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BINGOL UNIVERSITY
INSTITUTE OF SCIENCE

**INVESTIGATION OF THE EFFECT OF REDUCED MOB2 EXPRESSION ON THE
OLAPARIB RESPONSE OF PARP1/2 INHIBITOR-RESISTANT CANCER CELLS**

MASTER THESIS

SIDIKA ACIKGOZ

MOLECULAR BIOLOGY AND GENETICS

THESIS SUPERVISIOR

Assist. Prof. Dr. RAMAZAN GUNDOGDU

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CONTENTS

ACKNOWLEDGMENTS.....	iii
LIST OF ABBREVIATIONS	vi
LIST OF TABLES	vii
LIST OF FIGURES.....	viii
ÖZET.....	ix
ABSTRACT	x
1. INTRODUCTION	1
2. LITERATURE REVIEW	2
2.1. Cancer and DNA Damage.....	2
2.1.1. DNA Single-Strand and Double-Strand Breaks	2
2.2. hMOB2 Protein	3
2.3. Homologous Recombination	7
2.4. Non-homologous End Joining	9
2.5. Chemotherapy and Drug Resistance	9
2.6. Pharmacogenomics	10
2.7. PARP Enzymes.....	11
2.8. PARP Inhibitors	12
3. MATERIALS AND METHODS	15
3.1. Materials.....	15
3.1.1. The chemicals used in cell culture workings.....	15
3.1.2. The equipments used in cell culture workings	15
3.1.3. Olaparib Stock Preparing	16
3.1.4. Reagent of siRNA Transfection	16

3.1.5. The equipments used in SDS-PAGE and Western blot.....	16
3.1.6. The chemicals used in Chemicals SDS-PAGE and Western blot	17
3.1.7. Solution A.....	18
3.1.8. Solution B.....	18
3.1.9. Antibodies.....	18
3.2. Methods.....	19
3.2.1. Cell lines and culture conditions	19
3.2.2. Olaparib stock preparing and establishment of PARP inhibitor resistant (PIR) cell clones.....	19
3.2.3. Silencing of hMOB2 Gene Expression by siRNA Transfection	20
3.2.4. SDS-PAGE and Western blot	21
3.2.6. In vitro colony survival analysis (Clonogenic Assay)	25
 4. FINDINGS AND DISCUSSION	 26
4.1. Establishment of U2OS/PIR Cells	26
4.2. Loss Of hMOB2 Affects The Survival of U2OS/PIR Cells	28
4.3. Expression Profile of p53/p21 In U2OS PIR Cells.....	30
4.4. Loss hMOB2 Potentiates The Chemotoxicity of Olaparib in U2OS PIR cells ..	31
 5. CONCLUSIONS AND RECOMMENDATIONS	 33
 REFERENCES.....	 43

LIST OF ABBREVIATIONS

ATM	: Ataxia Telangiectasia Mutated
ATR	: Ataxia Telangiectasia and Rad3 Related
BRCA1/2	: Breast Cancer 1/2
CDK	: Cyclin Dependent Kinase
CHK1	: Checkpoint Kinase 1
CHK2	: Checkpoint Kinase 2
Cip1	: CDK-interacting Protein 1
CtIP	: C-terminal binding protein) Interacting Protein
DDR	: DNA Damage Response
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethyl Sulfoxide
DNA	: Deoxyribonucleic Acid
DNA- PKcs	: DNA-dependent Protein Kinase
DSB	: Double Strand Breaks
EDTA	: Ethylenediaminetetraacetic Acid
Exo1	: Exonuclease 1
GCCP	: Good Cell Culture Practice
H2AX	: Histone 2AX
hMOB2	: human Mps One Binder Proteins 2
HR	: Homologous Recombination
LATS	: Large Tumour Suppressor
miRNA	: microRNA
MOB	: Mps One Binder Proteins
MRN	: MRE11-RAD50-NBS1

MST1/2	: Macrophage-Stimulating 1/2
NAD	: NikotinamidAdeninDinükleotit
NDR	: Nuclear Dbf2-Related
NHEJ	: Non-homologous End Joining
p21	: Cyclin dependent kinase inhibitor 1A
p53	: Protein 53
PALB2	: Partner And Localizer of BRCA2
PARP	: Poly (ADP-ribose) polymerase
PBS	: Phosphate-Buffered Saline
RISC	: RNA-Induced-Silencing-Complex
RNA	: Ribonucleic acid
RNAi	: RNA interference
RPA	: Replication protein A
siRNA	: small interfering RNA
SSB	: Single Strand Breaks
U2OS	: Human Osteosarcoma Cells
XLF	: XRRC4 like factor
XRCC4	: X-Ray Repair Cross Complementing 4

LIST OF TABLES

Table 1. The content of standard lysis buffer.....	21
Table 2. The protease inhibitors using in the lysis buffer	22
Table 3. The content of 5X SDS-Laemmli Buffer	22
Table 4 (Continue). The content of 5X SDS-Laemmli Buffer.....	23
Table 5. The content of stacking and separation gels	23
Table 6. The dilutions of antibodies for Western blot	25

LIST OF FIGURES

Figure 2.1. The roles of hMOB2 gene in a cell.....	7
Figure 2.2. The activity mechanism of PARP enzymes during DNA damage repair....	13
Figure 3.1. Establishment resistant cells. U2OS cells treated with Olaparib for each 5 days, in increasing doses.....	19
Figure 4.1.1. The confirming of the resistance against to Olaparib	25
Figure 4.1.2. Verify the level of hMOB2 antibody in U2OS/Parent and U2OS/PIR cells.....	26
Figure 4.2.1. Confirmation of hMOB2 transfection in U2OS/PIR cells	28
Figure 4.2.2. Investigation of colony formation ability as a result of transient silencing of hMOB2 protein by transfection method	29
Figure 4.3. Investigation of hMOB2, p53 and p21 protein expressions levels after transfection.....	30
Figure 4.4. Clonogenic assay by co-administration of siMOB2 transfection with increasing doses of Olaparib therapy.....	31

PARP1/2 İNHİBİTÖR DİRENÇLİ KANSER HÜCRELERİNİN OLAPARİB YANITINDA AZALAN MOB2 EKSPRESYONUNUN ETKİSİNİN ARAŞTIRILMASI

ÖZET

PARP enzimleri DNA tek zincir kırık onarımı dahil olmak üzere çok sayıda hücrel süreçte rol oynamaktadır. PARP aktivitesinin inhibisyonu, özellikle DNA replikasyonu esnasında DNA çift zincir kırıklarına dönüşen DNA tek zincir kırıklarının birikmesine neden olur. Homolog rekombinasyon DNA çift zincir onarım yolağından yoksun olan kanser hücreleri, DNA kırıklarının tehlikeli birikimlerinden dolayı PARP inhibisyonuna karşı yüksek hassasiyet kazanmaktadır. Çeşitli PARP inhibitörleri klinikte etkili bir şekilde kullanılmasına rağmen, neticede çoğu hastada gelişen PARP inhibitör direnci klinik başarıyı sınırlandırmaktadır. Bu nedenle, farmakolojik olarak önem arz eden çeşitli hücrel hedeflerin ve prediktif biyobelirteçlerin tanımlanması, PARP inhibitörleriyle gerçekleştirilen güncel tedavi stratejilerini güçlendirebilir. hMOB2 proteini, homolog rekombinasyon onarım mekanizmasını destekleyen potansiyel bir DNA hasar yanıt regülatörüdür. Bundan dolayı, hMOB2-defektif kanser hücreleri PARP inhibisyonuna karşı hassas hale gelmektedir. Bu çalışmada, hMOB2 işlevsizliğinin, PARP inhibitör tedavisine dirençli kanser hücrelerini de PARP inhibisyonuna karşı hassas hale getirip getirmeyeceği araştırıldı. Araştırma sorularımızın test edilmesi için memeli hücre kültürü ve çeşitli moleküler biyoloji teknikleri kullanıldı. Deneylerimizde, U2OS osteosarkoma kanser hücreleri ve klinikte yaygın bir şekilde kullanılan PARP inhibitörü olaparib kullanıldı. PARP inhibitörüne dirençli klonların (U2OS/PIR) oluşturulması amacıyla U2OS hücreleri sürekli olarak artan dozlarda olaparib tedavisine maruz bırakıldı. hMOB2 ve p53 ekspresyonlarında bir fark gözlenmemesine rağmen, ilginç bir şekilde, parental hücrelere kıyasla, U2OS/PIR hücrelerinde p21 protein miktarının önemli oranda arttığı gözlemlendi. Azalan hMOB2 ifadesinin, olaparib tedavisi uygulanmaksızın U2OS/PIR hücrelerinin sağ-kalımını azalttığı belirlendi. hMOB2 ve PARP aktivitelerinin ikili inhibisyonunun, dirençli kanser hücrelerini olaparib tedavisine karşı daha hassas hale getirdiği ortaya çıkarıldı. Sonuç olarak, gerçekleştirdiğimiz deneyler PARP inhibisyonuna karşı, hMOB2 proteininin sadece parental hücrelerin değil aynı zamanda PARP inhibitörüne dirençli kanser hücrelerinin de sağ-kalımını destekleyici bir faktör olabileceğini göstermiştir.

Anahtar Kelimeler: hMOB2, olaparib-direnci, bireyselleştirilmiş kanser tedavisi.

INVESTIGATION OF THE EFFECT OF REDUCED MOB2 EXPRESSION ON THE OLAPARIB RESPONSE OF PARP1/2 INHIBITOR-RESISTANT CANCER CELLS

ABSTRACT

PARP enzymes play significant roles in various cellular processes, including DNA single-strand break repair. Inhibition of PARP activity results in the accumulation of DNA single-strand breaks, which are eventually converted to DNA double-strand breaks, especially during DNA replication. Cells with deficient homologous recombination DNA double-strand break repair pathway are hypersensitive to PARP inhibitors due to deleterious accumulation of DNA breaks. Although several PARP inhibitors have been effectively used in the clinic, PARP inhibitor resistance that ultimately develops in most patients limits clinical success. Therefore, identification of pharmacologically important cellular targets and predictive biomarkers may ameliorate the current treatment strategies with PARP inhibitors. hMOB2 protein is a potential DNA damage response regulator that supports homologous recombination repair mechanism. hMOB2-defective cancer cells, thus, become sensitive to PARP inhibition. In this research, it was investigated whether hMOB2 deficiency may also render PARP inhibitor-resistant cancer cells selectively sensitive towards PARP inhibition. Mammalian cell culture and various molecular biology techniques were used to dissect our research questions. U2OS osteosarcoma cancer cells and olaparib, a potent PARP inhibitor widely used in the clinic, were employed in our experiments. U2OS cells were subjected to continuously increased doses of olaparib to establish PARP inhibitor-resistant clones (U2OS/PIR cells). Interestingly, p21 protein levels were dramatically elevated in U2OS/PIR cells compared to parental counterparts, although no difference was observed in p53 and hMOB2 expressions. Notably, hMOB2 silencing significantly reduced the survival of U2OS/PIR cells in the absence of olaparib. Dual inhibition of hMOB2 and PARP activities further sensitized resistant cancer cells towards olaparib treatment. Taken together, our experiments revealed that, against PARP inhibition, hMOB2 protein is likely to be an important factor that supports not only parental cells but also PARP inhibitor-resistant cells to survive.

Keywords: hMOB2, olaparib-resistance, personalized cancer treatment.

1. INTRODUCTION

Uncontrolled cell division is the definition of cancer. The course of the cycle cannot be controlled if the genes responsible for the regulation of cell growth, development, differentiation, and death are damaged, mutated, or dysfunctional, and cells obtain the ability to divide indefinitely without control. Cancer cells have a number of characteristics, including maintaining proliferative signals, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan & Weinberg, 2011).

The eukaryotic cell cycle is regulated by CDK (cyclin dependent kinases) and Cyclins, and firmly controlled by cell cycle checkpoints. Cells need growth signals to pass from G0 to G1. A cell does not require growth factors after successfully completing the G1 phase. In every change of a cell cycle phase, there are checkpoints like G1-G1/S-G2/M, whose regulation is conducted by specialised kinases and phosphatases. Upon any type and level of DNA damage, cell cycle inhibitors are activated by specific cell cycle regulators such as p53, RB (retinoblastoma), BRCA1/BRCA2 at checkpoints, and the progression of the cycle is arrested, providing time for efficient damage repair. Because of mutant checkpoints has critical role to cell cycle arrest, imprisonment to growth factors, DNA damage repair pathways, apoptosis (Goldstein & Kastan, 2015; Gundogdu et al. 2021-b). Upon DNA damage induction, the well-defined DNA damage kinases ATM and ATR are activated. Once activated, ATM phosphorylates and activates one of its downstream components, the p53 tumour suppressor protein. The activated p53 protein binds to the DNA sequence of interest and promotes p21-mediated cell cycle arrest by inducing the expression of p21, one of the cell cycle inhibitors. After the cell cycle is stopped, the damage is tried to be repaired according to the signal of the damage. (Kucuksayan & Akca, 2018; Qu et al., 2003; Cabadak 2008; Otto & Scincski, 2017; Hanahan & Weinberg, 2000).

2. LITERATURE REVIEW

2.1. Cancer and DNA Damage

The mammalian cell cycle has certain checkpoints and complex repair mechanisms depending on the type of DNA damage. Finding an alternative repair mechanism for DNA damage repair can cause to gain resistance to chemotherapy and radiotherapy. That is why targeting DNA damage repair components is important for synthetic lethality and the specific elimination of cancer cells (Curtin, 2012).

In brief, DNA damage response (DDR) steps are classified as follows:

(1) Detection of lesions, (2) induction of cell cycle checkpoints, (3) activation of the corresponding signalling pathways, (4) repairing damaged DNA area, (5) alternatively, massive DNA lesions require the activation of death receptors and cell death.

The DNA double-strand breaks are mainly repaired by two different DNA repair mechanisms.

1. Homologous Recombination (HR)
2. Non-homologous End Joining (NHEJ)

2.1.1. DNA Single-Strand and Double-Strand Breaks

Among DNA damages, the most cytotoxics are DNA double-strand breaks and primary DNA lesions due to single-stranded breaks in the replication fork or intermediate strand crosslinking (ICL), which can develop into double-strand breaks in DNA, through exposure to cytotoxic agents. DNA double-strand breaks can also be caused by many different chemotherapy agents, such as IR (ionizing radiation) and topoisomerase inhibitors. There are two main way to deal with DNA double-strand breaks in the cell, the NHEJ and HR pathways. NHEJ can function to repair DNA strand breaks that occur at all

stages of the cell cycle, but this mechanism does not have access to the reference homologous base sequence, therefore repair is prone to error. Unlike NHEJ, HR only works in the late S and G2 phases of the cell cycle. Because for the repair of broken chain ends, access to base sequences showing homology to the broken chain is only possible in the S and G2 stages. In HR, the error rate is very low because during repair with this mechanism, there is a homologous sequence on sister chromatid (Chapman et al., 2012; Jackson & Bartek, 2009).

2.2. hMOB2 Protein

Cellular processes such as the Hippo signalling pathway, regulation of organ growth and tissue homeostasis in a living cell, signal reception and transmission are controlled by protein kinases involved in these pathways. In this regard, NDR (nuclear Dbf2-related) / LATS (large tumour suppressor) kinases, a family conserved from yeast to humans, attract great attention (Gomez et al., 2015; Gundogdu & Hergovich, 2019). Cell cycle progression, mitosis, apoptosis, and autophagy can be cited as examples of mechanisms by which NDR1/2 kinases play a role in mammalian cells. They are also known to have various functions in the immune system, embryogenesis and neurobiology, and DDR (Gundogdu & Hergovich, 2016; Jiang et al., 2020; Leng et al., 2012; O'Neill et al., 2018).

Four related kinases in human cells:

1. NDR1 ((serine/threonine kinase) STK38)
2. NDR2 (STK38L)
3. LATS1 (large tumoursuppressor-1)
4. LATS2

The Mps-one-binding (MOB) protein family, a regulator of the NDR/LATS family that modifies/influences cellular progression, is highly conserved in eukaryotic cells. Binding of MOB proteins is required for the full activation of NDR/LATS kinases. MOB proteins have been discovered in *Drosophila*, mammals, and yeast, and it is known that MOB proteins are involved in the regulation of mitosis, cell proliferation, controlled cell death (apoptosis), centrosome duplication, and morphological changes.

Three types of proteins, dMOB1, dMOB2 and dMOB3, are involved in cell cycle signalling in *Drosophila*. In recent years, geneticists working on flies have started to be more interested in Trc and Warts after realizing that NDR/LATS kinases play a role in the tumour suppressor Hippo signalling pathway. dMOB1 interacts with Trc and Wart kinases, but the interaction with Warts is better known. This interaction is one of the cornerstones of the Hippo signalling pathway in proliferation, controlled death and tumour suppression. It binds directly to the Tricornered protein, which is the NDR kinase ortholog. Tricornered interacts with the *Drosophila* NDR ortholog actin filaments and regulates dendritic laying and branching. In dMOB1 deficiency, an increase in cell proliferation, abnormal mitosis in the embryonic period, defective duplication of spindle fibres and a decrease in cell death are observed (Gomez et al., 2015; Gundogdu & Hergovich, 2016; Hergovich, 2012; Leng et al., 2012; O'Neill et al., 2018).

Yeast MOB1-MOB2 proteins are involved in mitotic emergence and morphogenesis by the regulation of NDR/LATS kinases-Dbf2p and Cbk1. Mob1, which has been observed to be involved in the regulation of mitotic exit and cytokinesis in Dbf2p, Cbk1p, Sid2p, and Orb6p in yeast, is one of the important components of the Hippo signalling pathway, which acts as a tumour suppressor in *Drosophila*, and binds directly to NDR kinases, supports cellular growth (Leng et al., 2012; O'Neill et al., 2018).

The highly conserved Cdk1 kinase phosphorylates Mob1p in yeast. However, these phosphorylation sites were not protected from yeast to humans. This difference leads us to think that different translational modifications may exist in the regulation of MOB1 protein in humans. Information about the regulators of hMOB2 and hMOB3A/B/C proteins is not clear yet (Hergovich, 2012).

In human cells; At least 7 different MOB proteins have been shown so far, hMOB1/A (human MOB1A), hMOB1B, hMOB2, hMOB3A, hMOB3B, hMOB3C and hMOB4 (Gundogdu & Hergovich, 2019). The two well-defined MOB proteins are hMOB1 and hMOB2. Luca and Winey identified MOB1 and MOB2 in 1998 and stated that MOB1 is a phosphoprotein. In 2001, it was discovered that Cdc15p, which counterparts of the MST1/2 kinases in humans, yeast, and fly hippo kinase, phosphorylate MOB1p (Mah et al., 2001). In other words, phosphorylated dMOB1 is involved in the Hippo signaling pathway. The

Avruch laboratory also investigated the phosphorylation of the hMOB1 protein by MST1/2 kinases (Proliferation, 2015). In contrast to the hMOB1A/B and hMOB3 proteins, the hMOB2 protein is mainly localized in the nucleus. MOB2 interacts with the NDR1/2 kinases but not LATS1/2. The binding sites of hMOB1 and hMOB2 on the NDR are the same, causing competition between them (Hergovich, 2012; Leng et al., 2012). hMOB2 is involved in the regulation of the NDR kinase signalling pathway (Gundogdu & Hergovich, 2016). hMOB2 competes with MOB1 when binding to NDR. If hMOB2 binds to the NDR, NDR becomes inactivated (Gomez et al., 2015).

MOB2 is critical in the NDR kinase signalling pathway in the context of the cell cycle progression and DNA damage response. The MOB1/NDR complex causes an increase in NDR kinase activity, while the MOB2/NDR complex reveals a decreased NDR kinase activity (Gomez et al., 2015; Gundogdu & Hergovich, 2016). From this perspective, hMOB2 is considered to be an inhibitor of the NDR signalling pathway; hMOB1 is recognized as a co-activator of NDR/LATS kinase signalling. As a result, while the role of MOB2 relative to MOB1 in the regulation of NDR1/2 kinases is not fully understood, it is highly likely that MOB2 has positive or negative activities in promoting the cellular cycle, DDR signalling. And this strong possibility suggests MOB2 as a DDR element in the regulation of DNA damage response and leads to a biological investigation. NDR1/2 controls the levels of p21/Cip1 proteins and regulates the G1/S phase (Gundogdu & Hergovich, 2016).

The expression and localization of the MOB2 protein is important in the cell's response to DNA damage. The MOB2 protein's interaction with proteins in distinct DNA damage repair pathways should be investigated in order to understand its biological function. According to Gundogdu et al. in 2021, upon expression of I-SceI by the DRGFP system, which allows HR-directed repair of DSBs induced by I-SceI endonuclease in a mutated GFP gene, in hMOB2-deficient U2OS cells, approximately 50 % of HR activity compared to control cells. They showed that the DNA cross-linker cisplatin and the DNA-damaging antibiotic bleomycin (which promotes DSB formation) sensitize cancer cells to these chemotherapeutics as a result of hMOB2 depletion, and cell viability was significantly reduced by bleomycin treatment (Gundogdu et al., 2021-a). They also discovered that depleting both hMOB2 and p21 proteins at the same time resulted in G1/S checkpoint arrest

as a result of cell cycle restoration. In hMOB2-depleted cells, the ATM-CHK2-p53-p21 DNA damage signalling pathway was activated, resulting in an increase in CHK2 and p53 phosphorylation and DDR signalling in direct proportion to damage (increased DNA damage by siMOB2), with exogenous DNA damage still at the G1/S point; they discovered that in the case of endogenous DNA damage, the hMOB2 defect induces p53/p21-mediated G1/S cell cycle arrest as a result of DNA damage accumulation. In addition, they observed that the uptake of active ATM into the damaged chromatin region was impaired (Gomez et al., 2015).

When the role of MOB2 in terms of DDR signalling is examined, it is known that MOB2 interacts with RAD50, which is the main component of the DNA damage sensor complex MRN (MRE11-RAD50-NBS1), independent of NDR signalling. In DNA lesions, the presence of this interaction is required for the activation of the DDR kinase ATM. When DNA is damaged, the MRN complex binds to the damaged DNA region in interaction with PARP and the DNA double-strand break signal complex Ku70/80. ATM is also connected to double chain breaks. ATM, one of the fundamental DDR components triggered by autophosphorylation, phosphorylates and activates the downstream DDR proteins. Active ATM activates p53, CHK2, KAP1 effector proteins as well as NBS1 in the MRN complex through phosphorylation by creating feedback to maintain its own activity (Gomez et al., 2015; Gundogdu & Hergovich, 2016).

The interactions indicated above explain DDR deficiency in MOB2-deficient cells (MRN inactivation). Based on these findings, it is reasonable to conclude that the MOB2 protein is required for cell survival, DDR response, and cell cycle control (Gundogdu and Hergovich, 2016). The key regulatory axis in DDR response is p53-p21 signalling, which is responsible for the G1/S transition point; hence, p53 activity may be tested for DDR signalling in cells with hMOB2 deletion. Given the link between p53-dependent cell cycle arrest and the hMOB2 deficiency, it is more than a reasonable assumption that DNA damage caused by hMOB2 deficiency can activate p53. It is vital to comprehend the mechanism by which it participates in two critical stages, namely cell viability and the cell cycle checkpoint (G1/S arrest). In this respect, it is very important to understand the mechanism by which the hMOB2 protein is involved in two critical stages: cell viability and cell cycle checkpoint (G1/S arrest).

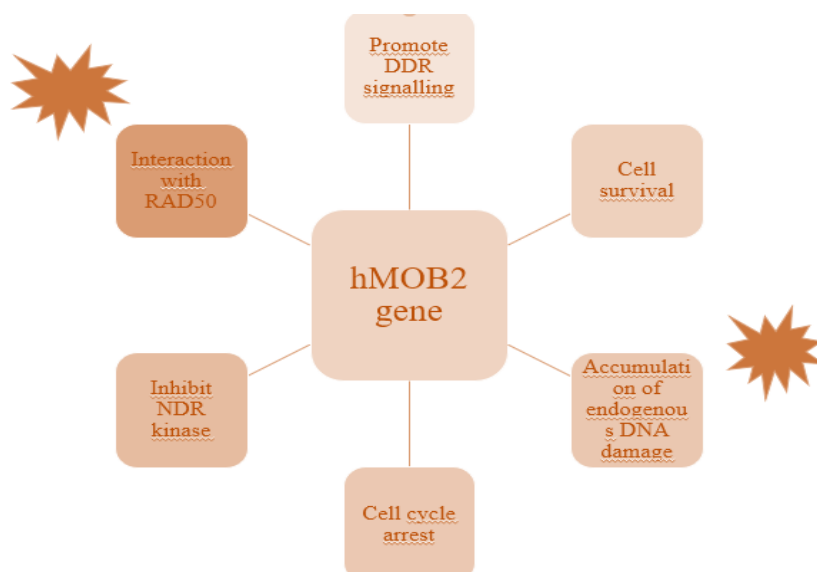


Figure 2.1. The roles of hMOB2 gene in a cell

Although the MOB protein family has been known to have various roles in carcinogenesis in preclinical studies conducted so far, the role of MOB2 in cancerization has not been clearly explained. Cancer-related databases such as the Cancer Genome Atlas show that expression of the human MOB2 gene is heterozygous in over 50% of bladder, ovarian, prostate cancers.

Taking into account that U2OS is the most frequently used cell line in cell survival experiments (clonogenic) using DNA-damaging agents, this cell line was chosen in present study (Gomez et al., 2015).

2.3. Homologous Recombination

HR is a well-established complex mechanism and active at S and G2 phases of the cell cycle since this mechanism needs to sister chromatid. The homologous sequence of broken ends acts as a template sequence during the repair. Most tumour suppressor proteins such as BRCA1/2, ATM are responsible for the efficient regulation of this pathway (Curtin, 2012). Upon DNA double strand breaks, the heterotrimeric MRN (DNA damage detection sensor) complex binds to the ends of DNA during the early stages of the DSB occurrence.

This complex consists of MRE11, RAD50 and NBS1. In the case of a double-strand break, the MRN complex, recognizes the broken site and initiates a series of biochemical signal transduction. MRE11 has the endo-exonuclease activity to facilitate the initial resection of DNA DSB ends. Together with CtIP, the MRE11 protein provides the first notch to the broken site, while RAD50 stabilizes the damaged ends at this stage, and the NBS1 protein activates ATM kinase, which eventually activates the downstream regulators, including the tumour suppressor p53 protein. Under physiological conditions, the inactive homodimer ATM molecules become active monomer ATM upon autophosphorylation and transmits the signalling of the damage to the sub-factors. Exo1 (exonuclease 1) extends DNA end resection. Following the completion of DNA end resection, the RPA complex surrounds resected single-strand ends of DNA in order to provide a protection against possible nuclease activities. In other words, the RPA heterotrimeric protein complex, which binds to the elongating 3' ssDNA, prevents the enzymatic degradation of the exposed DNA single-strand by nucleases. After 5' exonucleolytic resection, RAD51 (recombinase) nucleofilaments, extend to 3' ssDNA ends and, match with complementary strands by perform invasion. One of the most important steps of the HR system is the removal of the RPA protein complexes suspended on 3'ssDNA and the loading of the RAD51 recombinase enzyme into single-stranded DNA (Gundogdu et al., 2021-a). It is known that the BRCA1-PALB2-BRCA2 complex plays an important role in this process. RAD51 enzymes loaded on 3'ssDNA encodes the same genetic code on the sister chromatid and create a copy of the damaged region, and the DNA double-strand break is repaired. Thus, the Holliday connection remains free, and strands have change (Chapman et al., 2012; Goldstein & Kastan, 2015). Unless RPA protein bind to 3' ssDNA ends, the RAD51 could not bind to 3' ssDNA ends and also the BRCA2 protein provides the loading of RAD51 to the damaged area. If there is a problem in this loading phase, RAD51 cannot be loaded into the damaged area, and HR cannot be functional, double-strand breaks accumulate (Chapman et al., 2012; Goodarzi & Jeggo, 2013).

2.4. Non-homologous End Joining

Everyday almost 50 double strand breaks occur per cell per person. NHEJ take part in every phases of cell cycle and prone to error since a homologous sequence of broken ends is not available. About 50 pairs of strand breaks occur per cell per person each day. NHEJ is involved in every stage of the cell cycle and is error-prone because it lacks a homologous broken-end sequence. The first step in this repair mechanism is the binding of the Ku70/Ku80 complex to strand breaks in DNA. The Ku70/Ku80 complex has high affinity for broken DNA ends. The catalytic subunit DNA-PKcs is then aggregated and activated. Active DNA-PKcs phosphorylates and create a complex with XRCC4-XLF and Ligase 4 targets. Thanks to this complex, a connection is established between the two broken ends of the DNA and the broken ends are repaired (Chang et al., 2017; Goldstein & Kastan, 2015).

2.5. Chemotherapy and Drug Resistance

In response to DNA damage, normal cells stop cell cycle, organize a proper molecular response to damage, and finally either repair the corresponding DNA lesions or activate programmed cell death pathways. Cancer cells, however, mostly proliferate without a control. As a result, they can come up with alternative repair pathways to against the damages. In today's clinical cancer treatments, in addition to radiotherapy and surgical local cleansing methods, treatment is also carried out with chemotherapeutic agents that target rapidly proliferating cancerous cells and can be applied at every stage of the disease. However, cancer cells can rapidly change their genetic structures to ensure the continuation of life under stress, and by developing alternative damage repair pathways, they gain resistance to the drugs applied over time. In many cancer types, unfortunately, chemotherapy many times could be unsuccessful. Because cancer cells develop resistance to medications over time, drug resistance can arise as a de novo or acquired ability and this resistance is one of the biggest challenges limiting the clinical success. Overexpression of critical pathway proteins and efflux pump function, which limits drug accumulation and hinders active region activity, may be the major cause of DR (drug resistance).

Drug resistance mechanisms are basically of 2 types:

1. Multidrug resistance- P-gp

Drug resistance can occur by many different mechanisms or due to drugs and is called Multi-Drug Resistance (MDR). High doses of chemotherapeutic drugs or combination therapies that can create a synergetic effect are applied against MDR. P-gp (Permeability glycoprotein), also called multi-drug resistance protein, is localized throughout the gastrointestinal tract, which includes the small intestine. Especially for oral drugs, P-gp is known to reduce the bioavailability of chemotherapy. Likewise, overexpression of P-gp may be initiated by some antineoplastic drugs, thus preventing bioavailability.

2. Decreased drug activation (Pharmacokinetic problems)

Pharmacokinetics consists of absorption, dispersion, metabolism, excretion and is the body's reaction to the drug. In the case of decreased drug activation, the administered drug is absorbed by the cell. It cannot be accepted and sent out again or cannot be metabolized. In this case, treatment methods are developed by applying growth factors, kinases, and inhibitors against anti-apoptotic proteins (Alfarouk et al., 2015; Chakravarty et al., 2021; Li et al., 2021).

2.6. Pharmacogenomics

As responses to drugs administered in chemotherapy, the dose, combination, and duration of treatment to which every individual responds positively may be metabolically different, and the pharmacogenetics concept meets these differences. Pharmacogenomics research promises better treatments with stronger and safer drugs in chemotherapy, thanks to the clarification of patients' proteome profile (Demirkazik et al., 2010; Robert, 2004). Because drugs might effect diversity proteins interaction, post-translational modifications, the bioavailability of them and therefore there may occur unexpected responses such as toxicity, drug metabolism and metabolism pathway proteins troubles, receptor polymorphisms and finally drug resistance. With the application of small-molecule inhibitors in chemotherapy, genes that provide drug resistance can be blocked, the activities of proteins involved in damage repair mechanisms can be inhibited, and an attempt aims to induce cell death. Consequently, in pharmacogenomics, DNA damage and DDR (DNA Damage Response) compounds highly important (Izmirli, 2015; Nandal & Burt, 2017).

2.7. PARP Enzymes

Cell cycle, repair of DNA damage, proliferation, prevention of damage are important functions in carcinogenesis, and all of these functions are regulated by PARP. PARP enzymes are a large family of enzymes with approximately 17 members, using Nicotinamide Adenine Dinucleotide (NAD) as substrate, and the best-defined primary functional member is PARP1. PARP is a nuclear enzyme that mediates post-translational PARylation of proteins whose family members are involved in transcription and DNA damage repair and repair of single and double strand breaks (Gou et al., 2020). They form a platform for the uptake of proteins required for repair to the damaged area. An internal automodification domain contains a BRCA1 C-terminal region that mediates protein-protein interactions, and three lysine residues are targeted for automodification. Other domains on the enzyme also provide platforms for various protein-protein interactions such as transcription factors and DNA damage repair proteins.

PARP enzymes, which have a very short half-life, are found in both the cytoplasm and the nucleus, although some PARP enzyme activities occur in the cytoplasm. Nevertheless, cellular PARP enzyme activity is mostly localized in the nucleus. PARP enzymatic activity is induced by DNA damage and is also regulated by various signal transduction and protein modifications (Yang et al., 2020). It is a posttranslational modification that PARP enzymes change the function of their target proteins with ADP-ribose. After binding to the damaged area on the DNA, they incorporate target repair proteins into their body by autoPARylation. It binds NAD⁺ to nicotinamide (NAM) and ADP-ribose, forming the poly ADP-ribosylation chain. PARP activation is essential for damage repair. PARP1 can self-activate by autophosphorylation/autoPARylation in case of damage and can trap itself and inactivate by reversible phosphorylation after repair.

BRCA1/2 genes are known to be critical for the repair of DSBs by HR. Based on the synthetic lethal interaction between PARP inhibition and BRCA1 or BRCA2 mutation, Farmer and Bryant developed a new treatment strategy for BRCA-mutant tumours (Farmer et al., 2005; The et al., 2005). Preclinical PARPi studies applying the concept of synthetic

lethality have shown that PARPi can selectively target HR-deficient cells. When therapeutic inhibition of PARP enzymes occurs, since spontaneous DNA single-strand breaks cannot be repaired, this DNA damage accumulates and eventually turns into catastrophic DNA double-strand breaks, especially in the S phase of the cell cycle. The main purpose here is to inhibit PARP enzymatic activation in *BRCA1/2* defective cells and lead the cell to death (Gao & Guo, 2020). Therefore, pharmacological inhibition of PARP is considered a promising anticancer strategy. Various PARP inhibitors have been developed and evaluated clinically. Major PARP inhibitors include olaparib, veliparib, rucaparib, niraparib, and talazoparib. Olaparib has been approved by the FDA (Food and Drug Administration) for the treatment of patients with BRCA-defective metastatic ovarian and breast cancer. Inhibitors act by inhibiting the binding of damage repair proteins by interacting with β -nicotinamide adenine dinucleotide (β -NAD⁺) at the catalytic site of the PARP enzyme (Bai, 2015; Gao & Guo, 2020; Gou et al., 2020).

2.8. PARP Inhibitors

About 30 years ago, PARP inhibitors began to be developed with the discovery that 3-aminobenzamide (3-AB) competes with the PARP substrate NAD⁺. By targeting (inhibiting) PARP1 in the DNA repair pathway, regulating the transcriptions of the genes involved here, or preventing the repair of single-strand breaks and converting them to double-strand breaks, creating replication fork stress and driving the cell to death. In this way, genomic instability is triggered, replication stress occurs by inhibiting the damage repair ability, and antitumor treatment efficiency can be increased by precipitating the replication fork (Rajawat et al., 2017; Zhou et al., 2020).

Considering the studies of Farmer et al., it has been shown that PARP inhibition increases sensitivity to treatment and decreases survival in *BRCA1/2* deficient cells. Although *BRCA1/2* usually exhibits a homozygous mutation in cancer cells, selective lethality is encountered because homologous recombination works properly in normal cells and is not functional in cancer cells (Rajawat et al., 2017). This synthetic lethality mechanism does not harm healthy cells because homologous recombination specifically works in these cells. Tumour suppressor *BRCA1/2* proteins, which have important roles in regulating the cell cycle and maintaining genomic stability, have a critical role in repairing DNA double-

strand breaks by homologous recombination. Defective *BRCA1/2* also causes errors in homologous recombination. Therefore, mutations on the *BRCA1/2* genes increase the risk of cancer in those cells (Bai, 2015; Cetin et al., 2020; Gao & Guo, 2020; Zhou et al., 2020). PARP enzymes bind to damaged DNA regions and form a PARP-DNA complex. PARP competes with NAD in the catalytic region of the enzyme for PARP inhibition, interact with β -NAD⁺ (cofactor). Thus PARP trapping with inhibitors can block DNA repair by preventing the polymerization of PAR and consequently limit the signal formation for the proteins attracted to PAR-dependent DNA repair (Rajawat et al., 2017).

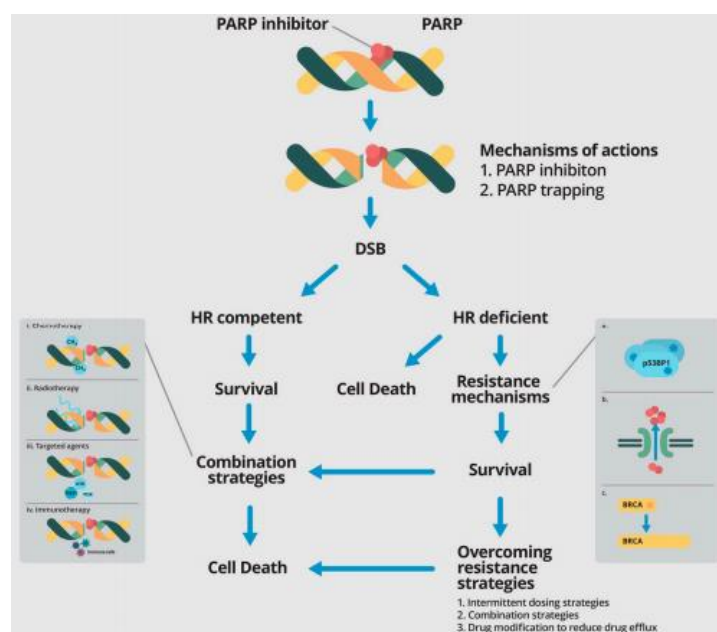


Figure 2.2. The activity mechanism of PARP enzymes during DNA damage repair (Lim & Tan, 2017)

In this way, on account of PARP inactivation, SSBs were unable to repair and develop into double-strand breaks; thus the replication fork collapsed. PARP inhibitors are β -NAD analogues. Among PARP inhibitors, PARP capture ability and level of cytotoxicity differ in proportion to their capacity to bind PARP. As a result of the clinical success of PARP inhibitors with some experience, olaparib, rucaparib, and niraparib have been approved by the FDA (Carrassa et al., 2020). It is known that five of them, which are widely used today, have good PARP trapping ability. These are veliparib, rucaparib, talazoparib, niraparib and olaparib. Olaparib is the first PARP inhibitor approved by the FDA. Olaparib was

developed predictively because it is more effective in cells with the *BRCA* mutations. Olaparib captures PARP from the site where it binds to DNA damage and inhibits polymerization and repair of the enzyme, so the replication fork collapses, and double strand breaks accumulate. Because when BRCA2 is mutant, DNA recombinase enzyme RAD51 will not be able to be loaded into the damaged area, and therefore the homologous recombination damage repair system it mediates will become dysfunctional.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. The chemicals used in cell culture workings

Chemicals	Manufacturer	Catalog Number
Dmem medium	SIGMA	D6429
Dulbecco's phosphate-buffered saline (dPBS)	GIBCO	14190094
Trypsin-EDTA	Biological Industries	03050
10% Fetal Bovine Serum (FBS)	GIBCO	10270106
1% penicillin (64 µg/mL)/streptomycin (100 µg/mL)	GIBCO	15140122
Trypan Blue	Thermo Fisher	15250061
Aquaguard 1	Biological Industries	018671B

3.1.2. The equipments used in cell culture workings

Equipment	Manufacturer	Catalog Number
CO ₂ incubator	ESCO	CCL170B8
Autoclave	HIRAYAMA	HVE-50
Centrifuge	Weightlab Instruments	WN-CL6500
Cell culture flasks: 75 cm ²	NEST	708003
Cell culture dishes: 60x18mm	LABSOLUTE	7696771
Biosafety cabinet	ESCO Class II	-
Micropipettes	ISO Lab	-
Hematocymeter	Thoma ISO Lab	-
Mini Shaker	ISO Lab	61311001
Electronic Balance	ISO Lab	S1909002

3.1.3. Olaparib Stock Preparing

Chemical	Manufacturer
Olaparib (MW: 434.46)	
DMSO (dimethyl sulfoxide)	MERCK KGaA (8.02912.2500)

3.1.4. Reagent of siRNA Transfection

Reagent	Manufacturer	Catalog number
Optimem	GIBCO	31985062
LipoRNAimax	Invitrogen	2209786
siRNA (siMOB2)	Qiagen	1027418

3.1.5. The equipments used in SDS-PAGE and Western blot

Equipment	Manufacturer	Catalog Number
Microcentrifuge tubes	ISOLAB	S.078.03.002.500
Centrifuge	Weightlab Instruments	WN-CL6500
Spectrophotometer	Shanghai Metash	UV-5100
SDS-PAGE apparatus	BIO-RAD	041BR307525
Transferring apparatus	BIO-RAD	041BR307525
Serological Pippette	Lab Marker	10082019
Water Bath	Thermomax	201910103425
PVDF Transfer Membrane, 0.2 μ m, 26.5 cm x 3.75 m	Merck Millipore	IPVH00010
X-Ray film (18x24 cm)	Carestream MXBE Film	7710783

3.1.6. The chemicals used in Chemicals SDS-PAGE and Western blot

Chemicals	Manufacturer	Catalog Number
Tris	Fisher Chemical	BP153-1
NaCl	ROTH	2618.1
Glycerol	SIGMA ALDRICH	15524
EDTA	SIGMA	E5134
EGTA	GLENTHOM LS	GE7249
NaF	CARL ROTH	2618.1
Bicinchoninic Acid Protein Assay kit (Bradford reactive)	BIO-RAD	5000006EDU
Spectrophotometer cuvettes	BİOSIGMA	20P1006010
Sodium dodecyl sulfate (SDS)	MERCK KGaA,	8.17034.1000
β-mercaptoethanol	SIGMA LS	M3148
Bromophenolblue	MERCK	536092
Acrylamide/bisacrylamide mix (30%)	CARLO ERBA	480141
Ammonium persulfate (APS)	MERCK KGaA	101201.0500
N,N,N',N'-tetramethylethylenediamine (TEMED)	SIGMA	110-18-9
Isopropanol	Riedel-de Haen	24137
Molecular weight marker (Protein)	Intron	24052
Skim milk powder	SIGMA ALDLRICH	70166
Methanol	MERCK KGaA	1.06007.2500
Tween-20	SIGMA	P1379
H ₂ O ₂	KarloErba	7722841
Luminol	SIGMA ALDLRICH	123072-5G
Cumaricacide	SIGMA	C9008-10G

3.1.7. Solution A

Content	Volume (10 ml)
Luminol	100 µl
Cumaricacide	44 µl
Tris pH: 8.5	1 ml
dH ₂ O	8.856 ml

3.1.8. Solution B

Content	Volume (10 ml)
H ₂ O ₂	6.5 µl
Tris pH: 8.5	1 ml
dH ₂ O	8.935 ml

3.1.9. Antibodies

Antibody	Kind	Manufacturer
Primary antibody		
MOB2	Polyclonal antibody	Home made
p53	Monoclonal antibody	Santa Cruz (sc-126)
p21	Polyclonal antibody	Santa Cruz (sc-397)
Tubulin	Polyclonal antibody	Home made
GAPDH (G-9)	Monoclonal antibody	sc-365062/Santa Cruz Biotechnology
Secondary antibody		
Anti-rat	Polyclonal antibody	
Anti-rabbit	Polyclonal antibody	
Anti-mouse	Polyclonal antibody	

3.2. Methods

3.2.1. Cell lines and culture conditions

Human osteosarcoma (U2OS) cells were obtained from Dr Alexander Hergovich (UCL Cancer Institute, London, UK). U2OS were routinely cultured in 5% CO₂ 37°C humidified incubator in DMEM/F12 medium including 10% FBS and 1% penicillin (64 µg/mL)/streptomycin (100 µg/mL) and L-glutamine. Cells allowed to grow exponentially. All cell culture studies were performed to GCCP standards. Cells were sub-cultured when confluency reach to 80% fullness. During subculturing, cells were laid away from culture medium with sterilized 1X trypsin-EDTA solution following washing with PBS, the cells in 4X10³ cell/mL resuspended and counted through a hematocymeter and transferred-resubcultured with fresh DMEM and saved in CO₂ incubator plates with the same conditions.

Cell counting formula:

cells/ml= average count per square X dilution factor X 10⁴

3.2.2. Olaparib stock preparing and establishment of PARP inhibitor resistant (PIR) cell clones

Olaparib stock preparing:

Olaparib (MW: 434.46) dissolved in DMSO with a stock concentration of 10 mM, separated into 20 µL aliquots and stored at -80°C for a maximum of 1 year. For each experiment, a fresh aliquot from this stock was removed and the final working concentration prepared in cell culture medium.

U2OS cell clones with resistance to PARP inhibitor Olaparib were established by treating Olaparib IC₅₀ doses determined according to the literature search. The IC₅₀ value is the concentration that inhibits the viability of half of the cells. Relevant sets of experiments were performed with continuous selection technique, as stated in the study published by Edwards et al. (Edwards et al. 2008). The cells seeded in 60 mm cell culture dishes were

treated with Olaparib for one week and first dose was 1 μM . U2OS cells were collected from culture plate and counted. As 2×10^5 were seeded into 60 mm plates. Cells allowed to attach plate face for 24 hours. The next day, cells attached to the cell culture dish surface were treated for 5 days with 1 μM of Olaparib prepared from Olaparib 10 mM stock solution. After 5 days, the U2OS cell line was re-treated and subcultured with increasing doses of Olaparib. As shown in Figure 2.1, increasing Olaparib treatment every five days resulted in a U2OS/PIR cell line resistant to the PARP inhibitor Olaparib. Part of the resulting U2OS/PIR cell line for use in studies continued to be cultured for use in analysis, while the other part was stored at -80°C .

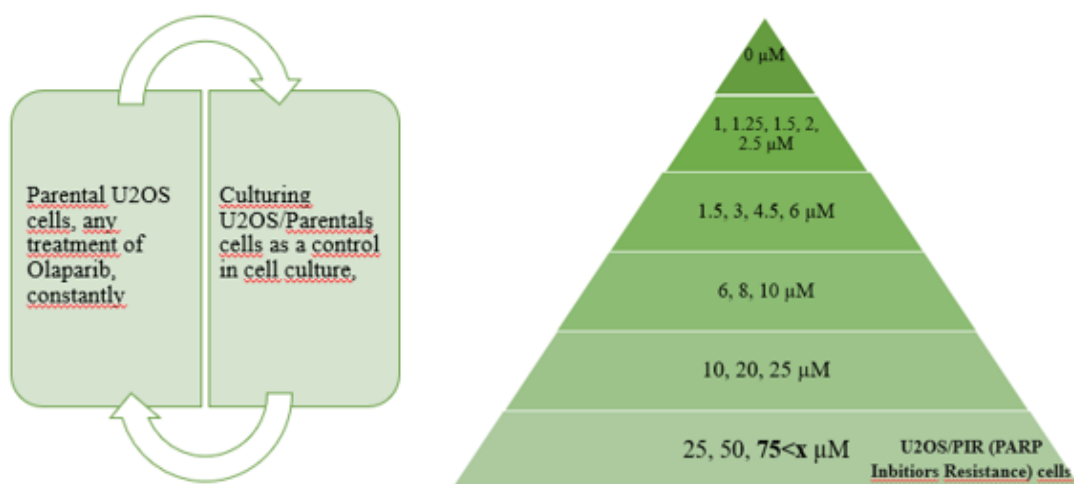


Figure 3.1. Establishment resistant cells. U2OS cells treated with Olaparib for each 5 days, in increasing doses

3.2.3. Silencing of hMOB2 Gene Expression by siRNA Transfection

RNA interference experiments were performed as instructed by the manufacturer and Gomez et al., (2015) (Gomez et al. 2015). Firstly, U2OS/PIR cells were collected with trypsinization from the cell culture plate and centrifuged at 2500 RPM at $+4^\circ\text{C}$ for 4 minutes and eventually resuspended in 4 mL fresh medium before being counted. Afterwards, 7.5 μL (75 μM) of siRNA (siCTL or siMOB2) and 15 μL of LipoRNAimax transfection reagent were diluted in 500 μL Optimem in two separate microcentrifuge tubes. Both siRNA/OPTIMEM and LipoRNAimax/OPTIMEM solutions were mixed carefully, and the final solution was incubated at room temperature for 15 minutes. Meanwhile, 1.5×10^6 cells were prepared in 2 mL fresh medium in two separate 15 mL falcons. At the

end of 15 minutes, 1 mL of siRNA/RNAimax/OPTIMEM suspension was mixed with 2 mL of cells/medium, and incubated at room temperature for another 15 minutes. The final mix contained 5×10^5 cells each mL and a concentration of 8,3 nm siRNA. 6 pcs of 6-cm petri dishes were prepared and labelled according to transfection time as follows: siCTL48h-72-96h and siMOB2 48h-72h-96h, then cells were seeded. At the end of associated transfection times, cells were collected day by day with trypsinization, washed with cold-PBS and saved at -80°C until Western blot analysis.

3.2.4. SDS-PAGE and Western blot

Cells were collected as follows; cells were exposed to trypsin-EDTA for 3-5 minutes in CO_2 incubator at 37°C and the detachment of cells was confirmed under invert microscopy. Cells were then pelleted by a centrifugation at 2500 RPM at $+4^\circ\text{C}$ for 4 minutes. Supernatant part was aspirated, and the cell pellet was resuspended in 1 mL of cold-PBS and subjected to a centrifugation at 2500 RPM at $+4^\circ\text{C}$ for 4 minutes. Later, cold-PBS was aspirated and cell were suspended and incubated in $100\mu\text{L}$ of Lysis buffer containing 1% protease and phosphatase inhibitors at $+4^\circ\text{C}$ on ice for 1 hour.

Table 1. The content of standard lysis buffer

Standard Lysis Buffer (150 mM)	
Content	Concentration
1 M Tris pH 8.0	20 mM
NaCl	150 mM
Glycerol	10 %
EDTA pH 8.0	0.5 M
EGTA pH 8.0	0.5 M
0.5 M NaF	50 mM
Na_3VO_4	1 mM

Table 2. The protease inhibitors using in the lysis buffer

Protease inhibitors	Concentration (%)
PMSF	1:1000
Benzamidine	1:1000
Leupeptin	1:1000
DTT	1:1000

100 μ L of Lysis buffer was used for each sample. Then, the cell suspension were centrifuged at 6600 RPM at +4°C for 10 minutes. Supernatants that contains soluble protein fractions were transferred into a new microcentrifuge tube labelled accordingly and pellet was discarded. Protein concentrations was detected by using a Bicinchoninic Acid Protein Assay kit (Bradford reactive). To prepare samples for the BCA test, 5 μ L of each samples was transferred into a spectrophotometer cuvette. Bradford reactive was diluted in ddH₂O (1:4) (reactive dye/dH₂O). Eventually, 1 mL of Bradford reactive solution was added in the cuvette containing the protein sample. Except protein samples, into another cryotube was added from lysis buffer as a blank solution. At 595 nm spectrum absorbances were detected. The concentrations of the samples were calculated with the help of the obtained spectrum absorbances. Afterwards, protein samples were mixed with the SDS-Laemmli buffer and boiled at 95°C for 5 minutes. Protein lysates were stored at -20°C until being analysed.

Table 3. The content of 5X SDS-Laemmli Buffer

5X SDS-LaemmliBuffer/100 ml	
Content	Volume
1 M Tris pH 6.8	12.5 ml
20% SDS	25 ml
dH ₂ O	57.5 ml

Table 4 (Continue). The content of 5X SDS-Laemmli Buffer

β -mercaptoethanol	5 ml
Glycerol	10 ml
Bromophenolblue (1%)	1 μ l

Before SDS-PAGE running stacking (5%) and separation (12%) gels were prepared.

Table 5. The content of stacking and separation gels

Kind of gel	Content	Volume (ml)
Stacking gel (5%) 3 ml	dH ₂ O	2.1
	30% Acrylamide mix	0.5
	1.0 M Tris pH 6.8	0.38
	10% SDS	0.3
	10% APS	0.3
	TEMED	0.003
Separation gel (12%) 10 ml	dH ₂ O	3.3
	30% Acrylamide mix	4.0
	1.5 M Tris pH 8.8	2.5
	10% SDS	0.1
	10% APS	0.1
	TEMED	0.004

To separate the proteins according to their molecular size in SDS-PAGE, a 12% separation gel and a 5% stacking gel were prepared. First, the separation gel was loaded into the gel chamber and left to polymerize, then the stacking gel was loaded and a 10-well comb was placed to wait for complete polymerization. After the polymerization was completed, the comb was removed and the SDS-Running buffer was loaded into the tank, and 5 μ L of molecular weight markers were loaded into the first well and samples were loaded into the other wells according to their absorbance values. Electrophoresis was started at 80 V and after passing to separation gel it upgraded to 120 V and left to run 1 hour. At the electrophoresis finished, transferring sandwich was prepared. PVDF membrane was activated in methanol. Respectively, a sponge-two filter papers-gel-membrane-two filter papers-a sponge were placed and so the proteins buried in the gel were transferred to a polyvinylidene fluoride membrane at the 400 mA 75 minutes within transfer buffer (EMD Millipore, Billerica, MA, USA). After transferring, membrane was washed in T-BST for 2X5 min.

Protein-transferred polyvinylidene fluoride membrane was blocked with 5% milk powder solution prepared in TBST; for 1 hour at room temperature to prevent non-specific antibody binding. In order to detect the target protein, the primary antibody specific for that protein was prepared by dissolving 5% milk powder in 5 ml of TBST and incubated overnight. Next, the primary antibody was removed from the membrane and the membrane was washed with T-BST for 3X10 min to remove the antibody residues. After the washing step, secondary antibodies specific to the primary targets were prepared by dissolving 5% milk powder in 5 ml of TBST and treated with the membrane at room temperature for 2 hours. After 2 hours, washing was done with T-BST for 3X10 minutes to remove unbound antibodies.

After whole incubations, membrane was incubated with A and B solutions (1:1) for 2 minutes by shaking then protein bands were detected by exposing X-ray film.

Table 6. The dilutions of antibodies for Western blot

Antibody	Dilution	Incubation time
Primary antibody		
MOB2	1:50	Overnight / +4°C
p53	1:1000	Overnight / +4°C
p21	1:500	Overnight / +4°C
Tubulin	1:500	Overnight / +4°C
GAPDH	1:1000	Overnight / +4°C
Secondary antibody		
Anti-rat	1:5000	2 hours/ room temperature
Anti-rabbit	1:5000	2 hours/ room temperature
Anti-mouse	1:5000	2 hours/ room temperature

3.2.6. In vitro colony survival analysis (Clonogenic Assay)

Cell colony formation assays were performed as indicated at Gomez et al. (2015). Pilot experiments were conducted to determine the number of cells to be treated, the dosages to be used during the treatment, and the duration of treatment. 1500 cells were seeded into 6 cm petri dishes in 3 mL complete medium and allowed to colony formation and waited for 9-12 days at 37°C in CO₂ incubator. Every 4 days, medium was changed with fresh medium carefully and followed under an invert microscope. When colony formation was completed (at least 50 cells per colony), medium was discarded and cells were washed with PBS (-Mg/-Ca). Firstly, cells were fixed using the methanol-acetic acid solution (3:1) for 5 min, then stained using 0.5% crystal violet dye for 15 minutes. Consequently, crystal violet dye was aspirated and all wells were washed with water and occurred colonies counted by an invert microscope.

4. FINDINGS AND DISCUSSION

4.1. Establishment of U2OS/PIR Cells

Olaparib resistant U2OS cell clone (PIR: PARP inhibitor resistant) was generated by continuous selection upon Olaparib treatment as explained previously. At the point of >75 μM , U2OS/PIR resistant cells were kept in DMEM medium containing 1 μM Olaparib to stabilize resistance.

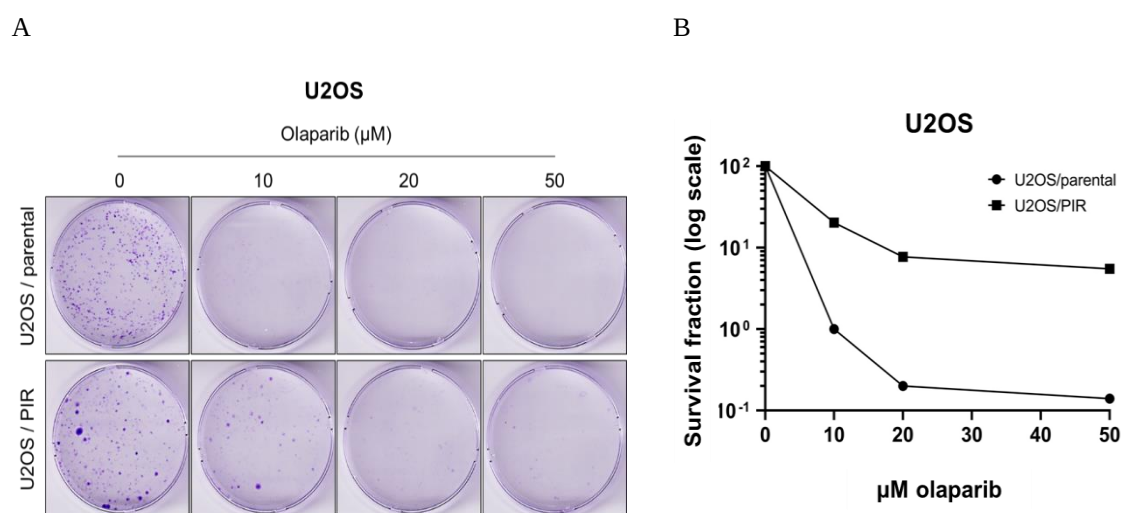


Figure 4.1.1. The confirming of the resistance against to Olaparib. (A) Resistance to Olaparib in U2OS cells was confirmed by clonogenic assay and colonies formed were compared with U2OS/Parental cells. After the cells were seeded in culture dishes, they were exposed to 0-10-20 and 50 μM Olaparib for 72 hours. Colonies formed at the end of 8-12 days were fixed, stained, dried and eventually calculated. (B) Line graph represents the survival fractions of treatment groups compared with parental cells

The generated Olaparib resistance was confirmed by colony survival assays. The U2OS/Parental and U2OS/PIR cells were seeded into 6-cm cell culture dishes in 3 mL medium and allowed to attach them for 24 hours. Later, the cells were treated with 0, 10, 20 and 50 μM Olaparib for 72 hours. Every 5 days the mediums were changed with fresh

medium. At the end of 8-12 days, the assay was stopped and the colonies formed were fixed and stained (Figure 4.1.1.A, B). It was observed that the overall survival rate of the Olaparib-resistant U2OS cells (U2OS/PIR) was significantly higher compared to parental counterparts upon increasing doses of Olaparib treatment. Before starting further analysis, U2OS/Parental and U2OS/PIR cells were harvested from cell culture using Trypsin-EDTA. Protein lysates were obtained from cells in lysis buffer containing protease inhibitors. Concentrations of proteins were determined in spectrophotometer using Bradford reagent. Then, the protein samples were prepared by adding 5X SDS-Laemmli Buffer and boiling them at 95 °C for 5 minutes. Protein samples were run in SDS-PAGE for 1 hour. Then, the proteins transferred to the PVDF membrane were exposed to blocking in 5% milk powder solution for 1 hour at room temperature. At the end of blocking, they were incubated with anti-MOB2 (1:50/ON) primary antibody and anti-rabbit (1:2500/2h) secondary antibody. After incubation, X-Ray results were obtained from the membrane incubated with solutions A and B and normalized to the GAPDH cleaning protein as shown in Figure 4.1.2. The hMOB2 protein levels between U2OS/Parental and U2OS/PIR cells were compared.

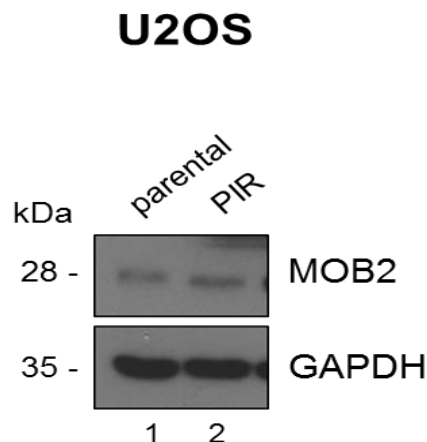


Figure 4.1.2. Verify the level of hMOB2 antibody in U2OS/Parent and U2OS/PIR cells. hMOB2 protein levels were tested with anti-MOB2 (1:50/ON) primary antibody and anti-rabbit (1:50/ON) in U2OS/Parental and U2OS/PIR ($p > 75 \mu\text{M}$ Olaparib) cells. :2500/2h) after membrane transfer, secondary antibodies and proteins were blocked after running on 12% gel by Western blot and results were normalized to GAPDH clearance protein

4.2. Loss Of hMOB2 Affects The Survival of U2OS/PIR Cells

To observe the effect of hMOB2 protein on the sensitivity of U2OS/PIR cells to PARP inhibitors, the MOB2 protein was transiently silenced by the siRNA transfection technique (Figure 4.2.1). RNAi is the technique of silencing through an endogenous or exogenous RNA molecule (miRNA, siRNA) with high homology to the target mRNA sequence. The increase in mRNA homology is directly proportional to the silencing of gene expression. As a result, it is employed as a therapeutic tool in the treatment of many cancers and diseases. siRNA are short RNA strands designed in vitro to trigger interaction with RNA in mammalian cells (Karagün et al., 2014; Rahmawati, 2009; Барышников, 2013). At the 5' and 3' ends, there is an unpaired 2-3 nucleotide terminal and an unstable cut area in the center of the chain. Dicer enzyme, a member of the RNase III family, cuts 70/80 bp double-stranded RNA at its 3' ends, leaving 2-3 ntds to form small siRNAs of 20-25 bp (Guzelgul and Aksoy, 2009). It allows RNase III enzymes to recognize the unpaired 2-3 ntd target mRNA at the ends and also offers stability to its own structure. The short siRNA strand is attached to the RISC complex. Taking the rope in the RISC complex as a guide; from the 5' end to the guide strand, the complementary is bound to the target mRNA strand, and the target mRNA is cleaved by endonuclease enzymes. Degraded mRNA strand fragments are digested and removed from the cell by cytoplasmic endonuclease enzymes (Gündoğdu and Çelik, 2009). Enzymatic and chemically synthesized siRNA molecules are often transfected into cells using lentiviral vectors. Many lentiviral vectors are employed in vector-mediated siRNA delivery methods. The most frequent are lentiviruses and adenovirus-dependent viruses. Some research have found that attaching different antibodies, target organ-specific ligands, or vitamins to the siRNA agent facilitates in vivo siRNA targeting (Rahmawati, 2009; Jonathan K. Watts 2012).

U2OS/PIR cells were harvested from cell culture and counted. 7.5 μ L (75 μ M) of siRNA (siCTL or siMOB2) and 15 μ L of LipoRNAimax transfection reagent were diluted in 500 μ L of Optimem in two separate microcentrifuge tubes. siRNA/OPTIMEM and LipoRNAimax/OPTIMEM solutions were mixed and incubated at room temperature for 15 minutes. Meanwhile, 1.5×10^6 cells were prepared in 2 mL of medium. 1 mL of siRNA/RNAimax/OPTIMEM suspension was mixed with cells and incubated for 15 minutes at room temperature. Cells were then seeded as 2 groups of 6 cm plates: siCTL48h-

72-96h and siMOB2 48h-72h-96h. At the end of the associated transfection times, cells were harvested. The effect of hMOB2-siRNA was evaluated 48, 72 and 96 hours after transfection.

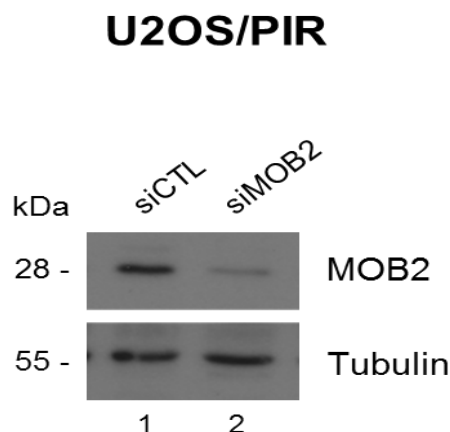


Figure 4.2.1. Confirmation of hMOB2 transfection in U2OS/PIR cells. U2OS/PIR cells were incubated with siCTL or siMOB2 transfection agents for 15 min at room temperature and seeded in 6 cm plates. The medium was refreshed 6 hours after transfection. 72 hours after transfection, cells were harvested using trypsin-EDTA for Western blot analysis and protein isolation was performed. Protein samples run in 12% SDS-PAGE were treated with the primary antibody MOB2 (1:50) overnight and exposed to anti-rabbit secondary antibody (1:5000) for two hours at room temperature after blocking to the membrane. It was confirmed whether hMOB2 transfection in cells was successful in the control (siCTL) and transfection (siMOB2) groups, and the results were normalized to Tubulin housekeeping protein

The efficiency of hMOB2 knockdown was confirmed by Western blot analysis. siMOB2-U2OS/Parental and siMOB2-U2OS/PIR cells were harvested from cell culture using trypsin-EDTA. Protein lysates were obtained from the lysed cells. Protein samples run on 12% SDS-PAGE were transferred to PVDF membrane and incubated with primary antibody MOB2 (1:50) overnight followed by anti-rabbit secondary antibody (1:5000) for two hours at room temperature. It was confirmed whether hMOB2 transfection in cells was successful in the control (siCTL) and transfection (siMOB2) groups, and the results were normalized to the Tubulin housekeeping protein (Figure 4.2.1). While hMOB2 protein expression was seen on control cells (siCTL group), the expression was not observed on siMOB2-treated cells (siMOB2 group), confirming the successful downregulation of hMOB2 mRNA. U2OS/Parental and U2OS/PIR cells transfected with the siRNA agent were seeded in 6 cm cell culture dishes and the colony formation assay was stopped at day 12. The colonies formed were compared with both non-resistant U2OS/Parental cells and the control (siCTL) group. Loss of hMOB2 expression in response to decreased colony

formation in U2OS/Parent cells resulted in decreased colony survival in U2OS/PIR cells (Figure 4.2.2.A, B).

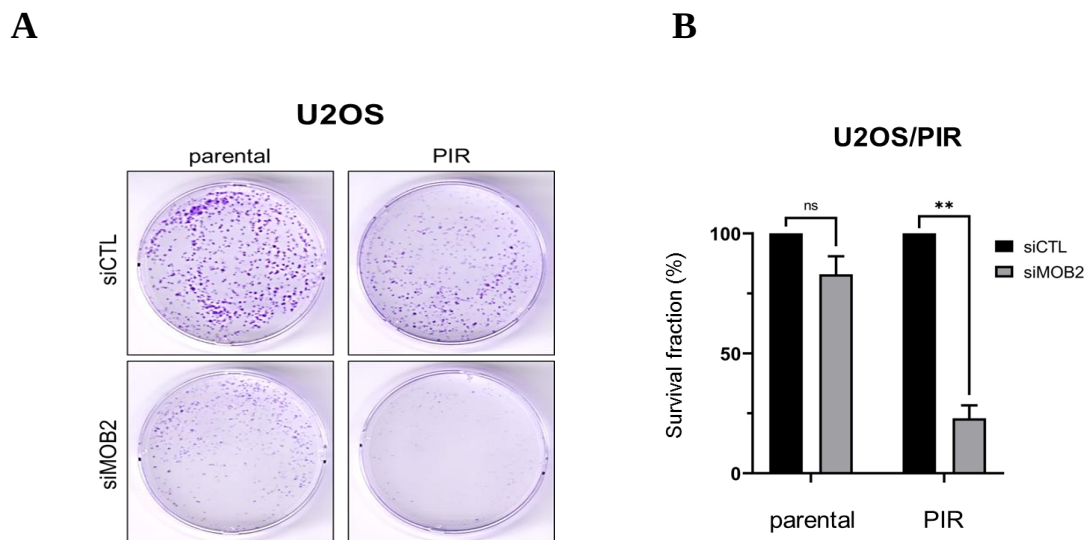


Figure 4.2.2. Investigation of colony formation ability as a result of transient silencing of hMOB2 protein by transfection method. A) Cells transfected with the siRNA agent was seeded into culture dishes and allowed to form colonies for 8-12days. The colonies formed were compared with both non-resistant U2OS/Parental cells and the control (siCTL) group. B) Representation of colonies formed after the effect of transfection on colony formation with bar bars. The decrease in colony formation in U2OS/Parental cells was not statistically significant. There was a significant decrease in colony formation in resistant cells

After transfection, a colony formation experiment was done to see if hMOB2-transfection had any influence on the survival fraction of cells. When U2OS/Parental cells were compared to U2OS/PIR cells in a colony formation experiment, colony formation was still present in the U2OS/Parental cells, but not in the U2OS/PIR cells. The presence of hMOB2 is required for cell survival and colony formation, according to this finding.

4.3. Expression Profile of p53/p21 In U2OS PIR Cells

We were curious about the effect of this combination which on proteins involved in DNA damage signalling, assuming that we produce a significant loss in DNA damage repair by reducing the expression of both the hMOB2 protein and PARP activity, which is implicated in DNA damage repair. However, with hMOB2 silencing, the mechanism underlying the reduction in colony formation as well as cell survival, we were able to verify using Western

blot the critical DNA damage protein p53 and the results were normalized to housekeeping protein Tubulin. As shown in Figures 4.3.A and B, hMOB2 and p53 protein levels were normal in parental U2OS cells. The transfection of hMOB2 was effective in transfected U2OS/PIR cells, therefore there was no protein band; similarly, the p53 protein level was the same in U2OS/PIR control cells. Surprisingly, when we examined cell cycle checkpoint proteins, we discovered that the expression protein level of p21 had significantly risen (Figure 4.3).

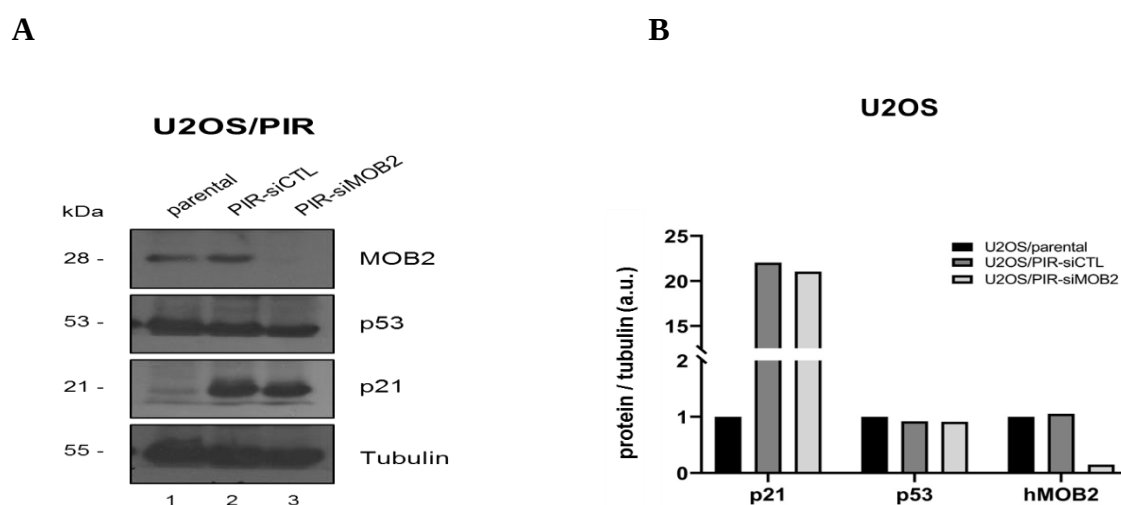


Figure 4.3. Investigation of hMOB2, p53 and p21 protein expressions levels after transfection. A) Determination of the effect of siMOB2 transfection on the expression of p53 and p21 proteins, which are important markers in DNA damage signalling, by Western blot. Protein samples run in 12% SDS-PAGE were treated with primary antibodies of p53 (1:1000), p21 (1:500), and MOB2 (1:50) overnight and after blocking to the membrane, afterwards, it was exposed to anti-mouse and anti-rabbit secondary antibodies for two hours at room temperature. The results were normalized to Tubulin housekeeping protein. B) Protein levels graphic via bars. Representation of the changes in p53, p21 and MOB2 protein expression levels after siMOB2 transfection with bar bars

4.4. Loss hMOB2 Potentiates The Chemotoxicity of Olaparib in U2OS PIR cells

To investigate the effect of co-inhibition of hMOB2 protein and PARP enzyme on the colony forming abilities of U2OS/PIR cells, clonogenic assays were performed. Cells transfected with siRNA reagents were seeded in 6 cm cell culture dishes and treated with different doses of Olaparib for 72 hours after adherence to the culture dish surface. The medium was refreshed every 3-4 days. After 8-12 days, the colony survival of hMOB2-effective and -defective U2OS/PIR-Control and U2OS/PIR-siMOB2 cell colonies cells was compared. As shown in Figures 4.4.A and B, colonies in the siCTL, 1 and 5 μ M

Olaparib groups were similar despite increasing Olaparib doses. However, with 10 μ M Olaparib treatment, a significant reduction in the formation of colonies was observed. No colony formation or statistically significant survival fraction was observed in the 25 and 50 μ M Olaparib treatment groups.

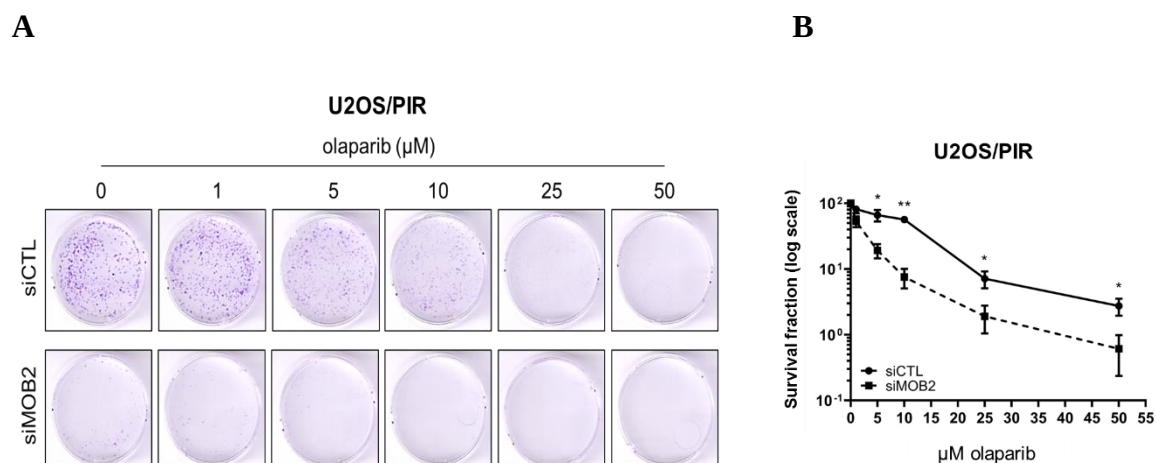


Figure 4.4. Clonogenic assay by co-administration of siMOB2 transfection with increasing doses of Olaparib therapy. A) U2OS/PIR cells transfected with siMOB2 were seeded into cell culture dishes and allowed to adhere for a day. Cells were then exposed to increasing doses of Olaparib treatment at 0, 1, 5, 10, 25 and 50 μ M for 72 hours. At the end of 8-12 days, the experiment was terminated and cells were fixed and stained with crystal violet dye and colonies were calculated. B) Representation of the survival fraction of colonies resulting from simultaneous administration of siMOB2 transfection and increasing doses of Olaparib therapy

The analysis of the group with siMOB2 transfection have shown that there was lower colony formation in the 0 μ M Olaparib group compared to the siCTL group. In the siCTL groups, colony formation in the 1 μ M Olaparib treatment group was similar to that in the control group. There was a partial decrease in colony formation in the 5 and 10 μ M groups. However, colony formation paused with 25 and 50 μ M Olaparib treatment (Figure 4.4.A). In the siMOB2 groups, there was a statically significant decrease in the survival fraction of U2OS/PIR cells with Olaparib treatment (Figure 4.4.B). Our experiments revealed that the targeting hMOB2 expression may offer a potential therapeutic option for selectively eliminating PARP inhibitor-resistant cancer cells. In addition, it can be said that hMOB2 protein is an important protein in terms of sensitivity to Olaparib treatment. Because the cell viability in the presence of hMOB2 continued, in the case of loss of hMOB2, colony formation decreased because there was greater sensitivity to Olaparib treatment.

5. CONCLUSIONS AND RECOMMENDATIONS

Cancer is a type of disease characterized by the spread of uncontrolled and irregularly proliferating cells to different tissues from the tissue they originated in the body (Lauren Pecorino, 2016). Cancer cells have the ability to achieve replicative immortality by generating spontaneous growth and division signals, to escape from growth suppressing factors, to survive controlled cell death apoptosis, to resist cell death signals, to invade other tissues from the point of origin, to metastasize, and to perform angiogenesis. These characteristic features of cancer lead to dynamic changes in the genome (Hanahan & Weinberg, 2000; 2011). One of the major characteristics of cancer is the build-up of genomic instability, which develops primarily as a result of poor DNA damage response to endogenously or exogenously produced genetic damages (Hanahan & Weinberg, 2011; Jackson & Bartek, 2009).

The DNA damage response is a complex mechanism developed to protect and maintain genomic integrity against DNA damage in mammalian cells. When DNA damage occurs, the damage signal is received by the receptors and transmitted to the corresponding sub-pathways. This signal transmitting, arresting the progression of the cell cycle or replication fork is also part of the DDR system (Curtin, 2013). Single or double strand breaks are the most dangerous kind of DNA damage (Jackson & Bartek, 2009). Upon receipt of the damage signal, first of all, ATM and ATR proteins are activated. ATM, which is activated in response to DSB, phosphorylates and activates various proteins, including CHK2, BRCA1 and p53 proteins (Jackson & Bartek, 2009). Another early and important activation in DDR is the binding of the DNA break sensor, the MRN complex, to the DSB ends, initiating biochemical signalling. The MRN complex is important for the binding of DDR components, such as ATM, to DSBs (Ciccia & Elledge, 2010; Goldstein & Kastan, 2015). DDR activations are required for the preservation of genome stability and the continuation of cell viability in the presence of DNA damage, but in the process of carcinogenesis,

defective or alternate DDR activities endanger genome stability and allow for the transfer of flawed DNA (Curtin, 2013; Jackson & Bartek, 2009). Cancer cells may choose other alternative DDR pathways as a result of the deficient DDR pathways of genomic instability and endogenous/exogenous DNA damage accumulating in comparison to normal cells. In this way, upregulation of DDR pathways promotes the gain of resistance to the chemotherapeutic agent (Connor, 2015). Therefore, determining the components of the DDR mechanism as markers is a critical starting point in terms of preventing tumour formation, suppressing it or increasing the effectiveness of chemotherapy. We can list the DDR components as ATM, ATR, PARP, CHK1, DNA-PK and WEE1 proteins in general. Knowing at what stage of the cell cycle and type of DNA damage occurs in the progression of the DDR mechanism is critical in therapeutically targeting DDR (Connor, 2015; Curtin, 2013; Desai et al., 2018).

NHEJ is mediated by DNA-PK. The DNA-PK protein kinase facilitates the recruitment of the corresponding repair proteins towards the vicinity of damaged chromatin by phosphorylating the relevant sub-pathway substrates upon the occurrence of a strand break at any stage of the cell (Cleary et al., 2020; Stover et al., 2016).

PARP, as another DDR component, polymerizes upon DNA strand break, thereby initiating signalling for strand break repair. Considering the effects of therapeutic targeting of DDR components on cancer cells, approaching these pathway components in anticancer treatments is a promising strategy (Curtin, 2013). In addition, PARP inhibitors are known to be more effective in BRCA1/BRCA2-defective cells because the BRCA1/BRCA2 proteins are required in HR-mediated repair of DSBs (Bryant et al 2005; Connor, 2015; Farmer et al., 2005; Trenner & Sartori, 2019).

The notion of synthetic lethality is based on the principle of targeting and suppressing components of potential alternative pathways based on a malfunction in one of the active DDR pathways. When the accumulated DNA damage becomes irreparable, cell viability is likely to terminate (Connor, 2015; Minchom et al., 2018; Nickoloff et al., 2017). Important developments in synthetic lethality were pioneered in 2005 by Farmer et al.'s work with PARP inhibitors in BRCA1 and BRCA2 defective breast cancer cells. They discovered that there is a synthetic lethal interaction between heterozygous mutations in

BRCA1/2 genes and PARP enzyme inhibition in cancer types such as ovarian, pancreatic, breast and prostate (Ashworth & Lord, 2018; Farmer et al., 2005).

PARP inhibitors inhibit the enzymatic activity by trapping the PARP enzyme on SSB, so that SSB cannot be repaired and increased SSBs turn into DSB. DSBs are highly toxic to the replication fork and cause the fork to stop expanding. DSBs cannot be repaired by the HR mechanism in BRCA1/2-defective cells. As a result, the cell cannot fight the accumulated DNA damage and HR-defective cells selectively cease to exist (Ashworth & Lord, 2018; Helleday, 2011; Huang et al., 2020; Neil et al., 2017).

Unfortunately, in practice, a positive response is not always possible. Although the concept of synthetic lethality is a promising clinical strategy, cancer cells may develop resistance to treatments through genetic or acquired mechanisms. There are several known potential mechanisms for resistance to PARP inhibitors. HR restoration is the best known of the possible resistance mechanisms. Under normal conditions, repair and recovery of damaged HR generates a secondary mutation in BRCA1/2 deficient cells (Neil et al, 2017; Rose et al, 2020). As a result, RAD51 recombinase DNA is loaded into damaged chromatin, HR is compensated and the replication fork is preserved, replication of damaged DNA continues (Andrea, 2018; Lord & Ashworth, 2013; Rose et al., 2020). Bunting et al. (2013) showed in a study that deficient activity or absence of 53BP1, which is also involved in the NHEJ pathway, restores HR and is required for cell death in BRCA-defective cells (Bunting et al., 2013). Pgp pump ATP Binding Cassette B Subfamily Members 1 and 2 (Abc1a/b) proteins are located at exit from the cell. Abnormal translocation of PARPi to the outside of the cell without being taken up by Abc1a/b proteins results in decreased intracellular PARPi activity and increased PARPi resistance (Andrea, 2018; Lord & Ashworth, 2013; Rose et al., 2020).

The human MOB (hMOB) proteins involved in the Hippo signalling pathway interact by directly binding to the NDR/LATS kinases of which they are regulators. The hMOB2 promotes cellular survival and cell cycle arrest by triggering DDR signalling in response to external DNA damage. hMOB2 deficiency leads to accumulation of endogenous DNA damage, Accumulated DNA damage induces the ATM/CHK2 signalling pathway and results in p53/p21-mediated G1/S cycle arrest. In the study by Gomez et al., increased

sensitivity to DNA-damaging agents and IR treatment were observed in hMOB2-defective U2-OS cells compared to control cells. In addition, a p53/p21 dependent cell cycle arrest was seen in the G1/S checkpoint as a result of hMOB2 depletion. This strengthened the hypothesis that hMOB2 supports survival against DNA damage (Gomez et al., 2015). The study of Gomez et al. in 2015 adds to the evidence supporting this notion. In the related study, IR and doxorubicin treatment caused DNA damage in hMOB2-depleted cells, and it was discovered that hMOB2 depletion increased DNA damage, ATM and CHK2 activation, and as a result, the p53-dependent G1/S loop checkpoint was activated (Gomez et al., 2015). The findings demonstrated that hMOB2 is needed for cell survival and G1/S phase arrest. Given these findings, the question of whether hMOB2 could be a DDR component becomes much more important. In response to this inquiry, RAD50 was discovered as hMOB2's binding partner by yeast 2-hybrid screening. This interaction is significant since RAD50 is the primary component of MRN. DDR degradation was detected in cells lacking hMOB2 as a result of decreased MRN activity. Because hMOB2 is required for the uptake of MRN and active ATM into damaged chromatin (Activated ATM protein, CHK2, phosphorylates NBS1, one of the MRN components, in addition to p53). These findings imply that the hMOB2 protein is required for genomic stability in the DDR pathway. Thus, in general, hMOB2 is required as a DDR element to support DDR signalling, cell cycle checkpoint activations, and cell survival. Therefore, elucidating the binding and working partners of hMOB2 will take mechanistic understanding to the next level (Gomez et al., 2015; Gundogdu & Hergovich, 2019, 2016).

Gundogdu et al. (2021-a) have recently demonstrated that hMOB2 promotes cell survival in response to bleomycin, which breaks DNA, mitomycin C, which generates DNA cross-links (ICL), and cisplatin therapies. They also discovered that hMOB2 deficiency in U2-OS cells impairs the replacement of RAD51 nucleofilaments in damaged DNA segments with RPA, resulting in HR-dependent DNA double-strand break repair defects. In other words, RAD51 phosphorylation is disrupted in hMOB2-defective cells, and interchange of RPA complex on the ssDNA end and RAD51 recombinase cannot occur, preventing HR repair. Another significant finding is that hMOB2-deficient cancer cells are sensitive to the PARP inhibitors Olaparib, Rucaparib, and Veliparib. Gundogdu et al. shown in 2021 that Upon expression of I-SceI with the DRGFP system, which allows HR-directed repair of DSBs induced by I-SceI endonuclease in a mutated GFP gene, cells lacking hMOB2

showed approximately 50% reduction in HR activity compared to control cells. DNA interchain cross-linker cisplatin and DNA-damaging antibiotic bleomycin (supports DSB formation) sensitized cancer cells to these chemotherapeutics as a result of hMOB2 depletion. As a result of hMOB2 degradation, cell viability was significantly reduced by bleomycin treatment. To observe a synthetic lethal interaction of hMOB2 loss with PARP inhibition, hMOB2-depleted cells were treated with the PARP inhibitors olaparib, veliparib, and rucaparib. In the HCT116 cell line, the combination of PARP inhibition and hMOB2 depletion promoted a decrease in cell viability and increased cytotoxicity in cells. The sensitivity created by hMOB2 deficiency in cells where PARP enzyme is inhibited provides us with a biomarker in cells with HR defects (Gundogdu et al., 2021-a).

Given that PARP inhibitor resistance through difference cellular mechanism has become a major challenge in the clinic, it is clinically significant to define and characterise novel molecular targets that may enhance the anti-cancer activity of PARP inhibitors (Rose et al., 2020). Loss of hMOB2 impairs the efficacy of HR-dependent double-strand break repair, and therefore, hMOB2 deficiency renders cancer cells sensitive to PARP inhibitors. We hypothesised that down-regulated hMOB2 expression is likely to re-sensitise PARP-inhibitor resistant cancer cells towards PARP inhibition.

In this study, a U2OS/PIR resistant clones was created to understand the mechanism that contributes to PARP inhibitor resistance and the effect of hMOB2 protein, which has a critical role in the DDR pathway. The continuous selection method was used in studies based on the 2008 publications of Edwards et al. when establishing a cell line resistant to PARP inhibitors. U2OS cells were subjected to continuous and gradually increasing doses of Olaparib in cell culture. The existing medium was replaced with a fresh medium containing the inhibitor every four days (Edwards et al., 2008).

In this study, for four months, U2OS/PIR cells were cultured with increasing doses of Olaparib. Beginning with 1 μ M, doses of Olaparib for treatment were increased to 1X1.25 and cells were treated for at least 1 week at each dosage. The U2OS parental cell line was also cultured to compare the results obtained at the molecular level. Meanwhile, when we examined the cell shape, we discovered that the resistant cells were slightly smaller in size and multiplied faster than the parent cells.

In accordance with the purpose of this study, firstly, the expression level of hMOB2 protein in U2OS/Parent and U2OS/PIR cells was examined. When hMOB2 expression levels were compared between the two cell lines, hardly any significant difference was observed (Figure 4.1.2). It was discovered that the hMOB2 protein levels were slightly higher in cells that developed resistance to Olaparib (U2OS/PIR) compared to U2OS/Parental cells. Colony formation assays in U2OS parental and U2OS/PIR cell lines subjected to escalating dosages of Olaparib as 0, 10, 20, and 50 M, verified the assessment of the resistant cell line. Here, Olaparib therapy resulted in a decrease in growth and an increase in the timeline of colony formation in the U2OS parental cell line. On the contrary, despite the higher dose of Olaparib, the resistant cell line's capacity to form colonies persists, as predicted (Figure 4.1.1.A, B). It is known that Olaparib inhibits damage-repair by trapping and blocking the PARP enzyme, which is activated along with the DNA damage signal and binds to the damaged region. Olaparib action is not seen in normal cells since homologous recombination is functional, but it has a DDR suppressive impact in malignant cells, particularly those with homologous recombination abnormalities. Consistent with the clonogenic analysis result of this study, cells whose DNA cannot be repaired are sentenced to death as a result of accumulated damage and a decrease in cell viability is observed. It indicates that the amount of DNA damage is higher in resistant cells. These cells have a DNA damage signal, but the cycle checkpoint is not reached, the damage is repaired or continues to proliferate without repair (Fig. 4.1.2).

In addition to these, in the resistant U2OS/PIR cells generated, the DDR pathway signalling in response to DNA damage allows the cell to survive the chemotherapeutic impact by removing the chemotherapeutic effect from the homologous recombination stage via whatever alternative pathway it has formed. It is critical to explore the biochemical components of the homologous recombination mechanism in terms of therapeutic effectiveness and resistance at this time.

To further understand the role of the hMOB2 protein in cell survival and DNA damage repair, this study investigated the effect of temporary silencing of hMOB2 mRNA via RNA interference. First, the hMOB2 protein was temporarily silenced using the siRNA/RNAimax agent, and the correctness of transfection with the hMOB2 antibody was

confirmed by Western blotting analysis (Figure 4.2.2.A, B). The hMOB2 protein band was found in the siCTL group, as predicted, but not in the siMOB2 group.

By transfection, the hMOB2 protein was temporarily silenced and the effect of hMOB2 protein on the maintenance of cell viability was observed. In the siCTL groups of cells transfected with the hMOB2 protein, there was colony formation in both U2OS/Parental and U2OS/PIR cells. As we expected, colony formation was observed in U2OS/Parental cells in siMOB2 groups but not in U2OS/PIR cells. Based on this, this study concludes, in agreement with the references in the literature review, that the hMOB2 protein is required for the maintenance of cell viability (Fig. 4.2.2.A, B). This is because, the DDR signal activated for DNA damage in the presence of hMOB2 and the interaction with the MRN complex component RAD50 cannot be regulated in hMOB2-defective cells, the damage cannot be repaired and the cell viability decreases (Gomez et al., 2015; Gundogdu & Hergovich, 2019). It is understood that the hMOB2 protein modulates the induction of DDR signalling, cell cycle arrest and viability suppression, damage accumulation, and cell cycle arrest by induction of exogenous or endogenous DNA damage, based on the obvious effect of the hMOB2 defect in suppressing colony formation in the resistant cell line. In this study, it was discovered that one or more of the aforementioned routes can lower the viability of the resistant cell line and diminish colony formation via transfection of hMOB2. In order to understand the mechanism behind this effect, detailed analyses on these pathways are required and these results support the idea that chemotherapeutic intervention of the hMOB2 protein in resistant cancer cells is a promising approach.

Interestingly, unlike the U2OS/Parent cells, there was no significant difference in the p53 protein level in the transfected Olaparib-resistant U2OS cell line, while an increase in the p21 protein expression level was reported (Figure 4.3A, B). By binding to Cdk4-6/Cyclin, Cdk2/Cyclin E, and Cdk2/Cyclin A, we know that p21 inhibits these complexes. The cell cycle is interrupted by inhibiting these cyclin-dependent kinases, which act as cell cycle checkpoints. p21 not only stops the cell cycle, but also stops DNA synthesis by binding to the proliferation-triggering cell nuclear antigen (PCNA, Proliferating cell nuclear antigen) complex, which is necessary for DNA replication, and inhibiting DNA polymerase (Kucuksayan & Akca, 2018). In our results, the key question is the mechanisms by which the increase in p21 protein level observed in Olaparib-resistant cells is induced. As we

mentioned above, the p21 protein is induced by p53 and is among the main regulators at the point of whether the cell will continue to live or not. In addition to mechanisms such as HR restoration, decreased HR versus increased NHEJ activity, and P-gp pumps over-activity which contribute to PARP inhibitor resistance, the difference in p21 protein level obtained in this study led to the questioning of whether there is another possible mechanism of resistance. Recent findings that the cytoplasmic p21 protein, which is increased in cancer cells, functions oncogenic and anti-apoptotic, shows that p21 protein is effective in the carcinogenesis process independently of p53 (Georgakilas et al., 2017; Ohkoshi et al., 2015). p21 protein, in various cellular settings, depending on its subcellular localization, it can exhibit both anti-apoptotic (cytoplasmic location needed) and pro-apoptotic (nuclear location required) action (Ohkoshi et al., 2015). It provides p53-independent regulation in cellular processes such as cell cycle arrest, apoptosis, and DNA repair (Georgakilas et al., 2017). Furthermore, clinical research on the presence of p21 in tumour tissues have revealed that elevated cytoplasmic p21 protein amounts result in a poor clinical picture, particularly in glioma, prostate, cervical, ovarian, and esophageal malignancies (Abbas & Dutta, 2009). Furthermore, as stated in (Gomez et al., 2015; Gundogdu & Hergovich, 2016), DNA damage accumulates as a result of hMOB2 protein suppression, and the levels of p53 and p21 proteins rise in response to increasing damage. As a result, the cell cycle is halted at the G1/S phase. At this stage, understanding the process will be more effective when DNA damage activates the ATM-p53-p21 pathway and the connection of the hMOB2 protein with RAD50, one of the MRN components of the damage sensor complex, are studied together.

Following the examination of the effects of transiently silencing the hMOB2 protein in U2OS/PIR cells, this study sought to explore how the combination treatment with sihMOB2 and Olaparib would affect these cells by administering escalating dosages of Olaparib to the transfected cells (0, 1, 5, 10, 25, and 50 μ M). For this purpose, clonogenic assay was performed (Figure 4.4.A, B). Using the siRNA-hMOB2 complexes, we transiently transfected U2OS/PIR cells using RNA interference technology.

First of all, our findings revealed that hMOB2 depletion negatively affected the colony forming ability of U2OS/PIR cells. Indeed, loss of hMOB2 expression induced a significant reduction in the number of colonies in U2OS/PIR cells in the absence of PARP

treatment. Furthermore, our clonogenic assays deciphered that the inhibition of PARP activity further sensitized U2OS/PIR cells towards hMOB2 knockdown. In fact, even 1 μ M of olaparib treatment showed a significant synergistic effect with hMOB2 inhibition to reduce the colony forming capacity of olaparib-resistant U2OS cells. Based on these findings, it can be concluded that co-inhibition of hMOB2 and PARP activities is more effective in reducing colony formation in resistant cancer cells compared to olaparib monotherapy.

In conclusion, the addition of hMOB2 loss to Olaparib therapy appears to be a viable target for overcoming drug resistance in the clinic, owing to improved sensitivity to therapy. Olaparib's selective lethal action can thus be used in this manner. Olaparib can be used to selectively screen hMOB2-defective cells during the DNA damage stage. Furthermore, when Olaparib is used in monotherapy to create toxic effects, smaller dosages can be employed, minimizing the utilization of harmful chemicals in the cell. There was a reduction in viability even at the lowest Olaparib dosages due to the synergistic impact of Olaparib with loss of hMOB2.

In addition to these research, the U2OS/Olaparib resistant cell lines generated in this work can be utilized to better understand the mechanisms of drug resistance in order to overcome drug resistance that has been formed or acquired. The results obtained here can be combined with different chemotherapeutic drugs. Furthermore, the possible effects of hMOB2 transfection and Olaparib combination on important cellular signalling pathways can be investigated. In this way, different factors or mechanisms affecting drug resistance can be clarified. Cell cycle analysis can be used to better understand cell cycle arrest. Apoptosis and caspase activity in cells can be investigated to discover if this combo treatment induces them.

In summary, in the light of the data obtained in our research, several critical points are important regarding clinical management of PARP inhibitor-based treatments: Olaparib treatment in BRCA1/2 defective cells provides efficiency with selective lethality, it widely introduces DNA damage by suppressing PARP enzyme activity. Cells that can establish an alternative pathway, however, continue to survive at this stage. Because of its close connection with the DNA damage sensor complex MRN-RAD50, the hMOB2 protein is

vital in DDR signalling and plays critical functions in DDR. When the cytotoxicity caused by the inhibition of PARP inhibitors combined with the hMOB2 deficiency that supports HR-dependent DSB repair, the specifically increased PARPi sensitivity shows us the synthetic lethality activity between PARPis and hMOB2 deficiency. The possible mechanism by which this interaction is based is that both the PARP enzyme and the hMOB2 protein are involved in DDR pathways. In the results we obtained here, we see that under normal conditions, when we target hMOB2, which activates the ATM/p53/p21 signalling pathway in response to DNA damage, which causes the cell cycle to stop in the G1/S phase, instead of repairing the damage in the cell, it leads to the accumulation of damage with impaired p21 activity. Meanwhile, PARP enzyme inhibition leads to accumulation of damage in cells with dysfunctional HR. In this way, sensitization can be created in PARPi-resistant cells by exploiting two separate defects in the HR pathway. Cell viability was reduced in the absence of hMOB2 in both non-resistant and resistant cells. However, the drop in resistant cells is statistically far more substantial. Combining these two routes resulted in the inhibition of two key processes of DNA damage in cells resistant to Olaparib, and a synergistic impact was seen. In conclusion, regarding PARP inhibitor treatment, the level of hMOB2 protein may serve as a predictive biomarker to specifically eradicate olaparib-resistant cancer cells.

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