Investigating the role of USP7 inhibitor, AD04, in multiple myeloma

CHAPTER 1: INTRODUCTION

Multiple myeloma is a hematological neoplasm characterized by growth of abnormal plasma cells (PC) that infiltrate the bone marrow (BM) (van Nieuwenhuijzen, et al., 2018). MM belongs to a group of disease with elevated paraproteins, called paraproteinemia (Siegel, et al., 2019). Paraproteins are monoclonal immunoglobin (Ig) or M proteins from post-germinal B-cell lineage (Kazandijian, 2016). MM cells require survival pathways to control protein production and homeostasis due to elevated Ig production (Crawford & Irvine, 2013; Barwick, Gupta, Vertino, & Boise, 2019). There might be some misfolded or non-functional peptides produced by MM cells that has to be disposed (Barwick, et al., 2019). The ubiquitin proteasome system (UPS) plays a role in targeted degradation of peptides, hence, the UPS can improve the survival of MM cells (Crawford & Irvine, 2013). A series of enzymes are responsible for the ubiquitination (Crawford & Irvine, 2016). The proteins were selected to be ubiquitinylated by a ubiquitin ligase, E3 (Crawford & Irvine, 2016). Proteasome degrades the K48-linked polyubiquitinated proteins (Crawford & Irvine, 2013). The ubiquitin process can be reversed by deubiquitinating enzymes (DUBs) (Crawford & Irvine, 2013).

The administration of proteasome inhibitors allows accumulation of misfolded or non-functional proteins, that can induce cell death through unfolded protein response that activates apoptotic proteins (Barwick, et al., 2019; Hetz, 2012). Three proteasome inhibitors are currently clinically approved to treat MM, bortezomib, carfilzomib, and ixazomib (Gandolfi, et al., 2017). However, these drugs can be limited by resistance and toxicities in some patients, highlighting the need to find other target enzymes within the UPS (Manasanch & Orlowski, 2017).

HECT domain containing E3 ligase (HUWE1, also known as Mule) is reported to be involved in tumorigenesis of MM through regulation of proto-oncogene and tumor suppressor gene (Kao, et al., 2018; Crawford & Irvine, 2016; Walker, et al., 2018; Janz, et al., 2018). K63-

Commented [iu1]: What is M protein?

Commented [iu2]: The above para is about the possible causes of MM, and that protein degradation mediated by UPS improve the survival of MM. MM cell death can be induced by inhibiting protein degradation → accumulation of misfolded/non functional protein → activate kinase in the ER membrane → phosphorylate eIF2a → increase uncharged tRNA → block protein translation alltogether → apoptosis.

Commented [iu3]: Doe HUWE1 always put Ub in K48? So the protein that is ubiquitylated always degraded?

linked polyubiquitinated of c-Myc, a proto-oncogene, via HUWE1, can enhance transcriptional activity, activate DNA replication, regulate proliferation, and alter tumor microenvironment (Jovanović, et al., 2018). HUWE1 also affects p53, a tumor suppressor gene, by targeting it for degradation, hence inhibiting apoptosis (Hao, et al., 2012). Small molecule inhibitors of HUWE1, BI8622 and BI8626, have been reported to downregulate Myc expression significantly, however these inhibitors have unfavorable pharmacokinetics (Peter, et al., 2014). Myeloid cell leukemia 1 (Mcl-1) has been reported to be overexpressed in multiple myeloma and is related to chemotherapy resistance, cancer progression, relapse, and lower survival (Wuilleme-Toumi, et al., 2005). It is an anti-apoptotic protein that is important for cancer cell survival (Tron, et al., 2018). HUWE1 regulates Mcl-1 during DNA damage by addition of K-48 polyubiquitin chains and then Mcl-1 is degraded (Myant, et al., 2017). Expression of p53 can inhibit the expression of Mcl-1 through promoter repression (Pietrzak & Puzianowska-Kuznicka, 2008). Therefore, HUWE1 can directly or indirectly affect Mcl-1 (Tron, et al., 2018; Myant, et al., 2017).

Ubiquitin specific protease 7 (USP7), a substrate of HUWE1, mediates deubiquitylation of hypoxia inducible factor (HIF)-1α during hypoxia, hence increasing angiogenesis and metastasis (Kao, et al., 2018). A study also shows that an isoform of USP7 controls the stability of HUWE1 by inhibiting its self-ubiquitylation and degradation (Khoronenkova & Dianov, 2013). The application of a USP7 inhibitor, P5091, has been reported to downregulate HUWE1 expression and overcome bortezomib resistance in a pre-clinical study (Bosshard, et al., 2017) (Chauhan, et al., 2012). A new selective allosteric USP7 inhibitor AD04, has recently been developed and is reported to stabilize p53, increase p21, and reduce mouse double minute 2 homolog (MDM2) levels in colorectal cancer and breast adenocarcinoma cell line (Gavory, et al., 2017).

In this study, we aim to elucidate the ability of USP7 inhibitor AD04 to reduce proliferation of MM cells *in vitro* and its related pathway, modulate HUWE1 expression and its downstream proteins, and affect cell cycle of MM cells.

Commented [iu4]: When the protein is polyubiquitylated, will the proteins then degraded by proteasome? But here, the polyubiquitylation of c-Myc enhance transcriptional activity, activate DNA replication, etc. Polyubiquitylation of c-Myc do not target it for degradation? Or is it because it is K63 that got Ubiquitylated?
In contrast, K63-linked chains regulate "proteasome-independent" processes such as inflammatory signal transduction, DNA repair, endocytosis, and selective autophagy

Commented [iu5]: How Myc expression is controlled by HUWE1?

Commented [iu6]: Does it mean that USP7 can be ubiquitylated by HUWE1 and then degraded?
Or USP7 is just a binding partner of HUWE1 that mediates deubiquitylation?

Commented [iu7]: At the gene-protein translation level? Or just downregulate it at the protein level? Enhanced HUWE1 degradation?

Chapter 2: Literature review

1.1. Multiple Myeloma

Multiple myeloma is a hematological neoplasm characterized by growth of abnormal PC that infiltrate BM (van Nieuwenhuijzen, et al., 2018). It is the second most common hematological malignancy (Siegel, et al., 2019). MM is commonly diagnosed above the age of 55, with 69 years as median age of diagnosis (Röllig, et al., 2015). MM belongs to a group of disease with elevated paraproteins, called paraproteinemia (Morgan, et al., 2012). Paraproteins are monoclonal Ig or M proteins from post-germinal B-cell lineage and are usually used as an indicator to differentiate between monoclonal gammopathy of unknown significance (MGUS) and smoldering MM (SMM) to malignant MM and PC leukemia (PCL) (Kazandijian, 2016; Morgan, et al., 2012). MGUS is indicated by less than 10% abnormal PC in BM with absence of myeloma-related organ damage (van Nieuwenhuijzen, et al., 2018). Approximately 1% of MGUS patients progress into MM every year and almost all cases of MM are preceded by MGUS (Landgren, et al., 2009). SMM is an intermediate clinical phase between MGUS and MM and is indicated by high level of M proteins, more than 10% abnormal PCs, and asymptomatic regarding organ damage (van Nieuwenhuijzen, et al., 2018). MM has similar characteristics to SMM, but it causes organ damage described as CRAB symptoms: hyper**c**alcemia, **R**enal failure, **A**nemia, and **B**one lesion (CRAB) (Crawford & Irvine, 2016). MM can develop into PCL when clonal PCs start to proliferate outside BM (Morgan, et al., 2012).

The risk of developing MM has been associated with environmental and occupational exposure (Kumar, et al., 2017). Sergentanis and their team reviewed that pesticide is correlated with development of MM. In addition, occupational exposure to methylene chloride, a solvent commonly found in paint strippers and adhesives, also increased the risk to MM (Sergentanis, et al., 2015). Several lifestyle factors, such as overweight and obesity, are implicated with increased MM incidence (Sergentanis, et al., 2015). Family history also has correlation to having MM (Kumar, et al., 2017). The risk of MGUS is increased twice in first-

degree relatives from an MM patient (Kumar, et al., 2017). Various single nucleotide polymorphisms have been identified to increase Myc activation (Kumar, et al., 2017). Moreover, several loci are also associated with poorer prognosis in MM patients (Kumar, et al., 2017).

The pathogenesis of MM occurs during B cell development. Initially, B cells go through several differentiation process in bone marrow and secondary lymphoid organs (Kumar, et al., 2017). In immature B cell, the variable, diversity, and joining gene segments (VDJ_H) are rearranged to generate various Ig repertoire in bone marrow (Gonzales, et al., 2007; Kumar, et al., 2017). B cells expressing B cell receptor on its surface migrate to secondary lymphoid organs to undergo affinity maturation after antigen presentation from antigen-presenting cells in germinal center (Kumar, et al., 2017). To produce specific antibodies, the hypervariable regions of Ig heavy chain gene (IGH) will go through somatic hypermutation (SHM) and class switch recombination (CSR) (Kumar, et al., 2017; Morgan, et al., 2012). CSR and SHM needs double-strand DNA breaks in the Ig loci, however the breaks can fuse with other breaks within the genome, causing DNA aberration and chromosomal translocations (Kumar, et al., 2017). The Ig genes of PC in MM patients are indicated by heavily mutated heavy chain variable regions without intraclonal variation and it has isotype switched IGH, which are IgG or IgA (Gonzales, et al., 2007). Myeloma cells have translocations at IGH genes due to DNA breaks (Gonzales, et al., 2007). The rearrangement causes closer proximity between IGH enhancers and oncogenes, causing overexpression of oncogenes, such as cyclin D genes (Nagaoka, et al., 2010; Crawford & Irvine, 2013). Hence, these cause plasma cell to regain its proliferative capability, and result in MGUS (van Nieuwenhuijzen, et al., 2018; Morgan, et al., 2012). One of the enzymes responsible for SHM and CSR is activation-induced cytidine deaminase (AID). AID can induce genomic instability, leading to translocation and mutations of protooncogenes (Nagaoka, et al., 2010). Secondary events such as mutations,

translocation, epigenetic changes, and microenvironment alteration can initiate evolution of PC into MM (van Nieuwenhuijzen, et al., 2018).

The interaction between MM cells and their bone marrow microenvironment have shown increase of MM survival and proliferation, support tumor progression and drug resistance (Blau, et al., 2018). Their communication mainly focuses on cell-cell, cell-matrix signaling, growth factors and cytokines (Röllig, et al., 2015). One of the most important components in the microenvironment is bone marrow stromal cells (BMSCs). BMSCs secrete cytokines, growth factors, and extracellular matrix factors (Markovina, et al., 2010; Garcia-Gomez, et al., 2014). The supportive cytokines include interleukin 6 (IL-6) and vascular endothelial growth (VEGF) (Blau, et al., 2018). Both of these cytokines play an important role in affecting multiple signaling pathways such as PI3K, JAK/STAT, Raf, and nuclear factor kappa B (NFκB) (Blau, et al., 2018). MM cells also produce proinflammatory cytokines, such as transforming growth factor beta (TGFβ) and tumor necrosis factor alpha (TNFα) that, in turns, activate protumorigenic effect of NFκB (Blau, et al., 2018). NFκB pathway activity affects the expression of adhesion molecules and are often implicated in drug resistance (Blau, et al., 2018). In addition, the activation of NFκB can increase the production of tumor-promoting soluble factors by BMSCs and lead to cell adhesion-mediated therapy resistance (Blau, et al., 2018).

The current standard care for MM involves proteasome inhibitor and immunomodulatory drugs (Rajkumar, 2018). Several immunomodulatory drugs are thalidomide, lenalidomide, and pomalidomaides (Kumar, et al., 2017). They can affect E3 ubiquitin ligase complex that are important in degradation of proteins that are critical for myeloma survival (Kumar, et al., 2017). In addition, immunomodulatory drugs can also upregulate functional cytotoxic T cells (Kumar, et al., 2017). Meanwhile, proteasome inhibitors lead to accumulation of misfolded or non-functional proteins, which can induce cell death through the unfolded protein response. Three proteasome inhibitors are currently clinically approved to treat MM, bortezomib, carfilzomib, and ixazomib (Kumar, et al., 2017). However, these drugs can be limited by resistance and

toxicities in some patients, highlighting the need to find other target enzymes within the UPS (Manasanch & Orlowski, 2017).

1.2. Ubiquitination

Ubiquitin is a conserved protein that can be found in cytoplasm and nucleus of all cells (Crawford & Irvine, 2016). Ubiquitination, a post-translational modification, is involved in regulation of cellular processes (Crawford & Irvine, 2016). The process of ubiquitination is mediated by three classes of enzymes (Crawford & Irvine, 2016). Ubiquitin attachment is initiated by ubiquitin-activating enzyme, E1 (Kao, et al., 2018). E1 activates ubiquitin, followed by ubiquitin transfer to a ubiquitin-conjugating enzyme, E2 (Kao, et al., 2018). The activated ubiquitin is transferred to a target protein selected by a ubiquitin ligase, E3. E3 transfers activate ubiquitin to a lysine (K) residue on the target substrate by forming an isopeptide bond between ubiquitin and protein (Kao, et al., 2018). The attachment of ubiquitin to target substrate can occur as a monomer or a polymer, resulting in different biological consequences (Crawford & Irvine, 2016). When a single ubiquitin is added to lysine residue of target protein, it is called mono-ubiquitination (Crawford & Irvine, 2016). The process is important to regulate receptor endocytosis, lysosomal degradation, and protein recycling (Crawford & Irvine, 2016; Kao, et al., 2018). Meanwhile, a chain of ubiquitin molecules can also attach to the protein in a process called polyubiquitination (Crawford & Irvine, 2016). The most widely studied polyubiquitination are K48 and K63-linked polyubiquitination. K48-linked polyubiquitination targets protein to be degraded, while K63-linked polyubiquitination regulates protein for downstream signaling, protein localization, DNA damage response, and protein-protein interaction (Crawford & Irvine, 2016). The process of ubiquitination is reversible, and ubiquitin can be removed by deubiquitinating enzymes (DUBs) (Crawford & Irvine, 2013). DUBs generate free ubiquitin from its precursors and remove ubiquitin chains (Crawford & Irvine, 2016). The balance between ubiquitination and deubiquitination plays a pivotal role in regulating the protein levels and their functions within the cells (Crawford & Irvine, 2016).

The ubiquitin proteasome system (UPS) plays a role in targeted degradation of the peptides (Crawford & Irvine, 2013). The process starts with ubiquitination followed by proteasomal degradation (Crawford & Irvine, 2013). The tagged protein will be degraded by protein to form small peptides (Crawford & Irvine, 2016). The most well studied ubiquitination is K48-linked polyubiquitination that tags proteins for degradation through 26S proteasome (Crawford & Irvine, 2016). The proteasome is a protease that are made of two distinct subcomplexes consisting of 20S core particle and 19S regulatory particle (Crawford & Irvine, 2016). The 19S regulatory particle recognize the K48-linked polyubiquitin-tagged proteins, remove the ubiquitin chains to be recycled, unfold the target protein, and leave it to 20S core particle to be degraded (Crawford & Irvine, 2016).

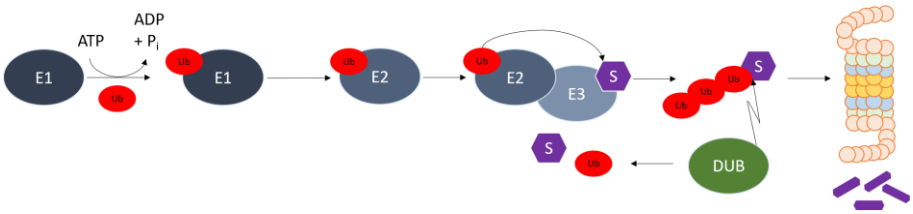

Figure 1. The ubiquitin proteasome system. E1 activates ubiquitin with ATP, followed with ubiquitin transfer to E2. The target protein which is the substrate of E3 will be attached to activated ubiquitin from E2. Proteasome degrades the polyubiquitinated protein. The process can be reversed with deubiquitinating enzyme.

Various E3 ligases are involved in tumorigenesis, because their substrates are oncogenes or tumor suppressor genes (Kao, et al., 2018). For example, HUWE1 regulates c-Myc, Mcl-1, and p53 that are implicated in tumorigenesis (Kao, et al., 2018)

1.2.1. HUWE1

HUWE1 is an E3 ligase that contains four domains, which are ubiquitin-associated domain, WWE domain, BH3 domain, and HECT domain (Kao, Wu, & Wu, 2018). WWE domain regulates ubiquitin-dependent proteolysis (Kao, Wu, & Wu, 2018). HECT domain is important to catalyze ubiquitin-thioester formation via its cysteine residue (Kao, Wu, & Wu, 2018).

HUWE1 is reported to be involved in tumorigenesis of MM (Kao, et al., 2018; Crawford & Irvine, 2016; Walker, et al., 2018; Janz, et al., 2018). It regulates several aspects of regulation within the cells, especially cell proliferation, apoptosis, DNA repair, and hypoxic stress (Kao, et al., 2018). Multiple substrates are regulated by HUWE1 activity, and most of them are implicated in tumorigenesis It regulates several aspects of regulation within the cells, especially cell proliferation, apoptosis, DNA repair, and hypoxic stress (Kao, et al., 2018). Multiple substrates are regulated by HUWE1 activity, and most of them are implicated in tumorigenesis (Kao, et al., 2018). K63-linked polyubiquitinated of c-Myc, a proto-oncogene, via HUWE1, can enhance transcriptional activity, activate DNA replication, regulate proliferation, and alter tumor microenvironment (Jovanović, et al., 2018). HUWE1 also affects p53, a tumor suppressor gene, by targeting it for degradation, hence inhibiting apoptosis (Hao, et al., 2012). Small molecule inhibitors of HUWE1, BI8622 and BI8626, have been reported to downregulate Myc expression significantly, however these inhibitors have unfavorable pharmacokinetics (Peter, et al., 2014). Myeloid cell leukemia 1 (Mcl-1) has been reported to be overexpressed in multiple myeloma and is related to chemotherapy resistance, cancer progression, relapse, and lower survival (Wuilleme-Toumi, et al., 2005). It is an anti-apoptotic protein that is important for cancer cell survival (Tron, et al., 2018). HUWE1 regulates Mcl-1 during DNA damage by addition of K-48 polyubiquitin chains and then Mcl-1 is degraded (Myant, et al., 2017). Expression of p53 can inhibit the expression of Mcl-1 through promoter repression (Pietrzak & Puzianowska-Kuznicka,

2008). Therefore, HUWE1 can directly or indirectly affect Mcl-1 (Tron, et al., 2018), (Myant, et al., 2017).

1.2.2. USP7

USP7, also known as HAUSP, is a 135 kDa DUB enzyme. It is involved in wide array of cellular processes and is involved in different disease progressions such as cancers, neurological disorders, metabolic dysregulation, and immune dysfunction (Bhattacharya, et al., 2018). USP7 is a cysteine protease that mediates hydrolysis of bonds formed by ubiquitin to the substrate (Bhattacharya, et al., 2018). The domain structure of USP7 involves conserved poly-glutamine (poly Q) stretch, tumor necrosis factor receptor-associated factor (TRAF)-like domain, catalytic domain, and ubiquitin-interacting regions (Bhattacharya, et al., 2018). TRAF-like domain has similar sequence homology to TRAFs and in this enzyme, the domain is responsible in protein-protein interactions and assists in nuclear localization (Bhattacharya, et al., 2018). This domain acts as the binding site of MDM2 and p53 (Bhattacharya, et al., 2018). The catalytic domain is the site of bond hydrolysis, while carboxyl-terminal domain is critical in facilitating protein-protein interaction (Bhattacharya, et al., 2018).

One of the early substrates found to be regulated by USP7 was p53 (Bhattacharya, et al., 2018). p53 half-life was increased due to deubiquitylation and hence, reactivation of apoptotic pathway (Bhattacharya, et al., 2018). Therefore, USP7 was attributed as a tumor suppressive protein (Bhattacharya, et al., 2018). However, later on, MDM2 was found to be regulated by USP7, followed by degradation of p53 (Bhattacharya, et al., 2018). This dynamic changes of deubiquitylation was affected by amount of genotoxic stress in cells, and its activity was context-specific in selecting the substrate to be deubiquitylated (Bhattacharya, et al., 2018).

In tumorigenesis, USP7 can regulates NFκB complex by deubiquitinating it or its upstream factors, such as TRAF6 and NEMO (Bhattacharya, et al., 2018). USP7 is selected

to NFκB promoters site following TNFα receptor (TNFR) activation and interacts with NFκB complex (Colleran, et al., 2013). NFκB regulates adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1). Inhibition of USP7 also showed decreased expression of IL-6, after reduced TNFR expression.

USP7 is also a substrate of HUWE1 and it mediates deubiquitylation of hypoxia inducible factor (HIF)-1α during hypoxia, hence increasing angiogenesis and metastasis (Kao, et al., 2018). A study also shows that an isoform of USP7 controls the stability of HUWE1 by inhibiting its self-ubiquitylation and degradation (Khoronenkova & Dianov, 2013). The application of a USP7 inhibitor, P5091, has been reported to downregulate HUWE1 expression and overcome bortezomib resistance in a pre-clinical study (Bosshard, et al., 2017; Chauhan, et al., 2012). A new selective allosteric USP7 inhibitor AD04, has recently been developed and is reported to stabilize p53, increase p21, and reduce MDM2 levels in colorectal cancer and breast adenocarcinoma cell line (Gavory, et al., 2017).

Chapter 3: Material and Methods

3.1. Drug

AD04 (Cpd4) drug (Almac Discovery) will be prepared in 0.1% DMSO and is diluted from stock solution to make different concentrations (1 nM, 10 nM, 100 nM, 1 μM, 10 μM).

3.2. Cell Culture

Human MM cell lines JJN3, OPM2, XG-1, and MOLP8; and human bone marrow stromal cell line HS-5 are cultured in RPMI 1640, 10% fetal bovine serum, and 1% penicillin-streptomycin. All cell lines are maintained at 37℃ in 5% CO_2 incubator. Suspension cells are cultured in T-80 flasks and passaged when they reached confluency (~1 x 10^6 cells/mL) to be subcultured to 3 – 5 x 10^5 cells/mL. XG-1 IL-6 dependent MM cell line was supplemented with 1 ng/mL of recombinant IL-6 (InvivoGen). Adherent cells are cultured in T-175 flasks

and passaged when they reached 80-90% confluency. To count the cells, the cells (50 µL) are diluted with 10 mL CASYton solution and counted with CASY Model TT Cell Counter and Analyzer (Innovatis). The viability of the cells is maintained at ≥95%.

3.3. Proliferation Assay

Drug dose response assay is set up with either stromal cell line HS-5 co-culture with MM cells, MM cells only, or HS-5 cells only. Stromal cells HS-5 for co-culture are seeded in 96-well flat bottom plate (1,000 cells/well) and incubated for 24 hours before seeding the MM cells (5,000 cells/well), subsequently treated with AD04 drug (1 nM, 10 nM, 100 nM, 1 µM, 10 µM) or vehicle (0.1% DMSO) for 24, 48, and 72 hours in 3 replicates. MM cells alone cultured in 96-well rounded bottom plate are treated in the same manner. Other 96-well plates of MM cells alone are cultured in conditioned media, consisting of media from HS-5 culture for at least 1 day that contains soluble factors secreted by BMSC. HS-5 alone is cultured in 96-well flat bottom plate (3,000 cells/well) and incubated for 24 hours before AD04 drug treatment. Cell viability for MM cells only is determined by adding equal volume of CellTiter-Glo® Luminescent Cell Viability Assay (Promega) to 25 µL of suspension cell mixture in 96-well white opaque microplate at each timepoint, while cell viability for co-culture and HS-5 only is determined by CyQUANT® Direct Cell Proliferation Assay Kit (Invitrogen), according to the manufacturer's instruction. The luminescence is read using GENios Microplate Reader (Tecan).

3.4. Western Blot

MM cells are collected and lysed with radioimmunoprecipitation (RIPA) buffer and protease inhibitor solution in ice and centrifuged. Meanwhile, albumin standard is prepared from stock solution (2 mg/mL) and diluted to 0.125, 0.25, 0.5, 1, and 2 mg/mL. 5 µL of each standard and sample are put into 96-well plate and added with 200 µL of Pierce™ BCA Protein Assay Working Reagent (ThermoFisher) per well and the absorbance is measured at

570 nm to calculate the concentration of the protein. The protein lysates are stored in -20℃ until further use.

Previously prepared protein lysates are mixed with 4x NuPage™ LDS sample buffer (Invitrogen). An amount of 30 µg of protein from each sample is heated at 95℃ for 5 minutes and separated through SDS-PAGE on 3-12% Tris-Acetate gels (Invitrogen). The gel is run at 150 V for an hour. The protein is transferred on polyvinylidene fluoride (PVDF) membrane (Merck) at 15 V at 4℃ overnight. Primary antibodies against GAPDH (Abcam), USP7 (Cell Signaling), HUWE1 (Abcam), Mcl-1 (Abcam), c-Myc (Abcam), p53 (Santa Cruz) were used at room temperature for an hour and secondary antibodies anti-mouse (DAKO) and anti-rabbit (DAKO) were used at room temperature for an hour. Protein bands were visualized with WesternBright™ ECL horseradish peroxidase (HRP) substrate (Advansta).

3.5. Isolation of Ubiquitinylated Protein

MM cells OPM2 and XG-1 are treated with AD04 drug for 6, 24, 48, and 72 hours. The cells are collected and lysed with RIPA buffer and protease inhibitor solution in ice and centrifuged. Protein concentration was determined with Pierce™ BCA Protein Assay (ThermoFisher).

250 µg of protein lysates were incubated with washed UbiQapture-Q® matrix (Enzo), overnight at 4℃. Matrix were harvested through centrifugation, washed twice with PBS, and boiled in 4x SDS NuPage™ LDS sample buffer (Invitrogen) for 10 minutes. Isolated proteins and 25 µg of the input were analyzed by Western blot, as described above.

3.6. Cell Cycle Analysis

OPM2 and XG-1 cells were seeded into 6-well plates and treated for 24 h, 48 h, and 72 h. The cells were harvested, fixed with 70% ice-cold ethanol for at least 4 h in 4℃. Subsequently, the cells were centrifuged, washed, and resuspended in 400 µL PBS and 100 µL solution containing 20 µL of 1 mg/mL PI and 10 mg/mL RNase A for 30 mins at 37℃. The

cells were then analyzed by flow cytometry. The cell cycle distribution is analyzed through FACS Diva (v.6).

3.7. Quantitative PCR

OPM2 and XG-1 cells are seeded into 6-well plates and treated for 24 h, 48 h, and 72 h. The cells are harvested. Total RNA will be extracted from cells. An amount of RNA will be converted into cDNA. The gene expression for VCAM1, ICAM1, USP7, and HUWE1 will be measured.

3.8. Immunofluorescence

The antibody will be tested against VCAM1 and ICAM1. DAPI dye will also be used

3.9. Adhesion Assay

HS-5 stromal cells will be seeded at 1 x 10^4 in flat-bottom 96-well plate and cultured overnight. MM cell lines will be co-cultured with adherent HS-5 in concentration of 5 x 10^5 and will be left to adhere for 2 h. Then, the plates will be gently inverted to remove the drug and media and washed for 3 times with RPMI 1640 medium to remove non-adherent cells. The fluorescence will be measured to check the amount of adherent before and after treatment.

3.10. Statistical Analysis

Data are presented as mean ± SD. All statistical analyses were conducted using GraphPad Prism (v.8). The statistical significance between two treatment groups was analyzed using unpaired Student's t tests with two-tailed p values. The significant difference between multiple treatment groups were analyzed using two-way ANOVAs. $P \leq 0.05$ was considered significant.

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