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The Effect of Circadian Clock Modulation on Cisplatin Cytotoxicity

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THE EFFECT OF CIRCADIAN CLOCK MODULATION ON CISPLATIN CYTOTOXICITY

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY **Nadeen Nibal Ahmad Anabtawi** ENTITLED **THE EFFECT OF CIRCADIAN CLOCK MODULATION ON CISPLATIN CYTOTOXICITY** BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

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Cisplatin is a DNA damage-based chemotherapeutic drug widely used to treat various types of cancers; however, the treatment's toxicity restricts its efficiency. Studies have shown that the circadian rhythm controls the DNA damage response and affects the repair pathways of cisplatin-induced DNA damage. Circadian clock modulation, therefore, has been proposed to be a potential mechanism for enhancing cisplatin tolerability. Here we used clock-enhancing molecules to evaluate the effect of pharmacological clock modulation on cisplatin cytotoxicity. Using cultured human cell lines, cisplatin cytotoxicity was found to be attenuated following treatment with circadian-enhancing molecules KS15 and SR8278. Moreover, the protein and mRNA levels of cell cycle and apoptosis regulators, as well as clock-controlled genes, were modified in response to KS15 and SR8278. Those molecules were also able to enhance cisplatin-induced DNA adducts removal and induce G1-phase cell cycle arrest. Our findings suggest that the use of circadian clock modulators has promising implications for improving cancer care and treatment outcomes.

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LIST OF ABBREVIATIONS

A549, Lung adenocarcinoma human cells
ANOVA, Analysis of variance.
BMAL, Brain and muscle aryl hydrocarbon receptor nuclear
BSA, Bovine Serum Albumin
cDNA, Complementary DNA
Clock, Circadian clock
Cry, Cryptochrome
DMEM, Dulbecco's modified Eagle's medium
DMSO, Dimethyl sulfoxide.
DNA, Deoxyribonucleic acid
E-Box, Enhancer box
EDTA, Ethylenediaminetetraacetic acid
F-12K, Kaighn's Growth Medium
FBS, Fetal bovine serum
gDNA, Genomic DNA
HaCaT, Immortalized human keratinocytes
IC50, Half maximal inhibitory concentration
mg, milligram
min, minute
ml, milliliter
mRNA, messenger RNA.
MTT, Methylthiazolyldiphenyl-tetrazolium bromide
NER, Nucleotide Excision Repair
PAGE, Polyacrylamide gel electrophoresis
PARP, Poly (ADP-ribose) polymerase
PBS, Phosphate buffered saline
PCR, Polymerase chain reaction
Per, Period
PI, Propidium iodide
qPCR, Quantitative PCR
RNA, Ribonucleic acid.
ROR, Related orphan receptor
rpm, Revolutions Per Minute
RT-PCR, Reverse transcription PCR
SCN, Suprachiasmatic nucleus
SDS, Sodium dodecyl sulfate
SEM, Standard error of the mean
TBST, Tris-buffered saline + 0.1% Tween
TTFL, Transcription-translation feedback loop
U2OS, Human bone osteosarcoma epithelial cells
µg, microgram
µM, micromolar
v/v, volume/volume
XPA, Xeroderma pigmentosum complementation group A

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1 INTRODUCTION

1.1 Cisplatin

Cisplatin is a platinum-based chemotherapeutic drug widely used to treat various types of cancers, including lung, breast, esophageal, ovarian, and pancreatic cancers. Despite its efficacy, cisplatin's clinical use is limited by its cytotoxicity to various tissues, such as renal toxicity, ototoxicity, myelosuppression, and gastrointestinal toxicity (1–3). These side effects could restrict cisplatin's efficiency and lead to therapeutic failure. For example, nephrotoxicity is a dose-limiting side effect, and it is one of the leading causes of treatment discontinuations, accounting for 25–30% of cisplatin-based chemotherapy discontinuations (4). Cisplatin exerts its effect by crosslinking with DNA purine bases and forming bulky DNA adducts (**Figure 1.1**); consequently, it induces cellular apoptosis and cell cycle arrest (4–6). The severity of cisplatin's toxicity and its efficacy are highly influenced by forming bulky DNA lesions. Those lesions are primarily repaired by the NER (Nucleotide Excision Repair) system to minimize the cisplatin-induced DNA damage. In addition to the DNA adduct formation and repair, other factors modulate cisplatin therapy, such as apoptosis pathway, drug uptake, and cellular efflux (7–9).

One promising strategy for enhancing cisplatin's tolerability and treatment outcomes is chronochemotherapy, which involves administering the chemotherapeutic agent at particular times of the day to optimize efficacy and reduce side effects (10). This suggests administering medications when healthy cells are least susceptible to toxicity, and cancer cells are more vulnerable to the drug's effects. It has been reported that platinum-based chemotherapeutic agents, including cisplatin, are good candidates for chronochemotherapy, and their toxicities could be modulated by changing the time of administration (11–13). For example, in patients with metastatic colorectal cancer, platinum drugs given in the afternoon were found to have substantially fewer adverse effects than traditional chemotherapy regimens (14). Furthermore, a randomized controlled trial evaluating the tolerability of cisplatin between lung cancer patients receiving regular doses and patients receiving chronotherapeutic doses found that the rate of leucopenia, neutropenia, and gastrointestinal toxicity in the chronotherapy group is significantly lower than in the regular chemotherapy group (15). Better chemotherapy tolerance observed with chronomodulated regimens has been associated with higher patients' survival and better quality of life.

In animal models, it has been shown that tolerance of platinum-based chemotherapy is influenced by administration time. Evening doses of oxaliplatin resulted in three times more tolerance than morning doses in a sample of mice treated with the drug for colorectal cancer (16). Similarly, in a melanoma mouse model, cisplatin renal and blood toxicities were less severe in the evening treated mice compared to the morning treated ones (17). In rodent models, more than ten classes of anticancer medications demonstrated significant differences in effectiveness or tolerance depending on the time of administration (11).

Although chronotherapeutic outcomes of cisplatin administration have been observed in animal models and human subjects, the molecular mechanism for these findings has only recently been explored. The latest evidence has shown that the circadian regulation of the NER system is responsible for cisplatin toxicity chronomodulation. The activity of Xeroderma pigmentosum complementation group A (XPA), an essential protein in the NER pathway, is regulated by the circadian clock in both mice and humans. The circadian oscillation of XPA influences the repair of cisplatin-DNA adducts by the NER system, and as a result, it modulates the associated cisplatin toxicity (18, 19). It has been reported that nucleotide excision repair activity in the mouse cortex was found to be the highest in the afternoon/evening hours and the lowest in the night/early morning hours (20). Similarly, there was an increased rate of cisplatin-DNA adduct removal via NER in the evening compared to the morning in melanoma mouse models and human subjects (17).

Although the concept of chronomodulation of anticancer agents is well supported in clinical trials and animal studies, the clinical application is not completely established yet. A proposed approach of implementing chronochemotherapy in cancer treatment is based on computational modeling targeting cell cycle phases and DNA repair pathways (21). Additionally, regulation of the circadian clock through bright light therapy has been investigated to increase chemotherapy tolerability in some cancers (22). However, there has not been much research done on using pharmacological modulation of the circadian clock to optimize chemotherapy efficacy and toxicity.

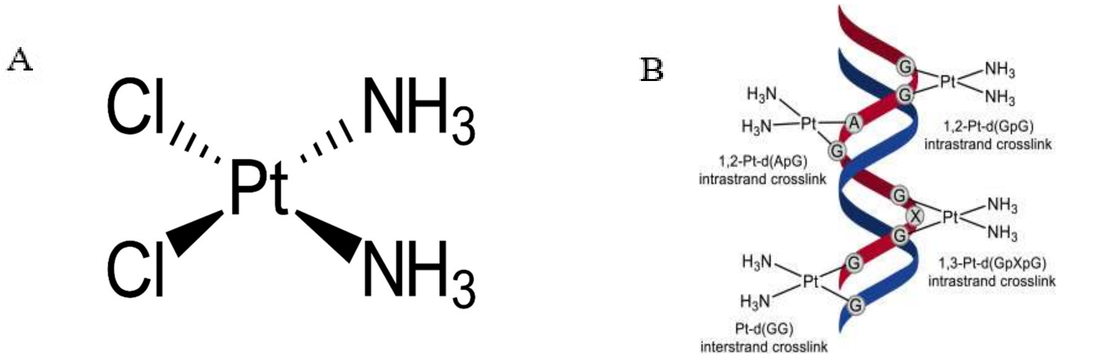


Figure 1.1: The structure of cisplatin and its mechanism of action. (A) The structure of cisplatin. **(B)** Cisplatin exerts its effect by crosslinking with DNA purine bases and forming bulky DNA adduct, so it induces cellular apoptosis and cell cycle arrest (4).

1.2 Circadian Clock

The circadian clock (from the Latin *circa* meaning "about" and *dies* meaning "day") is a molecular time-keeping system that controls the 24-hour cycle of several behavioral and biological functions. The discovery of the circadian system dated back to 1729 when Jean-Jacquesd'Ortous de Mairan observed a 24-hour periodicity of the *Mimosa* plant leaves' movement even without light stimulation. This indicates that the biological clock rhythm is endogenously controlled despite the absence of stimuli (23). Then several observations were reported to support the hypothesis that both animals and plants exhibit a particular circadian behavior. However, the mechanism of circadian rhythm remained unknown until the first clock mutation in *Drosophila melanogaster* was identified by Ron Konopka and Seymour Benzer, which was a mutation in the *period (Per)* gene (24). More clock genes involved in circadian timing were subsequently identified, and the basis of the molecular mechanism of the circadian clock was eventually established. In 2017, Jeffrey C. Hall, Michael Rosbash, and Michael W. Young were awarded the Nobel Prize in Physiology or Medicine for their effort in discovering the molecular mechanism controlling circadian rhythms. They independently cloned the *per* gene and conducted their experiments between the 1970s and 1990s (25).

In mammals, the circadian system is generated centrally by the master clock in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, which in turns regulates subsidiary clocks in peripheral tissues. Peripheral clocks play a crucial role throughout the body regulating the circadian expression of various genes, called clock-controlled genes, involved in a wide range of cellular and physiological functions (26, 27). At the molecular level, the circadian clock is composed of transcription-translation feedback loops that regulate 24-hour based oscillations of several clock components (**Figure 1.2**). At the core loop, a heterodimer complex known as CLOCK-BMAL1, a transcriptional factor that binds to a specific DNA sequence within the promoter region called E-box, turns on the expression of specific clock-controlled genes. Two of the gene products that are known to be regulated by the CLOCK-BMAL1 complex are the *Cryptochrome* (*Cry)* and *Period* (*Per)* genes. After translating the *Cry* and *Per* mRNAs in the cytosol, the CRY and PER proteins then re-enter the nucleus and interact with each other to block CLOCK-BMAL1 expression. The CRY and PER proteins' stability are regulated by different ubiquitin ligases that can induce protein degradation. At the stabilizing loop, *Bmal1* gene transcription is regulated by REV-ERBs and RORs, which are nuclear receptors that either suppress or activate *Bmal1* gene transcription. They are also considered targets for the CLOCK-BMAL1 complex. The interaction between the two loops ensures a robust regulation of the circadian clock output and provides a 24-h oscillation of the clock components (28–30).

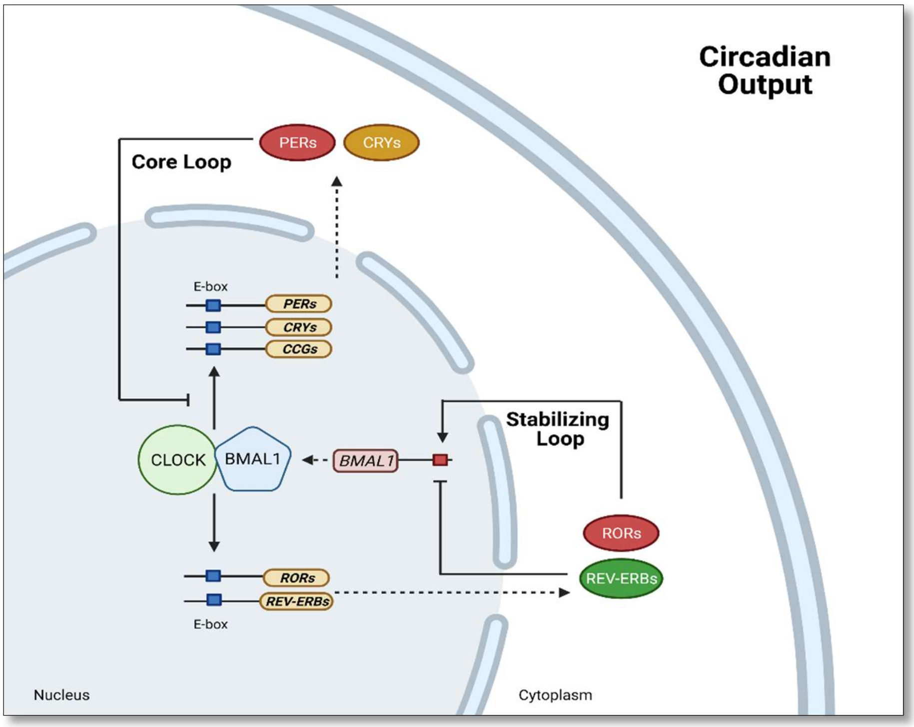


Figure 1.2: The molecular mechanism of the circadian clock. The circadian clock is composed of two interacting transcription-translation feedback loops. The core loop consists of the CLOCK-BMAL1 complex that regulates the transcription and translation of *Per* and *Cry* and other clock-controlled genes. In the other loop, *Bmal1* expression is regulated by REV-ERBs and RORs, which are nuclear receptors that either suppress or activate *Bmal1* gene transcription, and they are also considered targets of the CLOCK-BMAL1 complex. Adapted from Cha HK, et al. (2019) Small Molecule Modulators of the Circadian Molecular Clock With Implications for Neuropsychiatric Diseases (28).

1.3 Circadian Clock and Chemotherapy

The effect of genotoxic chemotherapeutic drugs like cisplatin on cancer cells is determined by the cellular response to DNA damage, which includes DNA repair, DNA damage checkpoints, and apoptosis. Since the circadian clock is known to regulate these responses, it is predicted that it will also affect the effectiveness and toxicity of chemotherapy.

1.3.1 Clock control of DNA repair

There are various DNA repair mechanisms, including nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), and double-strand break/crosslink repair. Out of those pathways, the NER system appears to be directly regulated by the circadian clock due to the circadian oscillation of XPA, the rate-limiting factor in the NER pathway that plays a significant role in cisplatin-induced DNA adducts repair. In mouse models, XPA transcription, XPA protein level, and excision activity all have shown a daily rhythm that increases during the evening and decreases during the early morning. Consequently, the removal rate of cisplatin-DNA adducts also exhibits circadian rhythmicity that follows a similar manner (18–20). Furthermore, XPA transcriptional levels and NER activity were higher and did not oscillate in *Cry* knockout mice, implying that the oscillation of NER activity and the XPA transcription is dependent on the circadian clock (18). Additionally, clock control of the NER system has previously been demonstrated in multiple tissues, including the kidney, which is the primary site of cisplatin's toxicity, making this finding clinically crucial in terms of cisplatin tolerability (17). Since the cell's ability to repair bulky DNA lesions influences the magnitude of cisplatin's toxicity and efficacy, controlling DNA repair mechanisms by the clock is expected to affect cisplatin therapeutic outcomes.

1.3.2 Clock control of DNA damage checkpoints

The evidence suggests that the clock controls the cell cycle by regulating the DNA damage checkpoints, which are the regulatory points within the cell cycle that hold the cycle progression when damage is detected within the DNA (31–34). There are multiple checkpoints through the cell cycle, G1/S checkpoint, which prevents DNA-damaged cells from starting S phase, intra-S checkpoint which stops late replication initiation; S-M and G2/M checkpoints which prevent starting mitosis phase to give the cell enough time for DNA repair before division. The circadian clock has been shown to control cell-cycle events' timing and efficacy by regulating cell-cycle-related genes involved in the checkpoint signals. In mouse liver cells and fibroblasts, the clock has been shown to control the G1/S checkpoint by regulating the cyclin-dependent kinase inhibitor protein p21, which was positively regulated by the CLOCK-BMAL1 gene (35). Moreover, the additional cyclin-dependent kinase inhibitor p20, which controls the G1/S transition of the cell cycle, has also been discovered to be a highly rhythmic gene (36). The circadian clock controls not only G1/S but also G2/M checkpoint through regulation of Wee1 expression profile. Wee1 is a clock-controlled gene that acts as a negative regulator of mitosis and plays an essential role in G2-M transition. Wee1 mRNA, protein, and kinase activity levels have all been elevated in *Cry* deficient mice, indicating that it is positively regulated by the clock (37). Although cisplatin is not a cell cycle-specific chemotherapy, cells tend to be most sensitive to it in the G1 phase and least sensitive in peak DNA synthesis, with a decrease in sensitivity as cells enter the S phase (38). Moreover, when cisplatin-induced DNA adducts are detected, cyclin-dependent kinase inhibitor proteins like p21 and Wee1 are induced, and cells are arrested in either G1 or G2 phases (39). Therefore, cisplatin cytotoxicity would be affected by the circadian regulation of the cell cycle and DNA damage checkpoints.

1.3.3 Clock control of apoptosis

Cisplatin's anti-tumor activity is primarily mediated by DNA damage-induced apoptosis. Following the DNA damage recognition, cisplatin is believed to activate the p53 protein (40). p53 is a tumor suppressor protein that plays an essential role in DNA damage-induced apoptosis, and it upregulates the expression of pro-apoptotic genes in response to genotoxic stress. It has been shown that cryptochrome mutation in mice sensitized p53 mutant cells to apoptosis, suggesting a connection between the circadian clock and apoptosis (41). Moreover, it has been observed that *Per2* downregulation resulted in decreased p53 mRNA expression and lower apoptotic activity in human oral squamous cell carcinoma (42). Since these findings indicate a correlation between the circadian clock and p53-apoptosis, it is to be expected that the clock would affect the cisplatin's cytotoxicity.

1.4 Circadian Clock Modulation

Disruption of the internal circadian clock by external factors such as shift work, jetlag, misaligned sleep-wake cycle, or irregular food intake has been linked to many diseases, including cancer, metabolic, and mood disorders (43–45). Additionally, since the clock controls various cellular functions and signaling pathways, which are often targeted by cytotoxic anticancer agents, a positive correlation has been established between a robust circadian rhythm and the therapeutic response to chemotherapeutic drugs (46–48). As a result, the value of a balanced circadian rhythm is becoming more commonly recognized, and the discovery of clock modulation strategies that maintain a stable circadian rhythm has rapidly evolved.

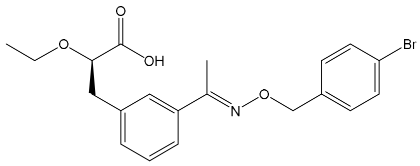
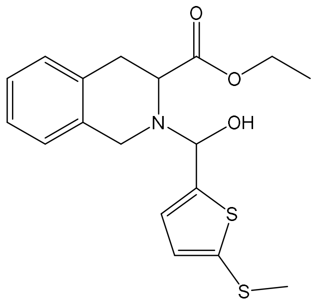
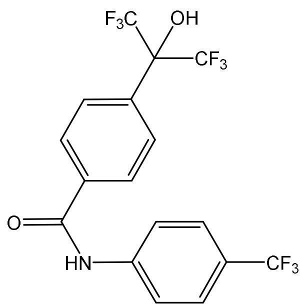
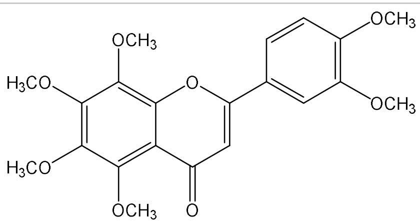
At the molecular level, a robust circadian rhythm can only be achieved if the quantities, localization, and activity of clock-related genes and proteins are accurately regulated. Understanding the molecular mechanism of circadian regulation of chemotherapy cytotoxicity is being used to develop behavioral and pharmacological approaches targeted to enhance the circadian output and improve cancer treatment outcomes. To begin, behavioral interventions in feeding-fasting, sleep-wake, or light-dark cycles were used to maintain a stable circadian timing. It has been shown that keeping a regular eating and sleeping schedule will support having a consistent circadian cycle and minimizing clock disruption (49, 50). Bright light therapy has been proposed to protect against circadian rhythm desynchronization and investigated to prevent chemotherapy and radiotherapy side effects in cancer patients, such as fatigue and oral mucositis (51–53). However, such intervention has not been widely adopted and is still being investigated in a clinical trial titled "The Effects of Light Therapy to Treat Cancer-related Side Effects" expected to be completed in 2024 (54).

The second approach for circadian clock modulation is chronotherapy, which aligns the drug administration time with the internal circadian cycle that gives the best efficacy and least side effects. This suggests administering medications when healthy cells are least susceptible to toxicity, and cancer cells are more vulnerable to the drug's effects. Chemotherapeutic agents have been investigated as targets for chronochemotherapy, and research has shown that treatment with cisplatin, oxaliplatin, doxorubicin, and fluorouracil was significantly affected by circadian drug administration, with substantial differences in adverse side effects and therapeutic outcomes (10, 55, 56). In the United States, one chronotherapy clinical trial is being conducted in the field of cancer therapeutics titled "A Randomized Feasibility Study Testing Temozolomide Chronotherapy for High-Grade Glioma," which is scheduled to be completed in 2022 (57). Nonetheless, it remains challenging to broadly incorporate the principle of chronotherapy in clinical settings, and concerns have been raised about the practical applications of chronotherapy in routine patient care (58, 59).

Considering the limitations of the above-mentioned circadian modulation approaches, strategies to pharmacologically regulate the clock have been developed over the past few years. Since the circadian clock components have natural ligands that often enhance their functions, small molecules have been designed to target several clock components and their regulators. Those molecules can offer a novel therapeutic approach for circadian rhythm-related diseases and could be combined with existing treatment modalities for efficacy and safety improvement. Pharmacological targets for circadian rhythm enhancement include CRYs, REV-ERBs, and RORs, which are key regulators in the molecular circadian clock (60, 61) (**Table 1.1**).

CRYs are among the common core clock proteins targeted by small molecules. CRYs are potent repressors for the clock-controlled gene transcription activated by the CLOCK-BMAL1 complex. Research has shown that a CRY inhibitor, KS15, enhances the circadian output by inhibiting the repressive effect of CRYs on CLOCK-BMAL1 transcriptional activity (62, 63). KS15 usage in cancer was investigated on MCF-7 human breast cancer cells, and it was discovered that it significantly reduced breast cancer cell proliferation and improved responses to doxorubicin and tamoxifen (64). RORs, which are nuclear receptors that activate *Bmal1* gene transcription, is another target for circadian rhythm modulators. RORs agonists, such as SR1078 and Nobiletin, have been shown to enhance the amplitude and duration of circadian output (65, 66). Research has shown that SR1078 treatment resulted in p53 stabilization and apoptosis induction in liver cancer cells (67). Moreover, down-regulation of RORs has been observed in various types of cancers, like breast, ovarian, and prostate cancer, suggesting that RORs agonists are potentially valuable for cancer management (68). Finally, REV-ERBs antagonism could be targeted to enhance the circadian rhythm output. SR8278, which is the only antagonist discovered so far, inhibits the transcriptional repression activity of REV-ERBs, thereby activating *Bmal1* gene transcription and enhancing circadian output (69). REV-ERBs activation has been shown to cause apoptosis, and it has anti-proliferative effects in human breast and gastric cancer cells (70, 71). Furthermore, several molecules with other targets are also being investigated and with the increasing number of studies, clock-enhancing molecules will not only be valuable research tools for further understanding the circadian clock regulation of cancer, but they will also provide novel therapeutic interventions for clock-associated diseases, including cancer prevention and treatment.

Table 1.1: Clock-Enhancing Molecules (72).

Clock Enhancing Molecule	Structure	Molecular Target	Action
KS15		CRY Inhibitor	Enhances CLOCK-BMAL1 transcriptional activity
SR8278		REV-ERBs Antagonist	Activates *Bmal1* gene transcription
SR1078		RORs Agonist	Activates *Bmal1* gene transcription
Nobiletin		RORs Agonist	Activates *Bmal1* gene transcription

1.5 Purpose and Significance

Cisplatin is a widely used chemotherapeutic agent for the treatment of various types of cancers. However, cisplatin's clinical use is limited by its cytotoxicity to off-target healthy tissues such as renal toxicity, ototoxicity, myelosuppression, and gastrointestinal toxicity. These side effects could restrict cisplatin's efficiency and lead to therapeutic failure. One promising strategy for enhancing cisplatin tolerability and treatment outcomes is circadian rhythm modulation that improves the cellular response to cisplatin-induced DNA damage. Among the circadian clock modulation strategies, there has not been much research done on using pharmacological modulation of the circadian clock to optimize chemotherapeutic use. Therefore, this project aims to test the hypothesis that the circadian modulation via clock-enhancing molecules can be used to improve cisplatin tolerability. Specific aims are to:

1. Define the impact of clock-enhancing molecules on cell viability post cisplatin treatment.
2. Characterize the changes in expression of clock-controlled genes and proteins that arise after exposing the cells to clock-enhancing molecules.
3. Identify the functional consequences of clock-enhancing molecules on DNA repair and cell cycle progression.

2 MATERIALS AND METHODS

2.1 Materials

Clock-enhancing molecules used were KS15 (Glixx laboratories), SR8278 (Sigma S9576), SR1078 (Calbiochem 557352), and Nobiletin (Sigma N1538) and all were prepared as 10 mM stock solutions in 100% dimethyl sulfoxide (DMSO; Fisher Chemical), stored at -20^{O} C, and diluted before each experiment with culture media. Cisplatin (Sigma) was prepared as a 3 mM stock solution in phosphate-buffered saline (PBS; HyClone), stored at -20^{O} C, and diluted with medium before each use. DMSO was used as a vehicle in all experiments at a final concentration of 0.1% (v/v). Methylthiazolyldiphenyl-tetrazolium bromide (MTT) reagent was used in cell viability assay, and it was dissolved in complete culture media at a concentration of 0.25 mg/ml.

2.2 Cell Culture

Since the individual mammalian cells maintain their circadian rhythmicity under a tissue culture environment (73), three cell lines were used as a model system throughout the project. U2OS cell line (Human Bone Osteosarcoma Epithelial Cells), which has robust circadian rhythmicity and has been widely used as an *in vitro* model to investigate clock genes modulation. A549 cell line (Adenocarcinomic Human Alveolar Basal Epithelial Cells), which is a model of non-small cell lung cancer, and HaCaT cell line (Human Epidermal Keratinocyte Cells), which has the basal cell characteristics and can respond to circadian rhythm modulators.

2.2.1 Cell Growth

Both U2OS and HaCaT cell lines were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM; HyClone) containing 10% fetal bovine serum (FBS; HyClone) (v/v), 2 mM L-glutamine (Gibco), 100 units/ml penicillin (Gibco), and 100 ug/ml streptomycin (Gibco). The A549 cell line was cultured in F-12K medium (Gibco) with 10% FBS, 100 units/ml of penicillin, and 100 ug/ml of streptomycin. All cells were preserved in a humidified atmosphere of 5% CO_2 at $37^{O}C$.

2.2.2 Cell Passage

Cells were passaged when they reached 80-90% confluency by aspirating the media from the plate and adding 5 mL of 1X PBS to wash away any remaining media. Then 1.5 mL of Trypsin-EDTA 1X (Gibco) (0.25% for HaCaT and 0.05% for U2OS and A549 cells) was added to each plate and incubated for 5-10 minutes. Then, 5 mL of media added to each plate to neutralize the trypsin, and 1 ml of cell suspension transferred to a new plate with 10 ml of fresh media.

2.2.3 Cell Count

Before splitting to the new plates, cells cultured for survival assays were counted using Trypan Blue staining (Gibco) and a Countess II Automated Cell Counter (ThermoFisher) to ensure an equal number of cells were cultured in each plate which then grown for the same duration to maintain consistency.

2.3 Cell Survival Assay

Cells seeded in 96-well plates at a density of 3×10^3 to 5×10^3 per well and incubated in 100 µl media for 48 hours in the presence or absence of the clock-enhancing molecules and cisplatin. After incubation, media was aspirated and cells were treated with 100 µl of methyl-thiazolyl diphenyl-tetrazolium bromide (MTT) containing medium by adding 1.25 ml of 5 mg/ml MTT stock solution to 23.75 ml of medium (for 0.25 mg/ml MTT final concentration). After incubation at 37^{O} C for 4 hours for the U2OS cell line and 1 hour for HaCaT and A549 cell lines, media was removed and MTT crystals were solubilized in 100 µl of DMSO. Absorbance from each well was detected at 570 nm using the Synergy H1 spectrophotometer (Bio-Tek). Relative cell viability was calculated by taking the average value of 3 replicate wells and normalizing to the non-treated wells.

2.4 Bio-Rad Protein Quantification Assay

The total protein concentration loaded in western blot experiments has been determined by Bio-Rad assay. BSA standards prepared by adding 0, 1, 2, 3, 4, and 5 µl of 2 mg/ml BSA to six microcentrifuge tubes containing 800 µl of PBS, and samples prepared by adding 4 µl of the cell lysate to 496 µl of PBS. Then, 200 µl and 100 µl of Bradford reagent were added to the BSA standards and samples, respectively. After well mixing and vertexing, absorbance was detected at 595 nm using the Bio-Tek plate reader.

2.5 Protein Immunoblotting

Cells were grown to 60-80% confluency in 6-well culture plates and then treated with DMSO, KS15, SR8278, or a combination of KS15+SR8278 in the absence or presence of cisplatin at different concentrations. After 24 hours, cells were harvested and then resuspended by adding 100 µl of Triton X-100 lysis buffer. Cells were disrupted by vortexing, and then soluble lysates were obtained by maximum-speed centrifugation. Total protein concentration was determined via the Bio-Rad protein assay, and equal amounts of protein were separated on 8% SDS-PAGE. Then, proteins were transferred to nitrocellulose membranes stained with 0.5% Ponceau S to ensure that equivalent amounts of protein were loaded. Blots were then washed 2-3 times with TBST (Tris-buffered saline containing 0.1% Tween-20) and incubated in 5% non-fat milk in TBST for 15 minutes to block irrelevant proteins. After washing and blocking steps, blots were incubated overnight at 4^{O} C with the primary antibodies probing for Actin at 1:5000 dilution (Bethyl A300-485), XPA at 1:1000 dilution (Santa Cruz Biotechnology sc-28353), Wee1 at 1:1000 dilution (Santa Cruz Biotechnology sc-5285) or for cleaved PARP at 1:2000 dilution (Cell Signaling 9542S) in TBST. The blots were next washed four times with TBST and probed with HRP-coupled anti-mouse or anti-rabbit IgG (Invitrogen by Thermo Fisher) secondary antibodies for one hour at room temperature. After multiple washes with TBST, either Clarity Western ECL substrate (Bio-Rad) or SuperSignal West Femto substrate (Thermo Scientific) were dispensed onto the blots according to the expected signal intensity. Finally, the chemiluminescence was detected by the Molecular Imager Chemi-Doc XRS+ imaging system (Bio-Rad), and the Image Lab (Bio-Rad) densitometry was used for band intensity quantification and normalization.

2.6 RNA Purification

Cells were grown to 60-80% confluency in 6-well culture plates and then treated with DMSO, KS15, SR8278, or a combination of KS15+SR8278. After 24 hours, cells were harvested as cell pellets and RNA was purified using RNeasy® Plus Micro Kit (Qiagen). Cell pellets were first homogenized in 350 µl Buffer RTL Plus by pipetting up and down several times. To eliminate the genomic DNA, the lysate was transferred to a gDNA Eliminator column placed in a 2 ml collection tube and centrifuged for 30 seconds at 10,000 rpm. The flow-through was saved to be mixed with an equal volume of 70% ethanol and transferred to an RNeasy MinElute spin column placed in a 2 ml collection tube then centrifuged for 15 seconds at 10,000 rpm. The column was washed with RW1 buffer, then with RPE buffer and 80% ethanol and centrifuged after each wash. To dry the column membrane before eluting the RNA, the column was centrifuged with an open lid for 5 minutes at full speed. Finally, RNase-free water was added to the center of the column, which was centrifuged for 1 minute at maximum speed to elute the RNA. To ensure equivalent quantities of RNA were reverse transcribed to cDNA, NanoDrop One spectrophotometer (Thermo Fisher) was used for RNA quantification, and 260/280 ratios were determined to be between 1.8-2.0.

2.7 Reverse Transcriptase Quantitative PCR (RT-qPCR)

Five hundred nanograms of purified RNA were reverse transcribed to cDNA using a QuantiTect Reverse Transcription Kit (Qiagen). The desired volume of RNA was added to small PCR tubes, and RNase-free water was used to bring the volume up to 12 µl. Then, 2 µl of 7X genomic DNA wipe-out buffer was added, and the samples were incubated at 42°C for 2 minutes in the Eppendorf Thermocycler. 6 µl of the master mix (consists of 4 µl RT buffer, 1 µl primer, and 1 µl RT enzyme) were added for each sample which was then incubated for 15 minutes at 42°C and 3 minutes at 95°C in the Eppendorf Thermocycler. PCR reactions were made using 2X TaqMan Fast Universal PCR Master Mix and TaqMan probes targeting XPA (Hs00902270), Wee1 (Hs01119384), and beta-2-microglobulin (B2M) (Hs0187842) (Applied Biosystems). Bio-Rad CFX96 Real-Time PCR Detection System was used to run the PCR reaction using an initial 3 min melting step at 95°C followed by 40 cycles of 95°C for 10 s and 55°C for 30 s. The ΔΔCt method was used to calculate fold changes in gene expression using B2M as an internal housekeeping gene.

2.8 DNA Isolation

Cells were grown to 80% confluency in 6-well culture plates and then treated with DMSO or a combination of KS15+SR8278 in the absence or presence of cisplatin at different concentrations. Cells were harvested as cell pellets at 2 and 24 hours post cisplatin treatment, and then genomic DNA was purified using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma). Cell pellets were first suspended in 200 µl of Resuspension Solution and 20 µl of RNase A solution added to get RNA-free genomic DNA. Then to lyse the cells, 20 µl of the Proteinase K added to the sample, followed by 200 µl of Lysis Solution. Homogenous mixture was ensured by vortexing, and then samples were incubated at 70 °C for 10 minutes. GenElute Miniprep Binding Column was prepared by adding 500 µl of the Column Preparation Solution and then centrifuging at 12,000 rpm for 1 minute. Samples were transferred into the column after mixing with 200 µl of 100% ethanol and well vortexing, then centrifuging at ≥ 6500 rpm for 1 minute. The column was then washed twice with 500 µl of Wash Solution and dried by centrifuging for 3 minutes at full speed. Finally, the DNA was eluted by adding 200 µl Elution Solution and centrifuging at ≥ 6500 rpm for 1 minute. To ensure equivalent quantities of DNA were loaded onto the dot blot apparatus, NanoDrop One spectrophotometer (Thermo Fisher) was used for DNA quantification, and 260/280 ratios were determined to range between 1.8 and 2.0.

2.9 DNA Immunoblotting

A hundred nanograms of isolated DNA was loaded in each well in triplicate, and samples were prepared by diluting the DNA in molecular biology grade water and heating at 95-100°C to denture the DNA. Samples were neutralized on ice, then an equal volume of 2M cold ammonium acetate (pH 7.0) was added to each sample, followed by vortexing and centrifuging at maximum speed for few seconds. The prepared samples were loaded onto a nitrocellulose membrane placed onto the dot blot apparatus (BRL Hybri-dot Vacuum Manifold), and pre-wet with 6X saline-sodium citrate (SSC) buffer. Then, gentle suction filtration was applied, and the membrane was baked at 80°C for 30 minutes, blocked with 5% non-fat milk in TBST, and incubated with primary antibodies targeting cisplatin modified DNA 1:10000 dilution (CP9/19; Abcam ab103261), or single-stranded DNA 1:5000 dilution (Millipore MAB3034). The membrane was next washed four times with TBST and probed with HRP-coupled anti-mouse (Invitrogen by Thermo Fisher) or anti-rat IgG (Abcam) secondary antibodies for one hour at room temperature. After multiple washes with TBST, Clarity Western ECL substrate (Bio-Rad) was dispensed onto the membrane. The chemiluminescence was detected by the Molecular Imager Chemi-Doc XRS+ imaging system (Bio-Rad), and the Image Lab (Bio-Rad) densitometry was used for dots' intensity quantification and normalization.

2.10 Flow Cytometry

To analyze cell cycle distribution by quantifying DNA content, cells were grown to 60-80% confluency in 6-well culture plates and then treated with DMSO, KS15, SR8278, or a combination of KS15+SR8278. After 24 hours, cells were harvested by trypsinization and centrifuged at 1500 rpm for 5 minutes. Then after washing with PBS, cells were fixed overnight with 70% ice-cold ethanol at -20°C. Fixed cells were washed with PBS, centrifuged at 4000 rpm for 5 minutes, and resuspended with propidium iodide (PI) staining solution. The PI staining solution was prepared by adding 1 µl of 10 mg/ml RNase A and 5 µl of 10 mg/ml propidium iodide to 1 ml of PBS. Cells were properly resuspended in 1 ml of the staining solution and analyzed for DNA content using an Accuri C6 flow cytometer. Cell cycle distribution was determined after appropriate gating of cell populations in FL-2-area of PI fluorescence.

2.11 Statistical Analysis

Statistical differences between groups were evaluated using either Student's t-test, one-way, or two-way ANOVA tests followed by Dunnett's post-hoc test for multiple comparisons. Data were considered statistically significant at P-values less than 0.05 and are interpreted as mean ± standard error of the mean (SEM). Outliers were excluded from datasets, and graphs were created using Graph Pad Prism (version 9.0).

3 RESULTS

3.1 Clock-enhancing molecules reduce cisplatin cytotoxicity in U2OS cells

To analyze the effect of the clock-enhancing molecules on cisplatin cytotoxic effect in U2OS cells, an MTT assay was performed to assess changes in cell viability. The circadian clock has been shown to control cisplatin-induced toxicity in mouse and human models (17); therefore, it was hypothesized that using specific clock-enhancing molecules to enhance the circadian output could improve the response to cisplatin-induced DNA damage. To test this hypothesis, U2OS cells were plated into 96-well plates then treated with CRY inhibitor KS15, the REV-ERB antagonist SR8278, or the ROR agonists SR1078 and Nobelitin and then exposed to different concentrations of cisplatin (1.25, 2.5, 5, 10, 20, 40, 60 μM). In addition to testing the clock-enhancing molecules individually, combinations of these molecules were also investigated. Since both REV-ERB inhibition and ROR stimulation increase BMAL1 expression and function (74–76), combining them with the CRY inhibitor KS15 can result in additional protection from the cisplatin cytotoxic effect.

Several experiments were conducted to optimize the timing of drug treatment in relation to cisplatin exposure and maximize the effect on treated cells (Supplemental Material **Figure 5.1**). The most significant effect was observed when cells were treated with 10 μM of circadian clock modulators, exposed simultaneously to cisplatin, and then incubated for 48 hours before MTT assays were performed to analyze the viability of the remaining cells. Therefore, conditions were fixed to 48 hours of simultaneous treatment with cisplatin and circadian modulators for all future experiments. Results revealed that clock-enhancing molecules improve U2OS cells survival rate compared to DMSO (**Figure 3.1**).

To further examine how cisplatin cytotoxicity would change in response to clock-enhancing molecules treatment, cisplatin IC50 (Half maximal inhibitory concentration) values were determined in the presence and absence of individual clock-enhancing molecules and their combination as previously described. U2OS cells were grown to confluence and then treated with a range of cisplatin concentrations (1.25, 2.5,5,10,20,40, 60 μM) in the presence of 10 μM clock-enhancing molecules or DMSO. Cells were exposed to the treatments for 48 hours before cell viability was detected by MTT assay. As shown in **Figure 3.2**, co-treatment with KS15+SR8278 significantly increased the IC50 value of cisplatin from 20.74 μM for DMSO treated group to 39 μM for the group treated with KS15+SR8278. These results indicate that higher cisplatin concentration was needed to achieve the same cytotoxic effect compared to DMSO. Thus, KS15 and SR8278 combination protects U2OS cells against cisplatin cytotoxicity.

Furthermore, dose-response curves for cisplatin were generated to compare the response rate of U2OS cells treated either with KS15+SR8278 or DMSO. Cells were exposed to different cisplatin concentrations (1.25, 2.5,5,10,20,40, 60 μM) and treated with either DMSO or 10 μM circadian clock modulators; then cell viability was evaluated by MTT assay. As displayed in **Figure 3.3**, treatment with clock-enhancing molecules KS15+SR8278 did shift the cisplatin dose-response curve to the right with a significant difference in cell viability compared to DMSO. These curves demonstrated that in response to KS15+SR8278, U2OS cells were less sensitive to cisplatin cytotoxicity.

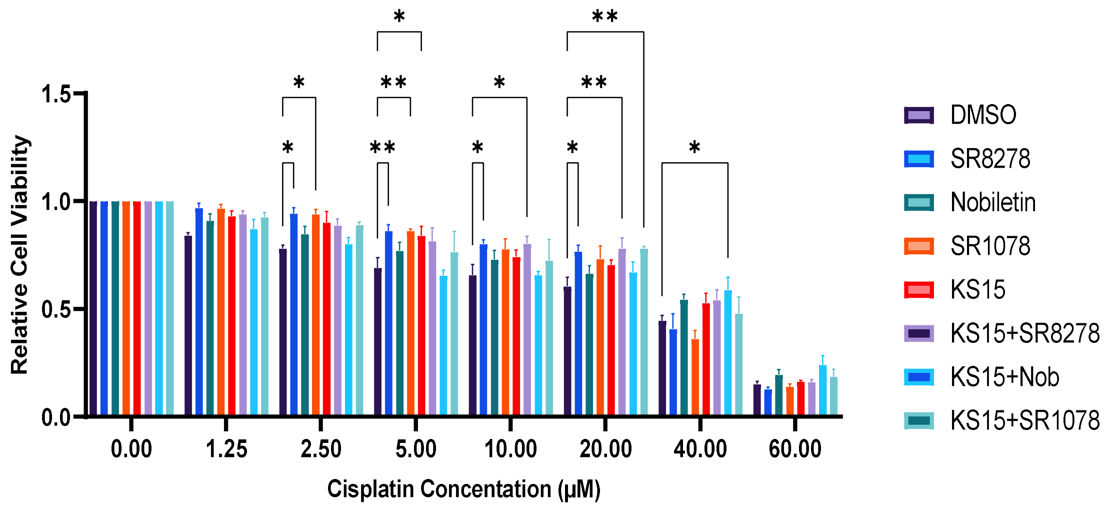


Figure 3.1: Clock-enhancing molecules improve U2OS cell survival. Relative cell viability of U2OS cells that were cultured and treated for 48 hours with vehicle (DMSO) or 10 µM clock-enhancing molecules simultaneously with the indicated concentrations of cisplatin. Cell viability assessed using MTT assay and data are presented as means ± SEM (n= 4). Groups were compared using two-way ANOVA and Dunnett's multiple comparison test. Differences in samples were considered significant compared to DMSO if $P<0.05$ (*), $P<0.01$ (**).

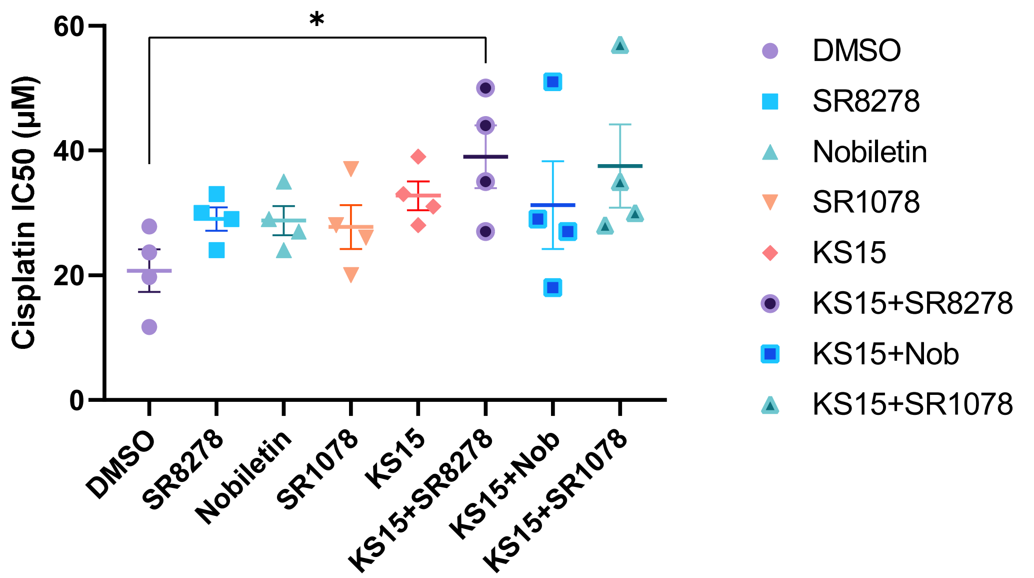


Figure 3.2: KS15 and SR8278 combination significantly increases cisplatin IC50 value in U2OS cells. Cisplatin IC50 values in U2OS cells that were cultured and treated for 48 hours with vehicle (DMSO) or 10 µM clock-enhancing molecules simultaneously with (1.25, 2.5, 5, 10, 20, 40, 60 µM) of cisplatin. Cell viability assessed using MTT assay and data are presented as means ± SEM (n= 4). Groups were compared using two-way ANOVA and Dunnett's multiple comparison test. Differences in samples were considered significant compared to DMSO if P<0.05 (*).

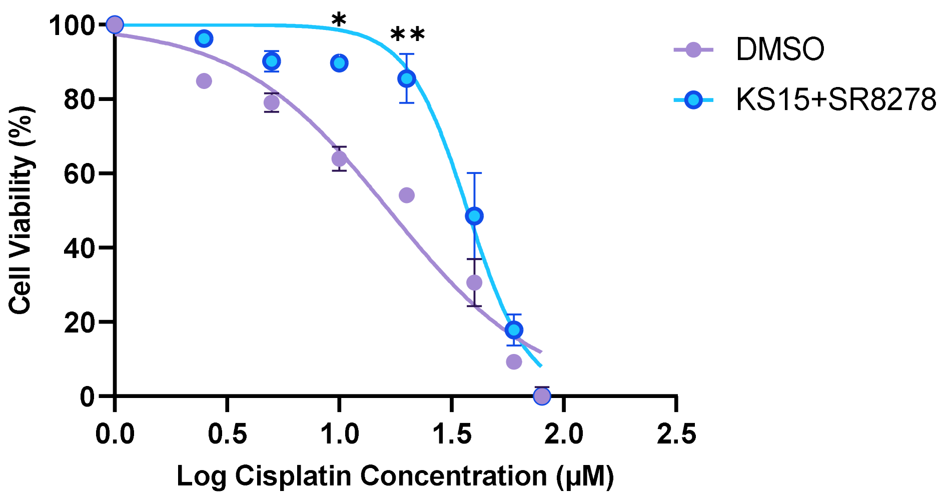


Figure 3.3: KS15 and SR8278 combination significantly improve U2OS cell survival. The dose-response curve of U2OS cells that were cultured and treated for 48 hours with vehicle (DMSO) or 10 μM clock-enhancing molecules simultaneously with 1.25, 2.5, 5, 10, 20, 40, or 60 μM cisplatin. Cell viability was assessed using MTT assays, and data are presented as means ± SEM (n= 4). Groups were compared using one-way ANOVA and Dunnett's multiple comparison test. Differences in samples were considered significant compared to DMSO if P<0.05 (*), P<0.01 (**).

3.2 KS15 and SR8278 improve U2OS cell survival in a dose-dependent manner

To optimize the dose that enhances the cellular response of U2OS cells to cisplatin, different concentrations of KS15 and SR8278 were used to generate dose-response curves and calculate cisplatin IC50 values. Cells were cultured to confluency and treated for 48 hours with vehicle (DMSO) or clock-enhancing molecules in the presence of various cisplatin concentrations (1.25, 2.5,5,10,20,40, 60 μM). KS15 was tested at 10, 20, and 50 μM either alone or in combination with 10 μM SR8278. As demonstrated in **Figure 3.4**.A, KS15 improved the U2OS cell survival rate in a dose-dependent manner, and the maximum response was seen at 50 μM. Also, the experiment revealed that combining SR8278 with KS15 had resulted in a greater increase in cell viability compared to treating with SR8278 alone (**Figure 3.4**.B).

Consistent with these results, a similar effect was observed on cisplatin IC50 values where a higher concentration of KS15 and the combination with SR8278 had resulted in a significant increase in cisplatin IC50 values (**Figure 3.5**). These results indicate that in response to KS15+SR8278, a higher concentration of cisplatin was needed to achieve the same cytotoxic effect compared to DMSO, and thus the U2OS cells were less sensitive to cisplatin cytotoxic effect (**Table 3.1**).

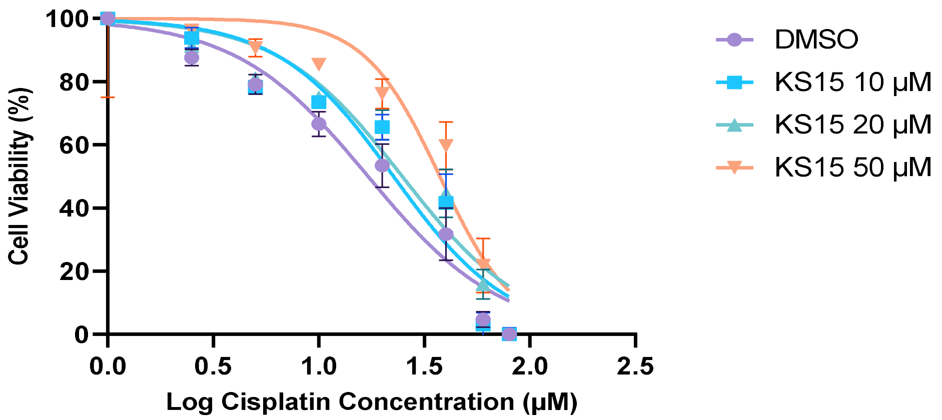
Moreover, to investigate whether the clock-enhancing molecules influence U2OS cell viability in the absence of cisplatin, cells were cultured to confluency and treated for 48 hours with vehicle (DMSO) or circadian clock modulators. Concentrations tested were 10, 20, and 50 μM of KS15 either alone or in combination with 10 μM SR8278. As illustrated in **Figure 3.6**, KS15+SR8278 have no significant effect on U2OS cell viability when they were not exposed to cisplatin.

In addition to the above experiments that were testing the increasing concentration of KS15 (10, 20, and 50 µM) with a fixed concentration of 10 µM SR8278, other concentrations were investigated using fixed KS15 concentration at 10 µM with an increasing concentration of SR8278 (10, 20, and 50 µM). Similar observations were reported where KS15 and SR8278 improve U2OS cell survival in a dose-dependent manner (Supplemental Material **Figure 5.2**).

Table 3.1: Effect of KS15 and SR8278 treatment on U2OS cells sensitivity to cisplatin.

Treatment	Cisplatin IC50 (µM)	Treatment	Cisplatin IC50 (µM)
DMSO	24	10 µM SR8278	29.3
10 µM KS15	35.6	10 µM KS15 + 10 µM SR8278	44.5
20 µM KS15	36	20 µM KS15 + 10 µM SR8278	45.6
50 µM KS15	43.4	50 µM KS15 + 10 µM SR8278	48.7

A



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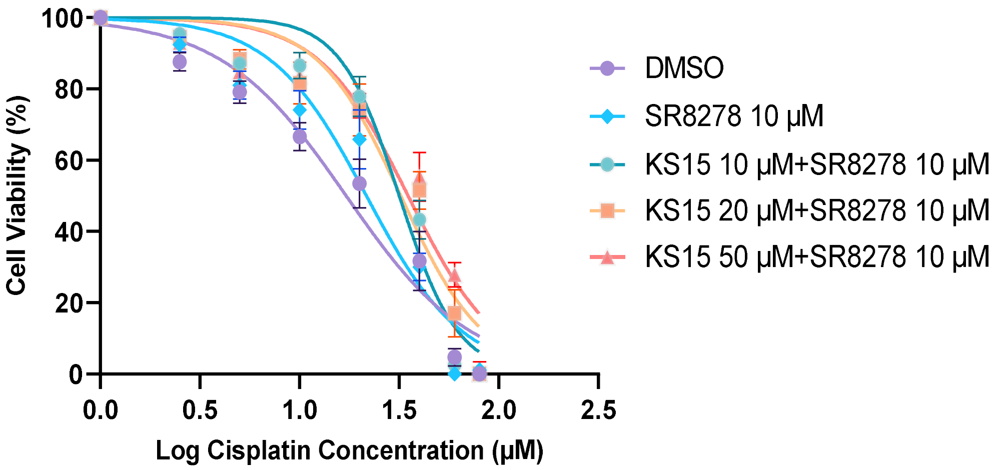


Figure 3.4: KS15 and SR8278 improve U2OS cell survival in a dose-dependent manner. (A) The dose-response curve of U2OS cells that were cultured and treated for 48 hours with vehicle (DMSO) or the indicated concentrations of KS15 with 1.25, 2.5, 5, 10, 20, 40, 60 μM cisplatin. **(B)** Cells were treated as in (A) except that KS15 was combined with SR8278 at the indicated concentrations. Cell viability was assessed using MTT assays and data are presented as means ± SEM (n= 4).

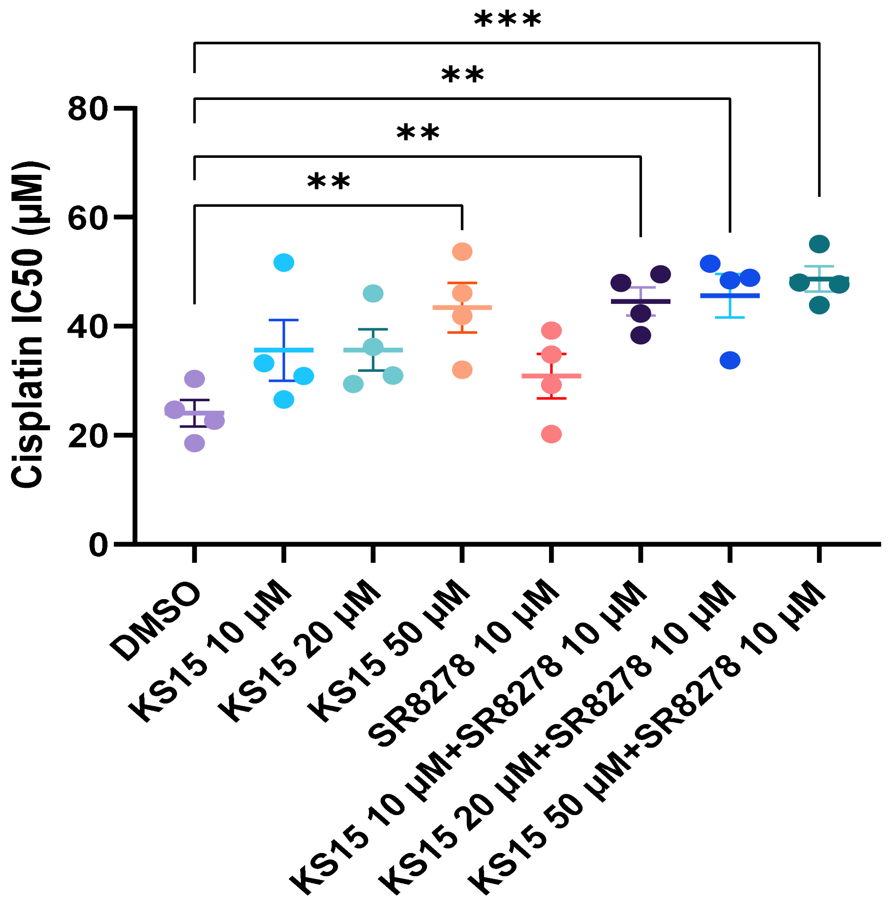


Figure 3.5: KS15 and SR8278 significantly increase cisplatin IC50 value in U2OS cells in a dose-dependent manner. Cisplatin IC50 values in U2OS cells that were cultured and treated for 48 hours with vehicle (DMSO) or the indicated concentrations of KS15 and SR8278 with 1.25, 2.5, 5, 10, 20, 40, 60 µM cisplatin. Cell viability was assessed using MTT assays and data are presented as means ± SEM (n= 4). Groups were compared using one-way ANOVA and Dunnett's multiple comparison test. Differences in samples were considered significant compared to DMSO if P<0.05 (*), P<0.01 (**), P<0.001 (***).

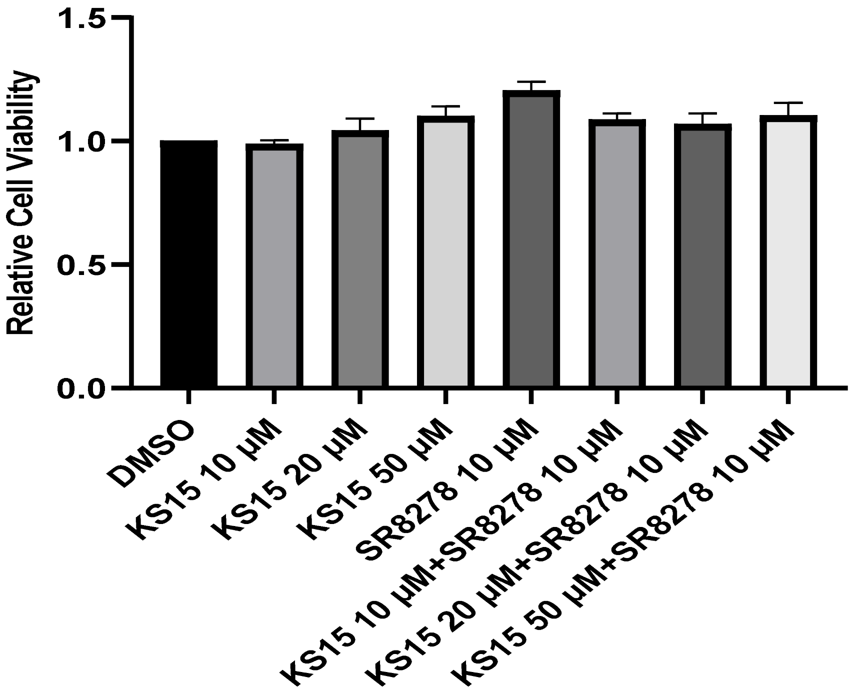


Figure 3.6: Clock-enhancing molecules have no significant effect on U2OS cell viability in the absence of cisplatin. Relative cell viability of U2OS cells treated with the indicated concentrations of KS15 and SR8278. Cells were cultured and treated for 48 h with vehicle (DMSO) or clock-enhancing molecules, and cell viability was assessed using MTT assays. Data are presented as means ± SEM (n= 4), and groups were compared using one-way ANOVA and Dunnett's multiple comparison test. No significant difference was detected compared to DMSO.

3.3 KS15 and SR8278 reduce cisplatin cytotoxicity in HaCaT cells

An MTT assay was used to evaluate changes in HaCaT cell viability to investigate the impact of clock-enhancing molecules on cisplatin cytotoxicity using other cell lines. HaCaT cells were grown to confluency in 96-well plates and then treated with KS15 and SR8278 at various concentrations in the presence of cisplatin (1.25, 2.5,5,10,20,40, 60 µM). After 48 hours, the cell viability was evaluated and used to create dose-response curves and determine cisplatin IC50 values.

KS15 was tested at concentrations of 10, 20, and 50 µM either alone or in combination with 10 µM SR8278. As demonstrated in **Figure 3.7**.A, the HaCaT cell survival rate was improved by KS15 in a dose-dependent manner, and the 50 µM concentration had the maximum effect. In addition, when SR8278 was combined with KS15, a greater increase in cell viability was observed compared to SR8278 alone (**Figure 3.7**.B).

A similar effect was detected on cisplatin IC50 values, where treating HaCaT cells with 50 µM KS15 combined with 10 µM SR8278 resulted in a substantial increase in cisplatin IC50 values from 5.2 µM for the DMSO group to 21.2 µM for the group treated with 50 µM KS15+ 10 µM SR8278 (**Figure 3.8**). These findings suggest that, in response to KS15+SR8278, a higher concentration of cisplatin was required to get the same cytotoxic effect compared to DMSO, and thereby the HaCaT cells were less susceptible to cisplatin cytotoxicity.

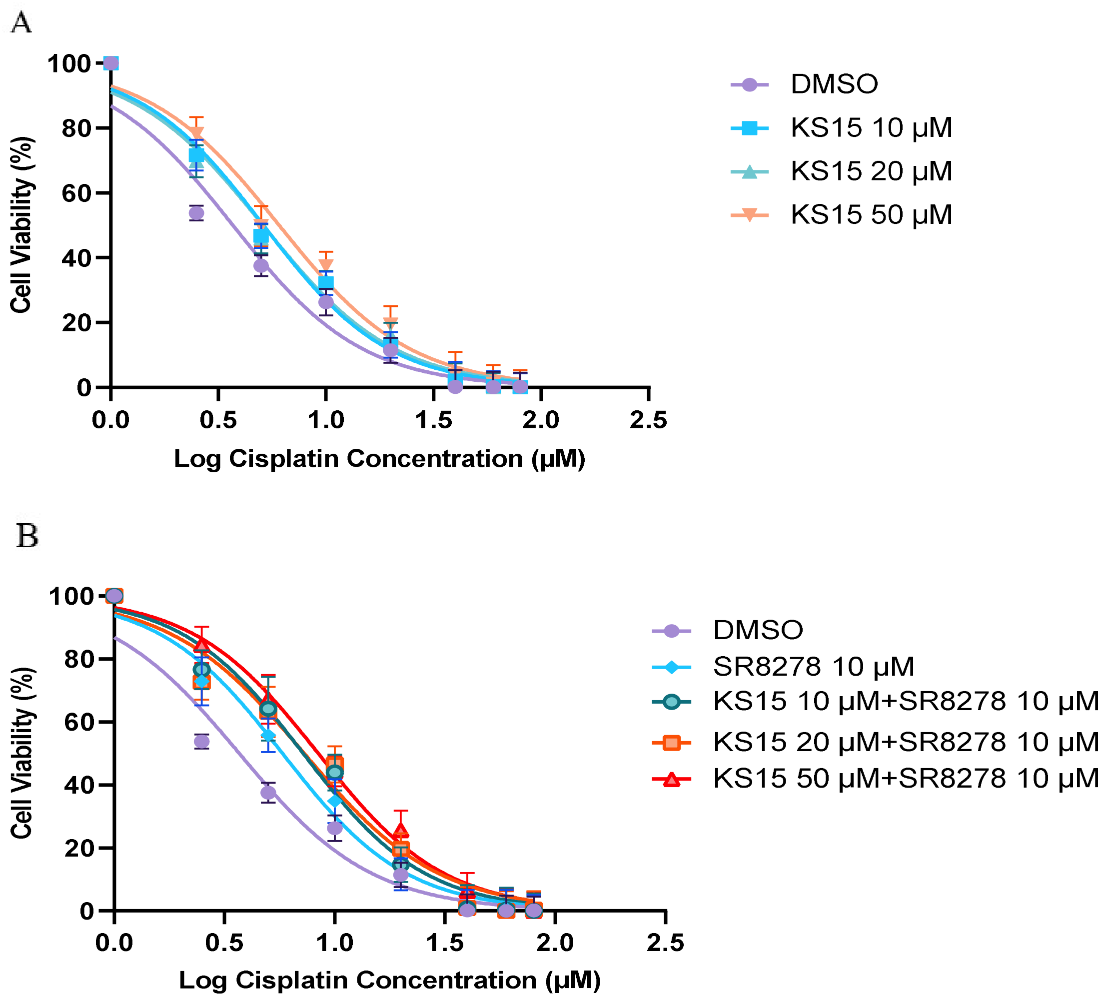


Figure 3.7: KS15 and SR8278 improve HaCaT cell survival rate in a dose-dependent manner. (A) The dose-response curve of HaCaT cells that were cultured and treated for 48 hours with vehicle (DMSO) or the indicated concentrations of KS15 with 1.25, 2.5, 5, 10, 20, 40, 60 µM cisplatin. **(B)** Cells were treated as in (A) except that KS15 was combined with SR8278 at the indicated concentrations. Cell viability was assessed using MTT assays, and data are presented as means ± SEM (n= 4).

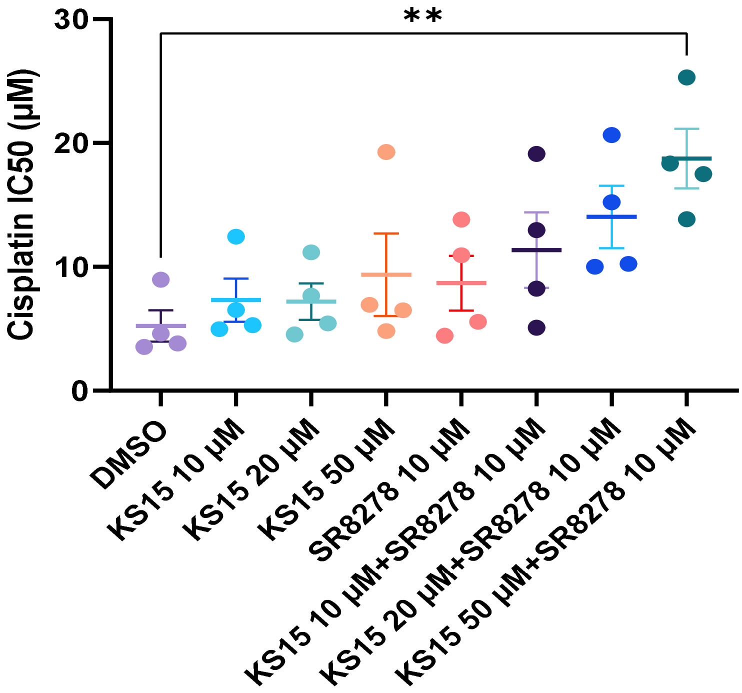


Figure 3.8: KS15 and SR8278 combination significantly increases cisplatin IC50 value in HaCaT cells in a dose-dependent manner. Cisplatin IC50 values in HaCaT cells that were cultured and treated for 48 hours with vehicle (DMSO) or the indicated concentrations of KS15 and SR8278 with (1.25, 2.5, 5, 10, 20, 40, 60 μM) of cisplatin. Cell viability assessed using MTT assay and data are presented as means ± SEM (n=4). Groups were compared using one-way ANOVA and Dunnett's multiple comparison test. Differences in samples were significant compared to DMSO at P<0.01 (**).

3.4 KS15 and SR8278 do not impact cisplatin cytotoxicity in A549 cells

To investigate the effect of clock-enhancing molecules on cisplatin cytotoxicity in lung cancer cells, an MTT assay was used to test changes in A549 cell viability. Cells were grown to confluency before being treated with KS15 and SR8278 at varying concentrations in the presence of cisplatin (1.25, 2.5, 5, 10, 20, 40, 60 M). The cell viability was determined after 48 hours, and data utilized to create dose-response curves and determine cisplatin IC50 values.

KS15 was tested either alone (at 10, 20, and 50 µM) or in combination with 10 µM SR8278. As shown in **Figure 3.9**, there was no significant change in A549 cell survival in response to clock-enhancing molecules and similarly as shown in **Figure 3.10**, no effect was observed on cisplatin IC50 values.

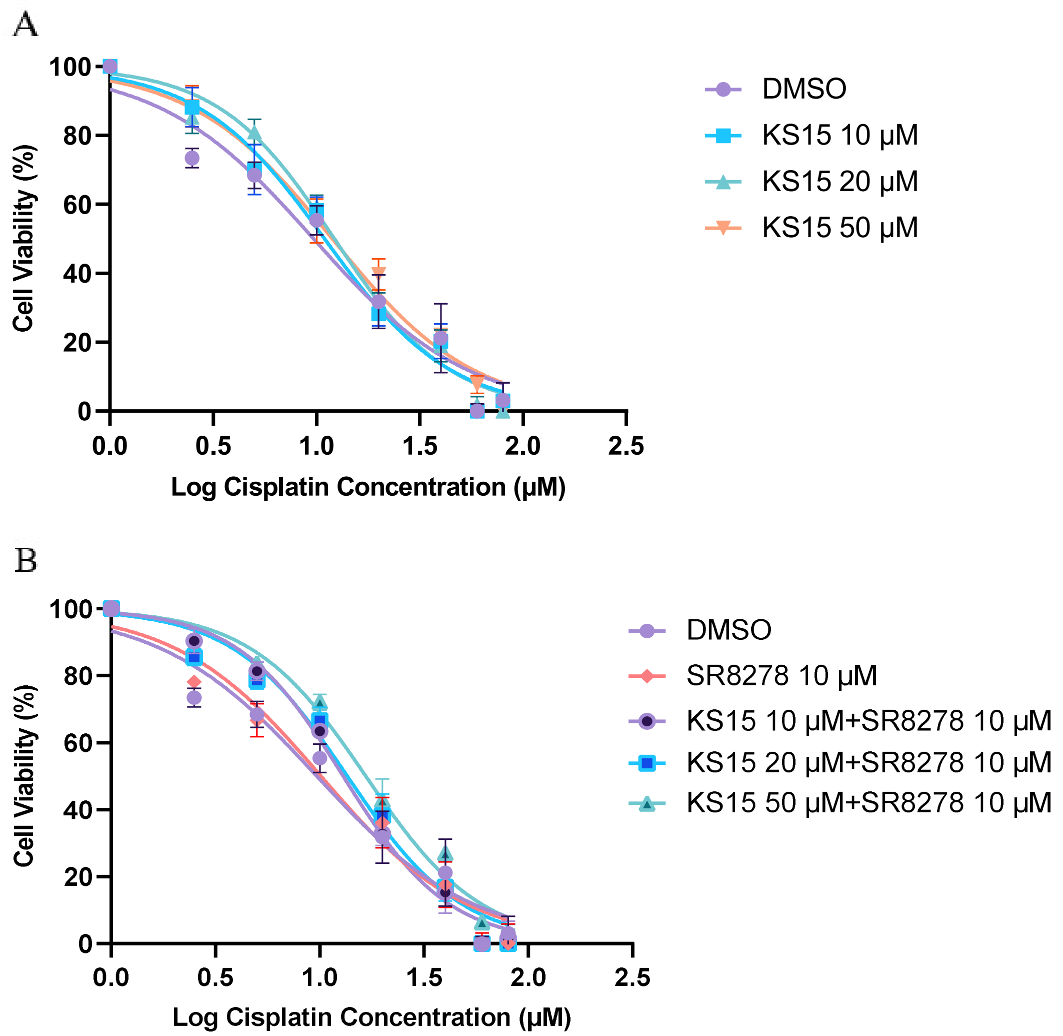


Figure 3.9: KS15 and SR8278 do not impact the A549 cell survival rate. (A) The dose-response curve of A549 cells that were cultured and treated for 48 hours with vehicle (DMSO) and the indicated concentrations of KS15 and cisplatin. **(B)** Cells were treated as in (A) except that KS15 was combined with SR8278 at the indicated concentrations. Cell viability was assessed using MTT assays and data are presented as means ± SEM (n= 3).

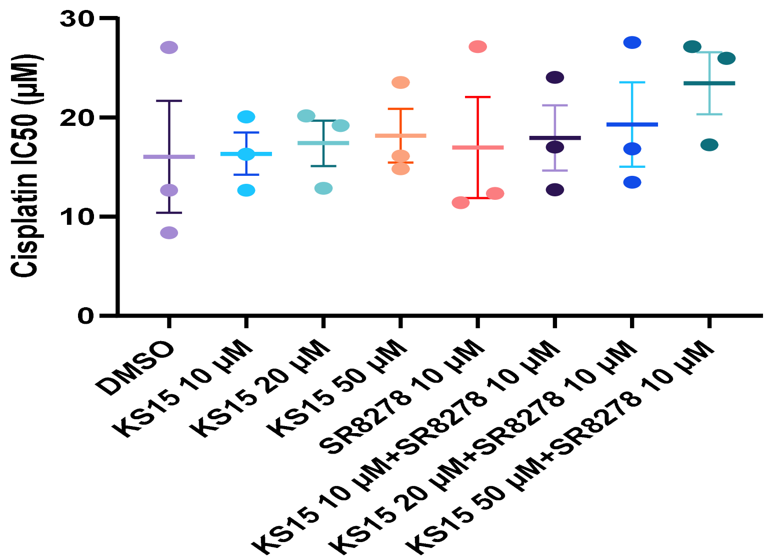


Figure 3.10: KS15 and SR8278 have no significant effect on cisplatin IC50 value in A549 cells. Cisplatin IC50 values in A549 cells that were cultured and treated for 48 hours with vehicle (DMSO) or the indicated concentrations of KS15 and SR8278 with 1.25, 2.5, 5, 10, 20, 40, 60 µM cisplatin. Cell viability was assessed using MTT assays and data are presented as means ± SEM (n= 3). Groups were compared using one-way ANOVA and Dunnett's multiple comparison test. No significant difference was detected compared to DMSO.

3.5 KS15 and SR8278 modulate the expression profile of clock-controlled genes

The expression profiles of clock-controlled genes were investigated to determine whether the protective effect of clock-enhancing molecules against cisplatin in U2OS and HaCaT cells (**Figure 3.3** and **Figure 3.7**) is related to circadian regulation of DNA repair and cell-cycle processes. It has been reported that XPA, which is a DNA repair gene and a rate-limiting factor in NER, and Wee1, which is anti-mitotic kinase and a cell cycle regulator, both exhibit circadian oscillation and their expression is regulated by the clock (20, 77). Therefore, it was hypothesized that KS15 and SR8278 could impact the expression of those genes as well as their corresponding proteins.

To test this hypothesis, U2OS cells were exposed to DMSO, 20 μM KS15, 10 μM SR8278, or a combination of KS15+SR8278 for 24 hours before being harvested for gene expression analysis by RT-qPCR. Dosing and time of treatment were selected based on the earlier dose-response analysis (**3.2**). Experiments showed that in comparison to DMSO, KS15+SR8278 treatment resulted in a 2.7-fold increase in the relative mRNA expression of XPA (**Figure 3.11**.A), and a 3.1-fold increase in Wee1 (**Figure 3.11**.B). However, neither KS15 nor SR8278 alone were able to significantly change the XPA and Wee1 gene expression.

After that, protein immunoblotting was performed to see if the changes in gene transcription were also detectable at the protein level. U2OS cells were treated for 24 hours with DMSO, 20 μM KS15, 10 μM SR8278, or a combination of KS15+SR8278. Protein lysates from these cells were immunoblotted and results are shown in **Figure 3.12**. A 2-fold increase in XPA and a 1.7-fold increase in Wee1 protein expression were observed with the KS15+SR8278 treatment, which is consistent with gene expression analysis.

However, the change in protein level did not reach a statistical significance as what was seen in gene expression because the time it takes for cellular signaling to affect gene expression is unlikely to be the same as it takes to change protein expression. Therefore, harvesting the cells at different time points or increasing the concentration of clock-enhancing molecules might be needed to achieve a significant difference at the protein level.

These findings demonstrate that KS15 and SR8278 increase the expression of XPA and Wee1 at both gene and protein levels, and this could correlate their protective effect against cisplatin cytotoxicity to the circadian regulation of DNA repair and cell-cycle processes. Interestingly, these results were not observed when the same experiment was done using HaCaT cells. According to **Figure 3.13**, KS15 alone tends to increase XPA and Wee1 protein expression, while SR8278 treatment causes a reduction. Thus, the stimulatory effect seen with the combination KS15+SR8278 in U2OS cells was not observed in HaCaT cells. The different responses observed in U2OS and HaCaT cells may be due to cell type and genetic background differences. However, because these clock modulators limit cytotoxicity in both cell lines, these results suggest that the protective effects of these compounds against cisplatin cytotoxicity may involve additional gene targets besides XPA and Wee1.

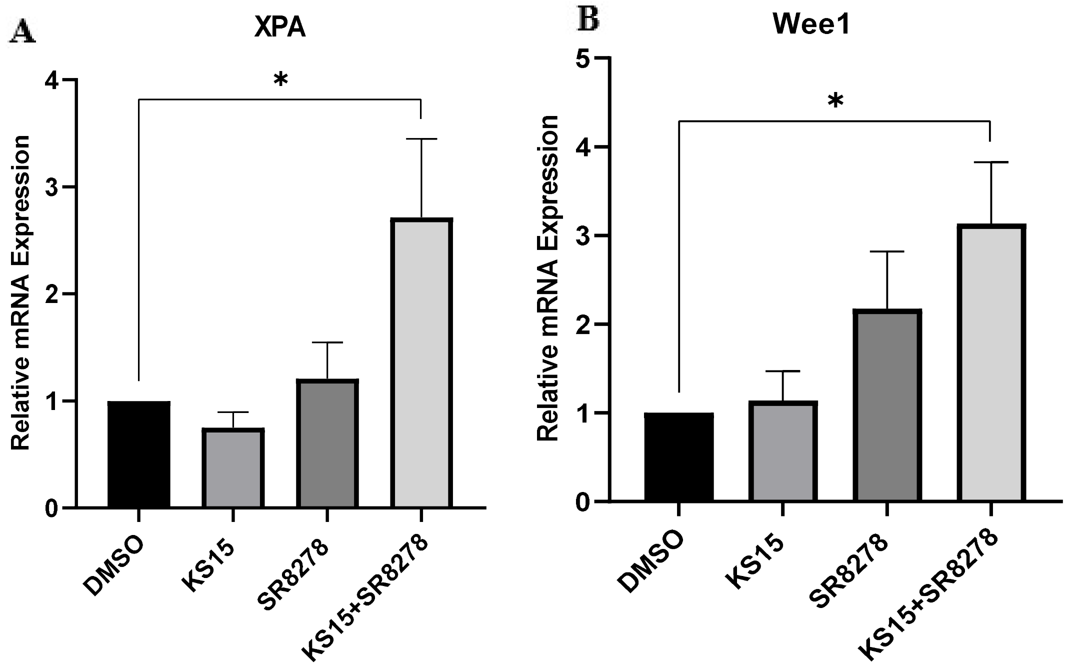


Figure 3.11: KS15 and SR8278 significantly increase mRNA levels of XPA and Wee1 in U2OS cells. Relative mRNA expression of XPA **(A)** and Wee1 **(B)** in U2OS cells treated with vehicle (DMSO), 20 uM KS15, 10 uM SR8278, or a combination. Data are presented as means ± SEM (n =4) for each group. Groups were compared using one-way ANOVA and Dunnett's multiple comparison test. Differences in samples were considered significant compared to DMSO if P<0.05 (*).

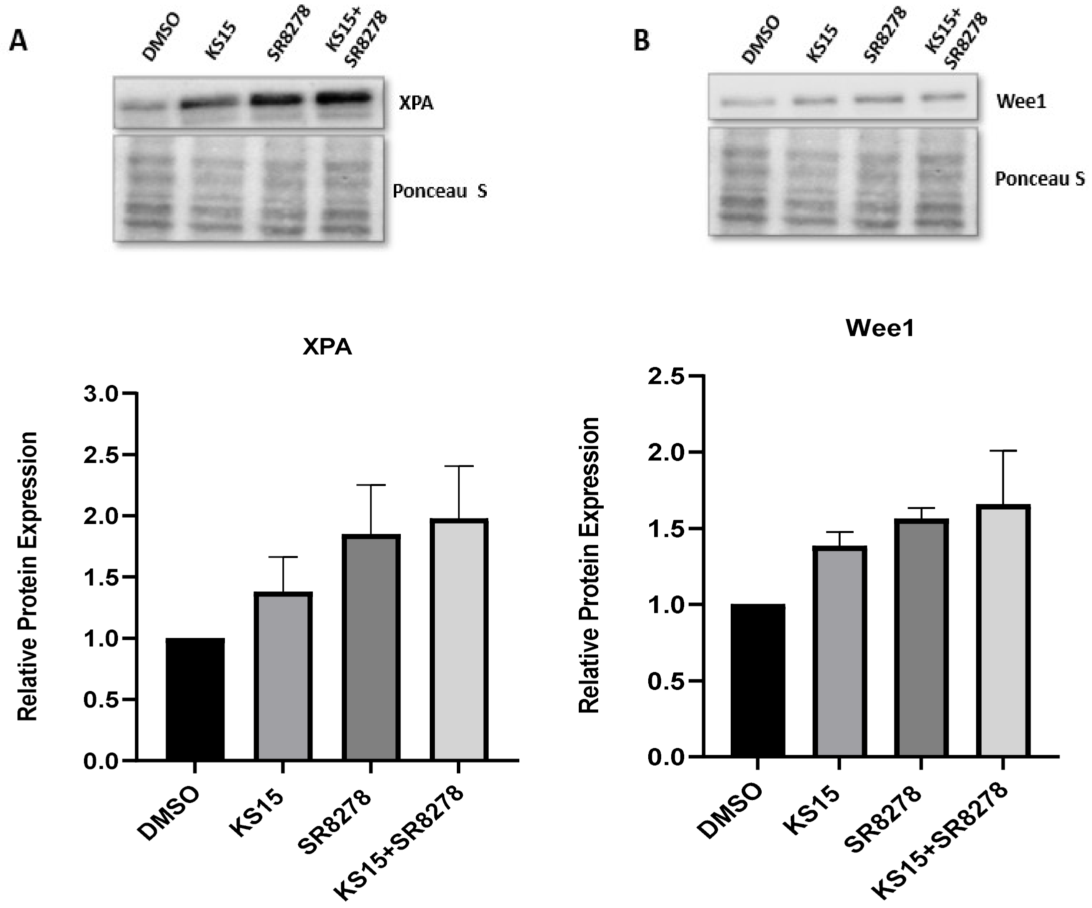


Figure 3.12: KS15 and SR8278 increase the protein expression of XPA and Wee1 in U2OS cells. Immunoblot analysis and quantification of XPA **(A)** and Wee1 **(B)** protein expression in U2OS cells, which were treated with vehicle (DMSO), 20 uM KS15, 10 uM SR8278, or a combination. Data are presented as means ± SEM (n=3). Groups were compared using one-way ANOVA and Dunnett's multiple comparison test.

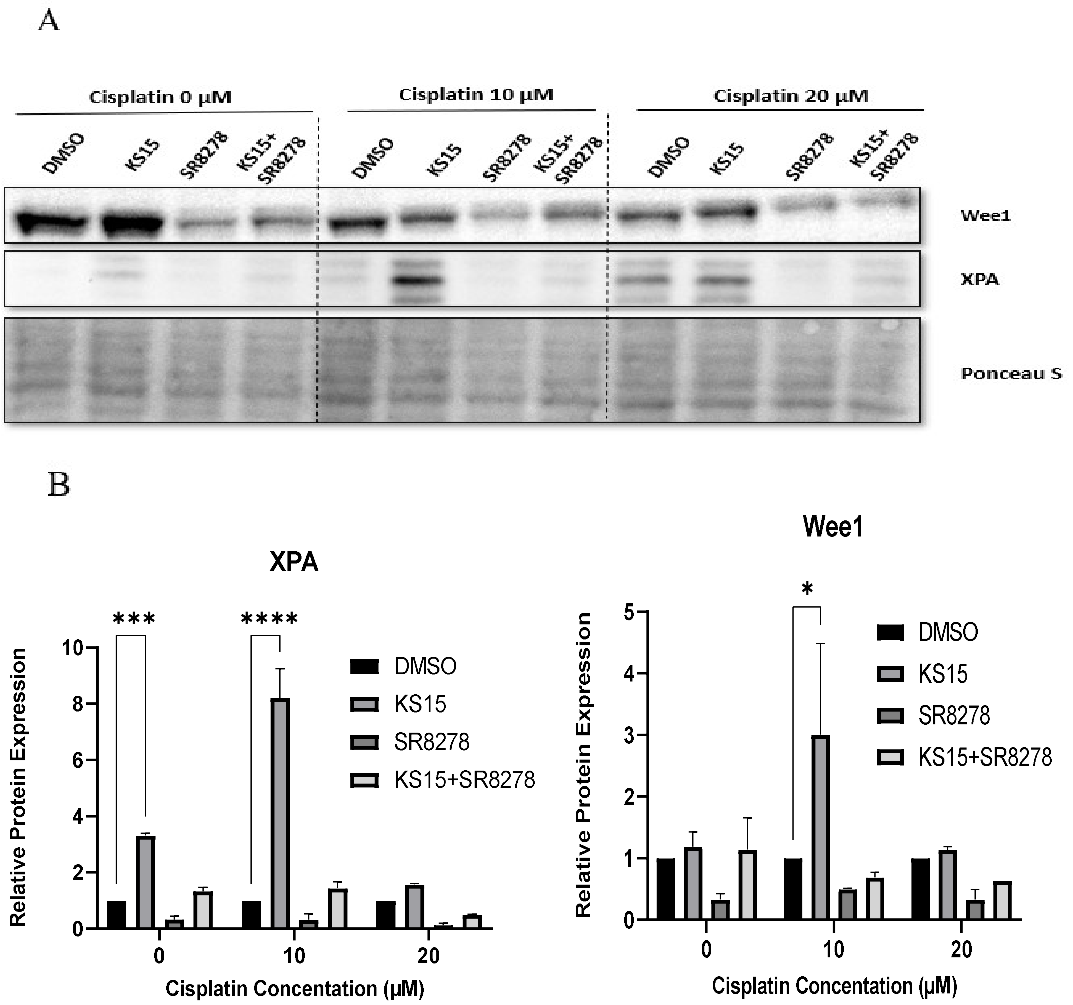
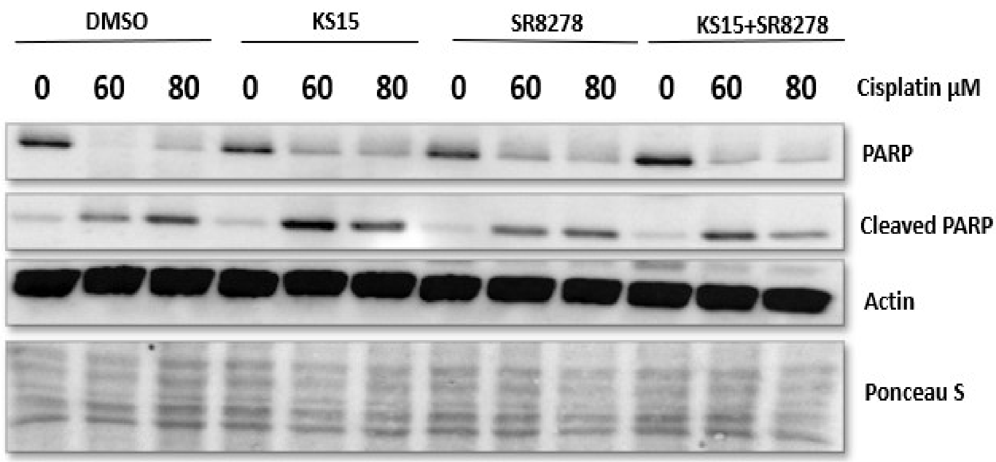


Figure 3.13: KS15 increases the protein expression of XPA and Wee1 in HaCaT cells. Immunoblot result **(A)** and quantification **(B)** of XPA and Wee1 protein expression in HaCaT cells which were treated with vehicle (DMSO), 20 µM KS15, 10 µM SR8278, or a combination in the presence and absence of indicated concentrations of cisplatin. Data are presented as means ± SEM (n=2). Groups were compared using two-way ANOVA and Dunnett's multiple comparison test. Differences in samples were considered significant compared to DMSO if $P<0.05$ (*), $P<0.001$ (***), $P<0.0001$ (****).

3.6 KS15 and SR8278 modestly reduce cisplatin apoptotic response in U2OS cells

Since cisplatin-induced cell death could be mediated by apoptosis, DNA damage-induced apoptosis following cisplatin treatment was evaluated by comparing PARP cleavage in response to circadian clock modulators. Circadian clock genes have been shown to play a role in cell proliferation, apoptosis, and cell cycle progression (42). Therefore, it was hypothesized that enhancing the circadian clock output using KS15 and SR8278 would impact cisplatin apoptotic response. To test this hypothesis, U2OS cells were treated with DMSO, 20 µM KS15, 10 µM SR8278, or a combination of KS15+SR8278 for 24 hours, then cell lysates were immunoblotted to detect apoptotic response using the cleavage of PARP as an indicator of apoptotic signaling. It was observed that KS15 and SR8278 resulted in 0.87-fold less PARP cleavage compared to DMSO at 80 µM cisplatin as shown in **Figure 3.14**. However, the difference was not substantial and higher concentrations of clock-enhancing molecules might be needed to detect a significant reduction in apoptotic response. It is also possible that the clock drugs limit cisplatin toxicity by preventing a non-apoptotic form of cell death. Nonetheless, these findings indicate that U2OS cells have slightly lower apoptotic signaling in response to clock-enhancing molecules following cisplatin treatment, and this observation is considered consistent with increased cell survival tested earlier (**Section 3.2**).

A



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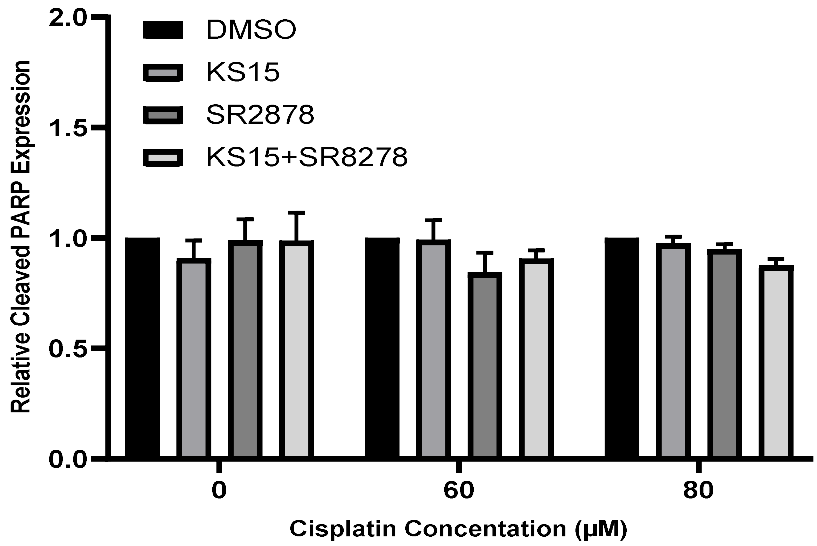


Figure 3.14: KS15 and SR8278 modestly reduce cisplatin apoptotic response in U2OS cells. Immunoblot result **(A)** and quantification **(B)** of PARP cleavage in U2OS cells, which were treated with vehicle (DMSO), 20 µM KS15, 10 µM SR8278, or a combination in the presence and absence of indicated concentrations of cisplatin. Data are presented as means ± SEM (n=3). Groups were compared using two-way ANOVA and Dunnett's multiple comparison test.

3.7 KS15 and SR8278 improve cisplatin-DNA adduct removal in U2OS cells

Cisplatin exerts its cytotoxic effect by crosslinking with DNA purine bases and forming bulky DNA adducts; consequently, it induces cellular apoptosis and cell cycle arrest. The severity of cisplatin's toxicity and its efficacy are highly influenced by forming the bulky DNA lesions. Those lesions are primarily repaired by the NER system to minimize the cisplatin-induced DNA damage. To determine whether the clock-enhancing molecules would affect the removal of cisplatin-DNA adducts, U2OS cells were treated with 0, 20, and 40 µM cisplatin in the presence or absence of 20 µM KS15 and 10 µM SR8278. Cells were harvested at 2 and 24 hours post cisplatin treatment and genomic DNA was purified for cisplatin-DNA adduct analysis by immunoblotting. Despite having more cisplatin-DNA adducts at 2 hours (**Figure 3.15**.B), the KS15 and SR8278 combination resulted in a significant improvement in the cisplatin-DNA adduct removal 24 hours post cisplatin treatment. At 40 µM cisplatin, 17% of unrepaired DNA- adducts remained in KS15 and SR8278 treated cells compared to 48% in DMSO treated ones (**Figure 3.15**.C). These findings are consistent with what was previously shown (**Section 3.5**) about how clock-enhancing molecules increase the expression of DNA repair protein (XPA), which is a key factor in the NER system.

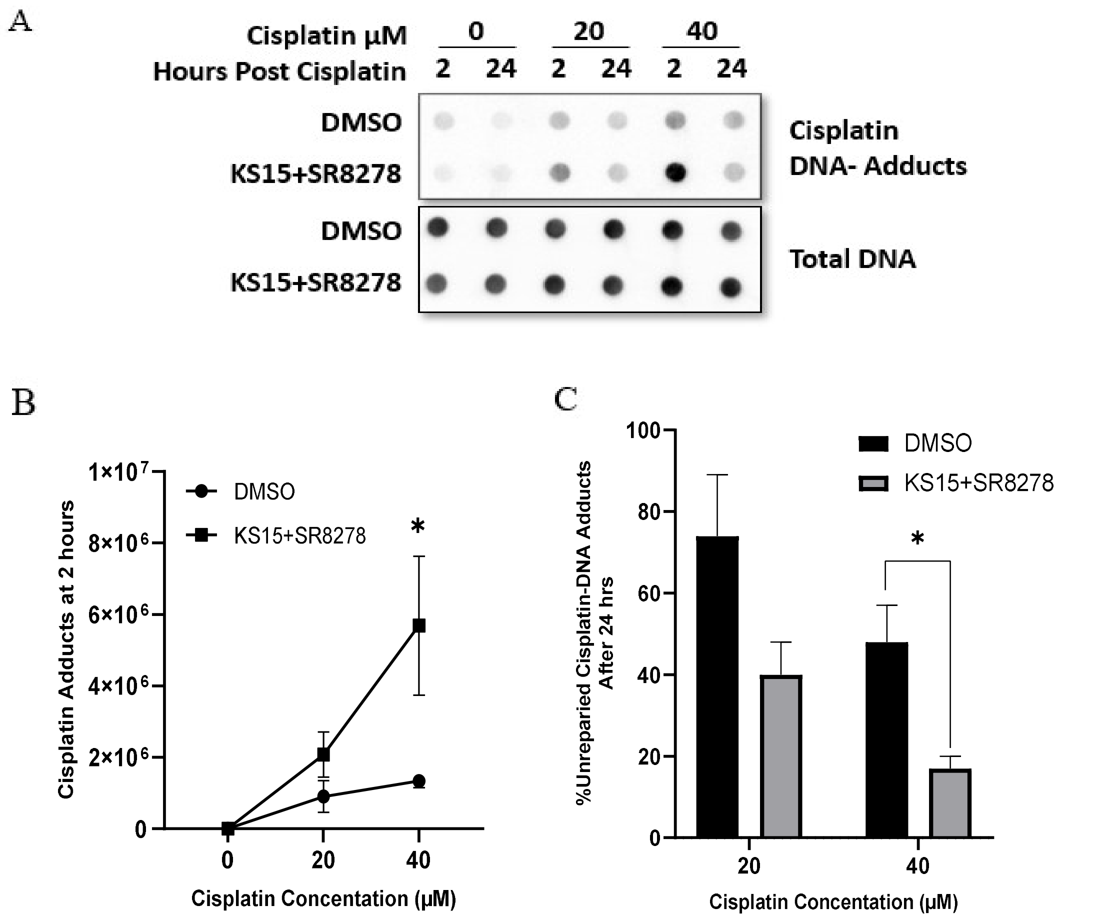


Figure 3.15: KS15 and SR8278 improve cisplatin-DNA adducts removal in U2OS cells. (A) Immunoblot result of cisplatin-DNA adducts in U2OS cells treated with vehicle (DMSO), 20 µM KS15, 10 µM SR8278, or a combination in the presence and absence of the indicated concentrations of cisplatin. **(B)** Quantification of cisplatin-DNA adducts at 2 hours post cisplatin treatment. **(C)** Quantification of cisplatin-DNA adducts 24 hours post cisplatin treatment. Data are presented as means ± SEM (n=3). Unpaired, two-tailed t-tests were performed for comparison, and differences in samples were considered significant compared to DMSO if $P<0.05$ (*).

3.8 KS15 and SR8278 induce G1-phase cell cycle arrest and inhibit cell cycle progression

Because cell cycle arrest maintains genome integrity and allows cells to repair DNA damage before replication occurs, it was hypothesized that KS15 and SR8278 would affect the cell cycle distribution as part of their protective effect against cisplatin-induced DNA damage. To test this hypothesis, flow cytometry was used to analyze the changes in cell cycle distribution following the KS15 and SR8278 exposure. U2OS cells were treated with DMSO, 20 μM KS15, 10 μM SR8278, or a combination of KS15+SR8278 for 24 hours and then harvested, fixed with ethanol, stained using propidium iodide solution and analyzed for their DNA content using the flow cytometer. As shown in **Figure 3.16**, a significant arrest in the G1 phase was observed after KS15 and SR8278 treatment. 68.8% of cells were accumulating in the G1 phase post KS15 and SR8278 exposure compared to 57.8% in DMSO treated cells. Additionally, treated cells with KS15 and SR8278 showed a concomitant decrease in the proportion of cells in the G2/M phase compared to DMSO, with 10.9% versus 16.9%, respectively.

Furthermore, the clock has been shown to control the G1/S checkpoint by regulating the cyclin-dependent kinase inhibitor protein p21, which inhibits cell cycle progression and has been shown to be positively regulated by the CLOCK-BMAL1 gene (35). Therefore, to further examine the effect of clock-enhancing molecules on cell cycle progression, the expression profile of p21 was investigated. U2OS cells were treated for 24 hours with DMSO, 20 μM KS15, 10 μM SR8278, or a combination of KS15+SR8278, then protein lysates from these cells were immunoblotted. As shown in **Figure 3.17**, KS15 and SR8278 combination increased the p21 protein expression up to 3.9-fold change compared to DMSO, which indicates that KS15 and SR8278 inhibit cell cycle progression by increasing the expression of the cyclin-dependent kinase inhibitor protein p21.

Overall, the results of this section support earlier findings (**Section 3.5**) about how clock-enhancing molecules increase the expression of the anti-mitotic kinase Wee1, and consistent with research studies that implicated the clock in the regulation of p20 and p21 proteins, which are expected to cause a cell cycle arrest (35, 36).

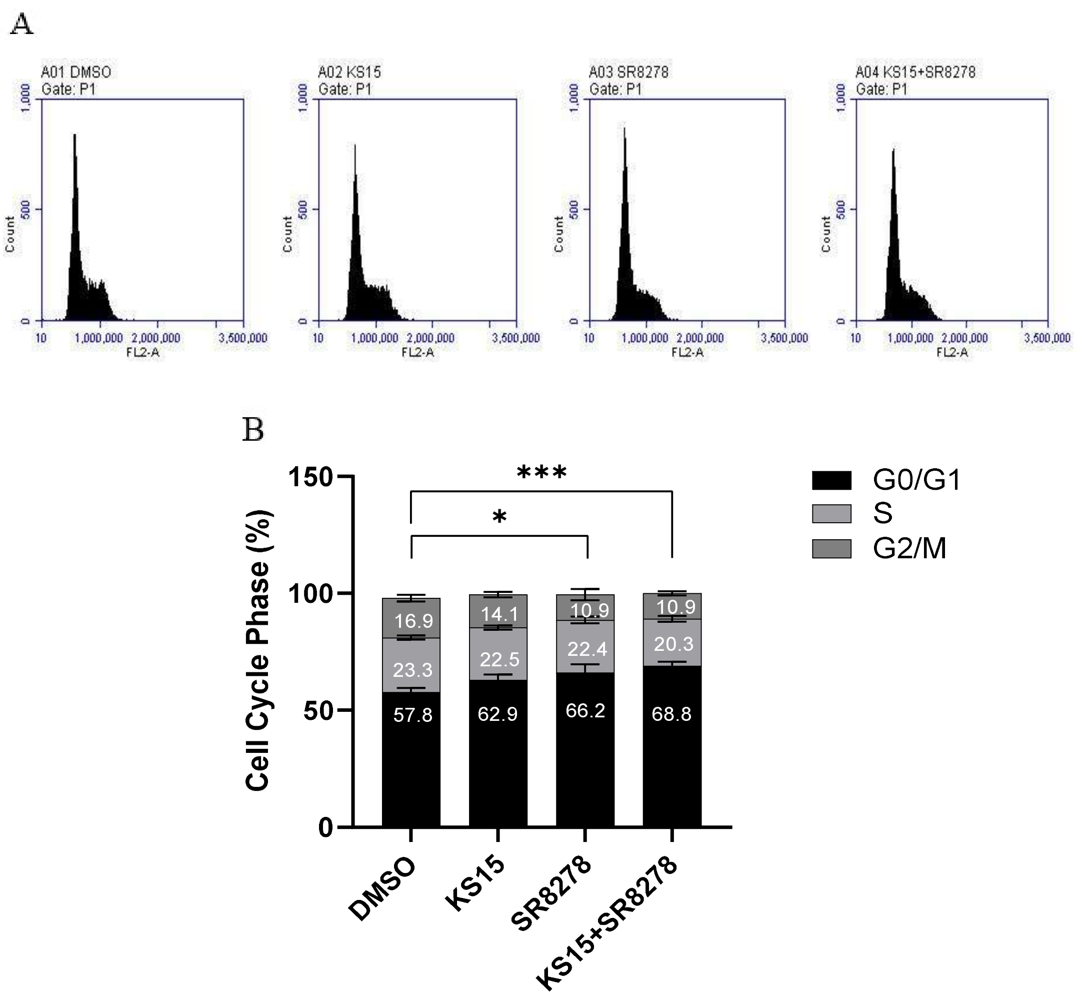


Figure 3.16: KS15 and SR8278 significantly induce G1-phase cell cycle arrest. (A) Representative histogram of the gated U2OS cells in the G0/G1, S, and G2/M phases analyzed by flow cytometry post 24 hours exposure to DMSO, 20 μM KS15, 10 μM SR8278, or a combination of KS15+SR8278. **(B)** Quantitative analysis of cell cycle phase distribution showing the proportion of cells in each phase. Data are presented as means ± SEM (n=4). Groups were compared using a two-way ANOVA test; asterisks indicate a significant difference in G0/G1 phase compared to DMSO at P<0.05 (*), P<0.001 (***).

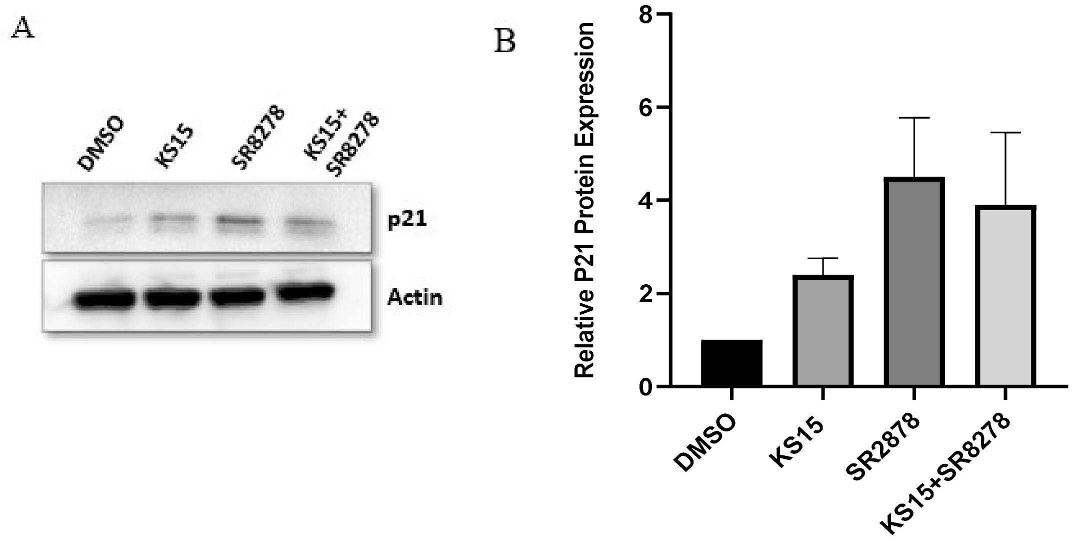


Figure 3.17: KS15 and SR8278 inhibit cell cycle progression by increasing the protein expression of p21. (A) Immunoblot analysis of p21 protein expression in U2OS cells treated with vehicle (DMSO), 20 uM KS15, 10 uM SR8278, or a combination of the two. **(B)** Quantification of the experiments performed as described in (A). Data are presented as means ± SEM (n=2). Groups were compared using one-way ANOVA and Dunnett's multiple comparison test.

4 DISCUSSION AND CONCLUSION

Due to the toxicity associated with cisplatin treatment, which is a significant limitation of cisplatin use, it is becoming essential to identify strategies that maximize its efficacy while minimizing side effects. Chronochemotherapy, which modifies cisplatin toxicity by changing the administration time to when it has the least DNA-damaging effect, is a promising approach for enhancing cisplatin therapeutic outcome (11–13). Cisplatin exerts its cytotoxic effect by crosslinking with DNA purine bases and forming bulky DNA adducts; consequently, it induces cellular apoptosis and cell cycle arrest, and since cisplatin-induced DNA damage is primarily repaired by the NER pathway, the circadian regulation of the NER system is thought to be responsible for cisplatin toxicity chronomodulation. Moreover, the circadian oscillation of XPA influences the repair of cisplatin-DNA adducts by the NER system, and as a result, it modulates the associated cisplatin toxicity (18, 19). Based on that, circadian rhythm modulation offers a potential mechanism for enhancing cisplatin tolerability and treatment outcomes by improving the cellular response to cisplatin-induced DNA damage. However, among the circadian clock modulation strategies, there has not been much research done on using pharmacological modulation of the circadian clock to optimize cisplatin use.

Here we use an in-vitro model to see whether the circadian modulation via clock-enhancing molecules can be used to improve cisplatin tolerability. We find that clock modulation using KS15 and SR8278 molecules significantly increases the cell viability of both U2OS and HaCaT cells post cisplatin exposure (**Figure 3.4** and **Figure 3.7**). This effect seems to be a dose-dependent effect and associated with higher cisplatin IC50 values, suggesting that KS15 and SR8278 clock enhancement protect those cells against cisplatin cytotoxicity (**Figure 3.5** and **Figure 3.8**). On the other hand, A549 cell viability and sensitivity to cisplatin are not affected by KS15 and SR8278 (**Figure 3.9** and **Figure 3.10**). Each cell line's different characteristics and genetic backgrounds could explain the variation in response to clock modulation. For example, A549 cells are deficient in cyclin-dependent kinase inhibitor 2A, a tumor suppressor gene that encodes two splice variants triggering G1-phase cell cycle arrest (78). Also, HaCaT cells have a p53 mutational spectrum similar to that of ultraviolet light-induced mutations (79). It will therefore be important to investigate the protective effects of KS15 and SR8278 in response to cisplatin on a broader panel of cell lines, including normal and primary cells, before being tested in mouse models.

To better understand the mechanism underlying the KS15 and SR8278 protective role against cisplatin toxicity, we investigate the changes in the expression profile of specific clock-controlled genes and proteins. U2OS cells are becoming less sensitive to cisplatin cytotoxicity post KS15 and SR8278 treatment, possibly associated with improved DNA repair capacity induced by enhanced circadian clock output. We find that clock enhancement via KS15 and SR8278 led to a significant increase in the expression of XPA and Wee1 at both the mRNA and protein levels (**Figure 3.11** and **Figure 3.12**). Our data suggest that the decreased susceptibility to cisplatin induced by KS15 and SR8278 results from increased expression of DNA repair protein XPA and anti-mitotic kinase Wee1. This is consistent with the role of XPA and Wee1 in cisplatin-induced DNA damage repair and in line with research studies demonstrated that both XPA and Wee1 exhibit circadian oscillation and their expression is regulated by the clock (20, 77). In HaCaT cells, on the other hand, KS15 alone appears to increase XPA and Wee1 protein expression, while

SR8278 treatment results in a decrease in expression (**Figure 3.13**). The variation in response observed in U2OS and HaCaT cells could arise due to the cell type and genetic background differences. Nonetheless, since the clock modulators reduce cytotoxicity in both cell lines, these findings indicate that the protective effect of clock-enhancing molecules against cisplatin toxicity may include genes other than XPA and Wee1. A large-scale genome-wide gene expression analysis by RNA tracking could be useful to better understand the diverse sets of genes impacted by these molecules.

Another finding is that clock-enhancing molecules modestly reduce the cisplatin apoptotic response in U2OS cells. Cisplatin-induced apoptosis was assessed by PARP cleavage, and KS15+SR8278 clock modulation resulted in a slightly lower apoptotic signaling detected 24 hours post cisplatin treatment (**Figure 3.14**). This observation is consistent with increased cell survival; however, it is still possible that the clock drugs are limiting cisplatin toxicity by preventing a non-apoptotic form of cell death. Furthermore, we identify the functional consequences of clock-enhancing molecules on cisplatin-DNA adduct repair and cell cycle progression. The formation of bulky DNA lesions and the cellular capacity to repair those lesions via the NER pathway significantly impact cisplatin's toxicity (18, 19). Based on the finding that the clock-enhancing molecules increase the expression of NER essential protein, XPA, it is expected that they would impact the removal of cisplatin-DNA adduct. Our data demonstrate that KS15 and SR8278 significantly improve cisplatin-DNA adduct removal 24 hours post cisplatin treatment (**Figure 3.15**.C). Paradoxically, at 2 hours after cisplatin exposure, more DNA adducts were detected with KS15 and SR8278 treatments (**Figure 3.15**.B), which is unexpected and warrants further investigation. It will be valuable to see whether this effect is caused by KS15, SR8278, or a combination of the two, and since it occurs rapidly within two hours of cisplatin therapy, it is unlikely to be due to changes in gene expression. Changes in transporter activity could have altered cisplatin cellular uptake, which may be a possible explanation of the two-hour effect. It has been shown that there is a circadian variation on the expression of cisplatin transporters, including OCT2, MRP1, and ATP7A, which might indicate that cisplatin cellular uptake is controlled by the clock (79).

Lastly, the evidence suggests that the clock controls the cell cycle by regulating the DNA damage checkpoints (31–34). Therefore, we investigate the effect of clock-enhancing molecules on the cell cycle progression, and our data suggest that the protective effect of KS15 and SR8278 against cisplatin cytotoxicity is at least partly due to their ability to inhibit cell cycle progression and induce a G1 phase cell cycle arrest, giving cells time to repair crucial DNA damage before replication. A significant arrest in the G1 phase was observed after KS15 and SR8278 treatment with a concomitant decrease in the proportion of cells in the G2/M phase (**Figure 3.16**). Furthermore, KS15 and SR8278 increased the protein expression of p21, which inhibits cell cycle progression through inhibiting the cyclin-dependent kinase activity (**Figure 3.17**). These findings are in line with the detected elevation in the anti-mitotic kinase Wee1 and consistent with research studies that implicated the clock in the regulation of p20 and p21 proteins, which are expected to cause a cell cycle arrest (35, 36).

It is noteworthy that while mammalian cells retain their circadian rhythmicity in a tissue culture setting, individual cells can be out of phase with one another resulting in a non-synchronized circadian rhythm (73). In this project, we do not use any *in-vitro* circadian synchronization by serum shock or dexamethasone that synchronizes the phases of the cells, and the culture as a whole becomes a synchronized circadian system (80, 81). However, our model responded well to the clock modulation, and changes in clock-controlled processes were detected. Nonetheless, it will still be interesting to test the clock-enhancing molecules in a synchronized system, and before moving to *in-vivo* studies either in mice or humans, it will likely be important to see if the clock modulator timing during the day will impact the outcome.

In conclusion, our results here indicate that circadian clock enhancement via small molecules KS15 and SR8278 attenuates cisplatin cytotoxicity by improving the cellular DNA repair capacity. This effect is likely due to the clock regulation of the DNA repair pathway and cell cycle progression, which results in a better repair of cisplatin-induced DNA adducts. Besides, our findings suggest that the use of circadian clock modulators has promising implications as a novel strategy for improving cancer care and treatment outcomes.

5 SUPPLEMENTAL MATERIAL

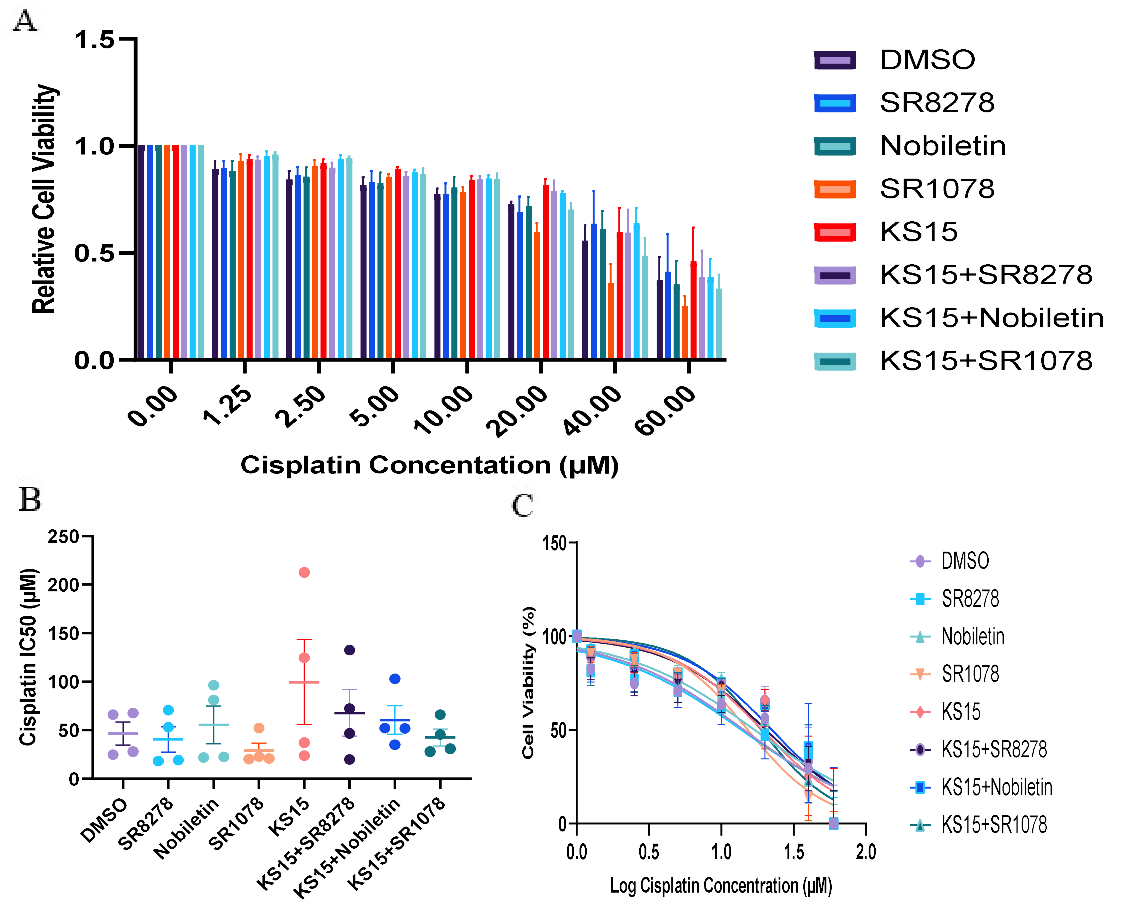
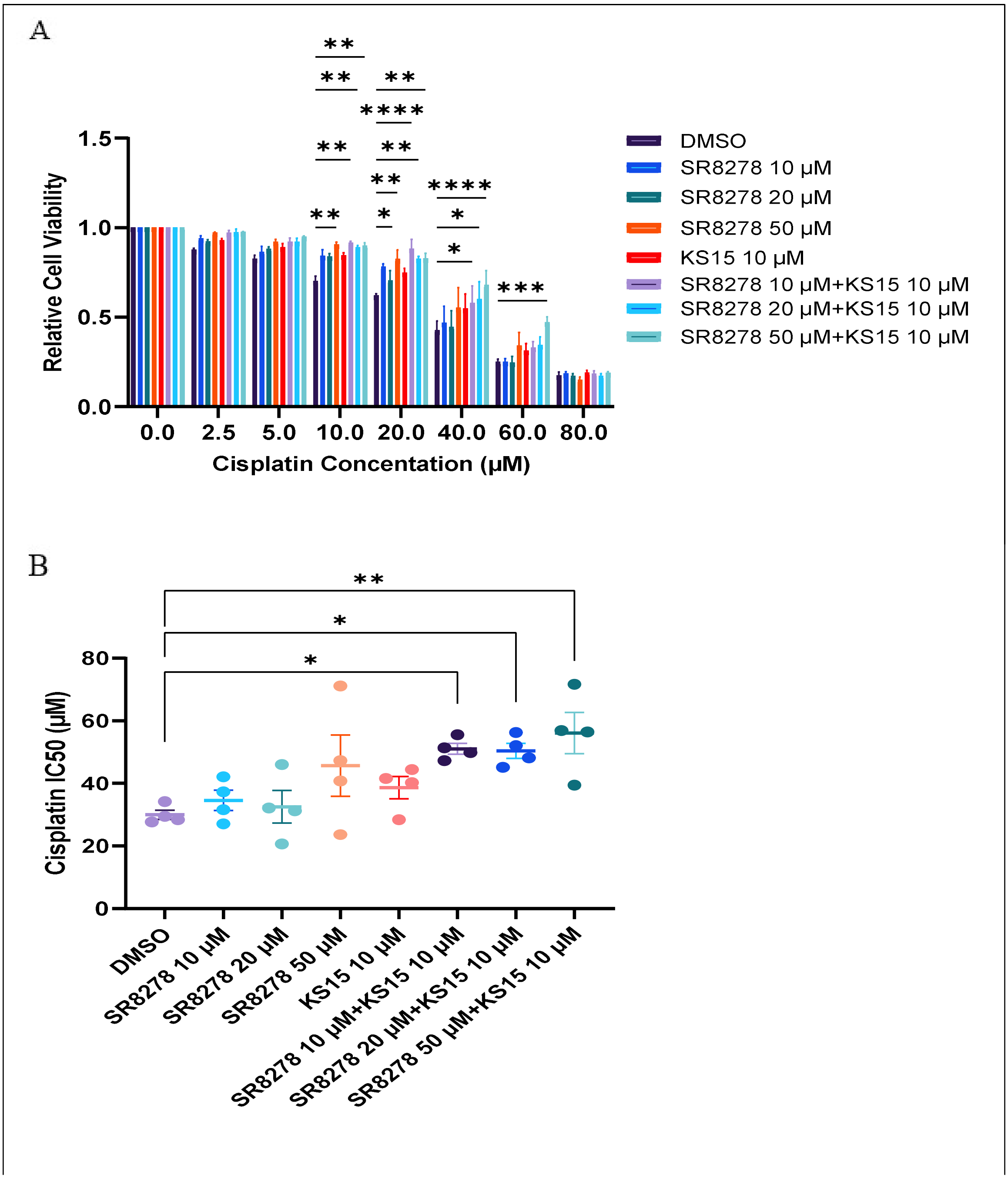


Figure 5.1: Optimizing concentrations, combinations, and timing of treatments. (A) Cell viability assay, **(B)** Cisplatin IC50 values, **(C)** Dose-response curves. U2OS cells were plated into 96-well plates then treated with 10 µM of clock-enhancing molecules for 24 hours, and then media was removed, and cells were exposed to different concentrations of cisplatin (1.25, 2.5, 5, 10, 20, 40, 60 µM). Cells were incubated for 24 hours before MTT assay was performed to analyze the viability of remaining cells compared to vehicle DMSO. Data are presented as means ± SEM (n= 4), and groups were compared using one-way ANOVA and Dunnett's multiple comparison test. No significant difference was detected.


A
Relative Cell Viability
1.5
1.0
0.5
0.0
0.0
2.5
5.0
10.0
20.0
40.0
60.0
80.0
Cisplatin Concentation (μM)
DMSO
SR8278 10 μM
SR8278 20 μM
SR8278 50 μM
KS15 10 μM
SR8278 10 μM+KS15 10 μM
SR8278 20 μM+KS15 10 μM
SR8278 50 μM+KS15 10 μM
B
Cisplatin IC50 (μM)
80
60
40
20
0
DMSO
SR8278 10 μM
SR8278 20 μM
SR8278 50 μM
KS15 10 μM
SR8278 10 μM+KS15 10 μM
SR8278 20 μM+KS15 10 μM
SR8278 50 μM+KS15 10 μM

C

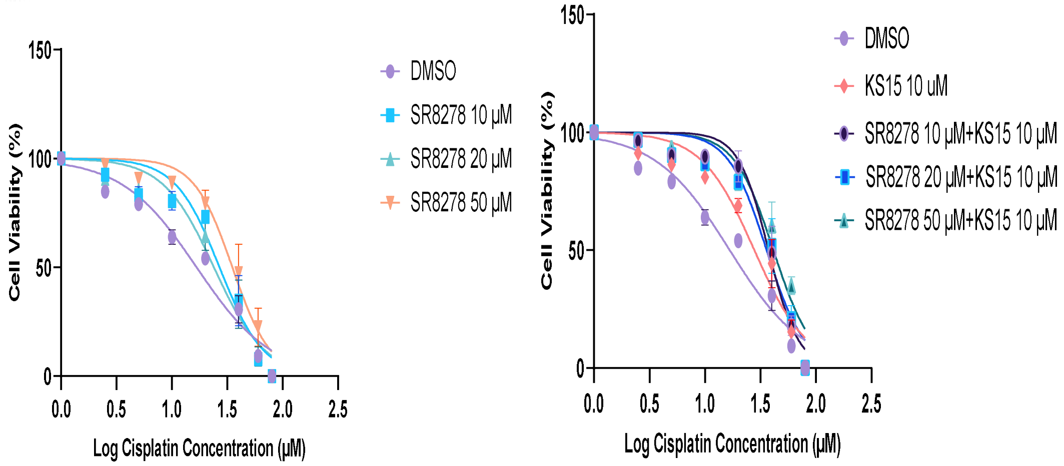


Figure 5.2: Testing different concentrations and combinations of KS15 and SR8278. **(A)** Cell viability assay, **(B)** Cisplatin IC50 values, **(C)** Dose-response curves. U2OS cells were cultured and treated for 48 hours with vehicle (DMSO) or the indicated concentrations of KS15 and SR8278 with (1.25, 2.5, 5, 10, 20, 40, 60 µM) of cisplatin. Cell viability assessed using MTT assay and data are presented as means ± SEM (n= 4). Groups were compared using one-way ANOVA and Dunnett's multiple comparison test. Differences in samples were considered significant compared to DMSO if P<0.05 (*), P<0.01 (**), P<0.001 (***), P<0.0001 (****).

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