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FOXO1 Inhibitor, AS1842856, induces cell cycle arrest and reverses anticancer drug-induced cytotoxicity in osteosarcoma cells

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FOXO1 INHIBITOR, AS1842856, INDUCES CELL CYCLE ARREST AND REVERSES ANTICANCER DRUG-INDUCED CYTOTOXICITY IN OSTEOSARCOMA CELLS.

THESIS

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Forkhead box class O (FOXO)-1 transcription factor controls cell proliferation, apoptosis, oxidative stress, and other cellular activities; FOXO1 has also been implicated in cell cycle regulation. This research project aims to determine the contribution of FOXO1 to cell cycle regulation and response to anticancer drug treatment in osteosarcoma. Osteosarcoma is the most common bone cancer, with most cases occurring in people younger than 30 years old. The study explores the impact of FOXO1 inhibitor AS1842856 on the cytotoxic effects of anticancer drugs in CCHOSD, Hos, and LM7 osteosarcoma (OGS) cell lines. Following chemical inhibition of FOXO1 and anticancer drug treatment, cell cycle and anticancer drug-induced cell death were determined using cell cycle analysis. It was observed that OGS cell lines do not naturally produce p21. However, FOXO1 suppression led to a G2/M cell cycle phase arrest, coinciding with an upsurge in p21 expression in CCHOSD and LM7 cell lines. FOXO1 inhibition increased p16, p21, and

p27 levels in CCHOSD cells, elevated p21 expression in LM7 cells, and reduced expression of p27 in LM7 cells. Interestingly, inhibition of FOXO1 counteracted cell death induced by anticancer drugs. The data generated in this project indicated that baseline expression of cell cycle inhibitors varies among OGS cell lines and influences cell cycle arrest differently. Additionally, findings suggest that the reversal of anticancer drug-induced cell death by FOXO1 inhibition is associated with induced arrest in the cell cycle.

Keywords: Osteosarcoma, FOXO1 inhibition, AS1842856 inhibitor, Cell cycle

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CHAPTER 1

INTRODUCTION

Osteosarcoma is a type of bone cancer that commonly affects children, teenagers, and young adults. It is the most prevalent form of primary bone sarcoma and usually originates in the osteoblast cells, responsible for forming bone tissue. This cancer leads to the development of tumors that create immature, irregular, and diseased bone. Osteosarcoma most frequently affects the long bones of the arms and legs, particularly near the ends of the bones. Common symptoms include limited movement, bone pain, and unexplained broken bones. The condition is categorized as low-grade, intermediate-grade, or high-grade, with high-grade tumors being aggressive. Treatment options may include surgery, chemotherapy, and radiation therapy. The survival rate is around 70% if the cancer has not spread to other body parts. Osteosarcoma is a complex condition that requires a multidisciplinary approach for effective management and treatment.

The Forkhead box (FOX) transcription factor is a family of evolutionarily conserved proteins that play an essential role in regulating gene expression in various biological processes. The FOX family consists of many members, classified into different subgroups (FOXA to FOXS) in the human species. These transcription factors regulate diverse biological processes, both during development and throughout adult life, and their

dysfunction is often associated with human diseases, including cancer.

The FOX family of transcription factors is characterized by a highly conserved DNA-binding domain called the forkhead domain (FOX-DBD). Members of the FOX family have been linked to a wide range of cancer types, highlighting their potential usefulness as therapeutic targets. At least 14 FOX subgroups are related to colorectal cancer (CRC) pathogenesis (Laissue, 2019). FOX proteins play essential roles in proliferation, differentiation, longevity, cell cycle control, and DNA repair. Members of class O (FOXO-proteins) regulate metabolism, cellular proliferation, stress tolerance, and possibly lifespan. Post-translational modifications control FOX proteins' activity, including phosphorylation, acetylation, and ubiquitination. In summary, the FOX transcription factor family is a critical player in various biological processes and has been linked to human diseases, making it an essential area of research for understanding disease pathogenesis and developing potential therapeutic strategies.

Cell cycle regulation

The cell cycle includes four phases: G0/G1, synthesis (S), G2, and mitosis (M). Cyclins and cyclin-dependent kinases facilitate progression through the cell cycle checkpoints. Cell cycle inhibitors, including p16, p21, and p27, regulate the activity of cyclins and cyclin-dependent kinases (Vermeulen et al., 2003).

p16. p16 functions as a cell cycle regulator by binding to cyclin-dependent kinases (CDK) 4, 6, and cyclin D. For the cell cycle to occur with cell proliferation, CDK4/6 binds to cyclin D to form an active protein complex that phosphorylates the retinoblastoma protein (Rb), therefore liberating a transcription factor that promotes the transition from G1 to S phase (Witkiewicz et al., 2011). With p16 binding to CDK4/6, the kinase activity responsible for phosphorylating Rb is inhibited, ultimately forcing the cell cycle to cease in the G1 phase and slow the cell cycle. Regulating p16 is relatively complex and involves the interaction of several transcription factors because of its low levels in normal proliferating tissue. Polycomb repression complex (PRC) 1 and 2 are two of the several transcription factors responsible for p16 regulation. PRC1/2 modifies p16 expression by activating or inactivating transcription pathways that cause methylation and other repression of p16's function (Cao et al., 2002). p16 has been recognized as a tumor-suppressor protein due to the prevalence of a mutated version of the gene in all types of human cancers. The inactivation or mutation of p16 causes an inability to form a stable complex with CDK 4, 6, or cyclin D, allowing unregulated cell progression.

p21. p21 functions as a cyclin-dependent kinase (CDK) inhibitor regulating cell proliferation by blocking the cell cycle via the cyclin kinase pathway. p21 is classified as a kinase inhibitor protein (KIP). It inhibits all cyclin-CDK activity via binding to the complexes, making it a universal cyclin-CDK inhibitor, blocking kinase activity and forcing the cell cycle to halt between the G1 and S phases (Xiong et al., 1993). Although it is considered a universal cyclin-CDK inhibitor, p21 is mostly associated with CDK2 inhibition. p21's nuclear form mediates CDK inhibition with p21 binding to CDK via the

N-terminal domain. At the N-terminal domain, there is cyclin binding motif 1 (Cy1); at the C-terminal half, there is a weaker Cy2. p21 can bind to cyclin-CDK complexes at the Cy2, preventing CDK activation. Regulation of p21 is done directly via p53, a tumor suppressor protein. p53 binds to p21's promoter, activating protein expression (Abukhdeir & Park, 2009). Ubiquitin ligases negatively regulate p21. During the G1/S phase, the E3 ubiquitin ligase complexes SCFSkp2 and CRL4Cdt2 induce the degradation of p21 (Yu et al., 1998). Although p21 functions to inhibit and control cell growth, it does not meet the classic criteria to be considered a tumor suppressor protein. Specifically, evidence has not shown that germline mutations of p21 increase susceptibility to tumor formation.

p27. p27 functions to inhibit cell cycle progression via inhibition of cyclin/CDK activity by binding to cyclin-CDK complexes. p27 also functions to regulate cell proliferation, cell differentiation, and apoptosis. p27 is classified as a kinase inhibitor protein (KIP), like p21, because it inhibits cyclin-CDK complexes. Regulation of p27 involves several independent pathways and mechanisms, including changing tumor cells between various modes of p27 inactivation as tumor progression continues. p27 protein levels are tightly controlled because cells undergoing cell deterioration (during G0/G1) have the highest levels of p27 protein. Proteolysis is another important mechanism for p27 regulation because p27 protein levels are reduced during the G1/S phase. This mechanism allows p27 to be marked for degradation via phosphorylation (Abukhdeir & Park, 2009). Functional loss of p27 has been implicated in human malignancies, and evidence even suggests tumor promotion, indicating that p27 is normally a tumor suppressor protein

CHAPTER 2

LITERATURE REVIEW

Osteosarcoma, the most common primary malignant bone tumor, poses significant challenges due to its aggressive nature and limited treatment options. Despite advancements in chemotherapy and surgery, the survival rates for osteosarcoma patients remain suboptimal, highlighting the need for novel therapeutic approaches. The FOXO1 transcription factor has emerged as a promising target in cancer therapy because of its role in cell cycle regulation, apoptosis, and DNA repair (Yang et al., 2021). The FOXO1 inhibitor, AS1842856, has been shown as a potential strategy due to its ability to induce cell cycle arrest and reverse anticancer drug-induced cytotoxicity in osteosarcoma cells (Zou et al., 2014).

Current Osteosarcoma treatment

Currently, treatment strategies for osteosarcoma include neoadjuvant chemotherapy and surgical resection followed by adjuvant chemotherapy (Isakoff et al., 2015). Current neoadjuvant treatments include cisplatin, doxorubicin, ifosfamide, and methotrexate, which target DNA and cause damage with hopes of shrinking the tumor before resection (Rathore & Van Tine, 2021). Doses of the medications must be heavily regulated due to potential nerve damage, organ damage, and cardiotoxicity. Cisplatin

causes DNA damage due to the platinum ion binding with DNA bases, inhibiting DNA replication and cell division. This medication tends to be very efficient in shrinking tumors and is widely used, but there are reports of adverse effects, including nerve damage and toxicity. Doxorubicin functions similarly to cisplatin, causing DNA damage by inhibiting topoisomerase II, which is vital for managing DNA coils and supercoils. Active and recruiting studies are underway to focus on treatment strategies that exploit cellular pathways that drive prognostic factors in osteosarcoma instead of causing DNA damage.

FOXO and Cancer

Targeting FOXO transcription factors has shown to be a potential therapeutic approach for treating cancer. FOXO3 is another human member of the FOXO family, like FOXO1, and is regulated by the PI3K-PKB signaling pathway. FOXO3 regulates cellular processes via targeted expression and effector gene activity. Because of this, FOXO3 has become a potential target of chemotherapy medication. BMS-345541 is a highly selective IκB kinase (IKK) inhibitor and indirectly targets FOXO3 in T-cell acute lymphoblastic leukemia (Liu et al., 2018). Evidence supports the expression of a cell cycle inhibitor, p21, being upregulated after treatment with an inhibitor. Because the upregulation of p21 is independent of the PKB pathway, the loss of FOXO3 is attributed to the overactivation of the IKK inhibitor (Buontempo et al., 2012).

FOXO3a functions downstream in a phosphorylated, inactive form in BCR-ABL-positive chronic myeloid leukemia. Studies have shown in cell lines, inhibition of the BCR-ABL oncoprotein (BCR-ABL tyrosine kinase) increases FOXO3a mediated

apoptosis via FOXO3-dependent cell cycle arrest along with expression of a pro-apoptotic gene, BIM (Kikuchi et al., 2007). Imatinib, a BCR-ABL tyrosine kinase inhibitor, converts FOXO3a from an inactive phosphorylated form to a dephosphorylated active form, inducing cell cycle arrest and apoptosis.

Similarly to FOXO3, FOXO1 plays significant roles in proliferation, differentiation, and cell survival. When in an active form, FOXO1 increases the transcription of the cyclin-dependent kinase inhibitor p27, which controls cell cycle progression. p27 majorly stops or slows the cell cycle, which has linked FOXO1 to normally functioning as a tumor suppressor protein (Lu & Huang, 2011).

FOXOs are generally accepted as tumor suppressors, but in certain contexts, FOXOs can promote cancer. The tumor suppressor idea is supported by evidence that suggests that FOXOs are deleted or completely inactivated in various human cancers by a signaling pathway, PI3-Akt, which is often deregulated in cancer (Farhan et al., 2020). When this signaling pathway is dysregulated, it results in an upregulation of protein kinase B (Akt), which can ultimately weaken the function and activity of FOXOs. Akt is responsible for cell growth, metabolism, and proliferation. Therefore, an upregulation of Akt can result in excessive cell growth, and FOXOs cannot keep up to regulate the cell growth.

FOXO1 inhibition has been linked to apoptosis in various cancer cells, including glioblastoma multiforme and basal-like breast cancer cells. FOXO1 inhibitors target FOXO1, including those dysregulated in cancer, aiming to disrupt the pathways promoting cancer progression and metastasis. FOXO1 inhibitors, including AS1842856,

can lead to reduced colony formation and increased apoptotic gene expression, ultimately triggering apoptosis in these cancer cells (Flores et al., 2023). AS1842856 is a selective FOXO1 inhibitor that reduces DNA binding and transactivation. In cancer, the inhibition of FOXO1 can induce pro-apoptotic genes like FAS and BIM, promoting cell death (Zhang et al., 2011). Additionally, the FOXO1-PLK1 pathway has been identified as a potential therapeutic target for advanced prostate cancer, where FOXO1's pro-apoptotic function is restored by inhibiting PLK1-dependent phosphorylation of FOXO1 in prostate cancer cells (Gheghiani et al., 2020). The FOXO1 inhibitor AS1842856 has been found to induce cell cycle arrest in various cancer cells, such as KMM cells, BCP-ALL cell lines, glioblastoma multiforme (GBM), and basal-like breast cancer cells. Specifically, AS1842856 treatment increased the number of G1-phase cells, indicating cell cycle arrest in KMM cells (Li & Gao, 2023). Moreover, the inhibition of FOXO1 by AS1842856 or AS1708727 resulted in reduced colony formation and increased apoptotic gene expression in GBM and basal-like breast cancer cells, ultimately triggering apoptosis (Flores et al., 2023). This result demonstrates the significant impact of FOXO1 inhibition by AS1842856 on cell cycle regulation and apoptosis induction in cancer cells

FOXO and Multidrug Resistance in Cancer

The reversal of anticancer drug-induced cytotoxicity is a significant area of research aimed at enhancing the effectiveness of chemotherapy in treating cancer. Studies have explored various approaches to overcome multidrug resistance (MDR) in cancer cells, a significant obstacle to successful chemotherapy. One strategy involves using phytochemicals as potential lead molecules for MDR reversal. For example,

phytochemicals like lobeline, harmine, nobiletin, and constituents of Carpobrotus edulis have shown promising effects in reversing P-glycoprotein (P-gp) dependent MDR in tumor cells, thereby increasing the cytotoxicity of anticancer drugs like doxorubicin and methotrexate (Tinoush et al., 2020). These findings highlight the potential of phytochemicals in combating drug resistance mechanisms and improving the efficacy of chemotherapy in cancer treatment.

The FOXO family in human cells (FOXO1, FOXO3, FOXO4, and FOXO6) have redundant activity and are thought to affect cell response to anti-cancer and antitumor treatments. Research has shown that silencing FOXO1 enhances angiogenesis in gastric cancer patients, while its activation reverses this by inhibiting angiogenesis (Shi et al., 2018). FOXO1 can exhibit anticancer actions via tumor inhibition characterized by differentiation and migration of tumor cells. Hepatocellular carcinoma (HCC) is enriched with small vessels with a high metastasis capability, and it was discovered that expression of miR183/96/182 induces activation of FOXO1, leading to a reduced number of blood vessels and metastasis (Leung et al., 2015). This evidence led researchers to discover that increased FOXO1 levels inhibit cell differentiation, leading to further blocking of HCC.

Additionally, the tissues from gastric cancer exhibit reduced suppression of FOXO1, which enhances proteins that increase angiogenesis and poor progression of gastric cancer. The knockdown of FOXO1 promotes the migration and invasion of gastric cancer cells, furthering the poor prognosis of the cancer (Ko et al., 2015). It was discovered that when FOXO1 is silenced, it boosts angiogenesis in gastric cancer by increasing the levels of HIF-1a and VEGF. Conversely, activating FOXO1 reverses this

effect and inhibits tumor advancement by reducing SIRT1 (Kim et al., 2022). These studies indicate that FOXO1 has anticancer effects by reducing cell proliferation and increasing cell apoptosis in digestive malignancies (Kim et al., 2016).

CHAPTER 3

DESIGN OF STUDY

Cell Lines, Cell Culture and Reagents

The Hos cell line represents non-metastatic human osteosarcoma cells, whereas CCHOSD, LM7, and OS17 cell lines denote metastatic human osteosarcoma cells. Cultivation and culture of cells occurred in a CO_2 incubator, maintaining conditions at 37℃ and 5% humidity. Drug treatments specified in the figures were administered accordingly. Camptothecin (CPT) was purchased from ChemWerth (Woodbridge, CN). Gemcitabine (GCB) was purchased from Eli Lilly (Indianapolis, IN). LC3 antibody was sourced from Novus Biologicals (Littleton, CO). Cleaved caspase-3 antibody was sourced from Cell Signaling Technology, Inc. (Danvers, MA). A beta-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO), and a pan-caspase inhibitor was purchased from ApexBio (Houston, TX). Gapdh antibody and buthionine sulfoximine (BSO) were purchased from Santa Cruz Biotechnology (Dallas, TX). Fetal bovine serum (FBS) was sourced from VWR International (Radnor, PA). The DMEM cell culture medium, cell culture supplements, and 2',7'-dichlorofluorescein diacetate (DCF) were purchased from Invitrogen (Carlsbad, CA).

Cell Viability

Cell death evaluation was conducted by quantifying cells residing in the sub-G0/G1 phase of the cell cycle. Propidium iodide (PI) was used as a dye, binding to double-stranded DNA by intercalating between base pairs. Cells exhibiting degraded DNA (non-viable) demonstrated inadequate PI binding, leading to their presence in the sub-G0/G1 population within the cell cycle histogram.

Western Blot Analysis

After drug treatment, the supernatant and cells were harvested and centrifuged at 300 g for 5 minutes at 4℃. The resulting pellet was lysed using RIPA lysis buffer supplemented with a protease and phosphatase inhibitor cocktail, followed by centrifugation at 10,000 g for 15 minutes at 4℃. The supernatants were then collected, and the total protein content was quantified using the BioRad reagent (BioRad Laboratories, Hercules, CA). The membranes were blocked with 5% nonfat milk before being subjected to primary antibody incubation. After washing, the membranes were exposed to the appropriate secondary antibody conjugated with HRP. After the secondary antibody incubation, the membranes were washed again, and the signal was detected utilizing an ECL detection reagent (Santa Cruz Biotechnology, Inc., Dallas, TX). The expression levels of Beta-actin or GAPDH proteins were employed as controls for protein loading.

Statistical Analysis

The results are expressed as means accompanied by the standard error of the mean (SEM). Analysis of experimental data was conducted utilizing a 2-tailed Student t-test. Statistically significant differences were defined as P values below 0.05.

CHAPTER 4

RESULTS AND DISCUSSION

To begin this study, the basal expression of cell cycle inhibitors, p16, p21, and p27, were analyzed in the cell lines; CCHOSD, LM7, and Hos. p16 had relative expression in CCHOSD and LM7 cell lines, with Hos showing no expression. p21 had relative protein expression in CCHOSD and LM7 cell lines, with Hos showing no expression. p27 showed protein expression in all three cell lines, with CCHOSD having the least expression (Figure 1).

Figure 1. Basal expression of cell cycle inhibitors. **A.** p16 basal protein expression. **B.** p21 basal protein expression. **C.** p27 basal protein expression. Actin or gapdh served as a protein loading control.

The impact of AS1842856 on cell cytotoxicity was analyzed. Cell lines were treated with AS1842856, FOXO1 inhibitor, and cell death percentage was analyzed. Increasing the treatment dosage for the CCHOSD cell line showed decreased cell death (Figure 2). Increasing the treatment dosage for the Hos cell line increases cell death. However, the Hos cell line showed significantly lower cell death than the CCHOSD and LM7 cell lines (Figure 2). Increasing the treatment dosage for the LM7 cell line showed a slight increase in cell death (Figure 2).

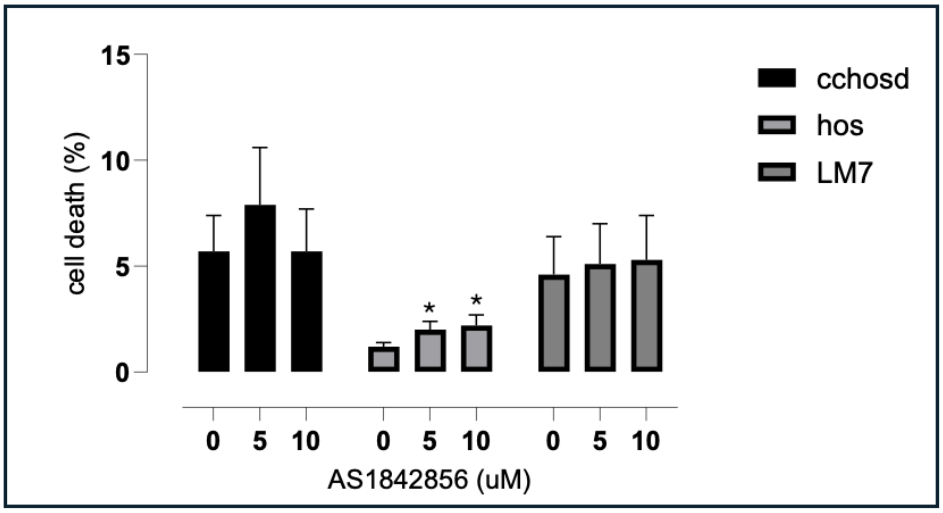

Figure 2. Graph presentation of AS1842856 effect on cell cytotoxicity. The asterisk indicates a significant difference between the treatment and the control groups.

Next, the effect of camptothecin (CPT) on cell cytotoxicity was analyzed in cell lines. Cell lines were treated with CPT, and cell populations were analyzed. Across all three cell lines, CCHOSD, Hos, and LM7, cell death percentage increased significantly as the CPT dosage increased (Figures 3 and 4).

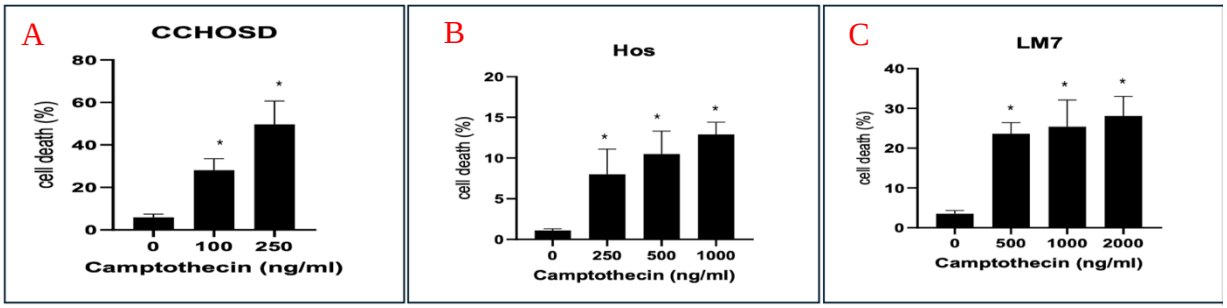

Figure 3. Graph presentation of CPT-induced cell death in all three cell lines. **A.** CCHOSD cells. **B.** Hos cells. **C.** LM7 cells

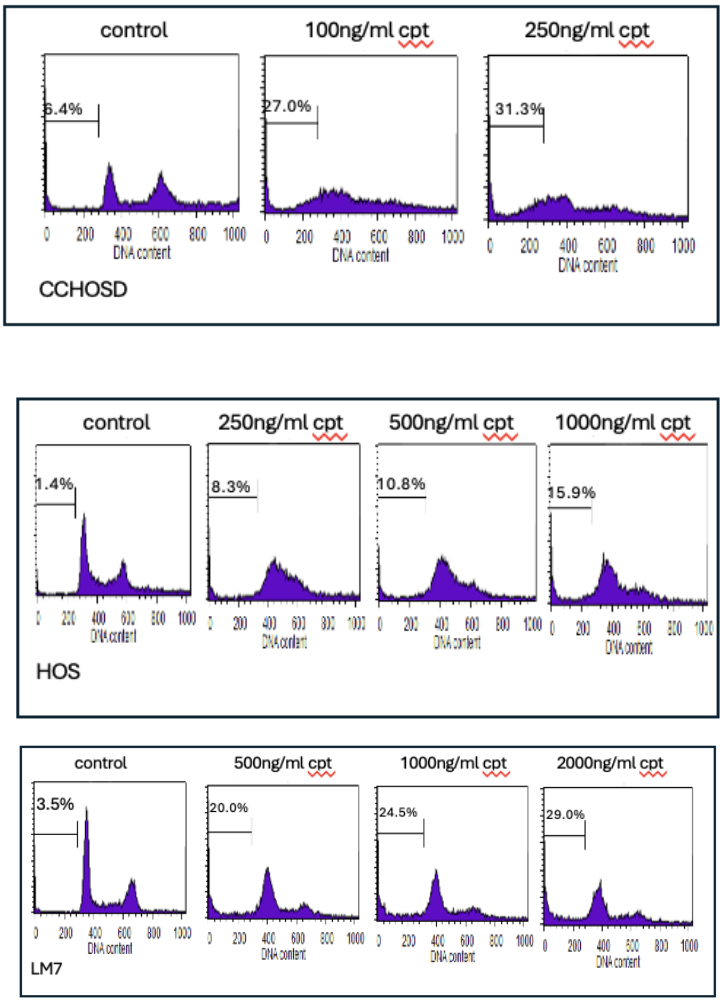

Figure 4. Histogram presentation of CPT-induced cell death in all three cell lines.

Samples were pretreated with the FOXO1 inhibitor AS1843856 to analyze the reversal of CPT-induced cell death**.** Following AS1842856 pretreatment, camptothecin (CPT) was used to induce cell death in cell lines. All three cell lines, Hos, LM7, and CCHOSD, showed that AS1842856 can reverse cell death/damage in CPT-induced cells due to a significant decrease in cell death percentage (Figures 5 and 6).

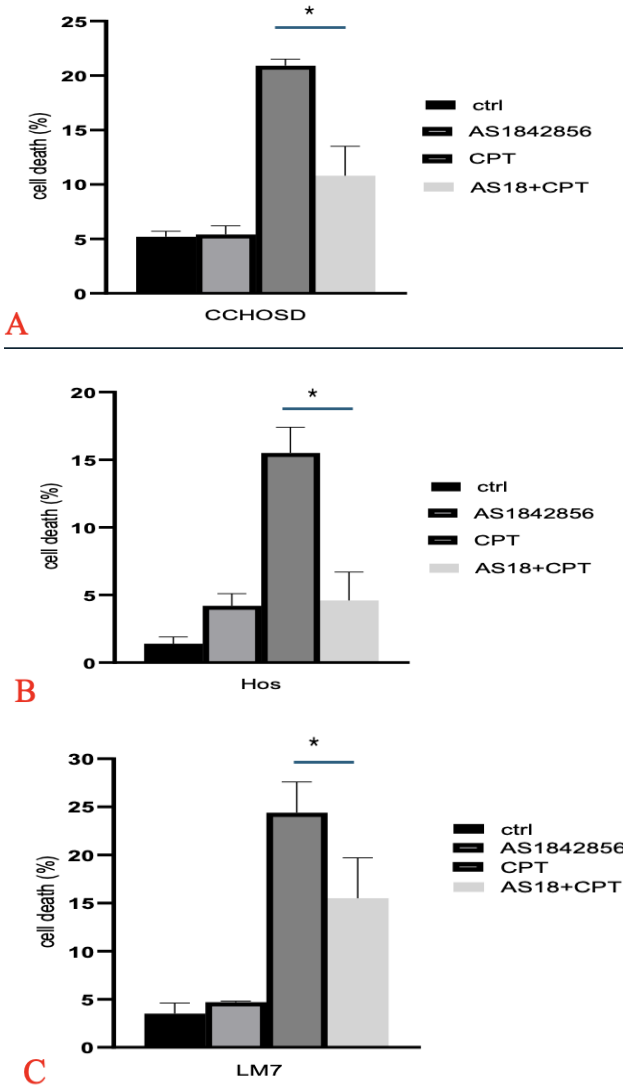

Figure 5. Graph presentation of AS1842856 of reversal of CPT-induced cell death. Cells were pretreated with AS1842856 for 1 hr to inhibit FOXO1 activity, followed by CPT treatment. Following CPT treatment, cells were collected, and the sub-G0/G1 cell population was determined, as described in the design of study section. **A.** CCHOSD cells. **B.** Hos cells. **C.** LM7 cells.

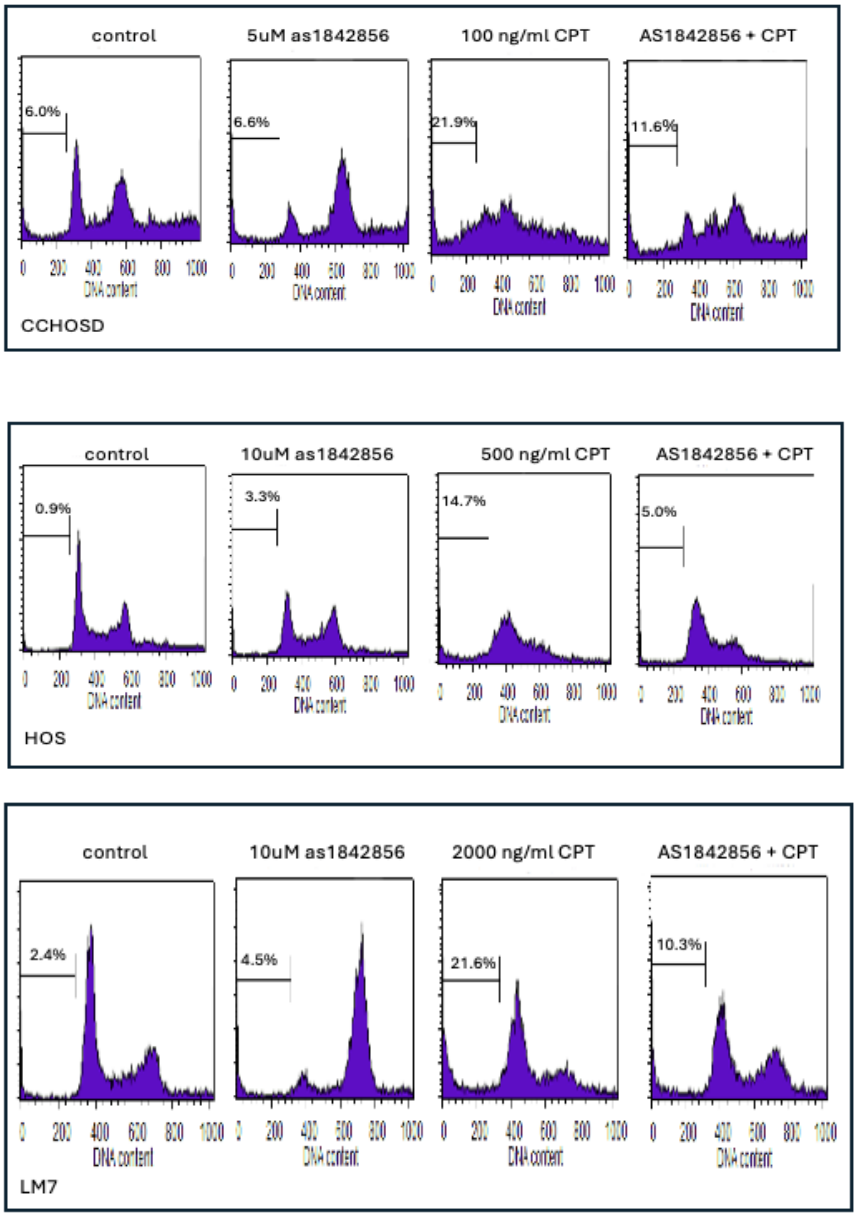

Figure 6. Histogram presentation of AS1842856 reversal of CPT-induced cell death in the sub-G0/G1 phase. Cells were treated with 0.01% DMSO (control) or AS1842856 or CPT or a combination treatment of AS1842856 + CPT. Following drug treatment, cells were collected, and the cell cycle was analyzed as indicated in the design of study section. Cells in the sub-G0/G1 population were considered non-viable.

Cell lines were then treated with FOXO1 inhibitor, AS1842856, and following treatment, the cell cycle was assessed. Figure 8 illustrates the different stages in the cell cycle with a control sample, whereas the population of cells in the sub-G0/G1 phase is considered non-viable or dead (Kuksin, 2013). Cell arrest occurs in the CCHOSD cell line and is most prevalent at the G2/M point because of the increased cell population between control and treated samples (Figures 7 and 9). Cell arrest occurs for the Hos cell line and is most prevalent at the G2/M point due to the increased cell population between control and treated samples. Cell arrest occurs for the LM7 cell line and is most prevalent at the G2/M because of the increased cell population from the control to treated samples. However, in points G0/G1, G1, and S across all three cell lines, the cell population decreases from control samples to treated samples, indicating that cell arrest is not occurring with the FOXO1 inhibitor.

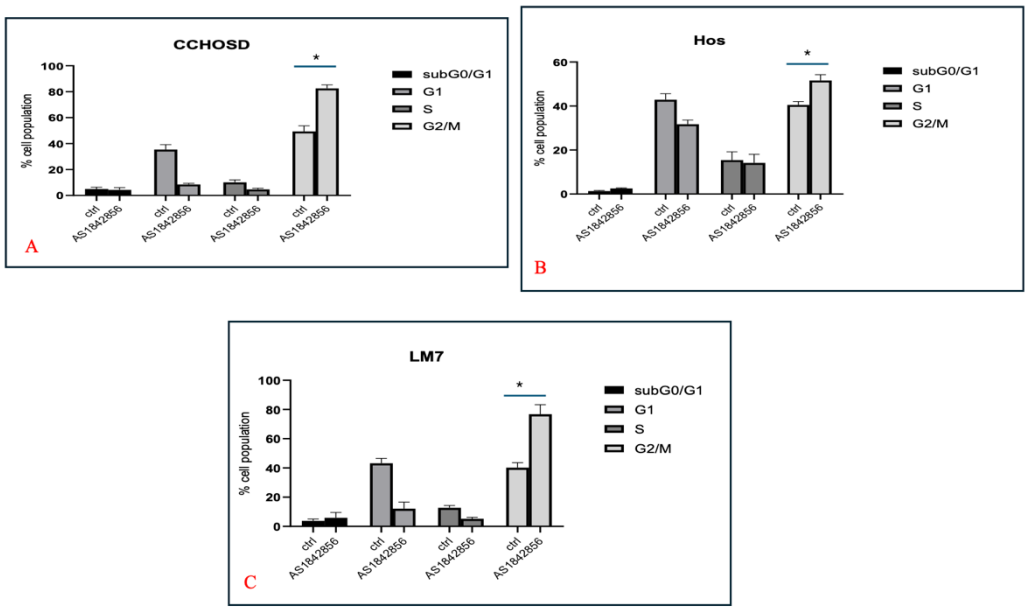

Figure 7. Graph presentation of AS1842856 inducing G2/M cell cycle arrest at in **A.** CCHOSD cells **B.** Hos cells **C.** LM7 cells. Cells were treated with 0.01% DMSO (control) or 10μM AS1842856 for 24 hr (CCHOSD and Hos) or 48 hr (LM7). Following AS1842856 treatment, cells were collected, and the cell cycle was analyzed as described in the design of study section.

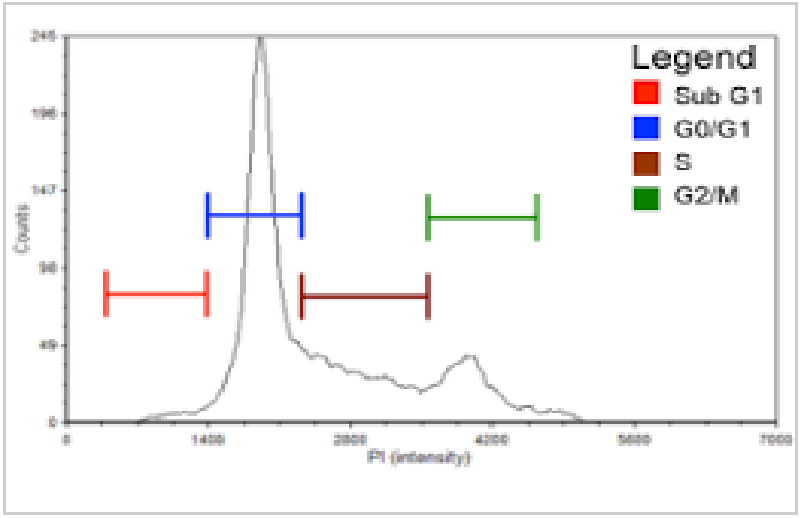

Figure 8. A control population histogram for a control sample generated with FCS Express 4 software. The sub-G1 population is in red, the G0/G1 population is indicated in blue, the S population is in brown, and the G2/M population is indicated in green (Kuksin, 2013).

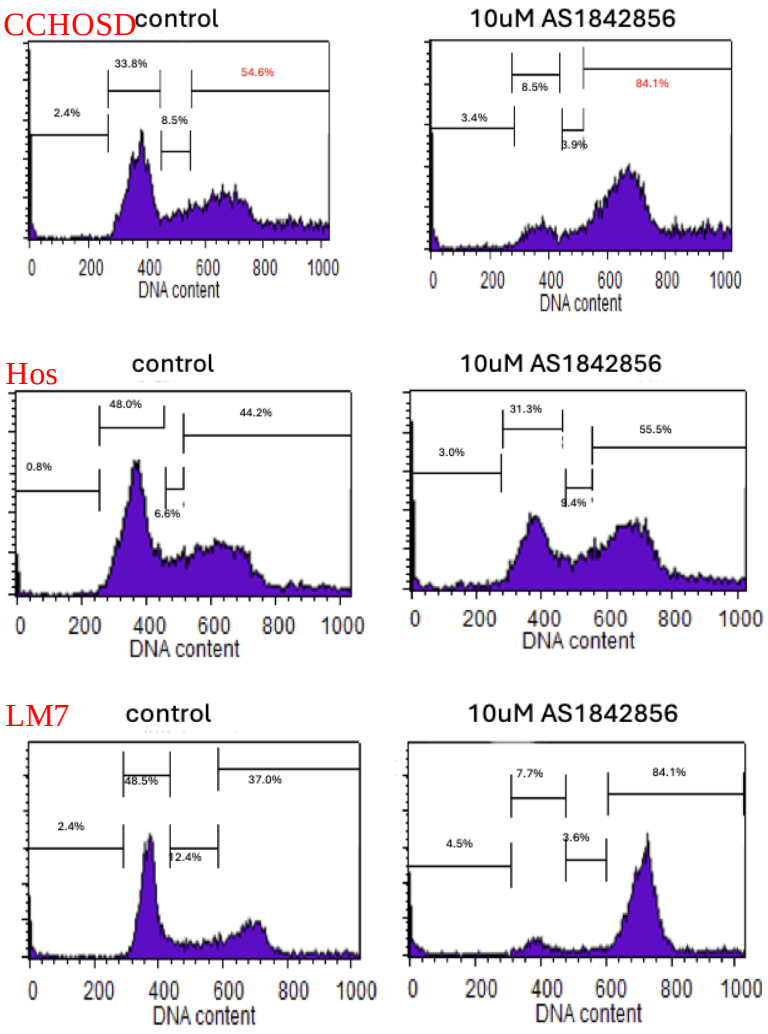

Figure 9. Histogram presentation of AS1842856 inducing G2/M cell cycle arrest. Cells were treated with 0.01% DMSO (control) or 10μM AS1842856 for 24 hr (CCHOSD and Hos) or 48 hr (LM7). Following AS1842856 treatment, cells were collected, and the cell cycle was analyzed as described in the design of study section.

Western blots were done to assess the effect of AS1842856 on the expression of cell cycle inhibitors p16, p21, and p27. The expression of cell cycle inhibitor proteins in the LM7 cell line treated with AS1842856 decreased as opposed to the initial western blot analysis (Figure 10). Hos cell line showed no decrease in p27, considering that inhibitor was the only inhibitor present before treatment (Figure 10). CCHOSD-treated cell line showed a significant increase in the expression of p21.

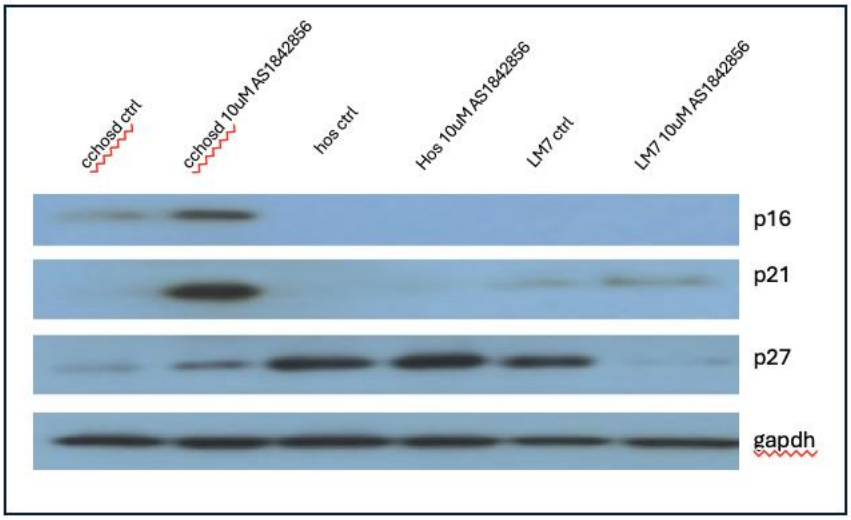

Figure 10. Western blot showing AS1842856 effect on cell cycle inhibitor expression. Gapdh served as a protein loading control.

Western blot analysis was used to investigate the potential mode of cell death by analyzing the activation and cleavage of caspase-3. Cells were pretreated with AS1842856 along with CPT. Full-length and cleaved caspase-3 were used as a marker for caspase activation. Results indicate that both CPT-treated and AS18-CPT-treated samples induce caspase activation in the CCHOSD line (Figure 11). Results also showed that only CPT-treated samples induce caspase activation in the Hos cell line. The reversal is shown to be the greatest in the Hos cell line (Figure 11), which indicates that apoptosis is the

mode of cell death. The CCHOSD cell line also showed some reversal, but not as great as the Hos cell line (Figure 11).

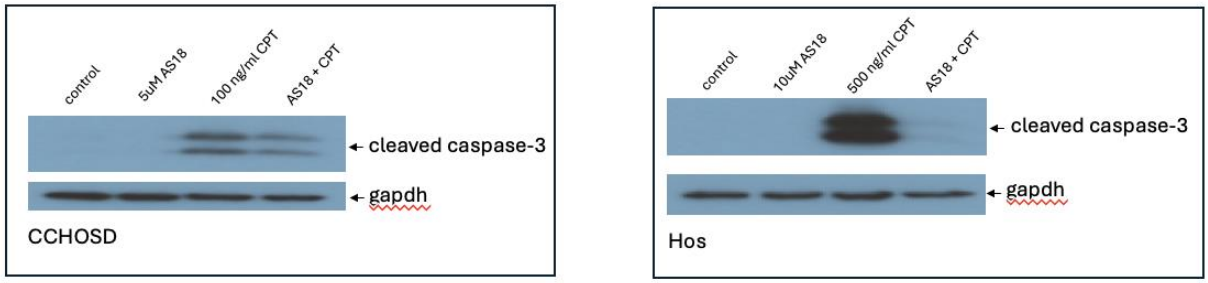

Figure 11. Western blot showing AS1842856 reversal of CPT-induced caspase-3 activation.

Western blot analysis was also used to confirm that the cleaved caspase-3 was indeed active with PARP1 activation in Hos and CCHOSD cells. PARP1 is a target of active caspase-3. Full-length and cleaved PARP1 were used as a marker for PARP1 activation. Results indicate that CPT-treated and AS18-CPT-treated samples in the CCHOSD line induce PARP1 activation (Figure 12). Results also indicated that the CPT-treated sample in the Hos line induces PARP1 activation (Figure 12).

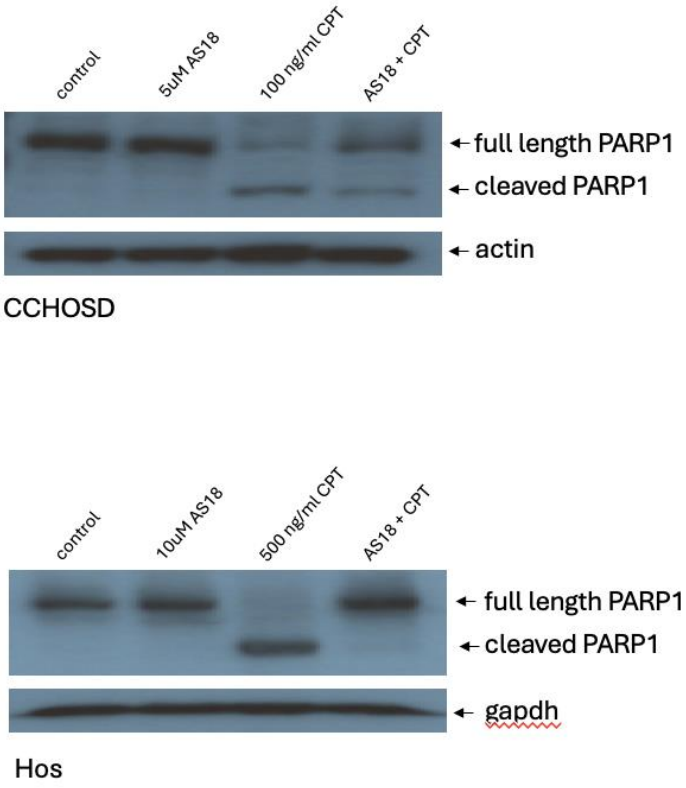

Figure 12. Western blot showing AS184285 reversal of CPT-induced PARP1 cleavage.

CHAPTER 5

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Research studies have shown the ability of FOXO1 inhibition to play a role in inducing cell cycle arrest and reversing the harmful and damaging effects of anticancer therapies. Research demonstrated that inhibition of FOXO1 can lead to pro-apoptotic expression and reduce colony formation in cancer cell lines (Flores et al., 2023). This inhibition of FOXO1 highlights a novel targeted cancer therapy. It provides evidence of a promising approach to induce cell cycle arrest to combat cancer and attempt to reverse the harmful effects of anticancer medications. In this study, we investigated the effects of the FOXO1 inhibitor, AS1842856, on cell cycle regulation and reversal of anticancer drug-induced damage and death in CCHOSD, Hos, and LM7 osteosarcoma cell lines. Results of this study demonstrated that FOXO1 inhibition induces cell cycle arrest at the most optimal opportunity in the cell cycle, G2/M phase, and FOXO1 inhibition will reverse CPT-induced cell death.

The results suggest inhibiting FOXO1 may be a promising approach for treating osteosarcoma. Cells were pretreated with a FOXO1 inhibitor, AS1842856, for one hour following CPT treatment. This pretreatment illustrated a significant increase in cell viability in all three cell lines. This evidence aligns with prior studies that suggest that FOXO1 inhibition can protect cancer cells from drug-induced death and overcome

chemotherapy resistance by attenuating apoptotic responses (Beretta et al., 2019). The ability to rescue cells from drug-induced death indicates that the FOXO1 pathway may play an essential role in mediating the cytotoxic effects of certain chemotherapies, such as camptothecin (CPT). Cells were treated with AS1842856 for 24 hours, followed by an assessment of cell cycle status. Following this treatment, it was evident that the cells arrested at every cell cycle stage but most significantly at the G2/M phase. The G2/M phase is a critical checkpoint in preventing DNA-damaged cells from entering mitosis. At the other checkpoints and phases, such as G1, cells prepare for DNA replication and go through DNA replication, as in the synthesis (S) phase, making those phases not optimal for cell cycle arrest as cellular processes can be disrupted. By inducing cell cycle arrest at the G2/M, the FOXO1 inhibitor slowed the growth and division of the cancer cells.

Initially, the different basal levels of cell cycle inhibitors' expression were measured across all three cell lines, with CCHOSD and LM7 expressing all three inhibitors while Hos did not express p16. Another experiment was conducted to measure the effect of AS1842856 on the expression of those same cell cycle inhibitors in the cell lines. AS1842856 increases the expression of all three cell cycle inhibitors in the CCHOSD cell line. However, in the Hos and LM7 cell lines, it was difficult to get a clear read of an increase in the inhibitors' expression, which could be explained by other cell cycle regulating components, such as cyclins being affected by FOXO1 inhibition treatment.

Moving forward, we investigated the potential mode of cell death by analyzing the activation of caspase-3 in CPT-treated CCHOSD and Hos cell lines. Caspase-3 is a

critical caspase that plays a central role in the apoptotic cell death pathway. Once activated, caspase-3 cleaves and activates other downstream caspases to induce cell death via apoptosis. Pretreatment of AS1842856 reversed the CPT-induced activation of caspase-3, with the greatest reversal in the Hos cell line. PARP1 is a direct target of caspase-3, and a follow-up experiment was conducted to confirm that caspase-3 was indeed active and signaling apoptosis. The cleavage of full-length PARP1 into smaller fragments is a critical event in apoptosis signaling, leading to inactivation of DNA repair abilities and ultimately promoting cell death. Pretreatment of AS1842856 reversed CPT-induced PARP1 cleavage in CCHOSD and Hos cell lines, with the greatest reversal in the Hos cell line, indicating that caspase-3 was active and signaling apoptosis in CPT-treated cells.

In summary, the findings underscore the potential of FOXO1 inhibition in inducing cell cycle arrest and mitigating the detrimental effects of anticancer therapies. The experiments revealed the efficacy of AS1842856 in inducing cell cycle arrest at optimal opportunities and reversing CPT-induced cell death in osteosarcoma cell lines. Moreover, pretreatment with AS1842856 increased cell viability alongside CPT treatment, suggesting a protective role against drug-induced apoptosis and helping overcome chemotherapy resistance. The protective role could be particularly valuable, as drug resistance is a significant challenge in cancer treatment. The data is indicative of FOXO1 being a critical regulator of osteosarcoma cell proliferation and survival. Targeting this transcription factor with small molecule inhibitors or other therapeutic approaches could represent a novel strategy for treating osteosarcoma patients as a monotherapy or combined with existing chemotherapies.

To build on the promising results from this project, several avenues must be explored, including the effect of FOXO1 inhibition on the response of osteosarcoma to other anticancer drugs. A recent study has shown that inhibition of the FOXO1 pathway promoted autophagy and apoptosis in osteosarcoma cells, including those resistant to methotrexate, a standard chemotherapy drug (Luo et al., 2023). This study suggests that inhibition of FOXO1 and chemotherapeutic drugs could be a promising approach to overcoming drug resistance in osteosarcoma (Guan et al., 2015). Numerous studies indicate that FOXO1 can play the role of both tumor-suppressive and tumor-promoting in osteosarcoma, depending on the specific context and the anticancer drug involved. Inhibition of FOXO1 can sensitize osteosarcoma cells to specific chemotherapeutic agents, while its activation can confer resistance.

REFERENCES

Abukhdeir, A. M., & Park, B. H. (2008, July 1). *P21 and p27: Roles in carcinogenesis and drug resistance*. Expert reviews in molecular medicine. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2678956/

Beretta, G. L., Corno, C., Zaffaroni, N., & Perego, P. (2019, January 14). *Role of FoxO proteins in cellular response to antitumor agents*. MDPI. https://www.mdpi.com/2072-6694/11/1/90

Brown, A. K., & Webb, A. E. (2017, November 15). *Regulation of FOXO factors in mammalian cells*. Current topics in developmental biology. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6383790/

Chen, X., Luo, X., & Cheng, Y. (2018, December). *TRIFLUOPERAZINE prevents FOXO1 nuclear excretion and reverses doxorubicin-resistance in the shg44/dox drug-resistant glioma cell line*. International journal of molecular medicine. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6202074/

Farhan, M., Silva, M., Xingan, X., Huang, Y., & Zheng, W. (2020, June 30). *Role of Foxo transcription factors in cancer metabolism and angiogenesis*. MDPI. https://www.mdpi.com/2073-4409/9/7/1586

Farhan, M., Wang, H., Gaur, U., Little, P. J., Xu, J., & Zheng, W. (2017, July 6). *Foxo signaling pathways as therapeutic targets in cancer*. International journal of biological sciences. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5555100/

Flores, D., Lopez, A., Udawant, S., Gunn, B., & Keniry, M. (2023, February). *The FOXO1 inhibitor AS1842856 triggers apoptosis in glioblastoma multiforme and basal-like breast cancer cells*. The FOXO1 inhibitor AS1842856 triggers apoptosis in glioblastoma multiforme and basal-like breast cancer cells. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9900086/

Gheghiani, L., Shang, S., & Fu, Z. (2020a, July 23). *Targeting the PLK1-FOXO1 pathway as a novel therapeutic approach for treating advanced prostate cancer*. Scientific reports. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7378169/

Gheghiani, L., Shang, S., & Fu, Z. (2020b, July 23). *Targeting the PLK1-FOXO1 pathway as a novel therapeutic approach for treating advanced prostate cancer*. Scientific reports. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7378169/#:~:text=The%20forkhead%20box%20protein%20O1,cycle%20arrest%2C%20and%20DNA%20damage.

Goto, T., Takano, M., Hirata, J., & Tsuda, H. (2008, March 25). *The involvement of FOXO1 in cytotoxic stress and drug-resistance induced by paclitaxel in ovarian cancers*. British journal of cancer. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2275483/

Guan, H., Tan, P., Xie, L., Mi, B., Fang, Z., Li, J., Yue, J., Liao, H., & Li, F. (2015). FOXO1 inhibits osteosarcoma oncogenesis via Wnt/β-catenin pathway suppression. Oncogenesis, 4(9), e166. https://doi.org/10.1038/oncsis.2015.25

Jiramongkol, Y., & Lam, E. W.-F. (2020, September). *Foxo transcription factor family in cancer and metastasis*. Cancer metastasis reviews. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7497309/#CR94

Kim, S. Y., Ko, Y. S., Park, J., Choi, Y., Park, J. W., Kim, Y., Pyo, J. S., Yoo, Y. B., Lee, J. S., & Lee, B. L. (2022, February 25). *Forkhead transcription factor FOXO1 inhibits angiogenesis in gastric cancer in relation to SIRT1*. Seoul National University College of Medicine. https://snucm.elsevierpure.com/en/publications/forkhead-transcription-factor-foxo1-inhibits-angiogenesis-in-gast

Kodani, N., & Nakae, J. (2020, March 13). *Tissue-specific metabolic regulation of FOXO-binding protein: FOXO does not act alone*. MDPI. https://www.mdpi.com/2073-4409/9/3/702

Kuksin, D. (2013, April 17). *Fluorescence-based cell cycle analysis-part 1*. Nexcelom Bioscience. https://www.nexcelom.com/blogs/fluorescence-based-cell-cycle-analysis/

Laissue, P. (2019, January 8). The forkhead-box family of transcription factors: Key molecular players in colorectal cancer pathogenesis - molecular cancer. BioMed Central. https://molecular-cancer.biomedcentral.com/articles/10.1186/s12943-019-0938-x

Liu, Y., Ao, X., Ding, W., Ponnusamy, M., Wu, W., Hao, X., Yu, W., Wang, Y., Li, P., & Wang, J. (2018, July 25). *Critical role of Foxo3a in carcinogenesis*. BioMed Central. https://molecular-cancer.biomedcentral.com/articles/10.1186/s12943-018-0856-3#ref-CR134

Lloyd, R. V., Erickson, L. A., Jin, L., Kulig, E., Qian, X., Cheville, J. C., & Scheithauer, B. W. (1999, February). *P27KIP1: A multifunctional cyclin-dependent kinase inhibitor with prognostic significance in human cancers*. The American journal of pathology. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1850003/

Lu, H., & Huang, H. (2011, August). *FOXO1: A potential target for human diseases*. Current drug targets. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4591039/

Luo, M., Su, Z., Gao, H., Tan, J., Liao, R., Yang, J., & Lin, L. (2023, December 12). *CIRSILIOL induces autophagy and mitochondrial apoptosis through the AKT/FOXO1 axis and influences methotrexate resistance in osteosarcoma - journal of translational medicine*. BioMed Central. https://translational-medicine.biomedcentral.com/articles/10.1186/s12967-023-04682-7

Rathore, R., & Van Tine, B. A. (2021, March 12). *Pathogenesis and current treatment of osteosarcoma: Perspectives for Future Therapies*. Journal of clinical medicine. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8000603/#:~:text=Osteosarcoma%20is%20the%20most%20common,in%20more%20than%2040%20years.

Shi, F., Li, T., Liu, Z., Qu, K., Shi, C., Li, Y., Qin, Q., Cheng, L., Jin, X., Yu, T., Di, W., Que, J., Xia, H., & She, J. (2017, September 28). *FOXO1: Another avenue for treating digestive malignancy?*. Seminars in Cancer Biology. https://www.sciencedirect.com/science/article/pii/S1044579X17301839

Taylor, S., Lam, M., Pararasa, C., Brown, J. E., Carmichael, A. R., & Griffiths, H. R. (2015, January 24). *Evaluating the evidence for targeting FOXO3A in breast cancer: A systematic review - cancer cell international*. Evaluating the evidence for targeting FOXO3a in breast cancer: a systematic review. https://cancerci.biomedcentral.com/articles/10.1186/s12935-015-0156-6

Vermeulen, K., Van Bockstaele, D. R., & Berneman, Z. N. (2003, June 17). *The cell cycle: A review of regulation, deregulation and therapeutic targets in cancer*. Cell proliferation. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6496723/

Witkiewicz, A. K., Knudsen, K. E., Dicker, A. P., & Knudsen, E. S. (2011, August 1). *The meaning of p16(ink4a) expression in tumors: Functional significance, clinical associations and future developments*. Cell cycle (Georgetown, Tex.). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3685613/

Xing, L., Zhang, X., Feng, H., Liu, S., Li, D., Hasegawa, T., Guo, J., & Li, M. (2019, April 19). *Silencing FOXO1 attenuates dexamethasone-induced apoptosis in osteoblastic MC3T3-E1 cells*. Biochemical and Biophysical Research Communications. https://www.sciencedirect.com/science/article/abs/pii/S0006291X19307569

Xing, Y., Li, A., Yang, Y., Li, X., Zhang, L., & Guo, H. (2017, November 20). *The regulation of FOXO1 and its role in disease progression*. Life Sciences. https://www.sciencedirect.com/science/article/abs/pii/S0024320517306070

Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R., & Beach, D. (1993, December 16). *P21 is a universal inhibitor of cyclin kinases*. Nature News. https://www.nature.com/articles/366701a0

Yadav, R. K., Chauhan, A. S., Zhuang, L., & Gan, B. (2018, June). *Foxo transcription factors in cancer metabolism*. Seminars in cancer biology. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5986595/#:~:text=FoxO%20transcription%20factors%20serve%20as,an%20emerging%20hallmark%20of%20cancer.

Yang, S., Pang, L., Dai, W., Wu, S., Ren, T., Duan, Y., Zheng, Y., Bi, S., Zhang, X., & Kong, J. (2021, April 28). Role of forkhead box O proteins in hepatocellular carcinoma biology and progression (review). Frontiers. https://www.frontiersin.org/journals/oncology/articles/10.3389/fonc.2021.667730/full

Yu, Z. K., Gervais, J. L., & Zhang, H. (1998). Human CUL-1 associates with the SKP1/SKP2 complex and regulates p21(CIP1/WAF1) and cyclin D proteins. Proceedings of the National Academy of Sciences of the United States of America, 95(19), 11324–11329. https://doi.org/10.1073/pnas.95.19.11324

Zhang, W., Duan, N., Song, T., Li, Z., Zhang, C., & Chen, X. (2017, June 3). *The emerging roles of forkhead box (Fox) proteins in osteosarcoma*. Journal of Cancer. https://www.jcancer.org/v08p1619.htm

Zhang, X., Tang, N., Hadden, T., & Rishi, A. (2011, March 31). *AKT, FoxO and regulation of apoptosis*. Biochimica et biophysica acta. https://pubmed.ncbi.nlm.nih.gov/21440011/

Zou, P., Liu, L., Zheng, L., Liu, L., Stoneman, R. E., Cho, A., Emery, A., Gilbert, E. R., & Cheng, Z. (2014). *Targeting FOXO1 with AS1842856 suppresses adipogenesis*. Cell cycle (Georgetown, Tex.). https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4613185/