susceptibility protein
BRCT	BRCA1 carboxy-terminal
CaMKII	Calcium/calmodulin-dependent protein kinase II
CAT	C-terminal catalytic domain
CBP	CREB binding protein
CHK1/2	Checkpoint kinase 1/2
cNHEJ	classical nonhomologous end-joining
COVID-19	Coronavirus disease 2019
CTG	CellTiter-Glo

CSB	Cockayne syndrome group B
CXCL1	C-X-C Motif Chemokine Ligand 1
DCFDA	2', 7' - dichlorofluorescein diacetate
DDR	DNA Damage Repair
DMF	3 ',4 '-dimethoxyflavone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPQ	3,4-Dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone
DSB	DNA double strand breaks
ECACC	European Collection of Authenticated Cell Cultures
EMA	European Medicines Agency
ERK	Extracellular regulated kinase
FANCA	Fanconi Anaemia Complementation Group A
FANCC	Fanconi Anaemia Complementation Group C
FANCD	Fanconi Anaemia Complementation Group D
FBS	Foetal Bovine Serum
FDA	US Food and Drug Administration
HCl	Hydrochloric acid
HD	Helical subdomain
HPS	Histidine, Proline, Serine
HTS	High-throughput screening
HR	Homologous recombination repair
JNK1	c-Jun N-terminal kinase 1
KDa	Kilodalton
KIF4	(Chromosome-associated) Kinesin Family4
LIG3	DNA Ligase 3
MAC	Macro domains
MAR	Mono (ADP-ribose)
MARylation	Mono ADP-ribosylation
mg	Milligrams
MIF	Migration inhibitory factor

ml	Millilitres
mM	Millimolar
MMR	Mismatch repair
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
MNNG	<i>N</i> -Methyl- <i>N'</i> -nitro- <i>N</i> -nitrosoguanidine
NAD ⁺	Nicotinamide adenine dinucleotide
NBS1	Nijmegen breakage syndrome 1 (Nibrin)
NER	Nucleotide excision repair
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	Non-homologous end joining repair
NHS	National Health Service
NLS	Nuclear localisation sequence
nM	Nanomolar
NMNAT-1	Nicotinamide mononucleotide adenylyltransferase 1
NSCLC	Non-small cell lung cancer
NTR	N-terminal region
p53	tumour protein p53
PAAN	PARP1-dependent, AIF-associated nuclease
PAR	Poly (ADP-ribose)
PARP	Poly (ADP-ribose) polymerase
PARP1	Poly (ADP-ribose) polymerase 1
PARPi	PARP inhibitor(s)
PARylation	poly ADP-ribosylation
PD	Parkinson's disease
PDB	Protein Data Bank
PDBQT	Protein Data Bank, Partial Charge (Q), & Atom Type (T)
Polθ	DNA polymerase theta
PBS	Phosphate Buffered Saline
PTM	Post-translational modification
RAP80	Receptor-associated protein 80
RBBP8	RB Binding Protein 8, Endonuclease
RCS	Reactive carbonyl species

RFC	Replication factor C
RMSD	Root-mean-square deviation
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPA	Replication factor A
rpm	Revolutions Per Minute
RRM	RNA recognition motifs
SAM	Sterile alpha motif
SAR	Structure-activity relationship
SARS CoV 2	Severe acute respiratory syndrome coronavirus 2
SDF	Structure-Data File
SIRT6	Sirtuin 6
SSB	DNA single strand breaks
tPA	Tissue plasminogen activator
UIM	Ubiquitin-interacting motif
UV	Ultraviolet
WGR	Tryptophan-Glycine-Arginine
WHO	World Health Organization
WWE	Tryptophan-Tryptophan-Glutamate
XPA	Xeroderma pigmentosum complementation group A
XRCC1	X-Ray Repair Cross Complementing 1
Zn	Zinc Finger
z-VAD-fmk	Benzyloxycarbonyl-Val -Ala-Asp(OMe)-fluoromethylketone

Amino Acid Codes

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Cys	Cysteine
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

Chapter 1: Introduction

1.0 General Introduction

The PhD work reported in this thesis explored the potential to develop novel classes of inhibitors of the poly (ADP-ribose) polymerase (PARP) enzyme, a druggable therapeutic target that underpins the clinical use of the first class of targeted cancer therapeutics (PARP inhibitors, PARPi), with four such drugs approved within the last eight years as monotherapy for the treatment of *BRCA*-mutated cancers. PARP is a key component of the DNA damage response (DDR), and the use of PARPi to treat *BRCA*-mutated cancers is based on the genetic principle of synthetic lethality, a situation whereby the simultaneous loss of function of two genes or proteins causes cell death but the loss of function of either of them alone does not lead to cell death and is thus tolerated. The work especially focused on natural products as a potential source of novel PARPi that might overcome challenges with the current PARPi, such as chemoresistance. The focus of the work is further justified by the fact that PARPi are now considered for repurposing for non-oncological indications, for example, as disease-modifying therapeutics for treating neurological and neurodegenerative conditions.

In this introductory chapter, the alarming scourge of cancer as an area of unmet clinical need is explored, followed by an exploration of the roles of PARP in the mechanisms of DNA repair (DNA damage response), with a major focus on PARP1, the most-important and thus most-studied member of the PARP superfamily. The underlying molecular (including genetic) mechanisms underpinning the use of PARP inhibitors are then discussed, and the profiles of

experimental and clinical PARPi are presented, including the emerging concern of chemoresistance to the clinical PARPi. In addition, opportunities for repurposing (repositioning) PARPi for non-oncological diseases are highlighted. Finally, the exploitation of the prospect of natural products, especially flavonoids, in drug discovery, and in this case as potential PARPi, is discussed. The aims and objectives of the work are then stated.

1.1 Cancer

Cancer is a major public health burden and the second leading cause of mortality worldwide, only behind cardiovascular diseases. With nearly 10 million deaths in 2020, it represents one in six deaths, and over 20 million new cancer cases are anticipated in 2025. The most common type of cancer is breast cancer, with 2.26 million cases, while lung cancer is the most lethal, with 1.8 million deaths (Figure 1) (Sung et al., 2021). In 2016, cancer chemotherapy was the single biggest expenditure of the NHS in England (UK), costing an estimated £1.4 billion a year (about a tenth of their entire central budget) (NHS, 2016). Despite the decades of advancement in understanding and treatment, cancer is an ever-growing global burden, hence the constant and urgent research for better cancer therapy or cure.

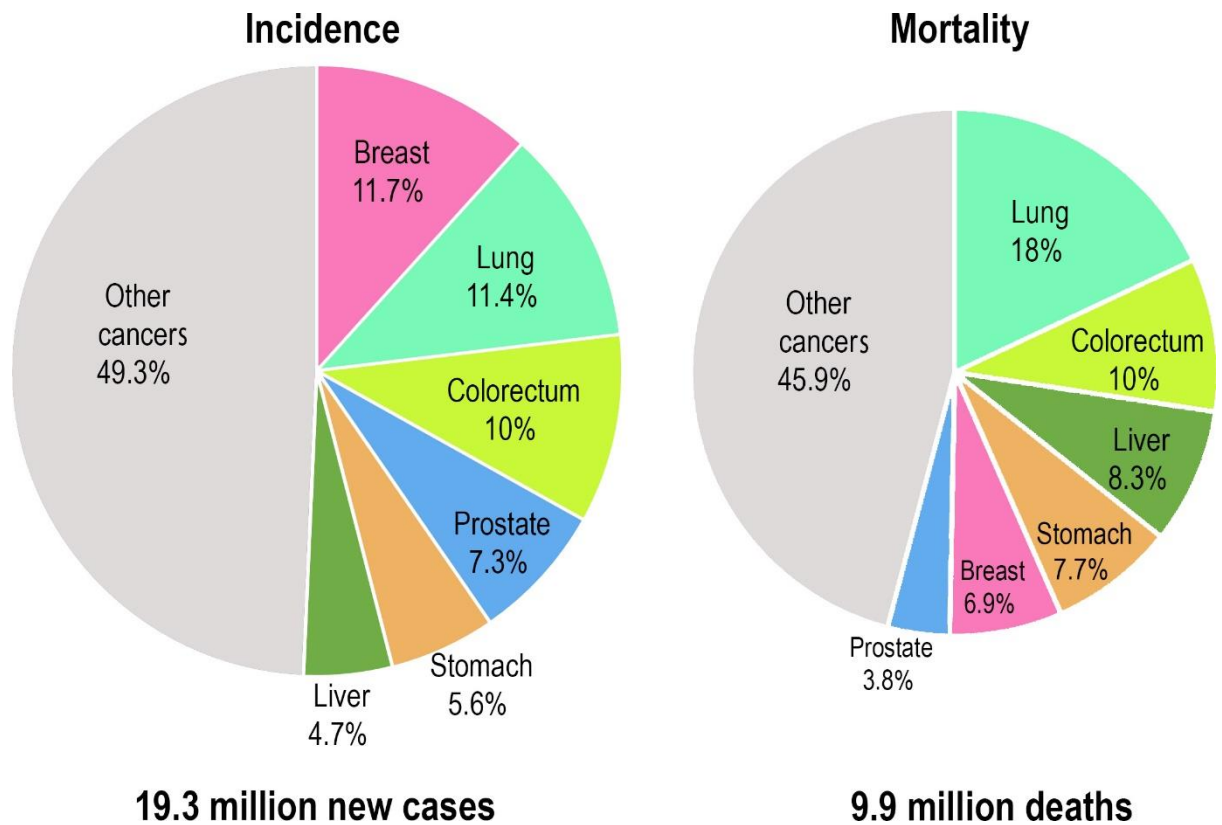


Figure 1: Incidence of cancers and deaths from the six most common cancers in 2020, according to WHO reports. Statistics are from (Sung et al., 2021) but images were made by the author.

Cancer refers to a large group of diseases that are characterised by uncontrollable growth of abnormal cells which can invade other parts of the body. The abnormal proliferation can happen in any part of the body, hence there are over 100 different types of cancers. However, it is the ability to invade surrounding normal tissues and spread throughout the body through the circulatory or lymphatic systems (metastasis) that makes cancer very dangerous and that accounts for most mortalities (Cooper and Hausman, 2013). The development of cancer (carcinogenesis) can be attributed to a variety of non-genetic factors (lifestyle (such as smoking), exposure to carcinogens, infections, etc.) and genetic factors. There are three stages involved in carcinogenesis, namely initiation, promotion, and progression ([Figure 2](#)). The primary driver of cancer is genetic mutations. While most cancers (about 80%) arise from somatic mutations (DNA alteration after

conception), some are inherited (germline mutations). Germline mutations in DNA repair genes such as *BRCA1*, *BRCA2*, and *p53* increase the risk of developing certain types of cancers, such as breast, ovarian, prostate, and pancreatic cancers (Turk and Wisinski, 2018).

From the initiation of a neoplasm to metastasis, cancers exhibit a wide and complex range of molecular, biochemical, cellular, and physiological traits. Hanahan and Weinberg (2000) initially proposed a list of six capabilities that are shared by most cancers—if not all known human tumours. However, the list of hallmark capabilities was updated to ten in 2011. According to the review, one of the emerging hallmarks and enabling characteristics of cancers is genomic instability ([Figure 3](#)) (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). This genomic instability is often due to an aberration in the DNA repair pathway in the tumour cells.

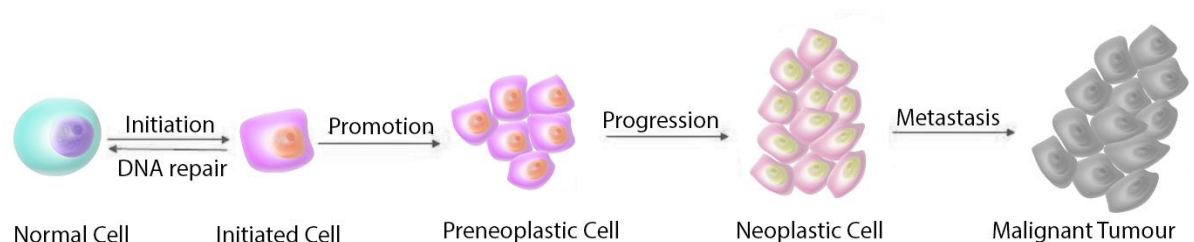


Figure 2: The multistep process of carcinogenesis, the transformation of normal cells into cancer cells. This process occurs in three stages: initiation, promotion, and progression. Initiation involves the non-reversible DNA modification and mutation in a critical gene that controls cell proliferation. Although initiation is irreversible, the initiated cell may die without replicating the neoplasm. At the promotion stage, the initiated cell epigenetically modifies the gene expression and stimulates proliferation. This is usually a protracted and reversible process in promoters such as reactive oxygen species (ROS). The final step is progression, where further mutations occur and the pre-neoplastic cell transforms into a malignant cell which proliferates with different genetic and phenotypic traits. Figure was developed by the author.

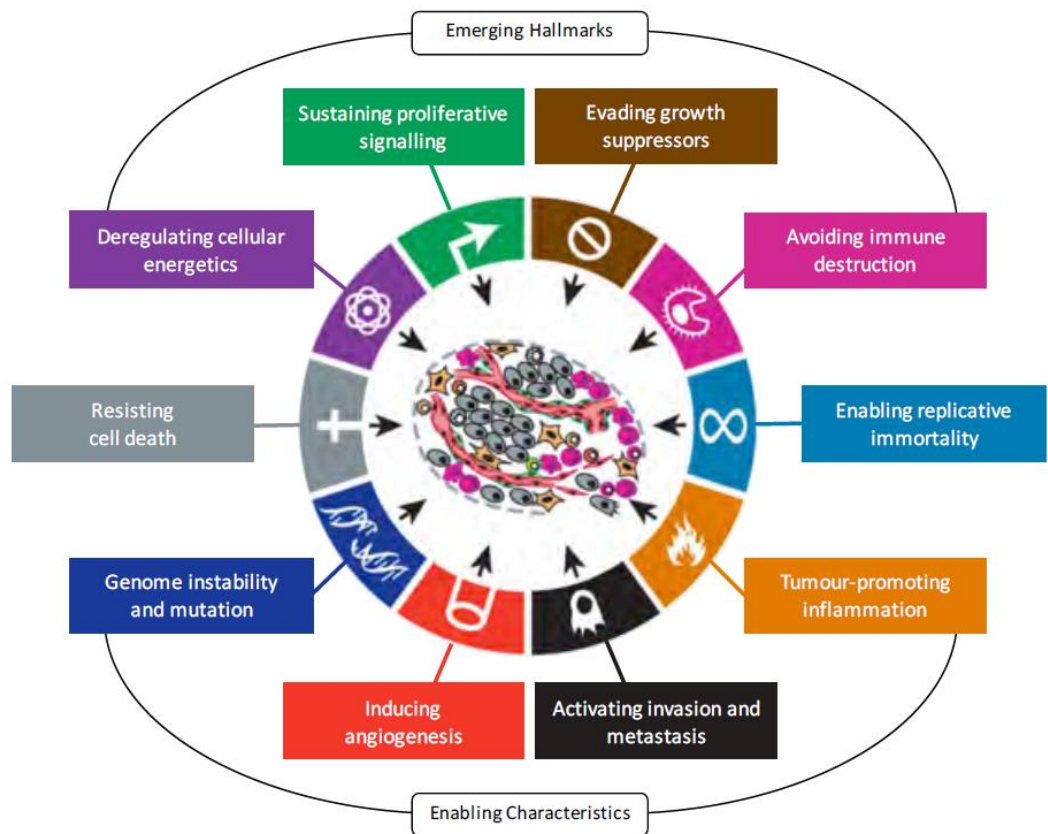


Figure 3: Hallmarks and enabling characteristics of cancer. These traits are acquired during the multistep process of tumour development (carcinogenesis) and provide the rationale behind the complexities of neoplastic disease. The hallmarks are illustrated in the diagram above. However, underlying all these traits is genome instability, which produces the genetic diversity that facilitates their acquisition. Adapted from Hanahan & Weinberg (2011).

1.2 DNA Damage Repair (DDR) Pathway

Human DNA is regularly exposed to exogenous and endogenous insults that can result in DNA damage. Exogenous sources are mainly environmental factors, such as ultraviolet (UV) light exposure, ionising radiation, and various genotoxic chemicals. On the other hand, endogenous insults, such as replication errors, oestrogen and cholesterol metabolites, reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive carbonyl species (RCS) are generated as by-products of normal cellular metabolic processes or spontaneous mutations. The human body contains over 10^{13} cells and each cell may experience tens of

thousands of DNA lesions daily, which can cause DNA single-strand breaks (SSB) and DNA double-strand breaks (DSB). Regardless of the cause of the DNA damage, the accumulation of these SSBs and DSBs can lead to serious genomic instability. Thus, an appropriate response is crucial to the survival of the individual cells and ultimately the organism. To preserve genomic integrity, a network of at least 450 proteins is involved in various DNA repair mechanisms collectively known as the DNA damage response (DDR) pathways (Cortesi, Rugo and Jackisch, 2021). There are five major DDR pathways, depending on the type of damage. SSBs are repaired by base excision repair (BER), nucleotide excision repair (NER), or mismatch repair (MMR), while DSBs, the most devastating form of DNA damage, are repaired by homologous recombination (HR) or non-homologous end joining (NHEJ) ([Figure 4](#)) (De Bont and van Larebeke, 2004; Jackson and Bartek, 2009; Wei and Yu, 2016; Jin and Oh, 2019; Prasad et al., 2019; He, Kawaguchi and Toi, 2020).

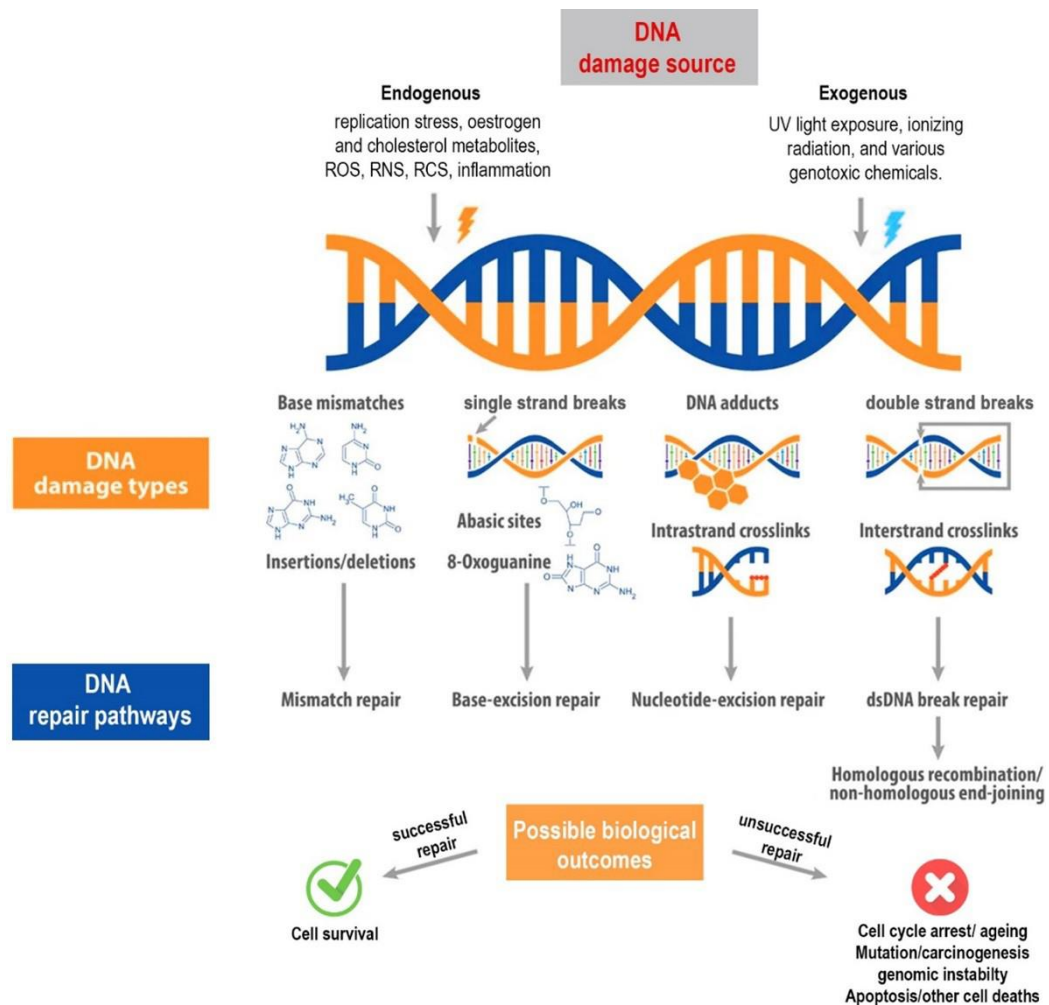


Figure 4: An overview of the DNA Damage Repair (DDR) pathways. The figure highlights the sources of DNA damage, the damage types, and the appropriate repair mechanisms.

The most common form of DNA damage is SSBs, and these are mostly caused by endogenous factors, occurring at a rate of about 10,000 spontaneous breaks in each cell per day. They are repaired by base excision repair (BER), nucleotide excision repair (NER), or mismatch repair (MMR), in a process that involves using the unbroken complementary strand as a template. However, the BER pathway is the main repair mechanism of SSBs and is facilitated by a family of enzymes called poly (ADP-ribose) polymerases (PARPs) (Underhill, Toulmonde and Bonnefoi, 2011). Thus, inhibition of PARP will impair the repair of SSBs, leading to

accumulation of the SSBs and a consequent generation and accumulation of DSBs.

In the DNA repair pathway for DSBs, HR occurs mainly in the synthesis phase (S phase) and G2 phase of the cell cycle, while NHEJ is operative throughout the cell cycle. During the S and G2 phase, HR utilises available sister chromatids as a template for accurate repair of damaged DNA, which makes the DDR very efficient and error-free. On other hand, NHEJ is more prone to error, which renders this mechanism for fixing damaged DNA less effective, especially for the repair of serious DNA damage (Cerrato, Morra and Celetti, 2016; Tempka et al., 2018; Parkes, McCabe and Kennedy, 2019). Although DSBs are generally repaired by HR and NHEJ in humans, there is a minor pathway similar to alternative nonhomologous end-joining (aNHEJ) known as single-strand annealing (SSA). SSA involves the annealing of homologous repeats (homology) flanking the DSB to join the DNA at the breakpoint junctions. This repair pathway is error-prone, just like the NHEJ (Bhargava, Onyango and Stark, 2016; Blasiak, 2021).

Ultimately, the cellular decision on whether to use the HR or NHEJ pathway is essentially dependent on the phase of the cell cycle and whether there are any aberrations in the DDR pathway. Consequently, any defect or impairment in one of these essential DNA repair pathways would lead to over-reliance on another pathway to mitigate loss. For example, the loss of the HR pathway will leave the cell solely dependent on NHEJ to repair its DSBs. Accordingly, if the alternative pathway cannot adequately compensate for the loss of function of the defective pathway, this could lead to severe genomic instability, and ultimately cell death. In

addition, defects in the expression of DDR genes, which cause dysregulation of DNA damage signalling and/or DNA damage repair pathways, have been associated with cancer (Ferreira and Dutreix, 2019; He, Kawaguchi and Toi, 2020).

In cancer cells with a defective HR pathway, such as those with a loss-of-function mutation in their *BRCA1* and/or *BRCA2* (tumour suppressor genes), it has been demonstrated that the inhibition of the enzyme poly (ADP-ribose) polymerase (PARP) leads to cell death through a genetic mechanism known as synthetic lethality (Karakashev et al., 2017; Zhong et al., 2019). Synthetic lethality is a situation whereby a simultaneous inactivation or loss of function of two genes from complementary pathways results in cell death, while the loss of either of the two genes is not lethal by itself (Figure 5) (Nijman, 2011). Since one of the hallmarks of cancer cells is alteration in the DDR alongside uncontrolled growth and rapid proliferation, developing therapeutics that explore these vulnerabilities via synthetic lethality is extremely attractive. A unique success story in this regard is the development of PARP inhibitors, as explained later.

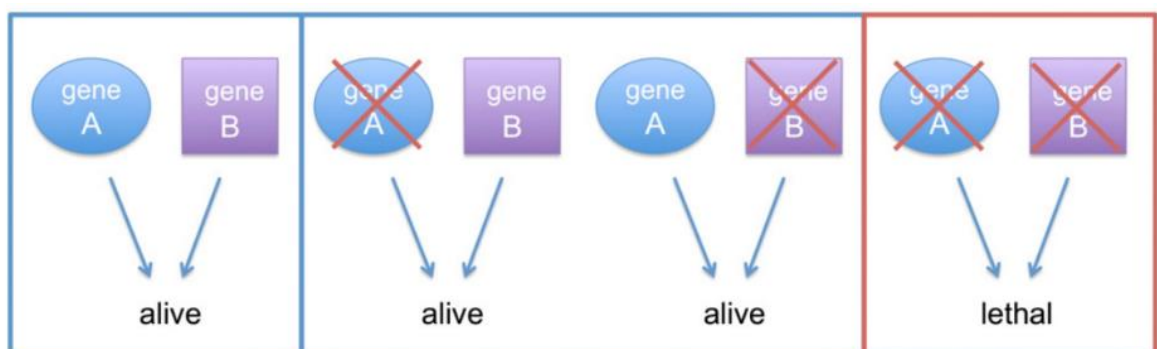


Figure 5: Synthetic lethality of two genes. The deletion or inactivation (indicated as red X symbol) of either gene A or gene B has no effect on the cell's viability, but their concomitant inactivation is lethal, resulting in cell death. Adapted from Nijman (2011).

In response to DNA damage, these major DDR pathways engage a range of complex and overlapping signalling pathways, which either promote genomic stability or initiate programmed cell death, depending on the situation. In these signalling pathways, post-translational modification (PTM), the biochemical alteration of proteins after synthesis, plays a very important role in the recruitment and dissociation of DDR proteins at the DNA damage sites. PTMs in DDR pathways can take numerous forms, including acetylation, phosphorylation, ubiquitination, and ADP-ribosylation. The addition of these PTMs is typically achieved by certain enzymes; for example, ADP-ribosylation is performed by poly (ADP-ribose) polymerases (PARPs) (Kryston et al., 2011).

1.3 The PARP superfamily

Poly (ADP-ribose) polymerases (PARPs) are a family of post-translational modification enzymes that regulate an array of biological processes, including DNA repair, DNA replication, chromatin remodelling, transcriptional regulation, antiviral response, programmed cell death, and mitosis (Wei and Yu, 2016; Guo et al., 2020; Wu et al., 2021; Padella et al., 2022). In humans, this family comprises 17 members that share a conserved C-terminal catalytic domain and a very diverse N-terminal domain ([Figure 6](#)). This superfamily's members are all encoded by different genes (Hottiger et al., 2010). PARPs catalyse the transfer of ADP-ribose from nicotinamide adenine dinucleotide (NAD⁺) onto acceptor proteins such as histones, topoisomerases, p53, and PARP itself. This is known as ADP-ribosylation. During this process, PARPs covalently attach their ADP-ribose units onto the carboxyl group of arginine, aspartic acid, glutamic acid, cysteine, lysine, serine, and tyrosine

residues of target proteins via an ester bond (Wei and Yu, 2016; Chen et al., 2018b; Wang et al., 2019; Li et al., 2020b).

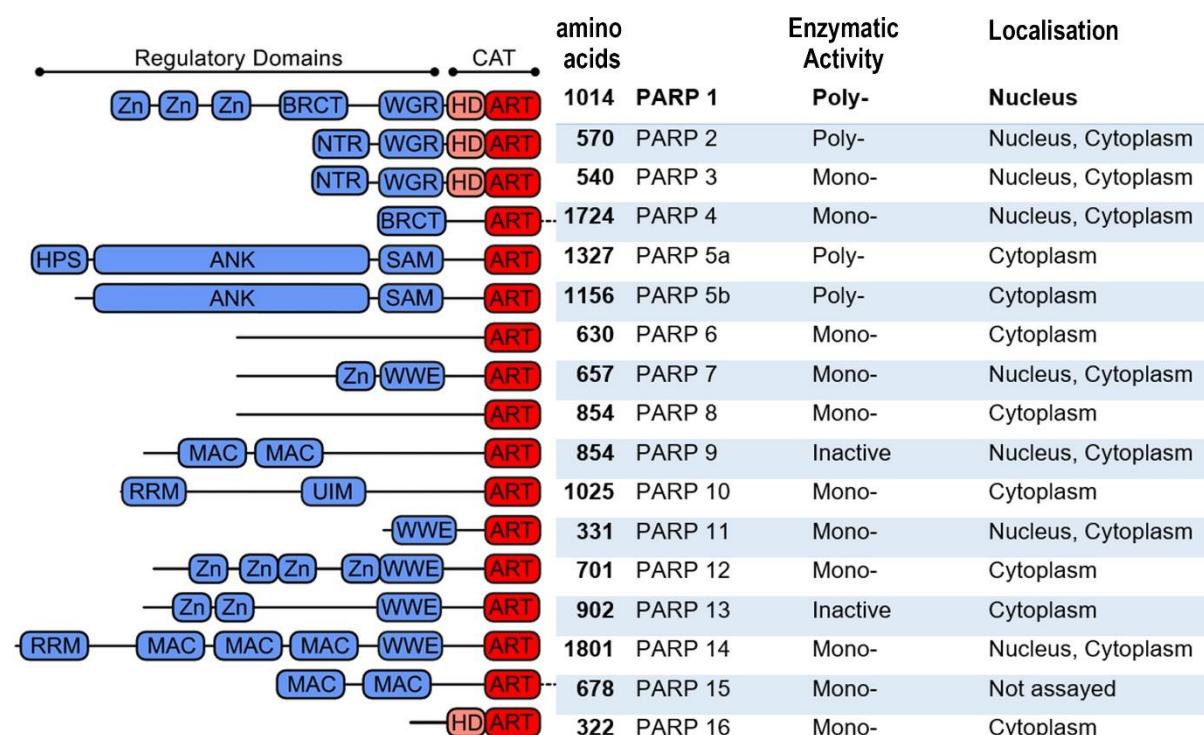


Figure 6: Domain architecture, protein size, ribosylation pattern, and subcellular localisation of the PARP superfamily. The 17 members share a conserved C-terminal catalytic domain (CAT), shown in pink/red, and a very diverse N-terminal domain, denoted in blue. The CAT domain comprises the helical subdomain (HD) and the ADP-ribosyl transferase (ART) subdomain. PARP1, PARP2, and PARP3 are DNA-dependent PARPs and share a common tryptophan, glycine, and arginine (WGR) motif that interacts with DNA. Some PARPs have zinc fingers (Zn) which are involved in sensing and binding DNA strand breaks. The BRCA1 carboxy-terminal motifs (BRCT) in PARP1 are important for protein-protein interaction. PARP5a and PARP5b are known as the tankyrases, with their unique sterile alpha motif (SAM) and ankyrin domain (ANK) repeats. The ankyrin domain is a 33-residue motif comprising of two α -helices separated by loops. Tankyrase 2 (PARP5b) has a histidine, proline, serine (HPS) region of unknown function. Macro domains (MAC) are found in PARP9, PARP14 and PARP15 and they have high-affinity ADP-ribose-binding ability. (Langelier et al., 2018; Eisemann and Pascal, 2020; van Beek et al., 2021). NTR means N-terminal region; WWE means Tryptophan-tryptophan-glutamate motif; RRM means RNA recognition motifs; and UIM means ubiquitin-interacting motif. Figure created by author.

Though they are called poly (ADP-ribose) polymerases, only 4 members (PARP1, PARP-2, PARP-5a, and PARP-5b - the last two known as the tankyrases) are responsible for poly ADP-ribosylation (PARylation) that leads to the synthesis of linear or branched poly (ADP-ribose) (PAR) polymer. The other members, with the exception of PARP-9 and PARP13, are only capable of mono ADP-ribosylation (MARylation). This means they are only able to transfer a single ADP-ribose unit (i.e., mono (ADP-ribose) (MAR) unit) onto their target proteins (Wei and Yu, 2016; Langelier et al., 2018). All catalytically active members of the PARP family are able to 'transfer' the ADP-ribose moiety from NAD⁺ onto various acceptors, but not all engage in the polymerisation of ADP-ribose units (ADPrs); thus, all PARPs are 'transferases' but not all are 'polymerases'. Due to this transferase activity, PARP enzymes are sometimes referred to as ADP-ribosyltransferases. Lüscher et al. (2021) recommended a change in nomenclature that better reflects the biochemical and structural diversity of this superfamily of enzymes which are involved in mono- and poly (ADP-ribose)-mediated cellular processes. In addition, the term 'polymerase' is often associated with enzymes involved in DNA or RNA synthesis, not proteins involved in protein-protein modification (Lüscher et al., 2021). Based on evolutionary similarity in the structures of bacterial ADP-ribosyltransferases and mammalian PARPs, the term "ADP-ribosyltransferase diphtheria toxin-like" (ARTD) has been suggested to be more accurate than "PARP" (Kuny and Sullivan, 2016). However, currently, most recent publications tend to use both ARTD and PARP nomenclature simultaneously. For example, PARP1 is described as ARTD1/PARP1. Apart from the irksomeness of writing two names for the same enzyme at the same time, there is a potential for confusion, as the new ARTD isoform numbering does not always correspond with the PARP

nomenclature isoform numbering. For instance, ARTD14 is PARP7, while ARTD7 is PARP15. Consequently, for ease of reading and for consistency, this thesis adopts the 'PARP' nomenclature and classification.

During PARylation, multiple ADPrs are transferred to acceptor proteins and are joined through 1'-2' or 2'-1' glycosidic ribose-ribose bonds, which lead to a long linear poly (ADP-ribose) (PAR) polymer structure or a complex, branched structure, respectively ([Figure 7](#)) (Kalisch et al., 2012; Bian et al., 2019). PARylation is a transient and reversible post-translational modification of proteins that plays an important role in various cellular processes, such as maintaining genomic stability, regulating transcription, telomere length homeostasis, chromatin structure stabilisation, DNA methylation, and cell signalling. At the molecular level, PARylation modifies the biochemical and functional properties of its target proteins, which in turn dictate the protein-protein or protein-nucleic acid (both DNA and RNA) interactions, thus promoting the formation of scaffolds that direct DDR proteins to a precise site and regulating other protein modifications such as ubiquitylation (Krietsch et al., 2013).

On the other hand, little is known about the biological importance of MARYlation, even though most members of the PARP superfamily are involved only in this type of enzymatic reaction (Vyas et al., 2013). Recent studies have revealed that most of these PARP 'monoenzymes' are localised in the cytosol and regulate cellular processes such as RNA metabolism, cellular transport, focal adhesion, and stress responses (Challa, Stokes and Kraus, 2021). Of the at least 17 members of PARP

now identified in eukaryotic cells, PARP1 is the most abundant and extensively studied (Di Girolamo and Fabrizio, 2019; Wang et al., 2019).

It is important to note that while PARPs are responsible for the formation of MAR or PAR, the degradation of the PAR chains is mainly catalysed by poly(ADP-ribose) glycohydrolase (PARG). PARG is the only enzyme that can hydrolyse the ribose-ribose bonds of PAR on PARylated proteins (Slade, 2020). PARG counteracts the action of PARP enzymes, thereby preventing the build-up of PAR which could have detrimental consequences for the cell. Several studies have demonstrated that the deficiency or pharmacological inhibition of PARG led to increased sensitivity to PARP inhibitors and chemotherapeutic agents (Steffen et al., 2011). Similarly, ADP-ribosylhydrolase 3 (ARH3) has been shown to catalyse the exoglycosidic hydrolysis of PAR and MAR. Both PAR hydrolysing enzymes, but predominantly PARG, play important roles in regulating PARP enzyme activities through their dePARylation of modified proteins (O'Sullivan et al., 2019; Harrison et al., 2020).

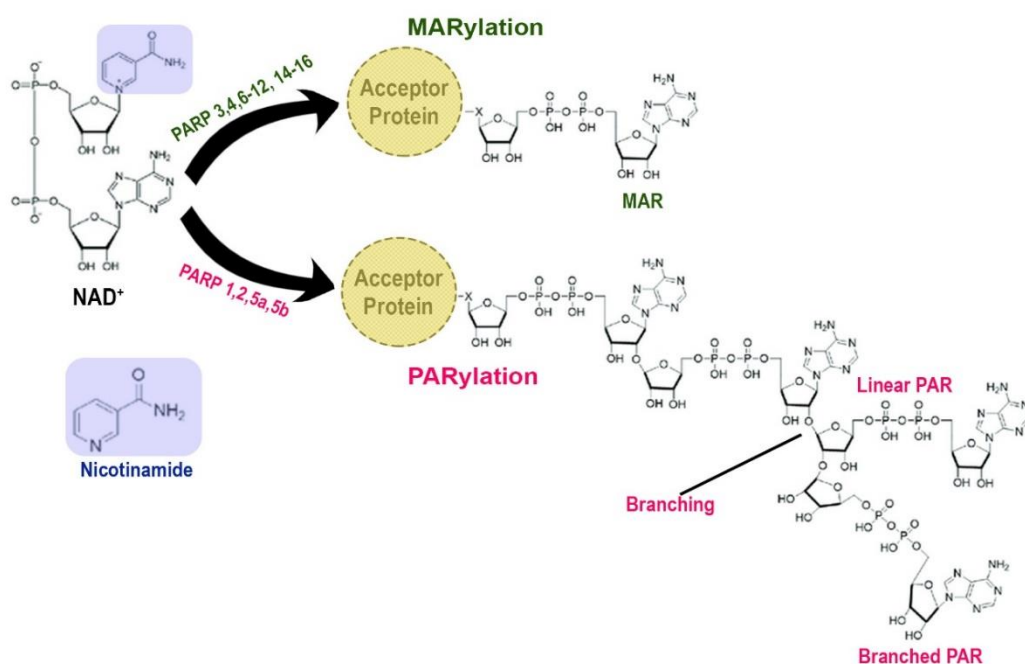


Figure 7: Poly ADP-ribosylation and Mono ADP-ribosylation by PARPs. PARPs can produce mono (ADP-ribose) (MAR) and poly (ADP-ribose) (PAR) from NAD⁺, releasing nicotinamide as a by-product. PARP1, the most prolific member of the PARP family, can produce PAR chains of up to 200 ADP-ribose units with branching occurring every 20–50 units. This causes a huge variation in the PAR chain's length and branching shape (Kamaletdinova, Fanaei-Kahrani and Wang, 2019). Figure created by author

1.4 Poly (ADP-ribose) polymerase-1 (PARP1)

PARP1 (EC 2.4.2.30) accounts for 85-90% of total PARP enzyme activity and the production of over 95% of total PAR polymers in most cells (Cayuela et al., 2001). PARP1 is a 116 KDa nuclear protein consisting of 1014 amino acid residues and is involved primarily in DNA damage signalling by detecting and rapidly binding to the site of DNA breaks, especially single-strand breaks. Apart from the initiation of DNA damage response, PARP1 has also been implicated in the surveillance of genomic integrity and other cellular homeostatic activities such as cell division, apoptosis, and regulation of gene transcription and amplification (Kumar et al., 2021). Upon sensing DNA damage, PARP1 activity can be stimulated up to 500-fold over basal levels, allowing it to transfer 50 to 200 ADPrs to numerous nuclear proteins, including PARP1 itself (Alvarez-Gonzalez and Althaus, 1989; Yu et al., 2002; Jannetti et al., 2020).

PARP1 structure is divided into three main domains: the N-terminal DNA-binding domain, a central auto modification domain, and a C-terminal catalytic domain ([Figure 8](#)). The N-terminal DNA-binding domain has two homologous zinc fingers (Zn1 and Zn2) and a nuclear localisation sequence (NLS) used for DNA binding. Zn1 and Zn2 recognise and bind DNA breaks, which increases PARP1 activity up to 500-fold. The NLS acts as a DNA-nick sensor. There is another zinc finger in

this domain, Zn3, which is structurally and functionally distinct from the previous two. Zn3 is uniquely found in PARP1 compared to the other PARPs. These Zn fingers play different roles in the enzymatic function of PARP1. Zn1 is involved in the sensing and binding of DSBs, interacting with the catalytic site and activating PARP1. Zn2 plays a crucial role in the recognition of SSBs, while Zn3 is responsible for protein-protein interactions during autoPARylation by initiating hydrolysis of the NAD⁺ substrate (Wang et al., 2017; Jannetti et al., 2020).

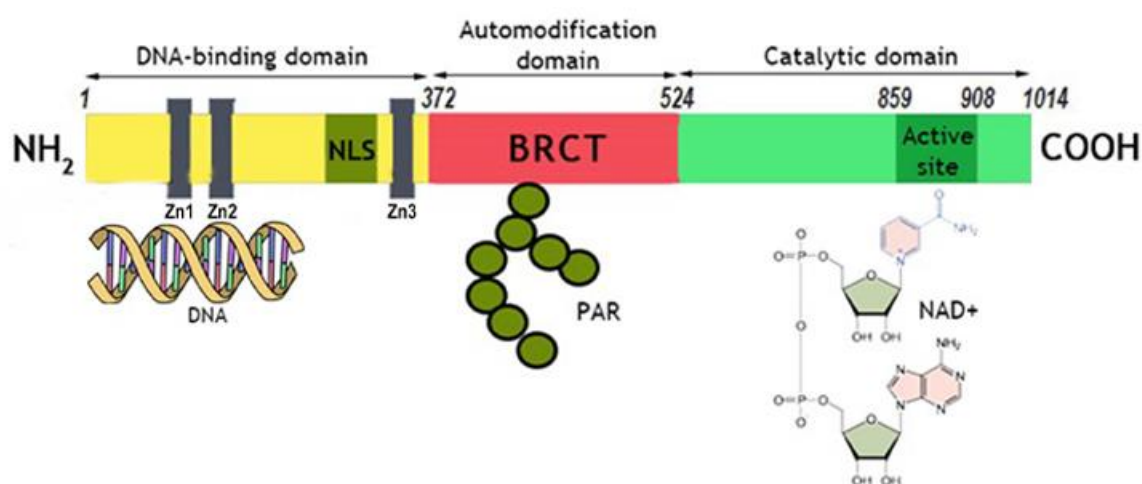


Figure 8: The primary structure of PARP1, with three main domains. (1) An N-terminal DNA-binding domain containing two zinc-finger motifs (Zn1 and Zn2), which are responsible for detection of DNA breaks, a nuclear localisation sequence (NLS) containing a caspase-cleavage site, and a third zinc-finger (Zn3) motif for protein-protein interactions ; (2) a central automodification domain with a breast cancer-associated gene 1 (BRCA1) C-terminal (BRCT) motif for protein-protein interaction and which plays an important role in regulating PARP1 activity; and (3) a C-terminal catalytic domain containing the nicotinamide adenine dinucleotide (NAD)-binding site and the poly (ADP-ribose) polymer (PAR)-synthesising domain (Fatokun, Dawson and Dawson, 2014).

The central auto modification domain serves as an acceptor of ADP-ribose units, thereby allowing the enzyme to transfer poly (ADP-ribose) to itself (automodification or autoPARylation). AutoPARylation occurs after DNA repair and causes the PARP1 enzyme to dissociate from the DNA due to the accumulated

high negative charge of the PAR chain. This dissociation allows the other DDR proteins recruited by PARP1 to have access to the DNA damage site (Murai et al., 2012; Mitra et al., 2019). This domain also contains a BRCA1 carboxy-terminal (BRCT) repeat motif which plays an important role in protein targeting in the DNA damage and repair response.

The WGR is the tryptophan-glycine–arginine-rich domain that participates in binding DNA near the 5-terminus and mediates domain-domain contact essential for DNA-dependent activity. Several studies have indicated that the WGR domain is critical for PARP1 activation. The WGR domain has been observed to move closer to form a tighter-binding complex after binding to a DNA break. This is accompanied by the transmission of binding information to the active site to yield the catalytic activation of PARP1 (Li et al., 2013; Rudolph, Mahadevan and Luger, 2020).

PARP1 has been reported to be about 62% identical in its catalytic domain to PARP2, its closest homologue. It has been revealed that PARP2 is responsible for 10-15% of human cellular PARP activity (Hoch and Polo, 2019). A closer study of the PARP1 catalytic domain showed that it has two sub-pockets, which are occupied by the substrate NAD⁺. One sub-pocket (NI site) binds to the nicotinamide-ribose part of NAD⁺, while the other (AD site) binds to the adenine-ribose moiety. The NI site allows NAD⁺-mimicking PARP1 inhibitors to bind through hydrogen bonds with residues Ser904 and Gly863, while residue Tyr907 provides supplementary π - π stacking interaction. On the other hand, the AD site is large enough to tolerate the binding of structurally diverse PARP inhibitors. Several

potent PARP inhibitors, such as olaparib, have been reported to exploit the AD sub-pocket to increase their potency (Zhong et al., 2019). PARP1's catalytic domain is involved in three different stages of PAR synthesis, namely: initiation – this is the attachment of the first ADP-ribose unit to the amino acid residue in an acceptor protein; elongation – the formation of a (2'-1'') ribose-ribose glycosidic bond; and branching – the formation of a (2''-1''') ribose-ribose bond between ADP-ribose units (Alemasova and Lavrik, 2019). A better understanding of the enzymatic structure of PARP1, especially the catalytic site, will help in the development of more potent PARP inhibitors that will have less undesirable effects on other NAD⁺-consuming enzymes such as sirtuins or HDACs (histone deacetylases), CD38 (Cluster of Differentiation 38), CD157, CD73, SARM1 (sterile alpha and TIR motif containing 1), and NNMT (nicotinamide N-methyltransferase) (Wei and Yu, 2016).

1.4.1 Roles of PARP1 in DNA repair

The PARP1 protein initiates and modulates multiple DNA repair pathways, which maintain the genomic stability, even though it is not directly involved in any of them.

1.4.1.1 Base excision repair (BER)

Base excision repair is the major DNA repair pathway that corrects the damage that occurs from oxidative stress, alkylation, deamination, and hydroxylation. These lesions damage the bases but cause minimal distortion to the DNA helix structure. 8-oxoguanine-DNA glycosylase initiates the BER by sensing and removing the damaged base, leaving an abasic site. PARP1 interacts with 8-oxoguanine-DNA glycosylase to recruit X-ray repair complementing factor 1

(XRCC1) to the site of the lesion through poly ADP-ribosylation. XRCC1 in turn provides a platform and plays a coordinating role for other BER enzymes, such as DNA polymerase- β and DNA ligase III, which eventually lead to repair (Krokan and Bjørås, 2013).

1.4.1.2 Nucleotide excision repair (NER)

Nucleotide excision repair is responsible for the excision of bulky, helix-distorting DNA lesions generated by UV irradiation and mutagenic compounds. This pathway involves the recognition and removal of a short, single-stranded DNA segment that contains the lesion, and subsequently restores it with a normal nucleotide sequence with the help of the complementary template. Even though the involvement of PARP1 in NER is not clear, studies have shown that at least two NER proteins, the DNA lesion recognition protein xeroderma pigmentosum group A (XPA) and the DNA-dependent ATPase Cockayne syndrome group B (CSB) protein, are PAR-binding proteins. Also, it has been demonstrated that poly ADP-ribosylation of CSB inhibits its DNA-dependent ATPase activity (Thorslund et al., 2005; Mangerich and Bürkle, 2012)

1.4.1.3 Mismatch repair (MMR)

Mismatch repair is the main pathway that corrects the erroneous deletion, small insertion (loops), and spontaneous base-to-base mispairs that can occur during DNA replication. Although the role of PARP1 in MMR is yet to be understood, physical interaction with four mismatch repair proteins MutS α , exonuclease 1, replication protein A (RPA), and replication factor C (RFC) has enhanced the mismatch dependence of 5'-directed excision (Liu, Kadyrov and Modrich, 2011).

1.4.1.4 Non-homologous end joining repair (NHEJ)

NHEJ is one of the two main pathways of DSB repair. Though less reliable and accurate because of its direct ligation approach to repair without a 'homologue' template, this mechanism plays a crucial role in tumour development and its dysfunction can lead to malignancies. In conventional non-homologous end joining repair (cNHEJ) which occurs mainly in G0/G1 interphase, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), a key NHEJ factor, is activated via poly ADP-ribosylation. The DNA-PKcs is then recruited to the site of DSB by Ku70/80 heterodimer to form a DNA-PK complex that initiates the rest of the repair. DNA-PKcs' kinase activity is required for NHEJ, which is not possible without the stimulation by PAR (Sishc and Davis, 2017). Similarly, in the S and G2 phases of the cell cycle, PARP1 plays a crucial role in the alternative non-homologous end joining repair (aNHEJ). This pathway is initiated by PARP1 which senses the DNA breaks and recruits a MRN/CtIP complex (Mre11-Rad50-Nbs1 /C-terminal-binding protein interacting protein) on the DNA ends, through endonuclease/exonuclease activity of MRN, and exposes the microhomology sequences flanking the DSBs. This is followed by bridging and alignment of the microhomologies on the DNA ends promoted by PARP1, MRN complex, and the DNA polymerase θ (Pol θ) activities. The non-homologous flaps are removed by ERCC1 and XPF nucleases. The last step involves filling in the gaps by Pol θ -mediated DNA synthesis and ligation by DNA ligase 3 (LIG3)/XRCC1 complex. PARP1 and MRN complex play an important role in this last step by bringing LIG3 into proximity with DNA breaks through physical interaction with the scaffold protein XRCC1 (Caracciolo et al., 2019; Padella et al., 2022).

1.4.1.5 Homologous recombination repair (HRR)

Homologous Recombination (HR) repair (HRR) is the cell's preferred choice of DSB repair because of its accuracy. In the S and G2 phases of the cell cycle, sister chromatids use identical templates during replication. The synthetic lethality relationship between PARP1 and HR proteins, especially BRCA1/2, raises interest in the role of PARP1 in this pathway. BRCA1 is a key HR protein that promotes DSB ends resection. Although the recruitment of BRCA1 to the DSB site is mainly facilitated by the ubiquitylation of CtBP-interacting protein (CtIP or RBBP8), it has now been shown that the localisation of BRCA1 to the damage site is enhanced by the activity of PARP1. Poly ADP-ribosylation of BRCA1 and BRCA1-containing nuclear protein complexes such as RAP80 complex stabilises BRCA1 and suppresses HR. Several studies have demonstrated that inhibition of PARP1 does not prevent the HR pathway (Helleday, 2011). This implies that even though PARP1 binds to DSBs, it is not required for the HR repair of DSB. However, when BRCA1 poly ADP-ribosylation is dysfunctional, this causes excessive HR and manifestations of genome instability that have been implicated in tumour development, especially in individuals with *BRCA1*^{+/+} (Hu et al., 2014; Padella et al., 2022).

1.4.2 Other non-DNA repair roles of PARP1

Apart from its DNA repair roles, PARP1 is involved in various biological activities. For example, its pro-inflammatory roles have been reported in different cancers. PARP1 regulates the inflammatory process by acting as transcriptional coactivator/corepressor of nuclear factor- κ B, a family of proteins that are involved

in acute and chronic inflammatory processes. For instance, the acetylation of PARP1 by acetyltransferases P300/CBP leads to the activation of NF- κ B. Moreover, the activation of the NF- κ B pathway is involved in proliferative signalling, tumour invasion and metastasis, and tumour-promoting inflammation. Similarly, PARP1 activates extracellular regulated kinase (ERK) signalling, which promotes metastasis and angiogenesis. Furthermore, studies have demonstrated that PARP1 is involved in chromatin compaction, de-condensation and remodelling. This is achieved either by direct poly ADP-ribosylation of histones and other chromatin-associated proteins, or indirect control of the recruitment of chromatin remodelling factors. Additionally, PARP1 can regulate transcriptional processes by directly binding to promoters or other transcription factors. For example, in healthy melanocytes, inactive PARP1 binds to the CXCL1 promoter, thus preventing the binding of NF- κ B. However, in melanoma, the activation of PARP and PAR modification release the CXCL1 to bind NF- κ B. This enhances the expression of CXCL1 and the production of CXCL1 chemokine, a key effector of tumour growth (Ke et al., 2019).

In conclusion, PARP1 activity has been linked to lipid metabolism, ageing, and cell death (parthanatos, which will be discussed in detail later) (Mangerich and Bürkle, 2012; Fatokun, Dawson and Dawson, 2014; Kiss et al., 2015). These numerous roles of PARP1 show its significance as a molecular target for the development of therapeutics beyond the current application in cancer treatment.

1.4.3 PARP1 expression and activity

The PARP1 protein is encoded by the *PARP1* gene located on chromosome 1 (1q42.12) in human cells (Auer et al., 1989). PARP1 is the most abundantly

expressed member of the PARP superfamily, being responsible for about 85% of total cellular PARP enzyme activity and the production of over 95% of total PAR polymers in most cells (Mitra et al., 2019). It operates by sensing SSBs and utilising nicotinamide adenine dinucleotide (NAD⁺) as a substrate to catalyse the formation of poly(ADP)-ribose (PAR) chains, which recruit other DNA repair proteins and nucleases to the site of the DNA damage (Yu et al., 2002).

The nuclear concentrations of PARP1 range from 2×10^5 to 1×10^6 enzymes/nucleus in eukaryotic cells. Within thirty seconds of detecting DNA damage, PARP1 autoPARylates itself, which leads to about a 500-fold increase in its activity over its basal level (Alvarez-Gonzalez and Althaus, 1989; Jannetti et al., 2020). The increase in PARP1 activity correlates to higher PAR levels in the cells. In cancerous cells, where the PARP1 enzyme activity is found to be higher than in healthy cells, remarkably increased PAR formation has been reported compared to the levels in adjacent non-cancerous cells (Nosho et al., 2006).

Interestingly, PARP1 enzymatic activity and functions are regulated through various post-translational modifications. PARP1 can be modified through ADP-ribosylation by itself (automodification) and by other members of the PARP family such as PARP2 and PARP3, which enhances its activity. Likewise, other ADP-ribosylating enzymes such as sirtuin 6 (SIRT6) can act on PARP1, promoting double-strand DNA repair in oxidative stress. Phosphorylation of PARP1 by the kinases ERK1/2, JNK1 and activated calcium-dependent protein kinase (CaMKII) can modulate its catalytic activity. Other post-translational modifications are

acetylation and ubiquitylation/SUMOylation by protein acetyltransferases (p300 and CBP) and E3 ligases, respectively (Luo and Kraus, 2012).

The binding of PARP1 with DNA, nucleosomes and other nuclear proteins such as KIF4, histone variant mH2A1.1, Ku, and condensin I/XRCC1 repair complex can activate its enzymatic activity. NMNAT-1, a cellular NAD⁺ synthase, catalyses the final step of NAD⁺ (the substrate for PARP1) biosynthesis. NMNAT-1 binds to PAR on activated PARP1, thereby stimulating poly ADP-ribosylation (Berger, Lau and Ziegler, 2007).

PARP1 enzymatic activation consumes high amounts of NAD⁺. As a result, its end product, PAR, exhibits mild regulation of PARP1 by conferring on it a strong negative charge, which repels the enzyme from the negatively charged DNA, thereby stopping ADP-ribosylation. This action limits the consumption of NAD⁺. With a similar goal of converting ATP which requires NAD⁺ for its production, PARP1 activity is regulated during apoptosis by its cleavage. Caspase 3, a key enzyme in the apoptotic pathway, breaks PARP1 into two fragments between the second and third zinc-binding domains, one larger 89-kDa, and a shorter 24-kDa. This alters its DNA binding ability, consequently affecting its activity. PARP1 cleavage has also been reported in autophagic and necrotic cell death pathways by the action of various cathepsins (Ko and Ren, 2012).

PARP1 has been reported to be overexpressed in several types of cancer, including neuroblastoma, hepatocellular carcinoma, malignant melanomas, breast cancer, endometrial carcinoma, HPV-positive oropharyngeal carcinoma, gastric

cancer, malignant lymphoma, testicular and germ cell tumours, and Ewing's sarcoma (Min et al., 2019; Abuzenadah et al., 2022). PARP1 mRNA is overexpressed in up to 70% of colorectal adenoma cancer tissues and pT1 cancer tissues, but is only faintly detected in neighbouring non-tumour and hyperplastic polyps tissues. This overexpression is detected in a substantially larger percentage of pT1 cancer tissues than in adenoma tissues and correlates strongly with tumour size. Thus, PARP1 overexpression appears to characterise the early stage of sporadic colorectal carcinogenesis (Nosho et al., 2006). In leukaemia, PARP1 overexpression correlates with a poor survival rate (Hurley et al., 2019). Similarly, several studies have shown hyperactivity of PARP1 in cancers harbouring a *BRCA* mutation because of their significant reliance on PARP1 to repair SSBs through the BER pathway. Consequently, PARP1 is considered a critical target in the development of anticancer drugs, and the overexpression of PARP1 could serve as a biomarker for patients that could gain optimally from this therapy.

1.4.4 Mechanisms of action of PARP inhibitors

PARP inhibitors work as targeted anti-cancer agents on the basis of synthetic lethality. The genetic concept of synthetic lethality was first described in 1946 by Dobzhansky as a situation where a mutation in either one of two genes alone has no effect but combining mutations in both genes leads to cell death. However, it was not until 1997 that this concept was introduced into cancer therapeutics (Hartwell et al., 1997). Although initially used only as chemosensitizers in chemotherapy and radiotherapy, PARP inhibitors were later identified as synthetically lethal partners with cancers arising from a *BRCA* gene mutation, and

thus represent the first class of targeted cancer therapeutics to explore synthetic lethality.

PARP1 is a nuclear protein that plays a vital role in sensing DNA damage and facilitating DNA base excision repair (BER), which is key in SSB repair. Research has demonstrated that knocking down PARP1 expression with siRNA or inhibiting PARP1 activity with small molecules significantly reduced the cell's ability to repair its SSBs. This implies that PARP1 must be required for this DDR pathway (Mateo et al., 2019; Amin, Chaabouni and George, 2020). Tumour cells carrying germline or somatic *BRCA1* and/or *BRCA2* gene mutations, such as in some breast, prostate and ovarian cancers, utilise NHEJ for the repair of DSBs due to their deficiency in the HR pathway. The error-prone NHEJ is not effective and cannot cope with a large-scale DSB repair. Blocking the action of PARP1 in these cancer cells will cause excessive SSBs in them, which will eventually lead to DSBs. The sheer level of DSBs will then overwhelm the NHEJ pathway, causing serious genomic instability and ultimately resulting in cell death via apoptosis (Shah et al., 2014). Thus, PARP1 inhibition in HR-deficient cells is synthetically lethal. The synthetically lethal effect of PARP1 inhibition is not limited to cancer cells with *BRCA1/2* mutations alone but to cells that show other key gene dysfunctions in the HR pathway. For example, it has been demonstrated that 30%–50% of high-grade serous tumours may be sensitive to PARP inhibitors due to mutations in other HR repair genes (Luo et al., 2020b). Tumours with loss of HR proteins, including RAD51, RPA1, NBS1, ATR, ATM, CHK1, CHK2, FANCD2, FANCA, and FANCC, have similar biological and clinical behaviours to those with *BRCA1/2* mutations. This HR pathway deficiency confers a unique vulnerability to PARP inhibition and

is described as “BRCAness”. Studies have demonstrated that BRCAness can be induced. For example, Luo et al. (2020) created double BRCAness in NSCLC cell models (which lack *BRCA1/2* mutation) by knocking down the *BRCA1* and *RAD50* genes. The clonogenic survival assay revealed that the knockdown cells were significantly sensitive to the effect of fluzoparib (a PARP1 inhibitor) (Luo et al., 2020a). Recently, genome-wide profiling of synthetic genetic lethality revealed cyclin-dependent kinase 12 (CDK12) as one of the additional genes conferring sensitivity to PARPi (Keung et al., 2020). Similarly, the therapeutic benefit of this BRCAness has been highlighted in triple-negative breast cancers and such benefits include increased sensitivity to PARPi and overcoming chemoresistance (Underhill, Toulmonde and Bonnefoi, 2011; Lu et al., 2018; Boussios et al., 2019; Parkes, McCabe and Kennedy, 2019).

There are two distinct mechanisms that underlie PARPi-induced death of cancer cells (i.e., the efficacy of PARPi): enzymatic inhibition, which is the original mechanism that has been known for a while and which first underpinned the development of PARPi; and PARP trapping, which is a relatively recent understanding of the effects of PARP inhibition.

1.4.4.1 Enzymatic Inhibition

PARP1 recognises and binds to the sites of SSBs through the zinc-finger DNA-binding domains. It then catalyses the transfer of ADPrs from NAD⁺ to acceptor proteins that repair SSBs. The resulting poly (ADP-ribose) polymer (PAR) has a negative charge that helps recruit other positively charged DDR proteins necessary for the repair of SSBs. This enzymatic activity plays a major role in the BER

pathway. However, the enzymatic activity of PARP1 is targeted by PARPi, which compete with NAD⁺ at the catalytic site, thus preventing PARylation. Subsequently, PARP inhibitors disrupt the repair of SSBs, leading to the accumulation of double-strand DNA breaks (DSBs) through subsequent DNA replications. In normal cells, such DSBs are repaired mainly by homologous recombination repair (HR) using sister chromatids as a template, but in cancer cells with defective HR, DSB repair is attempted by an alternative pathway, non-homologous end-joining (NHEJ). NHEJ is error-prone and not effective in dealing with the sheer volume of DSBs that result from unrepaired SSBs. The accumulation of these DSBs leads to increased genomic instability, eventually resulting in cell death ([Figure 9](#)). This is a key mechanism by which PARPi exert cytotoxic activity (D'Andrea, 2018).

1.4.4.2 PARP Trapping

A turning point in the understanding of the mechanisms of action of PARPi was reached when Murai et al. (2012) first demonstrated that PARPi can trap the PARP1 and PARP2 enzymes at damaged DNA. They also showed that trapped PARP-DNA complexes were more cytotoxic than the accumulation of unrepaired SSBs caused by the pharmacological inhibition or genetic depletion of PARP. Although the precise mechanisms of locking or trapping PARP1 on damaged DNA are yet to be understood, there are speculations that PARP1 inhibitors prevent the release of PARP1 from DNA by inhibiting autoPARylation or causing allosteric changes in PARP1 structure that increase its stickiness to the DNA. Either way, the trapped PARP1 will cause stalling and the collapse of replication forks ([Figure 9](#)).

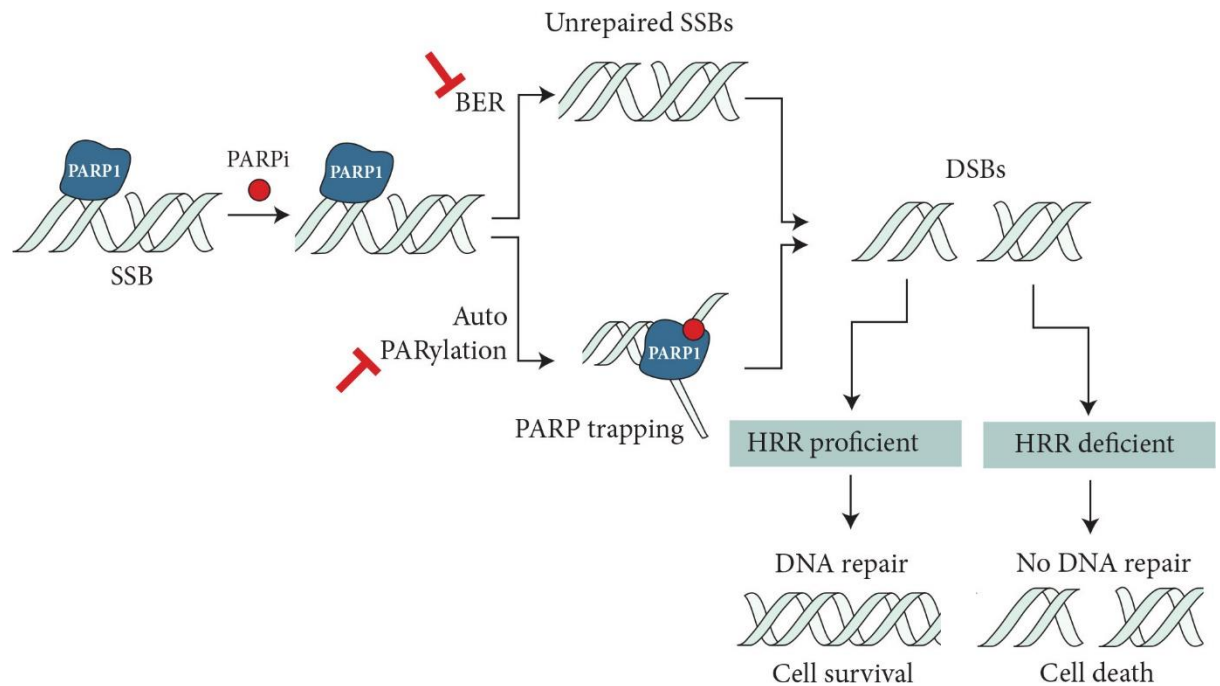


Figure 9: Mechanisms of PARP1 inhibition and trapping on the damaged DNA, leading to cell death. A PARP inhibitor will prevent the repair by PARP of single-strand DNA breaks (enzymatic inhibition of PARP) but could also trap PARP on the DNA damage site to form PARP-DNA complexes (PARP trapping), ultimately leading to double-strand breaks. In homologous recombination (HR)-deficient tumours, the resulting DSBs cannot be resolved, leading to cell death. Adapted from Mateo et al. (2019).

Further work has shown that the length of time for which PARP is trapped on DNA varies significantly based on the doses of the different PARPi. This implies that there is a link between their inhibitory potencies and their trapping potential. It has been demonstrated that weak PARP1 trappers, such as veliparib, do not give the same level of synthetic lethality in preclinical models when compared with effective trappers such as olaparib, rucaparib and niraparib. Consequently, veliparib, because of its lower trapping potency, has not demonstrated significantly increased antitumour activity when combined with neoadjuvant platinum-based chemotherapy. Current studies seem to show that new inhibitors with better PARP trapping are more potent PARPi, suggesting that PARP trapping is important in

improving the efficacy of PARPi in cancer treatment. Trapped PARP-DNA complexes were more cytotoxic than unrepaired, single-strand breaks caused by PARP inactivation (Murai et al., 2012). However, this high trapping potential might prove to be a liability for other promising non-oncological applications of PARPi, such as for neuroprotection (Boussios et al., 2019; Hoch and Polo, 2019; Mateo et al., 2019).

In mammals, the BER is accelerated by PARPs and XRCC1, a scaffold protein. PARPs detect SSB intermediates, while XRCC1 assembles DNA ligase III and interacts with protein complexes containing DNA polymerase β , which stalls the activity and excessive engagement of PARP1. During BER, excessive PARP1 engagement is a threat to genomic stability, as it becomes trapped on BER intermediates in a fashion similar to that facilitated by PARP inhibitors. This trapping results in BER intermediates becoming inaccessible to enzymes such as DNA polymerase β , so repair is stymied. Thus, XRCC1's role as an 'anti-trapper' that hinders this toxic PARP1 activity is critical. Unsurprisingly, in XRCC1-deficient cells, excessive PARP1 engagement occurs without restraint. In such cases, PARP1 deletion leads to the recovery of the BER pathway, and resistance to base damage can resume (Demin et al., 2021).

Other proposed mechanisms of PARPi-induced cell death include disrupting BRCA1 recruitment to damaged DNA and the enhancement of error prone NHEJ. All these mechanisms lead to the development of synthetic lethality in cancer cells with established BRCAness (Mirza, Pignata and Ledermann, 2018; Durmaz-Sahin, Jönsson and Hedenfalk, 2019; Mateo et al., 2019; Sato et al., 2019).

1.5 PARP inhibitors

The activity of PARP enzymes was first reported in 1963 (Chambon, Weill and Mandel, 1963). It was revealed that they were dependent on DNA and NAD⁺ as a substrate. In 1980, Purnell and Whish reported the first synthetic PARP inhibitor, 3-aminobenzamide (3-AB), a structural analogue of NAD⁺, following their observation that nicotinamide and 5-methylnicotinamide competed with NAD⁺ as a PARP1 substrate. 3-AB inhibits PARP to 96% but is required in millimolar concentrations, and its lack of specificity also inhibits de novo purine synthesis (Purnell and Whish, 1980; Milam and Cleaver, 1984). However, it was not until 1996 that the PARP-inhibitory activity of 3-aminobenzamide was noted as a chemosensitizer of cisplatin in the treatment of ovarian cancer (Bernges and Zeller, 1996). This led to the rise of the first generation of PARP inhibitors, which were merely substituted nicotinamide or benzamide. A major advancement in the use of PARP inhibitors as chemosensitizers in cancers occurred in 2005 when it was first demonstrated in a proof-of-concept trial that PARP inhibitors were synthetically lethal in *BRCA1*^{-/-} and *BRCA2*^{-/-} cells, but not in *BRCA*^{+/-} cells (Bryant et al., 2005; Farmer et al., 2005). Following this discovery, several analogues of benzamide with greater potency were developed as second-generation PARP inhibitors. Now, we have third-generation inhibitors with greatly improved properties belonging to a diverse family of chemical structures, such as derivatives of imidazopyridine, imidazoquinolinone, and isoquinolindione (Fatokun, Dawson and Dawson, 2014; Moreno-Lama et al., 2020).

Presently, four PARP inhibitors have been approved by the US Food and Drug Administration (FDA) and/or the European Medicines Agency (EMA) and are in

clinical use. These are (in the order of their approvals from 2014 to 2018): olaparib, rucaparib, niraparib and talazoparib ([Figure 10](#), [Table 1](#)). In order to identify patients who are eligible for and will benefit the most from PARPi, several companion diagnostic tests (CDx) have been developed to detect somatic or germline *BRCA1/2* mutations. The companion diagnostics not only identify *BRCA* mutations but also alterations in the other HR pathway genes, which are expressed as homologous recombination deficiency (HRD) status. Patients with *BRCA* mutations and/or HRD-positive status have shown significantly higher progression survival benefits from PARPi compared to patients with wild-type *BRCA*/HRD-negative or HR proficient tumours (Mateo et al., 2019; Kyo et al., 2022; Paulet et al., 2022). Currently, there are five FDA-approved, commercially available companion diagnostics used in determining the eligibility of cancer patients for any of the four clinically approved PARPi. These companion diagnostic tests include MyChoice HRD, BRACAnalysis, FoundationOne, FoundationFocus CDxBRCA Assay and FoundationOne Liquid CDx (Morice et al., 2021).

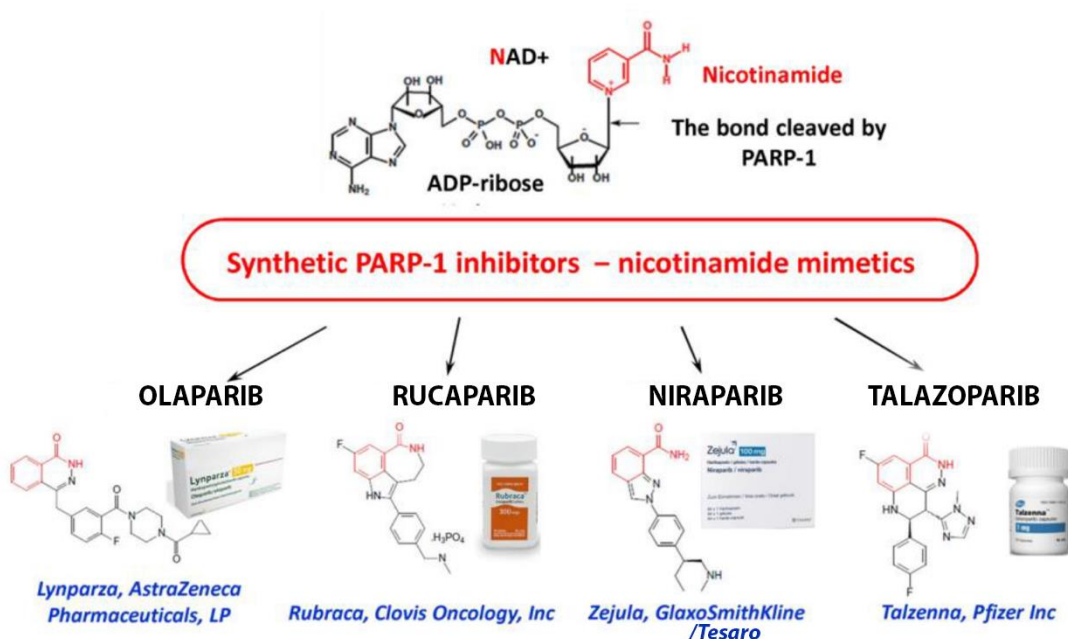


Figure 10: Chemical structures and manufacturers of the four approved and clinically used PARP inhibitors (PARPi). Most PARPi are designed to mimic the nicotinamide moiety of NAD⁺ (in red), enabling them to compete for the NAD⁺-binding site on PARP1. In niraparib, this mimicry is achieved using a rotationally constrained primary benzamide, while in olaparib, talazoparib, and rucaparib, the benzamide motif is embedded in a ring. Some PARPi possess a fluorine atom in the meta-position of their NAD⁺-mimicking benzamide moiety, which enhances their ability to bind to their target. All the approved PARP inhibitors are PARP1/2 inhibitors, while olaparib and rucaparib also inhibit PARP3. In addition, rucaparib inhibits PARP5a and PARP5b (tankyrases 1 and 2). The brand names and their pharmaceutical manufacturers are highlighted in blue. Adapted from Malyuchenko et al. (2021).

Table 1: US Food and Drug Administration (FDA) and European Medicines Agency (EMA): approval history of the four clinically approved PARP inhibitors. Adapted from Rose et al. (2020)

PARP inhibitor	Approving organization	Year of approval	Indication	Mutational requirement	Relevant studies
Olaparib	FDA and EMA	2014	Advanced ovarian carcinoma	Germline BRCA1/2 Mutation	NCT0107662 (Kaufman et al., 2015)
	FDA and EMA	2017	Reoccurring ovarian, fallopian and primary peritoneal carcinoma	Independent of BRCA1/2 Mutational Status	SOLO-2 (Pujade-Lauraine et al., 2011) and Study 19 (Friedlander et al., 2011)
	FDA EMA	2018 2019	HER-2 negative breast cancer	BRCA1/2 Mutated	OlympiAD (Robson et al., 2017)
	FDA EMA	2018 2019	First-line treatment of advanced ovarian, fallopian and primary peritoneal carcinoma	Germline BRCA1/2 Mutation Complete or partial chemotherapy response.	SOLO-1 (Moore et al., 2018)
	FDA	2019	Metastatic pancreatic cancer	BRCA1/2 Mutated	POLO (Golan et al., 2019)
	FDA	2020	First-line treatment of advanced ovarian, fallopian and primary peritoneal carcinoma in combination with Bevacizumab	HRD-Positive Complete or partial chemotherapy response.	PAOLA-1 (Ray-Coquard et al., 2019)
	FDA	2020	Metastatic castration-resistant prostate cancer	HRD-positive	PROfound (de Bono et al., 2020)
Rucaparib	FDA EMA	2016 2018	Advanced ovarian carcinomas, following multiple chemotherapy treatments	BRCA1/2 Mutated	ARIEL2 and Study 10 (Oza et al., 2017)
	FDA EMA	2018 2019	Reoccurring ovarian, fallopian and primary peritoneal carcinoma	Independent of BRCA1/2 Mutational Status	ARIEL3 (Coleman et al., 2017)
	FDA	2020	Metastatic castration-resistant prostate cancer	BRCA1/2 Mutated	TRITON2 (Abida et al., 2019)
Niraparib	FDA and EMA	2017	Reoccurring ovarian, fallopian and primary peritoneal carcinoma	Complete or partial chemotherapy response.	ENGOT-OV16/NOVA Study (Mirza et al., 2016)
	FDA	2019	Reoccurring ovarian, fallopian and primary peritoneal carcinoma	HRD-positive Independent of chemotherapy response	QUADRA Study (Moore et al., 2019)
	FDA and EMA	2020	Advanced ovarian carcinomas and primary peritoneal carcinoma	Independent of biomarker status Complete or partial chemotherapy response.	PRIMA Study (Gonzalez-Martin et al., 2019)
Talazoparib	FDA and EMA	2018	Advanced or metastatic HER2-negative breast cancer	Germline BRCA1/2 Mutated	EMBRACA Study (Ettl et al., 2018)

1.5.1 Olaparib (Lynparza)

Olaparib is an orally administered PARP inhibitor. Prior to its approval, in the Phase 1 trial olaparib (AZD2281) demonstrated antitumour activity at the maximum

tolerated dose of 400 mg twice daily, with reversible dose-limiting toxicity. 22 out of 60 patients in the cohort were carriers of *BRCA1* or *BRCA2* mutations in their ovarian, breast, or prostate cancers. Notably, only the patients with pathogenic *BRCA* mutations showed clinical improvement from the treatment with olaparib, with partial or complete radiologic response and stabilisation of disease for 16 weeks or more (Fong et al., 2009). Follow-up Phase 2 trials where objective response rate was the endpoint further validated previous results. Objective response rate was defined as the proportion of patients with reduction in tumour size of a predefined amount and for a minimum time period. Olaparib 400 mg was administered twice daily to a group of advanced breast or ovarian cancer patients with germline *BRCA1/2* mutations. They experienced an objective response rate of 41% and 33%, respectively. In a similar trial, high-grade serous and/or undifferentiated ovarian cancer patients showed an objective response rate of 41% (with *BRCA1/2* mutations) and 24% (without *BRCA1/2* mutations), but TNBC (Triple-negative breast cancer) patients showed no response (Audeh et al., 2010; Tutt et al., 2010).

Another critical Phase 2 trial was study 19. This was a randomised, double-blind, controlled trial with 265 platinum-sensitive, high-grade ovarian cancer patients (four times more in population size compared to the previous trials). All patients were relapsed and had recently received 2 or more rounds of platinum-based chemotherapy. The efficacy of olaparib was measured by progression-free survival (PFS), defined as the time from randomisation to objective disease progression or death. Patients received either olaparib capsules 400 mg twice daily or a placebo, with the olaparib group having 36% germline *BRCA* mutation. PFS for olaparib, 8.4

months, was significantly longer than for the placebo, 4.8 months. Also, of the patients with *BRCA* mutation, PFS was significantly longer in the olaparib group than in the placebo group, 11.2 vs. 4.3 months; likewise for the patients with wild-type *BRCA*, 7.4 vs. 5.5 months. 73% of the patients who received olaparib reported nausea (grade 1 to 3), compared to 32% on the placebo. Other grade 3 reactions in the olaparib group included fatigue (7%), anaemia (5%), and neutropenia (4%) (Ledermann et al., 2012; Ledermann et al., 2014). Based on the Study 19 findings, in October 2014 the EMA approved the use of olaparib as maintenance therapy in patients with a germline or somatic *BRCA* mutation who had previously responded to a platinum-based regimen. But the FDA was hesitant because earlier trials had reported that 2.2% of patients treated with olaparib later developed myelodysplastic syndrome (MDS) or acute myeloid leukaemia (AML) compared to 0% in the placebo group. The risk was later revised to below 1.5%, and consequently olaparib was approved by the FDA in December 2014 for the maintenance treatment of patients with deleterious or suspected deleterious germline *BRCA*-mutated advanced ovarian cancer who were treated with three or more previous lines of chemotherapy (Parkes, McCabe and Kennedy, 2019).

Further Phase 2 trials (Study 42 and TOPARP) and Phase 3 trials (OlympiAD, SOLO2, SOLO1 and POLO study) have led to additional approvals by the FDA and/or EMA. For example, the reduction of daily pill burden from 400 mg (16 capsules per day) to 300 mg (four tablets per day). In addition, olaparib has been approved for first-line maintenance of platinum-sensitive epithelial ovarian, fallopian tube, or primary peritoneal cancer, regardless of *BRCA* mutation status. Also, it has been approved for the treatment of germline/somatic *BRCA1/2*-mutated

human epidermal growth factor receptor 2 (HER2)-negative, high-risk, early and metastatic breast and metastatic pancreatic cancer patients who have been treated with neoadjuvant or adjuvant chemotherapy (Slade, 2020).

1.5.2 Rucaparib (Rubraca)

Rucaparib (AG014699) was the first PARP inhibitor to enter anticancer clinical trials and has its developmental origin in Newcastle University, United Kingdom (Curtin, 2020). Phase 1 and 2 trials established a 67% objective response rate in women with platinum-sensitive, relapsed, high-grade ovarian carcinoma when orally administered 600 mg twice daily. Further Phase 2 trials, Study 10 and ARIEL2, investigated 106 advanced, *BRCA*-mutant ovarian cancer patients who had received two or more prior chemotherapies. Patients were divided into three predefined HRD subgroups based on their tumour genetic analysis, namely (1) deleterious germline or somatic *BRCA* mutant, (2) *BRCA* wild-type and loss of heterozygosity-high (LOH-high) group, and (3) *BRCA* wild-type and LOH-low (LOH-low) group. PFS was the primary endpoint in ARIEL2 and objective response rates were used in Study 10. All the patients received 600 mg rucaparib tablets twice daily for 28 days. The median PFS for rucaparib in patients with *BRCA* mutations (12.8 months) was significantly longer compared with patients with high and low loss of heterozygosity (5.7 and 5.2 months, respectively). Study 10 showed an objective response rate of 54%. Similarly, the Phase 3 trial, ARIEL3, reported a median PFS of 26.8 months in high-grade, recurrent, platinum-sensitive ovarian carcinoma with germline or somatic *BRCA* mutation, compared to 5.4 months for the placebo (Coleman et al., 2017). Based on these data, rucaparib was approved in December 2016 by the FDA for the treatment of

germline/somatic *BRCA* mutation advanced ovarian cancer patients who have received two or more previous lines of chemotherapy. More recently, it has been approved by the FDA as monotherapy treatment for patients with *BRCA1/2*-mutant and metastatic, castration-resistant prostate cancer. The common grade 3 side effects included anaemia, nausea, constipation, diarrhoea, dysgeusia, abdominal pain, fatigue/asthenia, neutropenia, increased cholesterol, thrombocytopenia, decreased appetite, dyspnoea, vomiting, and increased need for liver function tests (Swisher et al., 2017; Swisher et al., 2021).

1.5.3 Niraparib (Zejula)

Niraparib is an oral PARP inhibitor with high selectivity for PARP 1/2. In a Phase 1 dose-escalation study, niraparib (MK4827) showed antitumor activity in platinum-resistant, high-grade, serous ovarian cancer and sporadic prostate cancer with the maximum tolerated dose of 300 mg daily (Sandhu et al., 2013). QUADRA, a Phase 2 study, assessed the activity of niraparib monotherapy in relapsed, high-grade, serous (grade 2 or 3) epithelial ovarian, fallopian tube, or primary peritoneal cancer patients who had received three or more previous chemotherapy regimens. The overall response was 28% according to RECIST (Response Evaluation Criteria in Solid Tumours). These findings led to the multinational Phase 3 trial, the NOVA Study, in which 553 platinum-sensitive, recurrent ovarian cancer patients were evaluated. The primary endpoint of the trial was the duration of PFS. The group was divided into two cohorts, those with germline *BRCA* mutation (gBRCAm), and those with wild-type *BRCA* with HR deficiency (HRD-negative). Patients were randomised to receive either niraparib capsules 300 mg twice daily or a placebo within 8 weeks of prior chemotherapy. The median PFS for niraparib, 21

months, in the gBRCAm subgroup was longer than for the placebo, 5.5 months. In the wild-type cohort, the median PFS for niraparib was 9.3 months vs. placebo's 3.9 months. The predominantly grade 1 or 2 treatment-related toxic effects were anaemia, neutropenia, thrombocytopenia, constipation, anxiety, dyspnoea, nausea, urinary tract infection, insomnia, fatigue, anorexia, hypertension, vomiting, and xerostomia. Another key point in this trial was that 1.4% of patients who received niraparib developed myelodysplastic syndrome, and there were three deaths during the follow-up period associated with the treatment (Mirza et al., 2016). In March 2017, niraparib became the first, once-daily PARP inhibitor to be approved by the FDA for the maintenance treatment of recurrent epithelial ovarian/fallopian tube and primary peritoneal cancers where there has been a partial or complete response to platinum-based therapy, regardless of *BRCA* or other HRD biomarker status (Scott, 2017).

1.5.4 Talazoparib (Talzenna)

Talazoparib (BMN 673) is the most potent oral PARP inhibitor to date. In Phase 1 trial, 110 patients with various advanced cancers, including 59 with *BRCA* mutation, were administered 1 mg once daily. The objective response rates for *BRCA*-mutated ovarian and breast cancer were 41.7% and 50%, respectively. Notably, there were no objective responses among the 14 Ewing sarcoma patients. In the follow-up Phase 2 (ABRAZO), talazoparib (1 mg/day) showed a 21% - 37% objective response rate in *BRCA*-mutated advanced breast cancer patients, while Phase 3 (EMBRACA) showed the PFS was longer in the talazoparib cohort (8.6 months) compared with the cohort that received standard therapy (5.6 months). The most common grade 3/4 toxicities were anaemia (39.2%), neutropenia

(20.9%), and thrombocytopenia (14.7%). Other side effects observed include fatigue, nausea, diarrhoea, vomiting, headache, and alopecia (Turner et al., 2017). Talazoparib was approved in October 2018 by the FDA for treating patients with deleterious or suspected deleterious, germline, *BRCA*-mutated (gBRCAm), HER2-negative, locally advanced or metastatic breast cancer.

1.5.5 Other PARP inhibitors in development

Currently there are more than 70 PARP inhibitors undergoing clinical trials (Deveshegowda et al., 2022). For example, veliparib showed an overall objective response rate (ORR) ranging from 14.3% to 79%. Veliparib is in Phase III of clinical development, still being investigated for its efficacy and safety as a single agent or in combination with chemotherapy (Ramalingam et al., 2021). Another example is fluzoparib, which has effectively inhibited PARP1 enzyme activity and sensitised both HR-deficient and HR-proficient cells to cytotoxic drugs. In on-going trials, fluzoparib has demonstrated good pharmacokinetic properties and a favourable toxicity profile. Fluzoparib in combination with another anti-cancer drug apatinib or paclitaxel showed remarkable antitumor activity without increased toxicity. Pamiparib has also displayed good bioavailability and has been shown to be 16-fold more potent than olaparib in an efficacy study using a *BRCA1*-mutated MDA-MB-436 breast cancer xenograft model (Boussios et al., 2019; Wang et al., 2019).

It is important to note that some potent PARP inhibitors did not progress to clinical trials but are now widely used as chemical tools for *in vitro* and *in vivo* probing of PARP activity. These PARPis are mostly PARP1 inhibitors and examples include 3-aminobenzamide (3-AB) ($IC_{50} = 30 \mu M$) (Ferraris, 2010), 4-hydroxyquinazoline

(IC₅₀ = 9.5 μ M) (Halmosi et al., 2001); 1,5-isoquinolinediol (IC₅₀ = 0.37 μ M) (Crockett et al., 2020); 3-methyl-5-AIQ (IC₅₀ = 0.23 μ M) (Sunderland et al., 2011), 4-ANI (IC₅₀ = 0.18 μ M) (Banasik et al., 1992), EB-47 (IC₅₀ = 0.045 μ M) (Jagtap et al., 2004), minocycline (IC₅₀ = 0.042 μ M) (Alano et al., 2006), NU1025 (IC₅₀ = 0.4 μ M) (De Soto and Deng, 2006), TIQ-A (IC₅₀ = 0.45 μ M) (Chiarugi et al., 2003), DPQ (IC₅₀ = 3.5 μ M) (Moroni et al., 2001), and INO-1001 (IC₅₀ = 0.05 μ M) (Brock et al., 2004). Others are inhibitors of both PARP1 and PARP2, such as PJ-34 (IC₅₀ = 0.11 and 0.086 μ M for PARP1 and PARP2, respectively), DR2313 (IC₅₀ = 0.2 and 0.24 μ M) (Nakajima et al., 2005); 3-(4-chlorophenyl)quinoxaline-5- carboxamide (IC₅₀ = 0.033 and 0.007 μ M) (Iwashita et al., 2004), UPF1035 (IC₅₀ = 9 and 0.15 μ M), and UPF1069 (IC₅₀ = 8 and 0.3 μ M) (Moroni et al., 2009). PJ34 and DPQ are among the most commonly used experimental PARPis (Scalia et al., 2013).

Most of these emerging or clinically approved PARPi are designed to mimic the NAD⁺ or nicotinamide structure (Deveshegowda et al., 2022). Recent studies have reported novel PARPi with non-NAD-like structure. 5F02, a non-NAD-like PARPi, exhibited strong anticancer activity in cell and animal models of human prostate cancer (Karpova et al., 2019). Similarly, a new class of PARP1 inhibitors that specifically target the histone-dependent route of PARP1 activation, a mechanism of activation that is unique to PARP1, elicited a significant decrease in clonogenic survival and cell viability of prostate and kidney cancer cells (Makhov et al., 2021). These represent some exciting future prospects for the new classes of PARPi that could help overcome the limitation of NAD-like PARPi, such as off-target effects and drug resistance.

1.6 PARP inhibitors and parthanatos

While PARP has a distinct and important physiological role, the understanding of which has been successfully harnessed for targeted cancer therapy as already explained, it has also been known to contribute to pathological scenarios. The switch from a beneficial physiological role to a detrimental pathological role is underpinned by the degree of PARP activation, which consequently determines the amount and complexity of the polymer (PAR) that it generates. PARP activation that generates mild-to-moderate levels of PAR is consistent with its physiological function, while PARP over-activation that produces a surplus amount of PAR is known to induce cell death. The latter is particularly relevant to neuronal death. In this context, the death that is caused by over-activation of PARP1 has been termed 'parthanatos,' a portmanteau word that comprises 'thanatos', the personification of death in Greek mythology, and 'par', the polymer generated by PARP, thus portraying 'parthanatos' as 'PAR-induced cell death', with PAR being generated by PARP, meaning that parthanatos could also be defined as 'PARP-mediated cell death'. It should be noted that while PARP does not leave the nucleus where it is localised, PAR as its messenger can exit the nucleus to act as a death signal (Andrabi et al., 2006).

Galluzzi et al. (2018) defined parthanatos as a form of regulated cell death driven by the hyperactivation of PARP1. Parthanatos is known to be caused by prolonged exposure to DNA-alkylating agents such as MNNG. Similarly, cellular insults that can trigger DNA damage such as oxidative stress, nitrosative stress, inflammation and glutamate-induced excitotoxicity could induce parthanatos, especially in neurones (Galluzzi et al., 2018).

Parthanatos has been widely implicated in many neurological conditions and neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, stroke, Amyotrophic lateral sclerosis, Huntington's disease and ischaemia-reperfusion injury (Yang et al., 2020; Park et al., 2022). When PARP1 is over-activated, it produces a prodigious amount of PAR of varying complexities. PAR, which has been demonstrated to be a death signal, causes nuclear translocation of the mitochondrially-localised apoptosis-inducing factor (AIF), resulting in large-scale DNA fragmentation and nuclear condensation, and ultimately cell death ([Figure 11](#)) (Andrabi et al., 2006; Fatokun, Dawson and Dawson, 2014). More recently, it has been further clarified that AIF causes the recruitment of the nuclease, macrophage migration inhibitory factor (MIF), to the nucleus, where MIF cleaves genomic DNA into large fragments (Wang et al., 2016). MIF has thus been recognised as a PARP1-dependent, AIF-associated nuclease (PAAN) (Wang et al., 2016). The entire process as described, from the insult-induced overactivation of PARP to the eventual parthanatic death of neurones is described as the PARP signalling or parthanatos cascade, which offers opportunities for pharmacological or similar interventions at various levels of the cascade in order to achieve neuroprotection. It should be mentioned that, previously, the death of neurones as a result of PARP overactivation was thought to be due to the excessive consumption and thus depletion of NAD^+ and ATP, which caused the neurones to die in a characteristically necrotic manner (as apoptosis could not proceed in the absence of energy) (the suicide hypothesis) (Ha and Snyder, 1999), but it is now known that, rather than the energy depletion, it is the PARP overactivation-mediated, PAR-induced triggering of AIF translocation and a raft of further downstream signalling events as earlier described that is principally responsible for

the cell death (Yu et al., 2002). Interestingly, parthanatos is a caspase-independent cell death and remarkably different from apoptosis. Although it shares some cytological and morphological features of apoptosis and necrosis, the co-involvement of PARP1, PAR and AIF still characterises parthanatos ([Table 2](#)) (Fatokun, Dawson and Dawson, 2014).

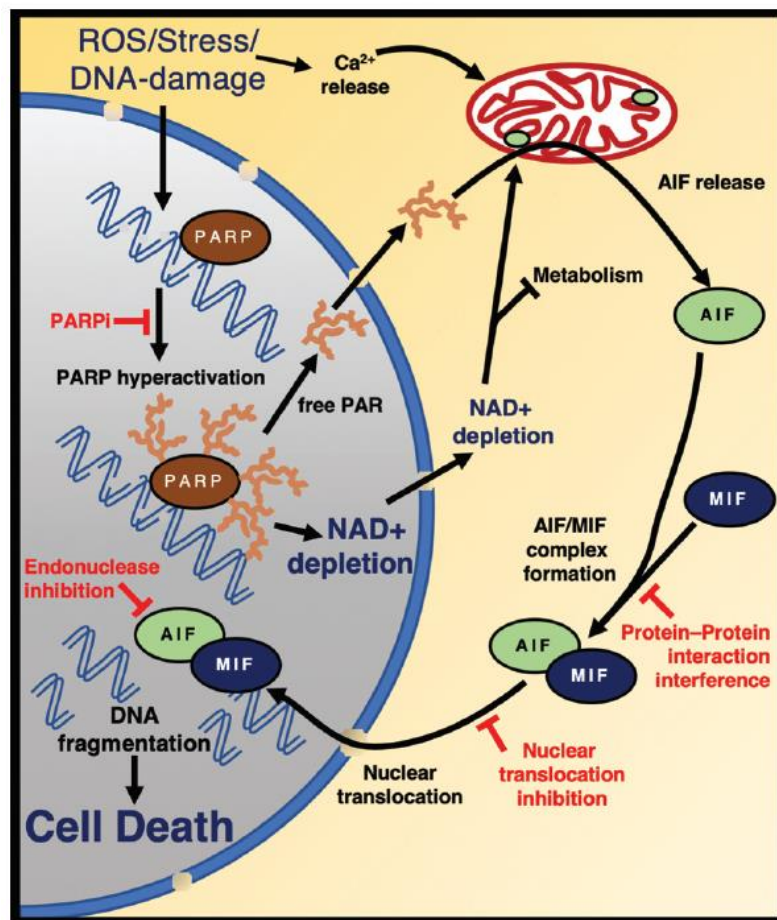


Figure 11: Schematic representation of PARP1-mediated cell death (parthanatos). Overactivation of PARP1 caused by oxidative stress leads to excessive production of poly (ADP-ribose) (PAR). PAR triggers a cascade that involves AIF release from the mitochondria and the translocation of the AIF/MIF complex to the nucleus where it ultimately causes cell death because of strong endonuclease activity (Hoch and Polo, 2019).

Table 2: Distinctive features of four different molecular mechanisms of cell death. While over ten cell death modalities have now been established, the table below compares parthanatos with the three major morphologically distinct types of cell death, namely apoptosis, necroptosis and autophagic cell death (Galluzzi et al., 2018). Adapted from Park et al. (2020).

Forms of death		Parthanatos	Apoptosis	Necroptosis	Autophagic cell death
Trigger		Genotoxic stress, Excitotoxicity	Death receptor (extrinsic), Intrinsic	Activation of death receptors, Inhibition of caspase-8	Starvation, Rapamycin
Cell morphology	Membrane	Loss of membrane integrity	Cell membrane blebbing Apoptotic body	Cellular swelling, Membrane rupture	Cell membrane blebbing
	Nucleus	Chromatin condensation, Large DNA fragmentation	Chromatin condensation, Small DNA fragmentation	–	–
	Cytosol	Mitochondrial depolarization, Cell shrinkage	Mitochondrial depolarization, Cell shrinkage	Mitochondrial swelling	Autophagic vacuoles, Increased lysosomal activity
Key regulators		PARP-1, AIF, MIF	Caspases, Bax, Bak, APAF1, cytochrome <i>c</i>	RIP1/3, MLKL	Atg proteins, Beclin-1
Inhibitors		PARP inhibitors, MIF nuclease inhibitors (putative), PARG, Iduna	Caspases inhibitors, Bcl-2	Necrostatin-1	3-Methyladenine, Wortmannin
Energy (ATP) requirement		No	Yes	No	Yes
Cellular markers		PARP1 activation, PAR accumulation, AIF and MIF translocation to nucleus	Caspase activation	RIPK3 activation	Autophagosome formation, Lysosomal degradation

Recent studies have exploited the unique features of parthanatos, with a view to using protection against it as a surrogate for anti-PARP activity or activity against the PARP signalling cascade. A PARP inhibitor in this context would then be neuroprotective. One of these studies prospecting for neuroprotective PARPi revealed that two natural or naturally derived compounds, 4'-methoxyflavone and 3',4'-dimethoxyflavone, protected against parthanatos and were thus characterised

as neuroprotective PARP inhibitors (Fatokun et al., 2013). Apart from being involved in neuronal cell death, the parthanatos pathway has been implicated in other diseases, including diabetes mellitus, heart attacks, pulmonary fibrosis, and acute respiratory distress syndrome (Yu et al., 2006; Künzi and Holt, 2019). The use of PARP inhibitors to prevent or treat disease conditions involving parthanatos, therefore, seems promising. However, certain aspects of the molecular mechanisms of parthanatos are yet to be clarified. A better understanding of this pathway could lead to the development of new treatments for a wide range of diseases (Hoch and Polo, 2019). This therefore raises the prospect of repurposing PARPi, and the concept of drug repurposing is now discussed.

1.6.1 Drug repurposing of PARP inhibitors in the treatment of non-oncological diseases

Drug repurposing aims to develop an existing drug (or drug candidate) for use in treating diseases other than the one(s) for which it was originally developed. This strategy is usually more systematic and considerably less expensive and time-consuming compared to conventional drug discovery programmes. Drug repurposing explores the pharmacological data of approved, preclinical and even failed therapeutics to discover new medical uses (Sahaboglu et al., 2016). Many *in vitro* and *in vivo* studies have confirmed the involvement of PARP in other pathologies apart from cancers. Therefore, although approved and preclinical PARPi are currently focused mostly on cancer therapy, there is now mounting evidence suggesting that PARPi could be repurposed for other diseases, especially in the context of achieving neuroprotection in neurological and neurodegenerative conditions such as stroke, Parkinson's disease, amyotrophic

lateral sclerosis, Huntington's disease and Alzheimer's disease (Martire, Mosca and d'Erme, 2015; Berger et al., 2018; Thapa et al., 2021; Zhao et al., 2021).

Cerebral ischaemia or ischaemic stroke is a global health issue that affects more than 3% of the adult population, resulting in lifelong disability or death. Up until now, the recombinant tissue plasminogen activator (tPA) is the only medicine approved by the FDA for the treatment of ischaemic stroke. However, the safety and effectiveness of tPA is limited to 3 to 4.5 hours after ischaemic stroke onset, and a vast majority of patients do not receive tPA administration due to its narrow therapeutic time window (Venkat et al., 2017). In view of this dire need for novel therapies for stroke, the reports of PARP1 inhibition significantly reducing post-ischaemia/reperfusion(I/R)-induced brain damage are reassuring. There is thus a promising opportunity to reposition PARPi for treating stroke. In 2016, Xu et al. demonstrated that DPQ, veliparib, rucaparib, olaparib and talazoparib each attenuated neuronal cell death in an ischaemic, cell-based model derived by oxygen and glucose deprivation (Xu et al., 2016). Hamby et al. (2007) reported that PJ34, a PARP inhibitor, reduced neuronal cell death by over 80%, even up to 8 hours after transient ischaemia–reperfusion in mouse models. This was corroborated more recently by Jiao et al. (2021) when they demonstrated that the inhibition of PARP1 by PJ34 mitigated I/R-induced brain damage via targeting over-activation of inflammatory response. Similarly, the PARPi JPI-289 was able to effect up to 53% decrease in infarct volume as late as 10 hours after stroke onset in a tMCAO (transient middle cerebral artery occlusion) stroke model in monkeys. Also, other PARP inhibitors, such as MP-124, nicotinamide, 3-aminobenzamide, 5-aminoisoquinolinone, 4-amino-1,8-naphthalimide, and 1,5 isoquinolinediol have

demonstrated protection against cell death induced by cerebral I/R injury and neurotoxins (Hamby et al., 2007; Han et al., 2020; Alhosaini et al., 2021; Jiao and Li, 2021; Yilmaz, Alkan and Ballar Kirmizibayrak, 2021).

Another neuropathology for which PARPi repurposing is promising is Alzheimer's disease (AD). This neurodegenerative disease is the most common type of dementia. AD is characterised by accumulation of extracellular amyloid- β ($A\beta$) peptide and hyperphosphorylated tau protein, which eventually trigger excessive production of intracellular free radicals. The increased oxidative/nitrosative stress leads to DNA damage and subsequently overactivation of PARP, which thus implicates PAR-mediated neuronal cell death (parthanatos) (Feng et al., 2019). The PARPi PJ34 has also been reported to suppress $A\beta$ -induced microglial activation and neurotoxicity in a mouse model (Kauppinen et al., 2011). Recently, Maggiore et al. (2022) suggested two different mechanisms of neuroprotection by PARP1 inhibition in *Drosophila* models of AD. They demonstrated that PARPi olaparib and MC2050 antagonised the aggregation of toxic $A\beta$ oligomers while also suppressing the AD-induced transposable element activation by epigenetically regulating chromatin structure and function (Maggiore et al., 2022). This finding notably reveals other mechanisms that might be involved in neuroprotection by PARPi in AD, apart from the PARPi preventing the accumulation of PAR.

Parthanatos has also been implicated in Parkinson's disease (PD), another common neurodegenerative condition and the most common movement disorder; therefore, PARPi can play a role in treating PD. In PD mouse models simulated by the injection of 6-hydroxydopamine (6-OHDA), genetic deletion of PARP1

prevented PD-related symptoms by suppressing AMP protein kinase (AMPK) and the nuclear translocation of AIF (Park et al., 2020). Kam et al. (2018) showed that the inhibition or deletion of PARP1 significantly reduced neurotoxicity induced by the transmission of pathologic α -synuclein in a PD murine model (Kam et al., 2018).

There are various studies indicating the potential benefits of PARPi in chronic inflammatory and immune-mediated diseases. For example, the PARPi minocycline reduces the apoptosis and alleviates inflammatory response after myocardial infarction through inhibiting PARP1 (Zhao, Zhang and Hong, 2018). PARP 1 inhibition has also been reported to improve microvascular function in type 2 diabetes mellitus, prevent hypertension-induced cardiac remodelling, block established asthma, mitigate elastase-induced lung inflammation/emphysema, and promote sleep (Choi et al., 2012; Ghonim et al., 2015; Wang et al., 2018; Zada et al., 2021). More recently, Curtin et al. (2020) reviewed the possibility of repurposing PARPi to combat the current global pandemic (COVID-19) caused by SARS-CoV-2. One of the serious complications of COVID is acute lung injury in which upregulation of PARP1 gene expression is involved in a cascade of events with other receptors. Murine models and preclinical trials have shown that PARP inhibitors can decrease key interleukins such as IL-1, IL-6, and TNF- α in SARS-CoV-2-induced cytokine storm and thus alleviate lung fibrosis after infection (Curtin et al., 2020; Ummarino, Hausman and Di Ruscio, 2021).

Although PARPi have shown potential therapeutic benefits in cellular and animal models of non-cancer related diseases, there are unresolved concerns about whether long-term exposure may have a detrimental effect on healthy cells.

Nevertheless, identifying PARPi with novel chemical scaffolds will enhance the prospect of developing new, more effective and safer drugs against cancer and drug repurposing them, especially for the treatment of neurodegenerative diseases.

1.7 PARP inhibitor resistance

Despite the promising prospect of PARPi, one of the emerging problems with their clinical use is the development of chemoresistance. PARPi resistance has been reported among patients who have initially benefited from PARPi, whether as monotherapy or combined therapy. Several studies have indicated a high rate of acquired and *de novo* resistance to PARPi. Human and murine cancer models have revealed that there is a positive correlation between the degree of initial response to PARPi and the extent of sensitivity to PARPi treatment over time. Studies showed that 30% to 80% of ovarian cancer patients harbouring germline or somatic mutations in the *BRCA* gene developed PARPi resistance (Hurley et al., 2019; Li et al., 2020a; Li et al., 2021). A number of mechanisms of resistance have been proposed, which mainly involve the restoration of the HR repair pathway, among others. These mechanisms are now discussed.

1.7.1 BRCA Reversion Mutation and HR Restoration

Research on PARP inhibition has shown better therapeutic benefit in HR-deficient cancers; hence, partial or complete restoration of the HR pathway could lead to resistance to PARPi. In *BRCA*-deficient cells, the loss of HR function is usually caused by short insertions, frameshift deletions and single-nucleotide mutations in their *BRCA1/2* genes. However, secondary somatic mutations in *BRCA1/2* could

restore the gene's open reading frame, thereby regaining its HR function. This is known as reversion mutation. These reversion mutations are often caused by deletions. According to a recent study of 327 patients with *BRCA1/2* mutations, 26.3% were identified with reversion mutations. Further analysis revealed that 58.1% and 77.6% of secondary mutations in *BRCA1* in *BRCA2*, respectively, were caused by deletions (Bonadio and Estevez-Diz, 2021). Coincidentally, these secondary mutations in *BRCA1/2* are usually associated with acquired resistance to platinum chemotherapy. This implies some degree of similarity between the mechanisms of acquisition of PARPi resistance and platinum resistance (Turk and Wisinski, 2018; Bonadio and Estevez-Diz, 2021; Spiegel, Van Houten and Durrant, 2021).

Furthermore, the loss or downregulation of key components of other DDR proteins that regulate HR and non-homologous end-joining (NHEJ) repair has been implicated in PARPi resistance. The loss of 53BP1 increased HR repair in *BRCA1*-mutant ovarian cancer cells by decreasing NHEJ, which in turn conferred diminished PARPi sensitivity (Hurley et al., 2019). Similarly, decreased expression of REV7 and other NHEJ enzymes such as Ku70, Ku80 and XRCC4, which impairs the NHEJ pathway, has been associated with poor response to PARPi. Conversely, an increased availability of other HR proteins apart from *BRCA1/2* can also cause PARPi resistance. For instance, an increase in ataxia telangiectasia mutated (ATM) activity will promote HR repair, thus leading to PARPi resistance. The overexpression of RAD51 has been associated with partial restoration of HR capacity in *BRCA1*-deficient cancer cells. Rucaparib resistance has been reported in high-grade ovarian carcinoma and colon carcinoma cells with RAD51

overexpression (Bonadio and Estevez-Diz, 2021; Spiegel, Van Houten and Durrant, 2021).

1.7.2 P-Glycoprotein overexpression and reduced Intracellular PARPi

The intracellular concentration of PARPi can affect their efficacy. P-glycoprotein, an ATP-binding cassette (ABC) drug efflux transporter, plays a crucial role in the uptake of biomolecules across the cell membranes. Just as in other drug resistance mechanisms, increased expression of the P-glycoprotein drug efflux transporter may lead to a reduction in a PARPi's intracellular concentration. In *BRCA1*-deficient mouse models, overexpression of drug-efflux transporter genes was found in the majority of breast tumours that exhibited PARPi resistance. Conversely, the inhibition of P-glycoprotein re-sensitised the PARPi-resistant cancer cells to PARPi. Similarly, overexpression of P-glycoprotein was observed in a PARPi-resistant human ovarian cancer cell line, and the resistance was reversed by concomitant treatment with PARPi and the MDR1 inhibitors verapamil and elacridar (potent inhibitors of P-glycoprotein) (Michelena et al., 2018; Noordermeer and van Attikum, 2019).

1.7.3 Loss of PARP1 Function

The ability of PARP1 to bind DNA is strongly associated with the cytotoxic effect of PARPi. Mutations both within and outside of the PARP1's DNA-binding zinc-finger domains have been reported in the alteration of PARP1 trapping competence, thereby inducing PARPi resistance. A *PARP1* mutation (R591C) that affects the WGR domain conferred *de novo* resistance to olaparib. Although the mutant PARP1 could bind DNA, it did not show trapping. Similarly, in a talazoparib-

resistant ovarian tumour, a R591C mutation in the *PARP1* gene reduced PARP1 trapping compared to the wild type (Pettitt et al., 2018).

Du et al. (2016) also described another mechanism of PARP1 activity loss that induces PARPi resistance. They showed that the receptor tyrosine kinase c-Met (the proto-oncogene receptor commonly overexpressed in many forms of cancer) associates with and phosphorylates PARP1 at Tyr907, thereby altering its catalytic site. The phosphorylated PARP1 (pY907) increases PARP1 enzymatic activity but reduces binding to a PARP inhibitor, thereby rendering cancer cells resistant to PARP inhibition. Upregulation of c-Met has been reported to confer resistance to olaparib, rucaparib and veliparib in preclinical breast cancer models (Du et al., 2016; Klotz and Wimberger, 2020; Spiegel, Van Houten and Durrant, 2021).

In addition, a low level of PARP1 expression is thought to be associated with decreased catalytic activity, hence reduced PARPi sensitivity. For example, colorectal-carcinoma HCT116 cells exhibiting PARPi resistance also have very low levels of PARP1 expression (Liu et al., 2009). On the contrary, Hurley et al. (2019) did not find any correlation between PARP1 expression and clinical response to PARPi. This observation seems to suggest that low PARP1 expression is not the only explanation for poor response to PARPi therapy. Other studies have also reported that cancer cell models with normal PARP1 expression levels but with decreased PARylation activity (as a result of other factors) showed more tendencies towards PARPi resistance (Hurley et al., 2019; Spiegel, Van Houten and Durrant, 2021).

1.7.4 Stabilization of Stalled Replication Forks

Increased stabilisation of stalled DNA replication forks prevents their collapse and the consequent formation of double-stranded breaks. PARPi can stall replication forks by trapping PARP1 to DNA during BER. On the other hand, BRCA1/2 proteins slow and repair the replication fork, so *BRCA*-deficient cells are sensitive to fork collapse. Recently, the re-establishment of replication fork stability has been implicated in acquired PARPi resistance. Chaudhuri et al. (2016) demonstrated for the first time that the absence of the MLL3/4 complex protein, Pax2 transactivation domain-interacting protein (PTIP), protected *BRCA*-deficient cells from replication fork stalling and induced PARPi resistance. The loss of PTIP did not restore the HR activity but inhibited the recruitment of the MRE11 to the site of stalled replication forks, consequently preventing extensive degradation of the nascent strand of DNA (Ray Chaudhuri et al., 2016). Enhancer of zeste homolog 2 (EZH2), a histone methyltransferase and catalytic subunit of PRC2, is involved in the degradation of replication forks. EZH2 is known to be activated by the catalytic activity of PARP1. Hence, a decrease or loss of EZH2 prevents degradation and stabilises the replication fork, facilitating DNA repair. This has been shown in *BRCA*-deficient cells to cause PARPi resistance. Low levels of expression or absence of other fork-stabilising proteins such as RADX, MUS81 and CHD4 has been reported to confer PARPi resistance (Rose et al., 2020; Bonadio and Estevez-Diz, 2021).

1.7.5 Loss of Poly (ADP-ribose) glycohydrolase (PARG)

Poly (ADP-ribose) glycohydrolase (PARG) (EC 3.2.1.143) counteracts the action of PARP enzymes, hydrolysing the ribose-ribose bonds present in poly (ADP-

ribose). PARG somehow acts as a natural PARP inhibitor by degrading PAR, the main product of PARP1 that is involved in the recruitment of BER proteins. PARP is known to be involved in post-translational modification by PARylation of other nuclear proteins, including itself (auto-PARylation). Auto-PARylation allows PARP1 to dissociate from DNA after recruiting relevant DDR protein and thus prevents it from being trapped on the damaged DNA. Considering this, the loss of PARG activity will lead to the accumulation of PAR and inadvertently enhance auto-PARylation, allowing the enzyme to detach easily from DNA. Subsequently, the impairment of PARG suppresses the PARP-trapping potential of PARPi, thereby inducing resistance. This resistance mechanism has been reported in a PARPi-resistant *BRCA2*-deficient mouse model. Similarly, loss of PARG by shRNAs in *BRCA*-mutant human breast cancer cells confers olaparib resistance. Recently, human triple-negative breast tumours and serous ovarian carcinomas with remarkably a high level of PAR have also been associated with low-level expression of PARG (Gomez et al., 2020; McMullen et al., 2020; Rose et al., 2020).

1.7.6 Overcoming PARP inhibitor resistance

The current strategies to overcome PARPi resistance are focused on using a combination of PARPi and other inhibitors of the HR and immune-checkpoint inhibitors. These combination therapies are usually designed to target the acquired or *de novo* vulnerabilities of PARPi-resistant cancer cells. Several preclinical studies have showed promising synergistic effects that have (re)sensitised tumours to PARPi. For example, a combination of the phosphoinositide 3-kinase (PI3K) pathway inhibition and PARPi has shown benefits, not only for overcoming PARPi insensitivity but also in the treatment of non-HR-deficient tumours (Juvekar et al.,

2012). Other preclinical assessments have demonstrated that PARP1i along with inhibitors of vascular endothelial growth factor (VEGF), androgen receptor (AR), Ataxia telangiectasia and Rad3-related protein (ATR), Checkpoint kinase 1 (CHK1), WEE1, cyclin-dependent kinase 1 (CDK1), CDK12, serine/threonine kinase (AKT), histone deacetylase (HDAC), DNA topoisomerase 2-binding protein 1 (TOPBP1), c-Myc, bromodomain and extraterminal protein (BET) and heat shock protein 90 (HSP90) were able to inhibit HR and sensitise HR-proficient cells to PARPi and/or platinum chemotherapy (Dulaney et al., 2017; D'Andrea, 2018; Fehling et al., 2020; Prados-Carvajal et al., 2022). Prados-Carvajal et al. (2022) also suggested that a combination of NHEJ and microhomology-mediated end-joining (MMEJ) inhibitors with PARPi could prevent the acquisition of PARPi resistance.

Another interesting approach to overcoming PARPi resistance is to identify novel PARPi. Finding PARPi that explore different catalytic inhibition mechanisms remains very attractive, considering the current clinical PARPi are competitive inhibitors of NAD⁺. One way of discovering new PARPi is by screening chemical libraries, especially of natural products. In fact, around 50% of all new drugs are derived from natural product compounds, either by the use of the original natural product, the direct derivation of active compounds, or their use as a scaffold for novel pharmacophores (Nobili et al., 2009).

1.8 Natural products drug discovery

Natural products are naturally occurring metabolites, even if they are produced synthetically for clinical studies or for the market (Butler, Robertson and Cooper,

2014). Natural products have been a tremendous source of bioactive compounds that have been and are still used as drugs to treat many human diseases. With rising interest, about 80% of people worldwide depend on the use of natural products as part of their primary healthcare. Although their use is more prevalent in developing countries through the practice of traditional herbal medicines, reports have indicated that over the past 75 years, more than 47% of all newly developed and approved drugs have been from natural origin. These new drugs have either been used in their authentic natural compound forms, as direct analogues of the active molecules, or as semi-synthetic novel structures inspired by natural products' pharmacophores. Natural products are successfully used and are being developed as analgesics, anaesthetics, antibacterials, antihypertensives, antimalarials, antitussives, antivirals, muscle relaxants, steroids, immunosuppressives and anticancer agents. For example, in 2015, the Nobel Prize in Physiology or Medicine was jointly awarded to William C. Campbell and Satoshi Ōmura for their discovery of avermectin and Youyou Tu for her discovery of artemisinin. Avermectins have revolutionised the treatment of river blindness and elephantiasis that are caused by parasitic roundworms, while artemisinins are used in the treatment of malaria. Both drug classes have reduced the mortality associated with these parasitic diseases. Therefore, natural products play a vital role in drug discovery (Gullo et al., 2006; Gordaliza, 2007; Shen, 2015).

Drug discovery is a long, costly, complex and oftentimes very risky process. The average developmental period of most drugs available today is 12 to 24 years. It costs an estimated US \$1.4 to \$2 billion for a single drug from the laboratory to make it to the launch of the product, and this is usually funded by private

pharmaceutical and biotechnology companies. Moreover, the major risk is that many of these financially intensive, long-term projects never produce a commercial product, as approximately only 1 in 15 to 25 drug candidates passes the rigorous safety and efficacy testing required for approval (Lombardino and Lowe, 2004). A new, successful FDA-approved drug in the market has passed through five main stages from initiation of the project to marketing ([Figure 12](#)). In addition, not all approved drugs become a commercial success. As a result, the manufacturers may never recoup the huge expenses incurred during the long, arduous process of drug discovery and development.

The high-throughput screening (HTS) of natural sources has a “hit rate” of 0.3%, which is much better than the <0.001% hit rate for the HTS of synthetic compound libraries. Furthermore, natural products have unique properties that grant both advantages and challenges over typical synthetic molecules in the drug discovery process. They are characterised by a higher molecular mass, higher numbers of H-bond acceptors and donors, lower partition coefficients, a larger number of sp³ carbon atoms and oxygen atoms, fewer nitrogen and halogen atoms, and greater molecular rigidity compared with synthetic compound libraries. These features allow them to be orally bioavailable, explore more chemical space, and have higher rigidity (which is advantageous in drug discovery when tackling protein–protein interactions). With the vast majority of drugs in the market being natural products or analogues derived from them, natural product drug discovery represents an unending reservoir of new drugs for various therapies (Li and Vederas, 2009; Atanasov et al., 2021).



Figure 12: Phases and stages in drug discovery and development. There are five crucial steps, including drug discovery and development, preclinical research, clinical trials, regulatory approval, and post-market safety monitoring. The drug discovery and development phase begins with identification of a specific molecular target, with the aim of meeting a medical need or improving existing treatment. Assays are then developed to screen and evaluate the effects of the new drug on its target. Potential ‘hits’ are identified from thousands, and in some cases millions, of compounds from large chemical libraries using high throughput screening (HTS) often incorporated with *in silico* approach. This first phase usually involves optimisation and structure-activity relationship study and the potential hits could then be run using *in vitro* experiments with cells to narrow down the hits into one or two lead compounds for the next phase. The second stage is preclinical research. The chosen drug leads are tested on non-human subjects such as zebrafish, rodents and monkeys for efficacy, toxicity, and pharmacokinetic analysis. Once the preclinical research stage is completed with a strong drug candidate, the clinical trials are initiated by the approval of an Investigational New Drug (IND) application (in the US, trials must be approved by the FDA). In the clinic, there are 3 major sequential human trials, namely Phase I, Phase II and Phase III. Phase I tests the new drug’s toxicities and pharmacokinetics on less than 100 healthy volunteers. Phase II trials are usually double-blind, randomised trials with 100 to 500 volunteers, including patients who may receive a placebo or a standard drug previously used as treatment to assess drug safety and the efficacy of the new drug. Phase III involves 1000 to 5000 patients who are further assessed to confirm the drug’s efficacy and any potential adverse effects. It is important to note that only 4-7% of drug candidates make it to this last phase, and the few drugs that reach this stage increase their chance of approval significantly by 50%. The fourth stage in the drug development process is approval from the regulatory body (FDA in the US and EMA in Europe), when all data from previous phases are submitted for review. This waiting period can take months. In the US, once approved, the FDA may grant up to 7 years of exclusive marketing rights to the pharmaceutical company or sponsor. Finally, following drug approval and commercial production, the last stage, also known as Phase IV, is post-market monitoring. This is long-term safety surveillance monitored by the manufacturers, health professionals, and consumers who report problems associated with new drugs (Lombardino and Lowe, 2004).

1.8.1 Natural products as sources of anticancer agents

Historically, naturally derived compounds have played a vital role in anticancer drug discovery. Currently, over 60% of anticancer drugs were discovered and developed in one way or another from natural sources (Newman and Cragg, 2020).

In the 1950s, vinca alkaloids were the first plant-derived compounds found to possess antitumour activity in mice with lymphocytic leukaemia. Since then, four major vinca alkaloids (vinblastine, vinorelbine, vincristine and vindesine) have been used in the treatment of various cancers, including advanced testicular cancer, breast cancer, lung cancers, leukaemias, lymphomas, and Kaposi's sarcoma. A more recent example is from the class of taxanes originally isolated from the bark of *Taxus brevifolia*, the Pacific yew tree. Paclitaxel, a taxane, is clinically effective in the chemotherapeutic treatment of breast, ovarian, and non-small cell lung cancer (NSCLC). Its semisynthetic derivatives such as docetaxel and cabazitaxel have also been approved. Other notable examples of natural products or analogues presently used in the clinic for different cancers include topotecan, irinotecan, belotecan, etoposide, teniposide, combretastatin A4 phosphate, synribo, picato, trabectedin, eribulin, rapamycins, carfilzomib, midostaurin and brentuximab vedotin (Cragg and Pezzuto, 2016; Huang, Lu and Ding, 2021). These examples indicate that natural products due to their structural and chemical diversity are good sources for new drug leads with better pharmacological efficacy and less side effects than synthetic products. A significant number of natural product or natural product-derived compounds are currently in drug development (preclinical development and clinical trials) and one of such is a class known as flavonoids. For example, recoflavone 122 (DA-6034) has completed a Phase III trial (NCT01813812) for the treatment of gastritis and is currently in a Phase III trial for the treatment of dry eye syndrome (Butler, Robertson and Cooper, 2014).

1.8.2 Flavonoids as anticancer agents

Flavonoids are a large class of ubiquitous polyphenolic compounds which represent the largest group of secondary metabolites in the plant kingdom. There are over 10,000 flavonoids, and they are mostly found as glycosides in herbs, fruits, and vegetables. It is estimated that the average intake of flavonoids in the western diet is 200 mg to 1 g per day. Flavonoids have a wide range of therapeutic effects, including antidiabetic, antimicrobial, anti-inflammatory, antioxidant, and antitumour activities. Several preclinical studies have reported their antiproliferative activities against different types of cancer such as breast, lung, prostate and colorectal cancers (Martínez-Pérez et al., 2016; Mitra et al., 2019). For example, the novel flavonoid derivative VI-14 has been demonstrated to inhibit migration and invasion in different cancer models. Likewise, another flavonoid derivative LZ-207 exhibited inhibitory effects on growth and viability in several human colon cancer cell lines and induced apoptosis in HCT116 cells. The different mechanisms of action of flavonoids against cancers are summarised as follows: acting as oestrogen signalling regulators, anti- and pro-oxidant regulators, cytochrome P450 regulators, ATP-binding cassette regulators, apoptosis inducers, cell cycle inhibitors and mitocans (mitocans are compounds that exert anti-cancer activity through their molecular targets within mitochondria) (Androutsopoulos, Li and Arroyo, 2009; Martinez-Perez et al., 2014; Sun et al., 2015).

The flavonoid structure consists of two fused rings, aromatic ring (A) and heterocyclic pyrene ring (C) connected to another aromatic ring (B) through a carbon-carbon bridge ([Figure 13](#)). Based on the level of hydroxylation or oxidation and pattern of substitution of the C ring, there are seven subclasses of flavonoids,

namely flavones, flavonols, flavanonols, isoflavones, flavanones, anthocyanidins, and chalcones. Although flavonoids are primarily known for their anti-inflammatory and antioxidant properties, recent studies have revealed that some flavones and flavonols are effective PARP inhibitors (Fatokun et al., 2013; Alqahtani et al., 2019).

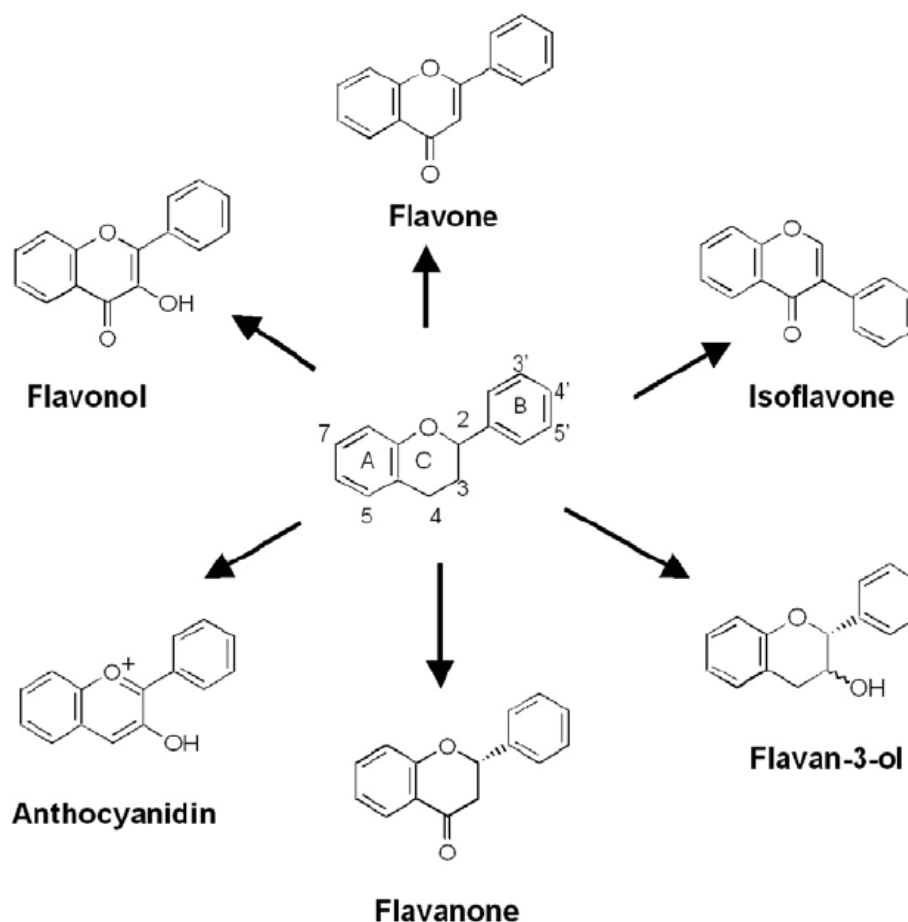


Figure 13: Basic structures of the flavonoid class and its subclasses.

1.8.3 Flavones with anticancer and PARP inhibition activity

The major dietary sources of flavones are tomato skin, celery, parsley, red wine, red pepper, buckwheat, mint, ginkgo biloba, and chamomile (Panche, Diwan and Chandra, 2016). Geraets et al. (2007) reported the PARP1-inhibitory activity of various dietary flavones in pulmonary epithelial cells. Flavones such as baicalein, tricetin, gossypetin and 3',5,7,-trihydroxy-3,4'-dimethoxyflavone all showed varying

degrees of PARP1 inhibition. Quercetin, fisetin (although flavonols), and tricetin exhibited moderate PARP1 inhibition at 100 μ M – 62%, 60% and 80% inhibition, respectively. Similarly, Fatokun et al. (2013) showed that 4'-methoxyflavone and 3',4'-dimethoxyflavone inhibited parthanatos (death induced by overactivation of poly (ADP-ribose) polymer) in HeLa and SH-SY5Y cells. The study concluded that flavones could act as anti-parthanatic neuroprotective agents, thus suggesting their use beyond anticancer and anti-inflammatory therapies. More recently, Alqahtani et al. (2019) identified two tea polyphenols (epigallocatechin gallate and theaflavin) to have PARP-inhibitory activity and selective cell toxicity towards *BRCA2*-deficient cells through synthetic lethality. Although the two polyphenols are not flavones, they share structural similarities to them, which seems to suggest that further research could yield more flavones or natural products with significant PARP inhibition activity (Geraets et al., 2007; Fatokun et al., 2013; Alqahtani et al., 2019).

1.8.4 Rationale for natural product PARP inhibitors

Classical chemotherapy often uses cytotoxic agents to interfere with DNA synthesis and mitosis, resulting in the death of rapidly growing and dividing cancer cells. These agents are non-selective and could cause some common side-effects such as fatigue, nausea, hair loss, anaemia, loss of appetite, sore mouth, insomnia, etc. They could also cause more serious side-effects such as permanent heart damage and the development of a second cancer called acute myeloid leukaemia. A major cause of the high mortality rate of cancer patients is the severe adverse effects of chemotherapeutic drugs on healthy tissues and organs. Moreover, due to the relatively poor bioavailability of these drugs, higher doses are sometimes required, which consequently increases their toxicity to healthy cells and raises the

risk of drug resistance by the cancers. In that regard, although conventional chemotherapy has made notable success, its non-specific targeting, adverse side effects, poor bioavailability, high-dose requirements, low therapeutic indices and the development of multiple drug resistance have limited its effectiveness (Senapati et al., 2018). Therefore, there is an increasing need for new, targeted therapies with much less side effects.

PARP inhibitors have emerged as one of the targeted therapies in the treatment of various cancers, especially those with HR deficiency. Preclinical and clinical studies have demonstrated that PARP inhibitors can be powerful adjuvants in chemotherapy, radiotherapy and immunotherapy, and also as monotherapy maintenance in tumours with defects in their DNA repair pathways. Therefore, the idea of PARP inhibitor therapy is very promising (Bruin et al., 2020; Moreno-Lama et al., 2020). Since 2014, four different PARPi have been approved, with a couple more at different clinical trial phases. Despite the accruing success stories of these targeted therapies, PARPi are not completely without their challenges. One of the major drawbacks is the development of resistance in approximately 40–70% of patients. Perhaps adverse effects could be another drawback, although due to their specificity PARPi have a relatively low toxicity or adverse effects profile compared to conventional chemotherapy. PARPi have shown common side effects such as anaemia, thrombocytopenia, neutropenia, nausea, constipation, vomiting, decreased appetite, fatigue, etc. Severe adverse events such as myelodysplastic syndrome and acute myeloid leukaemia are very rare, with an incidence of 0.5 to 1.4% after prolonged treatment. Fortunately, these toxicities are easily managed with dose modification (LaFargue et al., 2019). Another challenge is the huge cost

of treatment. Although it varies depending on the PARPi drug brand, duration of therapy, and access to healthcare benefits, among others, the average cost in the US is \$100,000 per progression-free year (Wethington et al., 2021). This implies that the benefits of such PARPi therapy may be out of reach for many patients, especially those in resource-limited countries.

With all these limitations and challenges of current PARPi, novel PARPi from natural sources can be of tremendous value. Presently, all the clinically available PARPi are synthetic and have their drawbacks, as mentioned earlier. It is conceivable that new PARPi scaffolds could be obtained from natural products, as they have better structural diversity to explore the chemical space. Also, natural PARPi drugs may be less susceptible to chemoresistance, and might additionally exhibit less toxicity or side effects. Various case studies have reported the preventative effects of the dietary intake of flavonoids on different cancers. The incidence and risk of cancers, including breast, colorectal, endometrial, gastric, hepatic, oral and pharyngeal, pancreatic, prostate, ovarian and lung cancers were reduced compared to the control groups (Romagnolo and Selmin, 2012). The lifelong or long-term effects of a diet enriched with these natural product PARPi might include a reduction in the incidence of cancers, especially in more susceptible people with the *BRCA* mutation and with TNBC, which is more prevalent among young people from black ethnicities (Hongthong et al., 2021). Therefore, finding new PARPi that are more affordable, less toxic or non-toxic, and with little or no susceptibility to drug resistance is currently an area of unmet clinical need in cancer precision medicine, either for chemoprevention or chemotherapy. This project contributes to meeting that need.

1.9 Aim and objectives

The aim of this research was to identify novel, natural product PARP inhibitors and characterise them as chemical tools for investigating the roles of PARP and as scaffolds for generating PARP inhibitor leads for therapeutics development, with a particular focus on cancer and neurodegeneration.

The objectives of this project were to:

1. Identify potential inhibitors of PARP or parthanatos through high-throughput screening (HTS).
2. Characterise hits from the HTS, as well as some emerging PARP inhibitors, for efficacy and potency in the contexts of cancer and neuronal death.
3. Explore structure-activity relationships (SARs) to identify moieties responsible for PARP-inhibitory activity and substitutions that could improve activity.
4. Model how the identified PARP inhibitors interact with their binding domains on the PARP1 and PARP2 protein crystal structures.
5. Assess the drug-likeness of the PARP inhibitors.

Chapter 2: Materials and Methods

2.0 Materials and Methods

2.1 Materials

2.1.1 Media, chemicals and reagents

The materials used and their suppliers and origins are shown in Table 3. The standard PARP inhibitors (PARPi), natural compounds, and other test compounds are shown in Tables 4 and 5, respectively.

Table 3: **Media, chemicals and reagents**

Material	Supplier
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific, UK
Dulbecco's Modified Eagle Medium, no phenol red	Thermo Fisher Scientific, UK
Ham's F-12 Nutrient Mix	Thermo Fisher Scientific, UK
Dulbecco's Phosphate Buffered Saline (PBS)	Thermo Fisher Scientific, UK
L-Glutamine	Thermo Fisher Scientific, UK
Antibiotic-Antimycotic (anti/anti)	Thermo Fisher Scientific, UK
Foetal Bovine Serum (FBS)	Sigma-Aldrich, UK
TrypLE™ Express Enzyme (recombinant trypsin)	Thermo Fisher Scientific, UK
Retinoic acid	Sigma-Aldrich, UK
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, UK
Benzyloxycarbonyl-Val -Ala-Asp(OMe)-fluoromethylketone (z-VAD-fmk)	Bio-Techne, UK
<i>N</i> -methyl- <i>N'</i> -nitro- <i>N</i> -nitrosoguanidine (MNNG)	Chem Service, PA, USA
AlamarBlue Cell Viability Assay Reagent	Thermo Fisher Scientific, UK

CellTiter-Glo® Luminescent Cell Viability Assay	Promega Incorporation, UK
MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)	Sigma-Aldrich, UK
Cisplatin	Merck Life Science, UK
Doxorubicin	Sigma-Aldrich, UK
Necrostatin-1	Bio-Techne, UK
Triton X-100	Fisher Scientific, UK
Hydrochloric acid (HCl)	VWR chemicals, France
Methanol	Fisher Scientific, UK
2,2-diphenyl-1-picrylhydrazyl (DPPH)	Sigma-Aldrich, UK
2', 7' - dichlorofluorescein diacetate (DCFDA)	Abcam, UK
Black 96 well microplate, F-bottom, sterile	Greiner Bio-one, UK
White 96 well microplate, F-bottom, sterile	Greiner Bio-one, UK
Opaque 96 well microplate, F-bottom, sterile	Greiner Bio-one, UK

Table 4: Clinical and experimental PARP inhibitors. All the four clinically approved PARP inhibitors and two experimental PARP inhibitors were used in this project.

Material	Supplier
Olaparib	Stratech, UK
Niraparib	Stratech, UK
Rucaparib	Stratech, UK
Talazoparib	Cambridge Bioscience, UK
PARPi -FL	Bio-Techne/Tocris, UK
3,4-Dihydro-5-[4-(1-piperidiny)butoxyl]-1(2H)-isoquinolinone (DPQ)	Sigma-Aldrich, UK

Table 5: Natural products and other test compounds. A total of 537 compounds (mainly natural products) were screened for PARP-inhibitory activity and some were further tested and characterised. This table shows the names and suppliers of these compounds, including a small, commercially available library of natural products. Purpurogallin was prepared by Dr Fyaz Ismail (LJMU, Liverpool, UK) using the method outlined by Kelly-Hunt et al. (2021). ^a Synthetic compounds and ^b derivatives of natural compounds

Material	Supplier
Screen-Well® Natural Product Library (BML-2865 Version 7.1), 502 structurally diverse natural products. Cat. No. BML-2865-0500	Enzo Life Sciences, UK
Ciclopirox ^a	Sigma-Aldrich, UK
Artemisinin	Sigma-Aldrich, UK
Dihydroartemisinin ^b	Sigma-Aldrich, UK
Zinc Pyrithione ^b	Sigma-Aldrich, UK
Warfarin ^a	Sigma-Aldrich, UK
Apomorphine ^b	Sigma-Aldrich, UK
Chloroxine ^a	Sigma-Aldrich, UK
Chrysosplenetin	Sigma-Aldrich, UK
6,2',4'-Trimethoxyflavone	Sigma-Aldrich, UK
CH 223191 ^a	Sigma-Aldrich, UK
Stemregenin 1 ^a	Sigma-Aldrich, UK
Ipriflavone	Sigma-Aldrich, UK
Indirubin	Sigma-Aldrich, UK
Aceroside VIII	Sigma-Aldrich, UK
Chlorogenic acid	Sigma-Aldrich, UK
Phloretin	Sigma-Aldrich, UK
Puerarin	Sigma-Aldrich, UK

Biochanin A	Sigma-Aldrich, UK
Laudanosine	Sigma-Aldrich, UK
Quercetin	Sigma-Aldrich, UK
Luteolin	Sigma-Aldrich, UK
5-hydroxytryptophan (oxitriptan)	Sigma-Aldrich, UK
Flavone	Indofine, USA
4'-Methoxyflavone	Indofine, USA
4'-Hydroxyflavone	Indofine, USA
3',4'-Dimethoxyflavone	Indofine, USA
3',4'-Dihydroxyflavone	Indofine, USA
3',4',7-Trimethoxyflavone	Indofine, USA
3',4',7-Trihydroxyflavone	Indofine, USA
7-Methoxyflavone	Indofine, USA
7-Hydroxyflavone	Indofine, USA
Resveratrol	Indofine, USA
Epigallocatechin gallate	Indofine, USA

2.1.2 Cell lines

Human cervical adenocarcinoma cells (HeLa) were purchased from the European Collection of Authenticated Cell Cultures (ECACC) (Salisbury, UK). Human neuroblastoma cells (SH-SY5Y) were procured from Thermo Fisher Scientific, UK, while human immortalised keratinocytes (HaCaT) were obtained from Dr Kehinde Ross (LJMU, Liverpool, UK).

2.2 Methods

2.2.1 Cell Culture - growing and maintaining cells

HeLa, SH-SY5Y, and HaCaT cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS), 1% L-glutamine (2 mM) and 1% Antibiotic-Antimycotic solution (this solution contains 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Gibco Amphotericin B). All cell culture procedures were carried out under aseptic conditions (Class 2 Biological Safety Cabinet) and media and reagents were warmed to 37°C in a thermostated water bath before cells were exposed to them. The cells were cultured usually in T75 cell culture flasks (occasionally in T25 flasks) and incubated in a humidified atmosphere of 37°C and 5% CO₂. They were allowed to grow and reach over 80% confluence before passaging. To passage the cells, the growth media (or media) was removed and the culture rinsed with 5 ml of pre-warmed PBS to remove any media left along with dead cells. In the case of HaCaT cells, this step was followed by adding freshly prepared 0.05% EDTA solution (in PBS) for 10 minutes and incubating at 37°C. This was done because HaCaT cells are more adherent than the HeLa and the SH-SY5Y cells. After aspirating the PBS solution from the flask (or EDTA solution in the case of HaCaT), 5 ml of pre-warmed trypsin (TrypLE, which is recombinant trypsin) was then added, and the flask was gently swirled to cover the entire surface area of the cells for 60-70 seconds before aspirating the trypsin. The flask was placed back in the incubator for 5 minutes to allow the adherent cells to fully detach. Cells were then observed under the light microscope to ensure that they were fully detached. Afterwards, 10 ml of media was added to flood the flask in order to detach the cells to form a suspension and to also inactivate trypsin. The cell suspension was then triturated (pipetted up and

down) several times to separate any clumps and ensure a suspension of single cells. The suspension was transferred into a 50 ml tube. To prepare a maintenance culture (culture to be put back in the incubator to keep growing for future use), 10 ml of usually a 1:10 or 1:5 dilution of the suspension (ratio depended on the cell line's growth rate (doubling time) and the time required before culture would be reused) was prepared and introduced (sub-cultured) into a new T75 flask.

To prepare cells for experiments the cell suspension in the 50 ml tube was diluted (1:1.5 or 1:2) with an appropriate volume of media, depending on initial confluency. Cell density was then determined by counting under a microscope using a haemocytometer (Improved Neubauer). An appropriate volume of the suspension needed to make 96-well plates was prepared at a desired seeding density (by dilution of the stock suspension whose density had been determined by using the haemocytometer). The seeding density was dependent on the cell type and the nature and duration of the assay to be carried out. For example, to develop a model assay for parthanatos, the suspension was diluted to a desired density of 1×10^5 cells/ml for HeLa and HaCaT, while 2.5×10^5 cells/ml was used for the SH-SY5Y, as it did not grow as fast as the other two cell lines. The working suspension was then seeded into the wells of 96-well plate(s) at 100 μ l/well, meaning 10,000 cells/well for the HeLa and HaCaT cells and 25,000 cells/well for the SH-SY5Y cells. Opaque plates were used for absorbance-based assays, black plates for fluorescence-based assays, and white plates for luminescence-based assays. The plates were placed in the incubator (37°C and 5% CO₂) for 24 h before treatment.

2.2.2 Cryopreservation of cells

The cultured cells were allowed to reach 80-90% confluence before being washed with PBS, trypsinised, flooded with media and counted, just as described earlier. They were then centrifuged at 1200 rpm for 5 minutes. The supernatant (medium) was removed and the pellet was resuspended in a cryoprotective freezing medium (90% growth media plus 10% DMSO) to avoid the lethal effect of freezing. The suspension was aliquoted into sterile cryovials (approximate 4 million cells/vial). The vials were then gradually frozen, first for 30-40 minutes in the fridge (4°C), followed by another 40 minutes at -20°C and then placed in -80 °C. Alternatively, after aliquoting, the vials were placed directly in a cryo-box, 'Mr Frosty' (filled with isopropanol), and stored at -80 °C for a few days. The frozen vials were subsequently transferred from -80 °C into liquid nitrogen (-196 °C) for long-term storage.

2.2.3 Resurrecting frozen cells

A cryovial of frozen cells was quickly thawed by placing it in a 37°C water bath for about a minute (with swirling). Once thawed, the suspension was transferred into a 15 ml tube, then 9 ml of growth medium was gradually added. This was followed by centrifugation: 1200 rpm for 5 minutes. The supernatant was carefully removed (to get rid of the cryoprotective medium). The cells were then resuspended in 10 ml of fresh medium and transferred into a T25 flask. The flask was then incubated in a humidified atmosphere (incubator) of 37°C and 5% CO₂ and monitored until the culture reached confluence (usually by 3 to 4 days, depending on the cell type).

2.2.4 Differentiating SH-SY5Y cells

The SH-SY5Y neuroblast-like cells are a subclone of the parental neuroblastoma cell line SK-N-SH and used as a neuronal cell model for investigating neurodegeneration, including in the contexts of Parkinson's disease, Alzheimer's disease, and other neurodegenerative diseases. Several studies have shown significant differences in the sensitivities of the undifferentiated and differentiated SH-SY5Y cells. Results from using the cells in both states have indicated that differentiated SH-SY5Y cells better mimic the *in vivo* models and are therefore more accurate for translation (Shipley Mackenzie et al., 2017).

To differentiate the SH-SY5Y cells into mature neurones, the cells were cultured normally as earlier described, using DMEM-F12 supplemented with 10% FBS, 1% L-glutamine (2 mM) and 1% Antibiotic-Antimycotic solution, and seeded at 7,500 cells/well (100 µl/well) in an appropriate 96-well plate. The medium was then removed 24 h later and replaced with differentiation medium (DMEM-F12 supplemented with 1% FBS, 1% L-glutamine (2 mM), 1% Antibiotic-Antimycotic solution, and 1% retinoic acid (retinoic acid is light sensitive and was therefore stored in the dark). On the third day, the media was gently aspirated from each well and a fresh 100 µl of differentiation medium was added. The cells achieved a differentiated, mature phenotype and were ready to use on day 7 post-differentiation.

2.2.5 Induction of parthanatos using MNNG

PARP-induced cell death (parthanatos) was used as a surrogate for PARP activity, meaning that a PARPi will show a protective (increase in cell viability) effect against

the cell death. The alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was used to induce parthanatos, as it has been shown to potently and selectively induce parthanatos, with the cell death induced almost completely blocked by a PARP inhibitor but not affected by a pan-caspase inhibitor (Fatokun et al., 2013). After seeding them into 96-well plates and incubating for 24 h, the cultured HeLa, SH-SY5Y, or HaCaT cells (originally seeded at a density of 1×10^5 , 2.5×10^5 and 1×10^5 cells/ml, respectively) were treated with MNNG. 150 μ M (3x dilution) MNNG was freshly prepared (in DMEM, prepared fresh every 15 min) in order to achieve the final desired concentration of 50 μ M by adding 50 μ l of the 3x solution to 100 μ l of medium already in the well. 50 μ l of MNNG was added to each well apart from the negative control wells (which received an equivalent volume of medium only), and the plate was incubated for 25 min at 37°C and 5% CO₂. Each treatment was done in triplicate. The wells were then fully but gently aspirated and 50 μ l of media was added to each well. Afterwards, 50 μ l of each tested compound (prepared as a range of concentrations from a stock solution) was added to each designated well, while media without any compound but containing the same %v/v of sterile DMSO as was in the highest concentration of the compounds tested was added to the negative (vehicle) control wells. Treatment with a positive control compound (a PARPi such as DPQ) was included. The cells were then incubated overnight. Protection against PARP-induced cell death was assessed 18-24 h later using various cell viability assays as described later. Previous studies have shown that a PARP inhibitor confers significant protection against the cytotoxic effect of MNNG, as evidenced by increased cell viability in the presence of the PARP inhibitor compared to the effect of MNNG alone (Fatokun et al., 2013).

2.2.6 PARP inhibitors and model parthanatos assay

Both experimental and clinically approved PARP inhibitors were included in the experiments. For the model assay, in the experiment described in the earlier section z-VAD-fmk (100 μ M) and DPQ (30 μ M) were used as negative assay and positive assay controls, respectively. z-VAD-fmk is a pan-caspase inhibitor that blocks caspase-mediated apoptosis, while DPQ is a potent experimental PARP inhibitor ($IC_{50} = 3.5 \pm 0.5 \mu$ M) (Moroni et al., 2001). DPQ has been reported to significantly attenuate the cytotoxicity of MNNG while z-VAD-fmk has no effect, demonstrating that the cell death caused by MNNG is not classical apoptosis but a different cell death; in this case, parthanatos (Yu et al., 2002).

Olaparib, PARPi-FL (a fluorescent version of olaparib), niraparib, rucaparib and talazoparib were tested at various concentrations up to 1 μ M, while the natural product compounds were tested up to 10 μ M, depending on their maximum concentration that could be used without them causing cytotoxicity on their own. The clinically approved PARPi were prepared from a 10 mM DMSO stock while most of the natural compounds were prepared from a 100 mM DMSO stock. Appropriate vehicle (DMSO) controls were always included in the experiments, and the final %v/v DMSO was never more than 0.2% v/v, which on its own was confirmed to be not toxic to the cells.

2.2.7 PARP inhibitors and necroptosis inhibition assay

Necrostatin-1 is a small molecule that inhibits necroptosis (Cao and Mu, 2021). The effect of necrostatin-1 on cell death induced by parthanatos was assessed. Parthanatos was induced by MNNG in HeLa cells as earlier described and, the

MNNG-treated cells were exposed to necrostatin-1, alone or concomitantly with DPQ or olaparib, as follows: Following the aspiration of the MNNG, 50 µl of media or a PARPi (DPQ 60 µM or olaparib 2000 nM, each of which represented a 2x concentration to achieve a final concentration of 30 µM and 1000 nM, respectively) was added to each well (treatment in triplicate). Then the cells were treated with 50 µl of necrostatin-1 (to have MNNG-treated wells with necrostatin-1 alone and MNNG-treated wells with necrostatin-1 in the presence of DPQ or olaparib) at a range of concentrations up to 100 µM (each prepared as a 2x concentration), while media only (with the same %v/v of DMSO as was in the highest concentration of necrostatin-1, DPQ or olaparib used) was added to the negative (vehicle) control wells. The cells were then incubated overnight. Protection against cell death was assessed 18-24 h later using the MTT assay.

2.2.8 Cell viability assays

18-24 h post-treatment (MNNG in the absence or presence of each test or standard compound), the ability of each agent to protect against MNNG-induced cell death (considered as evidence of PARP inhibition) was assessed by quantifying cell viability using one or more of three viability assays (as relevant) that relied on different readouts: MTT assay (absorbance-based), AlamarBlue assay (fluorescence-based) and CellTiterGlo (luminescence-based). This multi-pronged approach enabled the identification and exclusion of potential artefacts and confounding factors that could have arisen from the inherent deficiencies of each assay, e.g., potential interactions between assay reagents and test compounds, most of which were natural products and prone to such interactions. The

percentage cell viability was calculated as follows (blank values were found to be negligible):

$$\% \text{ cell viability} = (\text{absorbance of sample} / \text{absorbance of negative control}) \times 100\%$$

2.2.8.1 MTT Assay

This is a colorimetric assay based on the ability of viable cells to convert MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide], a yellow tetrazolium dye, to formazan, a purple insoluble crystal. Only live cells with active mitochondrial enzymes can reduce MTT to formazan ([Figure 14](#)) (Zhong et al., 2019) and the absorbance (as Optical Density (OD) values) correlates directly with cell viability when measured within the linear range. After treatment in an opaque 96-well plate, 10 μl (10% v/v) of pre-warmed MTT (5 mg/ml in PBS) was added to each well and the plate was further incubated for 2 to 3 h. The wells were then gently and completely aspirated and 100 μl of DMSO was added to each well to solubilise the formazan crystals. The plate was shaken on an orbital shaker for 5-10 min at 95 rpm (refrigerated centrifuge, 810R, Eppendorf, Germany). The absorbance was then read at 570nm on the CLARIOstar microplate reader (BMG LABTECH, Germany) or the Spark™ 10M multimode microplate reader (Tecan, Switzerland).

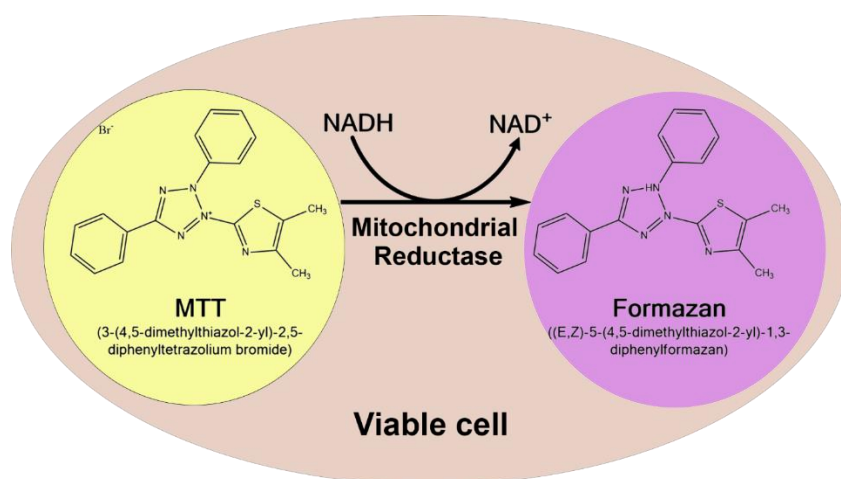


Figure 14: An overview of the MTT assay. MTT is taken up by the cells and live cells convert it to purple formazan crystals, which are then solubilised with DMSO before the absorbance (Optical Density, OD) is read at 570 nm. Absorbance correlates directly with cell viability.

2.2.8.2 AlamarBlue Cell Viability Assay (AB)

This is a fluorometric assay that measures cell viability by the ability of metabolically active cells to reduce resazurin, a weakly fluorescent blue dye (oxidised form), to resorufin, a highly fluorescent red dye (reduced form) ([Figure 15](#)). Unlike MTT, AB is a non-destructive assay which is non-toxic to the cells (Al-Nasiry et al., 2006) and, therefore, allows for multiplexing. 18-24 h after treatment, 10 μ l (10% v/v) of pre-warmed AB was added to each well and the plate (cultures were grown and treated in a black plate) was allowed to incubate for 3 h. The plate was then left at room temperature for 15-20 min before the fluorescence was read at an excitation wavelength of 530 nm and an emission wavelength of 590 nm, using the CLARIOstar microplate reader or the Spark™ 10M Tecan microplate reader (Fatokun et al., 2013).

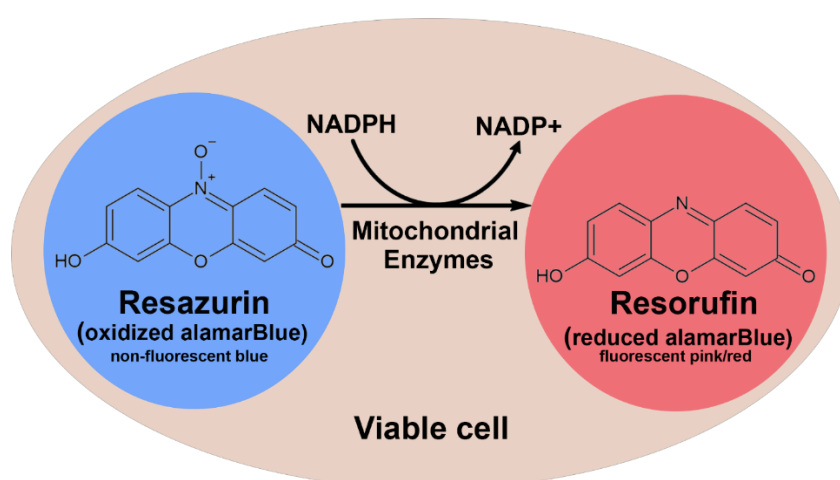


Figure 15: An overview of the AB assay. Viable cells maintain a reducing environment within their cytoplasm; therefore, the blue, non-fluorescent AlamarBlue reagent (oxidised form of redox indicator) is reduced to the red fluorescent form with an excitation wavelength of 540 nm and an emission wavelength of 590 nm.

2.2.8.3 CellTiter-Glo® Luminescent Cell Viability Assay (CTG)

The CellTiterGlo (CTG) assay is based on the quantification of ATP in the treated cells. Viable cells generate a luminescent signal that is directly proportional to the amount of ATP present, due to the conversion of luciferin to oxyluciferin by the luciferase enzyme ([Figure 16](#)) (Crouch et al., 1993). The treated culture plate (cultures were grown and treated in a white plate) was left at room temperature for 30 min, then 50 µl of the media was removed from each well (leaving 50 µl in the well) and replaced with 50 µl of the re-constituted CTG reagent (buffer added to lyophilised substrate), making a 1:1 ratio as recommended. The plate was then agitated for 2 min on an orbital shaker to fully lyse the cells and left at room temperature for 10 min to stabilise the luminescent signal (signal half-life >5 h). Plates were read using the CLARIOstar plate reader or the Spark™ 10M Tecan microplate reader. This assay was carried out as described by Fatokun et al., (2013).

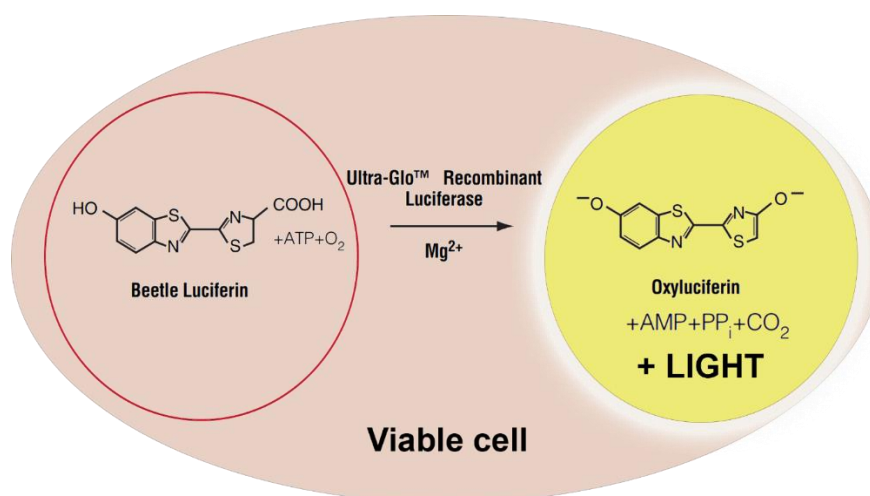


Figure 16: An overview of the luciferase reaction. Owing to the conversion of luciferin to oxyluciferin by the luciferase enzyme, viable cells produce a luminescent signal (which is then quantified) directly proportional to the amount of ATP present.

2.2.9 High-throughput screening of compound library (HTS)

High-throughput screening (HTS) is a crucial technique used in drug discovery to identify hits from compound libraries that may eventually produce drug leads. Following a high-throughput screening of 5,120 compounds, 4MF and DMF were shown to have PARP1-inhibiting properties (Fatokun et al., 2013). In the current study, a small commercial library (Screen-Well® Natural Product Library, BML-2865 Version 7.1) of 502 structurally diverse natural products (Cat. No. BML-2865-0500; Enzo Life Sciences, UK), in which each compound was supplied as a 2 mg/ml solution in DMSO (library stored at -80°C), was manually screened using an established, cell-based screening assay for detecting inhibitors of parthanatos (Fatokun et al., 2013). For the screening, HeLa cells were seeded at 10 000 cells per well (100 µl per well) in 96-well opaque plates (cell plates) which were allowed to incubate overnight. Aliquots (daughter plates) of the 2 mg/ml stock library (mother) plates (six 96-deep well plates) had been prepared in separate 96-well compound plates to avoid repeated freeze-thawing and accidental contamination of the stock library (mother) plates. On the day of the compound screening, the daughter compound plates were thawed and used as stock to prepare working compound plates as a 1:100 dilution (prepared as 2x – 1:50) in growth media. The cell plates were treated with MNNG as previously described (negative control wells without MNNG included), after which the wells were aspirated and 50 µl of fresh growth media was added to each well, followed by 50 µl of each library compound added at twice the desired final concentration (each compound added as

singleton). Each plate was set up to have medium with DMSO (vehicle) control treatment (column 1, top 4 wells), MNNG only-treatment (column 1, bottom 4 wells), MNNG treatment in the presence of a negative assay control (Z-VAD-fmk, 100 μ M) (column 12, top 4 wells), and MNNG treatment in the presence of a positive assay control (DPQ, 30 μ M) (column 12, bottom 4 wells). The library compounds then occupied the inner 80-well matrix (one well per compound), with the template design replicating their location on the stock compound plates. The cell plates were returned to the incubator for 18-24 h, after which the MTT assay was used to quantify cell viability. The results were analysed and the potential hits identified were re-screened for confirmation using the same screening procedure.

2.2.10 Time course of parthanatos and assessment of the protection elicited by PARP inhibitors

In order to assess the extent of protection against PARP-mediated cell death that could be elicited by PARP inhibitors when added to the cultures up to several hours after the initiation of the MNNG insult, cultured HeLa cells were treated with olaparib (10, 100, and 1000 nM) and DPQ (1, 10, and 50 μ M) using the parthanatos assay as previously described, with each inhibitor added once, either immediately after treatment with MNNG or at each subsequent 4 h time point up to 16 h post-insult. Protection against cell death (evidence of PARP inhibition) was assessed 18-24 h after MNNG treatment, using the MTT assay.

2.2.11 Cell-free PARP1 Inhibition Assay

A cell-free colorimetric assay kit (HT Universal Colorimetric PARP Assay Kit with Histone-coated Strip Wells, Trevigen; Cat. No 4677-096-K or PARP1 Colorimetric

Assay Kit, AMSBIO; Cat. No AMS.80580) was used to measure direct PARP enzyme inhibition of olaparib, PARPi-FL, DPQ, and thirty-three natural compounds, including 4MF and DMF. The key principle of this assay is the poly ADP-ribosylation of histone proteins coated on a 96-well plate in the presence of biotinylated NAD⁺ ([Figure 17](#)) (Brown and Marala, 2002).

PARP Buffer (50 µl) was added to each well to rehydrate the histones, then allowed to incubate at room temperature for 30 min. The PARP Buffer was then discarded from the wells by tapping the strip wells onto paper towels. 50 µl of each test compound prepared at a range of concentrations was added to appropriate wells. This was followed by the addition of diluted PARP enzyme (0.5 Unit/well, 15 µl), and the reaction was left to incubate for 10 min at room temperature. PARP cocktail, 25 µl (a mixture of NAD⁺ and activated DNA) was added to each well and the reaction was incubated at room temperature for 60 min. A negative control without the PARP enzyme (to indicate no PARP enzyme activity) and a positive control without the inhibitor (to indicate full PARP enzyme activity) were included. Afterwards, the wells were washed twice with PBS plus 0.1% Triton X-100 (200 µl/well), followed by washing twice with PBS (200 µl/well) which was then discarded. Diluted Strep-HRP (50 µl per well) was added and the reaction incubated at room temperature for 60 min. The washing step was then repeated. of pre-warmed TACS-Sapphire™ colorimetric substrate (50 µl) was added to each well and the reaction was incubated in the dark for 15 min at room temperature. The reaction was then stopped by adding 50 µl per well of 0.2 M HCl, which generated a yellow colour that was read at 450 nm using the CLARIOstar microplate reader or the Spark™ 10M Tecan microplate reader.

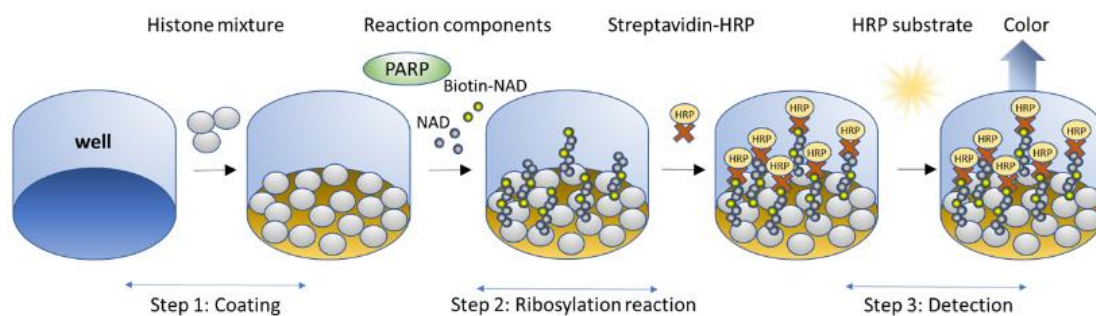


Figure 17: PARP1 Colorimetric Assay Kit Schematic. The key steps involve the coating of histone proteins on a 96-well plate, followed by the addition of biotinylated NAD⁺ (PARP1 enzyme substrate), activated DNA and PARP1. Finally, the production of PARylated histone is detected by the treatment of the plate with streptavidin-HRP followed by addition of the HRP substrate which produces colour that can be measured by a microplate reader. Adapted from BPS Bioscience website (Bioscience, 2022).

2.2.12 Potentiation of Doxorubicin and Cisplatin by PARP Inhibitors

HeLa cells were seeded at 2.5×10^4 cells/ml in an opaque 96-well plate. The cells were treated with five concentrations of 4MF and DMF (5, 10, 20, 50 and 75 μ M) each alone and concomitantly with cisplatin (6.25 μ M) or doxorubicin (1 μ M). The negative control was media alone, while cisplatin at 100 μ M and doxorubicin at 20 μ M were used to assess the maximum effect of each compound. Cell viability was measured after 96 h using the MTT assay.

2.2.13 Assessment of induction of reactive oxygen species (ROS)

A DCFDA Cellular ROS Detection Assay Kit (Abcam, UK; Cat. No. ab113851) was used to measure intracellular ROS. HeLa cells were cultured using growth media with no phenol red and seeded into a black, clear bottom 96-well microplate at 2.5×10^5 cells/ml (25,000 cells/well). The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and allowed to adhere overnight. The medium was thereafter aspirated and each well washed with 100 μ l of the buffer provided in the assay kit (1x was prepared by diluting the 10x stock in double-distilled water). The

cells were then stained with 100 μ l of a 25 μ M DCFDA solution (diluted with 1x buffer from a 20 mM stock) added to each well and the plate was incubated for 45 min at 37°C in the dark (inside the incubator). Blank wells (no cells, medium and buffer only) and non-stained control wells (cells, but no stain) were included. After 45 min, the DCFDA solution was removed, and the cultures were rinsed with the buffer. The rinse buffer was then aspirated and replaced with 50 μ l of the growth media without phenol red. Hydrogen peroxide (H_2O_2), 50 μ l (50 μ M or 100 μ M), and 4MF, 4HF, DMF and DHF (each at 1, 25, 75 μ M), each prepared in full growth medium without phenol red as double the indicated concentrations (2x) were added. Each of 4MF, 4HF, DMF and DHF was added alone and in the presence of hydrogen peroxide and a hydrogen peroxide-only treatment was also included. Each treatment was done in triplicate. Fluorescence was measured using the Spark™ 10M Tecan microplate reader at an excitation of 485 nm and an emission of 535 nm. Readings were taken at 1, 3, 6, and 24 h following treatment. The average fluorescence of the negative control (treatment with vehicle only) was set to '1' (unity) and the fluorescence of each other treatment was computed as fold change compared to the negative control.

2.2.14 DPPH free-radical scavenging assay

The stable DPPH radical model is a widely used, relatively quick method for the evaluation of free radical scavenging activity. The effects of antioxidants on DPPH radical scavenging are thought to be due to their hydrogen-donating ability. DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The absorption maximum of a stable DPPH radical in methanol is 517 nm. A decrease in the absorbance of a DPPH radical, caused

by antioxidants is due to the reaction between antioxidant molecules and radical, results in the scavenging of the radicals by hydrogen donation. It is visually noticeable as a change in colour from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the antioxidant activity of antioxidants (Zhang et al., 2020).

DPPH solution (80 µg/ml) was freshly prepared in methanol. The test compounds and the positive control (quercetin) were prepared in methanol as 2x concentrations. Each test compound, 50 µl (1, 25, 75 µM) or quercetin (1, 25, 75 µM) was added into an opaque 96-well plate. This was followed by the addition of 50 µl of the DPPH solution to the test compounds and controls. The standard ('no DPPH scavenging' control) was 50 µl of methanol and 50 µl of the DPPH solution. The mixtures were shaken for 2 min on the orbital shaker and the plate was left in the dark for 30 min. The absorbance was then read at 517 nm on the Spark™ 10M Tecan microplate reader. The capability for scavenging the DPPH radical was calculated using the following formula:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1)/A_0] * 100]$$

Where A₀ is the absorbance of the control reaction and A₁ is the absorbance of the test compound or the reference (quercetin). Each test was performed in triplicate and the triplicate values were averaged.

2.3 Molecular docking

Molecular docking has recently become a routinely used approach in drug discovery, especially complementarily with high-throughput screening (HTS) (Ferreira et al., 2010). The selected natural compounds for structure-activity relationship (SAR) studies were first evaluated using molecular docking to predict their PARP-inhibitory activities, based on their ligand-receptor interactions. Docking is a multistep process that involves various software and docking algorithms.

2.3.1 Ligand preparation

The .SDF format for the natural compounds (ligands) was downloaded from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>). The .SDF files were converted to .PDB format using the Chem3D 21.0.0 software (PerkinElmer Informatics Inc., Massachusetts, USA). Structures in the ligand.PDB format were then converted to the ligand.PDBQT format using AutoDock Tools Version 1.5.7 (ADT) (The Scripps Research Institute, San Diego, USA).

2.3.2 Receptor preparation

The three-dimensional structures of PARP1 (PDB ID: 5DS3 - Crystal structure of constitutively active PARP1) and PARP2 (PDB ID: 4TVJ - Human ARTD2 (PARP2) - Catalytic domain in complex with olaparib) were downloaded in .PDB format from the RCSB Protein Data Bank (<http://www.rcsb.org>) (last accessed 10th September 2022). Using notepad software, the co-crystallised ligand (olaparib) was separated from the X-ray crystal structures of PDB ID: 5DS3 and PDB ID: 4TVJ into the ligand.PDB and receptor.PDB files. The PARP2 receptor crystal had two chains, A

and B. The B chain was removed, leaving a single chain (A) that was required later for docking. All heteroatoms and water molecules from the receptor's PDB files were removed using notepad. The receptor.PDB files were loaded onto AutoDock Vina. Using the ADT software, polar hydrogens, Kollman charges, and Gasteiger charges were assigned to the protein structures. Finally, the receptor.PDB file was converted to receptor.PDBQT format and saved for docking (Trott and Olson, 2010; Dawicki-McKenna et al., 2015; Thorsell et al., 2017).

2.3.3 Docking

The grid coordinates and grid boxes (active site) of each receptor were determined using the ADT software and notepad. For PARP1, the grid size was set at $16 \times 16 \times 24$ (x, y, and z) points, and the grid centre was designated at x, y, and z dimensions of -2.541, 39.662, and 14.108, respectively, with a grid spacing of 1.000 Å. For PARP2, the grid size was set at $12 \times 14 \times 12$ (x, y, and z) points, and the grid centre was designated at x, y, and z dimensions of 18.881, -1.417, and 17.624, respectively, with a grid spacing of 1.000 Å. Ligands were docked individually to the receptor with grid coordinates and grid boxes calculated and prepared for each receptor. The ligand was in a rigid condition when interacting with macromolecules under rigid conditions. The AutoDock Vina docking was run using the appropriate command line. The results of the docking were given as ligand-binding affinities expressed as free energy (ΔG) scores (kcal/mol) calculated based on the AutoDock Vina scoring function. Out of 9 possible poses, the results with the lowest free energy of binding and a positional root-mean-square deviation (RMSD) of less than 2 Å were considered for further post-docking analysis. The

docking runs were performed 10 times for each compound on PARP protein receptors. An average binding affinity score of the best poses per ligand was taken.

2.3.4 Post-docking

The docking parameters and methods were validated by redocking the extracted co-crystal ligands against their respective receptors and comparing with the original X-ray crystal structures. Visualisation and ligand-protein interactions such as hydrogen-bond interactions and hydrophobic interactions were carried out using PyMOL (DeLano Scientific LLC, California, USA) and LigPlot⁺ Version 2.2 Software (European Bioinformatics Institute, Cambridge, UK) (Laskowski and Swindells, 2011; Schrödinger and DeLano, 2020).

2.3.5 Lipinski's Rule of Five

Lipinski's Rule of Five is a method used for evaluating the drug-likeness of chemical compounds. Also known as the rule of five, it estimates the bioavailability of a suspected drug candidate in the human body based on the following criteria - no more than 5 hydrogen bond donors, no more than 10 hydrogen bond acceptors, molecular mass less than 500 Da, and an octanol–water [partition coefficient](#) (log *P*) not greater than 5. A violation of 2 or more of these conditions predicts a molecule as a non-orally available drug (Lipinski, 2000). The structures of the selected natural compounds were accessed through the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) and downloaded as SDF files (last accessed 15th September 2022). The SDF files were converted to SMILES using the ChemDraw 21.0.0 Software (PerkinElmer Informatics Inc., Massachusetts, USA). The SMILES were then pasted into Molinspiration tool

(<https://www.molinspiration.com/cgi-bin/properties>) for evaluation (last accessed 15th September 2022). Molinspiration is a Java-based online cheminformatics software which can calculate all the parameters of the Lipinski's rule of five. The predicted bioactivity score and drug-likeness properties of the SAR study natural compounds were evaluated and compared.

2.4 Brightfield microscopy

Bright-field images of morphological changes to cultures induced by various treatments were captured on an inverted microscope, Olympus CKX41, using the Olympus cellSens Entry 1.16 imaging software (Olympus Corporation, Manila, Philippines), and saved. Appropriate scale bars were included in the acquired images.

2.5 Data presentation and statistical analyses

An average of each duplicate or triplicate treatment was taken. Each average value for the measured variable (e.g., viability) was then expressed as a percentage of the negative (vehicle) control value that was set to 100% or as otherwise stated. All data are reported as the Mean $\pm$ SEM (standard error of the mean) for $n = 3$ to 6 independent experiments, each run in triplicate or duplicate (except otherwise specified). All statistical analyses were performed using the GraphPad Prism Software, Version 8.0 (GraphPad Software Inc., CA, USA). IC₅₀ (half-maximal inhibitory concentration) or EC₅₀ (Half maximal effective concentration) is defined as the concentration of the test compound that produces 50% of the maximal response. IC₅₀ or EC₅₀ values were determined in Graphpad using the nonlinear

regression equation $\log(\text{inhibitor or agonist})$ vs. response (three parameters) or $\log(\text{inhibitor or agonist})$ vs. response (variable slope, four parameters).

T-test was used to assess the statistical significance of differences between two means, while Analysis of Variance (ANOVA) followed by Tukey's post-hoc test (or as otherwise stated) for multiple comparisons was used to assess the statistical significance of differences between more than two means, with $P < 0.05$ considered statistically significant.

Chapter 3: Results and Discussion I

3.0 Development, validation, and optimisation of a model assay for screening and characterising PARP inhibitors

3.1 Introduction

Developing a screening assay is one of the first major steps in the screening of chemical libraries to evaluate their effects on molecular targets in drug discovery and development (Lombardino and Lowe, 2004). PARP1 accounts for approximately 85% of the total cellular enzymatic activity of the PARP superfamily because it produces over 90% of PAR following its overactivation upon DNA damage (Mitra et al., 2019). Therefore, to assess the effect of PARP inhibitors on cellular PARP activity, PARP1 has been the main molecular target probed by researchers. The inhibition or activation of PARP activity has been determined using different methods based on the measurement of PAR production and/or consumption of NAD⁺ by using commercially available PARP assay kits (van der Heden van Noort, 2020; Belhadj et al., 2021). Various methods such as immunohistochemistry, flow-cytometry, western blot, and cell-based assays have been used to evaluate *in vitro* or *ex vivo* PARP1 activity (Geraets et al., 2007; Horvath, Zsengellér and Szabo, 2011). Furthermore, fluorescence- and luminescence-based phenotypic assays that exploit caspase independency of parthanatos (PARP1-mediated cell death) were developed and used to screen chemical libraries containing thousands of compounds for PARP1-inhibitory activities (Fatokun et al., 2013). Such an approach has been used in this project, in which MNNG, a DNA-alkylating agent known to induce PARP1-mediated cell death in HeLa cells, was used to stimulate parthanatos (Yu et al., 2002; Fatokun

et al., 2013). The ability of a compound to protect against the MNNG-induced cell death was then considered a valid phenotypic readout for its PARP1-inhibitory activity. This surrogate response was validated across a number of cell lines. In order to establish that the predominant cell death induced by MNNG was parthanatos and that contributions from other potential cell death mechanisms such as apoptosis and necroptosis were negligible or absent, z-VAD-fmk, a pan-caspase inhibitor that inhibits caspase-mediated apoptosis, and necrostatin-1, a necroptosis inhibitor, were used to rule out significant involvement of apoptosis and necroptosis, respectively.

This first results chapter focuses on the development, validation and optimisation of a model phenotypic assay for probing PARP1 activity, which was subsequently used to identify and characterise PARP1 inhibitors, whether in high- or low-throughput. The investigations were conducted using a range of cell lines and the experimental design included appropriate negative and positive controls. The positive controls were established experimental and clinically approved PARPi. This chapter presents data on the following, and also discusses the data in light of the original aim and objectives and the context of extant literature:

- The effect of the parthanatos inducing agent MNNG on the viability of HeLa cells in the presence or absence of DPQ, a potent experimental PARP-1 inhibitor, and Z-VAD-fmk, a pan-caspase inhibitor, using the MTT, AB and CTG assays (development of model assay).
- The effect of MNNG on the viability of HeLa cells in the presence or absence of necrostatin-1, a necroptosis inhibitor, using the MTT assay (assay validation for specificity of parthanatos induction).

- Assay validation by characterising a known (clinical) PARPi, olaparib, and PARPi-FL, its fluorescent derivative, for their potencies relative to one another in three different cell lines (HeLa, HaCaT and SH-SY5Y), using the MTT and CTG assays.
- Further assay validation by characterising the four clinically approved PARPi for their relative potencies in HeLa and HaCaT cells using the MTT assay.
- Using the context of a potential therapeutic application (protection against neuronal death in an acute neuropathological scenario exemplified by stroke) to further validate and optimise the assay by determining the extent of protection against PARP-mediated cell death (parthanatos) afforded by the experimental PARPi DPQ or the clinical PARPi OPB over a 16 h time course following MNNG induction in HeLa cells, using the MTT assay.

3.2 MNNG induces PARP1-mediated cell death in model assay

The model assay involved exposure of HeLa cells to MNNG (50 μ M) for 25 min, followed by restoration of the MNNG-treated cells to a test compound and incubation over a 24 h period before a viability assay was conducted. The PARPi DPQ (30 μ M) and the pan-caspase inhibitor Z-VAD-fmk (100 μ M) were used as positive and negative assay controls, respectively. It should be noted that, in the context of this assay, a PARPi is expected to protect against the reduction in viability induced by MNNG, thus increasing viability compared to MNNG alone. In the assay ([Figure 18](#)), MNNG alone reduced cell viability significantly to 16.3% compared to the negative control ($P < 0.0001$), while DPQ, which did not alter viability by itself alone, significantly protected against the reduction in cell viability induced

by MNNG. On the other hand, Z-VAD-fmk, while also not having any effect on its own, did not modify the toxic effect of MNNG, as the MNNG reduction in cell viability in the presence of Z-VAD-fmk (17.2%) was not different from the effect of MNNG alone. The observation that Z-VAD-fmk could not mitigate against MNNG-induced cell death suggests that apoptosis (caspase-mediated apoptosis) was not involved. DPQ and Z-VAD-fmk each by itself did not reduce cell viability, implying that they were not inherently cytotoxic. These findings are in line with previous studies which found that MNNG induced caspase-independent, PARP-dependent cell death (Yu et al., 2002; Fatokun et al., 2013). Furthermore, in this work, brightfield photomicrographs complemented the viability data, as negative control cultures were nearly completely confluent in a carpet-like network of attached cells, while cultures treated with MNNG showed significant loss of cells and shrinkage of the remaining cells, against which DPQ significantly protected by restoring morphology to near the negative control phenotype, while the phenotype in MNNG-treated cultures in the presence of Z-VAD-fmk was not different to the phenotype in the presence of MNNG alone ([Figure 19](#)).

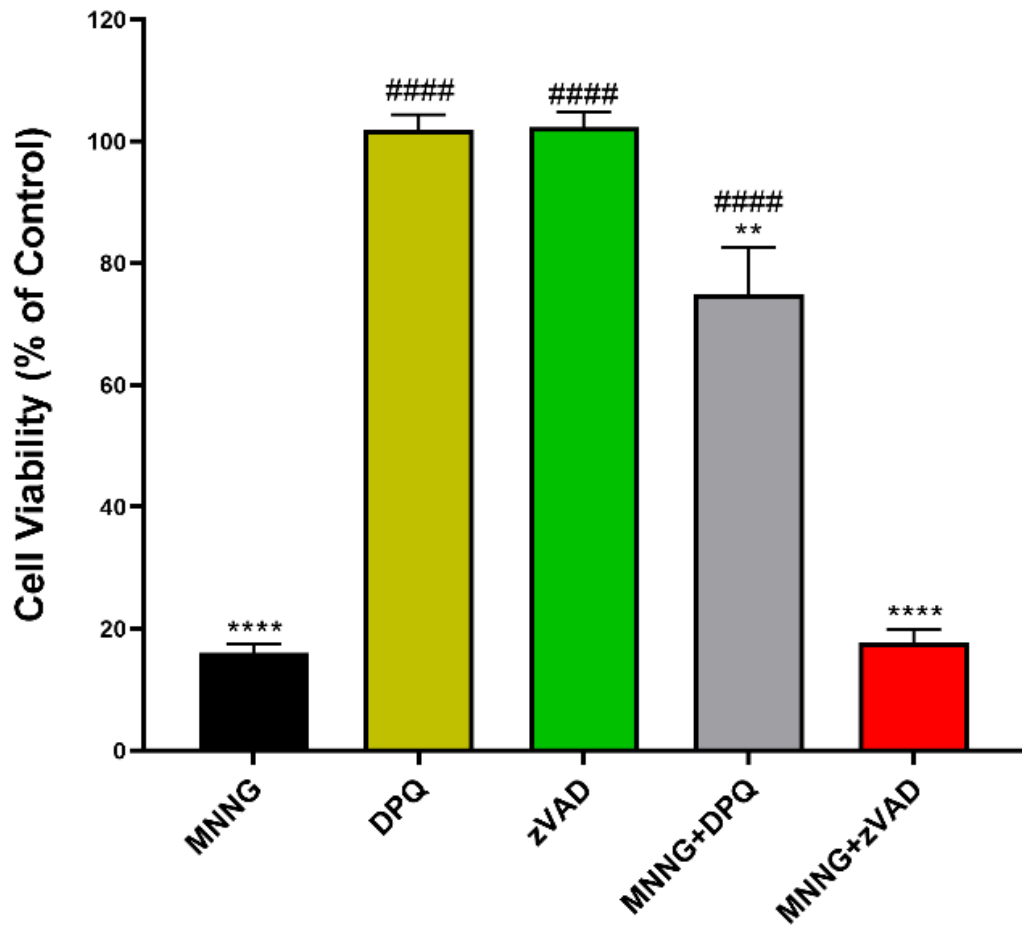
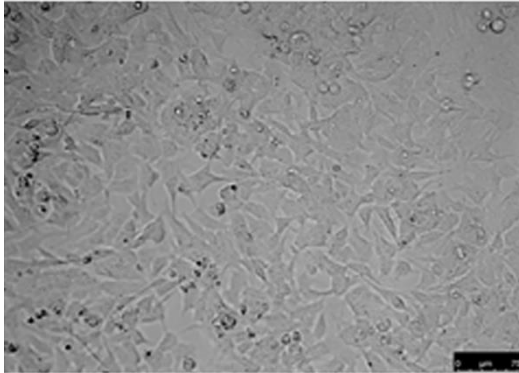
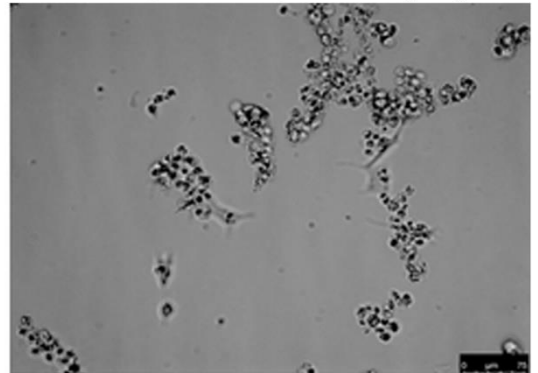


Figure 18: The model assay with MTT as viability reagent. The MTT assay shows that the PARP inhibitor DPQ (30 μ M) elicited very significant protection against cell death induced by MNNG (50 μ M, 25 min), whereas Z-VAD-fmk (zVAD) (100 μ M), a well-known pan-caspase inhibitor, elicited no protection. DPQ and Z-VAD-fmk each alone did not affect cell viability. ** $P < 0.01$ and **** $P < 0.0001$ with respect to the negative control; #### $P < 0.0001$ with respect to the effect of MNNG alone. N= 3 independent experiments, each run in triplicate.

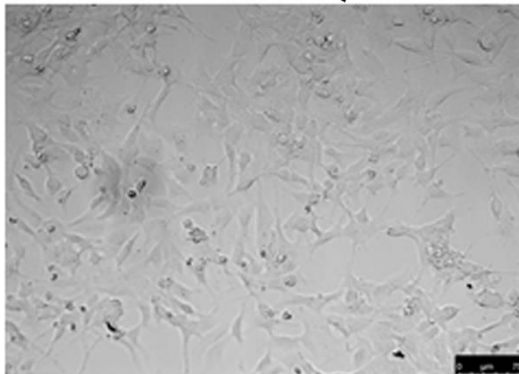
CONTROL (A)



MNNG ALONE (B)



MNNG + DPQ (C)



MNNG + ZvAD (D)

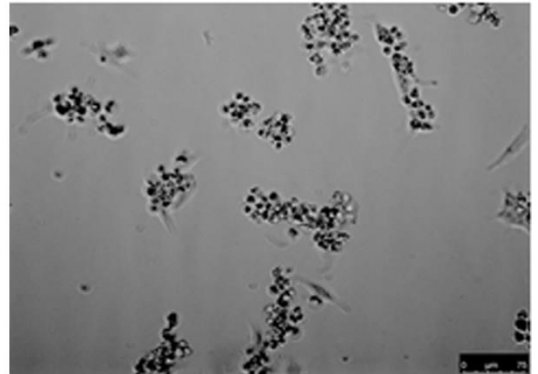


Figure 19: Cell morphology of MNNG-treated HeLa cells in the presence and absence of DPQ and Z-VAD-fmk (ZvAD). DPQ but not Z-VAD-fmk protected against morphological damage induced by MNNG. (A) Negative control cells (no MNNG, no test agent), (B) MNNG (50 µM) alone, (C) MNNG in the presence of Z-VAD-fmk (100 µM), and (D) MNNG in the presence of DPQ (30 µM). MNNG-treated cells appear rounded and shrunken compared to the control phenotype. DPQ ameliorates the damaging effect of MNNG by sustaining a phenotype that is similar to the control phenotype. The brightfield images were captured 24 h after treatment, using X10 magnification. Scale bar = 200 µm

To identify any potential artefacts that could be due to the use of the MTT reagent, and which could confound the results, the experiment was repeated and viability was quantified using two other viability assays whose readouts were not absorbance: the fluorescence-based AB assay and the luminescence-based CTG assay. [Figures 20](#) and [21](#) show a similar pattern to what was observed in the MTT assay: DPQ conferred significant protection against MNNG, while Z-VAD-fmk

lacked any effect. However, the baselines for the effects of MNNG alone, as well as the levels of protection by DPQ against MNNG-induced cell death, varied significantly across the three assays ([Figure 22](#)). MNNG alone lowered cell viability to 28.8%, 16.3% and 3.5%, for the MTT, AB and CTG assays, respectively, while the presence of DPQ increased those values to 82.4%, 59% and 47.1%, respectively ([Table 6](#)). Variations in cell viability or cytotoxicity results depending on the viability assay used have been reported (O'Donovan, 2012). The three different viability assays used in this work are based on different readouts and biochemical parameters as previously explained (Cell viability assays -Chapter 2.2.9). The differences in the endpoint results are consistent with other studies that have employed the MTT assay and other cell viability assays such as ATP and calcien assays. Although they differed in the endpoint measurements, there was a good correlation among these assays (Mueller, Kassack and Wiese, 2004), as found in our own work. In fact, the different endpoint assays can be considered to provide quantitative perspectives on how the levels of various biochemical indices and mediators of cell death, which might be involved simultaneously or sequentially in the complex death process, could be altered within the context of a defined cell death paradigm.

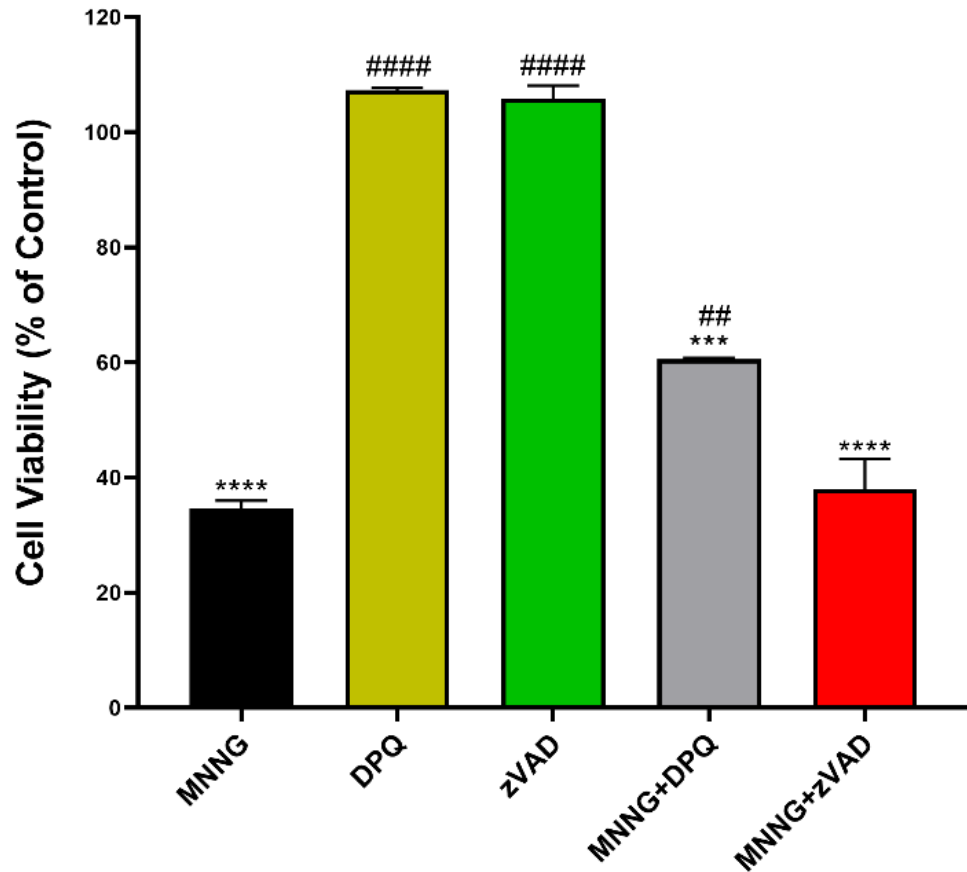


Figure 20: The model assay with AB as viability reagent. The AB assay shows that the PARP inhibitor DPQ (30 μ M) elicited very significant protection against cell death induced by MNNG (50 μ M, 25 min), whereas Z-VAD-fmk (zVAD) (100 μ M), a well-known pan-caspase inhibitor, elicited no protection. DPQ and Z-VAD-fmk each alone did not affect cell viability. *** P <0.001 and **** P <0.0001 with respect to the negative control; ## P <0.01 and ##### P <0.0001 with respect to the effect of MNNG alone. N=3 independent experiments, each run in triplicate.

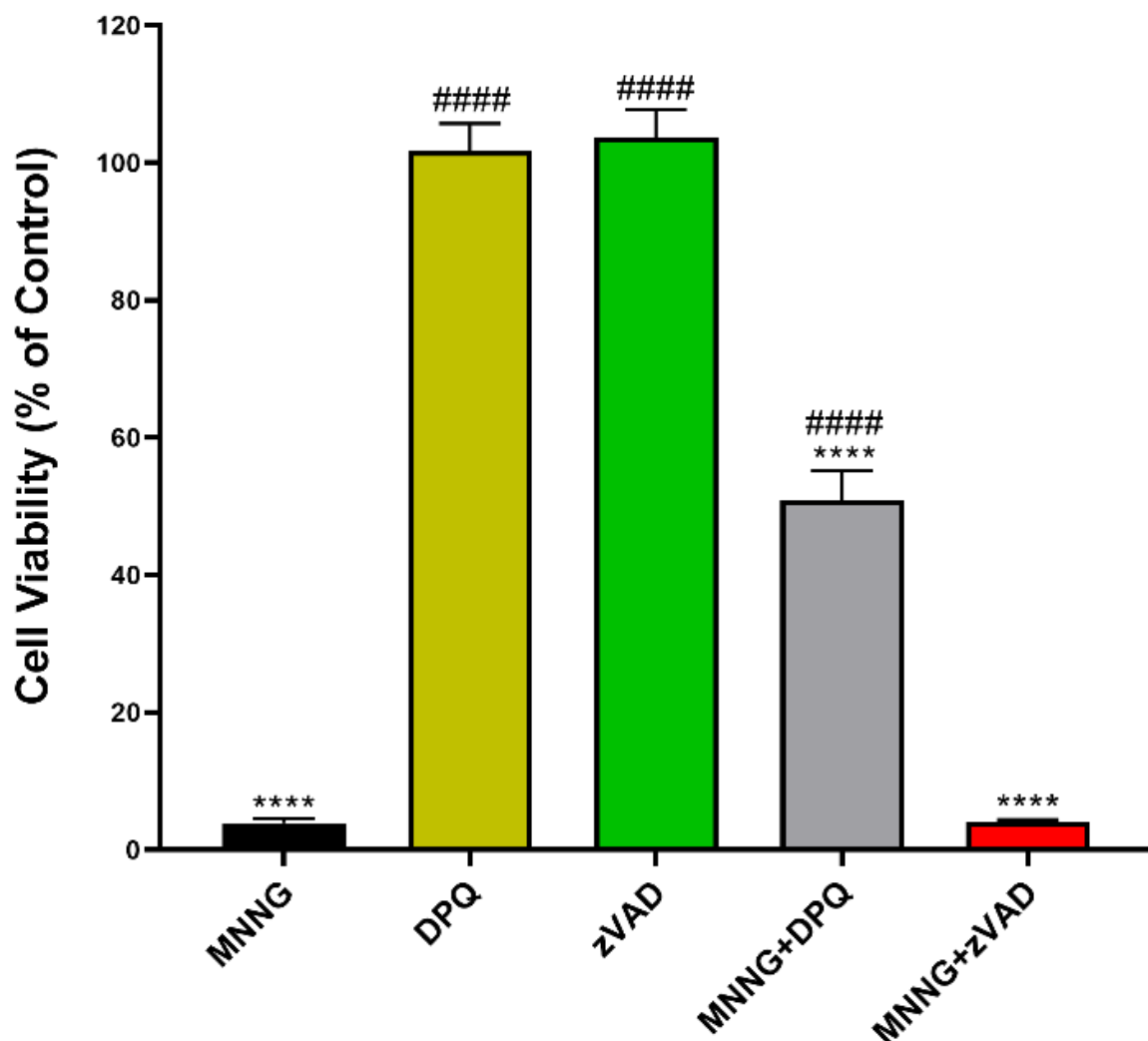


Figure 21: The model assay with CTG as viability reagent. The CTG assay shows that the PARP inhibitor DPQ (30 μ M) elicited very significant protection against cell death induced by MNNG (50 μ M, 25 min), whereas Z-VAD-fmk (zVAD) (100 μ M), a well-known caspase inhibitor, elicited no protection. DPQ and zVAD-fmk each alone did not affect cell viability. **** $P < 0.0001$ with respect to the control; ##### $P < 0.0001$ with respect to the effect of MNNG alone. N=3 independent experiments, each performed in triplicate.

The CTG assay appeared to be the most sensitive to the effect of MNNG, as the endpoint readings for MNNG-induced reduction in cell viability were significantly lower than for the MTT and AB assays ([Figure 22](#)). The same observation holds true when the fold increase in viability caused by DPQ is considered. To understand the differences in the results of these assays, it is important to note that

they capture different cellular activities or biomolecule(s) that serve as surrogates for cell viability. The CTG assay measured ATP levels in the wells after 18 – 24 hours of exposure of the treated cells to MNNG. Intracellular ATP concentration is known to decrease rapidly following MNNG induction of parthanatos (Huang et al., 2014). Within 15 min of MNNG insult, the resulting overactivation of PARP1 can cause up to 80% reduction in the NAD⁺ level due to elevated poly (ADP)-ribosylation of DDR nuclear proteins and PARP1 itself (Yu et al., 2006). Consequently, the depletion of the NAD⁺ leads to high consumption of ATP, as four molecules of ATP are required for the resynthesis of each molecule of NAD⁺ (Fatokun, Dawson and Dawson, 2014). In addition, NAD⁺ is a cofactor required by several metabolic pathways for generating ATP, for example, in glycolysis and the tricarboxylic acid (TCA) cycle (Sazanov and Jackson, 1994).

Mechanistically, it was initially thought within this research field that the cell death caused by PARP1 overactivation was exclusively as a result of a bioenergetics crisis initiated by NAD⁺ depletion (Berger, 1985). The rapid depletion of NAD⁺, followed by severe ATP starvation, can eventually lead to necrotic cell death. This proposed pathway was referred to as the “suicide hypothesis” (Ha and Snyder, 1999; Chiarugi, 2005). However, several studies have now suggested that the NAD⁺ depletion as a result of PARP-1 hyperactivation was not sufficient to account for cell death (David et al., 2009). Moreover, Huang et al. (2014) demonstrated that ATP levels were restored following an initial depletion triggered by MNNG within 4 h of insult in mouse embryonic fibroblasts (MEFs). Also, the MNNG-induced, early-phase ATP depletion was completely abated in the absence of PARP1 or by the pharmacological PARP1 inhibitors 3AB and DPQ (Huang et al., 2014). All these

suggest that the ATP levels measured after 18 - 24 h in this study might not necessarily have been as a direct result of the initial dip in the NAD⁺ with a consequential decrease in ATP. However, this initial drop in the energy level of the HeLa cells might have contributed to the cell death that was later captured as reduction in viability via the CTG readout. This is a possible explanation for the lower cell viability compared to the other assays.

Previous studies have reported that the ATP level is a good indicator of cell viability, as the intracellular concentration often correlates with the biomass of living cells. The MTT assay measures the activity of mitochondrial dehydrogenases, which is a good index of cell viability. MTT assay readouts have been shown to be higher than those of assays that measure ATP content in HeLa cells (Maehara et al., 1987; Mueller, Kassack and Wiese, 2004). This is consistent with the results of this study. Likewise, a comparison of the MTT and AB assays in the high throughput screening of a small chemical library using HepG2 cells suggested that the AB assay was more sensitive than the MTT assay (Hamid et al., 2004). In the context of the fold changes (increases) in cell viability elicited by DPQ against parthanatic cell death in the three assays ([Table 6](#)), the CTG assay demonstrated the highest signal-to-noise ratio ([Table 6](#)), a parameter that could be important as indicative of a wider effects window for identifying and discriminating between potential PARPi in a screening assay. However, all the three assays possess sufficient capacity to capture PARP1-mediated cell death and therefore could be used to screen compounds for PARP-inhibitory activity as well as characterise putative PARPi in subsequent, low-throughput mode. In fact, these assays could be seen as complementary to one another, revealing the different biochemical mechanistic

aspects of parthanatos. Furthermore, our results provided the opportunity to determine which of the assays could be deployed as a primary screening assay and which other one(s) could then be used as secondary (orthogonal) assay(s) to confirm the results from primary screening (Thorne, Auld and Inglese, 2010). Our approach is in alignment with that of a previous screening campaign for PARP inhibitors in which the AB and CTG assays were used in primary and secondary screenings, respectively, in HeLa and SH-SY5Y cells (Fatokun et al., 2013).

A key distinction of the MTT assay in this study compared to the AB or CTG assays was its profile for the effect of DPQ at 30 μ M, which was consistent with a near-complete blockade of the PARP-dependent toxic effect of MNNG, whereas the AB and CTG assays each showed a partial blockade of the toxic effect of MNNG at the same concentration of DPQ. MTT was, therefore, the assay that most clearly demonstrated the preponderance of parthanatos in the death paradigm elicited by MNNG. In addition, MTT was the most cost-effective of the three assays, and assay cost is a critical factor to consider in the choice of a screening assay, especially when the campaign includes a large set of chemical libraries. These qualities of the MTT assay, combined, made it the assay of choice as primary assay for our high-throughput screening, while the AB and CTG assays were used as secondary (orthogonal) assays to confirm the activity of hits obtained from the primary screen and weed out false positives (Thorne, Auld and Inglese, 2010).

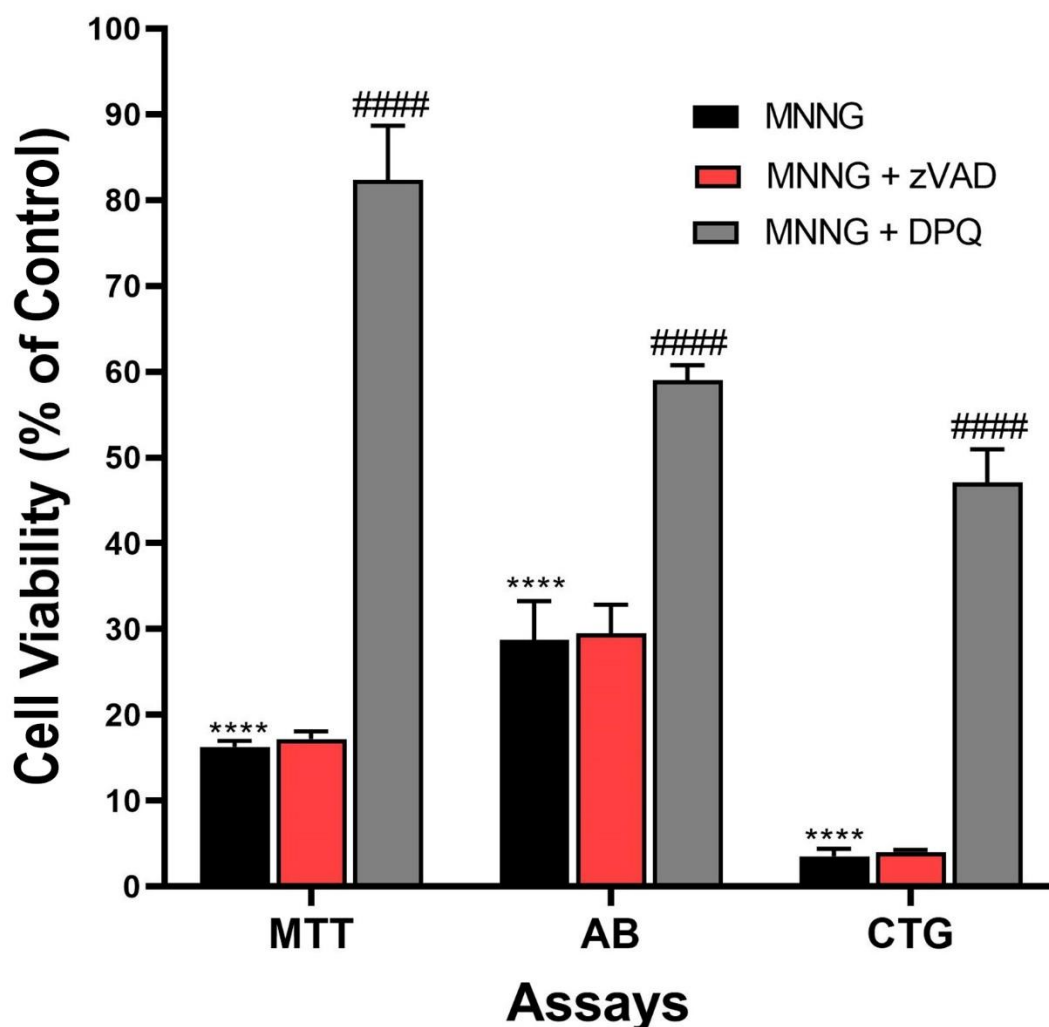


Figure 22: Model assay comparing the sensitivities of the three viability assays MTT, AB and CTG. The model assay shows the profiles of the three different assays when used to evaluate the effects of an established experimental PARP inhibitor DPQ (30 μ M) and a pan-caspase inhibitor Z-VAD-fmk (zVAD) (100 μ M) against cell death induced by MNNG (50 μ M, 25 min). In all the three assay modalities, DPQ elicited very significant protection against cell death induced by MNNG, whereas Z-VAD-fmk elicited no protection. This validates the model assay as robust enough for interrogating parthanatos (PARP1-mediated cell death), as parthanatos was the predominant cell death induced. *** $P < 0.001$ and **** $P < 0.0001$ with respect to the negative control; #### $P < 0.0001$ with respect to the effect of MNNG alone. N=3 independent experiments, each performed in triplicate.

Table 6: Cell viability values (mean $\pm$ SEM) for the model assay. Percentage (%) viability values obtained (each with respect to the negative control) following the indicated treatments and the respective assays are shown, as well as fold changes for the effects of DPQ vs. the effects of MNNG alone for the three assays. ^a $p < 0.0001$ with respect to MNNG alone for N= 3 experiments, each performed in triplicate.

	MTT	AB	CTG
MNNG alone	16.3 ± 0.7	28.8 ± 4.5	3.5 ± 0.9
MNNG + zVAD	17.2 ± 0.9	29.5 ± 3.3	4.0 ± 0.3
MNNG + DPQ	82.4 ± 6.3 ^a	59.0 ± 1.8 ^a	47.1 ± 3.9 ^a
Fold increase over the baseline (MNNG+DPQ vs. MNNG)	5.1	2.0	13.5

3.3 MNNG does not induce necroptosis in HeLa cells

Necroptosis is another form of programmed lytic cell death that is mediated by receptor-interacting serine/threonine kinase 1 and 3 (RIPK1 and RIPK3). RIPK3 and its substrate, phosphorylated mixed lineage kinase domain-like protein (pMLKL), have been demonstrated to be necessary for necroptosis, while RIPK1 plays an important upstream regulation of this pathway by activating RIPK3 via phosphorylation (Galluzzi et al., 2018). Poly ADP-ribosylation has been implicated in various cell death pathways (Aredia and Scovassi, 2014), and overactivation of PARP is suggested to cause necrosis by depleting NAD⁺ and ATP. Parthanatos has been described as regulated necrosis and is sometimes associated with necroptosis; for example, PARP1 activated by ischaemic damage and/or oxidative stress induced parthanatos and necroptosis in AML 12 mouse liver cells (Aredia and Scovassi, 2014; Haga et al., 2022).

The potential involvement of necroptosis in the cell death profile of the model assay earlier reported ([Figure 21](#)) was, therefore, examined by testing a pharmacological inhibitor of necroptosis, necrostatin-1, which has even been reported to prevent

necroptosis (induced by zinc-phthalocyanine) in HeLa cells (Soriano et al., 2014), against MNNG-induced cell death, with quantification by the MTT assay. As shown in [Figure 23](#), MNNG alone significantly reduced cell ability to 15.1% of the negative control, which was not different to viability values 14.5% and 11.8% in the presence of necrostatin-1 (NCS) at 10 μ M and 100 μ M, respectively. On the other hand, the addition of DPQ conferred significant protection against MNNG's toxicity, raising viability to 80.1%. Furthermore, the protection by a combination of DPQ and necrostatin-1 (NCS) against MNNG's toxicity was not different to that of DPQ alone against MNNG. Necrostatin-1 was confirmed not to have any effect on viability on its own.

With the involvement of apoptosis previously ruled out in this study, as the pan-caspase inhibitor Z-VAD-fmk did not ameliorate MNNG-induced cell death, the further finding that necrostatin-1 also failed to protect the cells against MNNG-induced cell death confirmed that the death profile induced by MNNG in the model assay was predominantly parthanatic due to PARP1 activation and did not involve significant apoptotic or necroptotic components. This was not just due to the fact that HeLa cells are claimed to lack RIPK3 expression - which implies that RIPK3-dependent necrosis (necroptosis) might not be possible to be induced (Xu et al., 2019b), as our results are consistent with previous studies that demonstrated that alkylating agent-induced parthanatos could be blocked by genetic deletion or pharmacological inhibition of PARP1, whereas inhibitors of autophagy, apoptosis, necrosis, and necroptosis had no effect (Zhang et al., 2011; Yang et al., 2022).

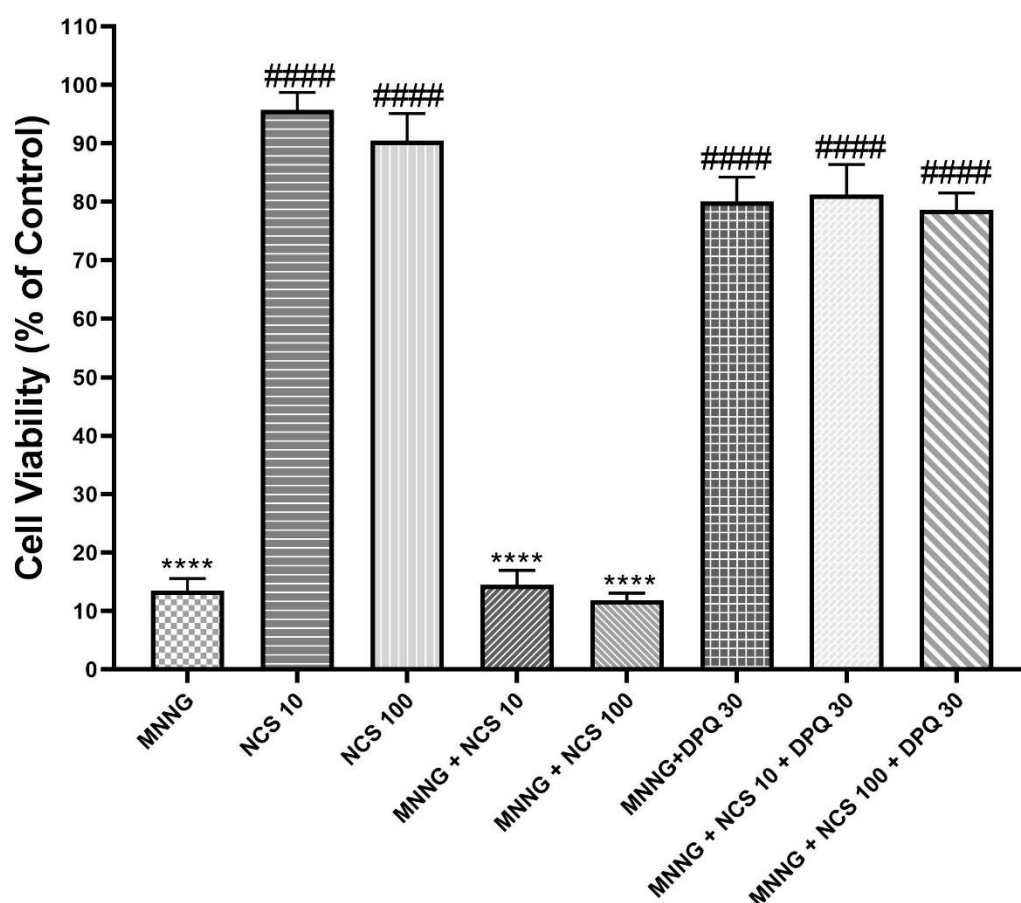


Figure 23: Lack of effect of the necroptosis inhibitor necrostatin-1 (NCS) on MNNG-induced cell death (MTT assay). The PARP inhibitor DPQ (30 μ M) elicits very significant protection against cell death induced by MNNG (50 μ M, 25 min), whereas necrostatin-1 (NCS) (10 and 100 μ M), a necroptosis inhibitor, elicits no protection. DPQ (30 μ M) or NCS (10 and 100 μ M) alone does not affect cell viability. **** P <0.0001 with respect to the control; ##### P <0.0001 with respect to the effect of MNNG alone. N=4 independent experiments, each performed in triplicate.

3.4 PARPi-FL, a fluorescent version of the PARP inhibitor olaparib, exhibits decreased potency but similar efficacy, compared to olaparib in a cell-based assay

PARPi-FL was employed in the validation of our model assay. PARPi-FL is a fluorescent version of olaparib (a clinically approved PARPi) ([Figure 24](#)), in which the cyclopropane moiety on olaparib has been replaced with the green fluorescent

BODIPY-FL dye to produce a PARP1 imaging agent (Irwin et al., 2014). A fluorescent version of a ligand is very useful as a chemical tool (probe) because, unlike the non-fluorescent parent ligand, it allows live and real-time visualisation and imaging of ligand-receptor (ligand-target) interactions in a wide range of scenarios, as well as the quantification of those interactions. PARPi-FL was advantageous in the validation and optimisation of the model assay, as it was used to assess whether the assay was sensitive enough to reveal the reported difference in PARP inhibition potency between itself and the parent olaparib. This is because it is known that the addition of a fluorophore (and a linker) to a parent ligand to derive its fluorescent version often changes some of the ligand's physicochemical and pharmacological properties, including efficacy and potency (Reiner, Carney and Kossatz, 2017). Both olaparib and PARPi-FL were then tested against MNNG in the model assay, using three different cell lines, and their potencies were compared

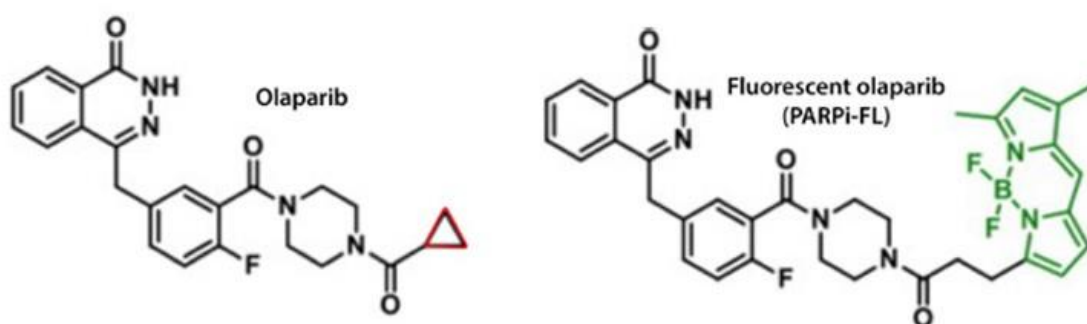


Figure 24: The chemical structures of olaparib and PARPi-FL. The cyclopropane component (red) in olaparib is substituted with a fluorescent dye, BODIPY-FL (green), in PARPi-FL.

The results showed that olaparib and PARPi-FL each elicited concentration-dependent protection against MNNG-induced reduction in cell viability in all the three cell lines: HeLa, HaCaT and SH-SY5Y (Figures [25-27](#)). The EC₅₀ values for PARP-FL were significantly higher than for olaparib (meaning olaparib's potencies were higher than PARPi-FL's) in all the three cell lines, with respect to both the MTT assay and the CTG assay ([Table 7](#)). In the MTT assay, while there were no significant differences in their efficacies ([Figure 28](#)), as they reached largely similar maxima, PARPi-FL was more than twice (2.4 times), more than thrice (3.4 times), and six times less potent than olaparib in the HeLa, HaCaT and SH-SY5Y cells, respectively ([Figure 29](#)). In a similar pattern observed with the CTG assay, the efficacies of both ligands were similar, but PARPi-FL was more than thrice (3.3 times), twice (2.1 times), and nearly eleven times (10.6 times) less potent than olaparib in the HeLa, HaCaT and SH-SY5Y cells, respectively ([Figure 30](#)). Differences in EC₅₀ or IC₅₀ values for the same compound using the same assay but in different cell lines have been demonstrated by various studies; for example, the IC₅₀ values of olaparib and veliparib were highly varied among 20 ovarian cancer cell lines (Stordal et al., 2013). Therefore, the variation in the EC₅₀ values of olaparib and PARPi-FL in the three different cell lines as seen in this study is in line with previous studies.

The EC₅₀ values in the nanomolar concentration range show that olaparib and PARPi-FL are very potent PARPi. Based on what is known in the literature, a decrease in the potency of PARPi-FL compared to olaparib, as was found in our assay, was expected – and that validated our assay - as the substitution of cyclopropane with green fluorescent BODIPY-FL increased the molecular weight

(PARPi-FL, 640 g/mol; olaparib, 435 g/mol), which altered binding affinity and cell membrane permeability (Reiner, Carney and Kossatz, 2017). The EC₅₀ values of PARPi-FL and olaparib obtained in this study's cell-based assays are higher than those reported in cell-free systems. This was not unexpected, as it is known that potency values obtained in cellular (functional) assays can be lower than those obtained by direct, target-based biochemical (cell-free) assays due to several factors such as intracellular permeability, bioavailability, etc. (Mateus et al., 2017). Despite the relatively higher cellular EC₅₀ values, the results from this study are consistent with previous reports of the two ligands' high potencies in cell-free systems, in which PARP-FL (12.2 nM) was 2.4 times less potent than olaparib (5 nM) (Irwin et al., 2014), which reveals a potency ratio largely similar to the potency ratios obtained in this study, e.g., PARPi-FL was also 2.4 times less potent than olaparib in the MTT assay in HeLa cells ([Figure 29](#)). Interestingly, this is the first direct comparison of olaparib and PARPi-FL in a cell-based, functional assay of parthanatos, as opposed to testing in cell-free systems. This thus represents one of the contributions to knowledge of this study, in addition to validating our parthanatos assay. It should be noted that although PARPi-FL exhibited reduced potency compared to the parent olaparib in our cell-based assay, both ligands are still relatively highly potent, as their potency (EC₅₀) values are low-to-medium nanomolar concentrations. With PARPi-FL being fluorescent, it represents a powerful imaging tool in the discovery and development of novel, more efficacious, potent, and safer PARPi to treat cancers and other conditions such as neurodegeneration in which PARP activation plays a role. It can also be deployed to develop relevant diagnostic tools and biomarkers to revolutionise the treatment of such diseases and related conditions. In fact, PARPi-FL is currently in Phase I/II

clinical trial to evaluate its usefulness in the imaging and screening of oral cancer (Kossatz et al., 2016). The findings in this study could, therefore, contribute to advancement in this field by proving useful in guiding the use of PARPi-FL in cell-based versus cell-free scenarios and, thus, by extrapolation, in *in vivo* contexts.

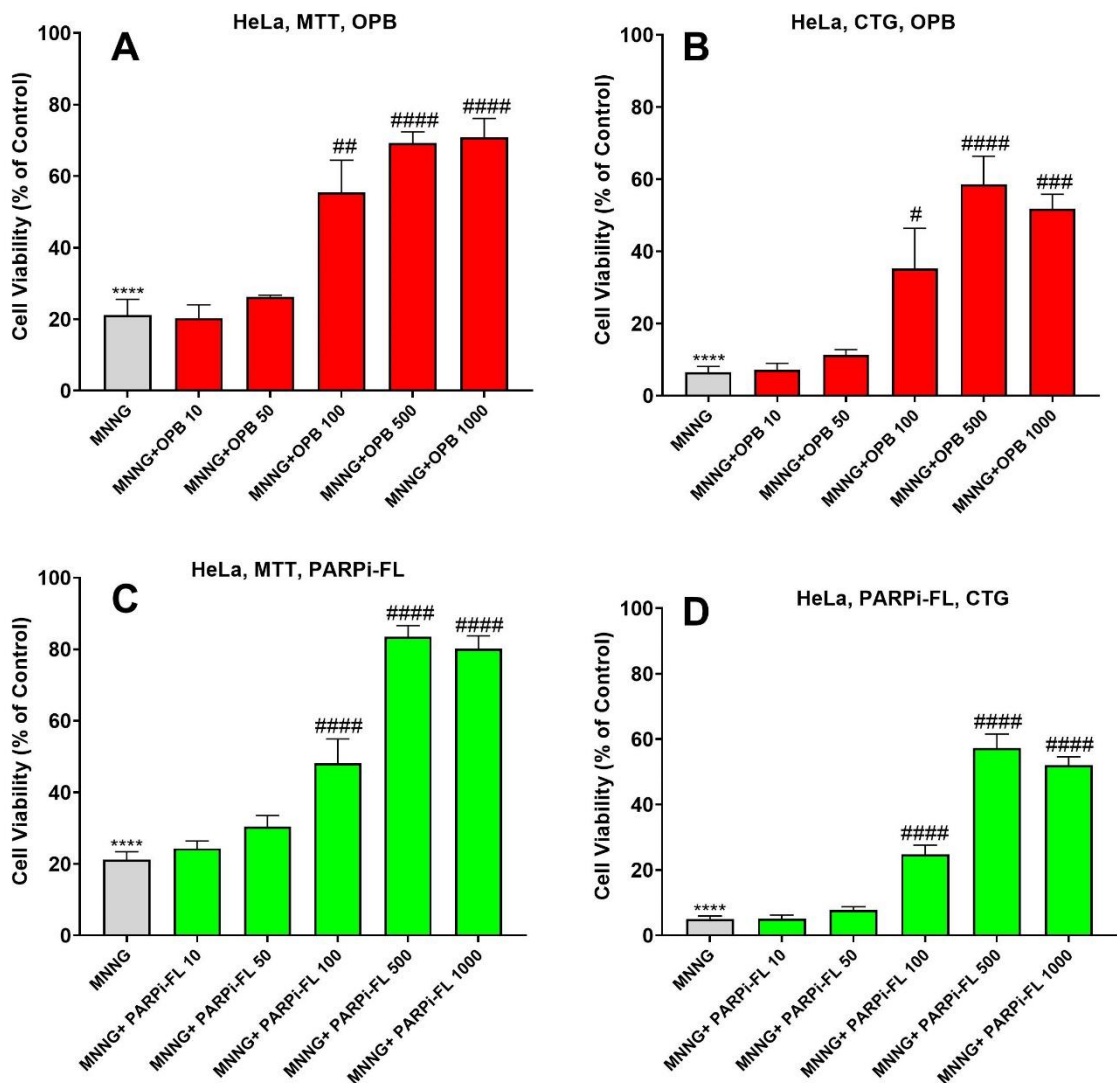


Figure 25: Protective effects of olaparib (OPB) and PARPi-FL against MNNG-induced cell reduction in viability (HeLa). OPB (red bars, A and B) and PARPi-FL (green bars, C and D), each up to 1000 nM, show concentration-dependent protection against MNNG-induced reduction in cell viability, as quantified by the MTT (A, C) and the CTG (B, D) assays. **** $P < 0.0001$ with respect to the negative control; # $P < 0.05$, ## $P < 0.01$, #### $P < 0.001$ and ##### $P < 0.0001$ with respect to MNNG alone. N=3-6 independent experiments, each performed in triplicate.

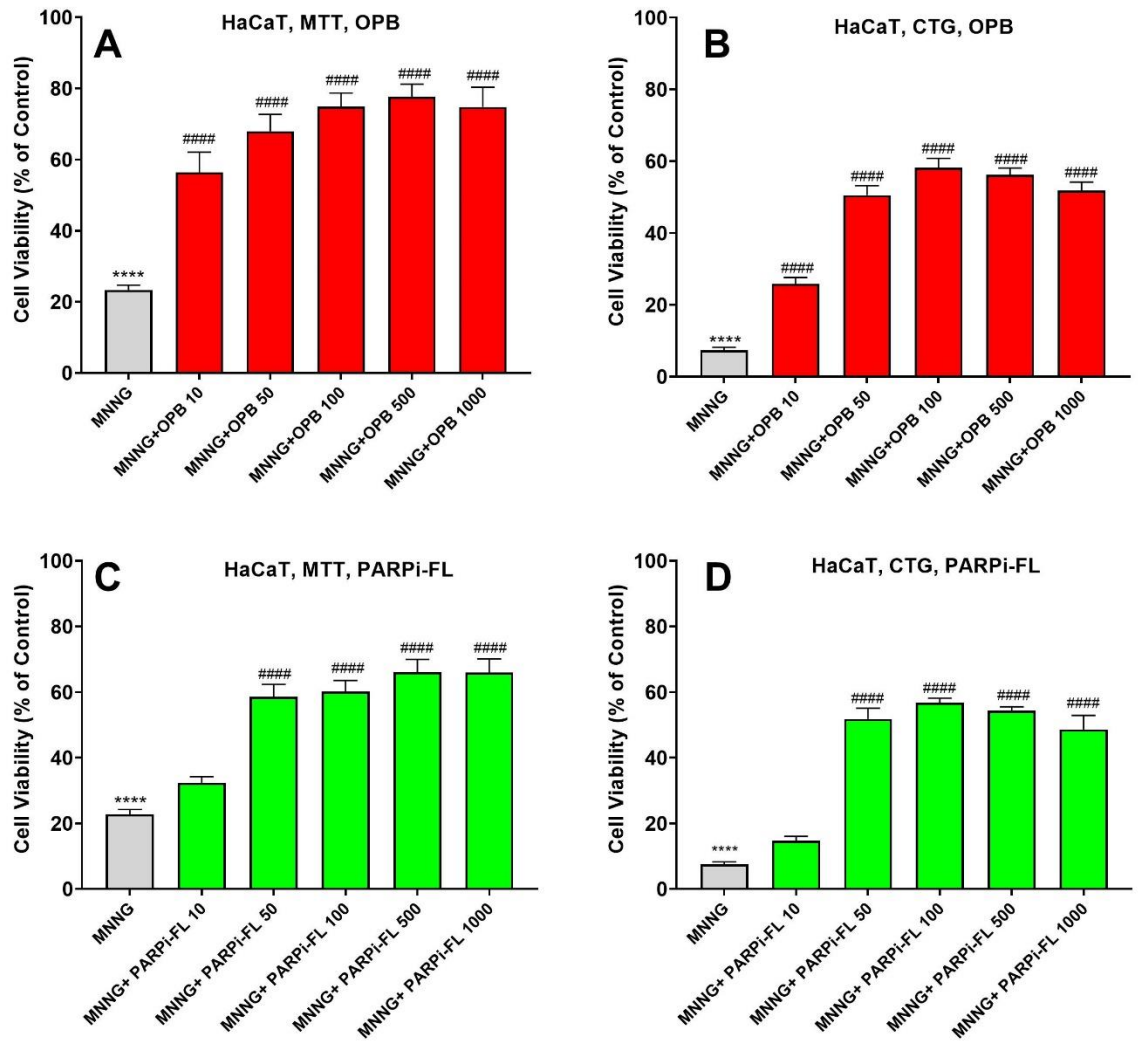


Figure 26: Protective effects of olaparib (OPB) and PARPi-FL against MNNG-induced cell reduction in viability (HaCaT). OPB (red bars, A and B) and PARPi-FL (green bars, C and D), each up to 1000 nM, show concentration-dependent protection against MNNG-induced reduction in cell viability, as quantified by the MTT (A, C) and the CTG (B, D) assays. **** $P < 0.0001$ with respect to the negative control ##### $P < 0.0001$ with respect to MNNG alone. N=3 independent experiments, each performed in triplicate.

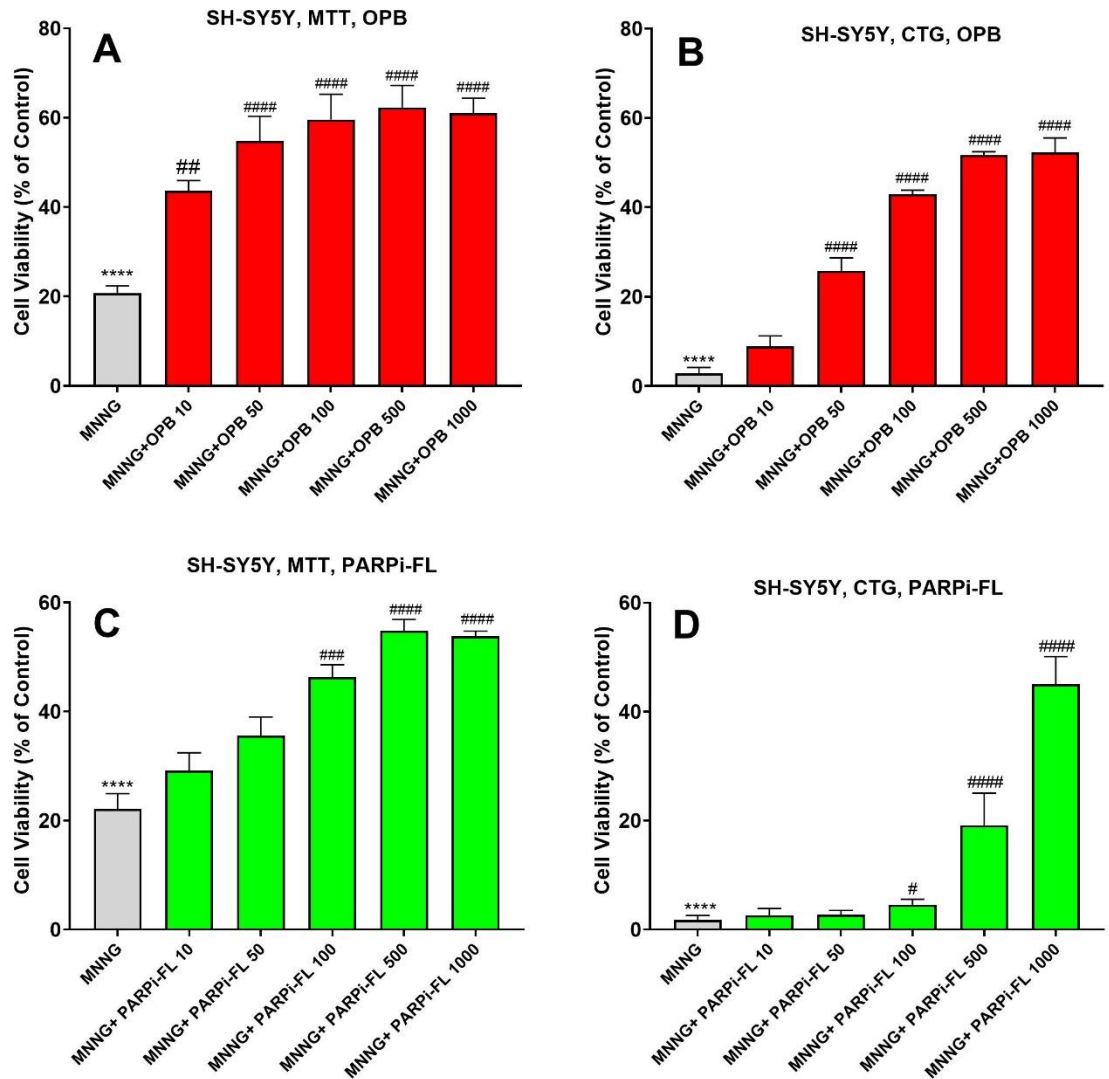


Figure 27: Protective effects of olaparib (OPB) and PARPi-FL against MNNG-induced cell reduction in viability (SH-SY5Y). OPB (red bars, A and B) and PARPi-FL (green bars, C and D), each up to 1000 nM, show concentration-dependent protection against MNNG-induced reduction in cell viability, as quantified by the MTT (A, C) and the CTG (B, D) assays. **** $P < 0.0001$ with respect to the negative control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ and #### $P < 0.0001$ with respect to MNNG alone. $N=3$ independent experiments, each performed in triplicate.

Table 7: EC₅₀ values in nM (Mean ± SEM) and corresponding pEC₅₀ values for olaparib and its fluorescent derivative (PARPi-FL) in the HeLa, HaCaT and SH-SY5Y cell lines. N=3-6 independent experiments, each performed in triplicate.

		Olaparib		PARPi-FL	
		EC ₅₀ (nM)	pEC ₅₀	EC ₅₀ (nM)	pEC ₅₀
HeLa	MTT	23.1 ± 8.9	7.64	55.7 ± 15.9	7.25
	CTG	38.9 ± 4.2	7.41	130.1 ± 16.9	6.89
HaCaT	MTT	6.23 ± 0.4	8.21	21.8 ± 3.9	7.66
	CTG	9.6 ± 0.1	8.02	19.7 ± 2.0	7.71
SH-SY5Y	MTT	8.3 ± 0.7	8.08	49.9 ± 6.7	7.30
	CTG	53.5 ± 3.8	7.27	567.0 ± 56.4	6.25

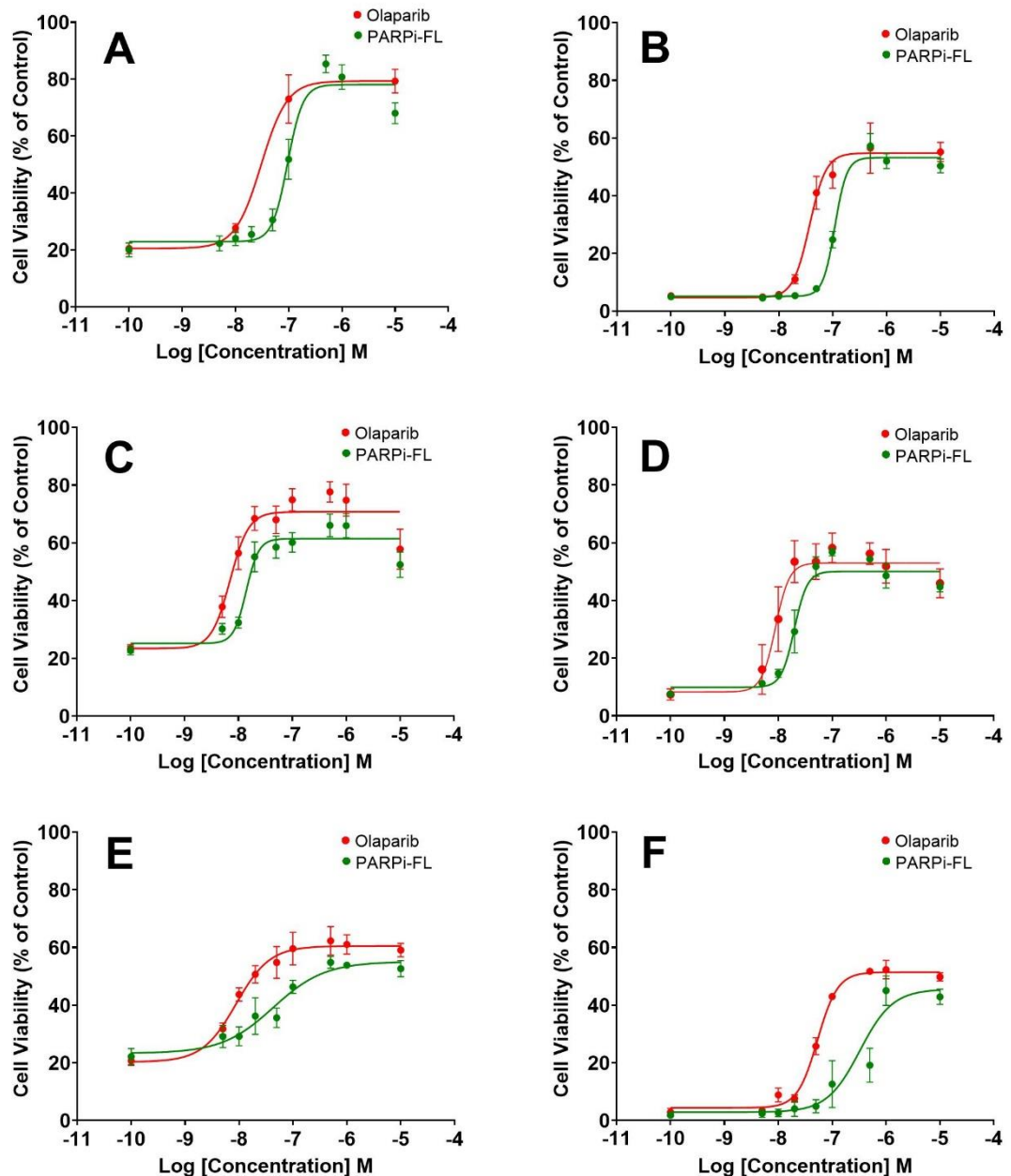


Figure 28: Log concentration-response curves for the protective effects of olaparib and PARPi-FL against MNNG-induced cell death in the HeLa, HaCaT and SH-SY5Y cell lines based on the MTT and the CTG assays. For the MTT and CTG assays, respectively: A and B are for the HeLa cells; C and D are for the HaCaT cells; and E and F are for the SH-SY5Y cells. N=3-6 independent experiments, each performed in triplicate.

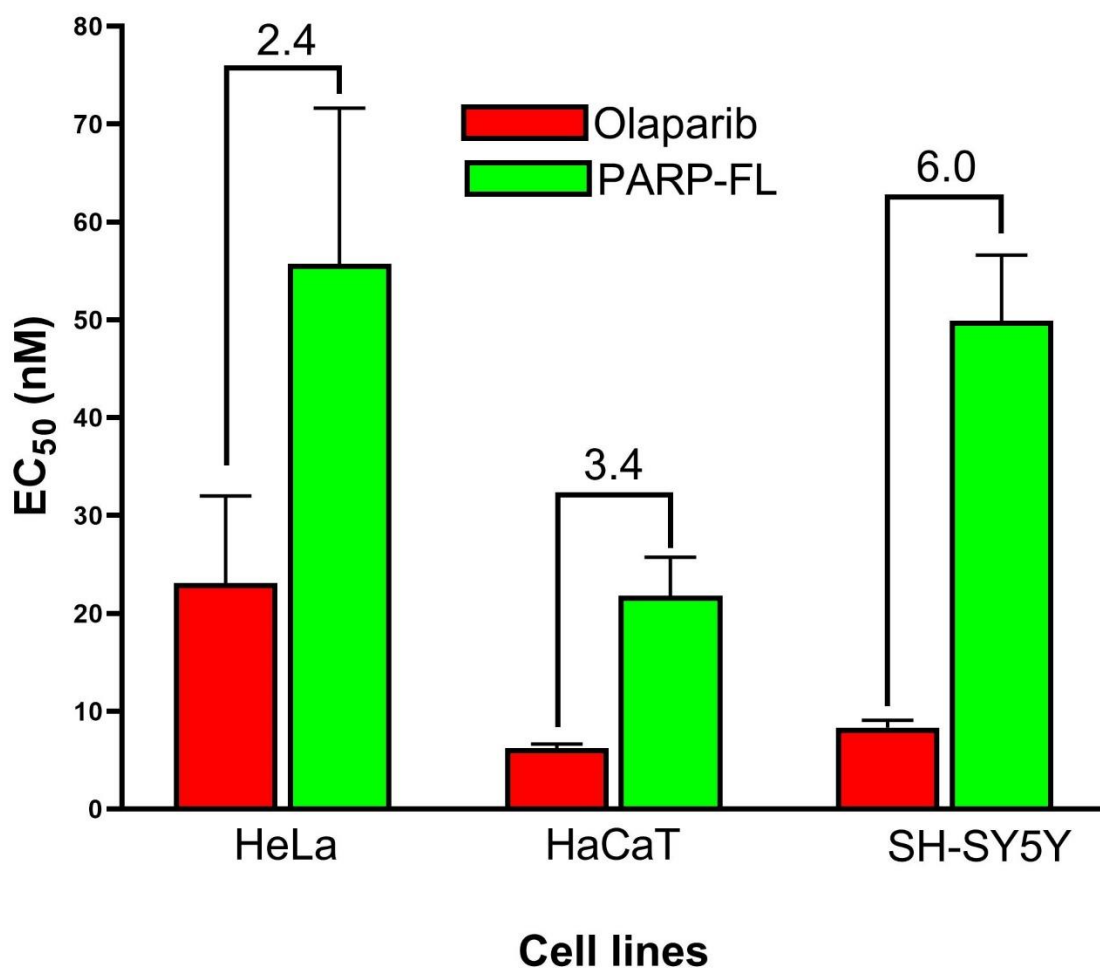


Figure 29: Comparison of the potencies of olaparib and PARPi-FL using the MTT assay. The plotted EC₅₀ values are as shown in Table 7, derived from the sigmoid curves in Figure 28 for the protection elicited by each of the two PARPi ligands against MNNG-induced death in the MTT assay. The EC₅₀ value for PARPi-FL in each assay was significantly higher than for olaparib, meaning PARPi-FL was consistently less potent than olaparib. Numbers on top of each red-green pair of bars indicate fold changes in EC₅₀ values (depicting potency changes) for PARPi-FL vs. olaparib. N=3-6 independent experiments, each performed in triplicate.

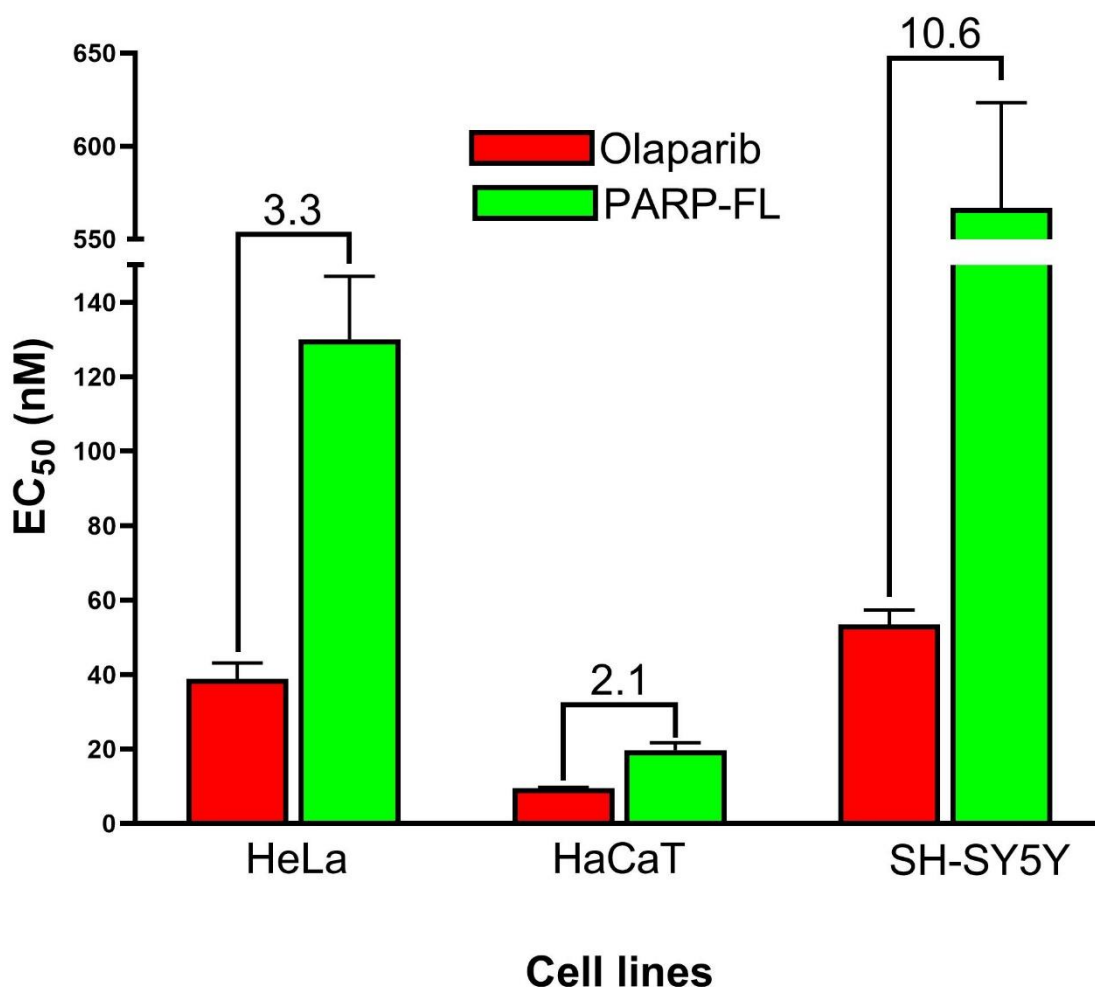


Figure 30: Comparison of the potencies of olaparib and PARPi-FL using the CTG assay. The plotted EC₅₀ values are as shown in Table 7, derived from the sigmoid curves in Figure 28 for the protection elicited by each of the two PARPi ligands against MNNG-induced death in the CTG assay. The EC₅₀ value for PARPi-FL in each assay was significantly higher than for olaparib, meaning PARPi-FL was consistently less potent than olaparib. Numbers on top of each red-green pair of bars indicate fold changes in EC₅₀ values (depicting potency changes) for PARPi-FL vs. olaparib. N=3 independent experiments, each performed in triplicate.

3.5 Model assay accurately reveals the relative potencies and the potency ranking of clinical PARP inhibitors

PARP inhibitors can selectively kill cancer cells that harbour deleterious mutations in tumour suppressor genes such as *BRCA1* and *BRCA2*. Currently, four PARP inhibitors have been approved by the US Food and Drug Administration (FDA) and/or the European Medicines Agency (EMA) and are in clinical use for the

treatment of various cancers, either as monotherapy (in most cases) or in combination adjuvant therapy. To further validate the model cell-based assay, the PARP-inhibitory activities of these clinically established PARPi: olaparib (2014), rucaparib (2016), niraparib (2017) and talazoparib (2018), were examined in HeLa cells using the MTT assay.

Each of the four PARPi showed concentration-dependent protection against MNNG-induced reduction in cell viability ([Figure 31](#)). They displayed similar efficacies and very high but different potencies all in the low nanomolar range ([Figure 32](#)). The EC₅₀ values (nM) revealed their rank order of potencies as: talazoparib (3.6 ± 0.3) > rucaparib (8.4 ± 1.8) > niraparib (17.1 ± 2.0) > olaparib (23.1 ± 8.9) ([Table 8](#)). As much as we are aware, this is the first time that the four PARPi have been so compared in such a cell-based assay. Although the cell-based EC₅₀ values are higher than the literature-reported IC₅₀ values in cell-free assays, the order of potencies in our cell-based assay is the same as what has been reported in cell-free assays and the relative potencies compared to olaparib as baseline are similar ([Table 8](#)) (Slade, 2020). This finding further confirms the accuracy and robustness of this study's model assay for identifying and characterising PARPi.

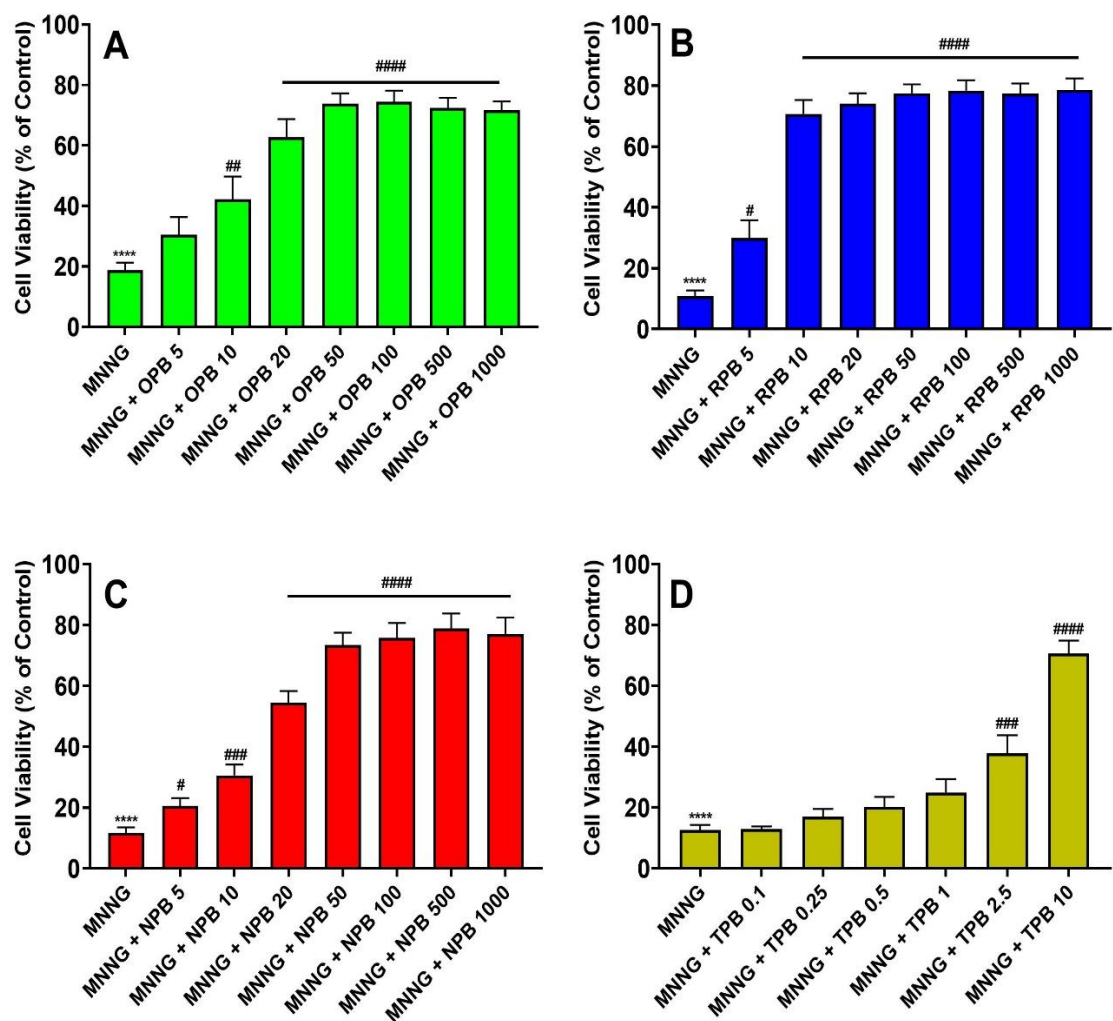


Figure 31: Protection by olaparib (A), rucaparib (B), niraparib (C) (5-1000 nM) and talazoparib (0.1-10 nM) against MNNG-induced reduction in cell viability in HeLa cells, as quantified by the MTT assay. The protection by each of the PARPi was concentration-dependent. **** $P < 0.0001$ with respect to the negative control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ and #### $P < 0.0001$ with respect to MNNG alone. N=6 independent experiments, each performed in triplicate.

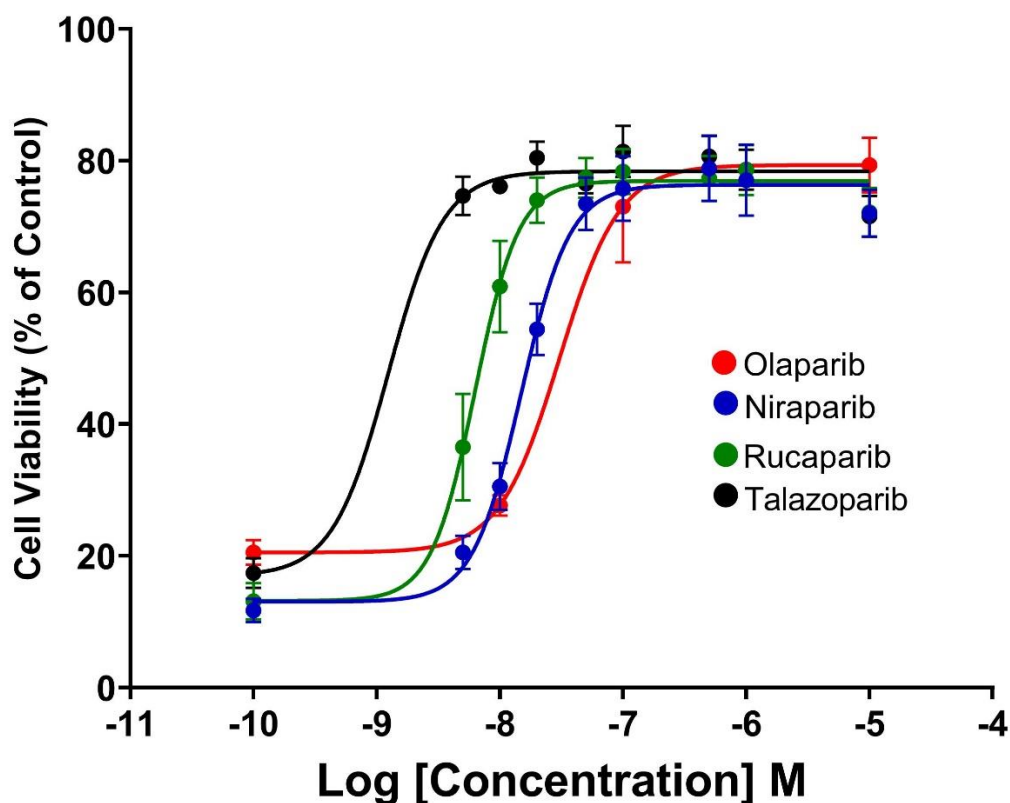


Figure 32: Log concentration-response curves for the protective effects of olaparib, niraparib, rucaparib and talazoparib against MNNG-induced reduction in viability in HeLa cells using the MTT assay. N=6 independent experiments, each performed in triplicate.

Table 8: EC₅₀ values (Mean $\pm$ SEM) for the four PARPi in cell-based and cell-free assays and their potency ratios relative to olaparib. The EC₅₀ values revealed their rank order of potencies as: talazoparib > rucaparib > niraparib > olaparib. N=6 independent experiments, each performed in triplicate.

	EC ₅₀ /IC ₅₀ (nM)		Potency ratio vs. olaparib	
	EC ₅₀ (our <u>cell-based</u> assay)	IC ₅₀ (literature-reported, <u>cell-free</u> assay)	Our Cell-based Assay	Literature- reported Cell-free Assay
Olaparib	23.1 $\pm$ 8.9	5	1	1
Rucaparib	8.4 $\pm$ 1.8	2	2.8	2.5

Niraparib	17.1 ± 2.0	3.8	1.3	1.3
Talazoparib	3.6 ± 0.3	0.57	6.4	8.8

3.6 PARPi elicited concentration- and time-dependent protection against MNNG-induced, PARP-mediated cell death when each inhibitor's addition was delayed for up to 12 hours post-insult

Functional recovery in patients who have suffered debilitating neurological events such as stroke and traumatic brain injury critically depends on the time window within which appropriate therapeutic interventions are initiated following the event, and delayed treatments are known to be associated with poorer functional recovery outcomes. One major pathway now identified to underlie neuronal death in neurological and neurodegenerative conditions is over-activation of PARP, leading to PARP-mediated neuronal death (parthanatos) (Venkat et al., 2018). PARP inhibitors are, therefore, being investigated for their potential as neuroprotective agents. However, the time course of potential protection by PARP inhibitors in the context of delayed intervention following the initiation of PARP-mediated cell death has not been examined in detail. Having established the reliability of our model assay for characterising a PARP inhibitor, as part of additional validation the assay was used to clarify for how long the addition of a PARPi could be delayed, following cell exposure to MNNG, before any protection by that PARPi ceases to be elicited. Two highly potent and selective PARPi, DPQ (an experimental agent) and olaparib (a clinical PARPi), were each added at various time points up to 16 h after the initiation of the MNNG insult, and their protection against MNNG-induced reduction in cell viability was measured in HeLa cells using the MTT assay.

DPQ and olaparib, when added immediately after the initiation of the MNNG insult, each elicited concentration-dependent protection against MNNG-induced reduction in cell viability, which was significant at 100 and 1000 nM ($P < 0.0001$) for olaparib and at 10 and 50 μ M ($P < 0.0001$) for DPQ (Figures 34 and 35, red bars). MNNG reduced viability to about 18% of the negative control, and olaparib and DPQ protected against this deleterious effect by raising viability to nearly 80% and 70% of the negative control, respectively, at their maximal effects. While the protection by olaparib progressively decreased as the number of hours that had elapsed post-insult (when it was added) increased up to 16 h, it continued to elicit significant, concentration-dependent protection up to 12 of those 16 hours post-insult ([Figure 33](#)). Its protection profile revealed that the highest significant decrease in its protection occurred between the 0 and 4 h time points, followed by less-steep decreases in protection between each of the subsequent 4 h time-point pairs. A profile similar to that of olaparib was exhibited by DPQ ([Figure 34](#)), although DPQ's concentration-dependent protection was significant only up to 8 h post-insult. Olaparib exhibited significantly higher potency of protection than DPQ in the time course.

Based on the literature, as far as this study's investigator is aware, this is the first experiment that investigated the time course of protection of a PARPi against MNNG-induced cell death. However, this finding is similar to the work of Hamby et al. (2007) who found that PJ34, a potent PARPi, elicited some reduction in CA1 neuronal death when administered up to 8 h post-ischaemic event. It is important to note, however, that the CA1 neuronal death in the rat model was caused by microglia/macrophage activation and not parthanatos (Hamby et al., 2007). This study's finding demonstrates that PARPi could elicit protection against PARP-

mediated cell death when added even as late as 8-12 h after the initiation of insult. This suggests that PARP inhibitors could provide significant therapeutic benefits and at least some functional recovery if used clinically to manage neurological condition scenarios that are exacerbated by delayed initiation of treatment. This investigation should, therefore, be expanded to assess the effects of a wider range of PARPi using *in vitro* and *in vivo* models of neurological conditions such as stroke and traumatic brain injury.

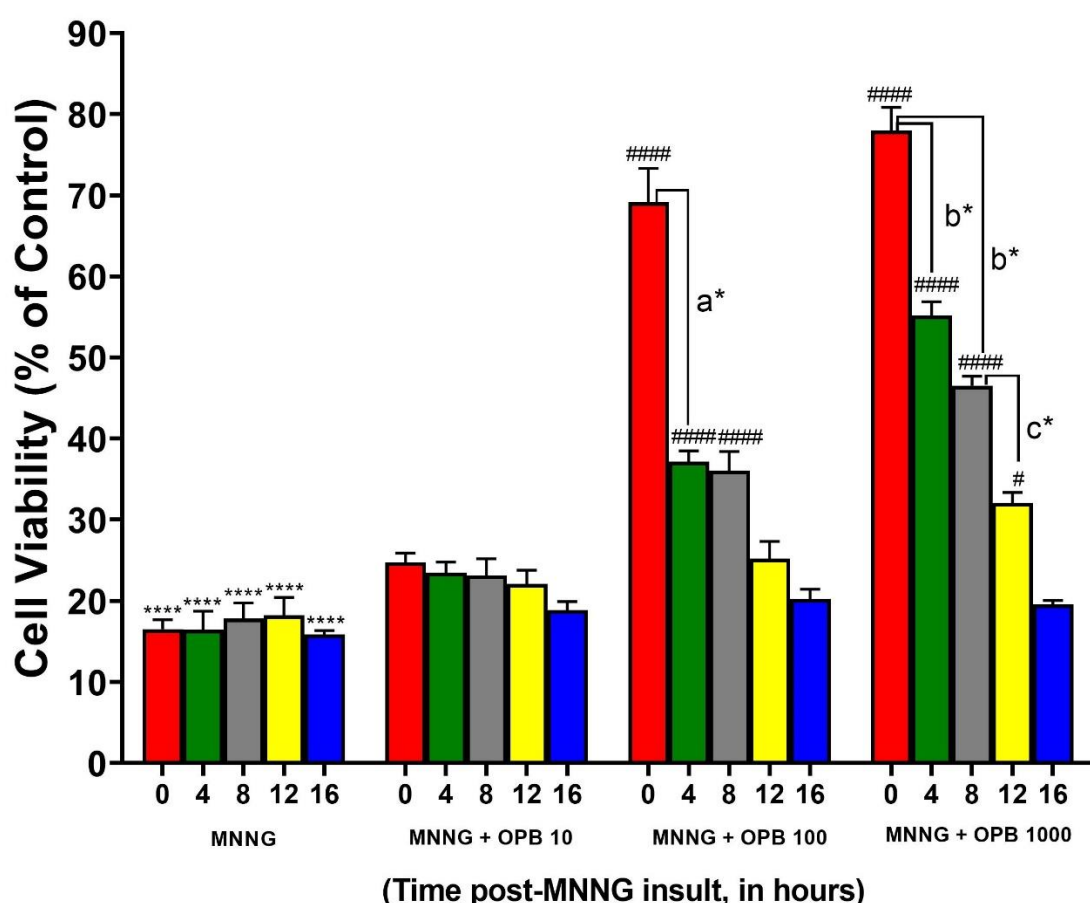


Figure 33: Time-course of protection by olaparib (OPB) against MNNG-induced reduction in cell viability when OPB was added at time points between 0 to 16 h post-MNNG insult. The protection by olaparib progressively decreased as the number of hours that had elapsed post-insult increased. Olaparib continued to elicit concentration-dependent protection up to 12 h post-insult. **** $P < 0.0001$ with respect to the negative (vehicle) control; # $P < 0.05$ and ##### $P < 0.0001$ with respect to the corresponding 'MNNG alone' treatment; a* $P < 0.001$ with respect to 0 h at 100 nM of OPB; b* $P < 0.0001$ with

respect to 0 h at 1000 nM of OPB; c* $P < 0.01$ for comparison of the effects of 1000 nM of OPB at 8 h and 12 h. N=5 independent experiments, each performed in triplicate.

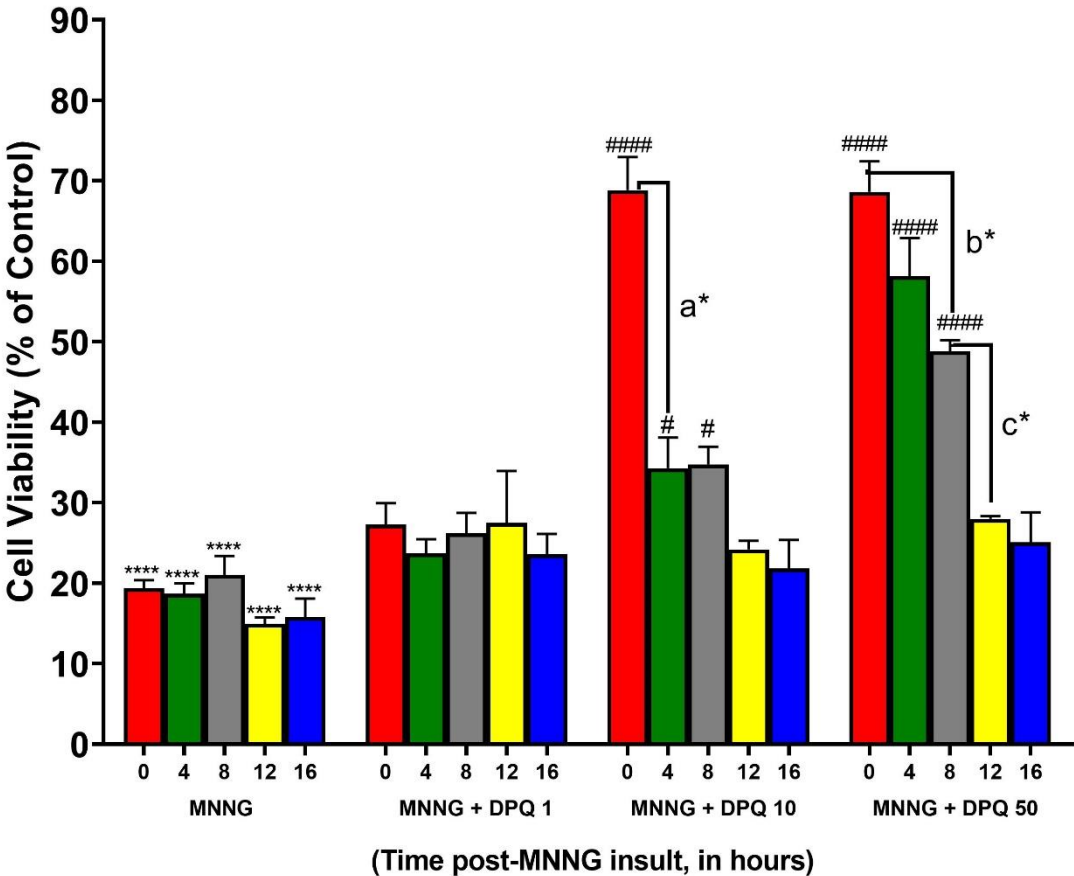


Figure 34: Time-course of protection by DPQ against MNNG-induced reduction in cell viability when DPQ was added at time points between 0 to 16 h post-MNNG insult. The protection by DPQ progressively decreased as the number of hours that had elapsed post-insult increased. DPQ continued to elicit concentration-dependent protection up to 8 h post-insult. **** $P < 0.0001$ with respect to the negative control; ##### $P < 0.0001$, # $P < 0.05$ with respect to the corresponding 'MNNG alone'; a* $P < 0.001$ with respect to 0 hours at 10 μM ; b* $P < 0.01$ with respect to 0 hours at 50 μM ; c* $P < 0.05$ with respect to 8 h at 50 μM . N= 5 independent experiments, each performed in triplicate.

Collectively, the findings in this chapter demonstrate that the model assay is robust and reliable for identifying and characterising novel and existing PARP inhibitors.

Chapter 4: Results and Discussion II

4.0 Screening of a small chemical library and a customised compounds set for novel PARP inhibitors

4.1 Introduction

High-throughput screening (HTS) plays an important role in the drug discovery process. It is a technique that allows a large library of compounds, usually a few thousands to millions, to be tested against a specific molecular target in a rapid way. The ultimate goal of HTS is to identify hits (compounds with desired pharmacological or biological activity) from compound libraries that can become potential drug leads, which may be further investigated in the drug discovery pipeline. In pharmaceutical and biotechnology companies where hundreds of thousands of compounds are screened daily, this process comprises relatively simple, robotic-assisted sample handling, automation-compatible assay designs, and automated data processing. When HTS is carried out within academia, or for relatively small numbers of compounds, assays are typically formatted in 96-well or 384-well microplates, while in the industry HTS (or UHTS, ultra-high-throughput screening) applications assays are done in 384-well, 1536-well or 3456-well microplates. However, while the potential of HTS in drug discovery involving large and diverse compound libraries is tremendous, its success depends on a couple of factors, such as the quality and effectiveness of the assay (Aherne et al., 2002; Hughes et al., 2011).

In developing and choosing an assay format for HTS, for example, a cell-based assay, several factors are considered. These include the pharmacological

relevance of the assay, the reproducibility of the assay, and assay cost and quality. Assay quality is usually determined by an assessment of the Z-prime factor (Z') (Zhang, Chung and Oldenburg, 1999). Z' is an important statistical value that is calculated using both the means and standard deviations of the samples. Assay quality could be considered in the context of robustness, which relates to the inherent ability of the assay to identify true positives from plate-to-plate iterative screening of a large number of compounds (or samples), usually in batches. Such quality is quite important, especially if one considers that each compound (or sample) in the library is usually screened once as a singleton. For this project, the Z' factor for the model assay was calculated using the standard formula:

$$\text{Z' factor} = 1 - \frac{3(S_{c+} + S_{c-})}{(m_{c+} - m_{c-})}$$

where S_{c+} and S_{c-} are the standard deviations of the positive and negative controls, respectively, and m_{c+} and m_{c-} are the mean values of the signals of the positive control (DPQ in this case) and the negative control (z-VAD-fmk in this case) in the assay (Zhang, Chung and Oldenburg, 1999). The Z' factor has become the industry standard for measuring assay quality on a plate-to-plate basis. The Z' factor ranges between 0 and 1. An assay with a Z' factor of greater than 0.4 is considered appropriately robust for HTS, although studies have shown that most researchers prefer to work with assays with a Z' factor of greater than 0.5, and a Z' factor beyond 0.7 is considered excellent (Hughes et al., 2011).

HTS assays are typically used for screening 100,000 or more samples per day. However, HTS assays can be classified based on the sample size(s) of the chemical library(ies) used. For 1-500 samples per day, this is described as low-throughput screening. Sample sizes of 500-10,000, 10,000-100,000 and above 100,000 are technically called medium-throughput, high-throughput and ultra-high-throughput screening, respectively (Wildey et al., 2017). In this chapter, the results of a medium-throughput screening of a small but diverse chemical library as well as a customised collection of compounds are reported and discussed. Also, the results of the secondary (orthogonal) screening of hits and other compounds with reported PARP-inhibitory activity, and their low-throughput characterisation, including through the use of a cell-free assay, are presented and discussed. Wherever in this thesis the expression “high-throughput screening” (HTS) is used to describe the library that was screened in this study, even though it is a small library (technically a ‘medium-throughput screening’ library), the meaning intended is the generic sense of screening any compounds collection designated as a chemical library, whether proprietarily or through a specific project-inspired assembling of a defined collection, regardless of its size.

4.2 High-throughput screening of a natural product library reveals a few PARPi hits

Having established a robust parthanatos model assay, as demonstrated in the previous chapter and earlier in this chapter, the assay was used to screen, in HeLa cells, using the MTT assay, a small chemical library of natural compounds. The library, Screen-Well® Natural Product Library (Enzo; Cat. No. BML-2865-0100), comprises 502 compounds that include terpenoids, peptolides, flavones,

coumarins, alkaloids, macrolides, isoflavones, synthetic derivatives and many more. The collection is housed in six 96-Deep Well plates.

[Figure 35](#) shows the pooled data for all the seven plates screened. It should be noted that seven daughter plates were prepared from the six originally supplied 96-Deep Well plates – the additional (7th) daughter plate was as a result of creating well locations on the screening plates for the positive (DPQ) and negative (Z-VAD-fmk) compound controls, as well as the ‘negative: no compound, no insult’ and the ‘insult (MNNG) only’ controls (each in quadruplicate wells, Column A and Column H used), as no allowance was made in the original library plates for such additional treatments (all 96 wells of the original library plates were occupied, except the 6th plate that housed less than 96 compounds). As expected, MNNG reduced viability significantly (the mean value for the effect of MNNG alone on cell viability across the plates was 7.7% of the vehicle (Media + DMSO only) control); the positive control compound (DPQ) elicited a protective response against the reduction in viability caused by MNNG, and the negative control compound (Z-VAD-fmk) had no effect on the MNNG-induced reduction in cell viability ([Figure 35](#)). This is consistent with previous results as reported in Chapter 3.

To confirm the robustness of the assay, the Z' factor was calculated for all the screening plates. Each of the 7 screening plates had a $Z' > 0.5$ ([Figure 36](#)), while the average Z' factor for the library was 0.7, implying that the assay was very robust and, thus, reliable, as the Z' factor indicates a screening assay's excellent discriminating power (Zhang, Chung and Oldenburg, 1999). The average Z' factor

is similar to that of a previous HTS campaign that used the parthanatos assay but with an alamarBlue fluorescence readout (Fatokun et al., 2013).

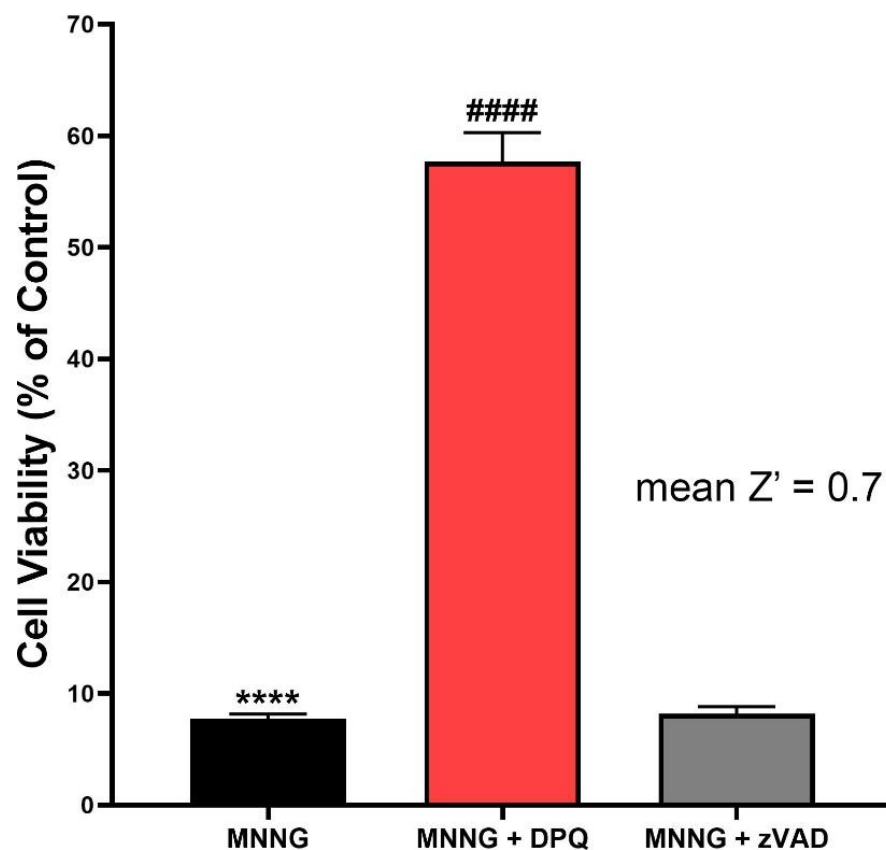


Figure 35: Pooled screening (model) assay data. Data was pooled from all the 7 plates in the library screening (using the MTT assay). The average Z' factor was 0.7. ****P<0.0001 with respect to the control; #####P<0.0001 with respect to the effect of MNNG alone.

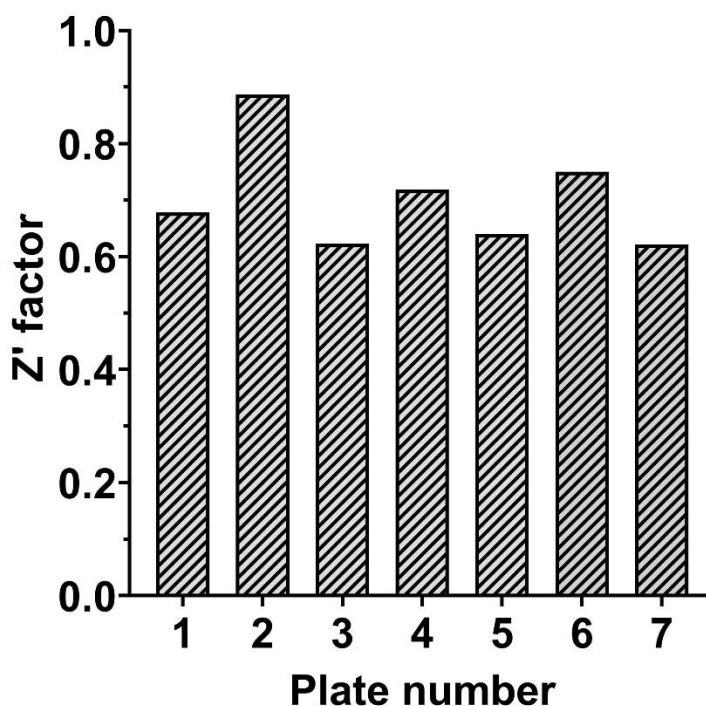


Figure 36: Z' factor values for the seven screening plates. The factor provides an indication of the robustness of the assay. The average value was 0.7.

[Figure 37](#) shows the results of the high-throughput screening of the library at 20 µg/ml for each compound (it should be noted that, as the stock concentration of each library compound was 2 mg/ml, screening the library at 20 µg/ml meant that the compounds were not screened at the same molar concentration (the molar concentration range for the screening was calculated as 13.2 – 196.0 µM). Compound 3B2 (i.e., screening plate position 3B2) showed the highest viability value at 24.6% which, compared to the MNNG baseline (average across the seven screening plates) of 7.7%, suggested some protection against the cytotoxic effect of MNNG, although the protection was lower than that of the positive control (DPQ, 57.3%). All the compounds that showed a cell viability value of at least 10% (red horizontal line, [Figure 37](#)) (i.e., at least 30% increase in viability compared to the average viability for MNNG alone at 7.7%) were re-screened (152 compounds met

this criterion). Following re-screening, only 7 compounds, including 3B2 (18.7%), emerged as putative PARP inhibitors, having shown some protection in the 12-18% viability range compared to the 9.1% viability (average across all re-screening plates) for MNNG alone ([Figure 38](#)). The 7 compounds (graveoline, pratol, catechine, salsoninol, usnic acid, laudanosoline, geraldol) were then further tested, each at three different concentrations (25 μ M, 50 μ M, and 100 μ M – the range spanned concentrations at which they were originally screened), but they did not retain significant protection against MNNG toxicity ([Figure 39](#)), although 3B2 (revealed as graveoline) showed some protection consistently in the original screens. It is noteworthy that graveoline ([Figure 40](#)) shares some structural similarity with 3',4'-dimethoxyflavone (DMF) and 4'-methoxyflavone (4MF), both of which have shown PARP-inhibiting activity from previous work by Fatokun et al. (Fatokun et al., 2013). This seems to suggest that structural modification of graveoline might confer on it a significant PARP-inhibitory activity. With this in mind, a wider range of concentrations of graveoline was tested using both the MTT and AB cell viability assays, in the hope that its PARP-inhibiting activity might be detected. The results, however, revealed there was no significant difference in viability following treatment with MNNG alone compared with viability following treatment with MNNG in the presence of any of the tested concentrations of graveoline ([Figure 41](#) and [42](#)).

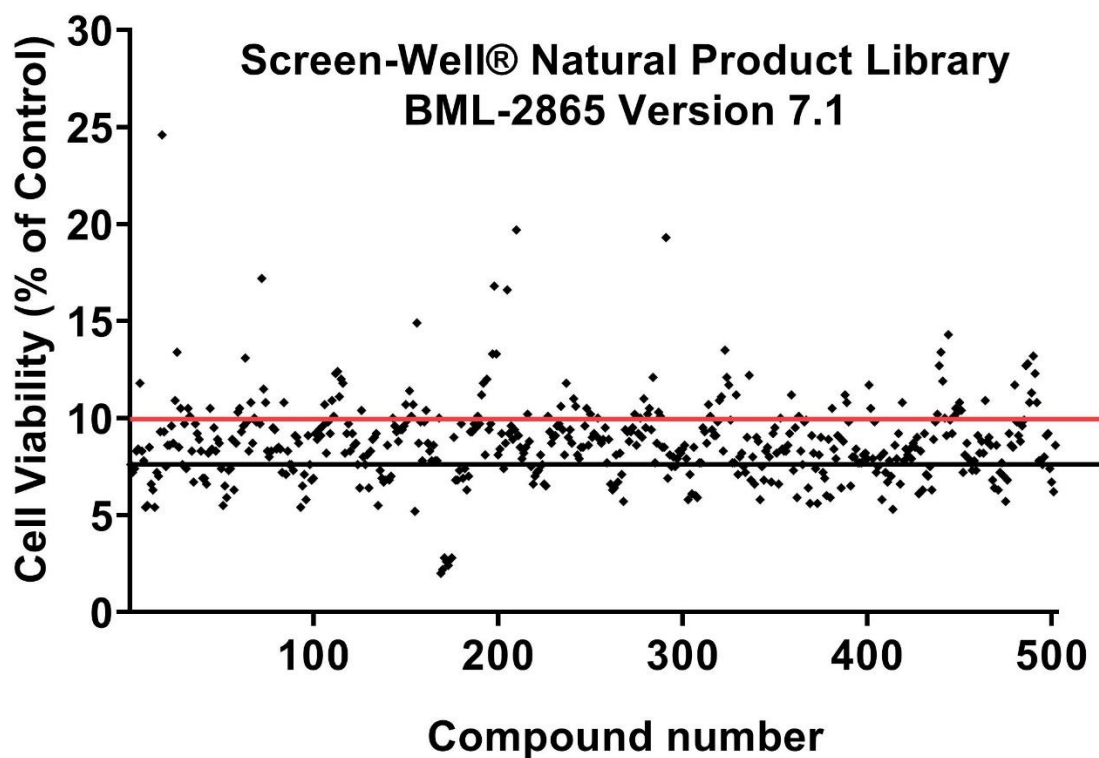


Figure 37: High-throughput screen (singleton) of the Screen-Well® Natural Product chemical library comprising 502 compounds. The library was screened manually. Each black dot on the plot represents the effect of a library compound. The black, horizontal line represents the MNNG (alone) effect baseline average at 7.7% and the red, horizontal line represents the threshold on or above which a compound was considered a hit.

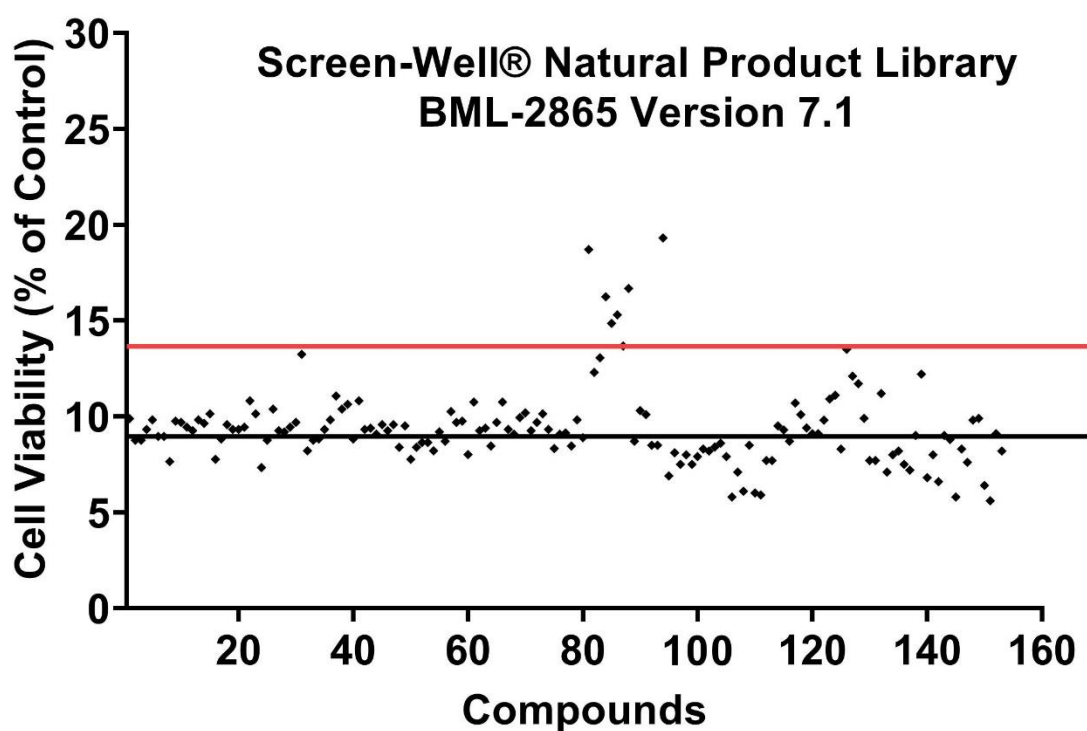


Figure 38: A re-screen of hits from primary screen. The 152 hits from the Screen-Well® Natural Product chemical library were re-screened for confirmation. Each black dot on the plot represents the effect of a compound identified as a hit from the previous screen. The black, horizontal line represents the MNNG (alone) effect baseline at 9.1% and the red, horizontal line (13.7%) represents the threshold on or above which a compound was considered a confirmed hit. The red, horizontal line (13.7%) represents a point beyond a 50% increase in viability compared to the viability in the presence of MNNG alone.

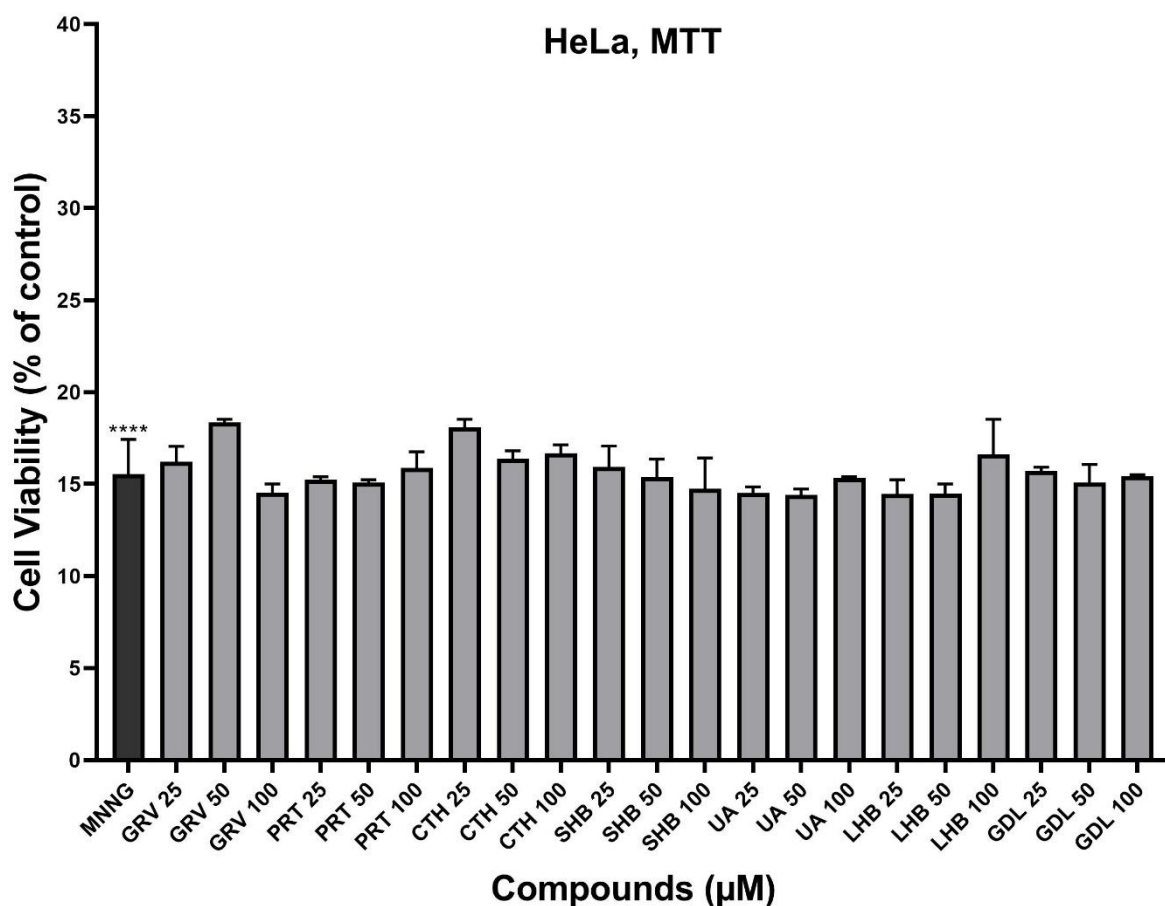


Figure 39: Lack of PARPi activity of primary screen hits following subsequent screening. Each of the seven hits from the re-screening did not elicit a significant difference in cell viability when present with MNNG compared to MNNG alone and hence demonstrated no detectable PARP-inhibitory activity. Each compound was tested at three different concentrations (25, 50, and 100 μM). The compounds are Graveoline (GRV), Pratol (PRT), Catechine (CTH), Salsoninol HBr (SHB), Usnic acid (UA), Laudanosoline HBr $3\text{H}_2\text{O}$ (LHB), and Geraldol (GDL). **** $P < 0.0001$ with respect to the negative control. $N=3$ independent experiments, each performed in triplicate.

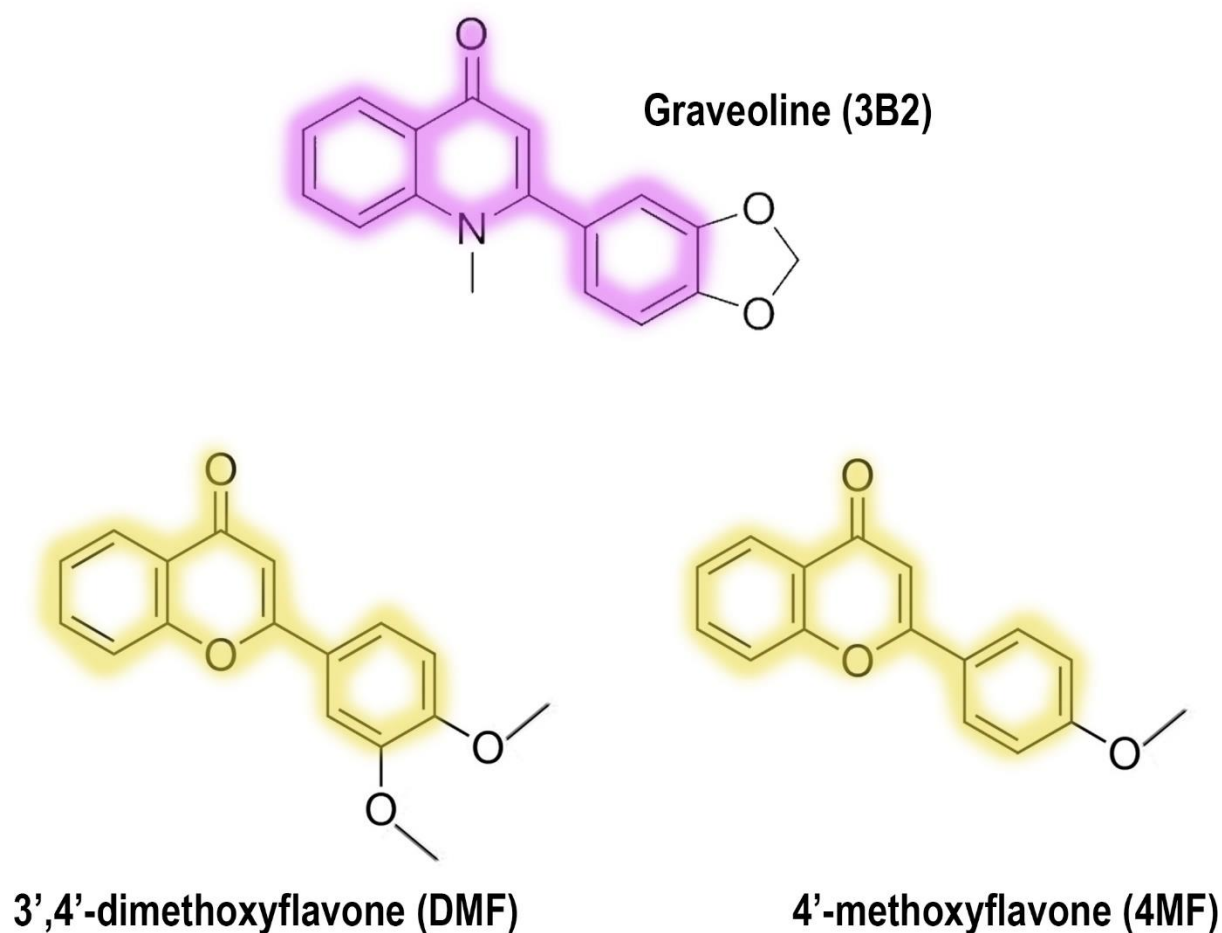


Figure 40: Chemical structures of 3B2 (Graveoline), 3',4'-dimethoxyflavone (DMF) and 4'-methoxyflavone (4MF). The quinolone structure attached to a benzene ring (purple) in graveoline is similar to the chromone structure attached to a benzene ring (yellow) in DMF or 4MF.

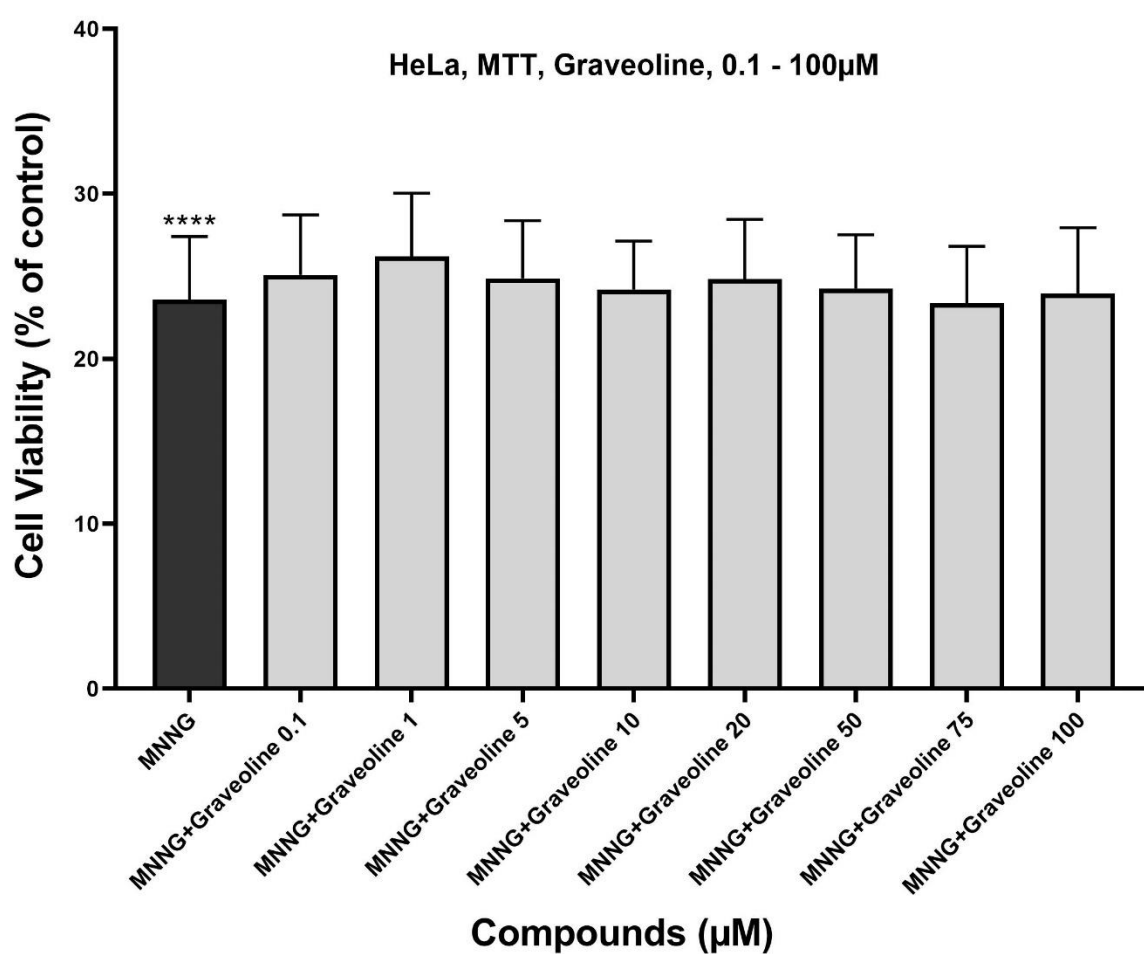


Figure 41: Lack of effect of graveoline against MNNG-induced reduction in viability (MTT assay). Graveoline did not show any significant protective effect against the toxic effect of MNNG. **** $P < 0.0001$ with respect to the negative control. N=4 independent experiments, each performed in triplicate.

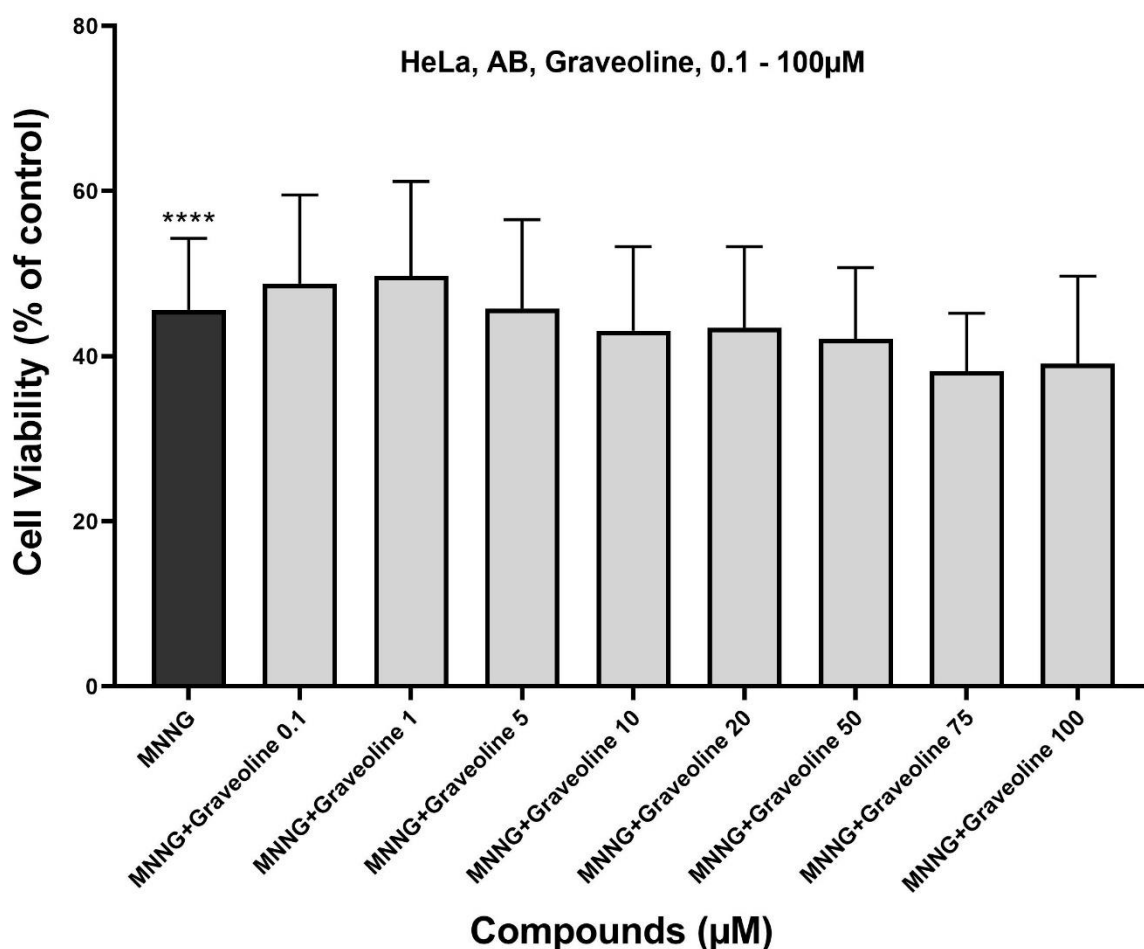


Figure 42: Lack of effect of graveoline against MNNG-induced reduction in viability (AB assay). Graveoline did not show any significant protective effect against the toxic effect of MNNG. **** $P < 0.001$ with respect to the control. $N=2$ independent experiments, each performed in triplicate.

4.3 Screening of a customised small collection of compounds

Having found no true hits from screening the Screen-Well® Natural Product library (502 compounds), information was sought from the literature about compounds or compound classes whose currently known biological activities or their chemical structures might suggest a potential or preliminary link with the PARP pathway, poly ADP-ribosylation or a related pathway, or activity against PARP. The literature interrogation led to the identification of two small groups of compounds, which were

then purchased individually and screened. Some of these compounds were not natural products. For example, Regdon et al. (2021) found that ciclopirox and apomorphine protected HaCaT keratinocyte cells against H₂O₂-induced necrotic cell death after screening a small library of 774 compounds. They also demonstrated that these two compounds inhibited the production of PAR polymer induced by H₂O₂ more potently than the PARP inhibitor PJ34 (Regdon et al., 2021). This seemed to suggest that ciclopirox and apomorphine have PARP pathway-inhibitory activities; therefore, it was decided that they should be screened using this project's parthanatos model assay.

[Figure 43](#) shows that MNNG alone significantly reduced cell viability in HeLa cells. However, none of ciclopirox, apomorphine and the other compounds as shown had any significant, protective effect against MNNG-induced parthanatic death. Similarly, none of the compounds from another group of 14 compounds had any effect against the death when screened using the MTT and AB assays ([Figures 44](#) and [45](#)).

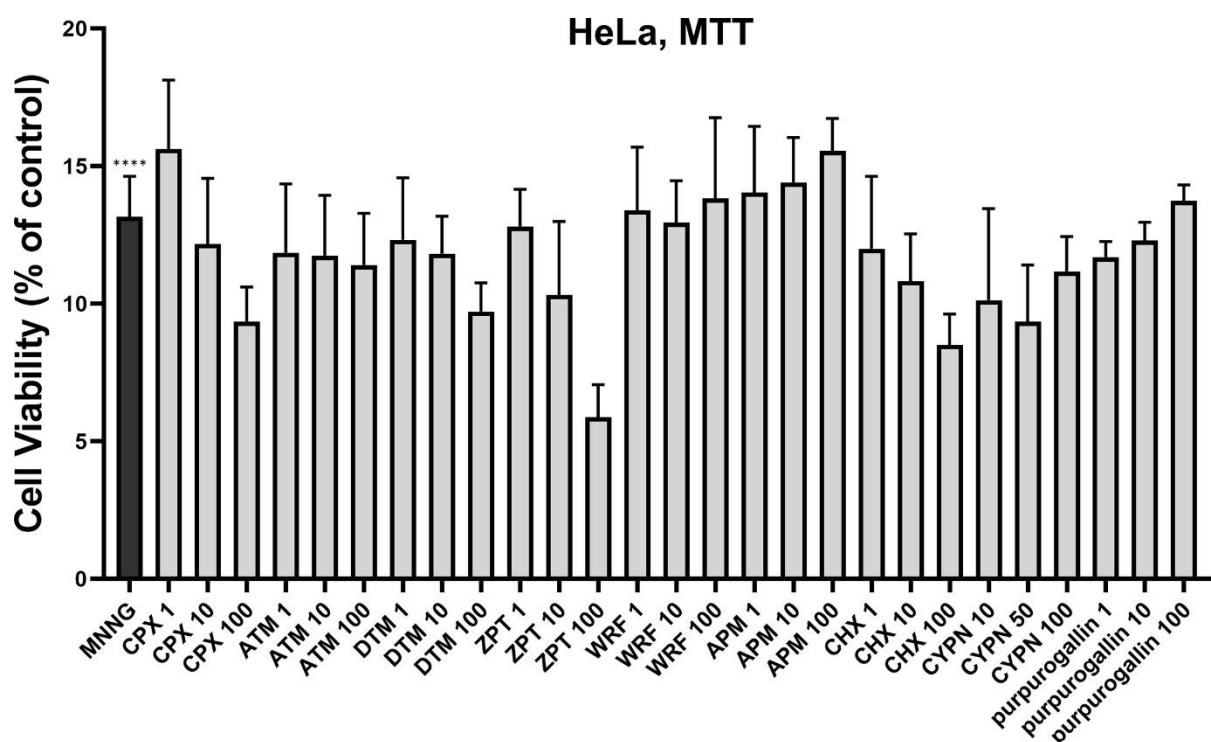


Figure 43: Lack of effect of compounds from a customised set (Set 1) against MNNG-induced reduction in cell viability. The MTT assay shows that ciclopirox, apomorphine and the other compounds did not protect HeLa cells against MNNG-induced cell death (reduction in cell viability). The compounds were Ciclopirox (CPX), Artemisinin (ATM), Dihydroartemisinin (DTM), Zinc Pyrithione (ZPT), Warfarin (WRF), Apomorphine (APM), Chloroxine (CHX), Chrysosplenetin (CYPN), and Purpurogallin. In the figure, numbers after the abbreviations indicate the concentrations tested in μM of each compound. **** $P < 0.0001$ with respect to the negative control. $N=3$ independent experiments, each performed in triplicate.

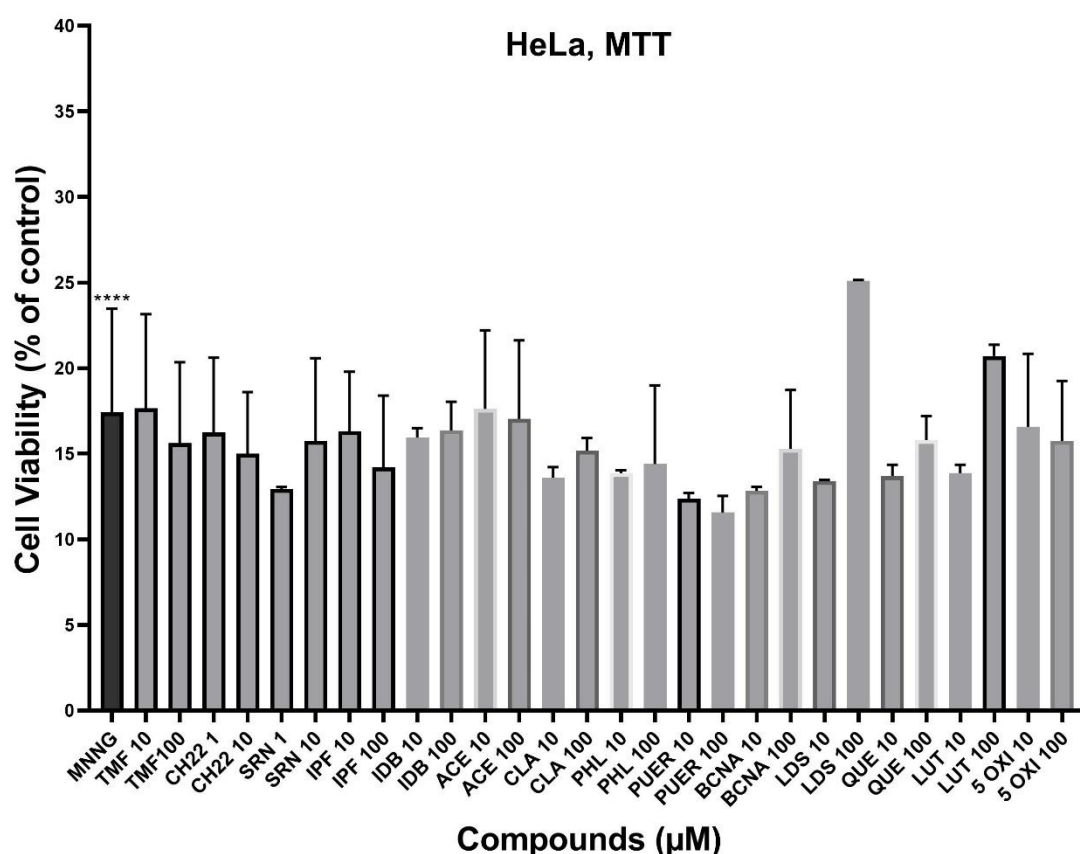


Figure 44: Lack of effect of compounds from a customised set (Set 2) against MNNG-induced reduction in cell viability (MTT assay). The assay shows that none of the small group of 14 compounds protected HeLa cells against MNNG-induced cell death (reduction in viability). The compounds were 6,2',4'-Trimethoxyflavone (TMF), CH 223191 (CH22), Stemregenin 1 (SRN1), Ipriflavone (IPF), Indirubin (IDB), Aceroside VIII (ACE), Chlorogenic acid (CLA), Phloretin (PHL), Puerarin (PUER), Biochanin A (BCNA), Laudanosine (LDS), Quercetin (QUE), Luteolin (LUT), and 5-hydroxytryptophan (oxitriptan) (5-OXI). In the figure, numbers after the abbreviations indicate the concentrations tested in μM . **** $P < 0.0001$ with respect to the control. $N=3$ independent experiments, each performed in triplicate.

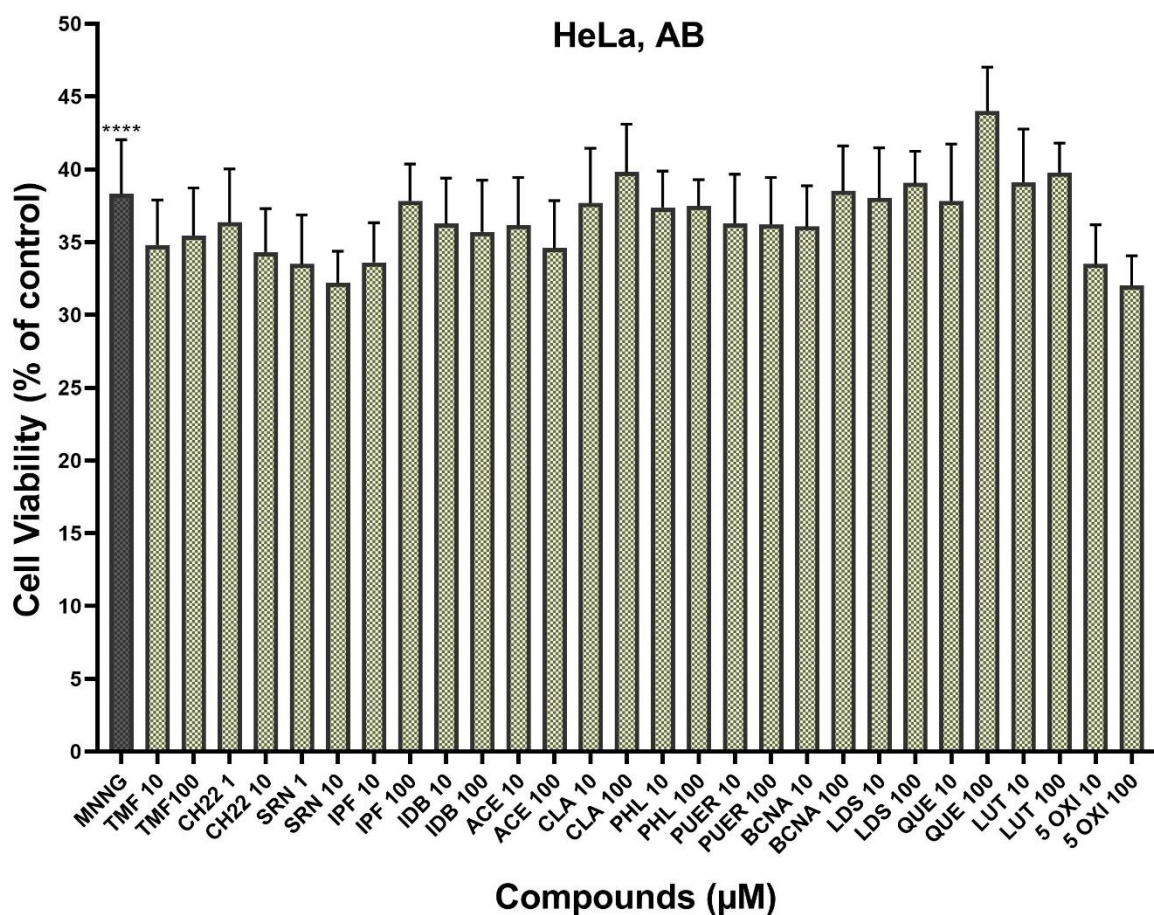


Figure 45: Lack of effect of compounds from a customised set (Set 2) against MNNG-induced reduction in cell viability (AB assay). The assay shows that none of the small group of 14 compounds protected HeLa cells against MNNG-induced cell death (reduction in viability). The compounds were 6,2',4'-Trimethoxyflavone (TMF), CH 223191 (CH22), Stemregenin 1 (SRN1), Ipriflavone (IPF), Indirubin (IDB), Aceroside VIII (ACE), Chlorogenic acid (CLA), Phloretin (PHL), Puerarin (PUER), Biochanin A (BCNA), Laudanosine (LDS), Quercetin (QUE), Luteolin (LUT), and 5-hydroxytryptophan (oxitriptan) (5-OXI). In the figure, numbers after the abbreviations indicate the concentrations tested in µM. ****P<0.0001 with respect to the control. N=3 independent experiments, each performed in triplicate.

4.4 4'-methoxyflavone (4MF) and 3',4'-dimethoxyflavone (DMF) protected against MNNG-induced cell death

Having found no hits from our screening so far, a keen focus was turned to two natural product compounds, 4'-methoxyflavone (4MF) and 3',4'-dimethoxyflavone (DMF), identified in a previous study as novel neuroprotective inhibitors of

parthanatos. 4MF and DMF were reported as protective against MNNG toxicity in HeLa and SH-SY5Y cells using the AB and CTG assays (Fatokun et al., 2013). A significant characterisation of these two compounds, beyond what had been reported in the previous study, was therefore undertaken, including revealing their mechanistic details, with a view to being able to describe them unequivocally as PARPi and then using their scaffold as inspiration to identify more natural compound PARPi. The characterisation of their PARP-inhibitory activity was done across three different cell lines (HeLa, HaCaT and SH-SY5Y). As shown in Figure 47, MNNG reduced viability to 15% ([Figure 46-A](#)) and 16% ([Figure 46-B](#)) of the negative control in HeLa cells, against which 4MF or DMF showed concentration-dependent protection. The significant protection against MNNG by 4MF began at 20 μ M and peaked at 75 μ M ([Figure 46-A](#)). However, the protection at 100 μ M, even though lower than at 75 μ M, was still significant. Similarly, DMF started to elicit significant protection at 10 μ M, which peaked at 50 μ M, although the protection at 75 μ M or 100 μ M, while lower than at 50 μ M, was still significant ([Figure 46-B](#)). These results are in agreement with the findings of Fatokun et al. (2013) that demonstrated using the alamarBlue assay that either 4MF or DMF at 10 and 20 μ M displayed significant protection against the reduction in HeLa and SH-SY5Y viability induced by MNNG. They found that the protective effects of 4MF and DMF peaked around 25 μ M, even though the compounds still conferred significant protection at 100 μ M (Fatokun et al., 2013). In this study, photomicrographs complemented the viability data to confirm that 4MF and DMF displayed some level of protection, albeit weak, against the deleterious effect of MNNG on cell morphology, shown as amelioration of MNNG-induced rounding up, clumping and loss of cells ([Figure 47](#) and [Figure 48](#)).

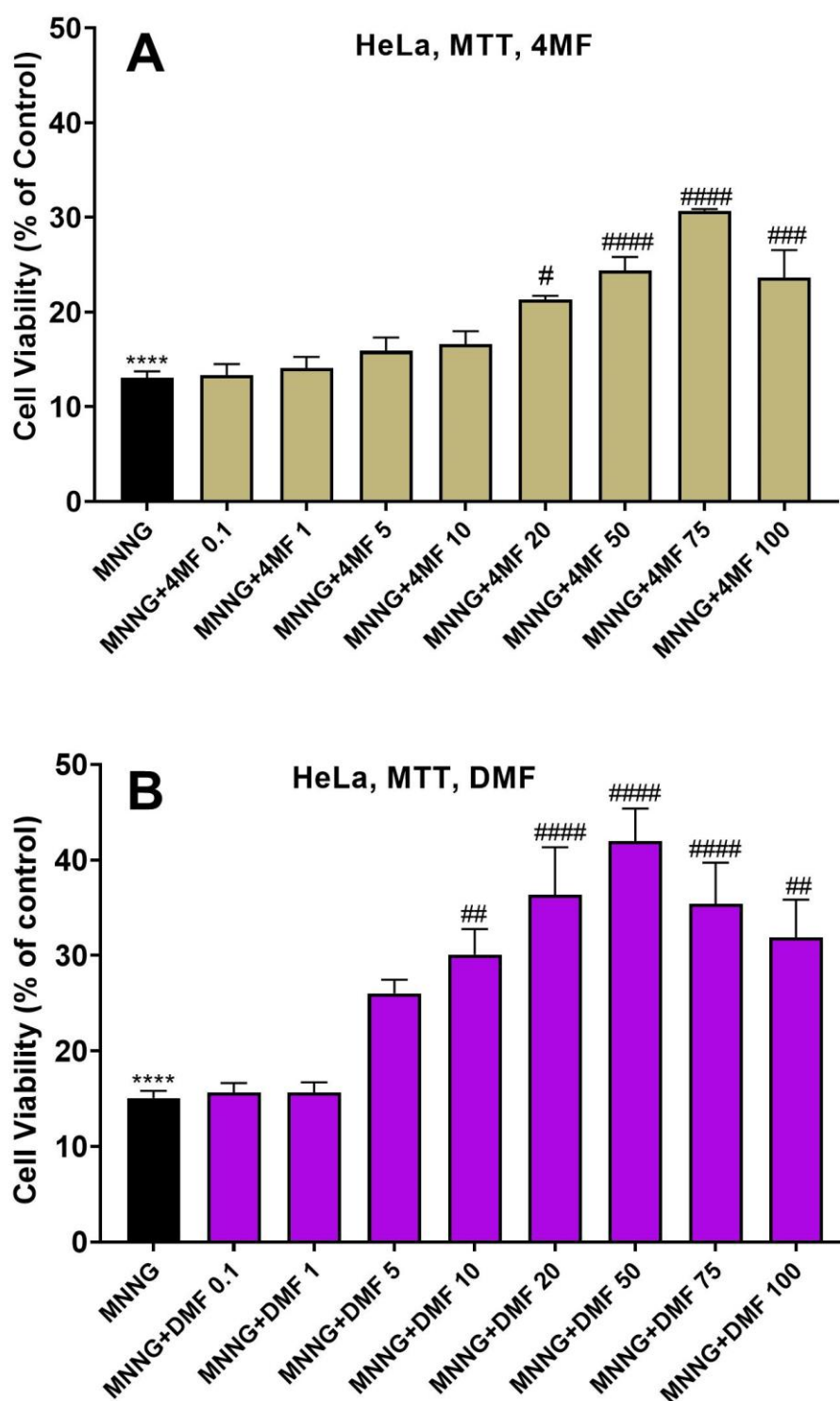
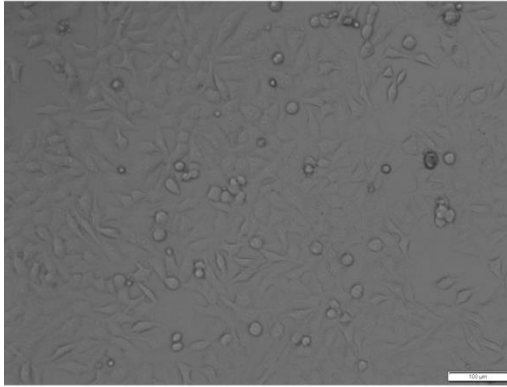
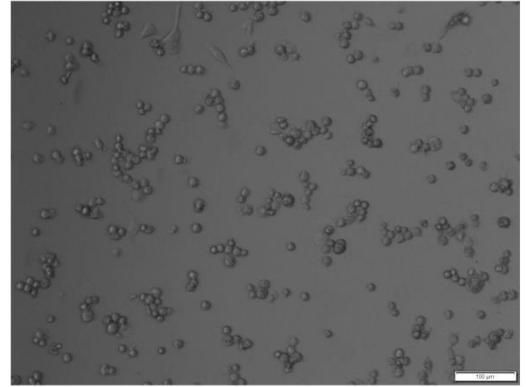


Figure 46: 4'-methoxyflavone (4MF) and 3',4'-dimethoxyflavone (DMF) protect against MNNG-induced reduction in cell viability. 4MF (A) or DMF (B) (0.1-100 μ M) elicits concentration-dependent protection against MNNG-induced reduction in cell viability, as quantified by the MTT assay in HeLa cells. **** P <0.0001 with respect to the negative control; ##### P <0.0001, ### P <0.001, ## P <0.01 and # P <0.05 with respect to MNNG alone. N=5 independent experiments, each performed in triplicate.

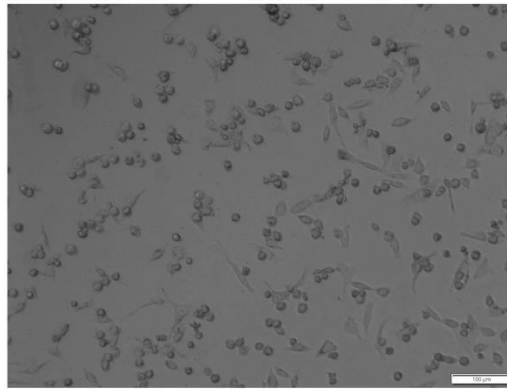
CONTROL



MNNG ALONE



MNNG + 4MF 10



MNNG + 4MF 20

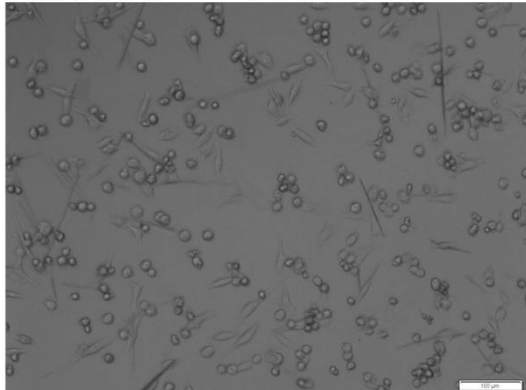
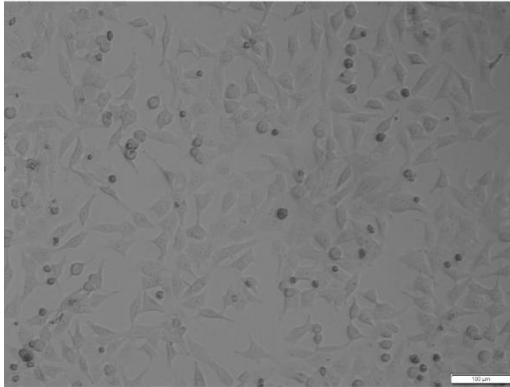
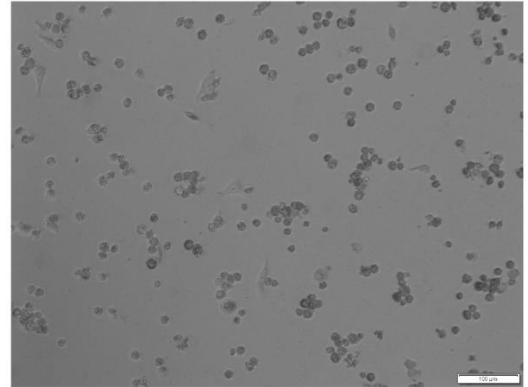


Figure 47: 4'-methoxyflavone (4MF) protects mildly against MNNG-induced morphological damage in HeLa cells. Photomicrographs show HeLa cell morphology following exposure to MNNG in the presence and absence of 4MF, in which 4MF protects against morphological damage induced by MNNG. Cultures treated with MNNG alone appear rounded and shrunken, compared to the control phenotype. Cultures treated with MNNG in the presence of 4MF show a slight reduction in the number of dead cells, with more cells with their processes, compared to those treated with MNNG alone. The brightfield images were acquired 24 h after treatments, with X10 magnification. Scale bar = 200 μ m.

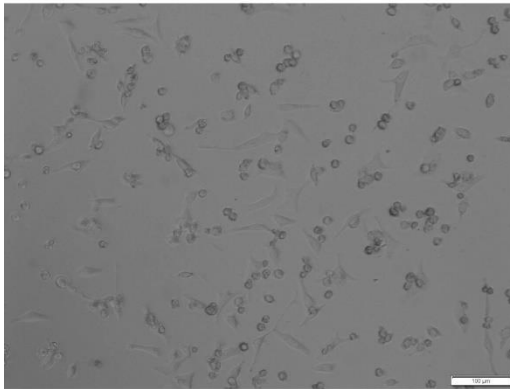
CONTROL



MNNG ALONE



MNNG + DMF 10



MNNG + DMF 20

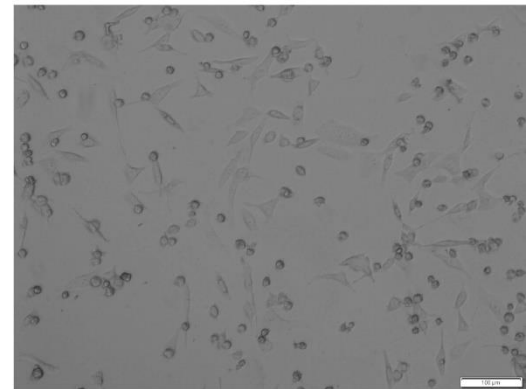


Figure 48: 3',4'-dimethoxyflavone (DMF) protects mildly against MNNG-induced morphological damage in HeLa cells. Photomicrographs show HeLa cell morphology following exposure to MNNG in the presence and absence of DMF, in which DMF protects against morphological damage induced by MNNG. Cultures treated with MNNG alone appear rounded and shrunken, compared to the control phenotype. Cultures treated with MNNG in the presence of DMF show a slight reduction in the number of dead cells, with more cells with their processes, compared to those treated with MNNG alone. The brightfield images were acquired 24 h after treatments, with X10 magnification. Scale bar = 200 μm .

In HaCaT cells, 4MF and DMF elicited significant concentration-dependent protection against MNNG in a similar pattern to what was observed in HeLa cells ([Figure 49](#)). 4MF showed a protective effect that started at 10 μM and peaked at 75 μM but extended to 100 μM ([Figure 49-A](#)). DMF began to show significant protection at 5 μM , which reached its peak at 50 μM . Interestingly, DMF did not show any protection at 75 and 100 μM but on the contrary reduced cell viability to

about 11%, which was slightly lower than the effect of MNNG alone (17%) ([Figure 49-B](#)). This seems to suggest that DMF at those concentrations was exerting its own toxicity on the HaCaT cells. It is important to note that the maximum protection conferred by either 4MF or DMF against MNNG toxicity was consistently higher in HaCaT cells than in HeLa cells. In HaCaT cells, 4MF and DMF protected significantly against the effect of MNNG, raising cell viability to up to 66% of the control, compared to 42% in HeLa cells, even though the 'MNNG alone' baseline values were similar in both cell lines (approximately 12-18%). Similarly, in SH-SY5Y cells, 4MF and DMF elicited their peak protection against MNNG at 50 and 75 μ M, respectively, although values for MNNG alone dipped to below 10%, which were less than in the other cell lines, suggesting the SH-SY5Y cells could be more sensitive to MNNG toxicity ([Figure 50](#)).

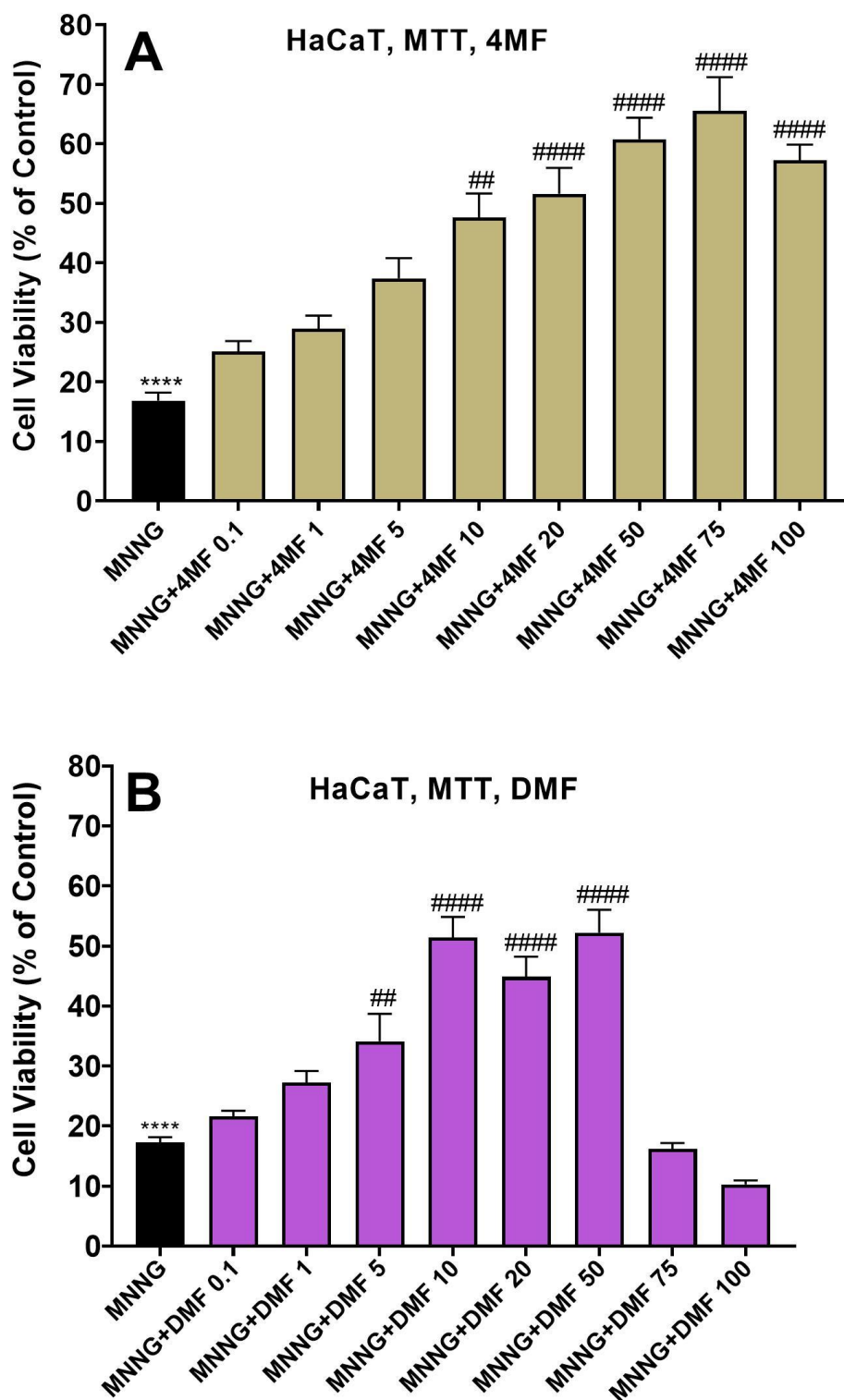


Figure 49: Protective effects of (A) 4'-methoxyflavone (4MF) and (B) 3',4'-dimethoxyflavone (DMF) against MNNG-induced reduction in HaCaT cell viability. 4MF and DMF (0.1-100 μ M) show concentration-dependent protection against MNNG-induced reduction in cell viability, as quantified by the MTT assay. Numerals after 4MF or DMF in the figure indicate their respective concentrations in μ M. ****P<0.0001 with respect

to the negative control; ##### $P < 0.0001$ and # $P < 0.05$ with respect to MNNG alone. N=3 independent experiments, each performed in triplicate.

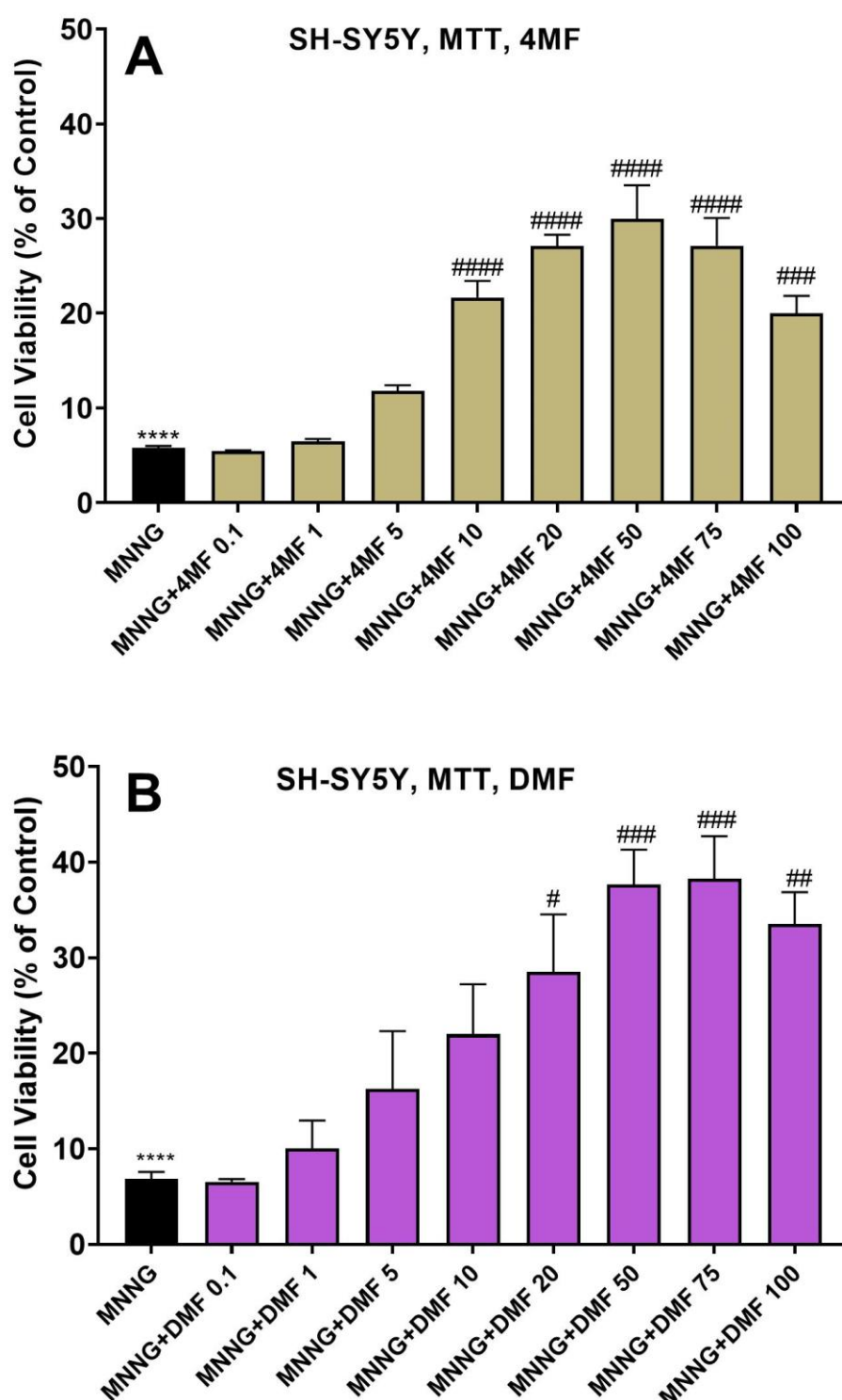


Figure 50: Protective effects of (A) 4'-methoxyflavone (4MF) and (B) 3',4'-dimethoxyflavone (DMF) against MNNG-induced reduction in SH-SY5Y cell viability. 4MF and DMF (0.1-100 μ M) show concentration-dependent protection against MNNG-

induced reduction in cell viability, as quantified by the MTT assay. Numerals after 4MF or DMF in the figure indicate their respective concentrations in μM . **** $P < 0.0001$ with respect to the negative control; ##### $P < 0.0001$, #### $P < 0.001$, ### $P < 0.01$ and # $P < 0.05$ with respect to MNNG alone. N=3 independent experiments, each performed in triplicate.

The data for both compounds and the PARPi DPQ were used to plot their log concentration-response curves ([Figure 51A-C](#)), from which the EC_{50} values for 4MF and DMF's protective effects against MNNG toxicity in the three cell lines were determined, as summarised in [Figure 51D](#). Unsurprisingly, DPQ, known to be a potent PARPi, was significantly more potent and efficacious than both compounds. This study's result demonstrating near-equipotency of 4MF and DMF is consistent with those from previous studies that showed they were nearly equipotent, with EC_{50} values of $11.41 \pm 1.04 \mu\text{M}$ and $9.94 \pm 1.05 \mu\text{M}$, respectively (Fatokun et al., 2013). The potencies of 4MF ($6.2 \pm 1.7 \mu\text{M}$) and DMF ($7.7 \pm 1.6 \mu\text{M}$) were also reasonably similar in HaCaT cells, although unlike in HeLa cells 4MF appeared marginally more potent than DMF. Their efficacies were similar in HaCaT cells, and, as expected and consistent with previous studies, DPQ was more potent and more efficacious than both compounds. In SH-SY5Y cells, the potencies of 4MF ($6.6 \pm 0.7 \mu\text{M}$) and DMF ($6.0 \pm 2.8 \mu\text{M}$) were similar to their potencies in HaCaT and HeLa cells, but DMF was marginally more potent than 4MF.

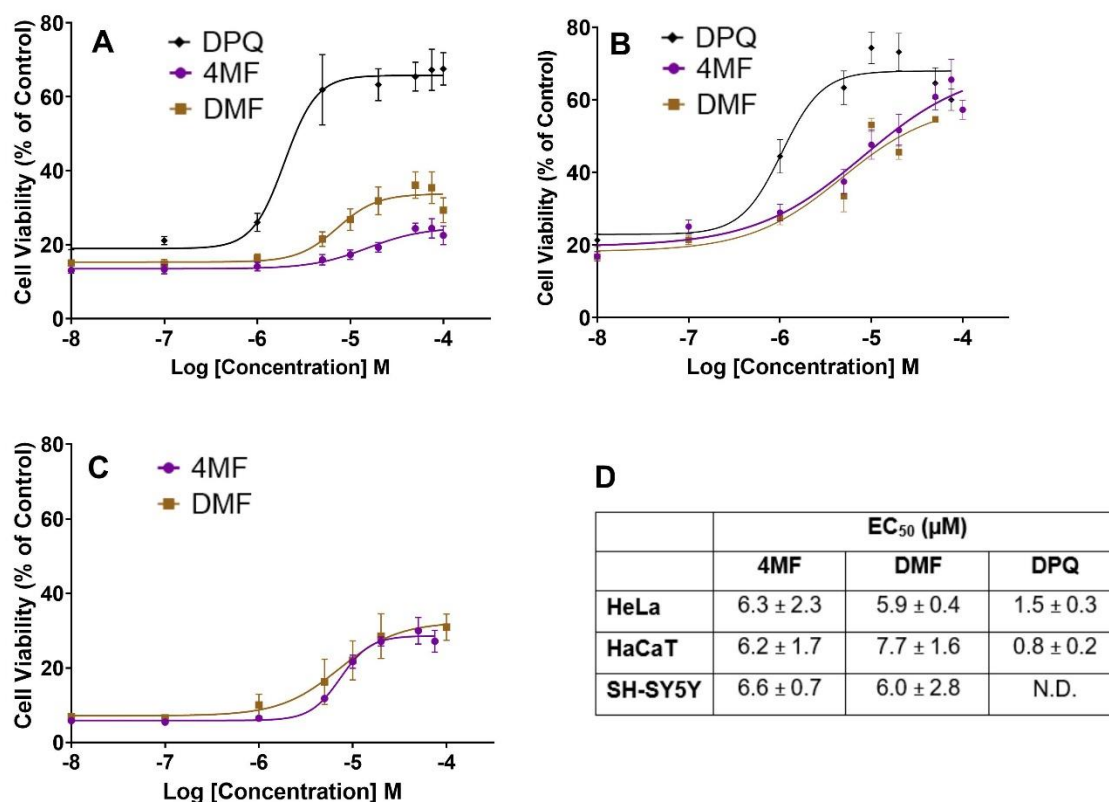


Figure 51: Log concentration-response curves and EC₅₀ values for the protective effects of 4'-methoxyflavone (4MF), 3',4'-dimethoxyflavone (DMF), and DPQ against MNNG toxicity. Curves are for (A) HeLa, (B) HaCaT and (C) SH-SY5Y cells, and (D) shows their EC₅₀ values. N.D. means not determined. N=3-5 independent experiments, each performed in triplicate.

Taken together, 4MF and DMF were nearly equipotent in the three cell lines and their effective concentrations were in the low-to-medium micromolar range. This result is consistent with findings from a previous study that several flavonoids inhibited PARP1 in MNNG-treated human pulmonary epithelial cells (A549 cells) at micromolar concentrations (up to 100 μM) (Geraets et al., 2007). Even though 4MF and DMF as shown in this study are not as potent as most experimental and clinical PARPi, they have potential not only as PARP drug leads but also as pharmacological tools for the elucidation of PARP1-mediated cell death. It is noteworthy that not all established PARPi are very potent (in the nanomolar range).

In fact, there are a number of PARPi that are usually used experimentally at micromolar and millimolar concentrations (Kuo et al., 1998; Radnai et al., 2012).

The characterisation of 4MF and DMF as true PARPi provides a unique inspiration to seek many more PARPi using their common scaffold as a template, and the work opens up avenues for a wide range of further translational studies, including the possibility of identifying dietary sources that are enriched in these compounds that could be used for chemoprevention as well as potentiation of chemotherapeutic drugs. Results from this study demonstrate that the additional methoxylation of 4MF at the 3' position to produce DMF did not affect its PARP-inhibitory activity. Interestingly, a previous study investigating the oxidative metabolism of some methoxyflavones and hydroxyflavones by human liver microsomes and recombinant cytochrome (P450) isoforms revealed that DMF was four times more stable to microsomal oxidation than 4MF (Walle and Walle, 2007). It also found that 4MF and 3MF both had relatively poor stability compared to the other methoxyflavones (Walle and Walle, 2007). Since 4MF is four times more liable to degradation by microsomal enzymes than DMF, the additional methoxylation of 4MF at the 3' position to produce DMF enhances the metabolic stability of DMF while retaining its PARP-inhibitory activity, thus improving the pharmacokinetic property of the scaffold and the prospect of its application as an investigational tool or a potential therapeutic agent in an *in vivo* scenario. To further demonstrate the therapeutics development and chemical biology opportunities that this study's findings have opened up, the effects on PARP-inhibitory activity of hydroxy- and methoxy- substitutions on the 4MF and DMF structures were

investigated in a structure-activity relationship (SAR) study, the results of which are reported and discussed in Chapter 5.

4.5 PARPi-FL exhibited decreased potency but similar efficacy compared to olaparib in a cell-free assay

To further characterise the mechanistic aspects of the PARP-inhibitory action of 4MF and DMF, biochemical assays that measure direct inhibition of PARP1 in a cell-free system were used to confirm if they were direct inhibitors of PARP. The cell-free assay was mentioned in Chapter 3 to have been used to assess the direct PARP inhibition ability of established PARPi which were being validated in the cell-based parthanatos model assay. The biochemical assay was carried out using a PARP1 Colorimetric Assay Kit purchased from Trevigen and Amsbio. The manufacturer's instructions stated that the PARP1 colorimetric assay kit was compatible with up to 1% final DMSO concentration. However, it is known that for cell-based assays a DMSO concentration higher than 1% v/v usually does have a significant adverse effect on cell viability. In fact, less than 0.5% DMSO is usually recommended (Chen and Thibeault, 2013), which was why for in this project the final DMSO concentration in the tests and controls in all the cell-based assays did not exceed 0.2% v/v, which was confirmed to have no effect on cell viability. Bearing this in mind, a range of DMSO concentrations was tested in the cell-free assay before proceeding with testing the different compounds for direct PARP binding activity. This allowed the detection and elimination of any unwanted effects of DMSO on the assay results that could have confounded data.

[Figure 52](#) shows that DMSO at 0.01 – 0.5% had no significant effect on PARP1 enzymatic activity, while at 1 – 10% it decreased enzymatic activity significantly in

a concentration-dependent manner, with 1% significantly reducing enzymatic activity to 40.6% (compared to PARP1 enzyme without DMSO or inhibitor). The final concentration of DMSO in the cell-free assays carried out in this project using the commercial kit was, therefore, kept at or below 0.1%.

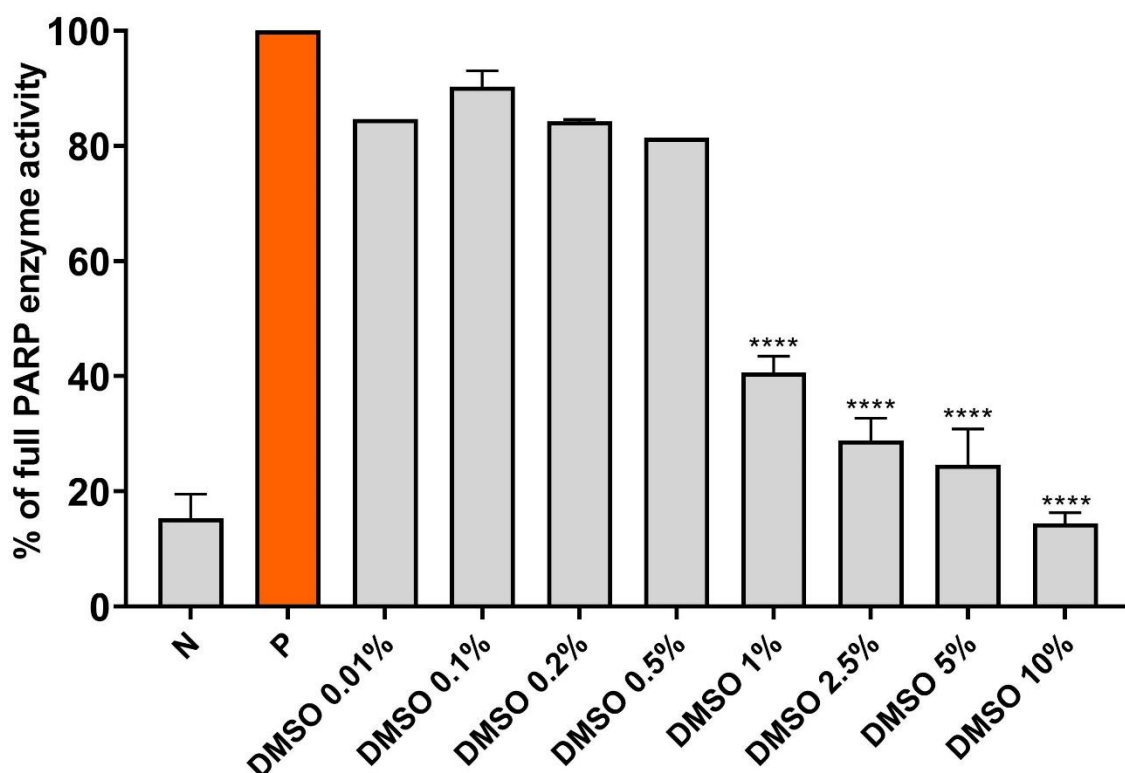


Figure 52: The effects of DMSO on PARP1 enzyme activity in a cell-free assay. N means negative control (no enzyme activity). **** $P < 0.0001$ with respect to full enzymatic activity (PARP1 enzyme activity without an inhibitor, indicated as P - the orange bar). N=1-3 independent experiments, each performed in duplicate.

The PARP-inhibitory activities of PARPi-FL and olaparib were then determined. As shown in [Figure 53](#), PARPi-FL and olaparib (up to 100 nM) each decreased PARP1 activity in a concentration-dependent manner, which reached significance from 0.1 nM and 0.01 nM for PARPi-FL and olaparib, respectively, and the IC_{50} values as calculated from the log concentration-effect plots ([Figure 54](#)) were 17.7 ± 0.5 and 5.4 ± 0.3 nM. These values revealed that the IC_{50} for PARPi-FL was significantly

higher than for olaparib, resulting in PARPi-FL being approximately thrice (3.2) less potent than olaparib ([Figure 54](#)). The IC₅₀ values obtained for both compounds in this study are quite similar to published values that were also obtained in cell-free binding assays, which were 12.2 and 5 nM for PARPi-FL and olaparib, respectively, representing a 2.4-fold decrease in potency for PARPi-FL vs. olaparib (Carney et al., 2016; Carney et al., 2018). Interestingly, the three-fold decrease in the potency of PARPi-FL vs. olaparib in the cell-free system as obtained in this study is in line with the study's cell-based assay results, in which olaparib was about three times more potent in a cell-based system: 3.4 times more potent in HaCaT cells and 3.3 times more potent in HeLa cells, with potency determined based on the MTT and CTG assays, respectively. The results also reveal that, although PARPi-FL exhibits decreased potency, its efficacy is similar to that of the parent olaparib, whether in a cell-free assay as shown here or in a cell-based assay as shown in the previous chapter.

The cell-free assay was then employed to assess the direct PARP binding activities of 4MF, DMF and other test compounds.

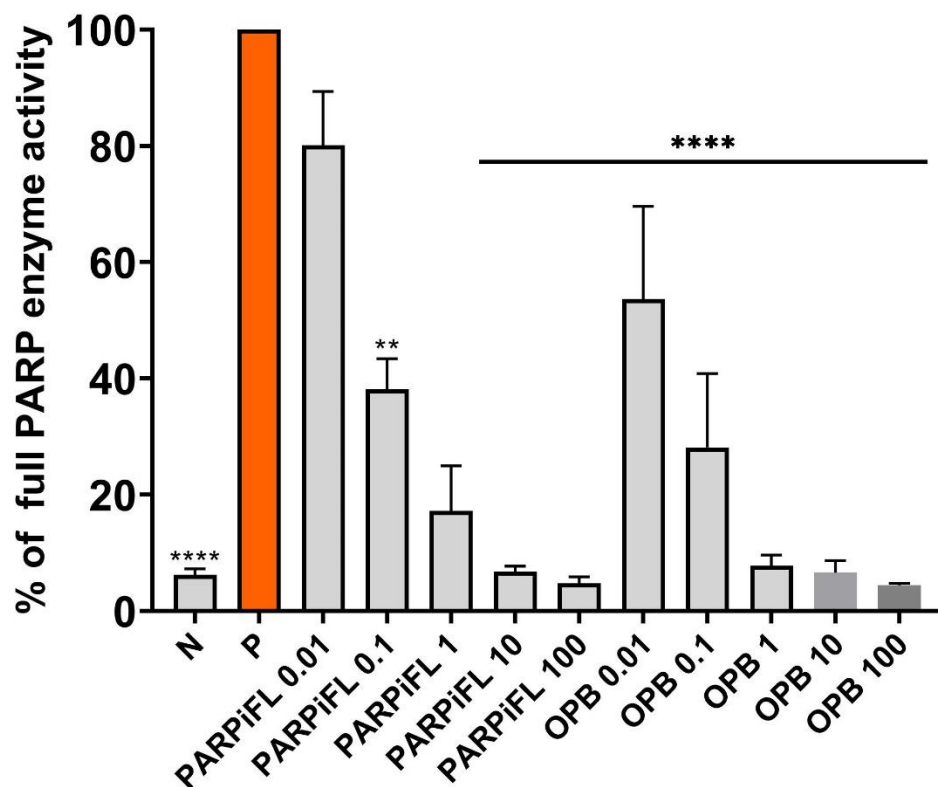


Figure 53: Effects of PARPi-FL and olaparib on PARP enzyme activity in a cell-free assay system. PARP-FL and olaparib (0.01-100 nM) inhibit PARP1 activity in a concentration-dependent manner. N means negative control (no enzyme activity) and numerals after the abbreviations of compounds indicate concentrations tested in nM. **** $P < 0.0001$ and ** $P < 0.01$ with respect to the full enzymatic activity (PARP1 enzyme alone, P, in orange). N=3-5 independent experiments, each performed in duplicate.

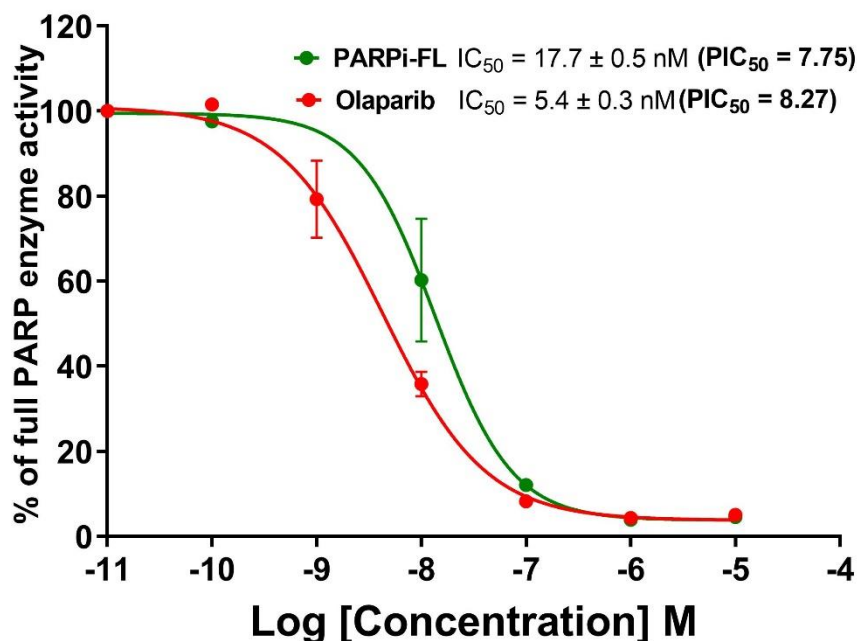


Figure 54: Log concentration-response curves for the effects of PARPi-FL and olaparib on PARP enzyme activity in a cell-free assay system. The plot was based on data in Figure 53. N=3-5 independent experiments, each performed in duplicate.

4.6 Effects of a small group of compounds on PARP1 enzyme activity

Compounds previously screened for PARP-inhibitory activity in the cell-based model assay were screened for their potential direct PARP enzyme binding activity. Most of the compounds did not have any significant effect on PARP enzyme activity ([Figure 55](#)). However, chlorogenic acid (CLA), laudanosine (LDS), quercetin (QUE), warfarin (WRF) and apomorphine (APM) significantly inhibited PARP1 enzyme activity, at 10 μ M (quercetin and apomorphine), and at 100 μ M (all the five compounds). While quercetin did not show any significant protection against PARP-mediated cell death in the cell-based model assay as reported earlier, a previous study has shown that it can inhibit human rPARP-1 enzymatic activity at 100 μ mol/L (100 μ M) in a cell-free assay (Geraets et al., 2007), consistent with this

study's cell-free PARP binding assay data for quercetin. The cell-based screening results from this study showed that laudanosine and apomorphine (100 μ M) might possess some capacity to protect against MNNG-induced, PARP-mediated cell death, although the potential effects did not reach significance ('MNNG alone' values were $17.4 \pm 5.2\%$ and $13.4 \pm 1.6\%$, compared to $25.7 \pm 0.5\%$ and $15.5 \pm 1.0\%$ in the presence of laudanosine and apomorphine, respectively). As there is notable structural similarity between laudanosine and apomorphine ([Figure 56](#)), perhaps some structural modification of these two compounds might confer on the derivative structures some significant PARP-inhibitory activity. Taken together, the data further confirmed the robustness of the cell-based model assay in terms of its ability to discriminate between compounds with significant PARP inhibition activity and those without such activity or with weak activity. Poor cell membrane permeability might underlie the lack of PARP-inhibitory activity of compounds that showed direct PARP binding activity in the cell-free assay but negligible or no PARP-inhibitory activity in the cell-based assay, as a compound that will bind PARP in the cell-based assay must be able to cross the lipid bilayer of the cell membrane to reach the cytosol and then access and bind PARP that is localised to the nucleus.

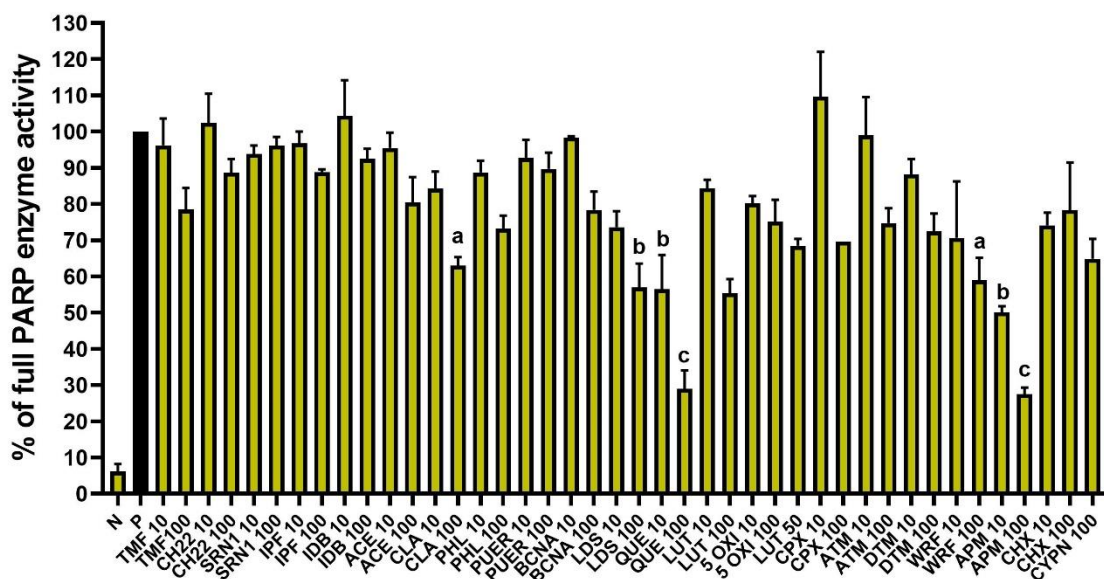


Figure 55: Effects of compounds on PARP enzyme activity in a cell-free assay system. Direct PARP1 binding activities of selected compounds from the study's previous cell-based screening were determined. Most of the compounds did not show any significant reduction in PARP1 enzyme activity. N on the figure means negative control (no enzyme activity) and numerals after the abbreviations of compounds indicate concentrations tested in μM . The compounds were 6,2',4'-Trimethoxyflavone (TMF), CH 223191 (CH22), Stemregenin 1 (SRN1), Ipriflavone (IPF), Indirubin (IDB), Aceroside VIII (ACE), Chlorogenic acid (CLA), Phloretin (PHL), Puerarin (PUER), Biochanin A (BCNA), Laudanosine (LDS), Quercetin (QUE), Luteolin (LUT), 5-hydroxytryptophan (oxitriptan) (5-OXI), Ciclopirox (CPX), Artemisinin (ATM), Dihydroartemisinin (DTM), Warfarin (WRF), Apomorphine (APM), Chloroxine (CHX) and Chrysosplenetin (CYPN). ^c $P < 0.0001$, ^b $P < 0.01$ and ^a $P < 0.05$ with respect to the full PARP1 enzymatic activity (PARP1 enzyme alone), indicated as P (black bar). N=3 independent experiments, each performed in duplicate.

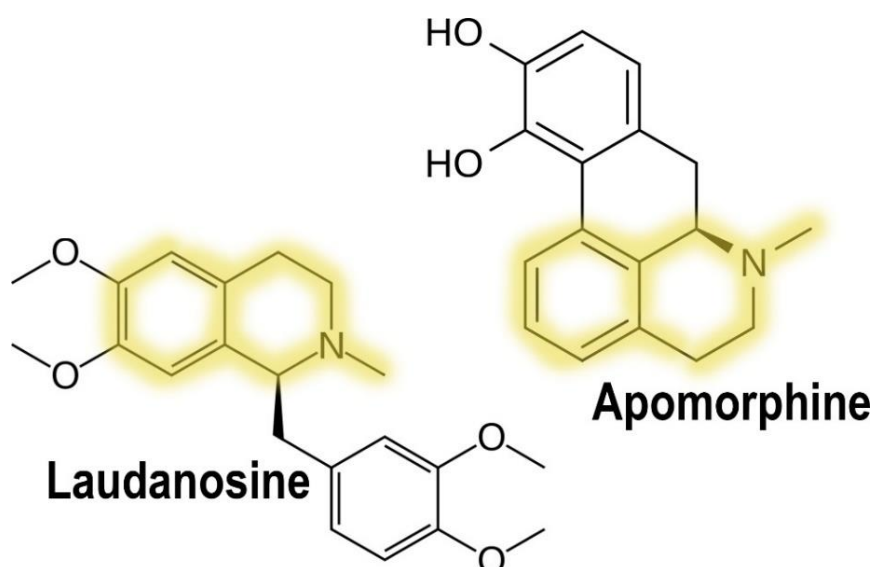


Figure 56: Chemical structures of laudanosine and apomorphine. The structural similarity between the two compounds is highlighted in yellow. Laudanosine and apomorphine both had significant direct PARP binding inhibitory activity at 100 μ M.

4.7 4'-methoxyflavone (4MF) and 3',4'-dimethoxyflavone (DMF) directly inhibited the PARP enzyme

4MF and DMF, alongside DPQ as a positive control, having shown significant inhibitory effects on PARP-mediated cell death in the phenotypic (cell-based) assay, were also screened in the cell-free PARP enzyme activity assay to assess their ability to bind directly to PARP to inhibit it. [Figure 57](#) shows that 4MF, DMF and DPQ each directly inhibited PARP enzyme activity in a concentration-dependent manner, with each compound showing significant effect from 1 μ M. The IC_{50} values for their effects, calculated from the derived log concentration-response curves ([Figure 58](#)), are shown in [Table 9](#). The results revealed that DPQ (IC_{50} : 1.77 ± 0.41 μ M) was slightly more potent than DMF (IC_{50} : 2.07 ± 0.99 μ M) and 4MF (IC_{50} : 2.54 ± 1.06 μ M) and also marginally more efficacious ([Figure 58](#)). The IC_{50} value obtained for DPQ in this assay is consistent with the 1 μ M reported by a previous work that also used a cell-free system (Takahashi et al., 1999). Similarly, Moroni et al. (2001) found that DPQ inhibited PARP activity in a concentration-dependent manner by measuring the conversion of radioactively labelled NAD^+ into PAR in an *in vitro* assay system, with an IC_{50} of 3.5 ± 0.5 μ M (Moroni et al., 2001).

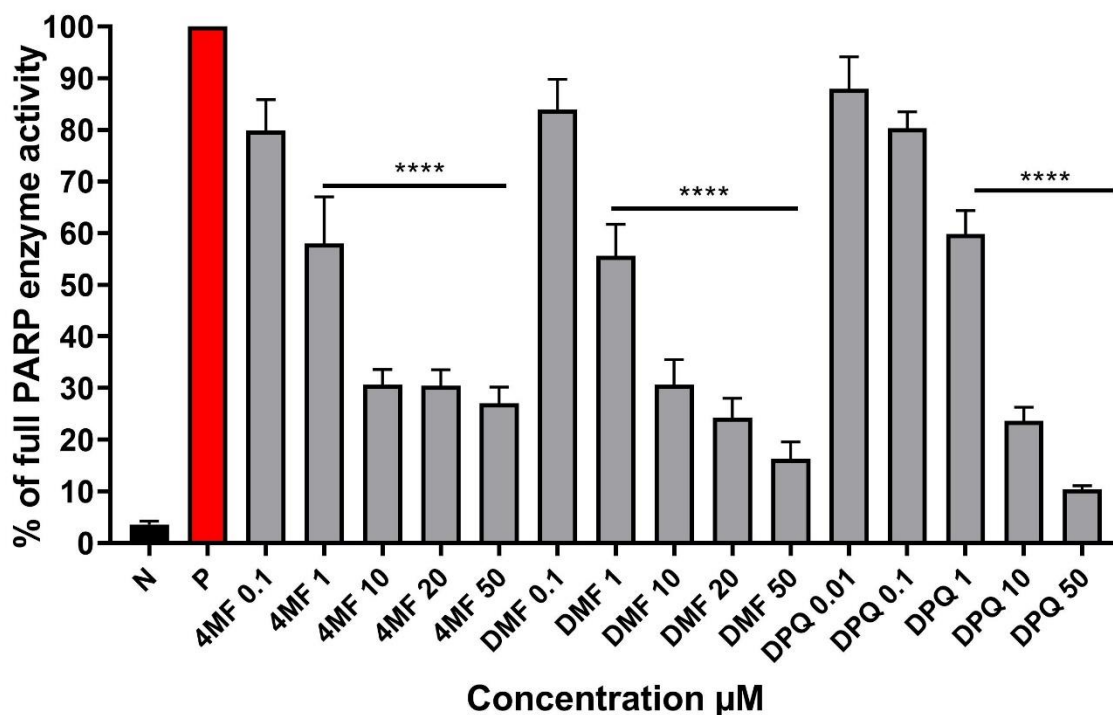


Figure 57: Effects of 4MF, DMF and DPQ on PARP enzyme activity in a cell-free assay. 4MF, DMF and DPQ (0.1-50 μ M) inhibit PARP1 enzyme activity in a concentration-dependent manner. N on the figure means negative control (no enzyme activity) and numerals after the abbreviations of compounds indicate concentrations tested in μ M **** P <0.0001 with respect to the full PARP1 enzymatic activity (PARP1 enzyme alone), indicated as P (red bar). N=3 independent experiments, each performed in duplicate.

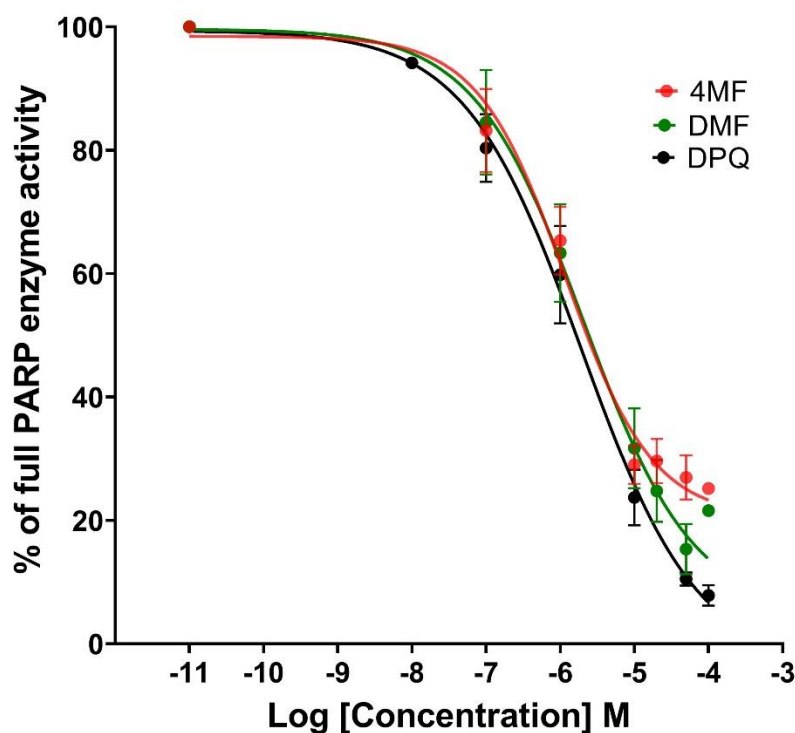


Figure 58: Log concentration-response sigmoid curves for 4MF, DMF and DPQ in a cell-free assay. The plot was based on data in Figure 57. N=3 independent experiments, each performed in duplicate.

In terms of the potencies of 4MF and DMF relative to DPQ's, the cell-free assay results are in contrast to the results from the cell-based assay. Based on IC_{50} values, whereas 4MF, DMF and DPQ were nearly equipotent in the cell-free assay, DPQ was thrice more potent than 4MF and DMF (Table 9). A comparison of the cell-based and cell-free assays with respect to each of the three compounds shows that while 4MF and DMF were each nearly three times less potent in the cell-based assay than in the cell-free assay, DPQ did not show any significant potency change across the two assays.

Table 9: IC₅₀ (potency) values (Mean ± SEM) for 4MF, DMF and the standard PARPi DPQ in cell-free and cell-based assays. N=3-5 independent experiments, each performed in duplicate (cell-free) and triplicate (cell-based).

	4MF	DMF	DPQ (positive control)
Cell-free Assay IC₅₀ (μM)	2.54 ± 1.06 (PIC₅₀ = 5.60)	2.07 ± 0.99 (PIC₅₀ = 5.68)	1.77 ± 0.41 (PIC₅₀ = 5.75)
Cell-based Assay EC₅₀ (μM)	6.32 ± 2.26 (PEC₅₀ = 5.20)	5.92 ± 0.41 (PEC₅₀ = 5.22)	1.45 ± 0.31 (PEC₅₀ = 5.84)
Fold difference in potency (cell-based vs. cell-free assay)	2.5	2.9	0.82

Taken together, the results confirm that 4MF and DMF, like DPQ, are able to directly bind at low micromolar concentrations to PARP to inhibit its activity, implying that they inhibited PARP-mediated cell death in the cell-based assay used in this study by binding directly to PARP. However, as 4MF and DMF exhibited reduced potencies in the cell-based assay compared to the direct binding cell-free assay, unlike DPQ that retained similar potencies across the two assays, it is possible that 4MF and DMF have cell membrane permeability issues that significantly reduce their concentration that reaches the cytosol compared to the extracellular concentration applied, while DPQ crosses the cell membrane with minimal or no hindrance.

4.8 4MF or DMF potentiated, and synergised with, the cytotoxicity of cisplatin or doxorubicin in HeLa cell

The experiments reported in this section were conducted to illustrate one of the potential therapeutic applications of PARPi, which is their use in combination (cancer) therapy, based on the ability of the PARPi to potentiate, or synergise with, the cytotoxic effect of the chemotherapeutic drug on cancer cells by preventing the cancer cells exposed to it from being able to repair their DNA damage. The inclusion of the PARPi could also prevent the cancer cells from developing resistance to the chemotherapeutic drug. The potentiation caused by the combination therapy could make it possible for the chemotherapeutic drug to be administered at a lower dose than if it were administered alone, thus resulting in reduced incidence and severity of side effects usually caused by the chemotherapeutic drug.

Cisplatin (*cis*-diamminedichloroplatinum II) is a well-known chemotherapeutic agent that is used in the treatment of various cancers. It is one of the most effective drugs for the treatment of both locally advanced and metastatic cervical cancer. However, its effectiveness is usually plagued by chemoresistance (Dasari and Bernard Tchounwou, 2014; Nguyen et al., 2022). Therefore, in order to overcome this drug resistance problem and improve clinical outcome, a combination treatment approach is often required. In several preclinical studies, it has been demonstrated that natural products not only enhance the therapeutic benefit of cisplatin but also mitigate its chemotherapy-induced toxicity (Dasari et al., 2022). Similarly, doxorubicin, another widely used anticancer drug, has been reported to have synergistic effects when combined with some natural products that help it to overcome drug resistance (Zhong et al., 2017; Al-Abbas and Shaer, 2021). Also,

there are reports of olaparib, a clinically approved PARP inhibitor, having a synergistic or potentiation effect in combination with cisplatin or doxorubicin (Minami et al., 2013; Park et al., 2018). Since the PARP-inhibitory property of 4MF and DMF has been established in this project, the potential effect of combining either of them with cisplatin or doxorubicin, as opposed to cisplatin or doxorubicin alone, was investigated in HeLa cells. The ability of 4MF and DMF to potentiate or synergise with existing chemotherapeutic drugs will be very promising, as they could be used as adjuvants in chemotherapy and possibly in other forms of cancer treatments too, such as radiotherapy and immunotherapy.

In order to be able to assess the combination effects of 4MF or DMF with cisplatin or doxorubicin, the cytotoxicity profiles of cisplatin and doxorubicin, each alone, were first determined. The results showed that cisplatin reduced cell viability after 24 and 48 h in a concentration- and time-dependent manner ([Figure 59-A](#)), and doxorubicin demonstrated a similar pattern of effect ([Figure 59-B](#)). Cisplatin, at the least concentration tested (6.25 μM), decreased cell viability significantly to 69% and 37% compared to the negative control after 24 and 48 h, respectively ([Figure 59-A](#)), while doxorubicin was significantly cytotoxic from 0.5 μM . [Figure 59](#) also shows that the cytotoxic effects of cisplatin or doxorubicin were significantly higher at 48 h than at 24 h. Based on these results, the cells were treated with cisplatin (6.25 μM) or doxorubicin (1 μM), alone or concomitantly with each of the various concentrations of 4MF or DMF, in order to identify any synergy or potentiation in the combination treatments. Cell viability was measured after 96 h (4 days) using the MTT assay.

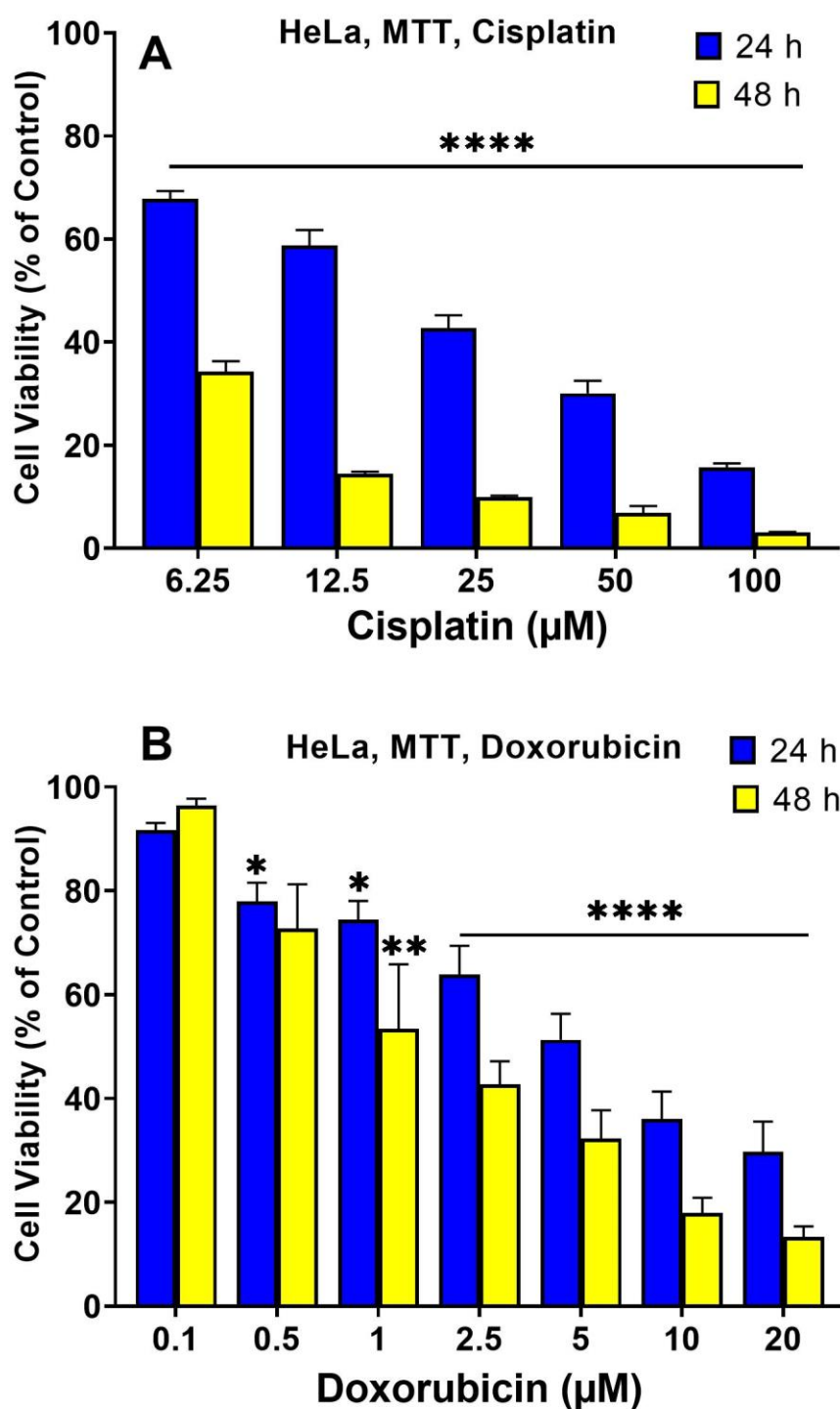


Figure 59: Effects of cisplatin and doxorubicin on HeLa cell viability. Cisplatin (A), (6.25 - 100 μM) and Doxorubicin (B), (0.1- 20 μM) show concentration-dependent cytotoxicity after 24 h and 48 h, as quantified by the MTT assay. **** $P < 0.0001$, ** $P < 0.01$ and * $P < 0.05$ with respect to the negative control. N=3 independent experiments, each performed in triplicate.

[Figure 60](#) shows that cisplatin (6.25 and 100 μM) and doxorubicin (1 and 20 μM) were cytotoxic as expected. Interestingly, the results also reveal that 4MF alone at 20 μM or above could be cytotoxic after 96 h. In addition, the results suggest that 4MF has a tendency to be toxic even at 5 and 10 μM , as the cell viability at those time points was slightly reduced, although not significantly. The cell viability for the corresponding combinations of 4MF (5 or 10 μM) and cisplatin (fixed concentration of 6.25 μM) (31.3% and 25.4%, respectively) were significantly lower than for 4MF alone at 5 and 10 μM (86.9% and 70.9%) or for cisplatin alone (43.8%) ([Figure 60-A](#)). In other words, the combination of cisplatin with 4MF was more effective (more cytotoxic) than the effect (cytotoxicity) of each agent individually. This indicates some potentiation of the cytotoxicity of cisplatin by 4MF. In addition, 4MF at 20 μM and above reduced viability significantly, but a combination of each of those concentrations with cisplatin did not achieve a further reduction in viability that was significant compared to 4MF alone, implying lack of synergy. The same pattern of potentiation was observed with the combination of 4MF and doxorubicin ([Figure 60-B](#)). As expected, doxorubicin was cytotoxic to the HeLa cells after 96 h. In line with the previous results, 4MF exhibited some toxicity on its own at 20 μM and above. There was also a more significant reduction in cell viability with the combinations of doxorubicin (fixed concentration of 1 μM) and 4MF at 5 or 10 μM (40% and 26.9%) compared to 4MF alone at 5 or 10 μM (86.9% and 70.8%), or doxorubicin alone (62.2%) – which suggests some potentiation of the effects of doxorubicin. Interestingly, the combinations of doxorubicin and 4MF at 20 μM and above of 4MF had significantly more cytotoxic effects than their corresponding 4MF alone concentrations. Taken together, this implies that the combinations of 4MF

and doxorubicin had some potentiating or synergistic effects that can be further explored in future work for therapeutics development.

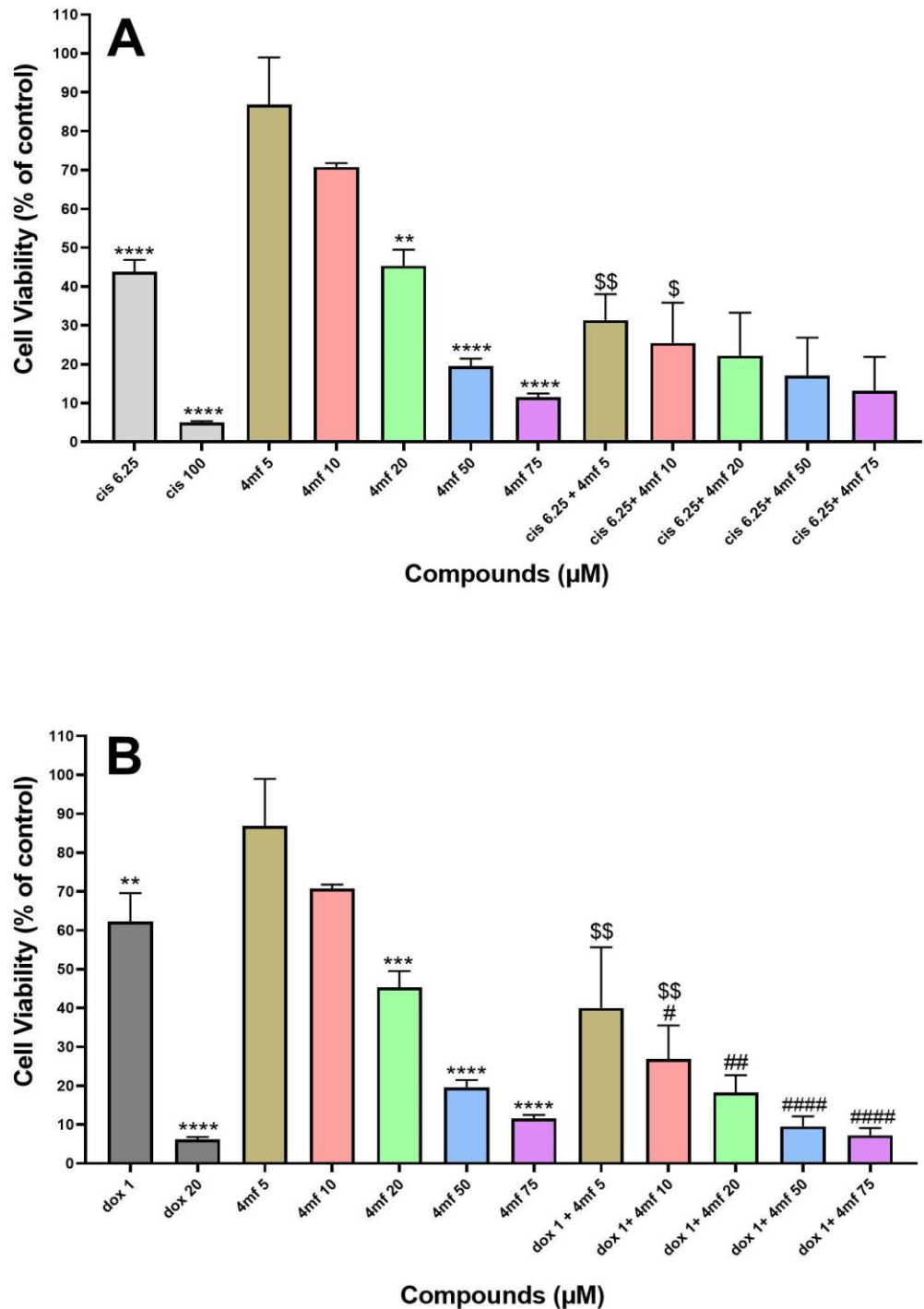


Figure 60: Effects on cell viability of a combination of cisplatin or doxorubicin with 4MF. 4MF shows some synergy or potentiation when combined with cisplatin, cis (A) or doxorubicin, dox (B) for 96 h in HeLa cells. Numerals after each abbreviated compound

name show the concentration tested in μM . **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ with respect to the negative control (media + DMSO); ##### $P < 0.0001$, ### $P < 0.001$, ## $P < 0.01$ and # $P < 0.05$ with respect to cis 6.25 or dox 1 alone; \$\$ $P < 0.01$ and \$ $P < 0.05$ with respect to the corresponding concentration of 4MF alone. N=2 independent experiments, each performed in triplicate.

On the other hand, the combination effect of DMF with cisplatin and doxorubicin showed a slightly different result from that of 4MF in combination with cisplatin or doxorubicin. [Figure 61](#) shows that after 96 h, cisplatin alone was cytotoxic but doxorubicin (1 μM) was not significantly cytotoxic. Also, in contrast to 4MF, DMF seemed not to be significantly toxic by itself up to 50 μM . In the cisplatin experiment, DMF alone at 50 μM was cytotoxic, but combinations of DMF and cisplatin produced viability values only significantly lower than those of DMF alone but not significantly lower than that of cisplatin alone ([Figure 61A](#)). Therefore, no potentiation of cisplatin by DMF could be confirmed. In the doxorubicin experiment, the seeming effect of doxorubicin alone did not reach statistical significance; therefore, the effect of a combination of DMF with doxorubicin could not be assessed.

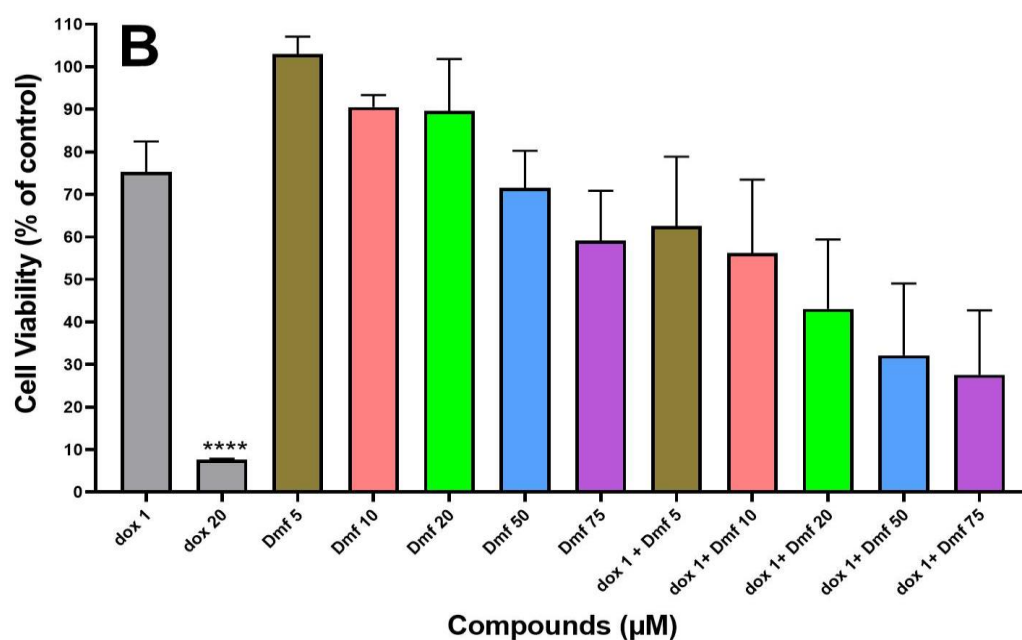
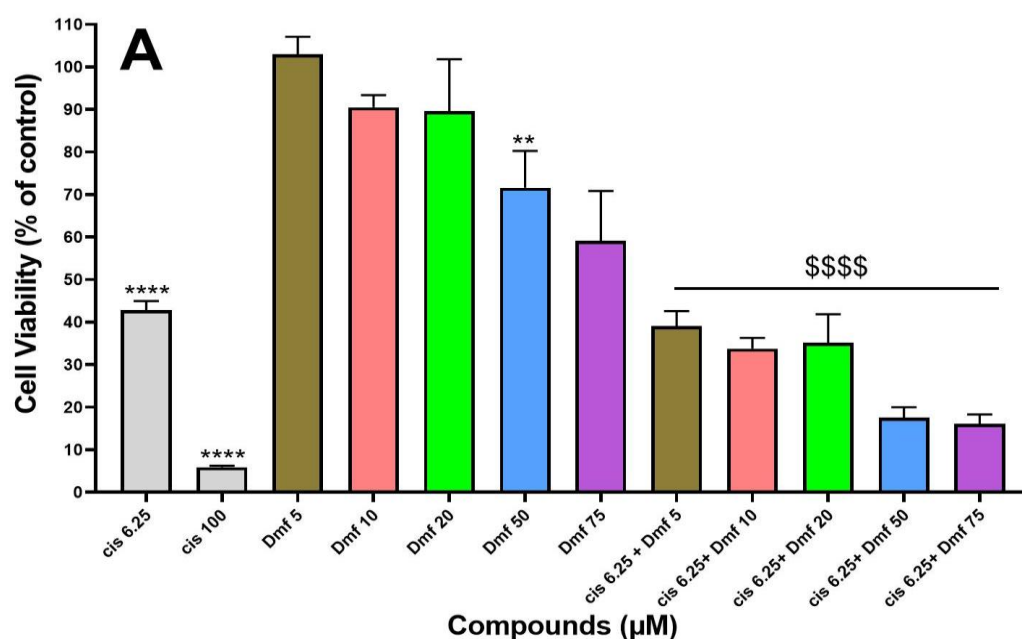


Figure 61: Effects on cell viability of a combination of cisplatin or doxorubicin with DMF. DMF shows some potentiation when combined with cisplatin (A) but no significant effect when combined with doxorubicin (B) for 96 h in HeLa cells. Numerals after each abbreviated compound name show the concentration tested in μM . **** $P < 0.0001$ and ** $P < 0.01$ with respect to the negative control (media + DMSO); \$\$\$\$ $P < 0.0001$ with respect to the corresponding concentration of DMF alone. $N = 2$ independent experiments, each performed in triplicate.

To conclude this chapter, the screening of the natural compounds and of the additional customised compounds did not produce any hits. However, 4MF and DMF lent themselves to a full characterisation of their PARP-inhibitory activities, both in cell-based and cell-free assays. The addition of another methoxy group to 4MF to generate DMF did not affect PARP inhibition potency and efficacy, but rather significantly improved metabolic stability. 4MF demonstrated ability to potentiate or synergise with the chemotherapeutic drugs cisplatin or doxorubicin.

Chapter 5: Results and Discussion III

5.0 Results of structure-activity relationship (SAR) study of 4MF and DMF

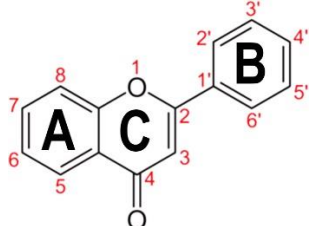
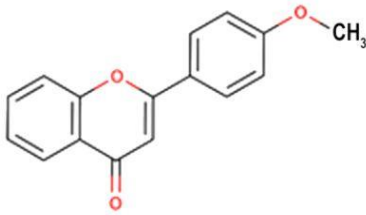
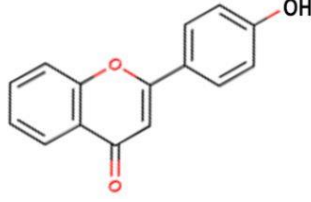
5.1 Introduction

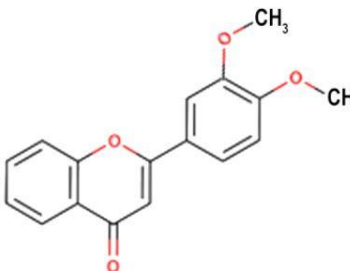
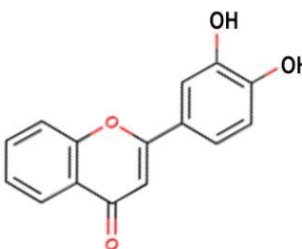
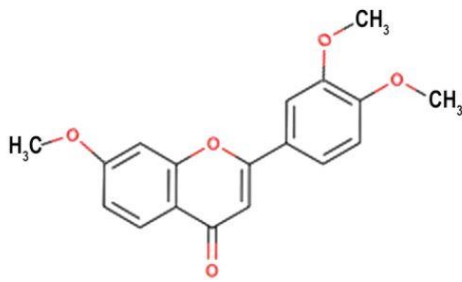
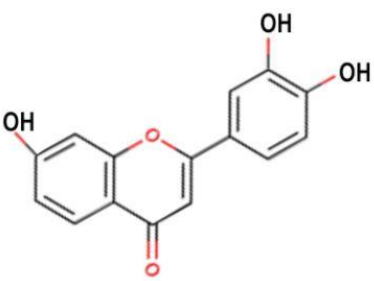
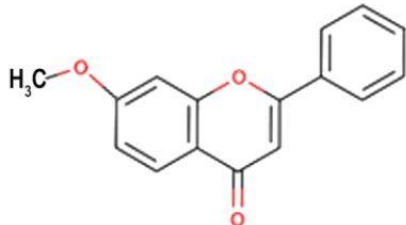
4MF and DMF are plant-derived polyphenolic flavonoids which have been reported to possess a wide range of biological characteristics (Proksa et al., 1990; Middleton, Kandaswami and Theoharides, 2000). These include antidiabetic, antimicrobial, antiatherogenic, anti-inflammatory, antioxidant, anti-hyperlipidaemic, neuroprotective and anti-cancer effects (Choi, Yokozawa and Oura, 1991; Datla et al., 2001; Martinez-Perez et al., 2014; Martínez-Pérez et al., 2016). Almost all flavonoids have the capacity to act as antioxidants, and the flavones subgroup has been shown to exhibit the most powerful defence against reactive oxygen species (Pietta, 2000). Furthermore, numerous research studies have linked many of the therapeutic benefits of flavonoids directly or indirectly to their antioxidant properties (Panche, Diwan and Chandra, 2016). Having established in this study that 4MF and DMF are PARPi, the next logical step taken in the direction of potentially developing them as novel therapeutics was the exploration of their SARs in order to determine whether their PARP-inhibitory activities can be improved upon and also to determine if there are any correlations between their antioxidant efficacy and their efficacy to protect against PARP1-mediated cell death (parthanatos). As the backbone flavone structure is shared by the two compounds, hydroxy- and methoxy-substitutions on the A-ring and/or B-ring were considered in assembling and testing a small, customised library of flavones. A total of 11 compounds,

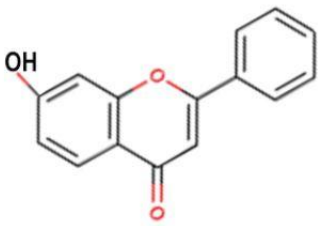
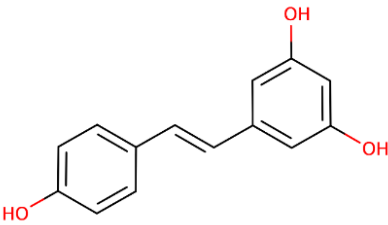
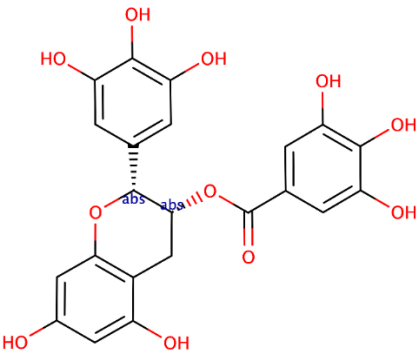
including 4MF, DMF, and two non-flavonoid polyphenolic natural products, were eventually selected for the SAR study ([Table 10](#)).

The antioxidant activities of this new library were determined using the DPPH assay (cell-free) and the intracellular ROS assay (cell-based), while the PARP inhibition activities were investigated using the direct PARP binding assay (cell-free) and the MNNG-induced, PARP-mediated parthanatos model assay (cell-based), with the use of the last two assays (PARP assays) having been reported in the previous chapters.

Table 10: Customised library of compounds for SAR study. The flavone structure (1) shows the names of the rings, and the numbers (in red) around the rings indicate potential substitution positions. Resveratrol and epigallocatechin gallate are non-flavones.

	Compound	Structure
1	Flavone (FLV)	
2	4'-methoxyflavone (4MF)	
3	4'-hydroxyflavone (4HF)	

4	3',4'-dimethoxyflavone (DMF)	
5	3',4'-dihydroxyflavone (DHF)	
6	3',4',7-trimethoxyflavone (TMF)	
7	3',4',7-trihydroxyflavone (THF)	
8	7-methoxyflavone (7MF)	

9	7-hydroxyflavone (7HF)	
10	Resveratrol (RSV)	
11	Epigallocatechin gallate (EPG)	

5.2 SAR compounds exhibited DPPH radical-scavenging activity

For the DPPH assay, quercetin (3,3',4',5,7-pentahydroxyflavone), a well-established antioxidant (Xu et al., 2019a), was used as a positive control. The results show that quercetin (75 μ M) exhibited a relatively high DPPH radical scavenging activity (82.5%) compared to the control (methanol) ([Figure 62](#)). The result is quite similar to those of previous studies, as Zhang et al. (2020) showed the DPPH radical scavenging activity (RSA) of quercetin as 80%, while Liang et al. (2021) and Adrah et al. (2021) reported it as 94.52% and 94.06%, respectively (Adrah, Ananey-Obiri and Tahergorabi, 2021; Liang et al., 2021). [Figure 62](#) also shows that all the 11 compounds had DPPH RSA, which implies that they all

possess some antioxidant property. However, 4'-hydroxyflavone (4HF), 3',4'-dihydroxyflavone (DHF), 3',4',7-trihydroxyflavone (THF), epigallocatechin gallate (EPG) and resveratrol (RSV) were found to possess relatively high DPPH RSA (55.9%, 77.9%, 76.6%, 73% and 67.2%, respectively, each at the highest concentration tested – 75 μ M). The rest of the compounds exhibited low or mild DPPH RSA, ranging from 5.5% to 17.3%. The parent flavone (FLV) structure had the least DPPH RSA (5.5%). Subsequently, to assess the contribution to antioxidant efficacy/potency of substitutions on the compounds relative to the parent flavone, the DPPH RSA (at 75 μ M) for each compound was compared with that of flavone, and the values for the fold change in their free radical-scavenging ability relative to flavone are summarised in [Table 11](#).

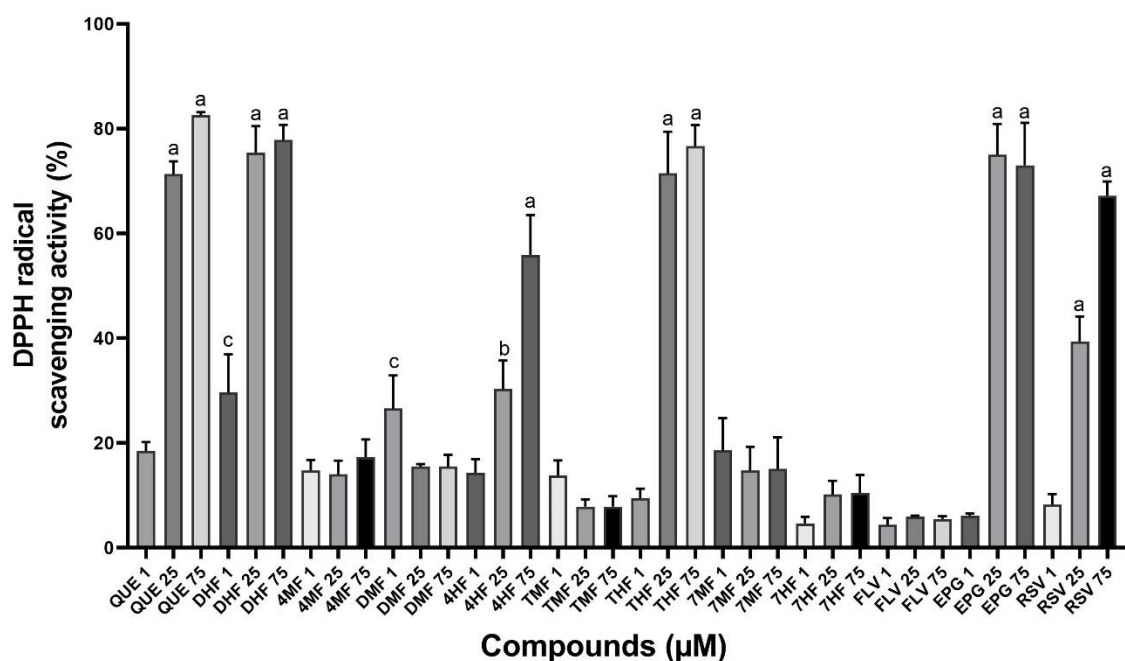


Figure 62: The DPPH radical scavenging activity of the SAR compounds. ^aP<0.0001, ^bP<0.001 and ^cP<0.01 with respect to the control (methanol alone). The eleven compounds were quercetin (QUE), 3',4'-dihydroxyflavone (DHF), 4'-methoxyflavone (4MF), 3',4'-dimethoxyflavone (DMF), 4'-hydroxyflavone (4HF), 7,3',4'-trimethoxyflavone (TMF), 7,3',4'-trihydroxyflavone (THF), 7-methoxyflavone (7MF), 7-hydroxyflavone (7HF), flavone (FLV), epigallocatechin gallate (EPG), and resveratrol (RSV). N=3 independent experiments, each performed in triplicate.

Table 11: The DPPH radical scavenging activities of the SAR compounds at 75 μ M relative to that of flavone (FLV) in yellow. N=3 independent experiments, each performed in triplicate.

	% DPPH RSA (Mean $\pm$ SEM)	Fold increase vs. FLV
QUE	82.5 $\pm$ 0.6	15.0
DHF	77.9 $\pm$ 2.9	14.1
4MF	17.3 $\pm$ 2.7	3.1
DMF	15.5 $\pm$ 2.2	2.8
4HF	55.9 $\pm$ 6.2	10.1
TMF	7.9 $\pm$ 2.0	1.4
THF	76.6 $\pm$ 4.0	13.9
7MF	15.1 $\pm$ 6.0	2.7
7HF	10.5 $\pm$ 3.4	1.9
FLV	5.5 $\pm$ 0.4	1
EPG	73.0 $\pm$ 8.1	13.2
RSV	67.2 $\pm$ 2.7	12.2

5.2.1 Effects of hydroxyl groups on antioxidant activity

All the five compounds that displayed strong antioxidant activity have at least two hydroxyl groups. In fact, EPG has eight hydroxyl groups, while quercetin (the positive control) has five. The number of hydroxyl groups present does not necessarily increase the DPPH RSA linearly. For example, DHF has only two hydroxyl groups but its RSA is higher than that of THF, which possesses three, or that of EPG, which is a non-flavone with eight hydroxyl groups. Like most other flavonoids, the number and positioning of the B-ring hydroxyl groups play a very crucial role in flavones' antioxidant activity mechanism, especially the 3',4'-catechol

structure in the B-ring, which has been reported to strongly enhance antioxidant activity (Heim, Tagliaferro and Bobilya, 2002). This might be a possible explanation for the strong scavenging activity observed in DHF and THF, as they both possess the 3',4'-hydroxyl groups. Moreover, the high antioxidant activity of quercetin has been attributed to the 3', 4'-hydroxyl groups on its B-ring, as they are mainly involved in hydrogen atom transfer reactions to DPPH (Zhang et al., 2020). Furthermore, several studies have demonstrated that the configuration of B-ring hydroxyl groups is the most important factor in the scavenging of both reactive oxygen species (ROS) and reactive nitrogen species (RNS) by flavonoids (Kerry and Rice-Evans, 1999; Pannala et al., 2001). The addition of a 7-hydroxyl group to the A-ring of DHF (77.9%) to produce THF (76.6%) did not improve antioxidant activity. Also, the DPPH RSA of 7-hydroxyflavone (7HF) (10.5%) compared with that of 4'-hydroxyflavone (4HF) (55.9%) suggests that the position of the hydroxyl plays a key role in the antioxidant properties of flavones. Moreover, Martínez-Pérez et al. (2016) proposed that the 4'-hydroxyl group could be the most important electron-donating group on the B-ring, due to its position in the aromatic ring. The ability of antioxidants such as quercetin or DHF to scavenge free radicals from DPPH is due to their polyphenolic scaffold, which generates extensive, conjugated π -electron systems, thus allowing them to donate hydrogen atoms or electrons from their hydroxyl moieties (Martínez-Pérez et al., 2016). Since the parent flavone (FLV) has no hydroxyl group, it is not surprising that its ability to donate electrons is not as strong as that of its derivatives with one or multiple hydroxyl groups. In fact, FLV was the weakest scavenger of DPPH ([Table 11](#)). These results strongly imply that possessing a 3',4'-dihydroxyl group is a key feature requirement for significant antioxidant activity of flavones in the DPPH assay. This is in line with

previous research (Zhang et al., 2020). There is also an indication that other functional groups, such as the methoxy groups, attached to the 3' and/or 4' position on the B-ring of flavones, might not only play a substantial role in antioxidation but also in other biological activities.

5.2.2 Effects of methoxy groups on antioxidant activity

The addition of a methoxy group to the flavone structure slightly increased the DPPH free radical-scavenging activity ([Figure 62](#), [Table 11](#)). 4MF (17.3%) had the highest DPPH RSA among the flavones with methoxy substitutions, while 3',4',7-trimethoxyflavone (TMF) (7.9%) possessed the least. Comparing the scavenging capacity of 4MF with those of DMF (15.5%) and 7MF (15.1%), it seems the positioning of the methoxy group, whether on the B-ring or A-ring, had no effect on the contribution of the methoxy group to DPPH RSA. However, a further incorporation of another methoxy moiety into DMF to produce TMF led to a significant decrease in the DPPH RSA, in contrast to the incorporation of a hydroxyl group to DHF to produce THF, which had no effect on the DPPH RSA. Methoxy groups on B-rings are still expected to be electron-donating but considerably weaker than the hydroxyl groups. Yet, adding 7-methoxy to the meta position of the A-ring causes it to be electron-withdrawing (Martínez-Pérez et al., 2016). This possibly underlies the reduction in scavenging activity observed in TMF compared to DMF.

A direct comparison of the DPPH radical scavenging activities of the hydroxyflavones with those of their methoxy counterparts showed that the hydroxyflavones were more antioxidant. For example, 4HF was about thrice more

DPPH radical-scavenging (or more antioxidant) than 4MF, while DHF and THF were five and about ten times more DPPH-radical scavenging (more antioxidant) than DMF and TMF, respectively. There was no significant change in DPPH RSA between 7HF and 7MF, which further suggests that the substitutions on the A-ring were not as important as those on the B-ring. Overall, methoxylation of the parent flavone yielded an increase of up to 3-fold in the DPPH RSA, while its hydroxylation led to up to a 15-fold increase. This is consistent with previous study that demonstrated that hydroxyflavones were more antioxidant than methoxyflavones (Takano et al., 2007). The biological activities of flavonoids depend on their chemical structures, which include the numbers of substitutions and substitution positions on the parent backbones. The results in this study show how number, position and the type of substitution (hydroxyl or methoxy) can affect the biological activities of flavones, in this case their direct antioxidant properties. However, it is important to recall that poor bioavailability is a major drawback for the therapeutic use of flavonoids. Some studies have reported that methoxyflavones have higher metabolic stability and intestinal absorption than their corresponding hydroxyflavones because methoxylation protects them from rapid metabolism, such as glucuronidation and sulfation (Wen and Walle, 2006; Walle and Walle, 2007; Walle, 2009). Research has also indicated that methoxylation can improve solubility and prevent both metabolic conjugation and auto-oxidation in the mitochondria, which can increase the bioavailability of these compounds (Martínez-Pérez et al., 2016). This suggests that while the hydroxyflavones possess higher DPPH free radical scavenging activity than the methoxyflavones, their successful application *in vivo* might be seriously hampered, and the

methoxylated derivatives, even if they possess less free radical scavenging activity, might be more efficacious in therapeutic applications *in vivo*.

5.3 Intracellular reactive oxygen species Assay (ROS)

Having established the direct antioxidant activities of the new customised SAR library, the intracellular antioxidant activities of 4MF, DMF and their hydroxylated derivatives were then determined, using 2',7' - dichlorofluorescein (DCF) fluorescence in HeLa cells. The ability of the flavones to reduce raised intracellular ROS levels generated by hydrogen peroxide was measured from 3 h up to 24 h post-treatment. [Figure 63](#) shows that peroxide (50 μ M) induced a significant 2.0 ± 0.2 -fold increase in ROS at the 3 h time point, an effect that was sustained up to 6 h time point but which decreased at the 24 h time point to 1.5 ± 0.1 fold. 4MF, DMF and 4HF each alone (without peroxide) did not have any significant effect on basal ROS levels. Interestingly, DHF significantly reduced the basal ROS levels to 0.6 ± 0.1 -fold ([Figure 63](#)). [Figures 64](#) and [65](#) show that 4MF, DMF and 4HF did not attenuate the effect of peroxide at any time point. On the other hand, DHF exerted a concentration-dependent effect and at 25 and 75 μ M was able to significantly reduce peroxide-induced intracellular ROS from 2.0 ± 0.2 -fold to 0.9 ± 0.1 - and 1.0 ± 0.1 -fold, respectively, at the 3 h time point, which are levels not different from negative control levels, suggesting DHF's complete abrogation of peroxide-induced intracellular ROS.

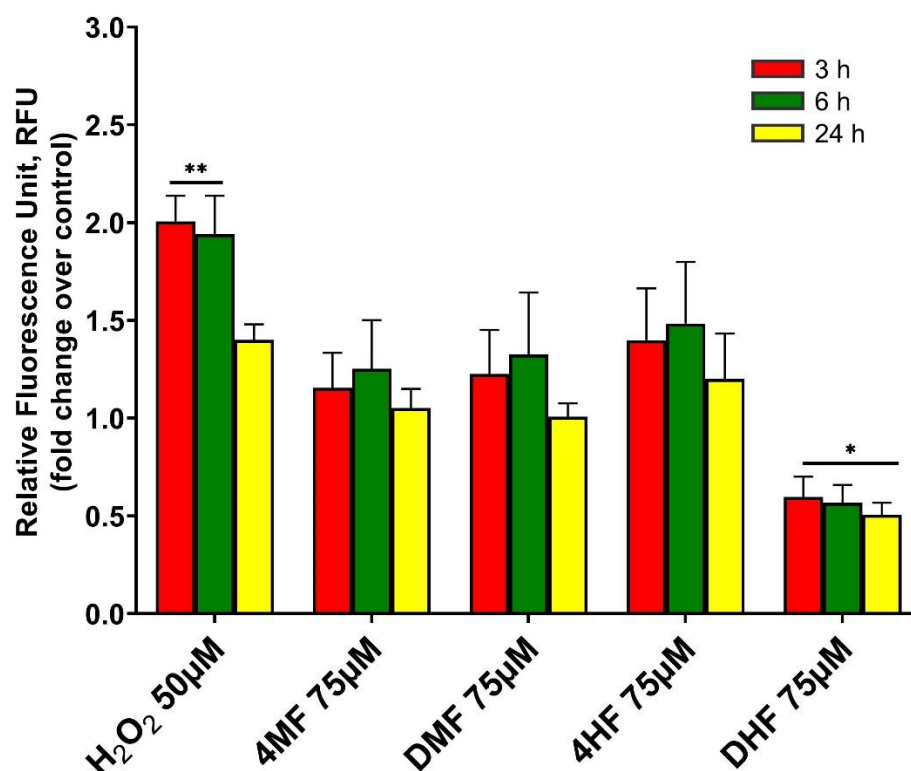


Figure 63: Effects of hydrogen peroxide, 4MF, DMF, 4HF and DHF on intracellular levels of reactive oxygen species (ROS). Intracellular ROS levels are presented as fold changes in fluorescence (Relative Fluorescence Unit, RFU) relative to the negative control, following 3 h, 6 h and 24 h exposures of HeLa cells to peroxide, 4MF, DMF, 4HF and DHF (values are shown for 75 µM of each of the flavones, which was the highest concentration subsequently tested against peroxide). *P<0.05 and **P<0.01 with respect to the negative control. N=4 independent experiments, each performed in triplicate.

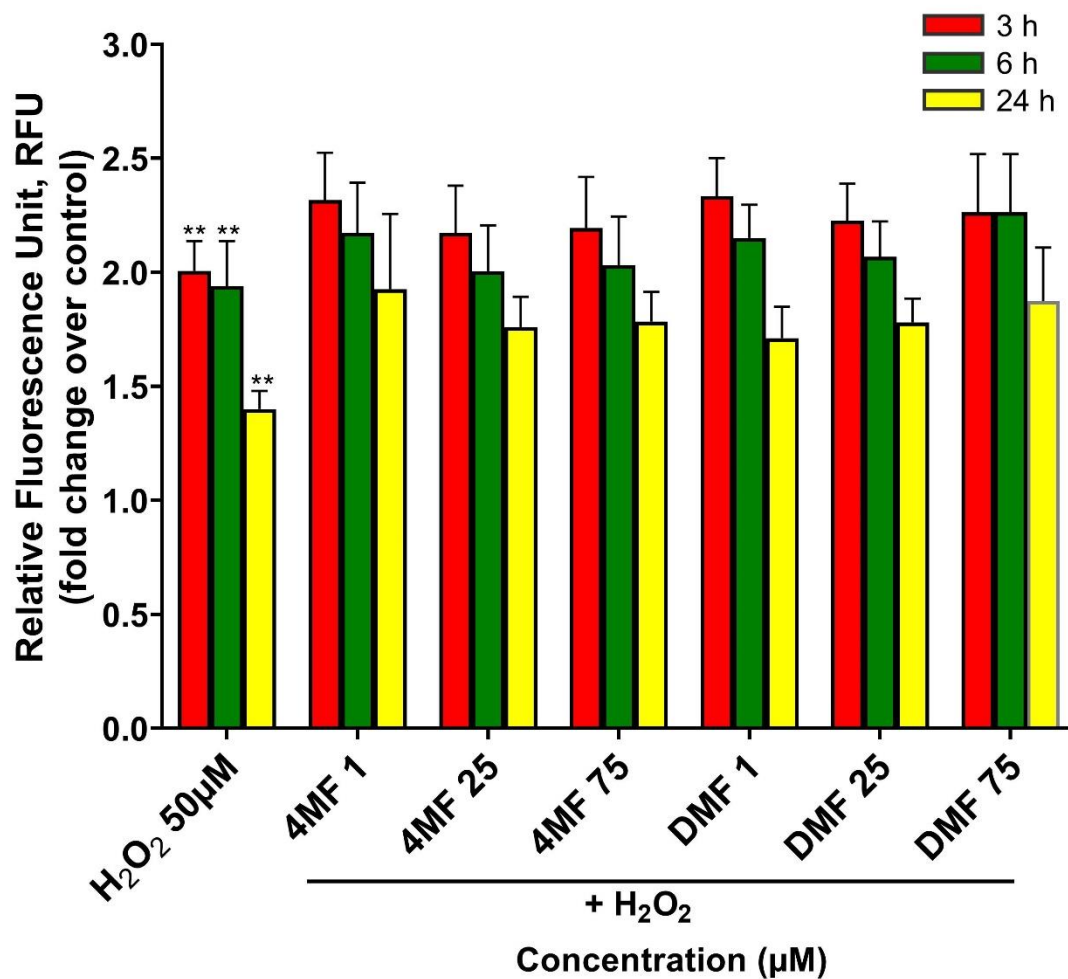


Figure 64: Effects of 4MF and DMF on hydrogen peroxide-induced intracellular ROS levels. Intracellular ROS levels are presented as fold changes in fluorescence (Relative Fluorescence Unit, RFU) relative to the negative control, following 3 h, 6 h and 24 h exposures of HeLa cells to hydrogen peroxide in the presence of each of 4MF and DMF. In the figure, numerals after the flavone name abbreviations indicate the concentration that was tested of the applicable flavone. **P<0.01 with respect to the negative control. N=4 independent experiments, each performed in triplicate.

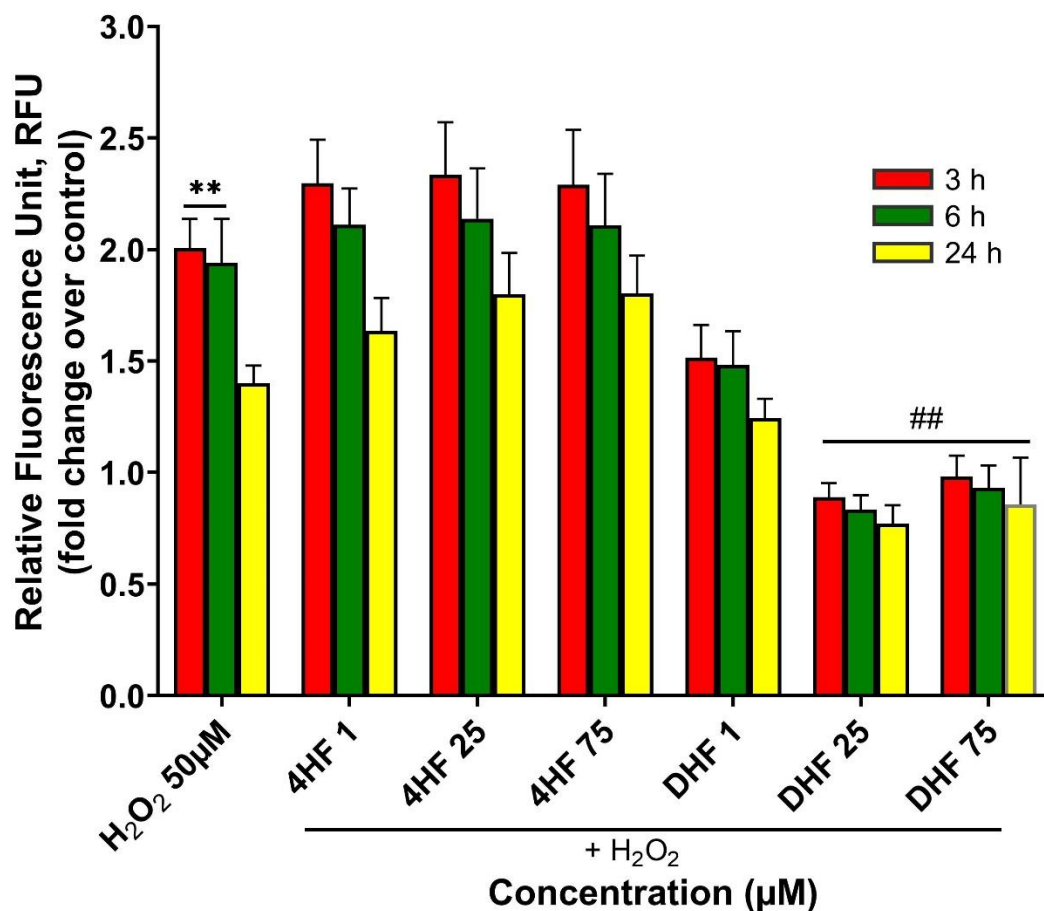


Figure 65: Effects of 4HF and DHF on hydrogen peroxide-induced intracellular ROS levels. Intracellular ROS levels are presented as fold changes in fluorescence (Relative Fluorescence Unit, RFU) relative to the negative control, following 3 h, 6 h and 24 h exposures of HeLa cells to hydrogen peroxide in the presence of each of 4HF and DHF. In the figure, numerals after the flavone name abbreviations indicate the concentration that was tested of the applicable flavone. **P<0.01 with respect to the negative control; ##P<0.01, with respect to peroxide alone. N=4 independent experiments, each performed in triplicate.

These results reveal that the mild DPPH free radical scavenging activity of 4MF and DMF earlier reported did not necessarily translate to a mild antioxidant activity in the more complex cellular milieu of raised reactive oxygen species levels (oxidative stress). It is interesting to note that while 4HF showed a relatively strong DPPH RSA (55.9%), it had no effect on peroxide-induced ROS levels in HeLa cells. However, for DHF, its significant free radical scavenging potential correlated with

its ability to elicit an antioxidant effect in a cellular environment. One possible explanation for the difference in the results of the direct antioxidant activity of 4HF and those of its cellular antioxidant property could be the influence of the absence of the 3'-hydroxyl on its B-ring. Studies have already shown that the lack of the 3'-hydroxyl moiety eliminates the *ortho*-3',4'-dihydroxyl structure which contributes significantly to the radical-scavenging effect of flavonoids, and this results in a decrease in antioxidant activity (Martínez-Pérez et al., 2016). This is corroborated by the DPPH results in this study. Additionally, a structure-antioxidant activity relationship study of 60 flavonoids revealed that the loss of the B-ring 3'-hydroxyl group decreased cellular antioxidant activity in HepG2 cells compared to the antioxidant activity measured by ORAC and DPPH assays (Zhang et al., 2020).

The inability of 4MF, DMF and 4HF to reduce ROS levels, despite their DPPH scavenging activities, could be due to the challenge of cell membrane permeability. Cellular antioxidant activity is influenced by two major factors: the efficiencies of membrane binding and cell uptake (Rice-Evans, Miller and Paganga, 1996). Considering this study's previous experience of differences in outcomes for 4MF and DMF in the parthanatos model assay compared to the PARP1 cell-free assay, (Chapter 4: Sections 4.3 and 4.5), the differences observed between their ROS assay results and their DPPH assay results might be underpinned by their membrane interactions and uptake in cells. In the cellular environment, the antioxidant mechanism of action can be accomplished in three major ways: (a) upregulating or protecting antioxidant defences; (b) direct scavenging of reactive oxygen species; and (c) suppressing ROS formation, either by inhibition of enzymes or by chelating trace elements involved in free radical production

(Halliwell and Gutteridge, 1986). It has been reported that ROS production is triggered by MNNG insults which cause enhanced DNA damage and PARP-1 overactivation (Chiu et al., 2011). This implies that for 4MF and DMF which have been shown to have PARP-inhibitory activity, their antioxidant properties as flavones might not contribute towards the mechanism by which they protect cells against MNNG-induced toxicity.

5.4 Cell-free PARP1 assay of SAR compounds

The direct PARP1-inhibitory activity of all the eleven compounds selected for the SAR study was evaluated using the cell-free PARP enzyme activity inhibition assay described earlier (Chapter 2: Section 2.2.12). PARP inhibition by three different concentrations (1, 10 and 20 μ M) of each compound was measured as a percentage of full PARP enzyme activity (enzyme activity without any inhibitor) and compared with that of flavone, the parent structure. The results showed that 4HF, DHF, THF, EPG and FLV (flavone itself) inhibited the activity of PARP1 in a concentration-dependent manner ([Figure 66](#)), whereas TMF, 7MF, 7HF and RSV did not significantly decrease the activity of PARP1. At 1 μ M, only 4MF, DMF and their hydroxyl derivatives 4HF and DHF exhibited significant PARP-inhibitory activity, decreasing activity to 53.9%, 32.2%, 56.9% and 39.7%, respectively ([Figure 67-A](#)). This observation further confirms the importance of the 3' and 4' positions in the B-ring of flavonoids to their biological activities, whether antioxidant or PARP inhibition. Also, the stronger antioxidant ability (DPPH and ROS) and higher PARP1 inhibition activity of the di-substituted 3', 4'-hydroxyl or -methoxy-flavones compared to their mono-substituted 4'-hydroxyl or -methoxy counterparts suggest some synergy between the effects of the functional group attachments on

these two positions. Even at 1 μ M, the addition of either a hydroxyl or a methoxy moiety at the C4' position of the flavone structure increased PARP-inhibitory activity by up to 25%. By contrast, the addition of either a hydroxyl or a methoxy group at the C7 position on the A-ring to produce 7HF or 7MF decreased PARP inhibition of flavone by up to 40%. This is consistent with earlier results in this study which strongly indicated that attachments to the A-ring did not improve the beneficial biological properties of flavones.

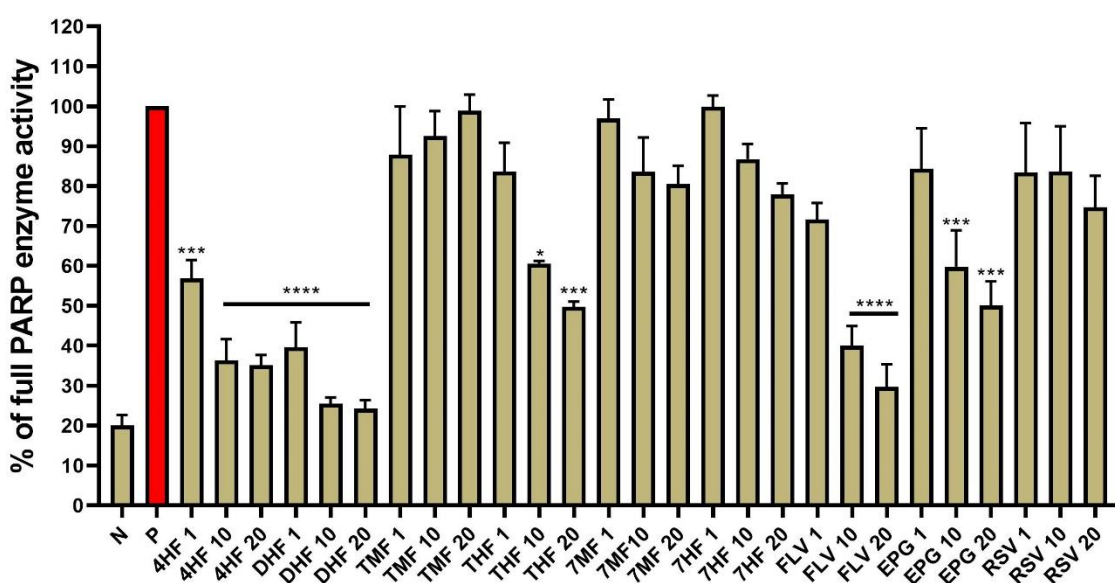


Figure 66: Effects of SAR compounds on PARP enzyme activity. The direct PARP1-inhibitory activities of all the eleven compounds selected for the SAR study are shown. **** $P < 0.0001$, *** $P < 0.001$ and * $P < 0.05$ with respect to full PARP enzyme activity indicated as 'P' (red bar). N indicates negative control (no enzyme activity). N=3 independent experiments, each performed in duplicate.

A similar pattern of PARP-inhibitory activity was observed at 10 μ M of the compounds, except that THF (60.6%), EPG (59.8%) and FLV (39.9%) also significantly reduced PARP activity ([Figure 67-B](#)). DMF (20.3%) had the highest inhibitory activity, followed by 4MF (25.6%), DHF (25.4%) and 4HF (36.4%). The introduction of the 3',4'-dimethoxy in flavone increased its activity by 49%.

Comparing 4MF to 4HF and DMF to DHF, the methoxylated flavones showed slightly better PARP1 inhibition than the hydroxylated flavones. This is quite a contrast to their DPPH radical scavenging-based antioxidant activity results. Having explored the literature, to the best of the author's knowledge this is the first study to directly compare the PARP-inhibitory activities of methoxylated flavones with those of hydroxylated flavones. Although it may not always be the case, some studies that investigated other biological activities (apart from antioxidant properties) of methoxylated flavones and hydroxylated flavones have reported that the hydroxylated ones were less potent and, in some cases, lost their activity (Walle and Walle, 2007). For example, methoxyflavones protected cells against endoplasmic reticulum stress-induced cell death *in vitro* and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine injection *in vivo* (a model of Parkinson's disease). Hydroxyflavones, however, did not have any protective effects in these conditions (Takano et al., 2007). Also, Dymarska et al. (2018) reported that 7-methoxyflavone had higher cytotoxicity than 7-hydroxyflavone in the MCF-7 breast cancer cell line (Dymarska, Janeczko and Kostrzewa-Susłow, 2018). Therefore, the results showing DMF and 4MF having higher PARP-inhibitory activity than their hydroxylated counterparts are, to some extent, in line with those previous studies. Quite importantly, the methoxy substitutions are more likely to improve bioavailability, as DMF and 4MF might be less prone to cellular modifications *in vivo* and will therefore be more chemically and metabolically stable, compared to DHF and 4HF.

[Figure 67-C](#) shows that 4MF, DMF, 4HF, DHF, THF, FLV, and EPG further decreased PARP1 activity at 20 μ M. It is interesting to note that while adding an additional C7 hydroxyl group to the A-ring of DHF to produce THF decreased its

PARP-inhibitory activity from 24.9% to 49.7% of full PARP activity, the introduction of a methoxy to DMF to make TMF completely quenched its PARP-inhibitory activity ([Figure 67-D](#)). Methoxylation at the C7 position in the A-ring of the flavone seemed to have an adverse effect on PARP activity, as 7MF and TMF did not show any significant reduction in PARP1 activity. Overall, DMF retains the highest PARP inhibition at 17.5% with respect to the full PARP enzyme activity control (100%). Compared with the parent flavone (FLV), DMF represented a 41% improvement in PARP inhibition activity, while 4MF and DHF gave 28% and 18% improvements, respectively. These findings indicate that the addition of one or two methoxy groups to the B-ring improved activity, while the addition of a third methoxy or the presence of a methoxy moiety on the A-ring significantly diminished flavone's PARP-inhibitory activity. Furthermore, the results suggest that methoxyflavones, with the exception of 7MF and TMF, have better PARP-inhibitory activity than hydroxyflavones. Linking this with earlier results, DMF and 4MF are less antioxidant but more PARP-inhibiting than DHF and 4HF. Based on the cell-free PARP activity assay results, it seems the hydroxyflavones, especially 4HF and DHF, are equally promising both as antioxidants and potential PARP inhibitors. However, their application in an *in vivo* scenario might be limited due to their poor bioavailability. Knowing that 4MF and DMF exhibit cellular PARP inhibition, the next step was to investigate if the promising activity of 4HF and DHF translated to success in a cell-based assay, too.

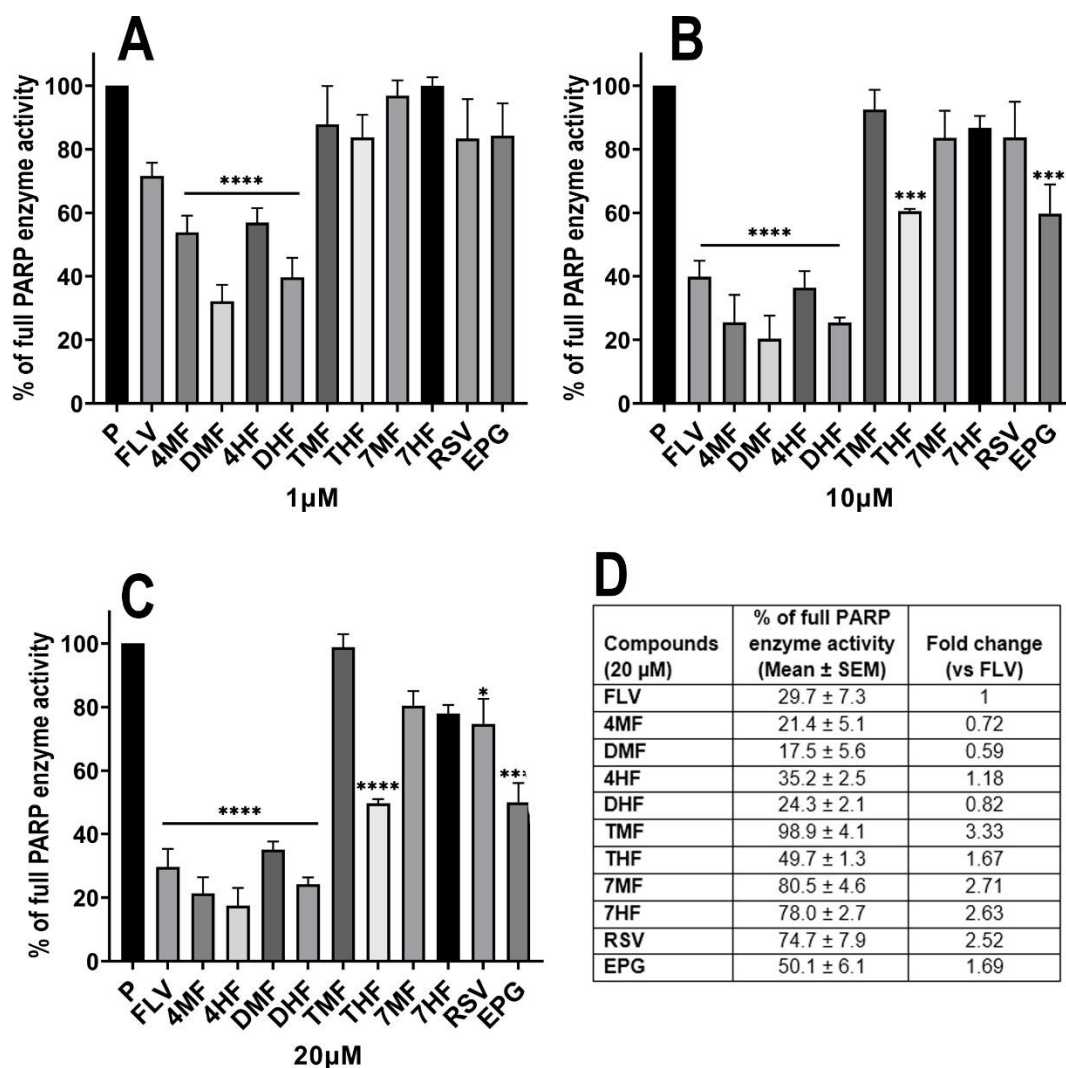
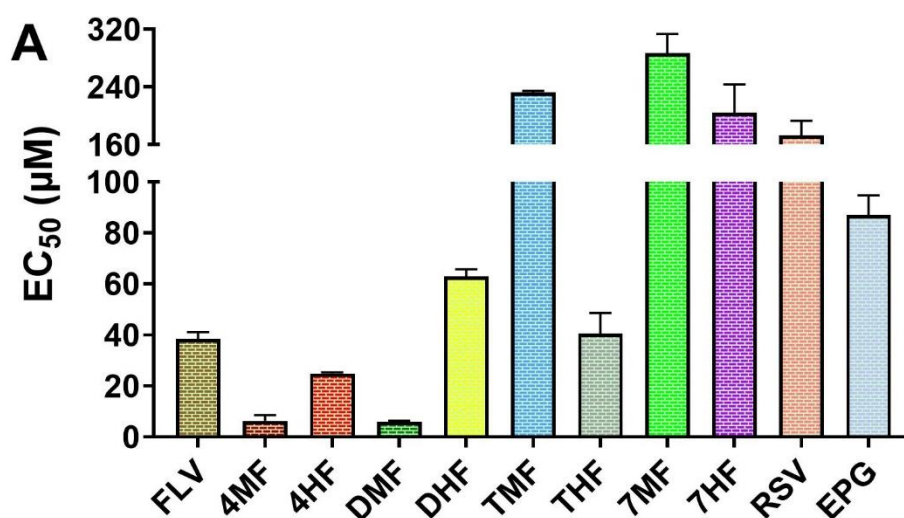


Figure 67: Effects of SAR compounds on PARP enzyme activity in a cell-free assay. The cell-free assay shows the decreases in PARP1 enzyme activity at (A) 1 μM, (B) 10 μM, and (C) 20 μM of the compounds. (D) shows comparisons of the PARP-inhibitory activities of the compounds with that of flavone, the parent structure. Fold change was calculated with respect to FLV (values below 1 represent better PARP-inhibitory activity than FLV and values above 1 represent less PARP-inhibitory activity than FLV). ****P<0.0001, ***P<0.001 and *P<0.05 with respect to the full PARP enzyme activity indicated as 'P' (black bar). N=3 independent experiments, each performed in duplicate.

5.5 Cell-based PARP inhibition activity

4MF and DMF have already been shown to have cellular PARP-inhibitory activity (Chapter 4, Section 4.3), and this was in agreement with previous research by Fatokun et al. (2013). Using the parthanatos model assay described earlier

(Chapter 3, Section 3.2.1), the ability of the SAR compounds to protect against MNNG-induced, PARP-mediated cell death was determined. The assay was carried out using HeLa cells and viability was measured using the MTT assay. The EC₅₀ value for the protective effect of each compound against PARP-dependent MNNG toxicity was determined and compared with that of the parent flavone ([Figure 68](#)).



B

Compounds	EC ₅₀ (μM)	Fold change vs. FLV
FLV	38.3 ± 2.8	1
4MF	6.3 ± 2.3	6.06
4HF	24.8 ± 0.6	1.55
DMF	5.9 ± 0.4	6.47
DHF	62.9 ± 7.4	0.61
TMF	232.6 ± 57.3	0.16
THF	40.55 ± 8.0	0.95
7MF	287.1 ± 26.2	0.13
7HF	204.3 ± 39.4	0.19
RSV	172.9 ± 20.1	0.22
EPG	86.99 ± 7.7	0.44

Figure 68: EC₅₀ values for the SAR compounds. The EC₅₀ values were determined in HeLa cells using the parthanatos model assay, with viability measured using the MTT assay. (A) shows the differences in the EC₅₀ values of the compounds and (B) shows the Mean ± SEM of the EC₅₀ values and the fold change in EC₅₀ for each compound relative to FLV. N=3 independent experiments, each performed in triplicate.

5.5.1 Effects of methoxy substitutions on protection against PARP-mediated cell death

The addition of a methoxy group to the 4' position of the B-ring in flavone increased its potency. The resulting 4MF ($EC_{50} = 6.3 \mu M$) was 6.3 times more potent than FLV ($EC_{50} = 38.3 \mu M$) ([Figure 68](#)). This is consistent with the result for the inhibitory activity of 4MF (20 μM) on PARP enzyme activity, where there was a 28% increase in 4MF's inhibition of PARP enzyme activity compared to FLV (20 μM) ([Figure 67D](#)). Furthermore, the introduction of another methoxy moiety at C3' to 4MF to produce DMF resulted in a slight increase in the potency of DMF ($EC_{50} = 5.9 \mu M$) in the cell-based assay. This also agrees with the cell-free assay results that showed that DMF (17.5%) had more inhibitory effect on PARP1 enzyme activity than 4MF (21.4%). The additional methoxy group at C3' of the B-ring did not change the potency drastically, unlike the first addition that led to a significant increase. The crucial role of the C3' and C4' positions in the B-ring of flavonoids has been reported in several studies (Kawaii, Ishikawa and Yoshizawa, 2018; Zhang et al., 2020), and this has been strongly corroborated by the findings in this study, including the earlier results of the DPPH assay and the cell-free PARP assay. However, it seems that the methoxy attachment at the C3' position might not be as important as the C4' methoxy group, since its addition did not create much change in the EC_{50} value of 4MF compared to that of DMF. In fact, previous work by Fatokun et al. (2013) revealed that methoxylation at the 3' position alone (3'-methoxyflavone) did not produce any significant protection against PARP-mediated cell death. This further underscores the significance of the functional group attachment at flavone's 4' position and suggests that it may be required for beneficial biological activities, such as antioxidant and PARP-inhibitory activities. Conversely, the introduction of an additional methoxy group to DMF's A-ring to

produce TMF ($EC_{50} > 200 \mu\text{M}$) nullified PARP inhibition. In a similar pattern, the presence of a methoxy group at the C7 alone (7MF) did not give rise to any significant protection against MNNG-induced cell death ($EC_{50} > 200 \mu\text{M}$). This is consistent with the previous results of the direct PARP enzymatic activity assay where 7MF and TMF did not show activity. These findings indicate that the methoxylation at the C7 position on the A-ring did not improve PARP-inhibitory activity but had detrimental effects on the PARP-inhibitory activities of flavones, whether in a cell-based or a cell-free scenario. Overall, the addition of methoxy group(s) to the B-ring on the flavone parent structure enhanced its cellular PARP inhibition potency, while the addition to the A-ring decreased the PARP inhibition potency.

5.5.2 Effects of hydroxyl substitutions on protection against PARP-mediated cell death

Interestingly, all the hydroxyflavones, apart from 7HF, showed some cellular PARP-inhibitory activity ([Figure 68](#)). This is in full agreement with the results of the study's cell-free PARP enzyme activity assay, where 4HF, DHF and THF showed significant PARP enzyme activity inhibition. 4HF ($EC_{50} = 24.8 \mu\text{M}$) exhibited the highest potency, followed by THF ($EC_{50} = 40.6 \mu\text{M}$) and DHF ($EC_{50} = 62.9 \mu\text{M}$). The order is different in the cell-free assay, where DHF (24.3%) had the most inhibition, followed by 4HF (35.2%) and then THF (49.7%). The addition of a hydroxyl group to the 4' position of the B-ring (4HF) in the parent flavone increased its potency slightly by 1.5 times, less than the methoxylation which caused a 6.3-fold increase. A further addition of hydroxyl at the C3' position decreased the potency of DHF, while another introduction of a hydroxyl at the C7 on the A-ring (THF) marginally improved DHF's PARP-inhibitory activity. This is almost an opposite effect to that of adding methoxy groups to 4MF at C3' and C7. A direct

comparison of the cellular PARP-inhibitory activities of 4MF and DMF with those of 4HF and DHF revealed that the methoxyflavones were more potent PARP inhibitors than their hydroxyflavone counterparts. Although this pattern of PARP-inhibitory activity was consistent with the results of the cell-free assay, it seemed that the superiority of 4MF and DMF to their hydroxyl equivalents was more pronounced in the cell-based assay. 4MF was 3.9 times more potent than 4HF, while DMF was 10.7 times more potent than DHF. It should be noted that, despite the fact that the hydroxylated flavones retained their PARP-inhibitory activity in a cellular environment, the addition of (a) hydroxyl group(s) decreased their potencies compared to that of the parent flavone, except in the case of 4HF. This suggests that the addition of hydroxyl groups, though it improved the antioxidant activity of flavone, might not necessarily be beneficial in enhancing its PARP-inhibitory activity. Moreover, as mentioned earlier, methoxyflavones such as 4MF and DMF are known to demonstrate higher bioavailability than their corresponding hydroxyflavones (Wen and Walle, 2006). This further commends 4MF and DMF, rather than 4HF and DHF, to further evaluation for potential therapeutic applications in the context of their PARP-inhibitory activity. Recalling that the position of a functional group on the flavonoid structure is very important to its activity, the results showed that hydroxylation at C7 alone (7HF, $EC_{50} > 200 \mu M$) completely eliminates the PARP-inhibitory activity of flavone. This is consistent with the results of the cell-free assay. Furthermore, it suggests that a hydroxyl- or methoxy- substitution on the A-ring of flavone, could adversely affect its antioxidant property or PARP-inhibitory activity. Results also showed that EPG ($EC_{50} = 87 \mu M$) had some PARP-inhibitory activity, while RSV ($EC_{50} = 172.9 \mu M$) did not show any appreciable PARP-inhibitory activity (protection against PARP-mediated cell

death). EPG and RSV are non-flavonoid polyphenolic compounds; therefore, their activities cannot be compared to FLV directly on the basis of their structural relatedness to FLV. However, studies have shown that epigallocatechin gallate (EPG) may selectively kill *BRCA2*-deficient cells through synthetic lethality induced by PARP inhibition (Alqahtani et al., 2019).

In summary, this chapter demonstrates that, within the context of the customised library of compounds used in the SAR study, the hydroxyflavones are more powerful antioxidants than the methoxyflavones in both cell-free and cell-based systems while, on the contrary, methoxyflavones, namely 4MF and DMF, are better PARP inhibitors in both cell-free and cell-based scenarios. Also, the 3' and 4' positions together exert a crucial effect on the biological activities of flavones.

Chapter 6: Results and Discussion IV

6.0 In silico evaluation of the binding of 4MF and DMF to the PARP enzyme

6.1 Introduction

Computational methods such as molecular docking have emerged as crucial tools in drug discovery because they provide insights about the ligand-receptor binding interactions of small molecules and target proteins (Meng et al., 2011). Molecular docking can predict the experimental binding modes and affinities of small molecules within the binding site of particular receptor targets. Ultimately, the main purpose in molecular docking is to suggest the correct binding poses and accurately predict binding affinity (Fu, Zhao and Chen, 2018). More recently, researchers have incorporated molecular docking and other *in silico* studies (e.g., virtual screening) into high-throughput screening (HTS) of large compound databases. Over the last decade, these techniques have featured in the drug discovery process of novel PARPi as well. *In silico* study has not only helped in the initial screening stages for more PARPi but has also been used in designing new drug leads and in the SAR studies of potential PARPi using existing scaffolds of proven PARPi such as olaparib. For example, in the development of veliparib, pamiparib, simmiparib and fluzoparib, which are currently undergoing clinical studies, several molecular docking studies were used to achieve a better understanding of their binding mechanisms with various PARP enzymes (Wang et al., 2020a; Lu et al., 2022; Yu et al., 2022).

To gain a deeper understanding of the key structural features involved in the binding to the PARP1 enzyme of 4MF, DMF and the other selected compounds in

the customised library used for the SAR study (Chapter 5), molecular docking studies were carried out. AutoDock Vina, an open-source program for molecular docking, was used to predict the protein–ligand binding affinities and best poses (RMSD of less than 2 Å) of each compound in the PARP1 active site. The post-docking analysis was done using PyMoL, LigPlot and Protein-Ligand Interaction Profiler. PARP2 shares about 60% homology of its catalytic domain with PARP1, and previous studies have demonstrated that PARP2 is involved *de novo* in poly ADP-ribosylation in the absence of PARP1, although at a much lower level (Chen et al., 2018a; Demin et al., 2021). Since all the currently approved PARP inhibitors are PARP1/2 inhibitors, the molecular docking studies were extended to the PARP2 enzyme to predict the selectivity of the compounds. In addition, the physicochemical properties of the compounds were assessed for their drug-likeness using Lipinski's rule of five.

Therefore, this chapter reports the binding affinities and the best poses for each compound's interactions with the PARP1 and PARP2 enzymes. The results of the docking were compared with the experimental results (potencies) of the cell-free and cell-based inhibition of PARP1.

6.2 Validation of the docking procedure

There are different ways to validate the docking procedure; however, a standard and commonly used method is known as the pose selection. This is a method in which the docking software is used to re-dock a ligand into the binding site of a protein target with a known conformation and orientation, usually from a co-crystal structure. The docking program is considered reliable if it generates a pose that is very close to the original conformation in the crystal structure. A successful docking should have a low root-mean-square deviation (RMSD) value between the docked

pose and the native conformation, usually less than 1.5 or 2 Å, depending on ligand size (Hevener et al., 2009; Almahmoud and Zhong, 2019).

Two high-resolution X-ray crystal structures of the PARP protein were used in this study, namely PARP1 (PDB ID: 5DS3) and PARP2 (PDB ID: 4TVJ) ([Figure 69](#)). Both enzymes were co-crystallised with olaparib at their catalytic domain (Dawicki-McKenna et al., 2015; Thorsell et al., 2017). Therefore, olaparib was used as the standard throughout the docking process. In order to ensure the accuracy of the AutoDock Vina program and the parameters used in this molecular docking, the co-crystallised ligand was removed from the active site and redocked. The results showed that the superposition of the docked olaparib was very close to that of the native olaparib from the X-ray crystal structure of both PARP1 and PARP2, with RMSD values of 0.469 Å and 0.409 Å, respectively ([Figure 70](#)). The low RMSD value (<2 Å) between the docked pose and the native structure in both models indicated that the docking procedure and AutoDock Vina's algorithms were valid and could therefore be reliably used to perform the binding conformations of the compounds in the customised library.

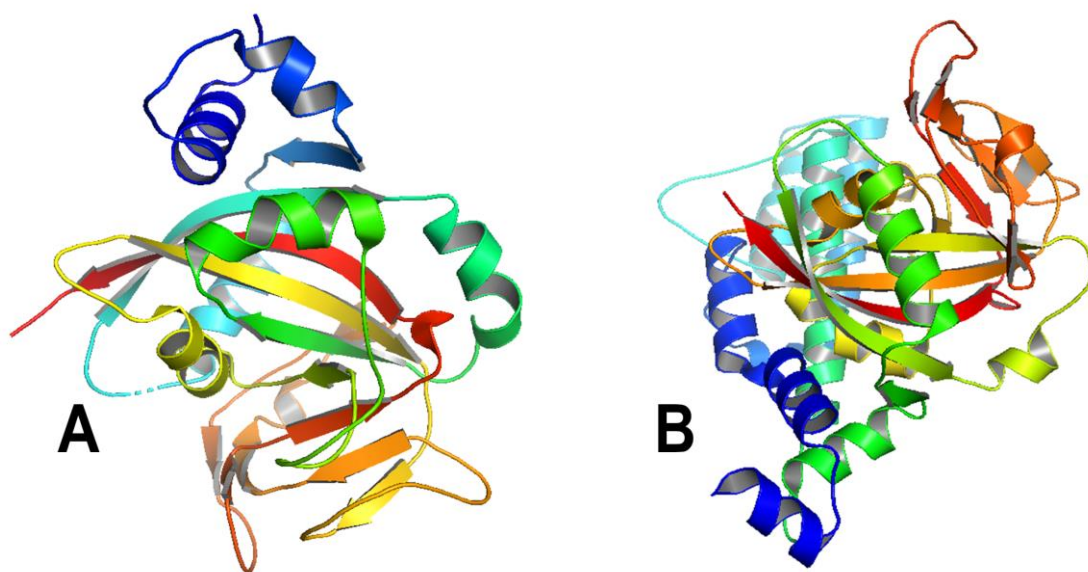


Figure 69: Crystal structures of PARP1 and PARP2. (A) PARP1 (PDB ID: 5DS3) with a resolution of 2.60 Å, obtained through X-ray diffraction; and (B) PARP2 (PDB ID: 4TVJ) with a resolution of 2.10 Å, also crystallised by X-ray diffraction. Images captured using PyMol software.

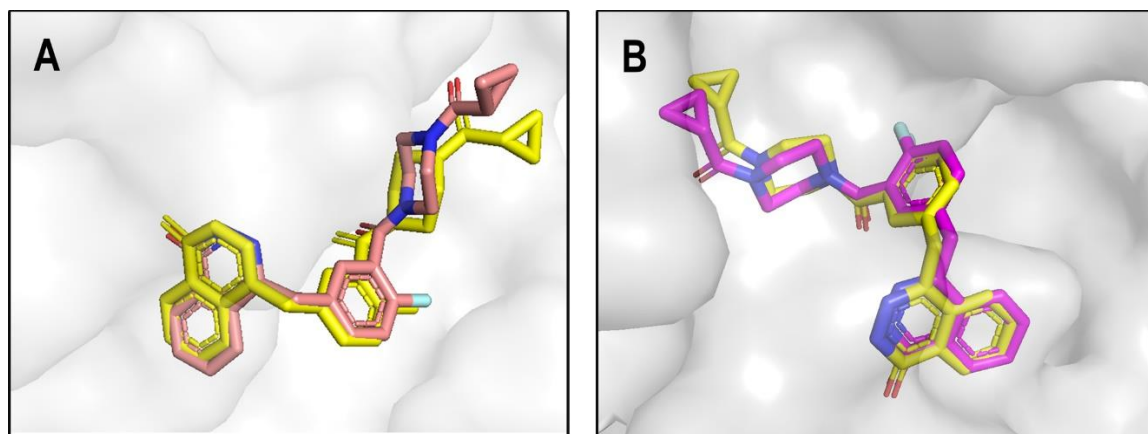


Figure 70: Validation of molecular docking results. Images show the superposition of redocked olaparib (yellow) and the native conformation of the co-crystallised olaparib (magenta or red). (A) In the PARP1 active site, the RMSD value of the two poses is 0.469 Å (B) In PARP2, the RMSD value is 0.409 Å.

6.3 The binding interactions of 4MF and DMF in PARP1 and PARP2

For a spontaneous protein-ligand binding to happen, the free energy change (ΔG) must be negative. Autodock Vina docking scores are expressed as binding affinity (kcal/mol), a surrogate of ΔG that indicates the efficiency of the ligand-protein complex. This implies that the higher the negative binding affinity, the more stable the ligand-protein complex, which can be roughly interpreted as the ligand's efficacy. The binding affinity scores of the best poses for all the docked compounds, including 4MF and DMF, are shown in [Table 12](#). Expectedly, olaparib, the reference structure and the most potent among the test compounds (it is a PARPi), had the lowest binding energy of -11.3 and -12.6 kcal/mol in PARP1 and PARP2, respectively, whereas 4MF and DMF both had the same score of -8.2 kcal/mol in PARP1, and -8.9 and -9 kcal/mol in PARP2, respectively. Olaparib's

binding affinity in PARP1 is consistent with previous studies that showed -11.5 kcal/mol for docked olaparib in the PARP1 crystal structure (PDB ID 5DS3) using Autodock Vina (Go et al., 2020; Guo et al., 2020). This further validates the docking parameters used in this study. The low binding scores of 4MF and DMF suggest that they can bind and interact with the catalytic site of the PARP enzymes.

Table 12: Average binding affinities of the best docking poses as assigned by Autodock Vina

	Binding affinity (kcal/mol)	
	PARP1	PARP2
Olaparib (OPB)	-11.31667	-12.63333
Flavone (FLV)	-8.8	-8.983333
4'-methoxyflavone (4MF)	-8.183333	-8.9
4'-hydroxyflavone (4HF)	-9.1	-8.7
3',4'-dimethoxyflavone (DMF)	-8.2	-9
3',4'-dihydroxyflavone (DHF)	-8.7	-9.6
3',4',7-trimethoxyflavone (TMF)	-7.82	-8.5
3',4',7-trihydroxyflavone (THF)	-8.7	-9.62
7-methoxyflavone (7MF)	-8	-8.683333
7-hydroxyflavone (7HF)	-8.7	-8.95
Resveratrol (RSV)	-7.92	-8.8
Epigallocatechin gallate (EPG)	-9.76	-10.36

Although binding affinity values are informative about the binding potential of the ligand at the target protein active site, the types of molecular interactions with key amino acid residues that generate the binding score are more indicative of the

favourability of the ligand-receptor complex. Also, the docking score function determines the binding affinity energy between the ligand and the protein by counting the number of favourable intermolecular interactions (Tiwari et al., 2022). Molecular interactions, such as hydrophobic π – π interactions, are more crucial to the stability of proteins than hydrogen bonds and electrostatic interactions (Pace et al., 2011). In this study, the best binding pose of olaparib in PARP1 showed five hydrogen bonds (Ser904, Gly863, Gly863, Arg878, Tyr896), two π -stacking (Tyr896, Tyr907), and four hydrophobic interactions (Tyr889, Tyr896, Tyr907, Tyr907) ([Figure 71](#)). These interactions are in agreement with previous studies (Yuan et al., 2017b). The catalytic pocket of PARP1 occupied by the substrate NAD⁺ can be divided mainly into two sub-pockets. The first sub-pocket is the nicotinamide-ribose binding site (NI site) and the second is the adenine-ribose binding site (AD site). It has been demonstrated that most of the potent PARPi, such as olaparib, bind to the NI site through hydrogen-bonding and π - π stacking interactions and further potentiate their inhibitory activity by more molecular interactions with the AD site, which is large enough to accommodate diverse structures (Zhong et al., 2019). Furthermore, several research studies have highlighted the significance of hydrogen bonds with residues Ser904 and Gly863 and π - π stacking interactions with residue Tyr907 in the NI site as mainly responsible for the effective and reversible inhibition of PARP1 (Yuan et al., 2017a; Wang et al., 2020b; Liu et al., 2022).

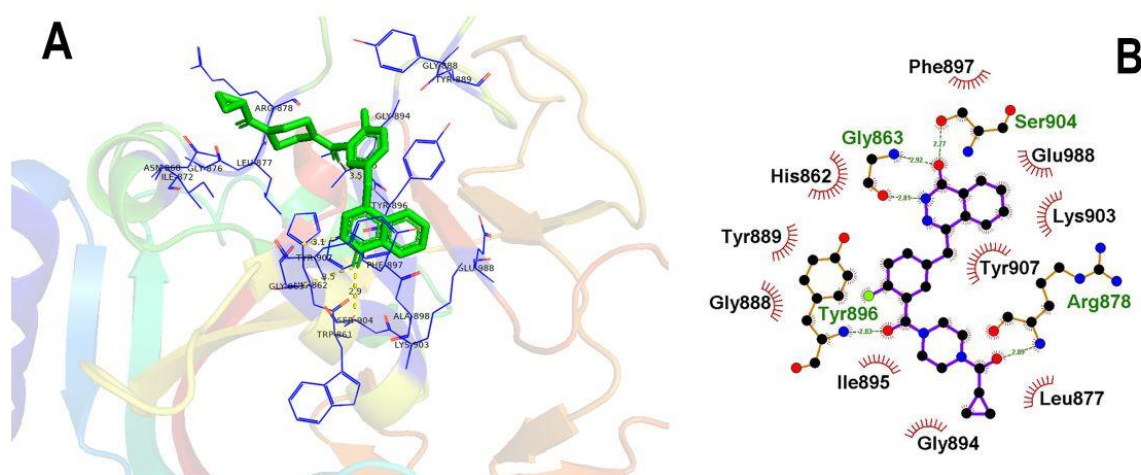


Figure 71: The predicted binding mode of olaparib to PARP1 (PDB ID: 5DS3). (A) The 3D docking model of olaparib (green) shows interactions with amino acid residues (blue) at the catalytic site of PARP1. Hydrogen bonds are shown as yellow dotted lines. The background cartoon structure is the secondary structure of PARP1. (B) shows 2D ligand interactions of olaparib (purple) with amino acid residues of PARP1. Hydrogen bonds are shown by green dashed lines, with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

The result of the molecular interactions of 4MF and DMF in PARP1 showed the presence of one hydrogen bond (Gly863), with a hydrogen bond distance of 3.31 and 3.22 Å, respectively ([Figures 72](#) and [73](#)). The formation of a hydrogen bond with this key amino acid residue (Gly863) in the NI site of PARP1 is a possible explanation for why 4MF and DMF possess some PARP-inhibitory activity. However, compared with olaparib, which also shares the hydrogen bond (H-bond), 4MF and DMF are much less potent. This is not a surprise, as the binding pose already revealed that olaparib had eleven interactions with the amino acid residues, including the key ones at the catalytic site, while 4MF and DMF formed only one. Also, a closer look at olaparib revealed that the two hydrogen bonds formed with Gly863 (2.92 and 2.81 Å) were shorter than those of 4MF (3.31 Å) and DMF (3.22 Å). This could contribute towards the huge discrepancy in their

potencies, as shorter bond length indicates better binding and stability. Perhaps the slight difference in H-bond length could also explain the marginal increase in the PARP-inhibitory potency of DMF over 4MF, both in the cell-free and cell-based experiments.

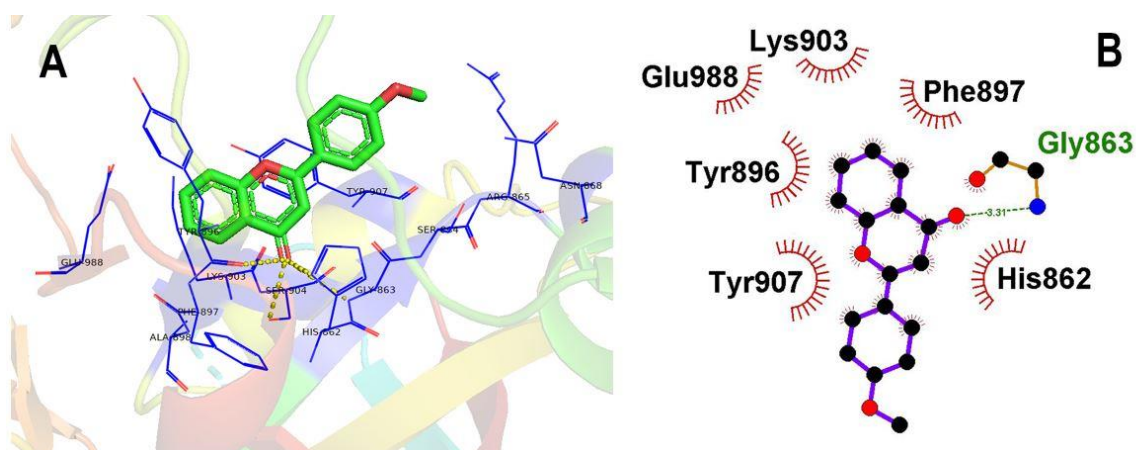


Figure 72: The predicted binding mode of 4MF to PARP1 (PDB ID: 5DS3). (A) The 3D docking model of 4MF (green) shows interactions with amino acid residues (blue) at the catalytic site of PARP1. Hydrogen bonds are shown as yellow dotted lines. The background cartoon structure is the secondary structure of PARP1. (B) 2D ligand interactions of 4MF (purple) with amino acid residues of PARP1. Hydrogen bonds are shown by green dashed lines, with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

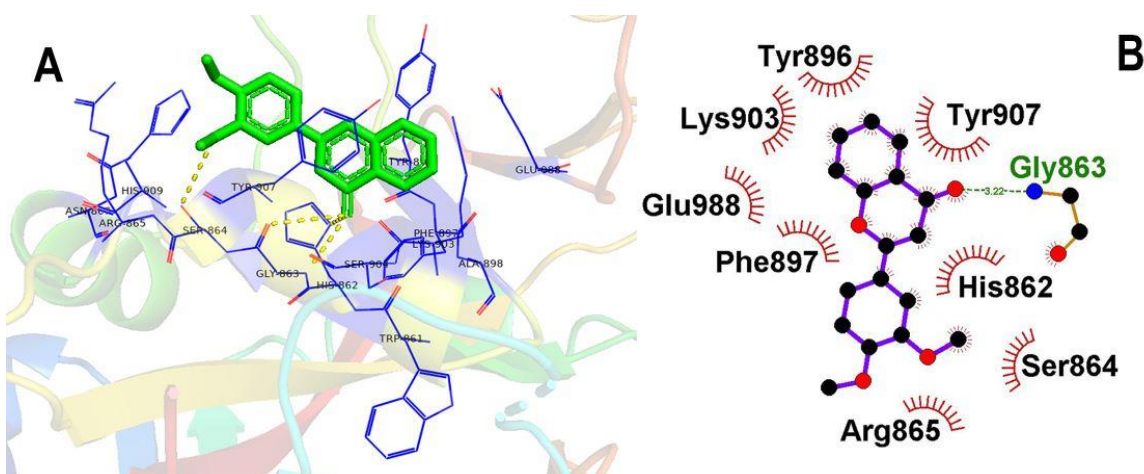


Figure 73: The predicted binding mode of DMF to PARP1 (PDB ID: 5DS3). (A) The 3D docking model of DMF (green) shows interactions with amino acid residues (blue) at the catalytic site of PARP1. Hydrogen bonds are shown as yellow dotted lines. The

background cartoon structure is the secondary structure of PARP1. (B) 2D ligand interactions of DMF (purple) with amino acid residues of PARP1. Hydrogen bonds are shown by green dashed lines, with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

It is interesting to note that the chromone structure (the A- and C-ring fused together) of 4MF and DMF fitted into the NI sub-pocket, mimicking the phthalazinone pharmacophore of olaparib ([Figure 74](#)). Most established PARPi, including olaparib, are designed to mimic the nicotinamide moiety of the natural substrate NAD⁺. The amide of the phthalazinone moiety of olaparib formed three H-bonds with Ser904 and Gly863, while the ketone group on the C-ring of 4MF and DMF was involved in H-bond with Gly863. This implies that even though the flavones do not contain a nicotinamide-like moiety, which is a common feature for most PARPi, 4MF and DMF bind competitively with NAD⁺ at the catalytic site of PARP1. Moreover, the 3D binding mode of 4MF and DMF showed that they were able to form a weak (bond length >5 Å) π - π stacking interaction with residue Tyr907. This weak π - interaction is corroborated with the proximity of residue Tyr907 to the A-ring of 4MF and DMF in the 2D representation ([Figures 72B](#) and [73B](#)). Even with a possible π -interaction and the imitation of olaparib by 4MF and DMF in the NI site, the methoxyflavones could not match the molecular interaction at the AD site which has been known to help potentiate PARPi. The 3D binding mode of 4MF showed that the 4'-methoxy has no interaction but the addition of 3'-methoxy (DMF) generates a H-bond with residue Ser864 ([Figure 73A](#)). This could add to the reason why DMF performed slightly better than 4MF. This also suggests that perhaps one way to improve the PARP-inhibitory activity of 4MF or DMF is to add substitution(s) to the B-ring that can engage the AD site.

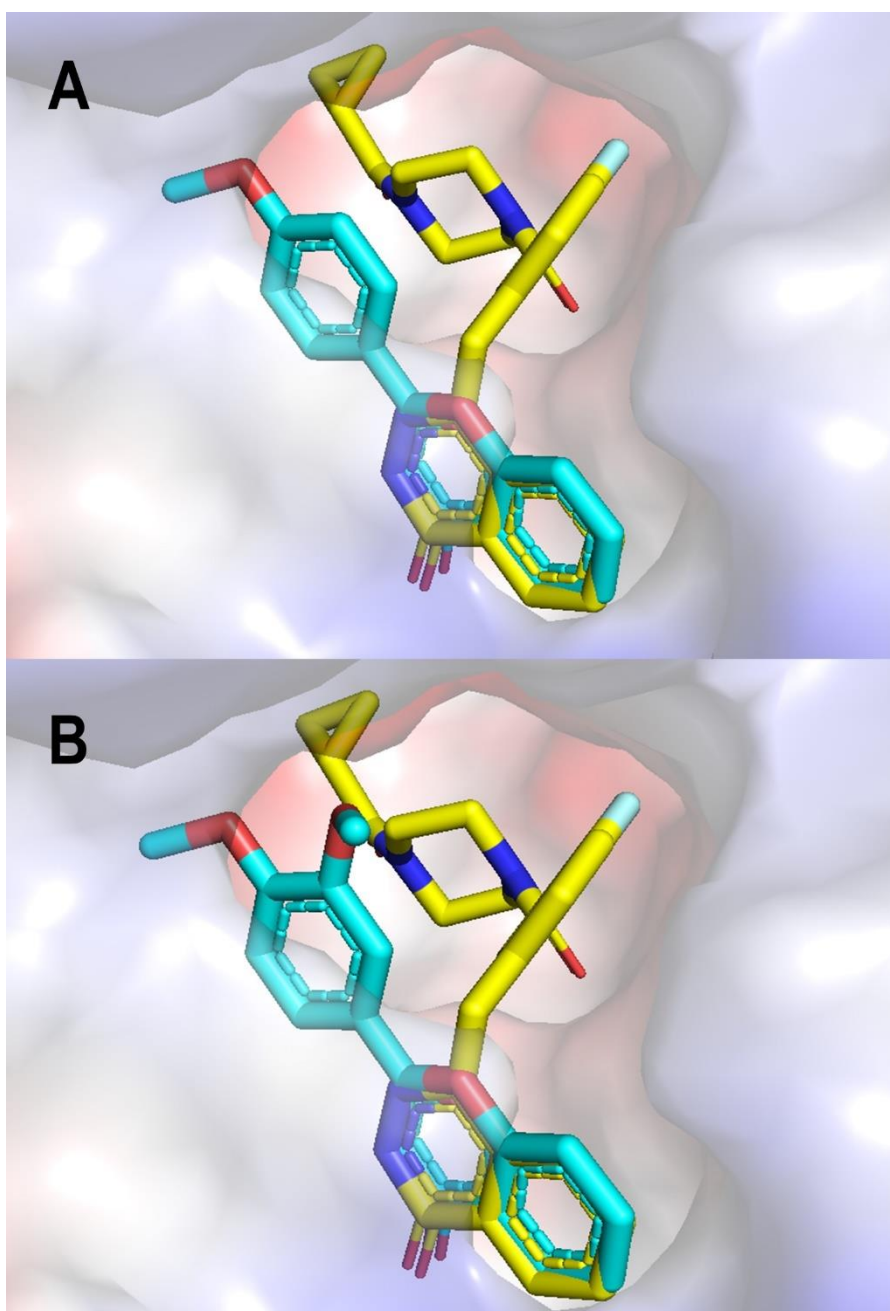


Figure 74: The superposition of 4MF (A) and DMF (B) with olaparib in PARP1. The 3D binding modes show that the chromone structure of 4MF (cyan) and DMF (cyan) fits into the NI sub-pocket, mimicking the phthalazinone pharmacophore of olaparib (yellow). Images generated by PyMol.

In PARP2, for olaparib five hydrogen bonds (Arg44, Tyr462, Gly429, Gly429, Ser470), five hydrophobic interactions (Tyr455, Tyr462, Tyr462, Tyr473, Tyr473), and two π - π stacking interactions (Tyr462, Tyr473) were formed ([Figure 75](#)). The

binding affinity and number of hydrophobic interactions of olaparib indicate that it could be more potent towards PARP2 than PARP1. This is supported by previous reports that olaparib is a more potent PARP2 inhibitor ($IC_{50} = 1$ nM) than a PARP1 inhibitor ($IC_{50} = 5$ nM). This proves that the Autodock Vina binding affinity in this study was reliable and could be applied to other ligands in predicting their PARP2 selectivity. The binding mode of 4MF and DMF showed that they formed one H-bond with Gly429 (Figures 76 and 77). Since Gly429 residue was also found in the olaparib binding mode, this suggests that it might be essential for PARP2 inhibition. Although there is no experimental data to prove the PARP2 activity of 4MF and DMF, the binding score and mode suggest that they could be more potent towards PARP2 than PARP1.

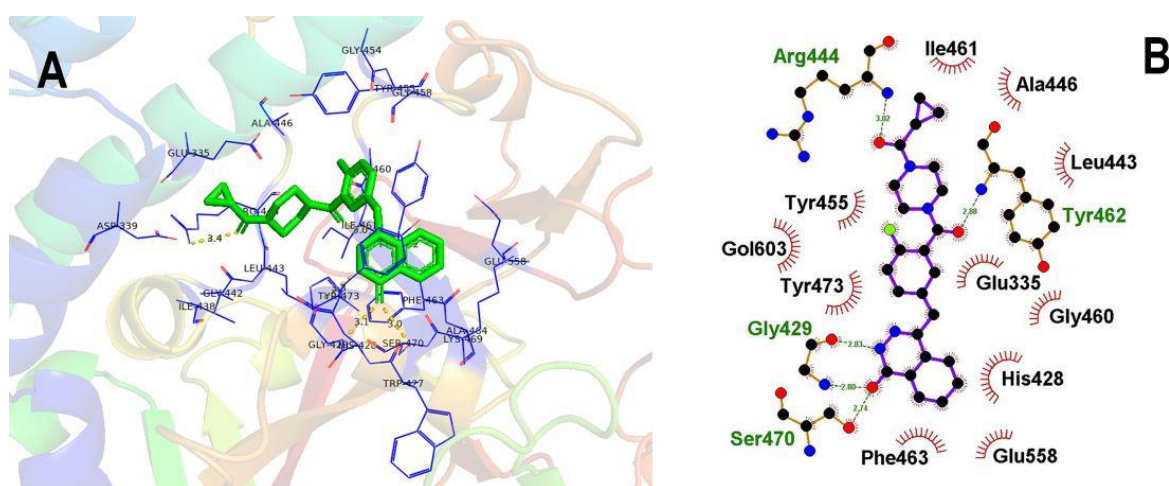


Figure 75: The predicted binding mode of olaparib to PARP2 (PDB ID: 4TVJ). (A) The 3D docking model of olaparib (green) shows interactions with amino acid residues (blue) at the catalytic site of PARP2. Hydrogen bonds are shown as yellow dotted lines. The background cartoon structure is the secondary structure of PARP2. (B) 2D ligand interactions of olaparib (purple) with the amino acid residues of PARP2. Hydrogen bonds are shown by green dashed lines, with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

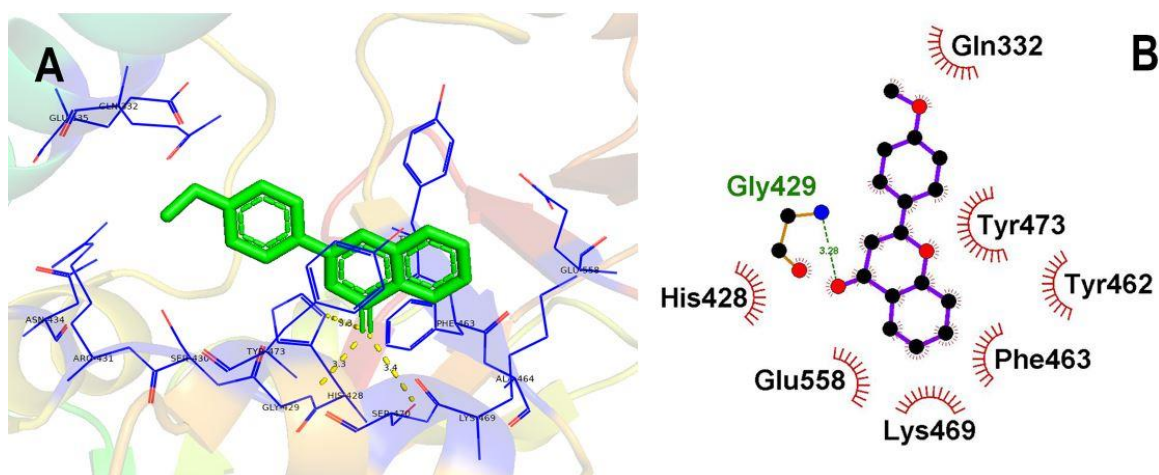


Figure 76: The predicted binding mode of 4MF to PARP2 (PDB ID: 4TVJ). (A) The 3D docking model of 4MF (green) shows interactions with amino acid residues (blue) at the catalytic site of PARP2. Hydrogen bonds are shown as yellow dotted lines. The background cartoon structure is the secondary structure of PARP2. (B) 2D ligand interactions of 4MF (purple) with the amino acid residues of PARP2. Hydrogen bonds are shown by green dashed lines, with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

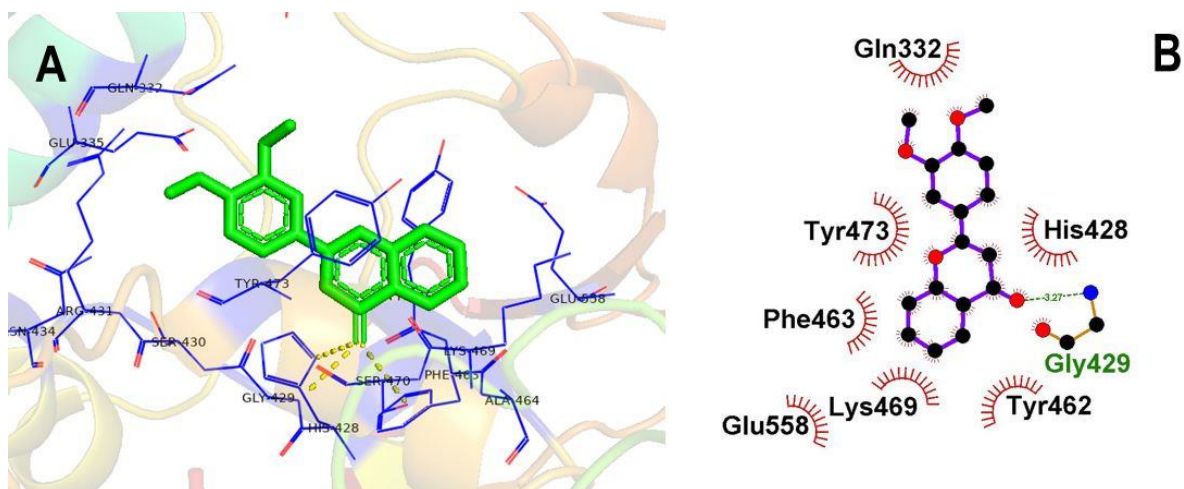


Figure 77: The predicted binding mode of DMF to PARP2 (PDB ID: 4TVJ). (A) The 3D docking model of DMF (green) shows interactions with amino acid residues (blue) at the catalytic site of PARP2. Hydrogen bonds are shown as yellow dotted lines. The background cartoon structure is the secondary structure of PARP2. (B) 2D ligand interactions of DMF (purple) with the amino acid residues of PARP2. Hydrogen bonds are shown by green dashed lines, with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

6.4 The binding interactions of other methoxylated flavones in PARP1 and PARP2

TMF had the highest binding affinity (-7.8 kcal/mol) among all the docked compounds ([Table 12](#)). The binding mode showed one possible hydrogen bond (3.41 Å) with residue Gly888 in the NI site ([Figure 78](#)). The interaction did not involve the key amino acid residues that are essential for PARP inhibition. The 3D binding mode also revealed that TMF did not properly fit into the NI sub-pocket the way olaparib, 4MF and DMF were able to tuck into the cavity. It seems the addition of the methoxy group at C7 on the A-ring caused some molecular hindrance that prevented TMF from effectively occupying the catalytic domain of PARP1. This could explain its lack of PARP-inhibitory activity in the earlier results of the PARP1 cell-free and cell-based assays. Similarly, 7MF had a binding affinity of -8.0 kcal/mol and the binding mode showed no H-bond interaction at all ([Figure 79](#)). Interestingly, the B-ring was fitted in the NI site while the chromone structure with the 7-methoxy attachment interacted with the AD site. The 2D binding mode revealed that there could be some weak hydrophobic interactions with three residues (Gly894, Gly888, Tyr889).

Compared with FLV (flavone itself), binding score -8.8 kcal/mol, all the methoxyflavones, including 4MF and DMF, had a higher binding affinity, suggesting that flavone could have better PARP-inhibitory activity than the methoxyflavones. The binding mode of FLV indicated that it occupied the NI site effectively and formed a H-bond with a key amino acid (Ser904) and two weak aromatic π - π stacking interactions (Tyr896, Tyr907), imitating olaparib more effectively than the methoxyflavones ([Figure 80](#)). Although experimental data from the cell-free and cell-based assays showed that 4MF and DMF were more potent

than FLV, the ability of FLV to form more hydrophobic interactions with important amino acid residues in the NI site explains its lower docking binding energy.

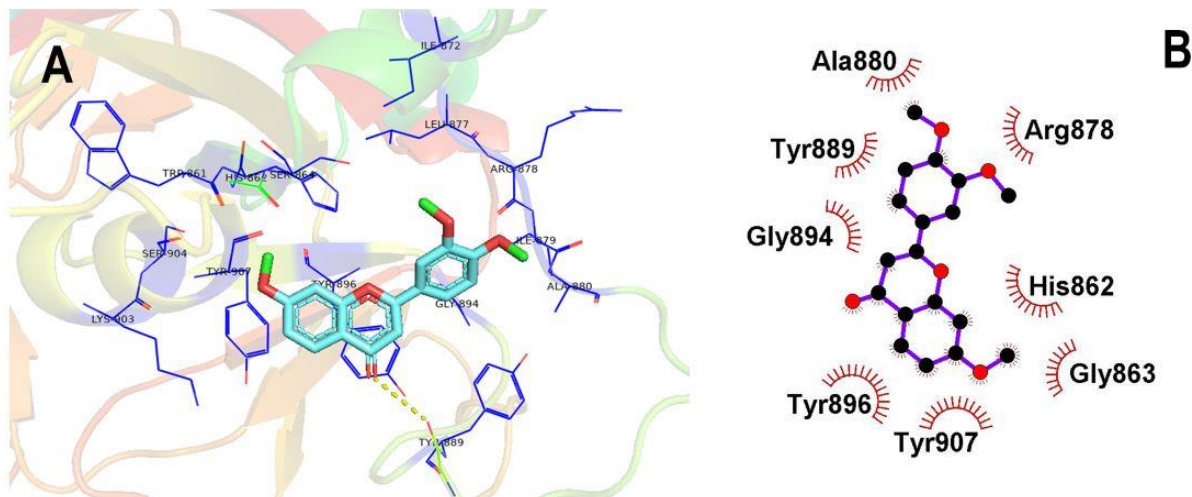


Figure 78: The predicted binding mode of TMF to PARP1 (PDB ID: 5DS3). (A) The 3D docking model of TMF (green) shows interactions with amino acid residues (blue) at the catalytic site of PARP1. Hydrogen bonds are shown as yellow dotted lines. The background cartoon structure is the secondary structure of PARP1. (B) 2D ligand interactions of TMF (purple) with amino acid residues of PARP1. Hydrogen bonds are shown by green dashed lines, with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

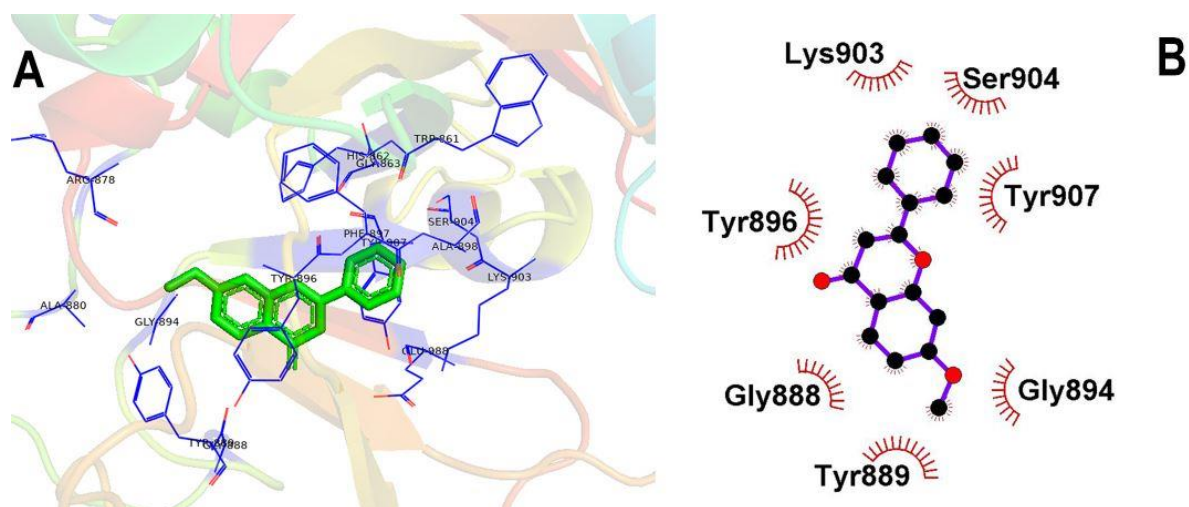


Figure 79: The predicted binding mode of 7MF to PARP1 (PDB ID: 5DS3). (A) The 3D docking model of 7MF (green) shows interactions with amino acid residues (blue) at the catalytic site of PARP1. Hydrogen bonds are shown as yellow dotted lines. The

background cartoon structure is the secondary structure of PARP1. (B) 2D ligand interactions of 7MF (purple) with amino acid residues of PARP1. Hydrogen bonds are shown by green dashed lines, with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

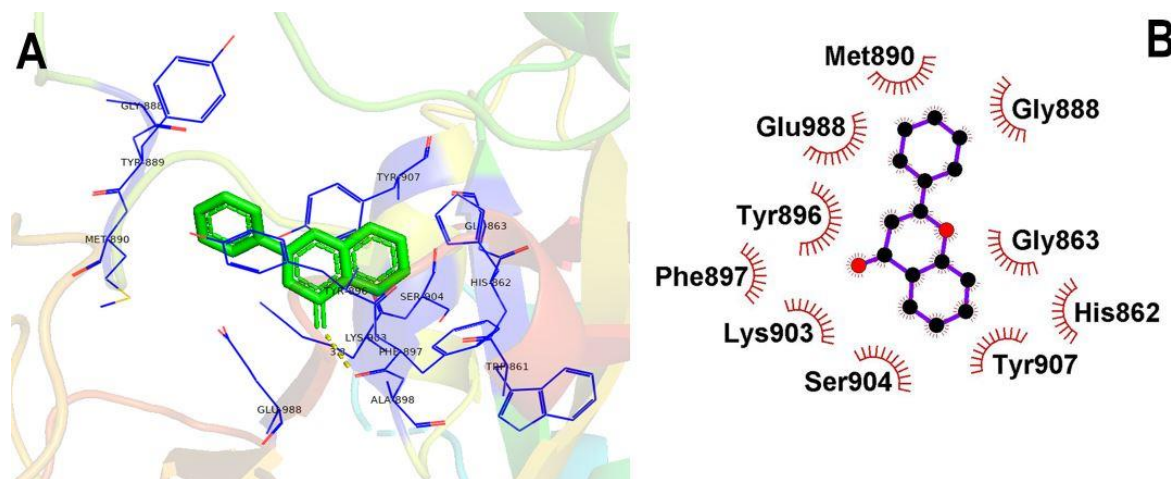


Figure 80: The predicted binding mode of FLV to PARP1 (PDB ID: 5DS3). (A) The 3D docking model of FLV (green) shows interactions with amino acid residues (blue) at the catalytic site of PARP1. Hydrogen bonds are shown as yellow dotted lines. The background cartoon structure is the secondary structure of PARP1. (B) 2D ligand interactions of FLV (purple) with amino acid residues of PARP1. Hydrogen bonds are shown by green dashed lines, with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

Comparing the Autodock Vina docking scores of the methoxyflavones alone, it is worth noting that the order of experimental PARP-inhibitory potency or activity was rightly predicted. 4MF and DMF were almost equipotent, while 7MF and TMF were lacking any significant activity. Therefore, the results of the molecular docking for the methoxyflavones can be relied on, although the binding affinity values did not necessarily reflect the magnitude of PARP-inhibitory activity or the lack thereof. Furthermore, the docking scores and interactions of 7MF (-8.7 kcal/mol) and TMF

(-8.5 kcal/mol) in PARP2 are shown in [Figures 81, 82](#) and [Table 12](#). Similarly, the score ranking for PARP1 showed that 7MF and TMF had the highest binding affinity among the methoxyflavones, suggesting that the presence of the C7 methoxy moiety might be hindering them from fitting well into the catalytic site of PARP2.

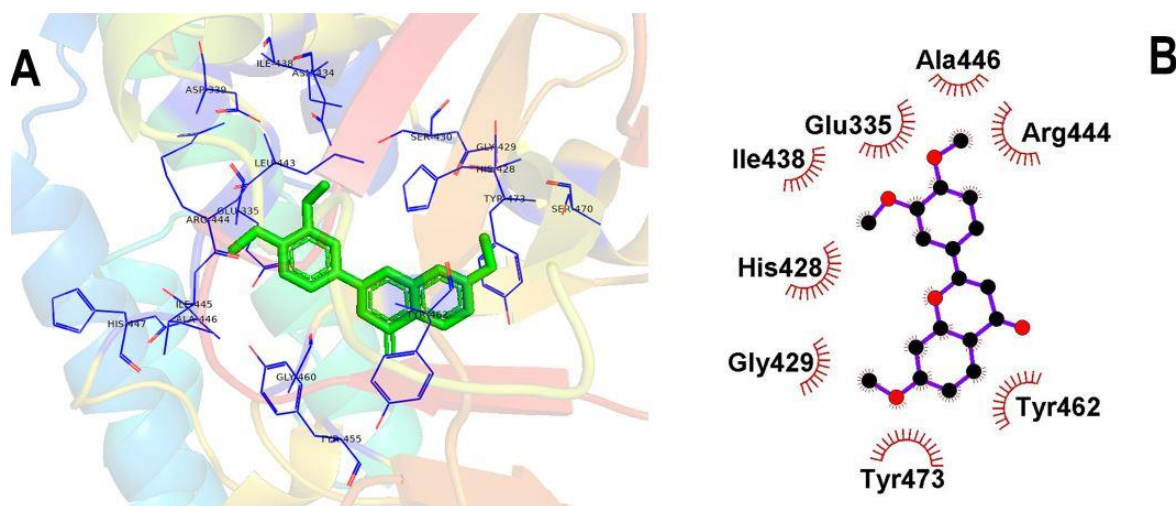


Figure 81: The predicted binding mode of TMF to PARP2 (PDB ID: 4TVJ). (A) The 3D docking model of TMF (green) shows interactions with amino acid residues (blue) at the catalytic site of PARP2. Hydrogen bonds are shown as yellow dotted lines. The background cartoon structure is the secondary structure of PARP2. (B) 2D ligand interactions of TMF (purple) with the amino acid residues of PARP2. Hydrogen bonds are shown by green dashed lines, with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

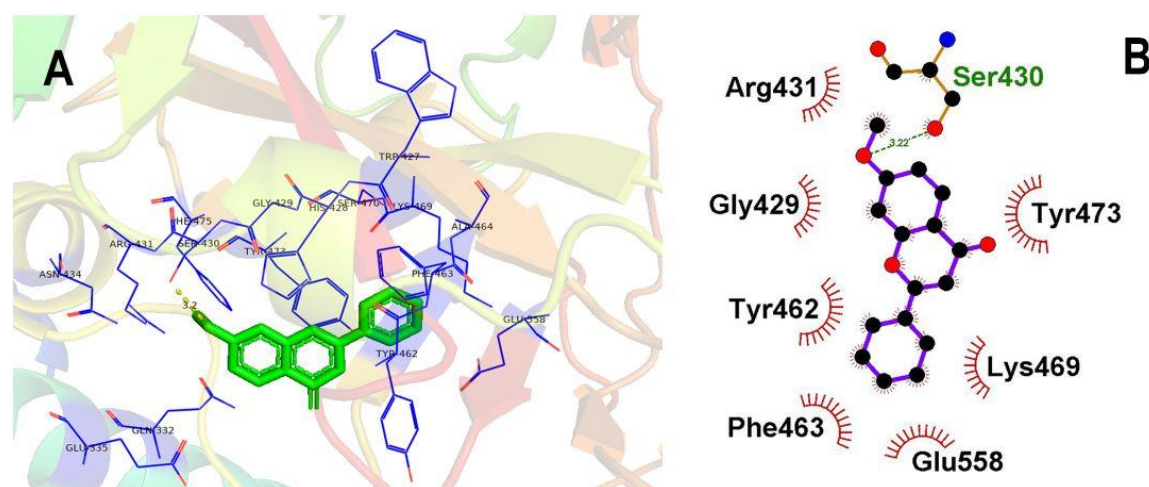


Figure 82: The predicted binding mode of 7MF to PARP2 (PDB ID: 4TVJ). (A) The 3D docking model of 7MF (green) shows interactions with amino acid residues (blue) at the catalytic site of PARP2. Hydrogen bonds are shown as yellow dotted lines. The background cartoon structure is the secondary structure of PARP2. (B) 2D ligand interactions of 7MF (purple) with the amino acid residues of PARP2. Hydrogen bonds are shown by green dashed lines, with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

6.5 The binding interactions of 4HF and DHF in PARP1 and PARP2

4HF and DHF have consistently shown PARP-inhibitory activity in both the enzymatic and cellular assays. In PARP1, the docking model with the lowest binding energy showed -9.1 and -8.7 kcal/mol for 4HF and DHF, respectively. The 3D binding pose for 4HF showed that the protein–ligand complexes formed two strong hydrogen bonds (Gly888, Met890) and π - π stacking interaction with residue Tyr907 ([Figure 83](#)). On the other hand, DHF had 2 H-bonds to residue Gly888 and a weak π - π interaction with Tyr907 ([Figure 84](#)). Interestingly, the hydroxyflavones had lower binding affinities than 4MF and DMF, suggesting that they may be better PARP1 inhibitors, even though this study's experimental data showed the reverse. Nonetheless, 4HF and DHF also showed *in vitro* PARP-inhibitory activity and these molecular docking results support the experimental observation.

A closer look at the binding modes of 4HF and DHF showed that their chromone structure fitted into the NI site of PARP1 very well, just like olaparib. However, their hydrogen bond interactions were not with either of the two key amino acid residues (Ser904, Gly863) that have been reported to be important for PARP1 inhibition among potent PARPi such as olaparib and talazoparib (Al-Sanea et al., 2022). This could be a possible reason why 4HF and DHF were less potent *in vitro* than 4MF

and DMF, even though 4MF and DMF had less interaction but with the critical amino acid residues. This also suggests that the number of molecular interactions influenced the docking scores, as a higher number of interactions gave lower binding affinity scores. Whilst the number of interactions is important in protein–ligand binding, equally important-if not more important in some cases-are the quality and type of interactions. The docking program is not aware of the peculiarities of the protein receptors and ligands; therefore, these are not factored into the scoring. For example, Autodock Vina did not give higher scores for key interactions with the NI site amino acid residues of PARP1, as opposed to other residues at the catalytic site. But, in reality, a strong H-bond with Ser904 or Gly863 and a strong π - π stacking interaction with residue Tyr907 translate to better PARP inhibition activity than ten H-bonds with other residues. This underscores the need to cautiously interpret the docking score in isolation. It is recommended to always complement with visualisation and detailed study of the binding modes. Besides, this approach could help reduce false positives in molecular docking studies.

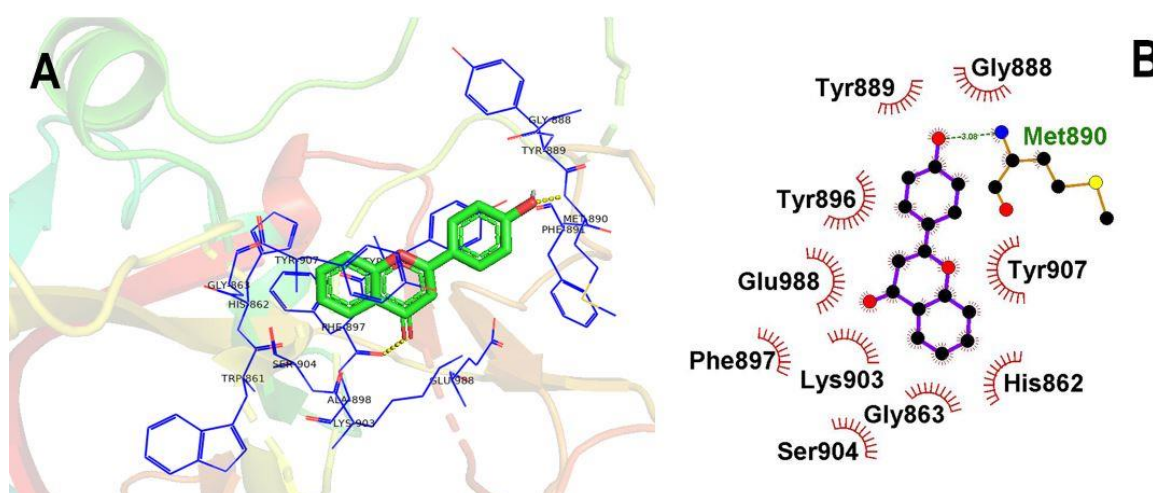


Figure 83: The predicted binding mode of 4HF to PARP1 (PDB ID: 5DS3). (A) The 3D docking model of 4HF (green) shows interactions with amino acid residues (blue) at the catalytic site of PARP1. Hydrogen bonds are shown as yellow dotted lines. The

background cartoon structure is the secondary structure of PARP1. (B) 2D ligand interactions of 4HF (purple) with amino acid residues of PARP1. Hydrogen bonds are shown by green dashed lines, with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

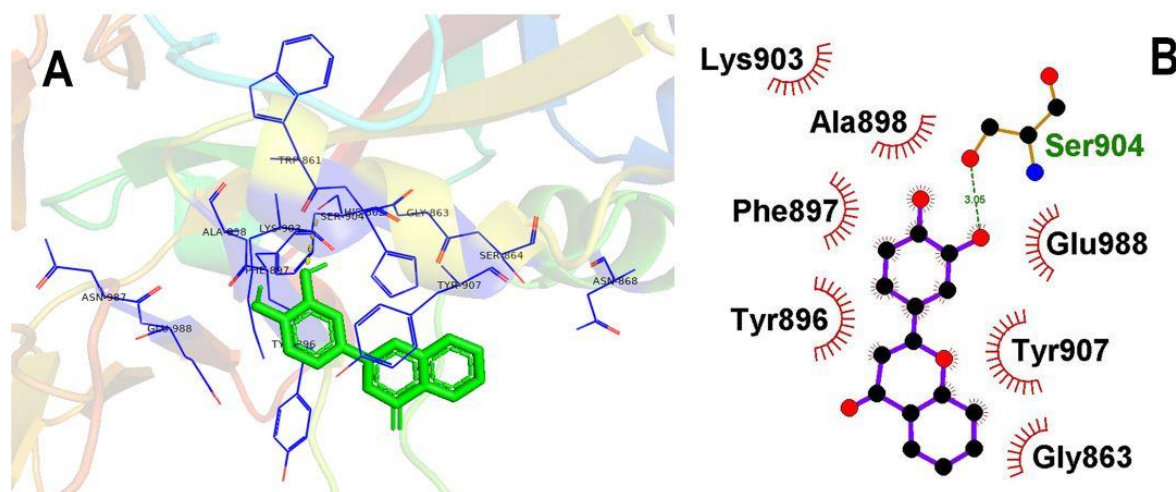


Figure 84: The predicted binding mode of DHF to PARP1 (PDB ID: 5DS3). (A) The 3D docking model of DHF (green) shows interactions with amino acid residues (blue) at the catalytic site of PARP1. Hydrogen bonds are shown as yellow dotted lines. The background cartoon structure is the secondary structure of PARP1. (B) 2D ligand interactions of DHF (purple) with amino acid residues of PARP1. Hydrogen bonds are shown by green dashed lines, with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

[Figures 85](#) and [86](#) show the molecular interactions of 4HF (-8.7 kcal/mol) and DHF (-9.6 kcal/mol) in PARP2. Though the general pattern observed in the docking scores was that the ligands had lower binding affinities for PARP2, it is interesting to note that only 4HF did not comply with that trend. In fact, the binding energy of 4MF was lower than that of 4HF in PARP2, despite sharing a similar binding mode. Surprisingly, DHF had a much lower binding affinity than FLV (-9.0 kcal/mol), in contrast with PARP1 docking. The binding mode of DHF revealed the presence of three H-bonds (Tyr473, Ser430, Arg431) and a weak π - π stacking interaction

(Tyr462). Overall, these results imply that 4HF and DHF could potentially be PARP2 inhibitors, just as with PARP1.

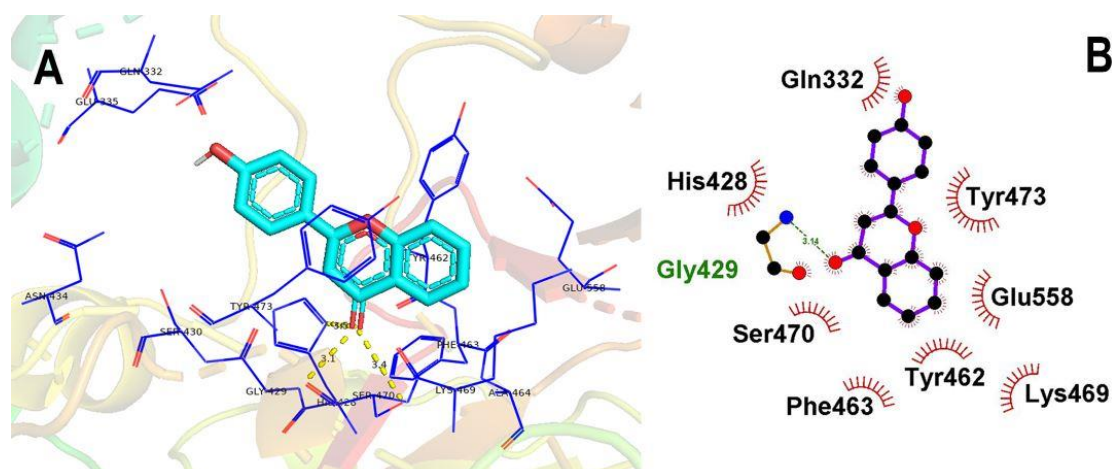


Figure 85: The predicted binding mode of 4HF to PARP2 (PDB ID: 4TVJ). (A) The 3D docking model of 4HF (green) shows interactions with amino acid residues (blue) at the catalytic site of PARP2. Hydrogen bonds are shown as yellow dotted lines. The background cartoon structure is the secondary structure of PARP2. (B) 2D ligand interactions of 4HF (purple) with the amino acid residues of PARP2. Hydrogen bonds are shown by green dashed lines, with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

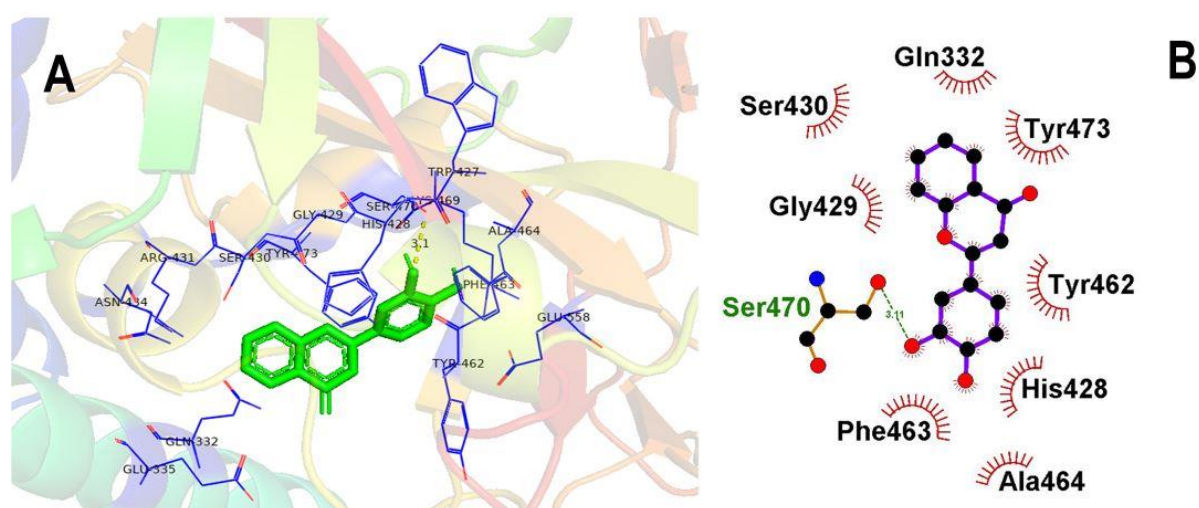


Figure 86: The predicted binding mode of DHF to PARP2 (PDB ID: 4TVJ). (A) The 3D docking model of DHF (green) shows interactions with amino acid residues (blue) at the catalytic site of PARP2. Hydrogen bonds are shown as yellow dotted lines. The background cartoon structure is the secondary structure of PARP2. (B) 2D ligand interactions of DHF (purple) with the amino acid residues of PARP2. Hydrogen bonds are

shown by green dashed lines, with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

6.6 The binding interactions of 7HF and THF in PARP1 and PARP2

In PARP1 docking studies, 7HF (-8.7 kcal/mol) and THF (-8.7 kcal/mol) surprisingly had lower binding affinities than 4MF and DMF, despite lacking *in vitro* PARP1 activity, especially 7HF. However, the binding mode of 7HF showed that the ligand did not properly fit into the NI sub-pocket with the A-ring, while the C7 hydroxy moiety formed two H-bonds with Trp861 and Ser904 (a key residue for inhibition) ([Figure 87](#)). Similarly, in PARP2, 7HF (-9.0 kcal/mol) and THF (-9.6 kcal/mol) had lower binding energies than FLV, 4MF and DMF. These results suggest that the docking scores cannot accurately predict the *in vitro* PARP1-inhibitory activity for the tested hydroxyflavones. Nevertheless, the binding modes gave insight into the mechanisms of the interactions of these compounds with the enzymes. As a result, this study could not draw a conclusion as to whether 4HF and DHF, or other hydroxyflavones, could be PARP2 inhibitors.

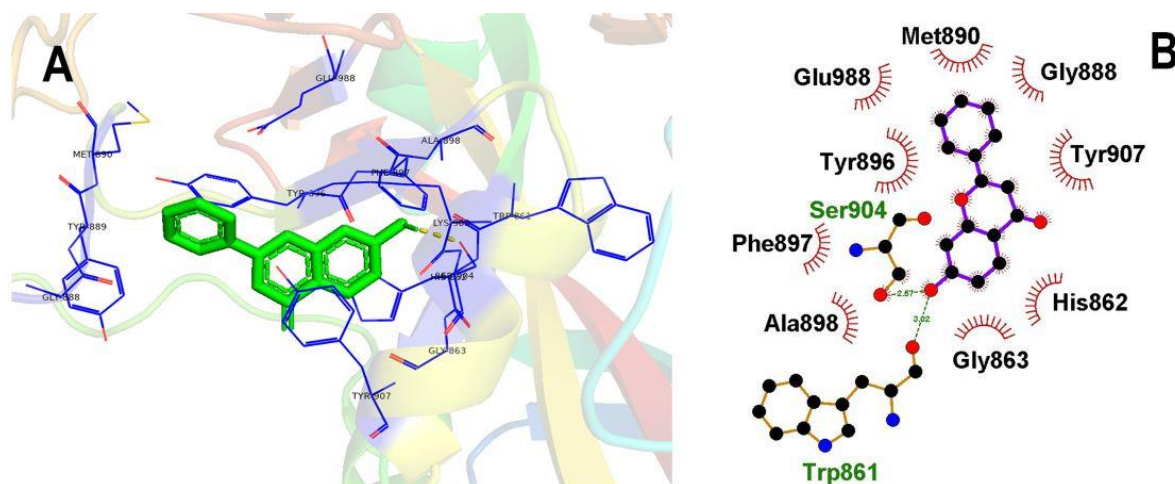


Figure 87: The predicted binding mode of 7HF to PARP1 (PDB ID: 5DS3). (A) The 3D docking model of 7HF (green) shows interactions with amino acid residues (blue) at the catalytic site of PARP1. Hydrogen bonds are shown as yellow dotted lines. The background cartoon structure is the secondary structure of PARP1. (B) 2D ligand interactions of 7HF (purple) with amino acid residues of PARP1. Hydrogen bonds are shown by green dashed lines, with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

6.7 The binding interactions of RSV and EPG in PARP1 and PARP2

Lastly in the molecular docking study, the results for the non-flavonoid polyphenols showed that RSV almost had the least favourable binding affinity: -7.9 and -8.8 kcal/mol in PARP1 and PARP2, respectively. Despite RSV forming key H-bonds with Ser904 and Gly863, the 3D binding mode showed the ligand fully inserted into the NI site cavity of PARP1 ([Figure 88](#)). There seemed to be no interaction, not even weak electrostatic ones, with the AD site. This might explain its failure to inhibit PARP1 *in vitro*. A similar observation was made in PARP2, although three H-bonds (Arg431, Trp427, Ser470) were found and the compound was completely swallowed by the catalytic domain sub-pocket, which prevented any intermolecular interaction with the AD site.

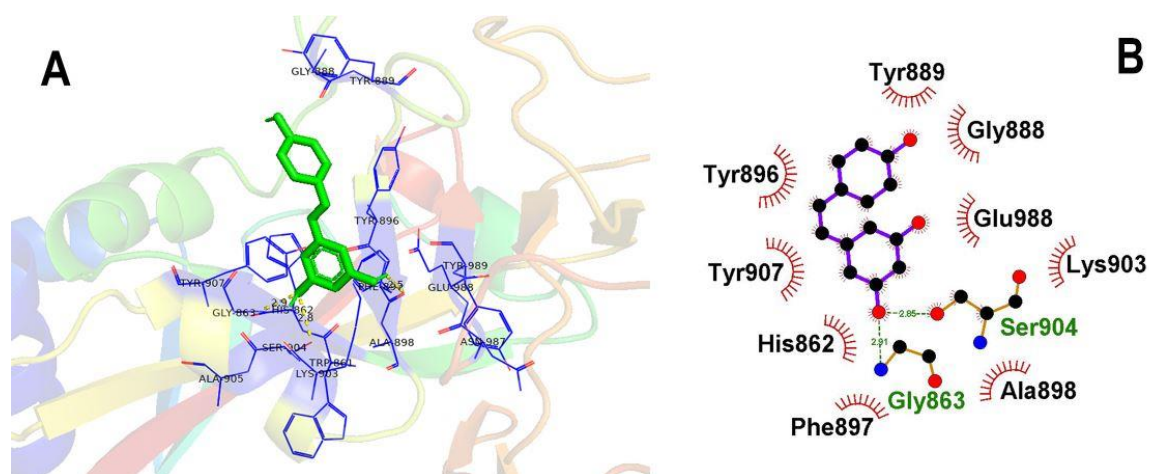


Figure 88: The predicted binding mode of RSV to PARP1 (PDB ID: 5DS3). (A) The 3D docking model of RSV (green) shows interactions with amino acid residues (blue) at the

catalytic site of PARP1. Hydrogen bonds are shown as yellow dotted lines. The background cartoon structure is the secondary structure of PARP1. (B) 2D ligand interactions of RSV (purple) with amino acid residues of PARP1. Hydrogen bonds are shown by green dashed lines, with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

It is worth noting that EPG had the lowest binding affinity after olaparib in both PARP models. Recalling the structure of EPG from the SAR study conducted earlier, EPG is relatively larger than all the other compounds in the customised library, possessing eight hydroxyl groups. Bearing this in mind, the high negative binding energy was not surprising, -9.7 and -10.4 kcal/mol in PARP1 and PARP2, respectively. Indeed, these docking scores translated to some PARP-inhibitory activity, as demonstrated in this study and previous studies (Alqahtani et al., 2019). Six H-bonds (Gly863, Gly863, Ser904, Gly894, Gly894, Tyr896) were found in PARP1, while PARP2 had five H-bonds (Glu335, Gly429, Ser470, Tyr462, Arg444) ([Figures 89](#) and [90](#)). In addition, EPG had more interactions with the NI and AD sub-pockets of PARP1 than other compounds in the SAR study. However, it had less potency compared to 4MF and DMF, which both had less interactions. As explained earlier, the nature and quality of these interactions also play a very significant role when it comes to the experimental translation of these bindings. Generally, hydrogen bonds can support protein stability, but hydrophobic interactions and π - π stacking are relatively stronger. Therefore, the absence of π - π stacking and favourable hydrophobic interactions in the PARP1-EPG complex may be responsible for its poor PARP-inhibitory activity.

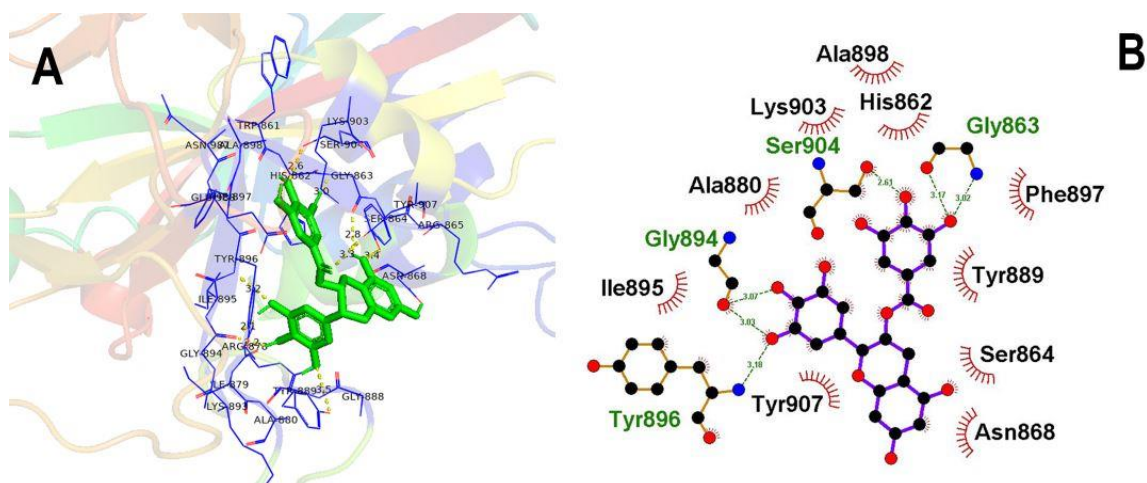


Figure 89: The predicted binding mode of EPG to PARP1 (PDB ID: 5DS3). (A) The 3D docking model of EPG (green) shows interactions with amino acid residues (blue) at the catalytic site of PARP1. Hydrogen bonds are shown as yellow dotted lines. The background cartoon structure is the secondary structure of PARP1. (B) 2D ligand interactions of EPG (purple) with amino acid residues of PARP1. Hydrogen bonds are shown by green dashed lines, with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

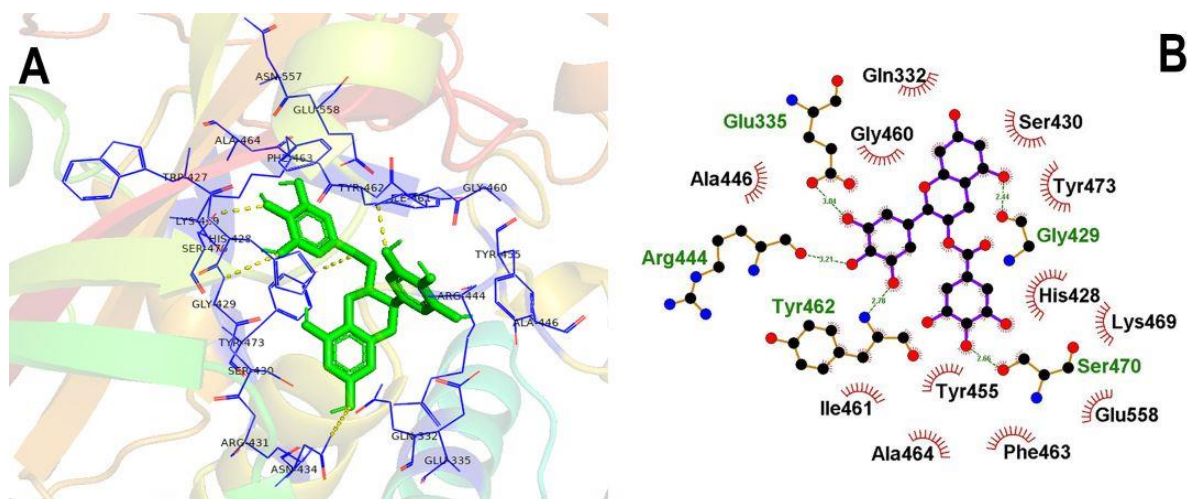


Figure 90: The predicted binding mode of EPG to PARP2 (PDB ID: 4TVJ). (A) The 3D docking model of EPG (green) shows interactions with amino acid residues (blue) at the catalytic site of PARP2. Hydrogen bonds are shown as yellow dotted lines. The background cartoon structure is the secondary structure of PARP2. (B) 2D ligand interactions of EPG (purple) with the amino acid residues of PARP2. Hydrogen bonds are shown by green dashed lines with the length of the bond printed in the middle. Red spoked arcs represent amino acids with possible hydrophobic contacts. Amino acids are identified with three letter codes. The 3D and 2D images were generated by PyMol and LigPlot software, respectively.

6.8 The physicochemical properties of the customised library

Lipinski's rule of five was used to determine the drug-likeness of 4MF, DMF and the other compounds. The physicochemical properties of the compounds are presented in [Table 13](#). Based upon Lipinski's rule of five, for a compound to be said to be drug-like, it must meet the following criteria: molecular weight ≤ 500 , number of hydrogen bond acceptors ≤ 10 , number of hydrogen bond donors ≤ 5 , and calculated logP ≤ 5 . logP is an octanol-water partition coefficient and can be defined as the relative concentration of the solute in octanol and water. logP is a measure of the degree of hydrophobicity and is well known to play an important role in drug absorption via the phospholipid bilayer (Lipinski, 2000). The violation of more than one of these rules implies that the molecule may have a problem with bioavailability. The logP scores of 4MF and DMF and their hydroxyl derivatives were well below 5, which suggests that they have good permeability across the cell membrane. Unsurprisingly, EPG, unlike the other compounds, did not comply with some of these rules. Due to the high number of hydroxyl groups on EPG molecules, its number of hydrogen bond acceptors exceeded 10 and the number of hydrogen bond donors was 8. These two violations strongly suggest that EPG could have poor bioavailability. In fact, numerous studies have reported the low bioavailability of EPG, despite its beneficial health effects (Alqahtani et al., 2019). In addition, the TPSA values (topological polar surface area) for all the compounds, except EPG, were within the acceptable range (0-140). TPSA indicates the surface area required to bind with the majority of the target protein and has been demonstrated to be a very good descriptor for characterising drug absorption, including permeability, intestinal absorption, bioavailability, and blood-brain barrier penetration (Jagannathan, 2019). Overall, the results indicate that 4MF, DMF, 4HF

and DHF, which have all shown some PARP-inhibitory activity, are drug-like and could be considered for further *in vivo* evaluations.

From this chapter, it can be concluded that molecular docking showed that 4MF and DMF can bind competitively to and interact with key amino acid residues in the PARP1 enzyme. Similarly, 4HF and DHF can bind with and form hydrogen bonds with PARP1, which could explain their *in vitro* activity. All the compounds, apart from 4HF, were more selective towards PARP2 in the docking model. Finally, 4MF, DMF, 4HF and DHF did not violate Lipinski's rule of five and could therefore be considered as drug candidates.

Table 13: Physicochemical properties of the SAR compounds. The violations are indicated in red. Red colour depicts a violation of the indicated rule. The properties of these structures were calculated using Molinspiration Chemoinformatics Software.

Lipinski's Rule of Five							
Compound	Molecular Weight (g/mol)	(cLogP)	H-bond Donors	H-bond Acceptors	Rule Violations	Drug-Likeness	TPSA
	≤500	≤5	≤5	≤10	≤2		
FLV	222.24	3.74	0	2	0	yes	30.21
4MF	252.27	3.79	0	3	0	yes	39.45
4HF	238.24	3.26	1	3	0	yes	50.44
DMF	282.3	3.38	0	4	0	yes	48.68
DHF	254.24	2.77	2	4	0	yes	70.67
TMF	312.32	3.42	0	5	0	yes	57.91
THF	270.24	2.27	3	5	0	yes	90.89
7MF	252.27	3.77	0	3	0	yes	39.45
7HF	238.24	3.23	1	3	0	yes	50.44
RSV	228.25	2.99	3	3	0	yes	60.68
EPG	458.38	2.25	8	11	2	no	197.36

Chapter 7: Conclusions and Future work

7.0 Conclusions

This project set out to identify and characterise, from natural products, novel inhibitors of the poly (ADP-ribose) polymerase (PARP) enzyme, which can then be deployed in the development of chemical tools for interrogation of the associated molecular pathways or chemical leads for therapeutics development for the treatment of cancer and other pathological contexts in which PARP is known to play a role, such as neurodegeneration. Only four PARP inhibitors (PARPi) have been approved to date, used orally for the monotherapy treatment of *BRCA*-mutated cancers, and they are susceptible to the growing challenge of chemoresistance (cancer cells becoming less sensitive (more resistant) to an anti-cancer drug to which they were once more sensitive). There is, therefore, a need to develop more PARPi drugs of different chemical scaffolds. As natural products are a veritable source of drugs, this project sought to identify novel, PARP-inhibiting natural compounds.

The work is significant in that it contributes to a better understanding of some of the cellular and molecular mechanisms that might underlie cancer and reveals novel scaffolds for potentially more efficacious and safer drugs. It also highlights opportunities for drug repurposing, as PARPi can be used in the future to treat non-oncological conditions such as neurodegenerative diseases, for which there are no disease-modifying agents to date.

The research proceeded from assay development and validation to high-throughput screening and then to extensive hits characterisation and some molecular modelling of interactions between the PARPi and the PARP1 or PARP2 protein. In order to screen a large number of natural products quickly and robustly, a cell-based assay was developed and validated. The results showed that the alkylating agent MNNG was able to induce PARP1-mediated cell death (parthanatos), which Z-VAD-fmk (an apoptosis inhibitor) and necrostatin-1 (a necroptosis inhibitor) could not prevent, but against which DPQ (a PARP inhibitor) elicited protection. The MNNG-induced, PARP-dependent model assay was further validated by being employed to characterise established PARPi such as PARP-FL, olaparib, niraparib, rucaparib and talazoparib. Their potencies were correctly revealed by the assay to be in the nanomolar concentrations range, and the ranking order of their potencies was consistent with that of previous studies. The assay was also able to capture in time course the protection elicited by DPQ and olaparib against MNNG-induced cell death to reveal that the PARPi if added as late as up to 12 h after the initiation of the insult could still elicit some significant protection.

The assay, with a Z' factor of 0.7, indicating robustness, was then used to screen the Screen-Well® Natural Product Library of 502 structurally diverse compounds and a small, investigator-customised set of natural products, but initial hits from the primary screen were not confirmed in secondary assays as true positives. Inspiration was then obtained from the findings of a previous work by Fatokun et al. (2013) that identified two natural compounds, 4'-methoxyflavone (4MF) and 3',4'-dimethoxyflavone (DMF), as novel PARPi, using a cell-based assay. An extensive characterisation of 4MF and DMF, using a range of cell lines (HeLa,

HaCaT, SH-SY5Y), was undertaken in this PhD study, which included assessment of structure-activity relationships (SAR).

Both cell-based (parthanatos) assay that assessed protection against MNNG-induced cell death as evidence of PARP activity and cell-free PARP enzyme assay that assessed direct binding to PARP revealed 4MF and DMF as true PARPi, with potencies in the low micromolar concentrations range. As one of the potential areas of investigation, the compounds were assessed for their potential for synergy with or potentiation of existing anti-cancer drugs. They were found to have some potentiation or synergistic effects when combined with cisplatin or doxorubicin.

A structure-activity relationship (SAR) study was carried out to determine whether the PARP-inhibitory activities of 4MF and DMF could be improved upon, and to determine if there were any relationships between their antioxidant properties and their ability to protect against PARP1-mediated cell death. A total of 11 compounds with different hydroxyl- and methoxy- substitutions, including 4MF, DMF) and two non-flavonoid polyphenols, were explored as a customised set. The SAR study found that while 4'-hydroxyflavone (4HF) and 3',4'-dihydroxyflavone (DHF) were better antioxidants, 4MF and DMF were more potent PARP inhibitors. The critical importance of the 3' and 4' positions on the B-ring were demonstrated.

Finally, an *in silico* study of the SAR compounds revealed the binding modes of 4MF, DMF, 4HF and DHF. These four compounds were able to effectively occupy the NI site of PARP1, forming hydrogen bonds and weak π - π stacking interactions with amino acid residues and, in some cases, with key ones. Molecular docking

indicated that the four compounds would bind competitively with NAD⁺ at the catalytic site of PARP1. In addition, docking scores predicted that 4MF, DMF and DHF could be more potent towards PARP2. Their physicochemical properties did not show any violation of Lipinski's rule of five. Therefore, these four compounds could have good bioavailability and can be further evaluated for drug development.

Overall, this study established a set of methoxyflavones and hydroxyflavones as natural PARPi. It contributes significantly to knowledge by revealing and characterising novel, natural product PARPi scaffolds for chemical biology and therapeutics development applications, providing key mechanistic aspects of their pharmacodynamic and pharmacokinetic properties, including their drug-likeness.

7.1 Future work

This study validated a robust and relatively inexpensive cell-based assay for the identification of novel PARPi. The assay was optimised and confirmed competent for high-throughput screening (HTS) applications. It could therefore be employed in future efforts to screen larger chemical libraries to identify more PARPi scaffolds.

The study clearly demonstrated that 4MF, DMF, and the newcomers, 4HF and DHF, are PARP1 inhibitors, although their potencies are in the micromolar range, making them mild-to-moderate PARPi compared to the potent, clinically approved ones such as olaparib. Perhaps synthetic and medicinal chemistry could be used to synthesise further derivatives of the structures for testing for any enhancement of potency.

However, regardless of their potencies, in the future the compounds should be tested in the various *in vitro* and *in vivo* models of cancer, neurodegeneration, and other diseases in which the repurposing of PARPi has been suggested as a promising therapeutic strategy. For example, in this study, for the first time, it was demonstrated that a PARPi could offer protection against a toxic insult even if the PARPi is added many hours after the insult has been initiated. This is quite relevant to neurological conditions such as stroke, in which the time window (a few hours) post-stroke within which a sufferer of stroke must be treated in order to achieve functional recovery is critical. PARPi could, therefore, be tested in *in vitro* and animal models of stroke for their ability to offer neuroprotection, with functional recovery outcomes, even if they are administered many hours after the stroke event. All these endeavours will provide a proof-of-concept for translating the pre-clinical work demonstrated in this study to clinical utility.

To demonstrate one of the potential applications of the identified PARPi, their efficacy in treating *BRCA*-mutated cancers on the basis of synthetic lethality should be determined. This can be done by testing their ability to kill *BRCA*-mutated cell lines more potently than they are able to kill normal cells without the *BRCA* mutation. They should also be tested for their potential to be effective against triple negative metastatic breast cancer, as a role has been established for PARP in Triple Negative Breast Cancer (Singh, Parveen and Yadav, 2021). In addition, the flavone PARPi can be investigated for their ability to potentiate or synergise with existing anti-cancer drugs, whether small molecules or biologicals such as monoclonal antibodies, as well as their ability to sensitise cancer cells to radiotherapy.

Furthermore, there is now an increasing emphasis on the use of dietary approaches for the management of disease conditions. Natural food sources containing high levels of the PARP-inhibiting flavones or food items specifically enriched for these compounds could be tested for their ability to achieve chemoprevention (prevention of the development of cancer) in those with or without the *BRCA* mutations.

List of conferences and publications

1. **Benson SO**, Ismail FMD, Fatokun AA. 2021. A cell-based neuroprotection assay targeting poly (ADP-ribose) polymerase (PARP) reveals oncology-based potency ranking of clinically used PARP inhibitors (PARPi) ELRIG's Annual Drug Discovery Meeting (Drug Discovery 2021), The Exhibition Centre, ACC, Liverpool. 19-20 October, 2021. *Poster presentation*
2. **Benson SO**, Ismail FMD, Fatokun AA. 2021. 4'-methoxyflavone (4MF) and 3',4'-dimethoxyflavone (DMF) directly bind to the poly (ADP-ribose) polymerase (PARP) enzyme to inhibit parthanatos British Pharmacological Society (BPS) Conference (Pharmacology 2021). 7-9 September, 2021. Online. *Poster presentation*
3. **Benson SO**, Ismail FMD, Fatokun AA. 2019. PARPi-FL, a fluorescent version of the PARP inhibitor olaparib, exhibits decreased potency but similar efficacy compared to olaparib in a cell-based assay British Pharmacological Society's Annual Meeting, Pharmacology 2019, 15-17 December 2019, Edinburgh International Conference Centre, Edinburgh, UK (poster was additionally competitively invited for a flash presentation (British Journal of Pharmacology 2020: 47-48)). DOI: 10.1111/bph.15035. *Poster and oral presentation*
4. **Benson SO**, Ismail FMD, Fatokun AA. 2019. PARP inhibitors elicit concentration-dependent protection against PARP-mediated cell death even with a delayed addition of up to 12 hours post-insult ELRIG Drug Discovery Conference 2019, 5-6 November, 2019, Liverpool, UK. *Poster presentation*
5. **Benson SO**, Fatokun AA. 2019. Cell-based optimisation of a novel, fluorescent PARP inhibitor for therapeutics development Faculty of Science Research Day, Liverpool John Moores University, UK. 11 June, 2019. *Poster presentation*

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