

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**IDENTIFICATION OF MOLECULES THAT AFFECT CRY1-
PER2 INTERACTION**



MEHMET SERDAR KOCA
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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THESIS SUPERVISOR
PROF. DR. NURİ ÖZTÜRK

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

CRY1-PER2 ETKİLEŞİMİNİ
ETKİLEYECEK MOLEKÜLLERİN
BELİRLENMESİ

MEHMET SERDAR KOCA
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
PROF. DR. NURİ ÖZTÜRK

GEBZE
2023



YÜKSEK LİSANS JÜRİ ONAY FORMU

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JÜRİ

ÜYE

(TEZ DANIŞMANI)

: Prof. Dr. Nuri ÖZTÜRK

ÜYE

: Prof. Dr. İbrahim Halil KAVAKLI

ÜYE

: Prof. Dr. Sedef TUNCA GEDİK

ONAY

Gebze Teknik Üniversitesi Fen Bilimleri Enstitüsü Yönetim Kurulu'nun

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SUMMARY

Circadian rhythms are biological patterns of approximately 24 hours in biological processes or systems. It is assumed that about 10% of transcriptome or 20-40% of the proteome shows circadian rhythmic patterns. The oscillation at RNA and protein levels indicates that the timing of treatment with drugs is important. Because of this relationship, it is possible to change the efficiency of the drugs by modulating circadian rhythms using small molecules. With the developments in computer-aided drug design in recent years, there has been an increase in the speed of reaching pre-clinical studies. In our study, using the crystal structure of the CRY1-PER2 complex, the interaction site of these proteins was chosen as the target, and molecular docking for, ~2 million molecules were performed. The pharmacokinetics of molecules with strong affinity were examined via *in silico* ADME. *In vitro* studies of 14 molecules were carried out by subjecting suitable molecules to molecular dynamics simulation and relative free energy calculation. The sub-toxic dose was determined by MTT. Molecules affecting circadian rhythm were determined by real-time bioluminescence assay. The effect of these molecules on CRY1 was investigated by Western blot. We report that the P09 molecule reduces the stability of CRY1.

Key Words: Biological Clock, Drug Discovery, Molecular Dynamics, Molecular Docking.

ÖZET

Sirkadiyen ritim, biyolojik işlemlerde veya sistemlerde yaklaşık 24 saatlik biyolojik desenlerdir. Transkriptomun yaklaşık %10'u ve proteomun %20-40'ı sirkadiyen ritmik desenler sergilediği varsayılır. RNA veya protein düzeyindeki salınımlar, ilaçların tedavi zamanlamasının önemli olduğunu gösterir. Bu nedenle, küçük moleküller kullanarak sirkadiyen ritmi düzenleyerek ilaçların etkinliği değiştirilebilir. Son yıllarda bilgisayar destekli ilaç tasarımındaki gelişmelerle, klinik öncesi çalışmalara ulaşma hızında artış yaşanmıştır. Çalışmamızda, CRY1-PER2 kompleksinin kristal yapısını kullanarak bu proteinlerin etkileşim bölgesi hedef olarak seçildi ve ~2 milyon molekül için moleküler docking yapıldı. Güçlü afiniteye sahip moleküllerin farmakokinetiği *in silico* ADME ile incelendi. 14 molekülün *in vitro* çalışmaları uygun moleküllere moleküler dinamik simülasyonu ve göreceli serbest enerji hesaplaması ile yapıldı. Alt-toksik dozlar MTT yöntemiyle ile belirlendi. Sirkadiyen ritmi etkileyen moleküller gerçek zamanlı biyoluminesans deneyi ile tespit edildi. Bu moleküllerin CRY1 üzerindeki etkisi Western blot ile incelendi. Bu çalışma, P09 molekülünün CRY1'in stabilitesini azalttığını göstermiştir.

Anahtar kelimeler: Biyolojik Saat, İlaç Keşfi, Moleküler Dinamik, Moleküler Kenetlenme.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
μg	: Microgram
μl	: Microliter
AD	: Alzheimer's Disease
ADME	: Absorption, Distribution, Metabolism, and Excretion
AMPA	: a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANI	: Accurate NeurAl network engINe for Molecular Energie
BAR	: Bennett Acceptance Ratio
<i>Bmal1</i>	: Bmal1 gene
Bmal1	: Bmal1 protein
cAMP	: Cyclic adenosine monophosphate
CCG	: Circadian-clock controlled genes
<i>Clock</i>	: Clock gene
Clock	: Clock protein
CREB	: cAMP-responsive element-binding protein
CREs	: cAMP-responsive elements
<i>Cry</i>	: Cry gene
Cry1	: Cry1 protein
CSNK1	: Casein kinase I
CSV	: Comma-separated values
DFT	: Density Functional Theory
DSPD	: Delayed sleep phase disorder
ESP	: Electrostatic Potential
FAD	: Flavin adenine dinucleotide
FASPD	: Familial advanced sleep phase disorder
FEP	: Free Energy Perturbation
FFT NLLS	: Fast Fourier Transform Non-linear Least Squares
GAFF	: Generalized Amber Force Field
GCL	: Ganglion cell layer
GPU	: Graphics processing unit

INL	: Inner nuclear layer
K	: Kelvin
MAPK	: Mitogen-activated protein kinase
MD	: Molecular Dynamics
MM-GBSA	: Molecular Mechanics-Generalized Born Surface Area
MM-PBSA	: Molecular Mechanics-Poisson-Boltzmann Surface Area
nm	: Nanometer
NMDA	: N-methyl-D-aspartate
NPAS2	: Neuronal PAS Domain Protein 2 protein
ns	: Nanosecond
P53	: P53 protein
PACAP	: Pituitary adenylate cyclase activating peptide
PDB	: Protein Data Bank
<i>Per2</i>	: Per2 gene
Per2	: Per2 protein
QM	: Quantum Mechanics
Rev-ERB	: Rev-ERB protein
ROR	: Retinoid-related orphan receptor
SCN	: Suprachiasmatic nucleus
SMILES	: Simplified molecular-input line-entry system
SNP	: Single Nucleotide Polymorphism
TTFL	: Transcription-Translation feedback loop
U2OS	: Human Bone Osteosarcoma Epithelial Cells

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1. INTRODUCTION

In Latin, “circa” and “dies” means about and one day, respectively therefore circadian rhythm is a term coined to express 24-hour cycles. Recent investigations have focused on understanding the effects of circadian clock on human health. Animal studies, particularly in mice, have provided valuable insights into the role of specific clock genes in disease development. For instance, the deletion of *Clock* and *Bmal1* genes in mice showed their importance in the development of hypoinsulinemia, diabetes, hyperglycemia, and premature [de Assis and Oster, 2021]. Disruptions in the *PER2* gene have been associated with familial advanced sleep phase syndrome, a condition characterized by a shift in sleep-wake cycles [Vanselow et al., 2006]. Moreover, structural alterations in circadian clock genes have been implicated in various neurological disorders, including schizophrenia, major depression, and bipolar disorder [Karatsoreos, 2014]. Notably, mutations in the *Cry1* gene have been linked to familial delayed sleep phase and attention deficit/hyperactivity disorders [Emre Onat et al., 2020]. Additionally, studies have demonstrated that the deletion of *Cry* reduces the risk of cancer caused by p53 mutation in mice [Cavga et al., 2019].

Given the importance of circadian clock proteins in numerous biological pathways, there is considerable interest in exploring their therapeutic potential. Manipulating the activity of clock proteins could have therapeutic applications in restoring or enhancing disrupted systems. For instance, that a CLOCK interacting small molecules called CLK8 enhances the amplitude of the cellular circadian rhythm by stabilizing the negative arm of the transcription-translation feedback loop without affecting period length, and this suggested that the molecule is a potential candidate for therapeutic development to manage disorders associated with dampened circadian rhythms [Doruk et al., 2020]. In this study, the missing residues in the crystal structure of the *PER2*/*CRY1* complex were completed using Swiss homology modeling. Subsequently, a 100-ns pre-molecular dynamics simulation was performed using Gromacs open-source software. Docking studies were conducted with approximately 2.5 million molecules from the ZINC15 database, which possessed lead-like properties and complied with Lipinski's 5 rule. The resulting ligands underwent *in silico* toxicology testing using SwissADME. Molecules identified as potential drug candidates were further evaluated through Protein-Ligand simulations, and non-toxic

doses were determined through *in vitro* studies. This strategy is sketched in Figure 1.1. Finally, the effects of these molecules on the circadian rhythm were examined through real-time bioluminescence assays and Western Blot analysis.

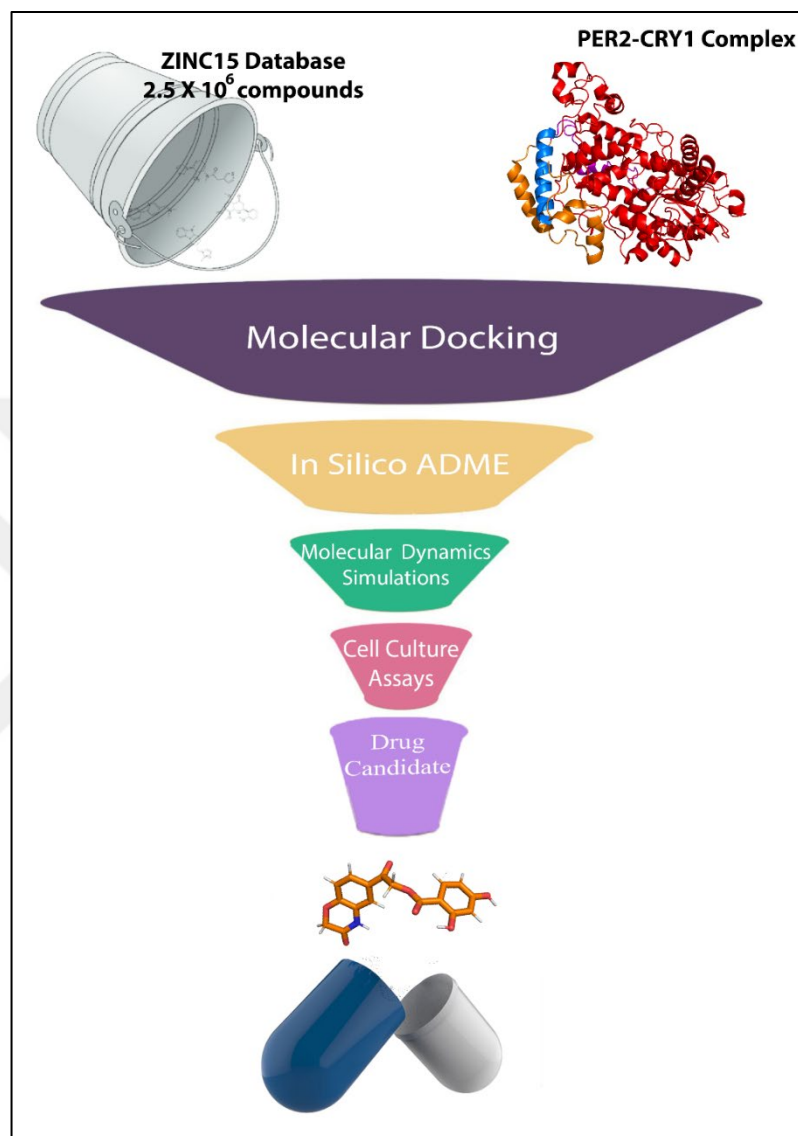


Figure 1.1: The workflow of the thesis.

Part 2 reviews detailed literature on the circadian clock, its mechanism, the components of the mammalian circadian clock, clock-related diseases, and structure-based drug design. Parts 3 and 4 describe materials and all experimental procedures. Part 5 provides a detailed description of all the experimental results obtained in this thesis. Finally, this thesis is concluded by giving conclusions and discussion in Part 6.

2. LITERATURE

2.1. SCN and Master Clock

Circadian clocks drive daily behavioural and physiological rhythms in organisms. The suprachiasmatic nucleus (SCN), located in the hypothalamus, positioned bilaterally against the third ventricle, acts as the timekeeper of the mammalian [Hastings et al., 2018]. Our nervous system also operates as a 24-hour machine. Although there are numerous cellular clocks in our body [Dallmann et al., 2014], [Reppert and Weaver, 2002], the SCN function mainly as the circadian pacemaker in the mammalian circadian clock. Each nucleus in the SCN located above the optic chiasm consists of about 10000 neurons and 3500 astrocytes [Patton and Hastings, 2018]. *In vivo*, the SCN exhibits rhythms that transmit metabolic and electrical activities in high-amplitude and transmits these circadian rhythms to the rest of the brain and body, and remarkably, these rhythms continue autonomously in *ex vivo* cultures [Hastings et al., 2020]. To be effective, the internal circadian clock time needs to be synchronized by the SCN to get advantages from or prepare for environmental cyclic changes. This synchronization is mediated by photosensitive retinal ganglion cells [Fernandez et al., 2016]. The electrical and metabolic signals in the SCN are strongly induced by the light presented by the solar system, thereby correcting the internal clock and local time [Hastings et al., 2020]. RGCs convey signals to the brain using glutamatergic neurotransmission which affects N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors on the membrane, which causes direct and indirect calcium influx [Ding et al., 1997], [Nahm et al., 2005], [Schurov et al., 1999]. Activated by solar light, ganglion cells secrete neuromodulator pituitary adenylyl cyclase activating peptide (PACAP) as well as glutamate [Hannibal, 2002], [Morin and Allen, 2006]. Released PACAP has a role to enhance the effect of secreted glutamate. Calcium, which is transferred to depolarized neuron cells by glutamate-stimulating NMDA and AMPA receptors, activates the cyclic adenosine monophosphate (cAMP) and mitogen-activated protein kinase (MAPK) pathways to phosphorylate cAMP-responsive element-binding protein (CREB) [Gau et al., 2002], [Ginty et al., 1993]. Activation of target gene promoters is dependent on CREB being phosphorylated, and

phosphorylated CREB binds to cAMP-responsive elements (CREs), initiating transcription. The core clock genes *Period 1* (*Per1*) and *Period 2* (*Per2*) contain E-box and CRE while *Period 3* (*Per3*) does not, indicating that *Per1* and *Per2* can be light induced in the clock machine. Expression of *PER1* and *PER2* is by light-induced CREB during the daytime, while at night it acts as a brain-muscle-Arnt-like protein 1 (BMAL1)/ circadian locomotor output cycle kaput (CLOCK) inhibitor. Thus, the BMAL1/CLOCK complex, which is not directly affected by light, transmits the light-dependent expression of *PER1* and *PER2* to BMAL1/CLOCK, thus resetting the clock in the SCN [Albrecht et al., 1997].

Cells in the SCN work together in a coordinated manner to generate a powerful oscillation [Herzog, 2007]. A single-cell circadian reporter system showed that individual SCN neurons in the SCN oscillate independently, with a wide range of phases and periods ranging from 20-30 hours [Liu et al., 1997], [Takahashi et al., 2010]. However, the coupling properties of SCN neurons give the SCN the ability to produce a robust and precise rhythm of locomotor activity in mice (with a ~12 min standard deviation) and maintain this rhythm even when there are perturbations from the environment [Herzog et al., 2004], [Mohawk et al., 2012], [Mohawk and Takahashi, 2011]. The coordinated activity of SCN cells is essential for the accurate and reliable production of the circadian rhythm, which is critical for the proper functioning of the body. Disruptions in the SCN and the resulting disruption of the circadian rhythm can have several negative consequences, including sleep disorders, mood disorders, and even metabolic disorders. Therefore, it is important to understand the mechanisms by which the SCN produces and maintains the circadian rhythm to develop therapies for those who suffer from disruptions to their body's internal clock.

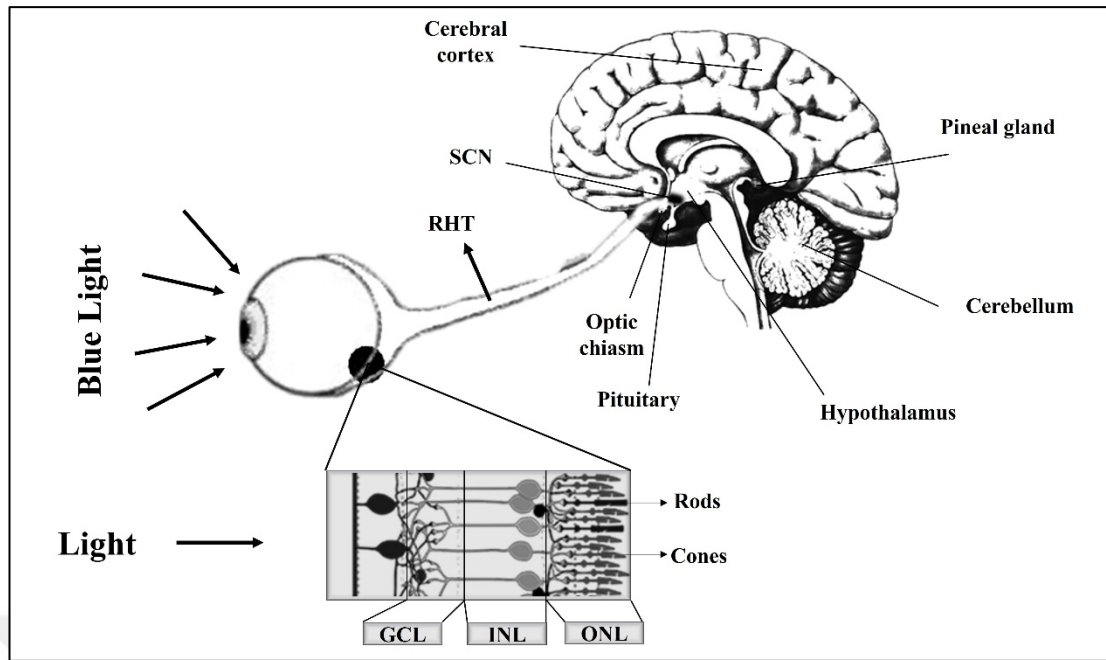


Figure 2.1: The suprachiasmatic nucleus (SCN) is a crucial region of the brain involved in the perception and transmission of light signals. It is situated in the brain and receives inputs from photoreceptor cells such as rods and cones, located in the inner retina, which are responsible for detecting light and relaying the information to the SCN. Apart from rods and cones, there are also other light-sensitive cells present in the inner nuclear layer (INL) and ganglion cell layer (GCL) of the retina, which possess opsins, specialized proteins responsible for light detection.

2.2. Peripheral Clock

The rhythmic expression of clock genes and/or proteins, which has been observed in a wide variety of cells and tissues in mammals, including suprachiasmatic nucleus (SCN), has been shown to persist in culture, thereby demonstrating that non-SCN cells also possess endogenous circadian oscillators that regulate various physiological processes [Balsalobre et al., 1998], [Yamazaki et al., 2000], [Yoo et al., 2004]. Even though the fundamental clock machinery is conserved among the various cellular clocks in mammals, there are marked discrepancies in the relative contributions of the individual clock components, as well as in the ways in which these peripheral clocks are reset and the output pathways that they regulate. These differences highlight the complexity and diversity of the mechanisms involved in the regulation of circadian rhythms at the cellular level. Although earlier research suggested that the intrinsic cellular clocks found in mammals regulate extensive rhythms of gene transcription, with a significant proportion of all mRNAs in a

particular tissue exhibiting circadian rhythms in their steady state levels, ranging from 3-10% [Akhtar et al., 2002], [Duffield et al., 2002], [Hughes et al., 2009], [Miller et al., 2007], [Panda et al., 2002], [Storch et al., 2002], more recent findings from studies conducted on 12 different mouse organs (including the adrenal gland, aorta, brainstem, brown fat, cerebellum, heart, hypothalamus, kidney, liver, lung, skeletal muscle, and white fat) indicate that an even larger proportion of genes, approximately 43%, that are translated into proteins in mammals are under the control of the circadian clock [Zhang et al., 2014]. The genes that are regulated by the body's internal clock (circadian control) differ significantly between tissues, indicating the need to coordinate cellular functions in each tissue type at specific times. The circadian clock, plays a crucial role in regulating a variety of biological processes, including xenobiotic detoxification [Gachon et al., 2006], glucose homeostasis [Lamia et al., 2008], [Marcheva et al., 2010], [Turek et al., 2005], and lipogenesis [Atger et al., 2017], [Le Martelot et al., 2009]. Indeed, these processes are all related to metabolism and are influenced by the circadian clock.

2.3. Mammalian Circadian Clock Elements

On September 1, 1971, an important paper was published which laid the foundation for the molecular mechanisms [Konopka and Benzer, 1971]. This work enabled us to start understanding the circadian clock in animals. Ronald Konopka and Seymour Benzer discovered circadian clock changes in pupal eclosion, and locomotor activity caused by three different mutations in the same gene in *Drosophila*. One mutant exhibited no rhythmicity, another had a short 19-h period, and a third had a long 28-h period. These mutations are mapped to the same locus on the X chromosome. This gene was named *Period* [Takahashi, 2021]. This work paved the way for the discovery of genes and their mechanisms involved in circadian biology. In subsequent studies, it was observed that the *per* gene oscillates in the brain of *D. melanogaster*. When it was observed that this oscillation was disrupted because of the nonsense mutation in the *Per* gene, the researchers proposed the auto-regulatory feedback loop model [Hardin et al., 1990].

In mammals, the circadian rhythm is generated by a transcription-translation feedback loop (TTFL). BMAL1 and CLOCK proteins on the positive arm induce

transcription, while the CRY and PER proteins on the negative arm suppress their own transcription by interacting with BMAL1 and CLOCK proteins. *CRY* and *PER* genes under the E-box element (CACGTG) begin to be produced by the activity of the heterodimer complex of BMAL1 and CLOCK proteins (Figure 2.2). PER and CRY proteins accumulating in the cytoplasm are phosphorylated by protein kinase (CSNK1) to form a heterodimer complex and migrate to the nucleus. PER/CRY complex suppress their production by inhibiting the BMAL1/CLOCK complex [Hastings et al., 2014]. The BMAL1/CLOCK complex also induces the transcriptions of REV-ERBs and RORs genes. On the other hand, the expression of *BMAL1* gene induced by RORs and suppressed by REV-ERBs. This produces another control mechanism. While the basic clock mechanism is formed in this way, there are many clock-controlled genes (CCG) under the E-Box element. Omics studies have shown that approximately 10% of transcripts are regulated directly and approximately 40% of transcripts are regulated by second-order genes and posttranscriptional modifications [Hughes et al., 2009].

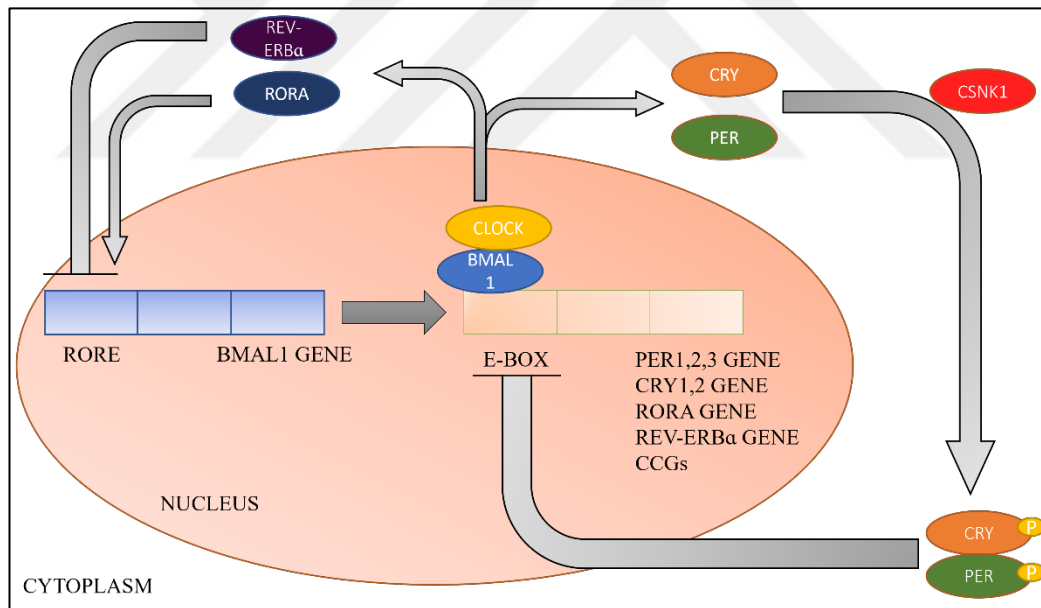


Figure 2.2: Transcription-Translation Feedback Loop of the mammalian circadian clock.

Although the CRY protein acts as a photoreceptor in many other taxa, mammalian CRYs do not have this function. CRY proteins, which are transcribed by two *CRY* genes (*CRY1* and *CRY2*), in BMAL1/CLOCK complex inhibition [Anand et al., 2013], [Griffin et al., 1999]. When discovered in mammals, it was named cryptochrome by the Sancar group because it was thought to have blue-light-dependent

activity [Hsu et al., 1996]. In further studies, it was observed that there was a high level of expression in the inner retina, and this expression changed over time, indicating that the CRY protein is involved in the molecular clock mechanism [Miyamoto and Sancar, 1999]. Based on this idea, they conducted an analysis on mice deficient in CRY1^{-/-}, CRY2^{-/-}, and CRY (CRY1^{-/-}, CRY2^{-/-}) in constant darkness conditions. The most notable finding was the complete absence of rhythm in CRY knockout mice compared to the wild type (WT). These results indicated that the CRY protein plays an essential role in the regulation of circadian rhythm.

As I explained above, PER protein, one of the other elements of the negative arm, was discovered in the *D. melanogaster*, and its mammalian orthologs were later discovered [Von Schantz et al., 2006]. The arrhythmic behaviour of mice in the absence of *PER1* and *PER2* genes, suggested that *PER1/2* genes are essential clock genes because *PER3* gene alone cannot support the circadian oscillation [Maywood et al., 2014], and the deficiency of the *PER2* gene appears to have greater effect on circadian rhythm. PER2, which can be found in the cytoplasm and nucleus, can act as a homodimer and heterodimer [Baidanoff et al., 2022]. Post-translational modifications of PER2 with more than 20 phosphorylation sites are regulated by CSNK1. This increases stability with specific phosphate groups, affecting ubiquitination [Smyllie et al., 2022]. One of the most important of these effects is the heterodimer structure formed with CRY protein.

The CRY1/PER2 (Figure 2.3) complex plays a role in inhibiting the transcriptional activation of the positive arm of circadian rhythm, BMAL1/CLOCK. CRY1 and PER2 interaction is influenced by zinc binding and disulfide bond formation [Schmalen et al., 2014]. CRY1 and PER2 interact with different domains of BMAL1, only PER2 interacts with CLOCK protein [Langmesser et al., 2008]. The repressor capacity of the CRY1 protein is higher than PER2 [Langmesser et al., 2008].

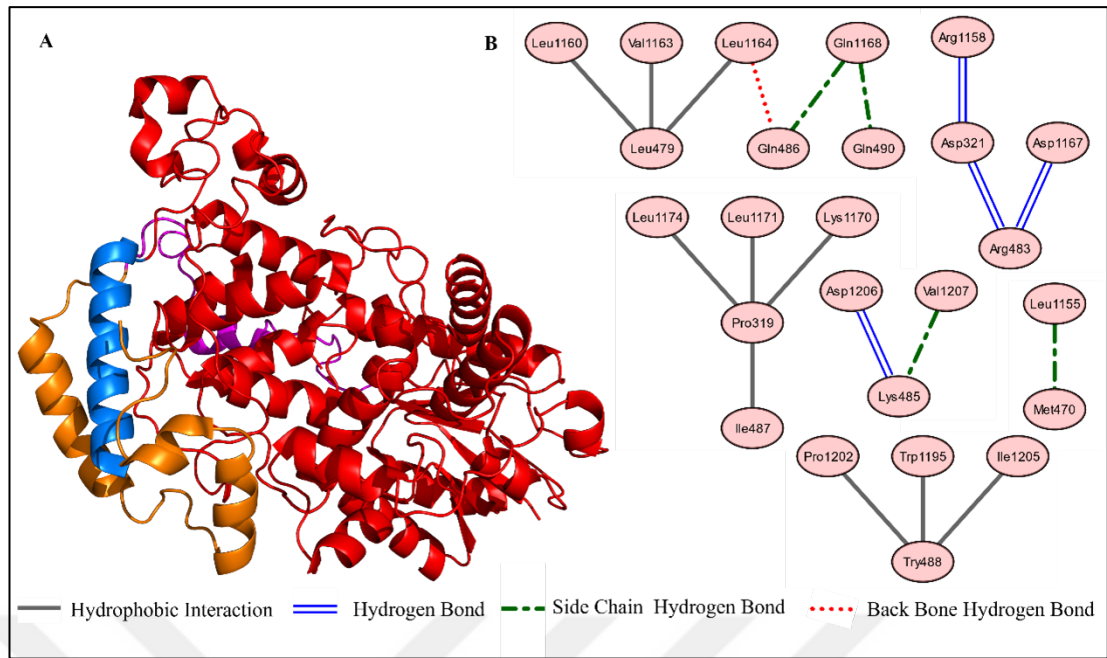


Figure 2.3: Crystal structure of CRY1-PER2(PDB ID: 6OF7) complex and interactions. (A) CRY1-PER2 are shown in red and magenta, respectively. Residues in CRY binding domain are coloured as orange. Residues in interaction with TIMELESS region are coloured as blue. (B) Interaction residues and types between mouse CRY1 and human PER2.

2.4. Circadian Clock-Related Diseases

Circadian rhythm controls hormone production, brain activity, and many other physiological events, and is a system above other biological mechanism [Dallmann et al., 2014]. The deterioration of circadian rhythm is a frequently occurring problem in modern society. Studies on shift workers have shown that the disruption of the circadian rhythms is associated with psychiatric illness, metabolic syndrome, dysplasia, and cancer (Boivin and Boudreau, 2014; Foster et al., 2013; Kuhn, 2001; Sack et al., 2007). Abnormalities in master regulator, SCN, have also been linked to numerous neurodegenerative diseases [Kuhn, 2001], [Leng et al., 2019], [Videnovic et al., 2014]. Studies with age-adjusted populations, showed that circadian disruption is higher in individuals with Alzheimer's Disease (AD) than in healthy people, and additionally, amplitude reduction and phase shift were also observed more frequently [Videnovic et al., 2014]. The most common circadian rhythm disorder in Alzheimer's patients is the patient's irregular sleep-wake cycle. The disease may be aggravated by wakefulness during the night and sleep disturbances during the day [Abbott and Zee,

2015], [Auger et al., 2015]. Disturbance of circadian rhythm in Parkinson's Disease, unlike AD, does not show a phase shift and is characterized by amplitude reduction [Berganzo et al., 2013], [Bolitho et al., 2014], [Breen et al., 2014].

Circadian genes are important in tissue homeostasis and tumorigenesis. Disruption of circadian rhythm has been associated with many cancer subtypes, and evidence continues to increase the association of this mechanism with cancer. The expression of *PER1* and *PER2* genes are decreased in sporadic and familial breast tumor compared to normal breast cells [Winter et al., 2007]. An increase in the survival of breast cancer cells was observed with methylation of the promoters of the *PER1* and *CRY1* genes which results in disruption of their cellular rhythm by inactivating their expression [Kuo et al., 2009]. In addition, in the study by Ozturk et al., it was observed that the life-spans of mice with the *p53* mutation increased by approximately 50% in the case of *Cry* double knockouts [Sancar et al., 2009].

In *Cry* KO studies, *Cry1*^{-/-} mice were observed to be resistant to a high-fat diet compared to wild-type [Griebel et al., 2014]. Mutations in *CRY1* and *CRY2* have also been associated with delayed sleep phase disorder (DSPD) and familial advanced sleep phase disorder (FASPD), respectively [von Schantz, 2017]. In studies on human keratinocyte cells, it has been observed that *CRY1* has a positive effect on human hair growth [Buscone et al., 2021]. The SNP of *CRY1* and *NPAS2* has been shown to be associated with unipolar major depression [Soria et al., 2010].

The *Per* genes, the element of the negative arm, has been shown to be associated with expression in the development and prognosis of cancerous cells [Fu et al., 2002], [Zhao et al., 2016] Expression of *Per1/2* gene was found to be significantly downregulated in colorectal, prostate and breast cancer [Wang et al., 2016a], [Winter et al., 2007], [Zhao et al., 2014], [Zhu et al., 2009]. In studies involving the overexpression of *Per2*, it has been observed that in K562 leukemia cells, the expression of *p53* is upregulated while the expression of *c-Myc* and *CyclinB1* is downregulated, leading to an increase in apoptosis. Additionally, in MG63 osteosarcoma cells, overexpression of *Per2* promotes an increase in the G0/G1 phase while inducing cell apoptosis [Cheng et al., 2015]. Furthermore, it has been demonstrated that downregulation of *PI3K* and *PKB* inhibits ovarian cancer [Wang et al., 2016b]. Knockdown studies of *Per2* have revealed that it leads to increase the expressions of β -catenin and *CyclinD*, promoting the proliferation of colon cancer cells [Wood et al., 2008], while also causing an increase in the phosphorylation of

AKT and mTOR, which decreases cell apoptosis in A549/cisplatin lung adenocarcinoma cells [Wood et al., 2008]. Additionally, Per2 knockdown activates the MDM2-p53 pathway, thereby regulating apoptosis in glioma cells [Zhanfeng et al., 2016].

The insights gained from the mentioned information highlight the potential of utilizing the components of the negative arm, namely Cry and Per proteins, as promising drug targets for numerous diseases. The findings suggest that targeting these proteins could have significant implications in the development of novel therapeutic approaches. Further research and exploration in this area could pave the way for innovative treatments and interventions that aim to modulate circadian rhythms and address the underlying pathophysiological mechanisms associated with various diseases. The utilization of Cry and Per proteins as drug targets holds promise for improving patient outcomes and advancing the field of personalized medicine.

2.5. Structure-Based Drug Design

Structure-based drug design, which has become much more popular with the Covid-19 pandemic, has overcome traditional drug design methods, and sought to meet the need for a fast, inexpensive, and accurate method. The protein structure-based drug design method has been widely used for predicting the molecule's binding mode and relative affinity. Computer-aided drug design has been used in the discovery of many drugs such as captopril [Talele et al., 2010] saquinavir, ritonavir, indinavir [Drie, 2007], and tirofiban [Hartman et al., 1992]. Recently, this method has become the pipeline of many pharmaceutical companies for drug discovery. This method, which is based on the principle of "Key and Lock" concept existing in textbooks, has been adapted as "Hand and Glove" to better explain protein ligand flexibility [Śledź and Caflisch, 2018].

Molecular Docking is the most common method to estimate the position of the molecule in the protein and its binding affinity. There is a wide variety of software (AutoDock vina [Trott and Olson, 2009], Glide [Friesner et al., 2006]), CCDC GOLD [Jones et al., 1997]) etc.) available for this method, which basically consists of 2 parts. These are search algorithms and scoring algorithms. The search algorithm is designed to find the best result by consuming the optimum power of the given molecule in the

relevant region. Scoring algorithms are based on testing the accuracy by scoring the created position. The basic search algorithms are Genetic algorithm, Exhaustive systematic search, Simulated annealing, Fragmentation algorithm as examples. Scoring algorithms are Semi-empirical calculation on free energy, Molecular force field, based on machine learning and quantum mechanics [Drie, 2007]. The time and success rates of these algorithms vary. Although most Molecular Docking programs are successful in finding the binding mode, their success in relative binding energy still raises doubts. Calculating the binding energy by defining the structure formed by the protein and ligand as rigid ignores the dynamic process of the complex. This leads us to another method, Molecular Dynamics (MD) simulations.

The first protein MD simulation was carried out in the late 1970s [McCammon et al., 1977], with work prioritized by Martin Karplus, Michael Levitt, and Arieh Warshel, who received the 2013 Nobel Prize in chemistry [Levitt and Lifson, 1969], [Lifson and Waeshel, 1968]. It has gained importance among experimental molecular biologists in recent years [Hollingsworth and Dror, 2018]. The underlying reasons for this are GPUs with increased processing power and MD simulation programs that can be used without requiring an expert. The idea of MD simulation is aimed at understanding the movement of a biological molecule in a cell. This system basically calculates the force exerted by atoms with known coordinates on other atoms, and the next position as a function of time can be calculated over Newton's laws of motion. By repeatedly calculating the forces on the atoms, one step in time is taken to find the atom's new velocity and position [Larsson et al., 2011]. The result is a movie of the simulated system. These simulations give us great power to understand the biological system. With these simulations, effects such as the effect of phosphorylation, the effect of mutagenesis or the conformational change of the protein in the presence of the ligand are observed. Molecular Dynamics uses Newton's laws (classical mechanics) to study the behaviour of molecules over time. Quantum Mechanics (QM) is valid for atomistic universe, but simulating a macromolecule such as a protein with QM is close to impossible.

Estimating accurate protein-ligand binding affinities *in silico* has been one of the major aims of structure-based drug design [Cournia et al., 2017]. Although the basic principles of ligand binding are relatively well understood, scoring is based on simplified approaches [Shoichet et al., 1999]. Some of the most frequently used methods such as Free Energy Perturbation (FEP) [Zwanzig, 1954], Bennett

Acceptance Ratio (BAR) [Bennett, 1976], Molecular Mechanics-Generalized Born Surface Area (MM-GBSA) and Molecular Mechanics-Poisson-Boltzmann Surface Area (MM-PBSA) [Srinivasan et al., 1998], are relatively successful approaches to estimating this energy. These calculations are made at the molecular mechanical level with electrostatic (coulombic) and Lennard-Jones potentials. Therefore, the electron potentials attributed to atoms have a very important. These binding energies are also estimated by machine learning based systems [Gu et al., 2020], [Kim et al., 2021], [Nguyen et al., 2019]. The systems are trained with experimental values and give interaction energy. Overall, these approaches contribute to the ongoing efforts in structural-based drug design, aiming to enhance our understanding of protein-ligand interactions and facilitate the development of novel therapeutic strategies.



3. MATERIALS

3.1. Buffers and Solutions

Table 3.1: List of solutions and chemicals.

10X SDS Running Buffer	250mM Tris-Base (pH:8.3), 1.9M Glycine, 1% SDS
10X TBS	20mM Tris-Cl(pH:7.6), 1.5M NaCl
1X Transfer Buffer	10% SDS Running Buffer, 20% Methanol
1X TBS-T	1X TBS, 0.1% Tween-20
5% Blocking Solution	5% non-fat dry milk in 1X TST-T
1X PBS	137mM NaCl, 2.7mM KCl, 4.3mM Na ₂ HPO ₄
4X Protein Loading Dye	250mM Tris-Cl (pH: 6.8), %8 SDS, 40% Glycerol, 20% β-mercaptoethanol, 0.008% Bromophenol Blue
Cell Lysis Buffer RIPA	750mM NaCl, 250mM Tris(pH: 8.0), 0.01% SDS, 1% NP-40
Cell Freezing Solution	10%FBS, 10%DMSO, 80% Growth Media
Protease Inhibitor Cocktail Tablets	S8830-2TAB (Sigma- Aldrich, USA)
Protein Molecular Weight Marker	S24052,(iNtRON Biotechnology, Korea)
Chemiluminescent HRP Substrate	K-12043-D10 (Advansta, USA)

Table 3.2: The SDS-Page preparation.

Separating Gel				Stacking Gel	
Materials (10ml)	8%	10%	12%	Materials (4ml)	5%
ddH ₂ O	5.3 ml	4.8 ml	4.3 ml	ddH ₂ O	2.92 ml
40% Acryl:Bis	2 ml	2.5 ml	3 ml	40% Acryl:Bis	0.5 ml
1.5 M Tris pH 8.8	2.5 ml	2.5 ml	2.5 ml	1.5 M Tris pH 6.8	0.5 ml
10% SDS	0.1 ml	0.1 ml	0.1 ml	10% SDS	0.04 ml
10% APS	0.1 ml	0.1 ml	0.1 ml	10% APS	0.04 ml
TEMED	0.006 ml	0.004 ml	0.004 ml	TEMED	0.004 ml

Table 3.3: List of western antibodies.

Name	Dilution	Source, Catalog No
Anti-CRY1	1:2000	Bethyl Laboratories, #A302-6164A
Anti- β -Actin	1:5000	CST, #4967
Anti-rabbit-HRP	1:5000	Thermo Fisher Scientific, #31460

Table 3.4: List of equipment.

Centrifuges	521-1647 refrigerated, (Micro Star 17R, VWR, USA)
	521-1646 (Micro Star 17, VWR, USA)
	521-2844 (Mini Star, VWR, USA)
	NF400 (Nuve, 02-1827)
Chemiluminescence Imaging System	ChemiDoc™ XRS+ System, (Bio-Rad, USA)
Incubator (CO ₂)	EC 160, (Nuve/TR)
Power Supply	(Bio-Rad, USA)
Transblot Transfer System	1704155, (Bio-Rad, USA)
Vortex	Scientific Industries, USA
Inverted Microscope	Nikon-Eclipse Series-E100

4. METHODS

4.1. Protein Structure Preparation

The co-crystallized structures of mouse CRY1 (mCRY1) and human PER2 (hPer2) proteins (PDB ID: 6OF7) were retrieved from the RCSB Protein Data Bank. A human CRY1 (hCRY1) structure with 93% similarity to mCRY1 was created using SwissProt homology modelling on the template 4k0r.1.A. The hCRY1 structure was superimposed onto the mCRY1 structure in the 6OF7 PDB and the mCRY1 structure was extracted. Molecular dynamics (MD) simulation was then performed and, after 100 nanoseconds (ns) of simulation, the final frame was extracted.

4.2. Molecular Docking Simulations

The ZINC15 database with approximately 700 million non-identified molecules was used to prepare the ligand library. Molecules were filtered according to the following criteria to eliminate non-relevant molecules: molecules should have no charge (total charge = 0), less than 350 Da more than 300 Da molecular weight, $\log P < 3.5$, in-stocks, and pH=mid. Finally, these criteria eventually retrieved approximately 2.3 million small molecules from the ZINC15 database as pdbqt format and these molecules were docked to CRY1-PER2 binding pockets by using the Autodock Vina software. The target site center was determined as between Interaction with TIMELESS on CRY1 region and 1155-1173 Helix on PER2 (center = Leu1158 N1 atom). The cubic box site was determined as X=30 Å Y=30 Å Z=30 Å. The exhaustiveness value was used as 8. In the results obtained, the molecules were selected for *in silico* ADME using Equation 4.1 analysis.

$$\frac{\text{CRY1PER2 binding pocket docking score}}{\text{CRY1 active pocket docking score}} > 1 \quad (4.1)$$

4.3. *In Silico* ADME

SwissADME is an online tool that proposes the physicochemical properties, and pharmacokinetic behavior, such as Absorption, Distribution, Metabolism, and Excretion, of molecules when presented in SMILES format. After molecular docking, molecules were converted to SMILES format using the openbabel tool [O'Boyle et al., 2011]. Molecules in the obtained SMILES notation were loaded into the SwissADME tool and the results were obtained in CSV (comma-separated values) format. While examining the pharmacokinetic properties of the molecules, a selection was made for the lack of a substrate for CYP450 enzymes and high absorption into the gastrointestinal system.

4.4. Molecular Dynamics Simulations and Binding Energy Calculations

Molecular dynamics (MD) simulations were conducted using the Gromacs 2018 software and the all-atom Amber ff99SB-ILDN force field for the receptors and GAFF for the ligands. The chosen docking results for the protein were generated using the Amber ff99SB-ILDN force field. The GAFF parameters for the ligands were generated using a custom script (github.com/mskoca96/mdutils) that performs density functional theory (DFT) calculations at the HF/6-31G* level using Gaussian16 to calculate the Merz Kollman electrostatic potential (ESP). The Restrained electrostatic potential (RESP) charges were then calculated using the antechamber and amber2gmx modules in the AmberTools21 software. The system was placed in the center of a cubic box and solvated with TIP3P water using periodic boundary conditions, with a margin distance of 20 Å between each periodic image. The system was neutralized with a 0.15 M NaCl concentration and energy was minimized using the steepest descent integrator until the maximum force reached 100 kJ/mol/nm. The NVT ensemble was then run at 310 K for 1 ns, followed by equilibration in 2 steps of the NPT ensemble: 200 ps using the Berendsen pressure coupling algorithm and 1 ns using the Parrinello-Rahman algorithm. Finally, the NPT ensemble MD simulations were run for 100 ns using the Parrinello-Rahman algorithm.

MD simulations, MM-GBSA, and ANI_LIE end-state free energy calculation methods were used on the molecules. The CRY1-PER2 interaction energy and

molecule-protein complex interaction energy were calculated for both algorithms using 1000 frames from each MD simulation. The gmx_MMPBSA script was used to calculate the MM-GBSA binding energy, using igb=2 and saltcon=0.15 in the sample input file. For ANI_LIE free energy, the deepQM (<https://github.com/otayfuroglu/deepQM>) script was used, running the bash script run_deepQM.sh included in the deepQM package without optimization and using the sp_grouped_multi_mol option and ANI2x neural network potential. Grimme DFT-D3 dispersion correction was not used while calculating the ANI_LIE binding energy. 1000 frames were generated from each MD simulation using the gmx trjconv function.

4.5. MTT Toxicity Assay

For the cytotoxicity assay, human osteosarcoma U2OS cell lines were used. The cells were cultured and passaged at 37°C under 5% CO₂ in 1X medium composed of filtered DMEM, 10% FBS, 100 µg/ml streptomycin, 100 µg/ml penicillin, and 2 mM L-Glutamine. The cells were seeded in triplicate into clear 96-well plates at a density of 4000 cells/well and allowed to grow for 48 hours. After that, the cells were treated with molecules at 10, 20, and 40 µM concentrations in DMEM, with a final DMSO concentration of 0.5%, and then incubated for another 48 hours. To measure cell viability, tetrazolium dye (MTT) was added to the cells and incubated for 3 hours, which was converted to an insoluble purple formazan as a result of mitochondrial activity. Before adding MTT reagent, the medium was aspirated, and the cells were washed once with 1X PBS. Then, the purple formazan crystals were solubilized with DMSO, and the absorbance of the wells was measured at 570 nm by the spectrophotometer. Cells treated with 5% final DMSO concentration, known to be toxic to cells, were used as negative controls.

4.6. Real-Time Bioluminescence Monitoring

This assay was performed Synergy H1 multi-mode plate reader (BioTek) with 96- well opaque plate. 5×10^4 U2OS Bmal1-dLuc cells were seeded on 96-well plate and cultured overnight. Next day, cells were reset by 100 nanomolar Dexamethasone for 2 hours, and then washed with 1X PBS. Homemade bioluminescence recording

medium was prepared by adding 0.1 mM luciferin (Promega cat no: E160C) into the recording medium, which contained low glucose DMEM powder (with L-glutamine) supplemented with 10 mM HEPES (Gibco, cat no: 15630-080), 0.35 mg/ml of sodium bicarbonate, 3.5 mg/ml of d-(+)-glucose powder, 5% fetal bovine serum, 0.1 mM NEAA, 100 units/ml of penicillin and 100 mg/ml of streptomycin. The bioluminescence recording medium was added as 200 μ l per well. Molecules were added at desired doses (maximum 0.04% final concentration). The plate was sealed and placed in the prewarmed Synergy H1. Luminescence values were recorded at 32°C for every 24 minutes with 15 seconds integration time for 103 hours.

For the detection of molecules on luciferase, the reverse transfection was conducted as follows: A total of 300 ng of plasmid 4 ng of Luc plasmid was mixed with 296 ng of empty Sport6 plasmids in serum-free DMEM. Subsequently, 0.6 μ l of PEI per well was added to the mixture and incubated for 20 minutes. The volume of the DMEM, DNA, and PEI mixture was adjusted to 50 μ l per well. During this 20-minute incubation period, HEK293T cells, seeded the day before the transfection and reaching 70-80% confluency, were trypsinized and resuspended in 2X medium (filtered DMEM, 20% FBS, 200 μ g/ml streptomycin, 200 μ g/ml penicillin, and 4 mM L-Glutamine). The total volume of the cell suspension was adjusted to 800×10^3 cells/ml. Then, 50 μ l of cells (40,000 cells/well) were added to each well of the 96-well plate, and at the end of the 20-minute incubation, the cells were mixed with 50 μ l of the DNA mixture. After 24 hours of transfection, the cells were treated with either molecules at 20 μ M or solvent (0.5% DMSO). Following 24 hours of post-molecule treatment, the cells were treated with luciferin (final concentration 0.4 mM) and HEPES (final concentration 10 mM and pH=7.2). After 2 hours, cycloheximide (final concentration 20 μ g/ml) was added to the wells to halt protein synthesis. The plate was sealed with optically clear film and placed into the Synergy H1 multi-mode plate reader. Luminescence readings were recorded every 10 minutes at 32°C for 48 hours. The half-life of the protein was calculated using the one-phase exponential decay fitting function in GraphPad Prism 5 software.

4.7. RIPA Lysis and Bicinchoninic Protein Assay

To prevent protein degradation, all tubes containing cells were maintained on ice or in a refrigerated centrifuge during the steps. The cells were seeded into 6-well plates at a density of 4×10^5 cells/well. After 24 hours, complete DMEM was aspirated and selected compounds were added the concentrations of 10 and 20 μM in the complete DMEM. Cells were harvested from 6-well plates after 48 hours. To collect the cells, a cell scraper was utilized to detach them from the surface of the 6-well plates, after which they were carefully transferred to a centrifuge tube for further analysis. The tubes were centrifuged for 4 minutes at 5000 rpm. After discarding the supernatant, 200-250 μl of 1X PBS was added to each tube and centrifuged under the same conditions. The resulting supernatant was then discarded, and the cells were washed by adding 100-150 μl of 1X PBS and centrifuging again. The supernatant was carefully removed with a micropipette, and the tubes containing only the cell pellets were centrifuged again. To obtain a pure cell mass, the remaining liquid at the bottom of the tubes was aspirated, leaving only the cell pellet.

To prepare 1 ml 1.5X RIPA buffer, 5 μl 100X Protease inhibitor, 650 μl double distilled water, and 300 μl 5X RIPA buffer are mixed. Based on the intensity of the cell pellet (50-70 μl), 1.5X RIPA buffer was added to each tube, and the mixture was incubated on ice for 20 minutes with brief vortexing every 5 minutes. The mixture was centrifuged at 13300 rpm for 20 minutes at 4°C , and the resulting supernatant was carefully transferred to a new tube to obtain the cell lysate.

To determine the protein concentration, the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, cat no: 23225) was used. A and B solutions from the kit were mixed at a 49:1 ratio, and 5 μl of each sample and RIPA buffer (blank) were transferred to a 96-well plate. 200 μl of BCA mix was added and briefly mixed, and the plate was incubated at 37°C for 30 minutes. After incubation, any bubbles on the surface of the wells were carefully removed with a needle, and colorimetric measurement was performed using a Varioskan microplate reader (Thermo Fisher Scientific, USA) at 562 nm. Each well was loaded with a total of 10 μg protein from the sample extracts and topped up with 1.5X RIPA buffer. Finally, a 4X Laemmli buffer containing 10% β -mercaptoethanol was added to each sample to achieve a final 1X concentration, along with loading dye. The protein samples were loaded onto an

8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel to assess CRY1 intensity.

4.8. Western Blotting

SDS-PAGE glass plates were inserted into a gel cassette and mounted on the electrophoresis apparatus. The mix for a 8% gel was prepared in a conical centrifuge tube following the recipe described in Table 3.2. First, the separating gel was poured into the cassette and isopropanol was added. After the acrylamide in the gel was polymerized with APS and TEMED, the gel was washed with dH₂O, and then the stacking gel was poured on top. Finally, the comb was quickly inserted into the gel to create wells for sample loading. After the stacking gel is polymerized, the gel cassette is removed from the apparatus and the comb is carefully taken out. The samples are boiled for 10 minutes in a heating block set at 95 °C. Next, the gel cassette is placed into the running cassette and the assembly is inserted into the running tank filled with 1X running buffer. The power source is connected to the tank and the device is turned on at 80V before sample loading. After the boiling process is complete, the samples and the protein marker (Gangnam Stain Protein Ladder (#24052)), are loaded into the wells with equal volumes, and the device is set to 80V. Once the proteins have passed through the border between the stacking and separating gel, the device's power is increased to 120V. The device is stopped once the proteins have run sufficiently for separation, which can be observed by the marker, and the gel is carefully removed from the cassette.

The next step involves transferring the proteins from the gel onto a nitrocellulose membrane, a process known as western blotting. Prior to the transfer, Whatman papers and nitrocellulose membranes were incubated in 1X transfer buffer for 10 minutes. Semi-dry western blot was performed using the Trans-Blot Turbo Transfer System (Serial No: 690BR011760, Biorad). The gel was carefully removed from the cassette and placed into the transfer buffer. A transfer cassette was assembled by placing two Whatman papers at the bottom, followed by the nitrocellulose membrane, the gel, and another two Whatman papers on top in a sandwich configuration. The cassette was then inserted into the transfer device, and the settings were adjusted to 12V and 60 minutes for the transfer.

After the transfer of the proteins from the gel to the nitrocellulose membrane, the membrane was incubated in a blocking solution to prevent non-specific binding of the primary antibody. In this study, a blocking solution was prepared using 5% skim milk dissolved in 20 ml of TBS-T buffer (TBS buffer with 0.1% Tween-20). The membrane was placed in the blocking solution and incubated for 1 hour on a shaker at room temperature. Subsequently, the membrane was cut into pieces if multiple antibody treatments were necessary and placed into a falcon tube containing the primary antibody diluted in TBS-T buffer supplemented with 1% sodium azide. The primary antibodies used in this study were, anti- β -Actin and anti-Cry1. The falcon tube was then placed on a shaker at 4°C overnight to allow the primary antibody to bind specifically to the protein of interest.

On the following day, the nitrocellulose membrane was washed with 1X TBS-T for a series of 5, 15, 5, and 5 minutes to remove any unbound primary antibody. Subsequently, the membrane was treated with a secondary antibody that binds specifically to the primary antibody used in the previous step. The choice of the secondary antibody (rabbit or mouse) depends on the origin of the primary antibody. After incubating the membrane with the secondary antibody for 1 hour at room temperature, the membrane was washed again with 1X TBS-T in the same manner as described earlier.

To visualize the protein bands, a Chemidoc™ XRS+ system with ImageLab™ Software (#1708265, Biorad) was used. A chemiluminescence agent, WesternBright Sirius HRP substrate (Advansta Inc., CA, USA), was used to generate the chemiluminescence signal from the protein of interest. The A and B solutions of Advansta were mixed in a 1:1 ratio, and 250 μ l of this mixture was used per membrane. The membrane was placed on a black paper and dried before adding the Advansta mixture. The membrane was then incubated for 3 minutes to allow the horseradish peroxidase (HRP) substrate to bind to the secondary antibody and generate the chemiluminescence signal. Finally, the signal was detected by the Chemidoc™ XRS+ system and the images were captured using ImageLab™ Software.

4.9. Statistical Analysis

The results were reported as means $\pm$ standard error of the means (SEM) for each variable studied. GraphPad Prism for Windows (GraphPad Software, California, USA) was employed for statistical analyses. To evaluate the significance of differences between groups, either Student's t-test or one-way analysis of variance (ANOVA) was used, followed by Tukey post hoc tests. The type of significant test used in each experiment and their corresponding p-values were clearly indicated in the figure legends.



5. RESULTS

5.1. In Silico Drug Design

The target CRY1-PER2 (PDB ID: 6OF7) crystal structure was obtained from the RSCB database. The human CRY1 protein was created using the SWISS-MODEL instead of mouse CRY1 in the crystal structure then superimposed with PyMOL (github.com/schrodinger/pymol-open-source). To determine the conformation of the cell in its physiological condition, firstly, the structure was performed to energy minimization in 0.15 M NaCl water solution, followed by 1 ns, 101 ns simulation in NVT and NPT ensembles at 310 K, respectively. As a result of the Molecular Dynamics (MD) simulation, it was observed that the root mean square deviation (RMSD) of the C α of the structure was converged (Figure 5.1). The last frame of the simulation was chosen to perform Molecular Docking.

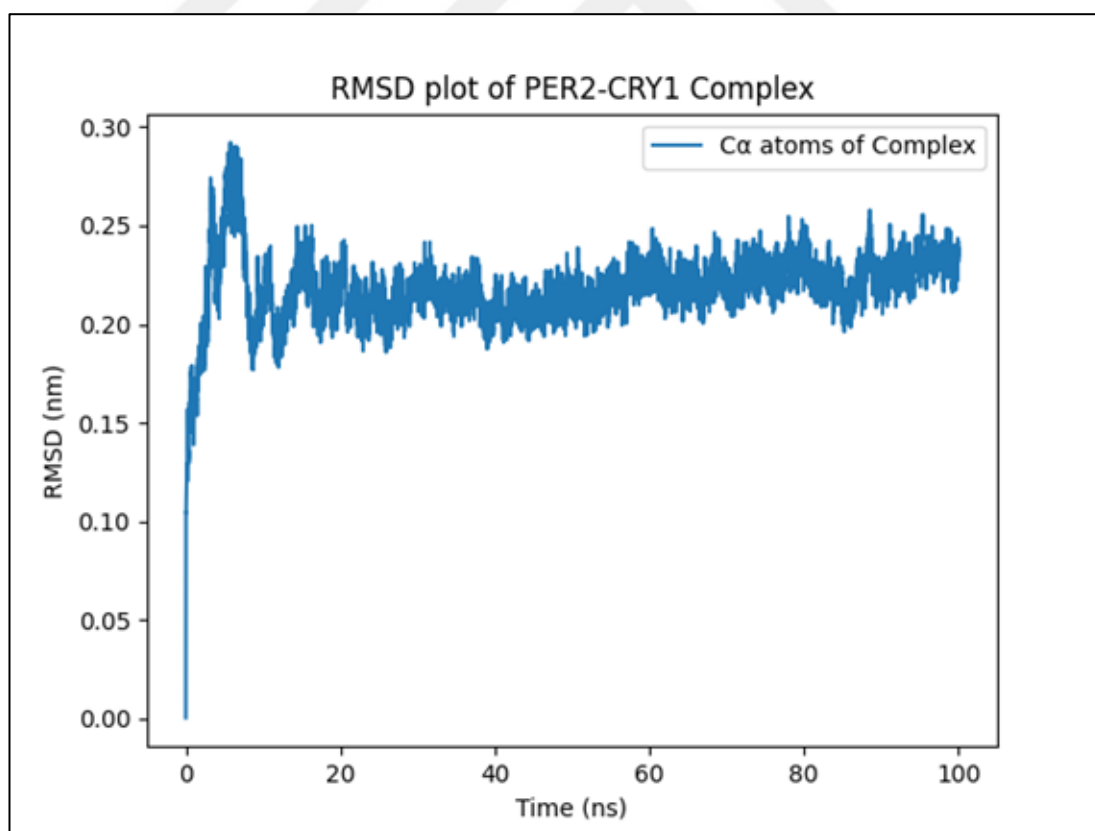


Figure 5.1: RMSD values of CRY1-PER2 complex MD simulation (100ns).

We targeted the region where CRY1 interacts with PER2 as the first pocket. As for the second pocket, the FAD binding region of CRY1 was targeted considering the possibility of performing non-pocket connections (Figure 2.3 and Figure 5.2).

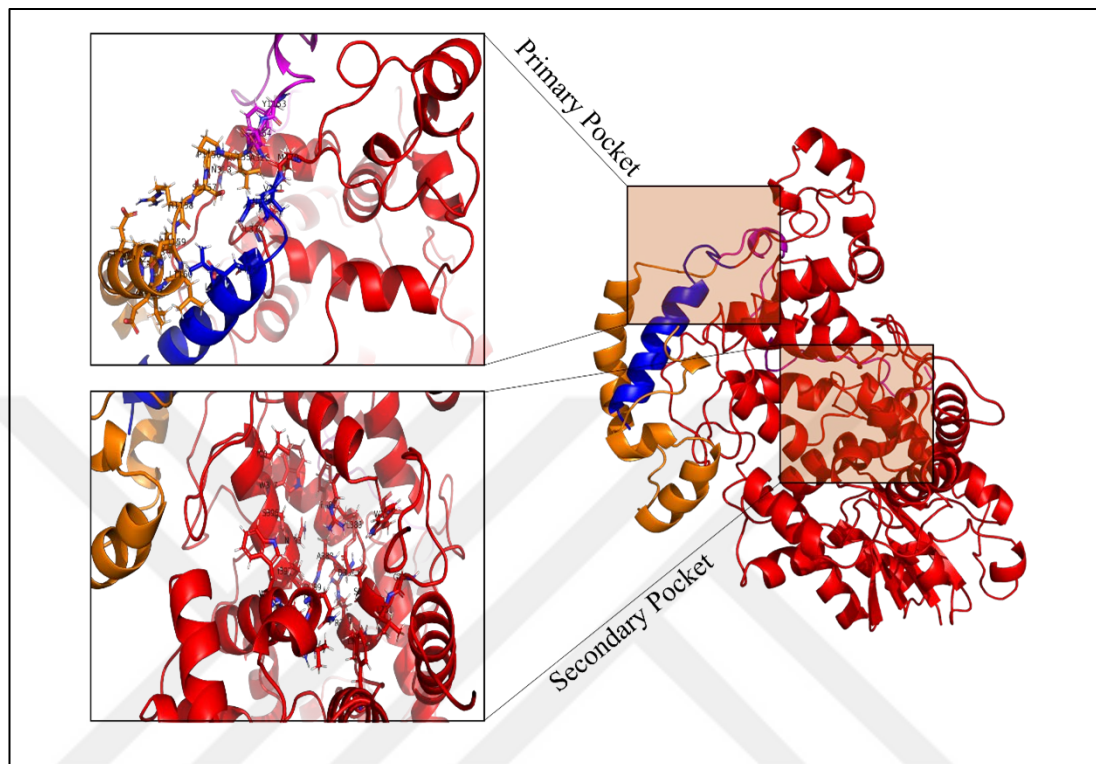


Figure 5.2: CRY1-PER2 complex and selected docking grids. (CRY1 is shown as red, PER2 as magenta. The Timeless interaction domain on CRY1 is shown in Blue, and the CRY1 interaction domain on PER2 is shown in orange.)

The ZINC15 database with approximately 1 billion molecules was used for the small molecules library. Ligand elimination criteria, 3-dimensional structure and stock molecules were determined as neutral, pH medium, $300 \text{ D} < \text{molecular weight} < 350 \text{ D}$, and log P value less than 3.5. After the criteria, ~2 million molecules obtained were docked to the primary pocket of CRY1-PER2 (Figure 5.2) with the Autodock vina software. After molecular docking, Autodock vina binding scores were ranked by using the cut-off value of -9 kcal/mol. The resulting molecules were also docked for the FAD-binding region. Using the Equation 4.1, the pharmacokinetic properties of the outstanding molecules were examined using SwissADME. Among the molecules that will not be substrate to CYP450 enzymes and will be well absorbed by the gastrointestinal system, 90 molecules with the highest docking scores were selected

for Molecular Dynamics (MD) simulation. The designed molecules were called the P series and were numbered from 1-90 such as P01, P45, P09.

MD simulations of 90 selected molecules were performed using Gromacs 2018 version. Docking poses obtained by Autodock vina were combined with the CRY1-PER2 complex. While using the all-atom Amber ff99SB-ILDN force field for the CRY1-PER2 complex, GAFF parameterization was performed for the ligands. Also, the ligand-free CRY1-PER2 complex was simulated. Energy minimization was performed in all simulations, and it was observed that the simulation started from the right place. It was observed that the volume and pressure reached a plateau in the 1 ns NVT ensemble and the 2-step NPT ensemble, respectively. Simulation was performed in the NPT ensemble for 100 ns. As a result of simulation, ligands, and receptor RMSDs were analyzed with gmx rms function (Figure 5.3 and Figure 5.4). It was observed that the interaction of the ligands with the complex was not broken.

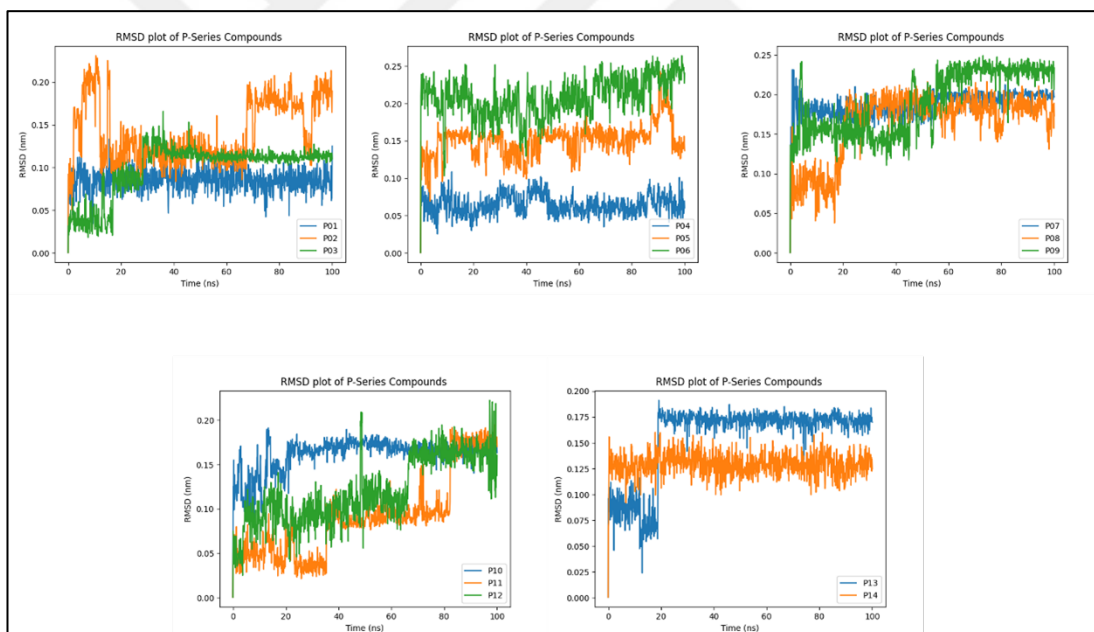


Figure 5.3: RMSD values for P-series molecules whole atoms in CRY1-PER2 complex.

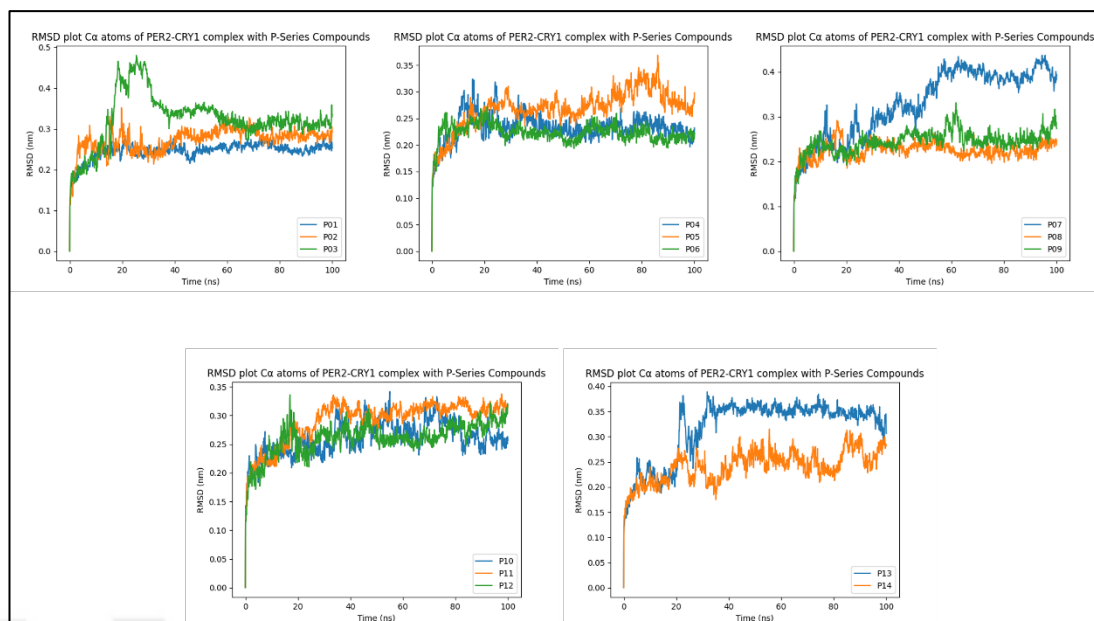


Figure 5.4: RMSD values of C $_{\alpha}$ of CRY1-PER2 complex with P-series molecules.

MM-GBSA and ANI_LIE methods were used to calculate the stabilization effects and binding energies of molecules targeting CRY1 and PER2 protein interactions on the complex (Table 5.1). The energy between ligand and receptor and the interaction between CRY1-PER2 were calculated separately. The interaction energies of CRY1 and PER2 in the simulation of the ligand-free complex were used as the cut-off value, and the variation of the interactions of these 2 proteins in the presence of ligand was investigated. Although ANI_LIE tested for ligand binding energy, there are no studies for proteins. This study also shows that the correlation between ANI_LIE and MM-GBSA can be used for ANI_LIE binding energy for proteins (Figure 5.5). Considering the binding energies of ligands and CRY1-PER2 interaction energies, 14 molecules (P1-P14) were purchased.

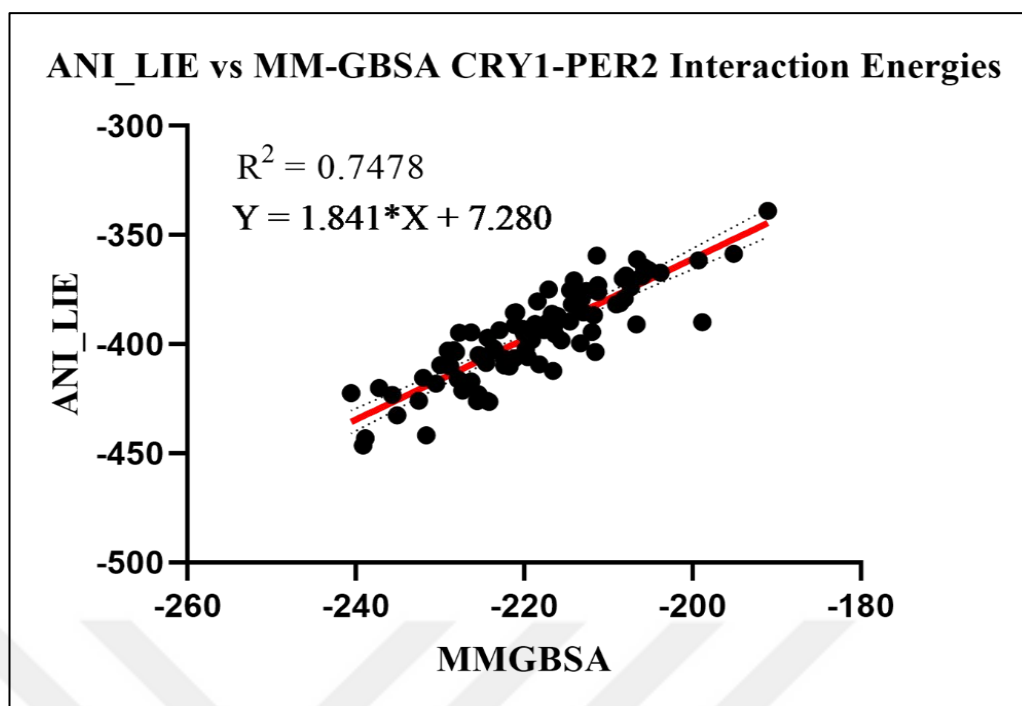


Figure 5.5: Correlation of ANI_LIE and MM-GBSA relative free energies of CRY1 and PER2 proteins in the presence of P-series molecule.

Table 5.1: ANI_LIE and MM-GBSA energies calculated as a result of 100ns MD simulation of selected P-series molecules.

Comp ounds	MMGBSA		ANI_LIE	
	Ligand Interaction Energy	CRY1- PER2 Interaction Energy	Ligand Interaction Energy	CRY1- PER2 Interaction Energy
P01	-35.50	-225.46	-40.10	-422.87
P02	-36.12	-226.30	-43.44	-394.63
P03	-24.77	-199.28	-35.46	-361.64
P04	-28.07	-214.06	-31.36	-370.72
P05	-34.77	-208.24	-37.18	-369.88
P06	-36.50	-227.69	-20.11	-394.83
P07	-28.78	-195.11	-37.70	-358.55
P08	-31.16	-228.77	-44.81	-410.20
P09	-29.72	-232.49	-44.88	-425.89
P10	-29.86	-217.10	-36.08	-374.94
P11	-30.00	-216.57	-48.75	-412.32
P12	-33.29	-211.93	-42.00	-394.34
P13	-29.07	-211.53	-55.89	-403.66
P14	-34.90	-205.20	-49.23	-366.08
Contr ol	N/A	-203.51 ± 2.34	N/A	-374.65 ± 7.87

5.2. Determining the Toxic Dose of Selected Molecules

Initially, the non-toxic dose of the P-series molecules was found by performing the toxicity assay. We used the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) test for this purpose. MTT is a colorimetric assay that determines the survival rate of cells based on mitochondrial activity. We started our toxicity test with at 40 μ M concentration of P-series. Since the U2OS cell line will be used in further studies, it was tested in this cell line. Cells were allowed to grow for 48 hours after seeding and MTT reagent was added 48 hours after treatment to determine relative viability by spectrophotometer. 5% DMSO, known to be toxic, was used as negative control. A control of 0.05% DMSO was also tested because all molecules were dissolved in DMSO, and this dose is just above of 0.04% DMSO concentration in molecule-treated wells. All molecules were observed to be non-toxic at 40 μ M concentration (Figure 5.6).

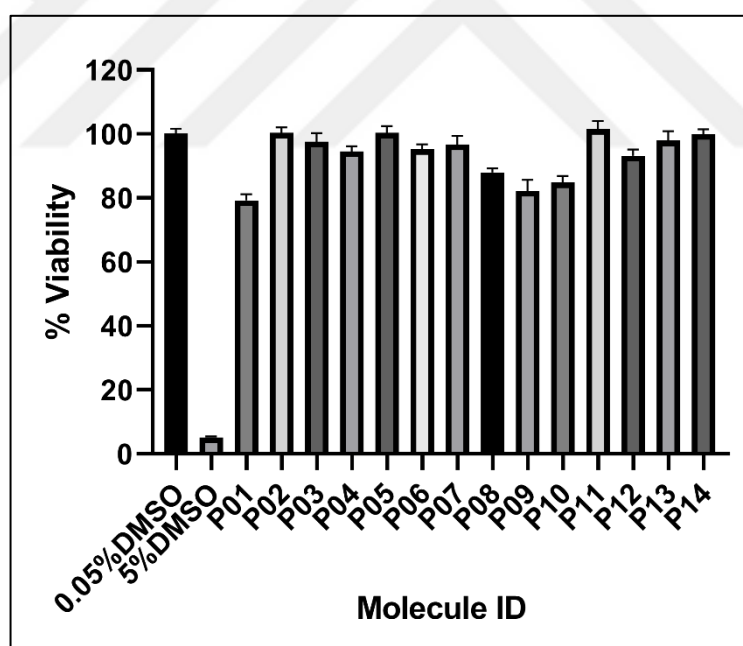


Figure 5.6: Cell viability effect of molecules at 40 μ M concentration. (Data represent the mean $\pm$ SEM n=3).

5.3. Effect of Non-Toxic Molecules on the Circadian Rhythm

Many agents that affect the stability or activity of the clock proteins also alter the circadian rhythm. Therefore, to examine the effect of P-series molecules on circadian rhythm, we tested on the U2OS cell line that stably expressing Bmal1-*dLuc*. The REV-ERBs and RORs proteins, whose expression is controlled by the core clock proteins, bind to the promoter region of BMAL1 and luciferase, thus producing a rhythmic circadian output which monitoring by luminometer device. With luminometric readings, circadian rhythm related data such as period, amplitude and phase are calculated. We tested P-series molecules initially at 20 μ M using a 96-well plate with the circadian reporter assay. Readings were performed every 24 minutes and 27 seconds for 5 days. Period and amplitude calculations were performed using Biodare2 (biodare2.ed.ac.uk) web tool and Chronostar [Maier et al., 2021].

With Biodare2 FFT-NLS analysis, it was observed that P01, P02, P03, P10, P11, and P13 molecules extend the period, while P01, P06, P09, P11, and P13 molecules enhance the amplitude (Figure 5.7). When analyzed with the Chronostar tool, it was observed that P01, P03, P09, P10, P13, and P14 molecules extend the period, while P06 and P10 molecules enhance the amplitude (Figure 5.7 and Figure 5.8).

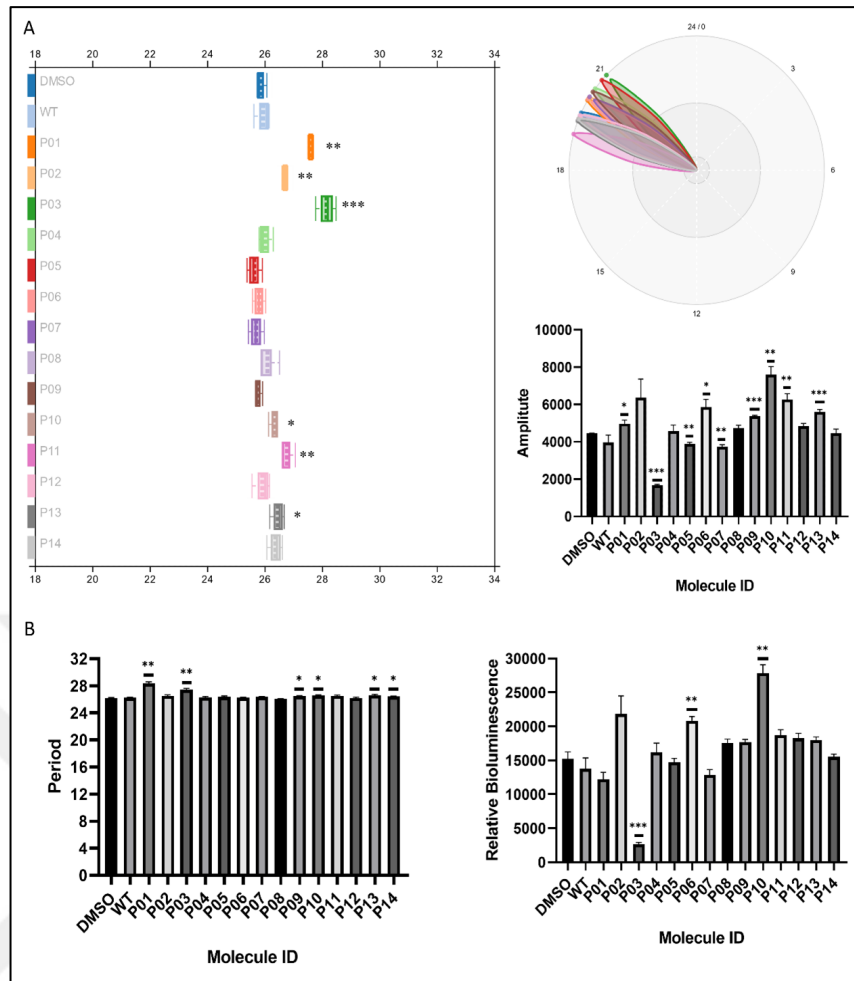


Figure 5.7: Effect of P-series molecules on circadian rhythm at 20 μ M. a) By using Biodare2 web tool, period, phase, and amplitude were calculated using Amp&Baseline as Detrending method and FFT NLLS as analysis method. b) Period, phase, and amplitude were calculated using the Chronostar tool. Significant changes were determined according to DMSO using unpaired t-test with Welch's correction (**** $p < 0.0001$ *** $p = 0.001$ ** $p < 0.01$, * $p < 0.05$, $n = 2$) for both tools.

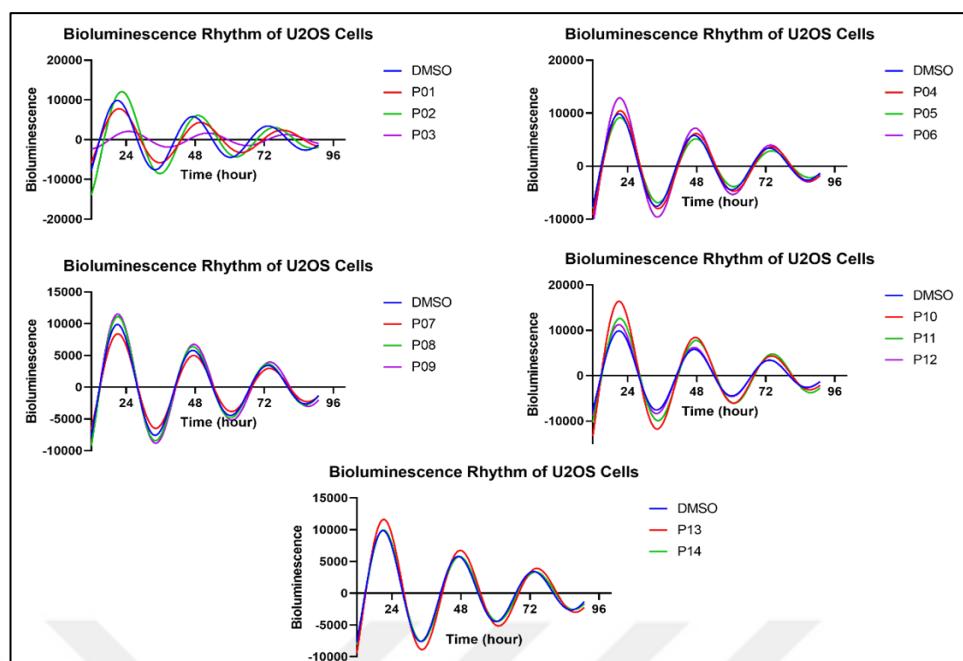


Figure 5.8: Bioluminescence rhythm of U2OS Bmal1-*dLuc* cells in the presence of molecules.

When analyzed dose-dependently, it was observed that all molecules except P09 enhanced the period in Biodare2 FFT-NLS analysis (Figure 5.9). The data analyzed with the Chronostar tool exhibited a similar profile in terms of the period. Regarding the amplitude, the P03 molecule showed a damped effect, while no significant effect was observed for the P01 molecule. On the other hand, the molecules P09, P10, and P11 were discovered to enhance the amplitude (Figure 5.10).

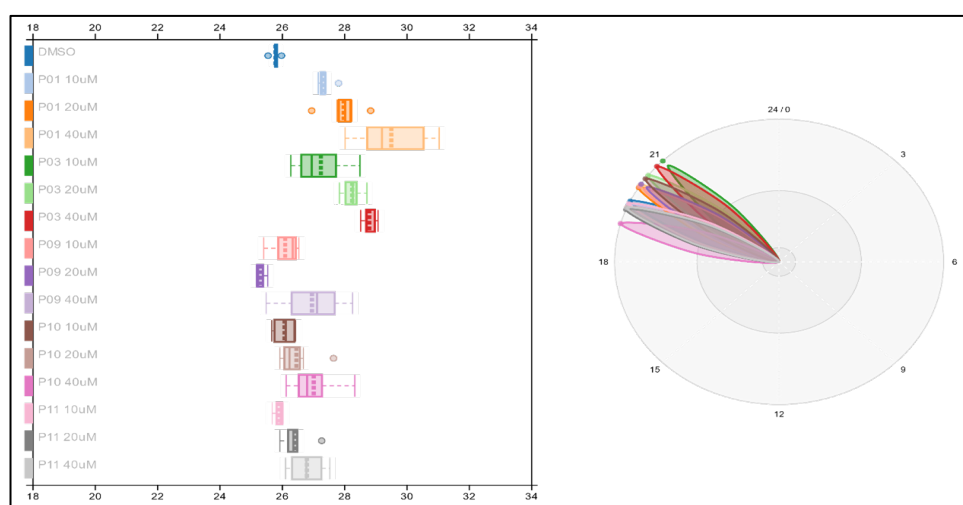


Figure 5.9: P-series dose dependent effect on the circadian rhythm of U2OS Bmal1-*dLuc* cells. Analysis was performed in Biodare2.

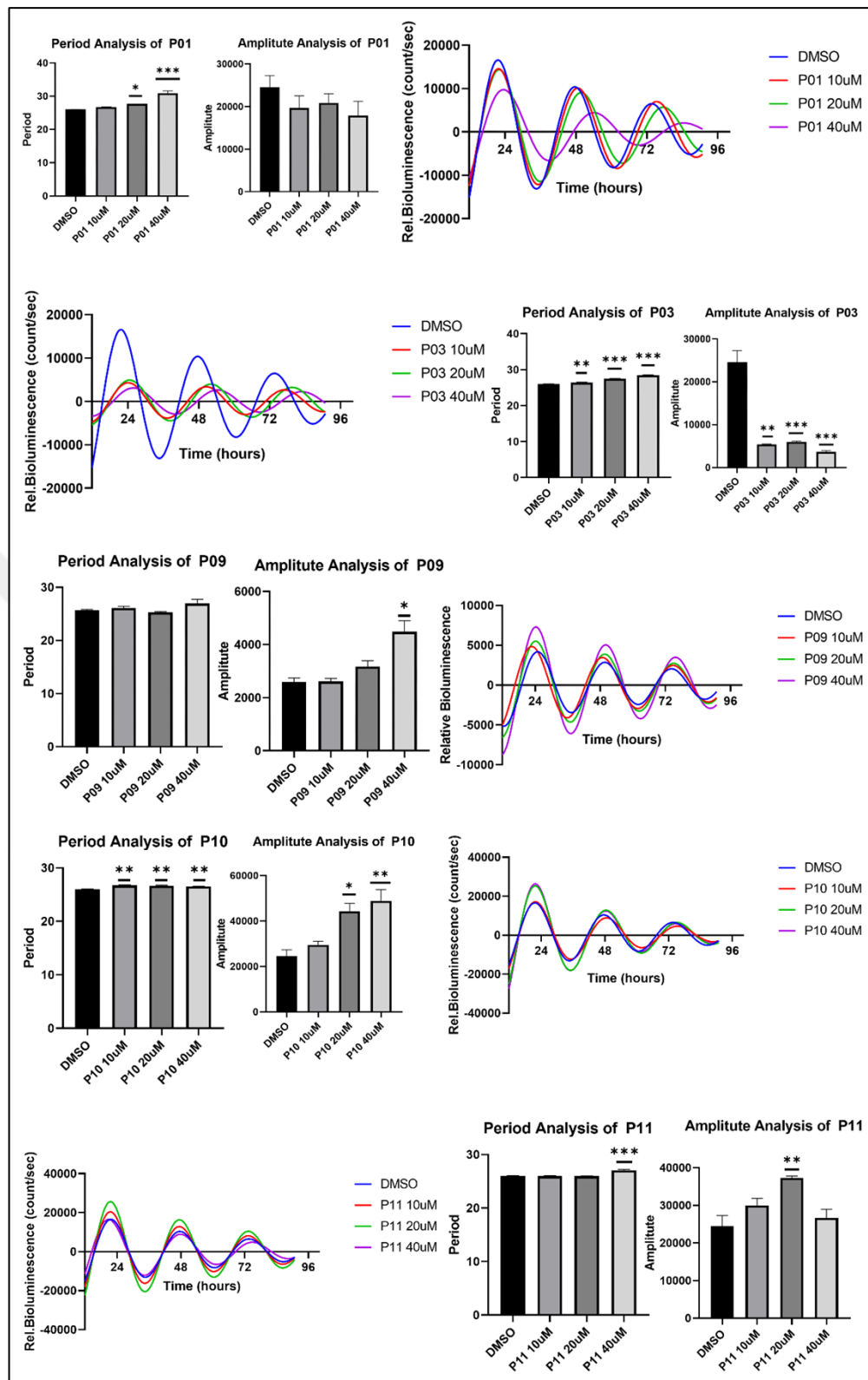


Figure 5.10: Effect of P-Series molecules as dose dependent on circadian rhythm in U2OS Bmal1-dLuc cells. Analysis was performed in Chronostar. (one-way ANOVA and Tukey post-hoc test was used for analysis. ***p = 0.001 **p < 0.01, *p < 0.05, n = 2)

Luciferase degradation assay results indicate a significant impact of the P03 molecule on the luciferase half-life. Additionally, it is possible that the P02 and P10 molecules also influence luciferase degradation (Figure 5.11).

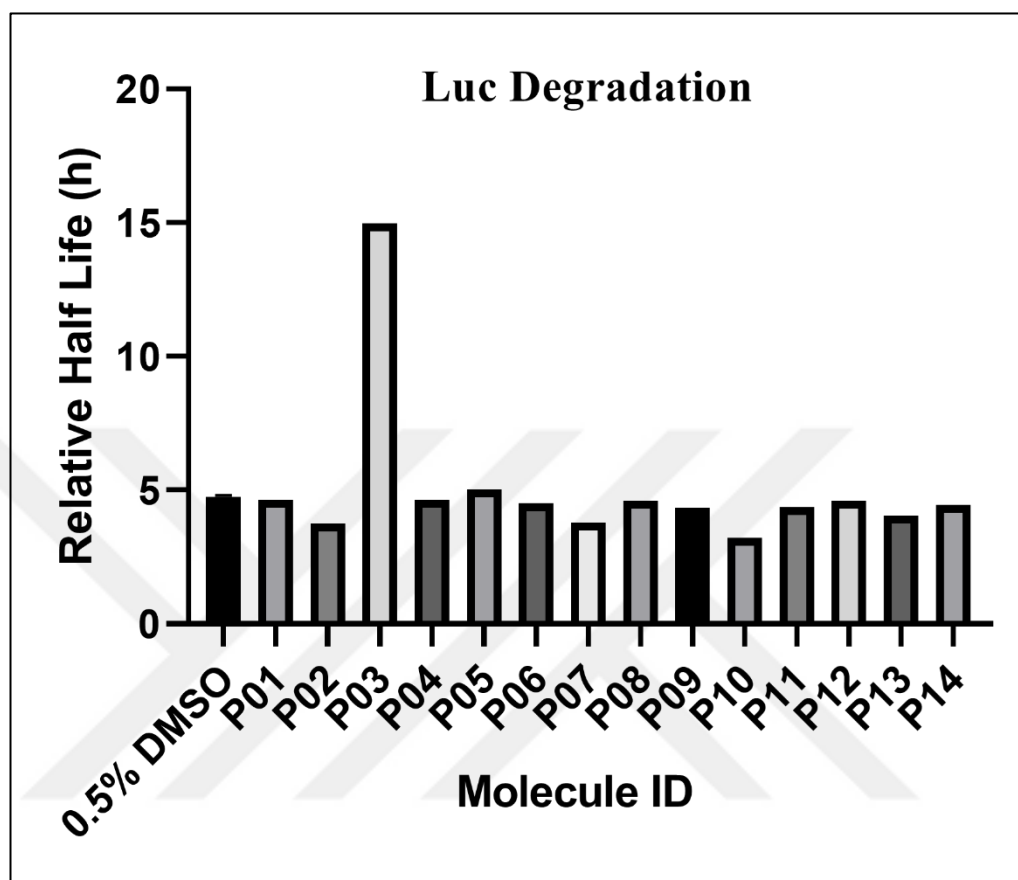


Figure 5.11: Effect of P-series molecules at 20 μ M concentration on the LUC degradation (n=1).

In this work, the molecule was extensively studied, and therefore the structure of only P09 was given in Appendices (Figure 8.1).

5.4. Western Blot Analysis of P09 Effect on CRY1

Initially, we looked at the effect of circadian rhythm regulating molecules on CRY1 by western blot. First, 4×10^5 U2OS cell/well were seeded in the 6-well. The next day, P-series molecules were added at 10 and 20 μ M (final DMSO 0.04%). After 48 hours, cells were harvested by scraping and then lysed with RIPA buffer. After BCA was made, it was loaded between 10-15 μ g per well.

Western blot results reveal that the P09 molecule accelerates the degradation of CRY1. The observation suggests that the P09 molecule may have a direct impact on CRY1 stability, leading to its faster degradation (Figure 5.12). Additionally, it is noteworthy that the P09 molecule forms strong hydrogen bonds with theoretically interacting residues, which could contribute to its regulatory effect on CRY1 degradation.

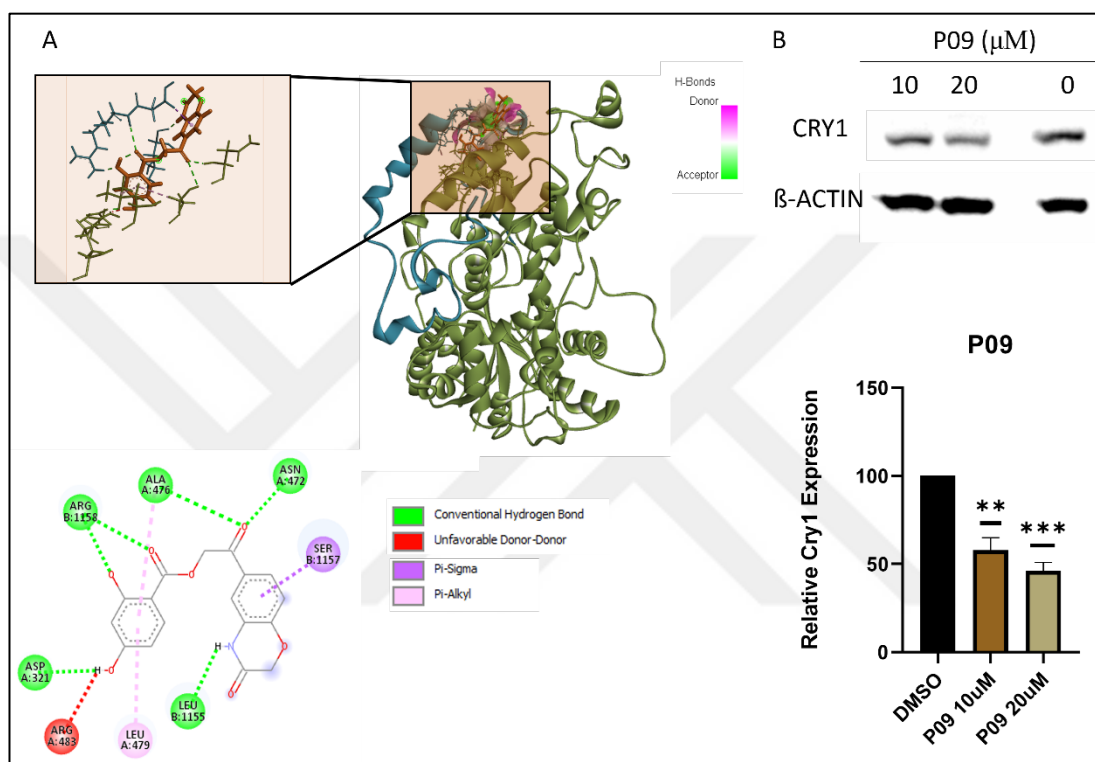


Figure 5.12: Effect of P09 molecule on CRY1. a) The pose and interaction diagram of the P09 molecule in the CRY1-PER2 complex at the end of 100ns MD simulation. b) Effect of P09 molecule on CRY1 level in non-synchronous U2OS cells (one-way ANOVA with host-doc Tukey, ***p = 0.001 **p < 0.01, n=3).

6. DISCUSSION

The biological clock is a system that has been conserved across taxa. Even 1 hour of change per year with Daylight Saving Time (DST) might result in shorter sleep time, increase in traffic accidents, increase in heart attacks in humans [Harrison, 2013], [Manfredini et al., 2018]. The cellular effects of the system, whose effects we see so highly in the physiological aspect, are being mostly revealed day by day. Therefore, small molecules that modulate the circadian rhythm might be therapeutic for many diseases. Molecules such as M47 [Gul et al., 2022], which destabilizes CRY1 by binding to the FAD binding domain, CLK8 [Doruk et al., 2020], which destabilizes the CLOCK/BMAL1 complex and increases its amplitude, and KL001 [Doruk et al., 2020], [Gul et al., 2022], which stabilizes CRY1, are promising for clinical use. P09 molecule may be among these molecules as an allosteric destabilizer of CRY1.

Our aim in our study was to allosterically target the CRY1 protein, preventing CRY1 from performing its functions only on TTFL. This will ensure direct circadian rhythm driven destabilization of CRY1 without affecting other functions in the cell. Targeting the CRY1/PER2 complex poses a particular challenge compared to other studies because only the crystal of the CRY1 interaction site of PER2 is insufficient to understand the behavior of the entire protein. To prevent this, a short MD simulation of 100ns was thought to be sufficient to start from the minimum energy structure and was performed before Molecular Docking.

Although many FDA-approved drugs have fallen outside of Pfizer's 5 rules in recent years, this was followed when preparing the ligand library. Due to the small size of the targeted pocket in the CRY1/PER2 complex and passage through the membrane independent of ATP, between 300-350 D and neutral molecules were preferred, respectively. According to the relevant criteria, ~2 million molecules were docked. Coincidentally, when a few molecules were docked into the FAD binding domain of CRY1, high affinity was shown for this pocket as well. Therefore, the number of molecules was reduced to ~5000 using the formula in equation 4.1.

Another challenge in drug design is pharmacodynamics which is to predict the modifications and effects that the molecule will undergo after entering the body. For this reason, we tried to understand this with *in silico* ADME, which has been used in recent years. Our priority was that it should not be modified by the Cytochrome P450

family. The xenobiotics that enter the body try to be metabolized by the liver at first. A few of the drugs on the market (e.g., Valacyclovir, Tenofovir alafenamide) are based on activating the inactive molecule (prodrug) using these modifications. Molecules that would not be inhibitors or substrates were selected using SwissADME. In addition, the molecule must have good absorption to reach the target. We also took this into account.

While Molecular Docking is very good at estimating the binding pose, it is a coarse method in binding affinity. Therefore, Molecular Dynamics simulation of the first 90 molecules was performed taking into computational cost. As a result of these simulations, the binding energy between ligand and protein was calculated by using the end-state free energy calculation methods MM-GBSA and ANI_LIE. In addition, the energy between the 2 proteins was calculated to understand the role of these molecules in the interaction of PER2 and CRY1. It was compared according to the binding energy in a ligand-free simulation. Although MM-GBSA is used for proteins, ANI_LIE was used for the first time to study protein interaction energy (Table 5.1 and Table 8.1). Interestingly, it has a ratio of $R^2 = 0.7478$ with MM-GBSA, suggesting that this method can be used with more detailed studies. In the MD simulation, all molecules retained their position in the target pocket. For the molecules in the complex, the molecules with the highest binding affinity were selected as priority, and those that stabilized and destabilized the complex were selected for both methods. 14 molecules were ordered from Ambinter company for *in vitro* studies.

The molecules were named P-series as they target the interaction of PER2 with CRY1 (e.g., P01, P02 etc.). Before proceeding with cell culture studies, it was necessary to establish the sub-toxic dosage. In the circadian rhythm-targeted studies in the literature [Doruk et al., 2020], [Gul et al., 2022], we looked at cell viability at a concentration of 40 μM since they mostly used 20 μM as the maximum dose. It was observed that none of the molecules had toxic properties by reducing the viability below the determined ~80% at 40 μM concentration.

The effects of molecules on the circadian rhythm were first scanned with a real-time bioluminescence assay at 20 μM concentration. The results obtained were analyzed with the Biodare2 and Chronostar tools. The results obtained were analyzed with the Biodare2 and Chronostar instruments. For Biodare2, data normalization was performed with the Amp&Baseline detrending method. The wave with the lowest error rate was selected using the FFT-NLS method to find the period. Averages amplitudes

were used for amplitude values. It was observed that P01 and P03 significantly extended the period more than 1 hour (Figure 5.7). It was observed that P02, P10, P11, P13 had a significant change according to the welch corrected t-test. It was observed that P01, P06, P09, P10, P11 and P13 molecules increased the amplitude significantly, while P03, P05, P07 molecules decreased it significantly. It was observed that P01, P03, P09, P10, P13 and P14 molecules significantly increased the significant period with the Chronostar tool. In the relative bioluminescence values, amplitude damping effect of P03 molecule and amplitude enhancer effect of P06 and P10 molecules were observed. It was observed that the molecules exhibited three fundamentally different behaviours. the first is period lengthening, the second amplitude enhances, and the last amplitude dumping.

Real-time bioluminescence assay was performed at 10, 20 and 40 μ M doses to examine the effect of P01 and P03 molecules on the period and the amplitude effect of P09, P10 and P11 molecules. In the analysis with the Biodare2 tool, a dose dependent period increase was observed in molecules other than P09 molecule (Figure 5.9). This may be due to the low resolution of Synergy H1. Analysis with Chronostar tool shows that P01 and P03 molecules prolong the period in a dose dependent manner and have amplitude dumping effect. P09, P10 and P11 molecules have an amplitude enhancer effect. The P10 molecule significantly increases the period, but this change may also be due to low solubility.

The effect of P-series molecules on luciferase, as measured by luciferase-based experiments for circadian rhythm, can lead to false positive results. The plasmid containing the Luc gene was transfected into cells, and protein synthesis was halted using cycloheximide. The results indicated that when compared to the control group, P02, P03, and P10 molecules influenced the half-life of luciferase, resulting in the misinterpretation of real-time bioluminescence assay results. These findings underscore the importance of careful interpretation and the necessity of control experiments in luciferase-based studies.

The effect of these molecules on CRY1 was examined by Western blot and it was seen that P09 molecule was a CRY1 destabilizer. According to our western blot result (Figure 5.12), there are 2 different cases. First, the P09 molecule stabilizes the CRY1/PER2 complex according to free energy calculations. Due to this stabilization, inhibition of the BMAL1/CLOCK complex is carried out, stopping the production of the CRY1 level in the medium. Over time, the CRY1 protein present in the complex

is ubiquitinated by FBLX3 and sent to destruction, and the decrease in CRY1 level is due to this. However, in the first case, the amplitude enhancer feature that we obtained from the real-time bioluminescence assay cannot be seen. Because, in the balance where the negative arm predominates, an increase in the luciferase level, which we measured on the BMAL1 promoter, cannot be expected. Therefore, we can say that the theoretical calculation is insufficient for both methods (MM-GBSA and ANI_LIE). Second, if it destabilizes the complex, the negative arm will have reduced capacity to fully suppress the positive arm so we will be able to observe the increase over amplitude like CLK8 molecule.

If we examine the interaction of P09 molecule with CRY1/PER2 (Figure 5.12), the conventional hydrogen bond of CRY1 with ASP321, ASN472, ALA476 establishes Pi-Alkyl with LEU479. It performs Unfavorable Donor-Donor interaction with ARG483. Conventional hydrogen bonding of PER2 with LEU1155 and ARG1158 establishes Pi-Sigma interaction with SER1157. The P09 molecule, which does not break its interaction in the complex with the strong hydrogen bonds it establishes with CRY1 and PER2, may cause the position change of ARG483. The ARG483 residue plays an important role in the binding of mouseCRY1 to the c-terminal of mouse Bmal1 [Czarna et al., 2013]. This unfavourable interaction can reduce the suppress capacity of the complex on the positive arm as a result of the altered conformation.

In summary, our study identified several promising molecules, including P01, P03, P09, P10, and P11, which exhibited significant effects on the circadian rhythm. Further investigations, including in vivo studies and additional characterization of the molecular interactions, are warranted to fully understand their therapeutic potential and mechanism of action. These findings contribute to the growing body of knowledge on circadian rhythm modulation and its potential applications in the treatment of various diseases.

REFERENCES

- Abbott S. M., Zee, P. C., (2015), "Irregular sleep-wake rhythm disorder.", *Sleep medicine clinics*, Elsevier, 10(4), 517–522.
- Akhtar R. A., Reddy A. B., Maywood E. S., Clayton J. D., King V. M., Smith A. G., Gant T. W., Hastings M. H., Kyriacou C. P., (2002), "Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus.", *Current Biology*, 12(7).
- Albrecht U., Sun Z. S., Eichele G., Lee C. C., (1997), "A differential response of two putative mammalian circadian regulators, *mper1* and *mper2*, to light.", *Cell*, 91(7).
- Anand S. N., Maywood E. S., Chesham J. E., Joynson G., Banks G. T., Hastings M. H., Nolan P. M., (2013), "Distinct and separable roles for endogenous CRY1 and CRY2 within the circadian molecular clockwork of the Suprachiasmatic Nucleus, as revealed by the *Fbxl3Afh* mutation.", *Journal of Neuroscience*, 33(17).
- de Assis L. V. M., Oster H., (2021), "The circadian clock and metabolic homeostasis: entangled networks.", *Cellular and Molecular Life Sciences*, Springer, 78(10), 4563.
- Atger F., Mauvoisin D., Weger B., Gobet C., Gachon F., (2017), "Regulation of mammalian physiology by interconnected circadian and feeding rhythms.", *Frontiers in Endocrinology*.
- Auger R. R., Burgess H. J., Emens J. S., Deriy L. V., Thomas S. M., Sharkey K. M., (2015), "Clinical practice guideline for the treatment of intrinsic circadian rhythm sleep-wake disorders: Advanced Sleep-Wake Phase Disorder (ASWPD), Delayed Sleep-Wake Phase Disorder (DSWPD), Non-24-Hour Sleep-Wake Rhythm Disorder (N24SWD), and Irregular Sleep-Wake Rhythm Disorder (ISWRD), an update for 2015.", *Journal of Clinical Sleep Medicine*, 11(10).
- Baidanoff F. M., Trebucq L. L., Plano S. A., Eaton P., Golombek D. A., Chiesa J. J., (2022), "Cysteine Oxidation Promotes Dimerization/Oligomerization of Circadian Protein Period 2.", *Biomolecules*, 12(7).
- Balsalobre A., Damiola F., Schibler U., (1998), "A serum shock induces circadian gene expression in mammalian tissue culture cells.", *Cell*, 93(6).
- Bennett, C. H., (1976), "Efficient estimation of free energy differences from Monte Carlo data.", *Journal of Computational Physics*, 22(2).
- Berganzo K., Díez-Arrola B., Tijero B., Somme J., Lezcano E., Llorens V., Ugarriza I., Ciordia R., Gómez-Esteban J. C., Zarranz J. J., (2013), "Nocturnal hypertension and dysautonomia in patients with Parkinson's disease: Are they related?", *Journal of Neurology*, 260(7).
- Boivin D. B., Boudreau P., (2014), "Impacts of shift work on sleep and circadian rhythms.", *Pathologie Biologie*.

Bolitho S. J., Naismith S. L., Rajaratnam S. M. W., Grunstein R. R., Hodges J. R., Terpening Z., Rogers N., Lewis S. J. G., (2014), “Disturbances in melatonin secretion and circadian sleep-wake regulation in Parkinson disease.”, *Sleep Medicine*, 15(3).

Breen D. P., Vuono R., Nawarathna U., Fisher K., Shneerson J. M., Reddy A. B., and Barker R. A., (2014), “Sleep and circadian rhythm regulation in early parkinson disease.”, *JAMA Neurology*, 71(5).

Buscone S., Mardaryev A. N., Westgate G. E., Uzunbajakava N. E., Botchkareva N. V., (2021), “Cryptochrome 1 is modulated by blue light in human keratinocytes and exerts positive impact on human hair growth.”, *Experimental Dermatology*, 30(2).

Cavga A. D., Tardu M., Korkmaz T., Keskin O., Ozturk N., Gursoy A., Kavakli I. H., (2019), “Cryptochrome deletion in p53 mutant mice enhances apoptotic and anti-tumorigenic responses to UV damage at the transcriptome level.”, *Functional & integrative genomics, Funct Integr Genomics*, 19(5), 729–742.

Cheng A. yuan, Zhang Y., Mei H. jun, Fang S., Ji, P., Yang J., Yu, L., Guo W. chun., (2015), “Construction of a plasmid for overexpression of human circadian gene period2 and its biological activity in osteosarcoma cells.”, *Tumor Biology*, 36(5).

Cournia Z., Allen B., and Sherman W., (2017), “Relative Binding Free Energy Calculations in Drug Discovery: Recent Advances and Practical Considerations.”, *Journal of Chemical Information and Modeling*.

Czarna A., Berndt A., Singh H. R., Grudziecki A., Ladurner A. G., Timinszky G., Kramer A., Wolf E., (2013), “XStructures of drosophila cryptochrome and mouse cryptochrome1 provide insight into circadian function.”, *Cell*, 153(6).

Dallmann R., Brown S. A., Gachon F., (2014a), “Chronopharmacology: New insights and therapeutic implications.”, *Annual Review of Pharmacology and Toxicology*.

Ding J. M., Faiman L. E., Hurst W. J., Kuriashkina L. R., Gillette M. U., (1997), “Resetting the Biological Clock: Mediation of Nocturnal CREB Phosphorylation via Light, Glutamate, and Nitric Oxide.”, *Journal of Neuroscience, Society for Neuroscience*, 17(2), 667–675.

Doruk Y. U., Yarpavar D., Akyel Y. K., Gul S., Taskin A. C., Yilmaz F., Baris I., Ozturk N., Türkay M., Ozturk N., Okyar A., and Kavakli I. H., (2020), “A CLOCK-binding small molecule disrupts the interaction between CLOCK and BMAL1 and enhances circadian rhythm amplitude.”, *Journal of Biological Chemistry*, 295(11).

Drie J. H., (2007), “Computer-aided drug design: The next 20 years.”, *Journal of Computer-Aided Molecular Design*, 21(10–11).

Duffield G. E., Best J. D., Meurers B. H., Bittner A., Loros J. J., Dunlap J. C., (2002), “Circadian programs of transcriptional activation, signaling, and protein turnover revealed by microarray analysis of mammalian cells.”, *Current Biology*, 12(7).

Onat E. O., Kars E. M., Gül Ş., Bilguvar K., Wu, Y., Özhan A., Aydın C., Başak N. A., Trusso A. M., Goracci A., Fallerini C., Renieri A., Casanova J. L., Itan Y., Atbaşoğlu C. E., Saka M. C., Kavaklı H., Özçelik T., (2020), "Human CRY1 variants associate with attention deficit/hyperactivity disorder.", *The Journal of Clinical Investigation*, American Society for Clinical Investigation, 130(7), 3885.

Fernandez D. C., Chang Y. T., Hattar S., Chen S. K., (2016), "Architecture of retinal projections to the central circadian pacemaker.", *Proceedings of the National Academy of Sciences of the United States of America*, National Academy of Sciences, 113(21), 6047–6052.

Foster R. G., Peirson S. N., Wulff K., Winnebeck E., Vetter C., Roenneberg T., (2013), "Sleep and circadian rhythm disruption in social jetlag and mental illness.", *Progress in Molecular Biology and Translational Science*.

Friesner R. A., Murphy R. B., Repasky M. P., Frye L. L., Greenwood J. R., Halgren T. A., Sanschagrin P. C., Mainz D. T., (2006), "Extra precision glide: Docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes.", *Journal of Medicinal Chemistry*, 49(21).

Fu L., Pelicano H., Liu J., Huang P., Lee C. C., (2002), "The circadian gene *Period2* plays an important role in tumor suppression and DNA damage response in vivo.", *Cell*, 111(1).

Gachon F., Olela F. F., Schaad O., Descombes P., Schibler U., (2006), "The circadian PAR-domain basic leucine zipper transcription factors DBP, TEF, and HLF modulate basal and inducible xenobiotic detoxification.", *Cell Metabolism*, 4(1).

Gau D., Lemberger T., Von Gall C., Kretz O., Le Minh N., Gass P., Schmid W., Schibler U., Korf H. W., Schütz G., (2002), "Phosphorylation of CREB Ser142 regulates light-induced phase shifts of the circadian clock.", *Neuron*, 34(2).

Ginty D. D., Kornhauser J. M., Thompson M. A., Bading H., Mayo, K. E., Takahashi J. S., Greenberg M. E., (1993), "Regulation of CREB phosphorylation in the suprachiasmatic nucleus by light and a circadian clock.", *Science*, 260(5105).

Griebel G., Ravinet-Trillou C., Beeské S., Avenet P., Pichat P., (2014), "Mice deficient in cryptochrome 1 (*Cry1*^{-/-}) exhibit resistance to obesity induced by a high-fat diet.", *Frontiers in Endocrinology*, 5(APR).

Griffin E. A., Staknis D., Weitz C. J., (1999), "Light-independent role of CRY1 and CRY2 in the mammalian circadian clock.", *Science*, 286(5440).

Gu G. H., Noh J., Kim S., Back S., Ulissi Z., Jung Y., (2020), "Practical Deep-Learning Representation for Fast Heterogeneous Catalyst Screening.", *Journal of Physical Chemistry Letters*, 11(9).

Gul S., Akyel Y. K., Gul Z. M., Isin S., Ozcan O., Korkmaz T., Selvi S., Danis I., Ipek O. S., Aygenli F., Taskin A. C., Akarlar B. A., Ozlu N., Ozturk N., Ozturk N., Ünal D. Ö., Guzel M., Turkay M., Okyar A., and Kavakli I. H., (2022), “Discovery of a small molecule that selectively destabilizes Cryptochrome 1 and enhances life span in p53 knockout mice.”, *Nature Communications*, 13(1).

Hannibal J., (2002), “Neurotransmitters of the retino-hypothalamic tract.”, *Cell and Tissue Research*.

Hardin P. E., Hall J. C., Rosbash M., (1990), “Feedback of the *Drosophila* period gene product on circadian cycling of its messenger RNA levels.”, *Nature*, 343(6258).

Harrison Y., (2013), “The impact of daylight saving time on sleep and related behaviours.”, *Sleep Medicine Reviews*.

Hartman G. D., Egbertson M. S., Halczenko W., Laswell W. L., Duggan M. E., Smith R. L., Naylor A. M., Manno P. D., Lynch R. J., Zhang G., Chang C. T. C., Gould R. J., (1992), “Non-Peptide Fibrinogen Receptor Antagonists. 1. Discovery and Design of Exosite Inhibitors.”, *Journal of Medicinal Chemistry*, 35(24).

Hastings M. H., Brancaccio M., Maywood E. S., (2014), “Circadian pacemaking in cells and circuits of the suprachiasmatic nucleus.”, *Journal of Neuroendocrinology*.

Hastings M. H., Maywood E. S., Brancaccio M., (2018), “Generation of circadian rhythms in the suprachiasmatic nucleus.”, *Nature Reviews Neuroscience*.

Hastings M. H., Smyllie N. J., Patton A. P., (2020), “Molecular-genetic Manipulation of the Suprachiasmatic Nucleus Circadian Clock.”, *Journal of Molecular Biology*, Academic Press, 432(12), 3639–3660.

Herzog E. D., (2007), “Neurons and networks in daily rhythms.”, *Nature Reviews Neuroscience*.

Herzog E. D., Aton S. J., Numano R., Sakaki Y., Tei H., (2004), “Temporal Precision in the Mammalian Circadian System: A Reliable Clock from Less Reliable Neurons.”, *Journal of Biological Rhythms*, 19(1).

Hollingsworth S. A., Dror R. O., (2018), “Molecular Dynamics Simulation for All.”, *Neuron*.

Hsu D. S., Zhao X., Zhao S., Kazantsev A., Wang R. P., Todo T., Wei Y. F., Sancar A., (1996), “Putative human blue-light photoreceptors hCRY1 and hCRY2 are flavoproteins.”, *Biochemistry*, 35(44).

Hughes M. E., DiTacchio L., Hayes K. R., Vollmers C., Pulivarthy S., Baggs J. E., Panda S., and Hogenesch J. B., (2009), “Harmonics of circadian gene transcription in mammals.”, *PLoS Genetics*, 5(4).

Jones G., Willett P., Glen R. C., Leach A. R., Taylor R., (1997), "Development and validation of a genetic algorithm for flexible docking.", *Journal of Molecular Biology*, 267(3).

Karatsoreos I. N., (2014), "Links between circadian rhythms and psychiatric disease.", *Frontiers in Behavioral Neuroscience*, Frontiers Research Foundation, 8(MAY), 87157.

Kim M., Park K., Kim W., Jung S., Cho A. E., (2021), "Target-Specific Drug Design Method Combining Deep Learning and Water Pharmacophore.", *Journal of Chemical Information and Modeling*, 61(1).

Konopka R. J., Benzer S., (1971), "Clock mutants of *Drosophila melanogaster*.", *Proceedings of the National Academy of Sciences*, National Acad Sciences, 68(9), 2112–2116.

Kuhn G., (2001), "Circadian rhythm, shift work, and emergency medicine.", *Annals of Emergency Medicine*, 37(1).

Kuo S. J., Chen S. T., Yeh K. T., Hou M. F., Chang Y. S., Hsu N. C., Chang J. G., (2009), "Disturbance of circadian gene expression in breast cancer.", *Virchows Archiv*, 454(4).

Lamia K. A., Storch K. F., Weitz C. J., (2008), "Physiological significance of a peripheral tissue circadian clock.", *Proceedings of the National Academy of Sciences of the United States of America*, 105(39).

Langmesser S., Tallone T., Bordon A., Rusconi S., Albrecht U., (2008), "Interaction of circadian clock proteins PER2 and CRY with BMAL1 and CLOCK.", *BMC Molecular Biology*, 9.

Larsson P., Hess B., Lindahl E., (2011), "Algorithm improvements for molecular dynamics simulations.", *Wiley Interdisciplinary Reviews: Computational Molecular Science*, John Wiley & Sons, Ltd, 1(1), 93–108.

Leng Y., Musiek E. S., Hu K., Cappuccio F. P., Yaffe K., (2019), "Association between circadian rhythms and neurodegenerative diseases.", *The Lancet Neurology*.
Levitt, M., and Lifson, S., (1969), "Refinement of protein conformations using a macromolecular energy minimization procedure.", *Journal of Molecular Biology*, 46(2).

Lifson S., Waeshel A., (1968), "Consistent force field for calculations of conformations, vibrational spectra, and enthalpies of cycloalkane and n-alkane molecules.", *The Journal of Chemical Physics*, 49(11).

Liu C., Weaver D. R., Strogatz S. H., Reppert S. M., (1997), "Cellular construction of a circadian clock: Period determination in the suprachiasmatic nuclei.", *Cell*, 91(6).

Maier B., Lorenzen S., Finger A. M., Herzel H., Kramer A., (2021), "Searching Novel Clock Genes Using RNAi-Based Screening.", *Methods in Molecular Biology*.

Manfredini R., Fabbian F., De Giorgi A., Zucchi B., Cappadona R., Signani F., Katsiki N., Mikhailidis D. P., (2018), "Daylight saving time and myocardial infarction: Should we be worried? A review of the evidence.", *European Review for Medical and Pharmacological Sciences*, 22(3).

Marcheva B., Ramsey K. M., Buhr E. D., Kobayashi Y., Su H., Ko C. H., Ivanova G., Omura C., Mo, S., Vitaterna M. H., Lopez J. P., Philipson L. H., Bradfield C. A., Crosby S. D., Jebailey L., Wang X., Takahashi J. S., Bass J., (2010), "Disruption of the clock components CLOCK and BMAL1 leads to hypoinsulinaemia and diabetes.", *Nature*, 466(7306).

Le Martelot G., Claudel T., Gatfield D., Schaad O., Kornmann B., Lo Sasso G., Moschetta A., Schibler U., (2009), "REV-ERB α participates in circadian SREBP signaling and bile acid homeostasis.", *PLoS Biology*, 7(9).

Maywood E. S., Chesham J. E., Smyllie N. J., Hastings M. H., (2014), "The Tau Mutation of Casein Kinase ϵ Sets the Period of the Mammalian Pacemaker via Regulation of Period1 or Period2 Clock Proteins.", *Journal of Biological Rhythms*, 29(2).

McCammon J. A., Gelin B. R., Karplus M., (1977), "Dynamics of folded proteins.", *Nature*, 267(5612).

Miller B. H., McDearmon E. L., Panda S., Hayes K. R., Zhang J., Andrews J. L., Antoch M. P., Walker J. R., Esser K. A., Hogenesch J. B., Takahashi J. S., (2007), "Circadian and CLOCK-controlled regulation of the mouse transcriptome and cell proliferation.", *Proceedings of the National Academy of Sciences of the United States of America*, 104(9).

Miyamoto Y., Sancar A., (1999), "Circadian regulation of cryptochrome genes in the mouse.", *Molecular Brain Research*, 71(2).

Mohawk J. A., Green C. B., and Takahashi J. S., (2012), "Central and peripheral circadian clocks in mammals.", *Annual Review of Neuroscience*.

Mohawk J. A., Takahashi J. S., (2011), "Cell autonomy and synchrony of suprachiasmatic nucleus circadian oscillators.", *Trends in Neurosciences*.

Morin L. P., Allen C. N., (2006), "The circadian visual system, 2005.", *Brain Research Reviews*, Elsevier, 51(1), 1–60.

Nahm S. S., Farnell Y. Z., Griffith W., and Earnest D. J., (2005), "Circadian regulation and function of voltage-dependent calcium channels in the suprachiasmatic nucleus.", *The Journal of neuroscience: the official journal of the Society for Neuroscience*, J Neurosci, 25(40), 9304–9308.

Nguyen D. D., Cang Z., Wu K., Wang M., Cao, Y., Wei G. W., (2019), "Mathematical deep learning for pose and binding affinity prediction and ranking in D3R Grand Challenges.", *Journal of Computer-Aided Molecular Design*, 33(1).

O'Boyle N. M., Banck M., James C. A., Morley C., Vandermeersch T., Hutchison G. R., (2011), "Open Babel: An Open chemical toolbox.", *Journal of Cheminformatics*, 3(10).

Panda S., Antoch M. P., Miller B. H., Su A. I., Schook A. B., Straume M., Schultz P. G., Kay S. A., Takahashi J. S., Hogenesch J. B., (2002), "Coordinated transcription of key pathways in the mouse by the circadian clock.", *Cell*, 109(3).

Patton A. P., Hastings M. H., (2018), "The suprachiasmatic nucleus.", *Current Biology*, Cell Press, 28(15), R816–R822.

Reppert S. M., Weaver D. R., (2002), "Coordination of circadian timing in mammals.", *Nature* 2002 418:6901, Nature Publishing Group, 418(6901), 935–941.

Sack R. L., Auckley D., Auger R. R., Carskadon M. A., Wright K. P., Vitiello M. V., Zhdanova I. V., (2007), "Circadian rhythm sleep disorders: Part I, basic principles, shift work and jet lag disorders: An American Academy of Sleep Medicine review.", *Sleep*, 30(11).

Sancar A., Ozturk N., Lee J. H., Gaddameedhi S., (2009), "Loss of cryptochrome reduces cancer risk in p53 mutant mice.", *Proceedings of the National Academy of Sciences of the United States of America*, 106(8).

von Schantz M., (2017), "Natural Variation in Human Clocks.", *Advances in Genetics*.

Von Schantz M., Jenkins, A., Archer S. N., (2006), "Evolutionary history of the vertebrate Period genes.", *Journal of Molecular Evolution*, 62(6).

Schmalen I., Reischl, S., Wallach T., Klemz R., Grudziecki A., Prabu J. R., Benda C., Kramer A., Wolf E., (2014), "Interaction of circadian clock proteins CRY1 and PER2 is modulated by zinc binding and disulfide bond formation.", *Cell*, 157(5).

Schurov I. L., McNulty S., Best J. D., Sloper P. J., Hastings M. H., (1999), "Glutamatergic Induction of CREB Phosphorylation and Fos Expression in Primary Cultures of the Suprachiasmatic Hypothalamus In Vitro is Mediated by Co-Ordinate Activity of NMDA and Non-NMDA Receptors.", *Journal of Neuroendocrinology*, John Wiley & Sons, Ltd, 11(1), 43–51.

Shoichet B. K., Leach A. R., Kuntz I. D., (1999), "Ligand solvation in molecular docking.", *Proteins: Structure, Function and Genetics*, 34(1).

Śledź P., Caflisch A., (2018), "Protein structure-based drug design: from docking to molecular dynamics.", *Current Opinion in Structural Biology*.

Smyllie N. J., Bagnall J., Koch A. A., Niranjana D., Polidarova L., Chesham J. E., Chin J. W., Partch C. L., Loudon A. S. I., Hastings M. H., (2022), "Cryptochrome proteins regulate the circadian intracellular behavior and localization of PER2 in mouse suprachiasmatic nucleus neurons.", *Proceedings of the National Academy of Sciences of the United States of America*, 119(4).

Soria V., Martínez-Amorós È., Escaramís G., Valero J., Pérez-Egea R., García C., Gutiérrez-Zotes A., Puigdemont D., Bayés M., Crespo J. M., Martorell L., Vilella E., Labad A., Vallejo J., Pérez V., Menchón J. M., Estivill X., Gratacòs M., Urretavizcaya M., (2010), "Differential association of circadian genes with mood disorders: CRY1 and NPAS2 are associated with unipolar major depression and clock and VIP with bipolar disorder.", *Neuropsychopharmacology*, 35(6).

Srinivasan J., Miller J., Kollman P. A., Case D. A., (1998), "Continuum solvent studies of the stability of rna hairpin loops and helices.", *Journal of Biomolecular Structure and Dynamics*, 16(3).

Storch K. F., Lipan O., Leykin I., Viswanathan N., Davis F. C., Wong W. H., Weitz C. J., (2002), "Extensive and divergent circadian gene expression in liver and heart.", *Nature*, 417(6884).

Takahashi J. S., (2021), "The 50th anniversary of the konopka and benzer 1971 paper in PNAS: 'Clock mutants of drosophila melanogaster.'", *Proceedings of the National Academy of Sciences of the United States of America*.

Takahashi J. S., Ko C. H., Yamada Y. R., Welsh D. K., Buhr E. D., Liu A. C., Zhang E. E., Ralph M. R., Kay S. A., and Forger D. B., (2010), "Emergence of noise-induced oscillations in the central circadian pacemaker.", *PLoS Biology*, 8(10).

Talele T., Khedkar S., Rigby A., (2010), "Successful Applications of Computer Aided Drug Discovery: Moving Drugs from Concept to the Clinic.", *Current Topics in Medicinal Chemistry*, 10(1).

Trott O., Olson A. J., (2009), "AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading.", *Journal of Computational Chemistry*.

Turek F. W., Joshu C., Kohsaka A., Lin E., Ivanova G., McDearmon E., Laposky A., Losee-Olson S., Easton A., Jensen D. R., Eckel R. H., Takahashi J. S., Bass J., (2005), "Obesity and metabolic syndrome in circadian Clock mutant mice.", *Science*, 308(5724).

Vanselow K., Vanselow J. T., Westermarck P. O., Reischl S., Maier B., Korte T., Herrmann A., Herzog H., Schloesser A., Kramer A., (2006), "Differential effects of PER2 phosphorylation: molecular basis for the human familial advanced sleep phase syndrome (FASPS).", *Genes & development*, *Genes Dev*, 20(19), 2660–2672.

Videnovic A., Lazar A. S., Barker R. A., Overeem S., (2014), "'The clocks that time us' - Circadian rhythms in neurodegenerative disorders.", *Nature Reviews Neurology*.

Wang Y., Cheng Y., Yu G., Jia B., Hu Z., Zhang L., (2016a), "Expression of PER, CRY, and TIM genes for the pathological features of colorectal cancer patients.", *OncoTargets and Therapy*, 9.

Wang Z., Li L., Wang Y., (2016b), "Effects of Per2 overexpression on growth inhibition and metastasis, and on MTA1, nm23-H1 and the autophagy-associated PI3K/PKB signaling pathway in nude mice xenograft models of ovarian cancer.", *Molecular Medicine Reports*, 13(6).

Winter S. L., Bosnyan-Collins, L., Pinnaduwegge D., Andrusis I. L., (2007), "Expression of the circadian clock genes Per1 and Per2 in sporadic and familial breast tumors.", *Neoplasia*, 9(10).

Wood P. A., Yang X., Taber A., Oh E. Y., Ansell C., Ayers S. E., Al-Assaad Z., Carnevale K., Berger F. G., Peña M. M. O., Hrushesky W. J. M., (2008), "Period 2 mutation accelerates ApcMin/+ tumorigenesis.", *Molecular Cancer Research*, 6(11).

Yamazaki S., Numano R., Abe M., Hida A., Takahashi R. I., Ueda M., Block G. D., Sakaki Y., Menaker M., Tei H., (2000), "Resetting central and peripheral circadian oscillators in transgenic rats.", *Science*, 288(5466).

Yoo S. H., Yamazaki S., Lowrey P. L., Shimomura K., Ko, C. H., Buhr E. D., Siepkas S. M., Hong H. K., Oh W. J., Yoo O. J., Menaker M., Takahashi J. S., (2004), "PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues.", *Proceedings of the National Academy of Sciences of the United States of America*, 101(15).

Zhanfeng N., Chengquan W., Hechun X., Jun W., Lijian Z., Dede M., Wenbin L., Lei Y., (2016), "Period2 downregulation inhibits glioma cell apoptosis by activating the MDM2-TP53 pathway.", *Oncotarget*, 7(19).

Zhang R., Lahens N. F., Ballance H. I., Hughes M. E., Hogenesch J. B., (2014), "A circadian gene expression atlas in mammals: Implications for biology and medicine.", *Proceedings of the National Academy of Sciences of the United States of America*, 111(45).

Zhao H., Zeng Z. L., Yang J., Jin Y., Qiu M. Z., Hu X. Y., Han J., Liu K. Y., Liao J. W., Xu R. H., Zou Q. F., (2014), "Prognostic relevance of Period1 (Per1) and Period2 (Per2) expression in human gastric cancer.", *International Journal of Clinical and Experimental Pathology*, 7(2).

Zhao Q., Zheng G., Yang K., Ao, Y. R., Su X. L., Li, Y., Lv X. Q., (2016), "The clock gene PER1 plays an important role in regulating the clock gene network in human oral squamous cell carcinoma cells.", *Oncotarget*, 7(43).

Zhu Y., Stevens R. G., Hoffman A. E., FitzGerald L. M., Kwon E. M., Ostrander E. A., Davis S., Zheng T., Stanford J. L., (2009), "Testing the circadian gene hypothesis in prostate cancer: A population-based case-control study.", *Cancer Research*, 69(24).

Zwanzig R. W., (1954), "High-Temperature Equation of State by a Perturbation Method. I. Nonpolar Gases.", *Journal of Chemical Physics*, 22(8).

BIOGRAPHY

Mehmet Serdar Koca from Gebze Technical University with a thesis titled "Bioinformatics Analysis of GSE125050 Dataset to Identify Therapeutic Targets and Pathogenesis for Alzheimer's Disease." Following his graduation, he joined the MSc Program in Molecular Biology and Genetics at the Graduate School of Natural and Applied Sciences in Gebze Technical University. For a duration of 2.5 years, he worked under the supervision of Prof. Dr. Nuri Ozturk.



APPENDICES

Table 8.1: All P-series molecules binding free energies(100ns).

Compounds	MMGBSA		ANI_LIE	
	Ligand Interaction Energy	CRY1-PER2 Interaction Energy	Ligand Interaction Energy	CRY1-PER2 Interaction Energy
P01	-35.5	-225.46	-40.09819	-422.86783
P02	-36.12	-226.3	-43.438425	-394.62781
P03	-24.77	-199.28	-35.4603	-361.63935
P04	-28.07	-214.06	-31.359037	-370.72387
P05	-34.77	-208.24	-37.175247	-369.87535
P06	-36.5	-227.69	-20.109587	-394.83211
P07	-28.78	-195.11	-37.702842	-358.54802
P08	-31.16	-228.77	-44.810368	-410.19865
P09	-29.72	-232.49	-44.883096	-425.88649
P10	-29.86	-217.1	-36.083604	-374.94024
P11	-30	-216.57	-48.749692	-412.31792
P12	-33.29	-211.93	-42.002813	-394.34078
P13	-29.07	-211.53	-55.89195	-403.66208
P14	-34.9	-205.2	-49.234806	-366.07566
P15	-31.07	-191.03	-28.429277	-338.86308
P16	-29.28	-203.81	-28.531193	-367.27807
P17	-36.36	-206.54	-40.704417	-361.15082
P18	-32.19	-206.66	-43.716127	-390.91666
P19	-33.58	-207.3	-38.344601	-373.91671
P20	-22.57	-207.91	-32.387695	-368.58581
P21	-26.57	-208.08	-36.667752	-379.07839
P22	-18.12	-208.58	-23.826119	-381.17011
P23	-28.41	-209.06	-33.139336	-381.67107
P24	-27.54	-211.21	-33.729679	-372.83656
P25	-34.93	-211.24	-41.209745	-376.04554
P26	-24	-211.35	-34.721827	-359.43037
P27	-26.41	-211.72	-28.586724	-386.78235
P28	-33.6	-212.62	-26.604436	-375.66418
P29	-27.87	-212.97	-26.150995	-385.39794
P30	-27.85	-213.2	-35.70431	-379.18862
P31	-32.73	-213.29	-22.55481	-399.66265
P32	-29.05	-213.67	-37.84944	-381.67362

Table 8.1: Continued

Compo unds	MMGBSA		ANI_LIE	
	Ligand Interaction Energy	CRY1-PER2 Interaction Energy	Ligand Interaction Energy	CRY1-PER2 Interaction Energy
P33	-23.09	-214.26	-27.79873	-375.61395
P34	-28.83	-214.27	-32.240868	-381.66776
P35	-27.48	-214.53	-28.427354	-375.22726
P36	-34.27	-214.55	-20.432424	-389.52029
P37	-34.84	-215.56	-54.855775	-398.18894
P38	-33.73	-216.02	-37.772345	-387.00726
P39	-33.96	-216.35	-45.857697	-395.43632
P40	-28.74	-216.43	-40.533634	-391.62324
P41	-26.26	-216.56	-30.187684	-387.27334
P42	-23.01	-216.67	-25.337087	-386.01336
P43	-28	-216.87	-39.87272	-389.24518
P44	-35.2	-217.22	-41.453055	-390.14176
P45	-24.66	-217.64	-39.324437	-393.66023
P46	-30.01	-217.85	-40.168245	-393.02527
P47	-26.14	-218.22	-28.49721	-409.19793
P48	-26.81	-218.41	-37.716379	-380.33983
P49	-17.14	-218.69	-17.620561	-390.72662
P50	-32.69	-219.11	-38.055317	-398.01884
P51	-19.51	-219.24	-25.9151	-394.34581
P52	-32.7	-219.57	-34.181485	-405.92299
P53	-27.28	-219.59	-28.990404	-396.23779
P54	-34.16	-219.79	-38.754202	-402.41482
P55	-33.4	-220.02	-46.676114	-394.99514
P56	-25.76	-220.05	-28.625189	-393.16237
P57	-31.39	-220.94	-23.803962	-385.34401
P58	-26.79	-221.09	-25.959244	-391.1979
P59	-22.82	-221.12	-25.548562	-406.49359
P60	-36.19	-221.2	-27.481897	-385.59257
P61	-24.27	-221.75	-34.909411	-410.2136
P62	-29.68	-222.35	-35.568599	-409.78919
P63	-32.35	-222.37	-41.780665	-406.63525
P64	-31.05	-222.89	-46.02065	-393.66664
P65	-29.53	-223.51	-40.344848	-402.73704
P66	-30.55	-223.62	-38.428355	-401.88867
P67	-26.48	-224.15	-44.417913	-426.39299
P68	-31.04	-224.33	-36.331377	-397.09169

Table 8.1: Continued

Compo unds	MMGBSA		ANI_LIE	
	Ligand Interaction Energy	CRY1-PER2 Interaction Energy	Ligand Interaction Energy	CRY1-PER2 Interaction Energy
P69	-23.14	-224.41	-34.277454	-425.8360406
P70	-32.73	-224.53	-20.050512	-408.5606125
P71	-26.26	-224.85	-41.236744	-406.5565624
P72	-20.22	-225.41	-26.811272	-404.9628891
P73	-26.57	-225.56	-36.156277	-426.1291713
P74	-14.54	-226.29	-24.200035	-417.0188984
P75	-26.79	-227.12	-35.633416	-417.7906776
P76	-30.49	-227.3	-43.336632	-421.3025169
P77	-31.88	-227.86	-39.191366	-416.0207188
P78	-27.09	-228.11	-31.240056	-403.6572553
P79	-26.12	-228.34	-30.461692	-402.7584977
P80	-25.07	-229.06	-25.787202	-402.9180236
P81	-24.2	-229.93	-35.830805	-409.5943152
P82	-26.98	-230.48	-35.484012	-418.1552572
P83	-20.43	-231.62	-21.194459	-441.6683694
P84	-26.48	-231.99	-37.715094	-415.4575671
P85	-34.29	-235.09	-21.251846	-432.5392436
P86	-26.27	-235.67	-31.90745	-423.215993
P87	-29.69	-237.24	-39.286361	-420.1424825
P88	-17.96	-238.88	-25.674287	-443.0127379
P89	-23.15	-239.13	-33.118142	-446.3422531
P90	-30.18	-240.56	-40.374517	-422.4585354
Control	0	-198.84	0	-390
Control	0	-205.99	0	-369
Control	0	-205.7	0	-365

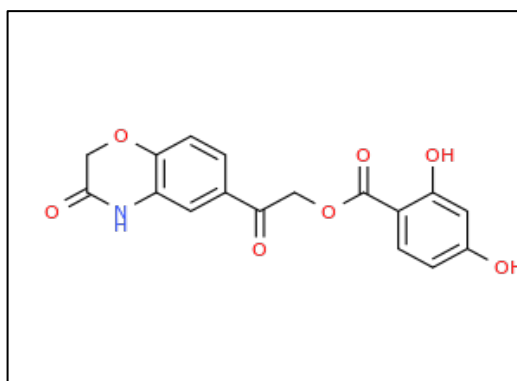


Figure 8.1: 2D structure of P09 molecule.