A DISSERTATION ON

Understanding the chronotherapeutic efficiency of anti-breast cancer pharmacological agents

SUBMITTED TO THE
DEPARTMENT OF BIOENGINEERING
FACULTY OF ENGINEERING & INFORMATION TECHNOLOGY
INTEGRAL UNIVERSITY, LUCKNOW

IN PARTIAL FULFILMENT
FOR THE
B.TECH-M.TECH DUAL DEGREE
IN BIOTECHNOLOGY

BY

Fatima Sarwar
B.Tech-M.Tech Dual Degree Biotechnology (X Semester)
Roll No: 1801101003

UNDER THE SUPERVISION OF

Dr. Sandipan Ray, Ph.D., MRSB
(Supervisor)
Assistant Professor and Group Leader
Circadian Rhythms and Disease Biology Laboratory
Indian Institute of Technology Hyderabad

भारतीय सांकेतिक विज्ञान संस्थ हैदराबाद
भारतीय प्रौद्योगिकी संस्थान हैदराबाद
Indian Institute of Technology Hyderabad

INTEGRAL UNIVERSITY, DASAULI, KURSI ROAD
LUCKNOW- 226026

DECLARATION FORM

I, **Fatima Sarwar**, a student of **B.Tech-M.Tech Dual Degree Biotechnology** (5th Year/ 10th Semester), Integral University have completed my six months dissertation work entitled **"Understanding the chronotherapeutic efficiency of anti-breast cancer pharmacological agents",** successfully from **Indian Institute of Technology Hyderabad (IITH)** under the able guidance of **Dr. Sandipan Ray.**

I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

Fatima Sarwar

Dr. Reena Vishvakarma
(Course Coordinator)

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Indian Institute of Technology Hyderabad

भारतीय प्रौद्योगिकी संस्थान हैदराबाद
कैंडी - ५०२ २८४, सांगरेड्डी, तेलंगाण, भारत
फोन : (०४०) २३०१ ६०३३; फेक्स : (०४०) ६००३ / ३२

Indian Institute of Technology Hyderabad
Kandi – 502 284, Sangareddy, Telangana, INDIA
Phone: (040) 2301 6033; Fax: (040) 2301 6003 /32

31 July 2023

TO WHOM IT MAY CONCERN

This is to certify that **Ms. Fatima Sarwar** (B. Tech + M.Tech dual degree Biotechnology, 5th year) of **Integral University, Lucknow**, worked as a dissertation trainee at the **Circadian Rhythms and Disease Biology Laboratory, Department of Biotechnology, Indian Institute of Technology Hyderabad**, from **1st Feb to 31st July 2023**. She successfully completed her dissertation project entitled **'Understanding the chronotherapeutic efficiency of anti-breast cancer pharmacological agents.'** During her tenure, she has learned metabolomics, mammalian cell culture maintenance, RNA and protein extraction, their quantification, and SDS-PAGE. Also, she was trained in different bioinformatic tools such as CircaDB, DAVID, and PANTHER.

Fatima demonstrated a solid commitment to learning during the training period and actively participated in various laboratory activities. She showed a keen interest in biotechnology and consistently exhibited high enthusiasm and dedication.

Any findings/data generated during this project are solely counted as a property of the Circadian Rhythms and Disease Biology Laboratory, Department of Biotechnology, Indian Institute of Technology Hyderabad.

This project could be submitted to the **Integral University, Lucknow** in partial fulfillment of the requirement for **Fatima's B. Tech + M.Tech dual degree Biotechnology.**

Yours faithfully,

Dr. Sandipan Ray, Ph.D., MRSB
Assistant Professor and Group Leader
Circadian Rhythms and Disease Biology Laboratory
Department of Biotechnology
Indian Institute of Technology Hyderabad
Kandi, Sangareddy 502285, Telangana, India
Email: sandipan.ray@bt.iith.ac.in
Mob: +91 7439865060

Dr. Sandipan Ray, Ph.D., MRSB
Assistant Professor
Department of Biotechnology
Indian Institute of Technology Hyderabad
Kandi, Sangareddy-502285, Telangana, India

INTEGRAL UNIVERSITY
LUCKNOW - INDIA
Phone No.: +91(0522) 2890812, 2890730, 3296117, 6451039, Fax No.: 0522-2890809
Kursi Road, Lucknow-226026 Uttar Pradesh (INDIA)

CERTIFICATE BY INTERNAL ADVISOR

This is to certify that **Fatima Sarwar**, a student of **B.Tech-M.Tech Dual Degree Biotechnology** (5th Year/ 10th Semester), Integral University has completed her six months dissertation work entitled **"Understanding the chronotherapeutic efficiency of anti-breast cancer pharmacological agents"**, successfully. She has completed this work from Indian Institute of Technology Hyderabad (IITH) under the guidance of Dr. Sandipan Ray, Ph.D., MRSB, Assistant Professor and Group Leader, Circadian Rhythms and Disease Biology Laboratory, Indian Institute of Technology Hyderabad (IITH). The dissertation was a compulsory part of her. **B.Tech-M.Tech Dual Degree Biotechnology** degree**.**

I wish her good luck and bright future.

Dr. Reena Vishvakarma
Assistant Professor
Department of Bioengineering
Faculty of Engineering & Information Technology

INTEGRAL UNIVERSITY
LUCKNOW - INDIA
Phone No.: +91(0522) 2890812, 2890730, 3296117, 6451039, Fax No.: 0522-2890809
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I wish her good luck and bright future.

Dr. Alvina Farooqui
Professor and Head
Department of Bioengineering
Faculty of Engineering & Information Technology

ACKNOWLEDGEMENT

Firstly, I am really grateful and fortunate to have the humble association, exemplary guidance and profuse supervision of **Dr. Sandipan Ray, Group Leader, Circadian Rhythms and Disease Biology Laboratory, Indian Institute of Technology Hyderabad (IITH),** for selecting and allowing me to carry out the dissertation project at Indian Institute of Technology Hyderabad (IITH). His constant encouragement throughout the training was a stimulus to achieve better every day.

I must express my very profound gratitude to the senior colleagues in the lab Ms. Apoorva Bhatnagar, Mr. Sourbh Rankawat, and Mr. Sandip Das for imparting their impeccable knowledge and unfeigned help and concern. They attended to all my queries and invested their precious time in making me learn the scientific techniques conscientiously during my stay at IIT Hyderabad.

Next, I would like to express my gratitude and special thanks to **Dr. Alvina Farooqui,** Professor and Head, Department of Bioengineering Faculty of Engineering & Information Technology for allowing me to join the laboratory of Indian Institute of Technology Hyderabad.

Also, I would like to extend the acknowledgement to the Chancellor, Prof. S. W. Akhtar, the Pro Chancellor, Dr. Syed Nadeem Akhtar, the Vice Chancellor, Prof. Javed Musarrat, and Prof. (Dr.) Tahseen Usmani, Dean, Integral University for their support and acceptance in undertaking this project at Indian Institute of Technology Hyderabad.

I am gratefully indebted to my Internal Advisor, Dr. Reena Vishvakarma for her constant support, encouragement, and guidance throughout the project.

Moreover, I would like to appreciate Prof. (Dr.) Mohd Kalim Ahmad Khan and Dr. Ashish for their valuable guidance and suggestions. In addition, I would like to thank Dr. Reena Vishvakarma, course coordinator, Dr. Khwaja Osama, U.G. coordinator, Prof. (Dr.) Roohi, P.G. coordinator, the faculty members of the department and the support staff, for their co-operation, affection, and encouragement during my project pursuit.

Above all, I give thanks to the Almighty Allah for all the countless blessings which helped me in completing the present project work. My upmost gratitude to my parents Sarwar Ahmed and Anjum Muzaffar, and sister Dr. Umme Kulsoom for their unconditional support, love, and coherent suggestions which helped me to navigate the ideas to the project.

Date: 31 July 2023 Fatima Sarwar

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LIST OF ABBREVIATIONS AND SYMBOLS

- TTFL: Transcriptional–Translational Feedback Loop
- SCN: Suprachiasmatic Nucleus
- CLOCK: Circadian Locomotor Output Cycles Kaput
- BMAL1: Brain and Muscle Arnt-Like Protein 1
- IARC: International Agency for Research on Cancer
- MPP: Mass Profiler Professional
- NAD/NADH: Nicotinamide Adenine Dinucleotide/reduced form of Nicotinamide Adenine Dinucleotide
- NADP/NADPH: Nicotinamide Adenine Dinucleotide Phosphate/reduced form of Nicotinamide Adenine Dinucleotide Phosphate
- AMP/ATP: Adenosine Monophosphate/Adenosine Triphosphate
- TNBC: Triple-Negative Breast Cancer
- µM: Micromolar
- µl: Microlitre
- µm: Micrometre
- ml: Millilitre
- rpm: Revolution per minute

1. INTRODUCTION

Breast cancer is a type of cancer that develops from breast tissue. Breast lumps, breast shape changes, dimpling of the skin, milk rejection, fluid leaking from the nipple, or a red or scaly patch of skin are all signs of breast cancer. Breast cancer has a huge global impact with 2.3 million women diagnosed in 2020, accounting for 685 000 deaths globally (Li Y *et al.*, 2022). With four times as many cases in Western Europe as in Middle Africa and Eastern Asia, the disease is more common in the developed world. This shows that elements of a contemporary western lifestyle might affect the development and spread of breast cancer (Blakeman V *et al.*, 2016).

An internal biological clock known as a **circadian rhythm** regulates an organism's physiology and behavior by anticipating day/night cycles. Circadian rhythms are physiological or behavioral oscillation with a period of ~24 h, which is sustained in constant conditions and are entrained by external cues such as light. The word circadian stems from the Latin '*circa'* meaning 'about' and '*diem*' meaning 'day' (Patke A *et al.*, 2020). Circadian rhythms are evolutionarily conserved from cyanobacteria to fungi, plants, and animals (Bell-Pedersen D *et al.*, 2005). Although it has long been known that organisms adjust their physiology and behavior in a circadian manner in response to the time of day, the presence of an endogenous circadian clock was proven in the 20th century. In 2017, Jeffery C. Hall, Michael Rosbash, and Michael Young were awarded Nobel Prize in Physiology or Medicine for their discoveries of the molecular mechanisms regulating the circadian rhythm (Huang RC, 2018).

The fundamental components of a circadian system are a self-sustaining 24-hour rhythm generator or oscillator, entraining mechanisms that connect the internal oscillator to external stimuli (referred to as zeitgebers, i.e., timekeepers), such as light, and output mechanisms to enable the timely scheduling of physiological processes. The circadian clock is controlled genetically by a set of genes known as 'clock genes,' and mutations in clock genes can alter rhythmic behavior in various animals, including humans and insects, as well as in plants, fungi, and bacteria (Patke A *et al.*, 2020). The circadian clock orchestrates many aspects of human physiology, and disruption of this clock has been implicated in various pathologies, ranging from cancer to metabolic syndrome and diabetes.

Circadian metabolism includes 24-h oscillating biochemical changes in tissue, cell, and sub-cellular compartment throughout human physiology (Dyar KA and Eckel-Mahan KL, 2017). Many intermediates of carbohydrate, protein, and lipid metabolism show 24-hour rhythmicity under the influence of cellular core clock machinery. The rhythmic metabolites are essential because they provide key mechanisms by which circadian clocks across the body communicate- whether in alignment or misalignment. Consequently, the blood levels of the majority of intermediary metabolites, such as glucose, amino acids, and lipids, fluctuate, reaching their highest levels during wakefulness and activity. Our body uses clock-controlled mechanisms of cellular metabolite uptake and release to align with the external cues and to dampen excessive fluctuations since these oscillations are synchronised with environmental time (Reinke H & Asher G, 2019). Additionally, clock genes have a significant impact on metabolism by regulating gluconeogenesis, insulin sensitivity, and systemic blood glucose oscillation. In numerous organisms, it has been shown that several small metabolites are crucial for the 24-h rhythmicity of the cell (Dyar KA and Eckel-Mahan KL, 2017). Also, tissue rhythmicity is driven by oscillating metabolites in the circulation. The rhythmic circulating metabolites are linked to metabolic disruption when aberrantly released. The cellular concentration of certain circadian metabolites such as heme, NAD/NADH, NADP/NADPH, AMP/ATP, acetyl co-enzyme A, polyamine, etc can affect the function of several circadian transcriptional regulators by modulating post-translational modifications, protein-protein interactions, and protein-DNA interactions (Panda S, 2016). This will help in regulating gene expression. A study conducted in 2012 demonstrated that nearly 15% of all metabolites identified in human plasma and saliva are controlled by circadian clocks (Dallmann R *et al*., 2012). Many more studies that have thus been conducted are important in navigating towards the recognition of new metabolic pathways which are under circadian control.

Disruption of homeostasis may contribute to the tumorigenesis process. There are numerous metabolic anomalies in cancer cells as they modulate their metabolism to survive better in hypoxic conditions which persist in the cancer microenvironment. Cancer cells predominantly use ‘aerobic glycolysis’ rather than the 'usual' citric acid cycle and mitochondrial oxidative phosphorylation for energy production resulting in lactic acid fermentation, which is known as the Warburg effect. Hence is accompanied not only by abnormalities in proliferation, metastasis, and invasion but also by metabolic disorders.

Factors such as drinking alcohol, smoking tobacco, consistent late-night eating, radiation exposure, shiftwork, and genetic mutations, are some risk factors for breast cancer. Shift work has been recently classified as "probably carcinogenic to humans" (Group 2A) (Erren TC *et al*., 2010). Also, it is one of the many reasons for circadian disruption. Circadian disruption is a significant factor in breast cancer development. In a recent study conducted revealed that modern life including hypercaloric diets, sedentary routines, artificial illumination at night, disrupted sleep, shiftwork, and jetlag may cause circadian disorganization promoting higher cancer risk and metabolic disorders (Wagner PM *et al.*, 2019). Changes in the activity of key regulators such as CLOCK and SIRT1 may lead to cancer by modulating metabolic pathways such as glycolytic and gluconeogenic enzymes (Sahar S and Sassone-Corsi P, 2009). The metabolic regulators i.e, SIRT1 and AMPK signaling pathways, which are both essential for regulating cell proliferation, apoptosis, and tumor suppressor pathways, could be directly impacted by clock disruption leading to breast cancer (Blakeman V *et al*., 2016).

Breast tumours that are more aggressive often exhibit higher rates of glycolysis, proliferation, and redox pathways. In triple-negative breast cancer (TNBC), the glucose transporter GLUT-1 is highly expressed. The metabolism of glutamine is crucial in meeting the energy requirements of cancer cells. Currently, more than 30 endogenous metabolites in breast tissue have been identified. In comparison to healthy tissue or benign tumours, breast cancer tissue had higher levels of choline, lower levels of glycerophosphocholine, and lower levels of glucose. The levels of glutamine, lipids, serine, protein translation, and cholesterol metabolism are also influenced by a number of pathways, all of which have been shown to be elevated in breast cancer. The impact of these metabolic changes on the development or prevention of breast cancer can be used as a biomarker for diagnosis and potential treatment (Subramani R *et al*., 2022).

Metabolomic analysis of cells and tissues has emerged as an important technique for studying cellular biochemistry. Circadian metabolomics relates to time prediction, which is successfully used in chronotherapy. Pharmacometabolomics is used to understand drug pharmacokinetics and pharmacodynamics. However, taking into account the toxicity and efficacy of the drug in the body, it might be easier to determine the best time to administer drugs if we had a better understanding of circadian variations of metabolites throughout the day and in particular environmental situations. The circadian metabolism is better understood by global metabolomics experiments. They reveal whole pathways of

related metabolites that are changing in concert throughout the circadian cycle. Mass spectrometry can be carried out in a high-throughput setting coupled with liquid chromatography (LC-MS), leading to enhanced sensitivity (Dyar KA and Eckel-Mahan KL, 2017).

Circadian misalignments can be treated and prevented by applying the chronobiology principles and techniques, aiming for a better chronotherapeutic mechanism. Apart from classical treatments, the new therapeutic approach, chronotherapy, includes: (i) 'clocking the drugs' and (ii) ‘drugging the clock’, which contribute towards ameliorating the existing drug administration and targeted drug delivery. Also, many small molecules have been discovered that pharmacologically modulate the circadian clock components and display potential as preventive and/or therapeutic strategies for chronic diseases such as different types of cancers.

In this study, we have attempted to understand the dosing-time dependency of drugs that are already in use for the treatment of breast cancer by performing an in silico analysis of rhythmic anti-breast cancer drug target. Secondly, we endeavoured to study the untargeted global metabolomics on mouse 4T1 cells treated with SR9009 (a REV-ERB agonist and clock modulator) versus DMSO (control). It is already present in published papers that SR9009 exhibits anti-tumor activity in glioblastoma (Wagner PM *et al*., 2019) and lung (Shen W *et al.*, 2020) cancer cells. Moreover, a recent study revealed SR9009 considerably affects metabolism and decreases fat mass, plasma triglycerides, and cholesterol levels in diet-induced obese mice (Solt LA *et al.*, 2012). However, it is unknown how SR9009 changes the rhythmicity of cell metabolites in a 24-hour period to affect metabolism, which was targeted in this study. This helped in establishing the connecting link between metabolism, breast cancer and clock circuitry.

Aim: <u>Understanding the chronotherapeutic efficiency of anti-breast cancer pharmacological agents</u>

Objectives:

(i) Analyzing the circadian clock control of therapeutic drug targets of anti-breast cancer drugs to understand their dosing time dependency

(ii) In silico docking analysis of clock modulator, SR9009 with anti-breast cancer drug targets

(iii) To comprehend the effect of SR9009 on the metabolism of breast cancer

2. REVIEW OF LITERATURE

2.1 Breast Cancer

Breast cancer accounts for 1 in 8 cancer diagnoses and 2.3 million new cases overall in females, overtaking lung cancer as the most frequently diagnosed disease in the world (Arnold M *et al*., 2022; Sung H *et al*., 2021). It was by far the most often diagnosed cancer in women in 2020, accounting for 25% of all female cancer cases, and its burden has been rising around the world, especially in transitioning nations (Heer E *et al*., 2020). About 685,000 female cancer deaths occurred in 2020 representing 1 in 6 or 16% of all female cancer fatalities. However, the age distribution of cases and deaths varied significantly across globe regions, with over 80% of cases and 90% of deaths occurring in Northern America as well as Western and Northern Europe, compared to 43% of cases and 49% of deaths occurring in Middle Africa at postmenopausal ages. Breast cancer varies greatly geographically between different nations and areas of the world. In women, breast cancer was the most diagnosed cancer in 157 (out of 185) countries, followed by cervical cancer in 23, mainly sub-Saharan African, countries and the leading cause of cancer death in 110 countries, followed by cervical cancer in 36 and lung cancer in 25 countries. Yet, great geographic variation exists in incidence and mortality rates (Arnold M *et al*., 2022).

Breast cancer is a disease wherein an individual's breast tissue's cells alter and divide out of control, frequently producing a lump or mass. The milk glands (lobules) or the tubes (ducts) connecting the milk glands to the nipple are where the majority of breast cancers start (Breast Cancer Facts & Figures 2022-2024).

Breast tumours typically begin as ductal hyperproliferation, and after being repeatedly stimulated by numerous carcinogenic stimuli, they progress to benign tumours or even metastatic carcinomas (Sun YS *et al.*, 2017). Breast lumps, altered breast shape, dimpling of the skin, milk rejection, fluid emerging from the nipple, an inverted nipple, or a red or scaly patch of skin can all be indicators of breast cancer. Patients with distant spread of disease may experience bone pain, swelling lymph nodes, shortness of breath, or skin that is yellow (Saunders CM and Jassal S, 2009).

Breast cancer is caused by a complex interplay of environmental, reproductive, and hormonal variables. Smoking, extended oral contraceptive usage, and ethnicity have also been linked to increased breast cancer risk. Additionally genetic predisposition, positive family history, chronic exposure to estrogen such as delayed age at first pregnancy, early menarche, low parity, and short-term breastfeeding practices have been implicated in the evolution of breast cancer (Olorunfemi G *et al.*, 2023).

2.1.1 Breast cancer types

There are various grading systems used to categorize breast cancers.

- **Ductal Carcinoma in Situ (DCIS):** DCIS is also called intraductal carcinoma or stage 0 breast cancer. DCIS is a non-invasive or pre-invasive breast cancer. In this, the mammary duct lining cells have transformed into cancer cells, but they haven't spread through duct walls into the close by breast tissue.
- **Lobular carcinoma in situ (LCIS):** In this, abnormal cells develops from lobules (glands in the breast that make milk). LCIS seldom develops into invasive carcinoma and is curable.
- **Invasive (infiltrating) ductal carcinoma (IDC):** The most typical form of breast cancer is IDC. Invasive (or infiltrating) ductal carcinomas (IDC) make up about 8 out of 10 cases of invasive breast cancer. IDC starts in the cells that line a milk duct in the breast. From there, the cancer spreads into the adjacent breast tissues after penetrating the duct's wall. The lymphatic and bloodstream of the body may allow it to spread (metastasize) to other areas of the body.
- **Invasive lobular carcinoma (ILC):** About 1 in 10 invasive breast cancers is an invasive lobular carcinoma (ILC). ILC starts in the breast's lobules (milk glands) and spreads to nearby healthy tissue. It can also spread to different body areas via the lymphatic and blood systems. It is the second most frequent form of breast cancer.
- **Paget disease:** The skin of the nipple and the areola (the black circle surrounding the nipple) are involved in this rare form of breast cancer.
- **Triple-negative Breast Cancer (TNBC):** It accounts for about 10-15% of all breast cancers. In this, breast cancer cells have tested negative for

hormone epidermal growth factor receptor 2 (HER-2), estrogen receptors (ER), and progesterone receptors (PR).

- **Inflammatory breast cancer (IBC):** It is rare and accounts for only 1% to 5% of all breast cancers. In this, the cancer cells infiltrate the skin and lymph vessels of the breast. It is aggressive and fast-growing breast cancer. It frequently results in no clear tumour or lump that is localised in the breast. However, symptoms start to show up when the breast cancer cells obstruct the lymph vessels.
- **Metastatic Breast Cancer:** Breast cancer in Stage 4 is metastatic cancer. Other bodily areas get affected by this cancer's spread. Typically, this involves the brain, bones, liver, lungs, or liver.
- **Medullary Carcinoma:** It accounts for 3% of all breast cancer types. While the tumour frequently appears on a mammography, it is not always felt as a lump. It can occasionally resemble a spongy breast tissue.
- **Tubular Carcinoma:** Tubular carcinoma cells exhibit a characteristic tubular form when seen under a microscope, accounting for 2% of all breast cancer diagnoses. Women over the age of 50 are more likely to develop this kind of breast cancer, which often responds well to hormone therapy.
- **Mucinous Carcinoma (Colloid):** Breast tumours between 1% and 2% are mucinous carcinomas. Mucus production and poorly defined cells are the major characteristics that set them apart from one another. It typically has a good prognosis as well.

Molecular subtypes

The four broad molecular subtypes are Luminal A, Luminal B, basal-like, and HER2-enriched (human epidermal growth factor receptor 2)**.** Cancers that test positive for estrogen receptor (ER), progesterone receptor (PR), or both are referred to as hormone receptor positive (HR+) cancers.

- **HR+/HER2- :** Surrogate for Luminal A. With 68% of cases, this is the most prevalent molecular subtype of breast cancer.
- **HR+/HER2+ :** Surrogate for Luminal B. It makes up around 10% of all breast malignancies, and is frequently of a higher grade than Luminal A.

- **HR-/HER2- :** Surrogate for basal-like. Because they test negative for all three biomarkers, these tumours are often known as triple-negative breast cancers (TNBC). This is more aggressive type of tumor.
- **HR-/HER2+ :** Surrogate for HER2-enriched. It accounts for 4% of all diagnoses and is least common breast cancer subtype.

2.1.2 Risk Factors

- **Lifestyle:** Even among very light drinkers (women who consume less than half of one alcoholic drink per day), drinking alcohol raises the risk of breast cancer (Choi YJ *et al*., 2018). The danger is greatest for heavy drinkers. About one in ten incidences of breast cancer worldwide are brought on by women who drink alcohol. Alcohol consumption is one of the most prevalent modifiable risk factors (McDonald JA *et al*., 2013). Smoking tobacco also raises the risk of developing breast cancer (Johnson KC *et al*., 2011; Knight JA *et al*., 2017). The extra fat in the modern western diet, particularly saturated fat, is linked to mortality and a poor prognosis in breast cancer patients (Makarem N *et al*., 2013). Obesity and breast cancer do not correlate in linear. According to studies, those who gain weight quickly as adults are more at risk than those who have been overweight since childhood. **Apart from this, circadian disruptions related to shift-work** (Wang X-S *et al*., 2011) **and routine late-night eating** (Marinac CR *et al*., 2016) **are also linked to breast cancer etiology**. Lack of physical activity, hormone therapy, and radiations are potential risk factors for breast cancer.
- **Genetics:** Almost 5 to 10 percent of cases are considered to be genetically based. Genetics has a larger impact by causing a hereditary breast-ovarian cancer syndrome in less than 5% of cases. Those with the BRCA1 and BRCA2 gene mutations are included in this. These mutations make up up to 90% of all genetic influences, and individuals who have them have a 60–80% increased risk of developing breast cancer. p53 (Li-Fraumeni syndrome), PTEN (Cowden syndrome), STK11 (Peutz-Jeghers syndrome), CHEK2, ATM, BRIP1, and PALB2 are further important mutations (Gage M *et al*., 2012). Other related genes include HER2, EGFR, c-Myc and Ras

(Sun YS *et al.*, 2017). Circadian clock molecules REV-ERB and TIM work to promote tumour growth in breast cancer, while CRY2 and BMAL1 prevent tumour growth (Liu H *et al.*, 2022).

- **Medical conditions:** Breast modifications such as atypical ductal hyperplasia and lobular carcinoma in situ that are present in benign breast disorders like fibrocystic breast modifications are associated with a higher chance of developing breast cancer. Breast cancer risk may also be increased by diabetes mellitus. The chance of developing breast cancer seems to be increased by autoimmune conditions like lupus erythematosus (Anothaisintawee T *et al.*, 2013).
- **Hormones:** Hormone levels are linked to the main causes of sporadic breast cancer. Both endogenous and exogenous estrogens are associated with the risk of breast cancer. The endogenous estrogen is usually produced by the ovary in premenopausal women and ovariectomy can reduce the risk of breast cancer. The main sources of exogenous estrogen are the oral contraceptives and the hormone replacement therapy (HRT). According to several studies, the use of HRT can increase the breast cancer risk (Sun YS *et al.*, 2017). Also, breast cancer risk is enhanced by elevated prolactin levels in the blood (Wang M *et al*., 2016).
- **Reproductive factors:** Early menarche, a late menopause, a late age at first pregnancy, and low parity are reproductive characteristics that can raise the risk of breast cancer. Breast cancer risk is increased by 3% for every additional year delay in menopause (Sun YS *et al.*, 2017).
- **Aging:** Due to the strong correlation between the prevalence of breast cancer and advancing age, ageing is one of the most significant risk factors for breast cancer. Women over 40 and 60 years old, respectively, accounted for 99.3% and 71.2% of all breast cancer-related fatalities in America in 2016 (Siegel RL *et al.*, 2017). Therefore, women aged 40 or older must get a mammogram screening in advance.
- **Family history:** About 25% of all breast cancer cases are related to family history. Women are more likely to contract this illness if their mother or sibling has a breast cancer. According to a UK cohort research involving over 113,000 women, women who have one first-degree relative who has

breast cancer are 1.75 times more likely to get the disease than those who don't have any affected relatives (Brewer HR *et al*., 2017). The mutations in breast cancer-related genes like BRCA1 and BRCA2 are partly responsible for the inherited propensity for breast cancer.

2.2 Circadian Rhythm

The circadian clock is a hierarchical timing system that coordinates with the environment to preserve temporal tissue homeostasis while regulating physiological, behavioural, and metabolic activities during a 24-hour day-night cycle (Dierickx P *et al*., 2018; Partch CL *et al*., 2014). For optimum fitness, most organisms prepare for daily changes in their environment, such as variations in light, temperature, and the availability of food. Animals, plants, fungi, and bacteria have all been found to exhibit prominent daily behavioral and/or physiological rhythms. These rhythms are known as circadian rhythm, which come from the Latin "circa diem," or "about a day," and are the consequence of an internal, autonomous timekeeping mechanism known as the circadian clock (Patke A *et al.*, 2020).

The French astronomer Jean Jacques d'Ortous de Mairan made the first scientific observation of circadian rhythm in 1729. He placed the mimosa plant in a light-tight dark room and observed that the plant continued to unfold its leaves in the morning and close them in the evening (Kreitzman L & Foster R, 2004; Ibáñez C, 2017). The first clock gene mutants were discovered by Ron Konopka and Seymour Benzer in the fruit fly Drosophila in the 1970s, providing genetic evidence for behaviour. The molecular biology era of chronobiology began with the cloning of the fly period (per) gene in the 1980s. The components of the molecular clockwork were successively discovered in different species (in mammals, about a dozen genes are involved) and the molecular mechanism of circadian rhythm generation, the TTFL, was deciphered. Michael Rosbash, Jeffery C. Hall, and Michael Young received the Nobel Prize in Physiology or Medicine in 2017—more than 25 years after these accomplishments were recognized (Kramer A *et al*., 2022).

2.2.1 Characteristics of Circadian rhythm

- **Periodicity:** rhythm with a periodicity of about 24 hours, even in the absence of an environmental cycle (called a free-running rhythm).
- **Self-sustained:** oscillations can persist even in the absence of environmental cues. They occur under natural conditions and continue to cycle under laboratory conditions devoid of any external time-giving cues from the physical environment (e.g., under constant light or constant darkness).
- **Entrainable:** circadian clocks can be synchronized by external cues known as a zeitgeber such as the light-dark cycle.
- **Temperature compensated:** Any moderate variations in ambient temperature do not affect the period of circadian oscillation.
- **Ubiquity:** Numerous biological processes and organisms have circadian rhythms, which share many of the same characteristics and even some of the same phase-response curves to light (Vitaterna MH *et al*., 2001).
- **Cellular level:** Because the rhythms of unicellular organisms (such algae or the dinoflagellate Gonyaulax) are so similar to those of highly complex mammals, circadian rhythms appear to be produced at the cellular level (Vitaterna MH *et al*., 2001).

2.2.2 Anatomy of Circadian clock organization

The human circadian system is hierarchically organized (Kramer A *et al*., 2022). In mammals, the input pathways, the central pacemaker, and output pathways make up the three parts of the molecular circadian clock in mammals. The suprachiasmatic nucleus (SCN) of the hypothalamus, which houses the central pacemaker, receives information from environmental stimuli (such as light) via input channels. To produce daily circadian outputs, the SCN synchronizes numerous single-cell circadian oscillators. Circadian oscillations are produced when output pathways translate commands from the central pacemaker into these oscillations, which control physiological and behavioral processes in peripheral organs and tissues (Lin HH & Farkas ME, 2018).

The Molecular Circadian oscillations in mammals are generated through two transcriptional/translational feedback loops (TTFLs). The core clock genes and proteins that make up circadian clocks are cellular entities. The core loop involves four core clock genes: Circadian Locomotor Output Cycles Kaput (CLOCK) and

brain and muscle Arnt-like protein 1 (BMAL1), are the activators; and Period (PER1, PER2, and PER3) and Cryptochrome (CRY1 and CRY2), are the repressors. The CLOCK-BMAL1 heterodimer attaches to an E-box DNA promoter in the morning, initiating the transcription of PER, CRY, and other clock-controlled genes. During night, PER and CRY proteins dimerize and translocate from the cytoplasm to the nucleus, where they associate with the CLOCK-BMAL1 complex and suppress its transcriptional activity at the E-box site. Through the degradation of PER and CRY by ubiquitin-dependent pathways and casein kinases (CKIδ and CKIε), which also regulate the time of PER and CRY's entrance to the nucleus, the suppression of CLOCK-BMAL1 is stopped. Following the degradation of PER and CRY, the cycle restarts with a 24-hour periodicity (Lin HH & Farkas ME, 2018).

The secondary TTFL is mainly carried out by transcriptional activation of the retinoid-related orphan receptors (RORs a, b, c) and repression of REV-ERBα/REV-ERBβ. REV-ERB binds to the ROR regions in the BMAL1 promoter, inhibiting BMAL1 transcription and driving the rhythmic oscillation of BMAL1. On the other hand, RORa and RORb promote BMAL1 expression. Robustness against environmental perturbations is provided by the collaboration between the two TTFLs with other kinases and phosphatases, which are essential for controlling the period, phase, and amplitude of oscillations. Additionally, this network aids in maintaining circadian timing and adjusting phase delays to align with internal physiology (Lin HH & Farkas ME, 2018).

2.2.3 Circadian rhythm disruption and consequence to human diseases

The interference with circadian rhythms is one of the main effects of a modern lifestyle. This causes a number of medical diseases, such as sleep disorders, depression and metabolic diseases. Human diseases such as sleep disorders, psychiatric and cardiovascular diseases, systemic chronic inflammation, impaired immune responses against pathogens, increased tumour risk, and worsened reactions to allergens and autoantigens have all been linked to circadian disruption brought on by extrinsic factors such as artificial light, shift work, travel across time zones, and social jetlag (the misalignment of biological and social time). For instance, the extensive epidemiological Nurses' Health Study revealed that shift work and irregular circadian rhythms are linked to cancer

(Schernhammer ES *et al.*, 2003). The risk of dyslipidemia, hypertension, type 2 diabetes, heart attacks, and multiple sclerosis is significantly increased by shift work. Shift work with circadian disruption or chronodisruption was identified by the International Agency for Research on Cancer (IARC) as a potential human carcinogen in October 2007 (Erren TC *et al.*, 2010). Social jetlag is linked to greater rates of obesity, diabetes, cardiovascular disease, and cancer, as well as increased alcohol and cigarette use. Social lifestyle influences the sleep timings in children and adult leading to health problems. Blue light is a predominate zeitgeber and its exposure is common in modern societies during night time (Ruan W *et al.*, 2021). This interferes with neuroendocrine circuits, disrupting sleep and delaying the rythms of melatonin and cortisol. Intrinsic factors such as ageing and genetics which involves the clock genes leads to various pathological. For instance, changes in the melatonin receptor 1B (MTNR1B) gene or variations in a number of clock genes, such as BMAL1 and PER2, are linked to an increased risk of certain metabolic illnesses (Kramer A *et al.*, 2022). With ageing, many metabolic rhythms show gradual dampening. Evidence of age-related declines in the amplitude of clock gene expression rhythms in clocks other than the master pacemaker highlights these changes. Dampening of these rhythms is thought to be a factor in the increased risk of metabolic disorders in older persons, including diabetes, dyslipidemia, and hypertension (Hood S & Amir S, 2017). Disturbances in the expression of regulators such as CLOCK and SIRT1 leads to cancer by causing higher defects in metabolic pathways, for example alterations of glycolytic and gluconeogenic enzymes (Sahar S and Sassone-Corsi P, 2009). The circadian gene malfunction caused by single nucleotide polymorphisms (SNPs), deletions, epigenetic change, and deregulation is significantly linked to cancer risk, according to research studies. Among the clock gene family members, PERs and BMAL1 are tumor suppressors, Whereas CLOCK and TIM act as tumor promoters in diversified cancers. Others, such as REV-ERBα and CRYs, play different or even opposite roles in other types of cancer (Liu H *et al.*, 2022).

2.2.4 Circadian rhythm and Breast cancer

The link between circadian rhythm abnormalities and an increased risk of breast tumour formation was initially discovered in the 1960s, and circadian genes may serve as tumour suppressors (Lin HH & Farkas ME, 2018). Studies from earlier

decades suggested that changes to circadian rhythms may also hasten the proliferation of breast epithelial stem cells, cause the development of the mammary gland, and enhance the occurrence of spontaneous breast tumours in mammals (Aubert C *et al.*, 1980). Using real-time bioluminescent imaging of mammary explants from PER2 :: Luciferase mice, clocks have been found inside the breast epithelium. The expression of the central clock proteins BMAL1 and PER2 varies rhythmically every day. In the mammary epithelium, clock gene abnormalities can cause cell cycle disruption. This interference results in incorrect cell division, greater vulnerability to breast cancer, and more aggressive tumour growth. Human sporadic and familial breast cancer cells express less Per genes than normal breast cells; this may be due to methylation of some Per promoter regions. Loss of Per2 impairs p53, which reduces apoptosis and causes a buildup of damaged cells. The circadian clock's influence on cell metabolism can both cause and spread cancer. The SIRT1 and AMPK signalling pathways, which are both essential for regulating cell proliferation, apoptosis, and tumour suppressor pathways, could be directly impacted by clock disruption (Blakeman V *et al.*, 2016).

Altered Clocks' Effects on Cancer by epidemiological evidences:

Since the light bulb was created in 1879, lifestyles have undergone a significant change. Since then, human daily activities have shifted into the night including the "night-shift" jobs. According to the U.S. Bureau of Labor Statistics, in 2016, the majority of those who were working in 2016 worked in the service sector (80.3%), which includes transportation, health care, and social assistance, followed by manufacturing (7.9%) —areas with high rates of shift work. These types of schedules can lead to disruption of the sleep–wake cycle and circadian time organization, in addition to exposure to light at night (LAN) for long periods of time (Lewy AJ *et al.*, 1980). Perturbations to sleep and circadian rhythms can cause metabolic changes and immune suppression, which can lead to various health problems, including diabetes, obesity, and cardiovascular disease, in addition to cancer. As a result, the International Agency for Research on Cancer has classified "shift-work that involves circadian disruption" as a "potential carcinogenic to humans (Group 2A)" (Baan R *et al.*, 2009).

Another environmental factor linked to abnormal circadian rhythms and an increased incidence of cancer is jet lag (Kettner NM *et al.*, 2016). Circadian

desynchrony, often known as jet lag, is a sleep problem caused by an imbalance between the internal body clock and the external light/dark cycle. This condition is frequently brought on by rapid transit through several time zones in short period of time. According to an early study conducted in Finland, flight attendants have a much higher prevalence of breast cancer (81.2/100,000) than women in general population (57.4/100,000) (Pukkala E *et al.*, 1995). A later, follow-up evaluation clearly suggested that the increased cancer incidence was connected to sleep rhythm disturbance brought on by excessive light exposure during the time when people should be sleeping, which led to dysregulated melatonin levels (Kojo K *et al.*, 2005). Additionally, a 2017 study that examined the impact of LAN exposure in the United States found that the greatest LAN was associated with a 14% higher risk of breast cancer than the lowest LAN (James P *et al.*, 2017).

Circadian Clocks and Breast Cancer Molecular Studies

Each tissue's clock genes have distinct roles and exhibit particular oscillation patterns. Clock gene expression levels vary depending on the extracellular milieu and the various stages of breast tissue development. Therefore, it is posited that clock gene disruption can impact normal breast biology and either cause or influence the development of cancer. Another link between circadian rhythms and breast cancer has been suggested by the alteration of nuclear hormone levels and signalling which alters the circadian rhythms. The disruption of PER2 in breast cancer has been associated with the oestrogen receptor-α (ER) signalling pathway. The relationship between PER2 suppression and ER stabilisation was initially discovered in 2007, and overexpression of PER2 in breast cancer cells was found to drastically restrict cell proliferation and increase apoptosis (Gery S *et al.*, 2007).

It has also been suggested that BMAL1 functions as a tumour suppressor. Loss of BMAL1 and hyperinsulinemia obesity were coordinated to speed up mitochondrial metabolism and alter the inflammatory tumour microenvironment by increasing macrophage recruitment and reducing CD8+ T cell infiltration. BC cells benefited from these actions in terms of survival and metastatic potential (Ramos CA *et al.*, 2020). In comparison to normal tissues, BC tissues exhibit considerably lower CRY2 expression and higher promoter hypermethylation of the CRY2 gene (Hoffman AE *et al.*, 2010). It has been suggested that CLOCK is a

tumour driver. Breast cancer tissues expressed CLOCK more than healthy breast patient tissues. Breast cancer growth was reduced and several cancer-related genes were downregulated when CLOCK was knocked down (Xiao L *et al*., 2014). REV-ERBα has a role in $HER2^+$ breast cancer. Poor clinical outcomes are associated with high TIM expression, particularly in patients with BC that is ER-positive. The modulation of TIM's impact on sphingosine metabolism which may be related to its carcinogenic potential (Liu H *et al.*, 2022). Role of clock genes is summarized in Table 2.1.

Table 2.1 Involvement of clock genes in breast cancer development (Lin HH & Farkas ME, 2018).

Circadian genes	Experimental study	Phenotype
CLOCK	Immunohistochemical assay(s) and qRT - PCR	Overexpressed in breast cancer cells; low expression in healthy breast tissue
	Knockdown(s)	Cell proliferation reduced; cancer-associated genes downregulated (CCL5, BDKRB2, and SP100)
BMAL1	qRT- PCR	Disrupted expression of mRNA in breast cancer cells
	Knockdown(s)	Cancer cell proliferation is promoted and *in vitro* invasion and *in vivo* tumor growth
PER1,2, and 3	Immunohistochemical assay(s) and qRT - PCR	Downregulation in ER-positive breast cancer cells
	Knockdown(s)	Disrupted circadian oscillation of other clock genes
	Overexpression	Inhibited cell growth and promoted apoptosis
CRY1 and 2	qRT - PCR	Disrupted expression of mRNA in breast cancer cells
REV-ERBα	RNAi screen	Co-expression in ERBB2-positive breast tumors [$HER2^+$ subtype]
REV-ERBβ	Overexpression	Provides protection to tumor cells against chemotherapy

2.2.5 Circadian Metabolomics

The disturbance of the equilibrium that underlies homeostasis is directly related to the tumorigenic process. The Warburg effect occurs when cancer cells primarily employ glycolysis rather than mitochondrial oxidative phosphorylation to produce energy for the growth of cancer cells even under aerobic conditions (Sahar S and Sassone-Corsi P, 2009). The imbalance in the intake and consumption of oxygen

caused by the rapid growth of cancer cells and the aberrant structure and function of the vascularization results in hypoxia, which forces cancer cells to adopt glycolysis as their primary energy source. While under normoxia, cancer cells prefer glycolysis as their main energy source due to abnormally activated oncogene signalling pathways and the tumour microenvironment, which means pyruvate is primarily converted into lactate rather than being incorporated into the tricarboxylic acid cycle (TCA cycle). Recently, more studies have proven that glycolysis is also involved in the activation of oncogenes such as phosphatidylinositol 3-kinase (PI3K) and hypoxia inducible factor-1 alpha (HIF-1A) shift in the tumor microenvironment such as hypoxia and acidosis (Liu C *et al*., 2021).

Circadian metabolites:

One of the most significant and enlightening areas of modern circadian biology study is the relationship between circadian and metabolic systems. In numerous organisms, it has been shown that a number of small metabolites are crucial for the 24-h rhythmicity of the cell. The metabolite nicotinamide adenine dinucleotide (NAD+), regulates the rhythmic circadian output at the level of gene expression of CLOCK-BMAL1 transcriptional complex and is also an activator of various NAD+-dependent sirtuin proteins. Moreover, the rhythmic activation of SIRT3 by NAD+ in the mitochondria induces oscillations in acetylation and activity of downstream enzymes crucial for mitochondrial oxidative function in addition to nuclear activation of the sirtuins (Dyar KA and Eckel-Mahan KL, 2017). Recently, it was discovered that polyamines oscillate and feedback the clock system. Effective glucose uptake into cells during nutritional abundance (active phase) or its release (as is the case for glucose-storing hepatocytes) during fasting (rest phase) are made possible by the rhythmic expression of glucose transporters (GLUTs). Due to the circadian activity of glutamine synthetase, the synthesis of glutamine, which is delivered in significant amounts from skeletal muscle as systemic fuel for other tissues, appears to be rhythmic. The extracellular lipases are rhythmically expressed and should facilitate the circadian mobilization of fatty acids for cellular uptake (Reinke H & Asher G, 2019). Hepatic gluconeogenesis, which is crucial for preserving glucose homeostasis in animals during periods of hunger, is controlled by the circadian cycle, most likely under the control of the cryptochromes (Cry1 and Cry2) via CLOCK and BMAL1. Acetyl-CoA is a

crucial molecule at the intersection of cytosolic and mitochondrial metabolism. It is involved in metabolism and also controls protein function by taking part in acetylation processes, which in turn controls gene expression (Ray S & Reddy AB, 2016). Hence most intermediary metabolites fluctuate in blood levels, with peak levels during awake and activity including glucose, amino acids, and lipids and important for clock functions. These studies give evidence for a bidirectional relationship between circadian rhythms and metabolism as well as a potential function for metabolic byproducts in directly changing and/or regulating the clock.

High-Throughput Metabolomics:

Global metabolomics research can help us understand circadian metabolism by showing entire pathways of linked metabolites that are changing in concert throughout the circadian cycle, just as genomics and transcriptomics studies have provided insight on mechanisms regulating gene expression. In order to comprehend how metabolism is altered in a time-dependent manner, it is necessary to grasp the relationship between metabolism and circadian function. Numerous favourable reviews of metabolomics experiments' technological components have previously been published. A high degree of circadian oscillation among metabolites has also been found by circadian metabolomics studies on rodent tissues, generally mirroring the diurnal rhythms of local tissue metabolism. Many human serum metabolomes have offered high-resolution results of significantly varying metabolic pathways across the circadian cycle. (Dyar KA and Eckel-Mahan KL, 2017).

Mass spectrometry can be carried out in a high-throughput setting with liquid chromatography (LC-MS), which often leads to enhanced sensitivity. Usually, the mass-to-charge value, retention time, fragmentation data, and isotopic detail are used to assign LC-MS data to a specific metabolite. Based on this knowledge, a number of programmes have been created to help in metabolite identification. It is still an active topic of research to determine the molecular processes that control circadian metabolite oscillations in cells and tissues and how closely they are related to cell/tissue-specific circadian clocks.

2.2.6 Circadian Chronomedicine

Different metabolic functions such as food intake, digestion, detoxification, breakdown, and storage of carbohydrates and fats are almost all regulated in a circadian way. Organs go through the classical absorption, distribution, metabolism, and elimination processes when exposed to xenobiotics (such as medications or environmental toxins), all of which are controlled by circadian clocks. Therefore, taking circadian rhythms into consideration when creating medicines and dosing plans has the potential to lead to improve illness outcomes (Lin HH & Farkas ME, 2018).

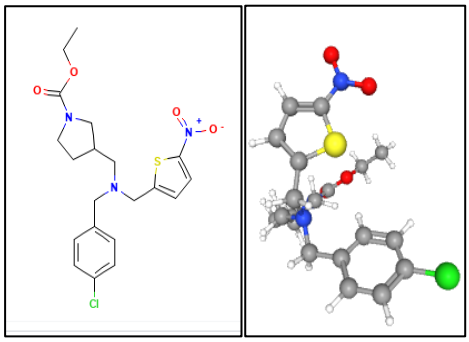
Chronotherapy is the practise of giving medication at a specific time of day when its effectiveness is greatest and its negative effects are at their lowest. It has shown potential in cancer treatment. Chronotherapy has three broad categories: (i) Interventions to maintain a strong circadian rhythm during feeding-fasting, sleep-wake, or light-dark cycles are referred to as "training the clock," (ii) to optimize the timing of the drugs to improve therapeutic efficacy referred to as "clocking the drugs," and (iii) targeting a circadian clock component using small molecule agent referred to as "drugging the clock" (Sulli G *et al*., 2018). The use of small molecules in research on disorders associated with the circadian rhythm has numerous benefits: Small molecules have several advantages over genetic techniques, which can lead to immutable alterations. They can be utilised in reversible, time- and dose-dependent ways, which can help us better understand the molecular circadian network. They can be used as lead structures for drugs development. CRYs, REV-ERBs, and RORs are some of the pharmacological targets of these small molecules.

SR9009: It is a synthetic pyrrole derivative i.e, derived from GSK4112, a selective agonist of REV-ERBα, that alters circadian rhythm and gene expression of BMAL1, PER1, and PER2 (Ruan W *et al*., 2021). Burris and associates created SR9009 that was more suited for in vivo applications in order to increase potency, effectiveness, and pharmacokinetics in the treatment of circadian-related metabolic illnesses, cancer and sleep disorders, the drug showed therapeutic efficacy of small molecule REV-ERB modulator (Cha HK *et al*., 2018). SR9009 in high concentrations was needed for anti-tumor effects in *in vivo* experiments, suggesting its therapeutic potential in the clinical practice (Battaglin F *et al*.,

2021). SR9009 considerably improves metabolic profile and decreases fat mass, plasma triglycerides, and cholesterol levels in diet-induced obese mice (Solt LA *et al.*, 2012). However, it is unknown how SR9009 changes the rhythmicity of metabolites in 24 hours to affect metabolism, which will be targeted in this study. This will help in establishing the connecting link between metabolism and clock circuitry.

Figure 2.1 represents SR9009 structure retrieved from PubChem database (National Center for Biotechnology Information, 2023).

Figure 2.1 2D structure and 3D conformer of SR9009 (PubChemCID:57394020)



Chemical Formula:

$C_{20}H_{24}ClN_3O_4S$

2D Structure **3D Conformer**

2.2.7 Metabolome coverage for untargeted metabolite profiling using LC-MS

The analytical chemistry technique known as liquid chromatography-mass spectrometry (LC-MS) combines the mass analysis capabilities of mass spectrometry (MS) with the physical separation capabilities of liquid chromatography (or HPLC).

Due to the high sensitivity and resolution, metabolomics techniques based on high performance liquid chromatography-quadruple-time-offlight mass spectrometry (HPLC-Q-TOF/MS) have been used to characterise changes in metabolites in cells, tissues, and biofluids under specific conditions as well as to identify biomarkers useful as therapeutic targets to reveal the metabolic processes and potential mechanisms of disease. These findings offer a solid scientific foundation for delving into the pathophysiological mechanisms behind breast cancer.

Liquid chromatography

In the process of physical separation known as liquid chromatography, the constituents of a liquid mixture are divided into two immiscible phases, referred to as stationary and mobile. Adsorption chromatography, partition chromatography,

ion-exchange chromatography, size-exclusion chromatography, and affinity chromatography are the five categories into which it is separated. The reverse-phase (RP) mode of the partition chromatography technique is widely used. It uses a nonpolar (hydrophobic) stationary phase and a polar mobile phase. In typical applications, the stationary matrix is made by attaching long-chain alkyl groups (such as n-octadecyl or C18) to the external and internal surfaces of irregularly or spherically shaped 5μm porous silica particles. The mobile phase is typically a mixture of water and other polar solvents (such as methanol, isopropanol, and acetonitrile). In HPLC, the sample of interest is typically injected into the mobile phase stream provided by a high-pressure pump in amounts of 10μl. The stationary phase bed is penetrated by the mobile phase carrying the analytes in a specific direction. Depending on their chemical affinity for the mobile and stationary phases, the mixture's constituent are separated. Up to 400 bar (5800 psi) of pressure is used to inject the liquid solvent (mobile phase) into a packed column containing the stationary phase. For reproducible chromatographic tests, a steady flow rate must be achieved under the high pressure. The components of the sample will flow out of the column at various times depending on how the mobile and stationary phases are divided. The most crucial part of the LC system, the column is built to resist the liquid's high pressure.

Mass spectrometry

The mass-to-charge ratio (m/z) of charged particles (ions) is determined by the analytical method known as mass spectrometry (MS). The ion source, mass analyzer, detector, data, and vacuum systems are the fundamental parts of a mass spectrometer.

The mass spectrum can be used to establish the mass of the analytes, as well as their elemental and isotopic makeup, or to clarify the sample's chemical composition. MS is an experiment that requires a vacuum ($1.33 * 10^{-2}$ to $1.33 * 10^{-6}$ pascal) and gas phase. There are different methods of ionization such as electrospray ionization (ESI), matrix-assisted laser desorption/ionisation (MALDI), Electron impact ionisation (EI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photo-ionization (APPI). Electrospray ionisation (ESI) is the most popular LC-MS ion source. The different kinds of mass analyzers used in LC-MS systems are the quadrupole, time-of-flight (TOF), ion traps, and hybrid quadrupole-TOF (QTOF) analyzers.

IONISATION- Electrospray Ionization (ESI)

It is utilised for the examination of compounds that are both moderately and even extremely polar, such as metabolites, xenobiotics, peptides, nucleotides, and polysaccharides. By spraying an analyte solution into an electrical field, ions are created in this process. It creates very small droplets of solvent-containing analyte. In a metal capillary, the liquid eluate from the LC column is directed. The fundamental idea behind ES is that atomization or nebulization creates a spray of charged liquid droplets. The strong electric field operating on the surface of the sample solution (about 4 keV at the capillary's end and 1 keV at the counter electrode) is what causes ESI. The size of the droplets gets smaller as the solvent evaporates in the high-vacuum area, and eventually just charged analyte (free of solvent) is left (Wilson K and walker J, 2010).

MASS ANALYSER- Q-TOF-MS

Ions enter a mass analyzer once they are produced and depart the ion source. In mass analyzer they are separated and their masses are determined. The Q-TOF-MS is a 'hybrid' device that combines quadrupole technologies with a time-of-flight mass analyzer. The first quadrupole (Q1) can be used as a mass filter to choose particular ions based on their mass-to-charge ratio (m/z), or it can be used in radio frequency (RF) alone mode to transmit all ions. In a process known as collision induced dissociation (CID), the second quadrupole (Q2) serves as a collision cell where ions are battered by neutral gas molecules like nitrogen or argon. Ions are reaccelerated after exiting the quadrupole and enter the time-of-flight analyser's ion modulator section where they are pulsed by an electric field and accelerated orthogonally to their initial direction. Then to the detector, lighter ions move more quickly than heavier ions. Ions with the same m/z can enter the detector at the same moment with help of the reflectron adjustment. Additionally, the reflectron device lengthens the flight path, which enhances mass resolution (Allen DR, McWhinney BC, 2019).

DETECTOR

A detector system called a time-to-digital converter, which converts the ion's flight time into a mass signal, is used to detect ions. A microchannel plate (MCP), a very thin plate with a number of tiny tubes that pass from the front surface to the back of the plate, is located at the surface of the ion detector. An electron escapes

from the MCP's front surface when an ion strikes it, starting the process of electrical signal amplification. A growing cascade of released electrons moves towards the back of the plate as they collide with the microscopic tubes' walls. Over ten times as many electrons leave the MCP as incoming ions make contact with the surface. These electrons are then concentrated onto a scintillator, which creates a flash of light when struck by electrons. Two small lenses focus the scintillator's light onto a photomultiplier tube (PMT), which generates the electrical signal recognised by the data system. A highly helpful parameter to measure during on-line MS is the total ion chromatogram (TIC), which indicates the sum of total intensity over the whole range of observed masses at each stage of the analysis. (Davison AS *et al*., 2019).

3. MATERIALS AND METHODS

3.1 OBJECTIVE 1: Analyzing the circadian clock control of therapeutic drug targets of anti-breast cancer drugs to understand their dosing time dependency

3.1.1 DATA COLLECTION

The data was collected from various databases including National Cancer Institute (NCI), DrugBank, CircaDB, **P**rotein **AN**alysis **TH**rough **E**volutionary **R**elationships (PANTHER) and **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery (DAVID).

National Cancer Institute (NCI)

The National Cancer Institute (NCI) is the top governmental organisation for cancer training and research. It is a part of the National Institutes of Health (NIH). To advance scientific knowledge and help everyone live longer, healthier lives, NCI directs, conducts, and supports cancer research across the nation. It carries out the majority of the country's research to enhance cancer prevention, detection, diagnosis, and survivorship and keeps publicly accessible cancer genomic and clinical data in long-term storage. It includes cancer drugs approved by the Food and Drug Administration (FDA) for cancer treatment.

DrugBank

The University of Alberta and The Metabolomics Innovation Centre, located in Alberta, Canada, created and manage this extensive, open-access online database, which contains data on drugs and drug targets. It provides thorough drug target data, including sequence, structure, and pathway information, together with detailed drug (i.e. chemical, pharmacological, and pharmaceutical) data. The pharmaceutical industry, medical chemists, pharmacists, doctors, students, and the general public all use it.

CircaDB

CircaDB is circadian gene expression profile database. It contains circadian transcriptional profiles from mouse and human time-course expression studies. Three different algorithms—JTK_Cycle, Lomb Scargle, and DeLichtenberg—can be used to assess the expression of each transcript. More accurate and effective separation of rhythmic from non-rhythmic transcripts is by Jonckheere-Terpstra-Kendall (JTK) or JTK_CYCLE. It has greater sensitivity and specificity. Additionally, JTK_CYCLE

efficiently facilitates downstream analysis by precisely measuring the period, phase, and amplitude of cycling transcripts. Users can use straightforward and effective full-text search terms to conduct queries on the gene annotations, limit the results to particular data sets, and specify probability thresholds for each algorithm. Data visualisations are simple graphs that communicate profile information better than a table of probabilities. It clearly has implications for chronotherapy, time-dependent drug administration, and improving the therapeutic index of drugs in the treatment of cancer, cardiovascular disease, and many other conditions.

Protein ANalysis THrough Evolutionary Relationships (PANTHER)

To enable high-throughput analysis, this Classification System was created to categorise proteins (and their genes) and identify the function of gene products. It is part of the Gene Ontology Reference Genome Project. It provides information about the evolution of protein-coding gene families, particularly protein phylogeny, function and genetic variation impacting that function. Proteins -coding gene are classified as follows: evolutionary groupings (Protein Class, Protein Family, Subfamily), and functional groupings (Gene Ontology and pathways). Functions are classified by the Gene Ontology (molecular function, cellular component, and biological process), and pathways (signaling and metabolic pathways) It is a comprehensive system that incorporates statistical analysis methods, pathways, gene function, and ontologies to allow biologists to analyse massive data from experiments generated across the entire genome. Phylogenetic trees, multiple sequence alignments, and statistical models (Hidden-Markov Models, or HMMs) are used to represent the evolutionary relationships between the gene families and subfamilies. The Thomas lab at the University of Southern California maintains PANTHER, which is supported by the National Science Foundation.

Database for Annotation, Visualization and Integrated Discovery (DAVID)

It gives researchers a complete range of functional annotation tools to help them comprehend the biological meaning of large gene-lists. These tools are supported by the extensive DAVID Knowledgebase, which combines functional annotations from many sources and is based on the DAVID Gene concept. The following capabilities of DAVID tools include: Finding enriched functionally related gene groups, Identifying enriched biological themes, particularly GO term,Clustering redundant annotation terms, Visualising genes on BioCarta and KEGG pathway maps, and many more.

The understanding of the molecular interaction, reaction, and relationship networks for metabolism (global/overview such as carbohydrate, energy, lipid, nucleotide, and amino acid), cellular processes, human diseases, drug development, etc. is represented by the KEGG PATHWAY which is a collection of manually drawn pathway maps. The Laboratory of Human Retrovirology and Immunoinformatics (LHRI) developed it as a free online bioinformatics tool.

3.1.2 METHODOLOGY

DRUG COLLECTION

To understand the dosing time dependency, anti-breast cancer drugs approved by the Food and Drug Administration (FDA) were fetched from National Cancer Institute (NCI) (https://www.cancer.gov/about-cancer/treatment/drugs/breast).

DRUG TARGET TABULATION

The anti-breast cancer drug targets were procured from DrugBank (Wishart DS *et al.*, 2008) (https://go.drugbank.com/).

RHYTHMICITY EXPRESSION PROFILE

The rhythmicity analysis of anti-breast cancer drug targets of mouse was performed using CircaDB (Pizarro A *et al.*, 2013), which is a Circadian Gene Expression Profile Database. The embedded JTK-cycle algorithm defines the significance of rhythmic gene expression. The probability filter used was JTK Q-value with 0.1 probability cut-off value and 0-40 JTK phase range. The rhythmic drug targets were selected based on periodicity and q-value.

GENE ONTOLOGY AND PATHWAY ANALYSIS

PANTHER (Mi H *et al.*, 2021) (http://www.pantherdb.org/) and DAVID (Sherman BT *et al.*, 2022) (https://david.ncifcrf.gov/) are bioinformatics resources that provide functional interpretation, annotations and pathway visualization of the large gene lists. PANTHER database was used for Gene Ontology (GO) annotation for classifications by molecular function, biological process and cellular component. The PANTHER Protein Class ontology includes commonly used classes of protein functions, many of which are not covered by GO molecular function.

DAVID database was used to study the KEGG pathways of the uploaded rhythmic genes.

The PANTHER website was accessed by entering the URL (http://www.pantherdb.org) in the web browser. The rhythmic gene ID list was uploaded in the ID box. *Mus musculus* organism was selected from the drop-down menu followed by selection of Statistical overrepresentation test i.e, GO biological process, cellular component, molecular function, and PANTHER Protein class. It finds the functional classes that are statistically over- or under-represented in the input list compared with the PANTHER reference list for the genome. The Fisher's exact test with FDR ($P < 0.05$) multiple test correction were selected for the statistical test for the over-representation analysis. Then the results from the statistical over-representation test for each of GO biological process, cellular component, molecular function, and PANTHER Protein class were displayed. The functional classification of the genes in the list was also displayed on pie chart by selecting the 'Functional classification viewed in graphic chart' option.

For the KEGG pathway analysis, the DAVID website was accessed by entering the URL (https://david.ncifcrf.gov/tools.jsp) in the web browser. The official gene symbols of rhythmic drug targets were uploaded for *Mus musculus* species. The DAVID Pathway Viewer of DAVID functional annotation tools displayed the KEGG pathway for rhythmic drug targets to facilitate biological interpretation in a network. The thresholds for minimum no. of genes count for each pathway term was set as 2 and maximum EASE score/ P-value was applied as 0.1. The significant result of KEGG pathway for rhythmic anti-breast cancer drug targets was displayed.

3.2 OBJECTIVE 2: In silico docking analysis of clock modulator, SR9009 with anti-breast cancer drug targets

3.2.1 DATA COLLECTION

PubChem

A database of chemical compounds and their responses to biological experiments is called PubChem (https://pubchem.ncbi.nlm.nih.gov/). The National Centre for Biotechnology Information (NCBI), a division of the National Library of Medicine, a branch of the National Institutes of Health (NIH) of the United States, is responsible for maintaining the system. Through a web interface, PubChem can be viewed for free. It is possible to obtain descriptive datasets and millions of compound structures for free using File Transfer Protocol **(**FTP). Small molecules with less than 100 atoms and 1,000 bonds can be found in PubChem along with numerous descriptions of various substances. Records of data provided to the PubChem databases are kept in the PubChem group: PubChem Substance, Compounds, and BioAssay. PubChem data can be searched through the common Web Search (Entrez) mechanism.

RCSB Protein Data Bank (RCSB PDB)

Large biological entities like proteins and nucleic acids have three-dimensional structural information stored in a database called the Protein Data Bank (PDB) (https://www.rcsb.org/). The information, which was provided by biologists and biochemists from all around the world and was typically collected by X-ray crystallography, NMR spectroscopy, or, increasingly, cryo-electron microscopy, is freely available online via the websites of its member organisations (PDBe, PDBj, RCSB, and BMRB). The Worldwide Protein Data Bank, or wwPDB, is the entity in charge of managing the PDB. A number of free and open source software applications, including Jmol, Pymol, VMD, Molstar, and Rasmol, can be used to view the structure files.A comprehensive list of web browser plugins and programmes for visualising molecules is available on the RCSB PDB website. Additionally, it offers a range of resources and tools. On the basis of sequence, structure, and function-related annotations, users can do basic and advanced searches. Understanding a biological macromolecule's function in human and animal health and disease, as well as its significance to other issues related to global prosperity and sustainability, requires knowledge of its 3D structure.

AutoDock Vina

A molecular docking programme called AutoDock Vina (Trott O & Olson AJ, 2010) is free and open-source. It aims to foresee how small compounds, such as substrates or drugs, would bind to a receptor with an established three-dimensional (3D) structure. In the Molecular Graphics Lab (now CCSB) at The Scripps Research Institute, it was initially created and put into use by Dr. Oleg Trott. The most recent AutoDock Vina version is 1.2.0. The AutoDock Suite's docking engines include AutoDock Vina. AutoDock Vina, has a significant increase in performance and accuracy. It is offered with the Apache licence. Vina utilises the MGLTools-created and viewed PDBQT molecular structure file format for input and output. It improves the average accuracy of the binding mode predictions. It has helped in the development of various drugs. X-ray crystallography, structure-based drug design, lead optimisation, and virtual screening (HTS) are among the fields where it has applicability.

PyMOL

Warren Lyford DeLano developed PyMOL, a proprietary yet open source molecular visualisation system. DeLano Scientific LLC, a private software company devoted to developing practical tools that become broadly accessible to scientific and educational groups, first commercialized it. Schrödinger, Inc. presently commercializes it. PyMOL has the ability to create stunning 3D images of both biological macromolecules like proteins and small compounds.

3.2.2 METHODOLOGY

Blind docking is used when a protein's binding pocket and catalytic/binding residues are unknown. Molecular docking of the top 10 rhythmic drug targets was performed with SR9009 (ligand) using AutoDock Tools 1.5.7, and the binding energies (ΔG) were compared with positive control NR1D1 (REV-ERB α).

Preparation of protein (drug targets) coordinate file:

The PDB file of the 10 drug targets (refer table 4.4.2 for PDB ID of drug targets) and a positive control NR1D1 (REV-ERB α) (PDB ID: 8D8I) was downloaded from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB). Heteroatoms and water molecules were deleted. AutoDock Tools 1.5.7 was launched, proteins were prepared for docking by deleting water molecules, adding polar hydrogens,

adding kollman charges, and finally adding atom assign AD4 type. The protein file was saved in PDBQT format, suitable for docking.

Preparation of ligand (SR9009) coordinate file:

The 3D structure of the ligand (SR9009) (PubChem CID: 57394020) was retrieved from the PubChem website in SDF format. Using the PyMOL software SDF file was converted to PDB format. Following this, AutoDock Tools 1.5.7 was used to add gasteiger charges to the ligand. The non-polar hydrogens were merged and rotatable bonds were detected. Then the torsion tree/ torsion angle was created on ligand from where it can be rotated and ligand file was saved as PDBQT format, the acceptable file format for docking analysis.

Preparation of grid parameter file:

In blind docking, there is no need to define a specific site in the protein for the ligand to bind, because the binding site is unknown, the whole protein is enclosed into the grid box. With the PDBQT files of protein and ligand on screen, the docking grid box was centered in position x, y, and z-direction with dimension of 40 x 40 x 40 Å. After that, the output file was saved as a "grid.txt" file.

Preparation of configuration file:

AutoDock Vina requires an input configuration file which contains all the information of the parameters used in configuring the docking including the name of the protein and the ligand. With the windows open after grid file preparation, Vina Configuration available under docking option was used to set the energy range = 4 and exhaustiveness = 8 to provide a text configuration file with the default name of "config.txt" file.

Molecular docking analysis:

AutoDock Vina was launched and the command prompt was opened to enter the folder where all the docking files will be placed. The molecular docking process was performed three times for each drug target, and the standard error of mean binding energy was considered. Nine protein-ligand docked models were obtained in each run following the molecular docking. Log text files containing the binding affinity and RMSD scores were also created for manual comparison for respective protein-ligand complexes. The first pose in the Vina output log file is regarded as the best because it has more binding

affinity than the other poses and no RMSD value. The best docking pose was chosen having the lowest binding energy (ΔG, kcal/mol). The molecular visualization of selected docked protein-ligand interaction was done in PyMOL.

3.3 OBJECTIVE 3: To comprehend the effect of SR9009 on the metabolism of breast cancer.

3.3.1 Cell line used

In this study, mouse 4T1 breast cancer cells were used. 4T1 resembles human metastatic triple-negative breast cancer (TNBC). The cells are adherent and have an epithelial morphology.

3.3.2 Drug used

To analyze the metabolic changes in mouse 4T1 cells, SR9009 was purchased from SigmaAldrich (554726). Dimethylsulfoxide (DMSO) was purchased from Thermo Fisher Scientific Inc. Invitrogen™ (D12345). 5mg of SR9009 was dissolved in 228.3µl DMSO to prepare 50mM stock solution, and stored at -20. From this, sub-stock was produced which was used for the experiment. The working concentration of SR9009 used for the experiment was 20µM.

3.3.3 Cultivation condition of 4T1 cell line

4T1 cells were cultured in Roswell Park Memorial Institute, RPMI 1640 Medium (Thermo Fisher Scientific Inc., Gibco^{TM} 1X, 11875093) containing 10% fetal bovine serum (Thermo Fisher Scientific Inc., Gibco^{TM}, 16010142) in a constant temperature cell culture incubator at 37 °C with 95% air and 5% CO_2.

3.3.4 Media preparation

Media composition (1 litre media) needed for culturing 4T1 cells was:

1. RPMI 1640 (Gibco^{TM} 1X)
2. 10% FBS (100ml)
3. 1% Penicillin-Streptomycin solution (10,000 U/mL) (10ml) (Thermo Fisher Scientific Inc., Gibco^{TM}, 15140122)
4. Gentamicin solution (SigmaAldrich, G1264) (Stock: 50mg/ml) – Working concentration 50µg/ml (1ml)

Media was sterilized immediately by Tarsons Membrane Filter Holder (filtration unit) using a PVDF membrane with a porosity of 0.22 microns. The filtered media was stored at 4°C.

3.3.5 Passaging 4T1 Adherent Cell line

4T1 cells should be passaged at 80% confluency. The following steps were employed:

1. The spent cell culture media was removed and discarded from the T-25 culture vessel.
2. Cells were washed using 1ml Dulbecco's phosphate-buffered saline (DPBS) (Thermo Fisher Scientific Inc., Gibco^{TM}, 14190136). Wash solution was gently added to the side of the vessel opposite to the attached cell layer to avoid disturbing the cell layer, and then the vessel was rocked back and forth several times.
3. The wash solution was removed and discarded from the culture vessel.
4. The pre-warmed dissociation reagent such as TrypLE™ Express Enzyme (1X) (Thermo Fisher Scientific Inc., Gibco^{TM}, 12604021), 1ml was added to the side of the flask. The container was gently rocked to get complete coverage of the cell layer.
5. The culture vessel was incubated in the incubator for approximately 2-3 minutes. The cells were observed under the microscope for detachment.
6. When ≥ 90% of the cells had detached, the vessel was tilted for a minimal length of time to allow the cells to drain. 1ml of the pre-warmed complete growth medium was added to neutralize the activity of trypsin. The medium was dispersed by pipetting over the cell layer surface several times. Trypsinized cells were transferred to a 15-mL conical tube and centrifuged at 1200 rpm for 5 minutes.
7. The supernatant was removed from conical tube and the cell pellet was resuspend in 1ml of pre-warmed complete growth medium.
8. Appropriate volume of cell suspension was pipetted into new T-25 cell culture vessel containing 5ml of pre-warmed complete growth medium
9. The flask was then kept in the incubator at 37℃ for optimal growth of the cells.

3.3.6 Sample preparation for metabolomics

4T1 cells were cultured in four 6-well plates (3×10^5/mL, 2 mL/well), with two plates seeded at 8am morning and 8pm evening, for 72 h and treated with SR9009 (20 μM) and DMSO (control) for 24 h. The SR9009 drug treated (20 μM) and control samples were prepared in triplicates for LC-MS analysis. 4T1 cells were washed with pre-cold PBS, scraped with 1 mL of pre-cold ultrapure water, transferred to 2.0 mL tubes. For the

experiment, 100 µL of this sample was added to a new tube, to which 1000 µL of extraction solution (methanol: acetonitrile: water = 2:2:1, v/v) was mixed and vortexed for 15s. The samples were ultrasonicated in an ice-water bath for 10 min and snap-frozen in liquid nitrogen for 1 min; these steps were repeated three times. The samples were incubated at -20°C for 1h, followed by centrifugation at 13,000 rpm for 15 min at 4°C. The supernatant was extracted, filtered using nylon membrane (0.22 µm), and transferred to new tubes, and dried in vacuum concentrator (SpeedVac concentrator) (Thermo Fisher Scientific Inc.) without heating for 30 minutes. The dried samples were dissolved and shaken vigorously for 30s in 100 µL acetonitrile: water (1:1 (v/v)) solution. The samples were then sonicated in an ice-cold water bath for 10 min followed by centrifugation at 13,000 rpm for 15 min at 4°C. The supernatants were carefully removed, filtered using nylon membrane (0.22 µm) and kept at -80°C until analysis. The samples were injected three consecutive times in the LC-Q-TOF/MS system for study.

3.3.7 LC-Q-TOF/MS Conditions

Metabolomic analysis was performed using Agilent 1290 Infinity/II LC (Liquid Chromatography) system (Agilent Technologies) coupled with an Agilent 6550 iFunnel Quadrupole Time-of-Flight (Q-TOF) mass spectrometer with a dual Agilent jet stream electrospray ionization (Dual AJS ESI) technology ion source (Agilent Technologies, Santa Clara, CA, USA). It was used in the ESI^+ mode to increase the number of detected metabolite ions. An Agilent InfinityLab poroshell HPH-C18 narrow bore LC column (2.1 x 150 mm, 2.7 µm) was used for separation. The column was maintained at 40°C at a flow rate of 0.400 $mL.min^{-1}$. The mobile phase consisted of a gradient system of (A) aqueous 0.1% formic acid in water and (B) aqueous 0.1% formic acid in methanol. The ion-mode gradient elution conditions were as follows: 5% B at 0–2 min; 95% B at 2–14 min; 95% B at 14–16 min; 95% B at 16-20 min. The sample injection volume was 10µL. The automatic samplers were maintained at 15°C.

Mass spectrometry (MS) data was collected in positive mode and the mass range was from 100 to 3000 m/z. The mass spectrometric conditions were carried out as follows: capillary voltage: 3.5 kV in positive ion mode; drying gas flow rate, 14 L/min; gas temperature, 200°C; fragmentor, 160 V; nebulizer, 40 psi; skimmer, 65 V; and acquisition rate, 1 spectra/s.

A total of 12 samples were injected in triplicate in the LC-MS system (drug vs control for morning and evening analysis). Blank analysis was inserted after analysis of each sample to avoid cross contamination.

3.3.8 Metabolomics Data Analysis

The Agilent LC/MS system is supported by the MassHunter software suite for effective data acquisition, qualitative data analysis, mass spectral library management, quantitative data analysis, simple access, and reporting. Agilent Mass Profiler Professional (MPP) software can be used in any MS-based differential analysis to identify correlations between two or more sample groups and factors.

Raw *.d files from the Mass Hunter acquisition software were processed using Agilent MassHunter ProFinder (version 10.0) ((Agilent Technologies, Santa Clara, CA, USA) which converted *d files to *cef files and also helped in feature extraction . The Batch Molecular Feature Extraction (MFE) function in Mass Hunter ProFinder (10.0, Agilent, Santa Clara, California, USA) was used to remove background noise and unrelated ions from the raw data acquired by the analytical instrument and extract features. The entire TOF mass spectrum data from the MFE are then used to compile a list of all potential components. According to mass, retention time (RT), and abundance, each compound is characterised. For feature extraction via the MFE, the background noise limit was set to 3000 counts, and the coeluting adducts of the same feature were identified using the following adduct settings: in the positive ion mode: +H, +Na, +K. Peak alignment is required to identify detected analytical signals as the same characteristics in all measured samples, which is important to rectify the common shift of retention time and observed monoisotopic mass during LC-MS studies. Mass Profiler Professional 15.1 (MPP) (Agilent Technologies, Santa Clara, California, USA) was used for the alignment process. Following alignment, the resultant data matrices were filtered, normalised, and log-transformed, and p-values were calculated using t-tests with a Benjamini-Hochberg FDR correction. Metabolites with p value <0.05 and fold change >2 were selected as final differential metabolites, which were validated using the Human Metabolome Databases (http://www.hmdb.ca/), METLIN (https://metlin.scripps.edu/) and KEGG (www.genome.jp/kegg). The validated differential metabolites were entered into the MetaboAnalyst 5.0 database (https://www.metaboanalyst.ca/) for metabolic pathway analysis.

4. RESULT

4.1 Rhythmicity analysis of anti-breast cancer drug targets to understand their dosing time dependency

To get insights into the dosing-time dependency of drugs that are already in use for the treatment of breast cancer, an in silico analysis of rhythmic anti-breast cancer drug targets was carried out. In this study, total 41 anti-breast cancer drugs were obtained from National Cancer Institute (NCI) (Table 4.1.1). This was followed by total 95 drug targets tabulated from DrugBank, among which 60 drug targets were non-redundant/significant. The rhythmicity analysis of 60 drug targets was performed in CircaDB for mouse, using JTK-cycle algorithm. For each queried gene, time-course vs expression level plots and statistical parameters such as p-value, q-value, period, and phase were returned. Genes with periodicity of 24±3 hours and minimum q-value were selected as circadian rhythmic drug target. Out of 60 drug targets, 37 anti-breast cancer drug targets were found to be rhythmic at the transcript level (Table 4.1.2). The circadian gene expression profile of ten rhythmic anti-breast cancer drug targets is shown in Figure 4.1. From the above analysis, it can be inferred that only 37 anti-breast cancer drug targets are clock-controlled and exhibit circadian rhythmicity. This rhythmicity analysis data can be used to optimize the drug administration time of anti-breast cancer drugs to increase the drug's therapeutic efficiency and reduce their toxic effects.

Table 4.1.1 Summary of anti-breast cancer drugs and their drug targets.

S.No.	Anti-breast cancer drugs	Source	Drug targets (Source: DrugBank)
1	Abemaciclib	NCI (National Cancer Institute)	CDK4, CDK6
2	Paclitaxel		TUBB1, BCL2, MAP4, MAP2, MAPT, NR1I2
3	Trastuzumab emtansine		ERBB2
4	Everolimus		MTOR
5	Alpelisib		PIK3CA
6	Anastrozole		CYP19A1

7	Pamidronic acid	NCI (National Cancer Institute)	FDPS, Hydroxylapatite, GGPS1, CASP3, CASP9
8	Exemestane		CYP19A1
9	Capecitabine		DNA, RNA, TYMS
10	Cyclophosphamide		DNA, NR1I2
11	Docetaxel		TUBB1, MAP2, MAP4, MAPT, BCL2, NR1I2
12	Doxorubicin		TOP2A, DNA, NOLC1, TOP1, TOP2B
13	Elacestrant		ESR1
14	Epirubicin		DNA, TOP2A
15	Trastuzumab deruxtecan		FCGR1A, TOP1
16	Eribulin		BCL2, TUBB1
17	Fluorouracil		DNA, RNA, TYMS
18	Toremifene		ESR1, SHBG
19	Fulvestrant		ESR1
20	Letrozole		CYP19A1
21	Gemcitabine		DNA, RRM1, TYMS, CMPK1
22	Goserelin		LHCGR, GNRHR
23	Trastuzumab		ERBB2
24	Palbociclib		CDK4, CDK6
25	Ixabepilone		TUBB3
26	Pembrolizumab		PDCD1, CD274
27	Ribociclib		CDK4, CDK6
28	Lapatinib		EGFR, ERBB2
29	Olaparib		PARP1, PARP2, PARP3, AKR1C3
30	Margetuximab		ERBB2
31	Megestrol acetate		PGR, NR3C1
32	Methotrexate		TYMS, ATIC, DHFR
33	Neratinib		EGFR
34	Pertuzumab		ERBB2
35	Sacituzumab govitecan		TACSTD2, TOP1, FUBP1
36	Tamoxifen		ESR1, ESR2, PRKCA, PRKCB, PRKCD, PRKCE, PRKCG, PRKCI, PRKCQ, PRKCZ, SHBG, EBP, AR, KCNH2, NR1I2, ESRRG, MAPK8
37	Talazoparib		PARP1, PARP2
38	Atezolizumab		CD274, PDCD1

39	Thiotepa		DNA
40	Tucatinib		ERBB2, ERBB3
41	Vinblastine		TUBA1A, TUBB, TUBD1, TUBG1, TUBE1, JUN

Table 4.1.2 Rhythmic expression of anti-breast cancer drug targets in mouse (*Mus musculus)*.

S.No.	Targets	Drugs	Rhythmic circadian expression
1	CDK4	Abemaciclib, Palbociclib, Ribociclib	Heart (JTK-Q = 0.003), Brown Adipose (JTK-Q = 0.01), SCN (JTK-Q = 0.01), Aorta (JTK-Q =0.02), Lung (JTK-Q =0.07)
2	CDK6	Abemaciclib, Palbociclib, Ribociclib	Kidney (JTK-Q = 0.006), Brown Adipose (JTK-Q = 0.01), Liver (JTK-Q = 0.03), Liver (JTK-Q = 0.07)
3	BCL2	Paclitaxel, Docetaxel, Eribulin,	SCN (JTK-Q = 0.01), Liver (JTK-Q = 0.07)
4	MAPT	Paclitaxel, Docetaxel	Adrenal Gland (JTK-Q = 0.001), Heart LD (JTK-Q = 0.04), Aorta (JTK-Q = 0.09), OST SCN (JTK-Q = 0.09)
5	NR1I2	Paclitaxel, Cyclophosphamide, Docetaxel, Tamoxifen,	Liver (JTK-Q = 0.00001), Liver (JTK-Q = 0.0004)
6	MTOR	Everolimus	Liver (JTK-Q = 0.00001), Kidney (JTK-Q = 0.00003), Liver (JTK-Q = 0.001), Kidney (JTK-Q = 0.05), Heart (JTK-Q = 0.08), SCN (JTK-Q = 0.09),
7	PIK3CA	Alpelisib	Kidney (JTK-Q = 0.001), Liver (JTK-Q = 0.002), Skeletal Muscle (JTK-Q = 0.02), Heart (JTK-Q = 0.05),
8	FDPS	Pamidronic acid	Kidney (JTK-Q = 0.003), Brown Adipose (JTK-Q = 0.03), Aorta (JTK-Q = 0.05), Liver (JTK-Q = 0.07)
9	GGPS1	Pamidronic acid	Brain Stem (JTK-Q = 0.03), Lung (JTK-Q = 0.04),
10	TYMS	Capecitabine, Fluorouracil, Gemcitabine, Methotrexate	SCN (JTK-Q = 0.03), SCN MAS4 (JTK-Q = 0.08)
11	TOP1	Doxorubicin, Trastuzumab deruxtecan, Sacituzumab govitecan	Heart (JTK-Q = 0.001), Lung (JTK-Q = 0.003), Liver (JTK-Q = 0.005), Aorta (JTK-Q = 0.007), Hypothalamus (JTK-Q = 0.05), Kidney (JTK-Q = 0.07), Macrophages (JTK-Q = 0.09)

12	ESR1	Elacestrant, Toremifene, Fulvestrant, Tamoxifen	Liver (JTK-Q = 0.000000004), Liver (JTK-Q = 0.0000001), Liver (JTK-Q = 0.0001), Kidney (JTK-Q = 0.005), White Adipose (JTK-Q = 0.01), Liver (JTK-Q = 0.01), SCN (JTK-Q = 0.05), Wild Type Muscle (JTK-Q = 0.08), Aorta (JTK-Q = 0.09)
13	PDCD1	Pembrolizumab, Atezolizumab	Wild Type SCN (JTK-Q = 0.07)
14	CD274	Pembrolizumab, Atezolizumab,	Lung (JTK-Q = 0.01), Heart (JTK-Q = 0.08)
15	EGFR	Lapatinib, Neratinib	Heart (JTK-Q = 0.009)
16	PARP2	Olaparib, Talazoparib	Liver (JTK-Q = 0.021), Heart (JTK-Q = 0.05)
17	PARP3	Olaparib	Liver (JTK-Q = 0.007), Liver (JTK-Q = 0.008), Brown Adipose (JTK-Q = 0.01), Pituitary (JTK-Q = 0.01), Liver (JTK-Q = 0.04), Pituitary (JTK-Q = 0.09)
18	PGR	Megestrol acetate	Aorta (JTK-Q = 0.09)
19	ATIC	Methotrexate,	Liver (JTK-Q = 0.004), Lung (JTK-Q = 0.01), Wild Type SCN (JTK-Q = 0.09)
20	DHFR	Methotrexate	Liver (JTK-Q = 0.00002), Liver (JTK-Q = 0.004)
21	FUBP1	Sacituzumab govitecan	Brown Adipose (JTK-Q = 0.006), Heart (JTK-Q = 0.01), SCN (JTK-Q = 0.02), Kidney (JTK-Q = 0.02), Hypothalamus (JTK-Q = 0.03), Aorta (JTK-Q = 0.09)
22	ESR2	Tamoxifen	Lung (JTK-Q = 0.01)
23	PRKCA	Tamoxifen	Lung (JTK-Q = 0.00002), SCN (JTK-Q = 0.04), Skeletal Muscle (JTK-Q = 0.05)
24	PRKCB	Tamoxifen	Lung (JTK-Q = 0.02)
25	PRKCD	Tamoxifen	Wild Type Muscle (JTK-Q = 0.00001), Skeletal Muscle (JTK-Q = 0.01)
26	PRKCE	Tamoxifen	White Adipose (JTK-Q = 0.00001), Brown Adipose (JTK-Q = 0.003), Aorta (JTK-Q = 0.01)
27	PRKCI	Tamoxifen	Liver (JTK-Q = 0.00009), Liver (JTK-Q = 0.008), Brown Adipose (JTK-Q = 0.009)
28	PRKCZ	Tamoxifen	Lung (JTK-Q = 0.03)
29	EBP	Tamoxifen	Liver (JTK-Q = 0.03)
30	AR	Tamoxifen	Aorta (JTK-Q = 0.003), Brown Adipose (JTK-Q = 0.006)

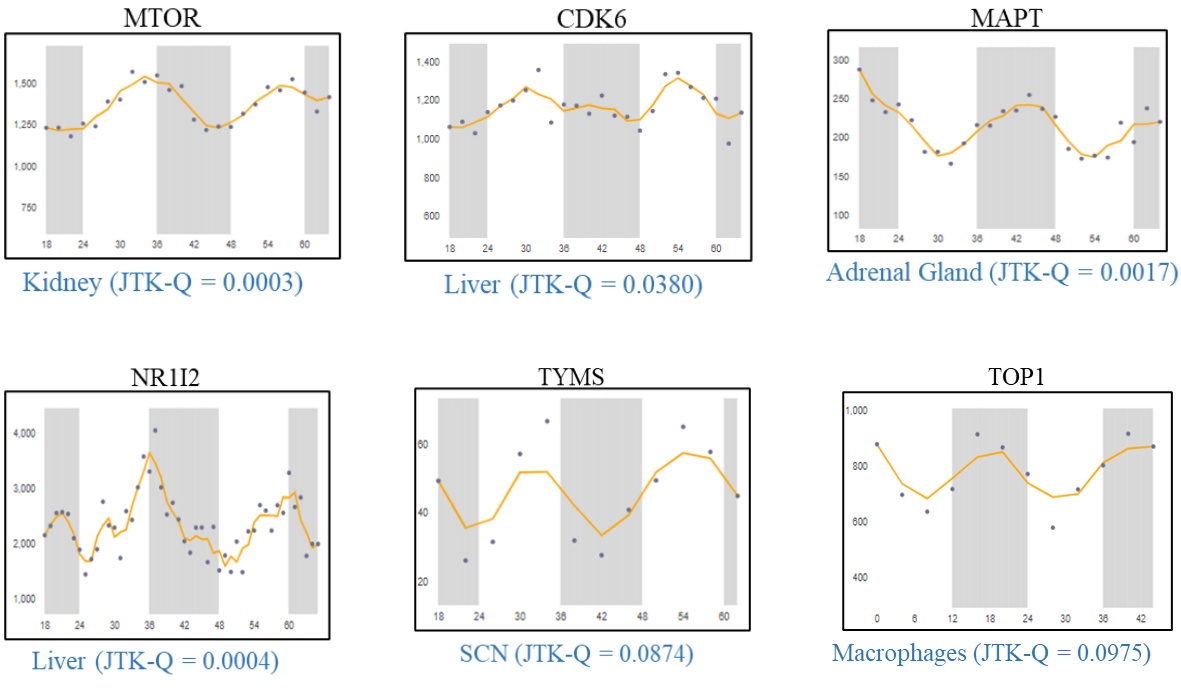
31	KCNH2	Tamoxifen	Heart (JTK-Q = 0.0001), Heart (JTK-Q = 0.02)
32	MAPK8	Tamoxifen	Wild Type SCN (JTK-Q = 0.02), Wild Type SCN (JTK-Q = 0.03)
33	ERBB3	Tucatinib	Liver (JTK-Q = 0.006), Kidney (JTK-Q = 0.006), Liver (JTK-Q = 0.008), Liver (JTK-Q = 0.09)
34	TUBA1A	Vinblastine	Brown Adipose (JTK-Q = 0.005), Heart (JTK-Q = 0.007), White Adipose (JTK-Q = 0.009), Liver (JTK-Q = 0.01), SCN (JTK-Q = 0.07), SCN (JTK-Q = 0.08)
35	TUBD1	Vinblastine	Lung (JTK-Q = 0.003), Brown Adipose (JTK-Q = 0.02), White Adipose (JTK-Q = 0.05), Liver (JTK-Q = 0.06), Liver (JTK-Q = 0.07)
36	TUBG1	Vinblastine	Liver (JTK-Q = 0.002), Wild Type Liver (JTK-Q = 0.04), Kidney (JTK-Q = 0.04)
37	TUBE1	Vinblastine	Liver (JTK-Q = 0.03)

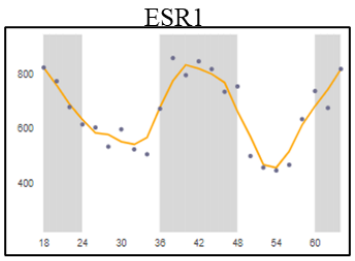
SCN= suprachiasmatic nucleus.

All rhythmic targets have been filtered out by probability filter- JTK-Q; probability cut off value: 0.1; JTK phase range 0-40; period length: 24±3 hours.

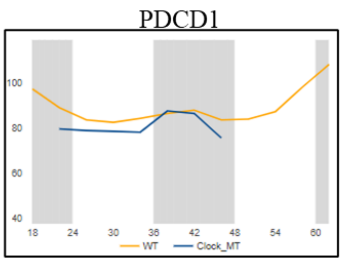
Targets are gene names represented in transcriptome data.

Figure 4.1 Circadian gene expression profile of anti-breast cancer drug targets in mouse (*Mus musculus)*. X-axis is the time in hours and the Y-axis reflects the expression levels measured on the individual gene chips.

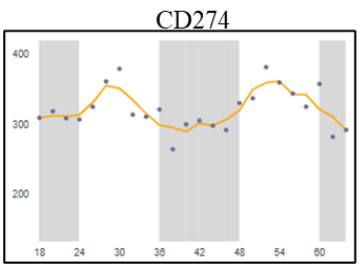




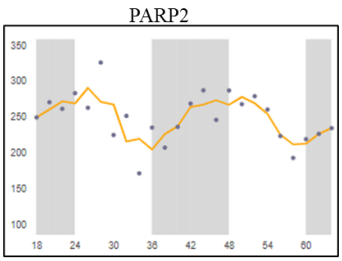
Liver (JTK-Q = 0.0001)



Wild Type SCN (JTK-Q = 0.0787)



Lung (JTK-Q = 0.0171)



Liver (JTK-Q = 0.0210)

4.2 Gene functional analysis using PANTHER classification system

To interpret the functional classes that are statistically over- or under-represented for *Mus musculus* organism in the input rhythmic gene list, statistical overrepresentation test of Gene Ontology terms such GO biological process, GO cellular component, GO molecular function, and PANTHER Protein class was performed. The biological systems that a gene product contributes are included in biological process. It entails the 'biological programmes' carried out by numerous molecular activities. The cellular component is where the gene product fulfils its molecular function. The function that a protein-coding gene performs on its specific molecular targets, or the molecular-level operations carried out by gene products, is known as molecular function. PANTHER Protein Class includes commonly used classes of protein functions, many of which are not covered by GO molecular function. The 37 rhythmic anti-breast cancer drug targets/gene ID's were uploaded for analysis and only top 10 from each functional annotation category were selected based on least False Discovery Rate (FDR<0.05). The result was tabulated (Tables 4.2.1, 4.2.2, 4.2.3, 4.2.4) containing information about top 10 names of the annotation data category (specific to each Gene Ontology), name and number of genes in the uploaded list that map to the annotation data category, fold enrichment of the genes indicating the category is overrepresented (if greater than 1) or underrepresented (if less than 1) and minimum False Discovery Rate (FDR<0.05) as calculated by the Benjamini-Hochberg procedure. The four ontologies were also represented on pie chart (Figure 4.2.1, 4.2.2, 4.2.3, 4.2.4) to understand the distribution proportion of rhythmic genes. Among the rhythmic drug targets, 22 drug targets are found to play a role in

carbohydrate, lipid, and protein metabolic processes. The functional gene analysis is summarized in Figure 4.2.5. Functional analysis of genes helps to dissect and analyze a collection of genes to determine the genes that are involved in the regulation of specialized biological processes/pathways. The analysis contribute to optimum dosing time of anti-breast cancer drugs and to link circadian metabolism in breast cancer. This analysis suggests that targeting these rhythmic genes in clinical practice can be a powerful therapeutic tool in treatment of breast cancer by majorly focusing on the chronotherapy i.e, dosing time dependency of drug.

Figure 4.2.1 Pie chart representing biological processes of rhythmic anti-breast cancer drug targets.

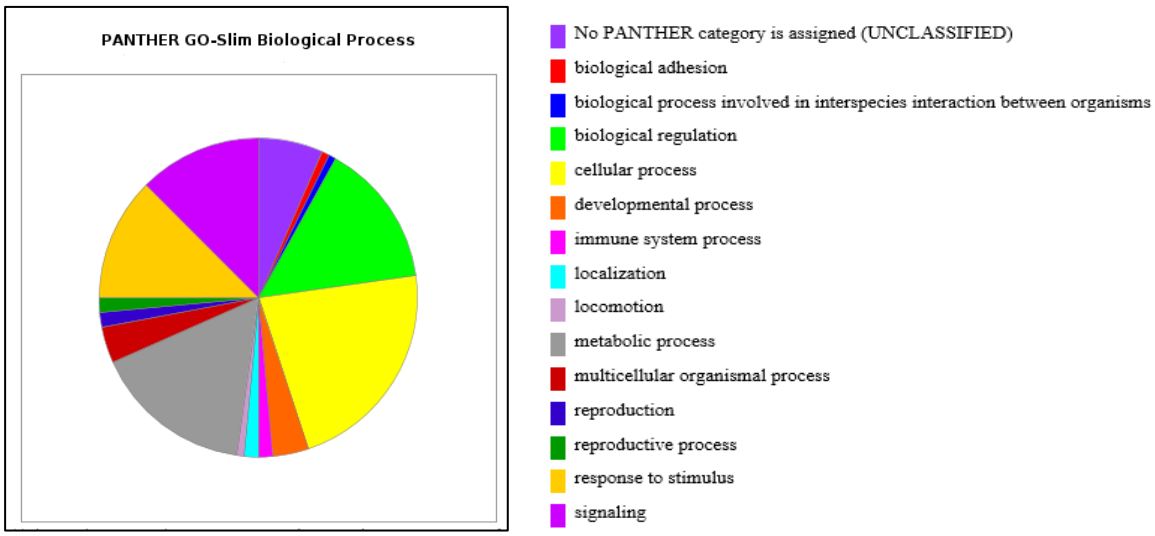


Table 4.2.1 Summary of biological processes of rhythmic anti-breast cancer drug targets.

S.No.	Biological process	Gene count	Genes	Fold enrichment	False Discovery Rate (FDR)
1	Response to oxygen-containing compound	21	PRKCA, TUBA1A, CD274, MAPK8, PIK3CA, AR, PRKCD, ESR2, PRKCI, PRKCB, EGFR, PRKCE, DHFR, PRKCZ, MTOR, CDK4, AR, PGR, BCL2, ESR1, AR	7.13	1.30E-09
2	Negative regulation of cell death	18	PRKCA, ERBB3, MAPK8, PIK3CA, AR, PRKCD, ESR2, PRKCI, PARP2, EGFR, PRKCE, PRKCZ, MTOR, AR, PGR, PDCD1, BCL2, ESR1	8.44	4.88E-09

3	Cellular response to chemical stimulus	23	PRKCA, KCNH2, TUBA1A, CD274, MAPK8, PIK3CA, MAPT, AR, PRKCD, ESR2, PRKCI, PRKCB, EGFR, PRKCE, PRKCZ, MTOR, ATIC, CDK4, AR, PGR, BCL2, ESR1, NR1I2	5.16	5.70E-09
4	Positive regulation of cell population proliferation	17	PRKCA, ERBB3, CDK6, CD274, MAPK8, PIK3CA, AR, PRKCI, EGFR, PRKCZ, MTOR, CDK4, AR, PGR, BCL2, ESR1, AR	8.62	6.39E-09
5	Peptidyl-serine phosphorylation	10	PRKCA, MAPK8, PRKCD, PRKCI, PRKCB, PRKCE, TOP1, PRKCZ, MTOR, BCL2	28.78	7.23E-09
6	Regulation of cell death	21	PRKCA, ERBB3, CD274, MAPK8, PIK3CA, MAPT, AR, PRKCD, ESR2, PRKCI, PARP2, EGFR, PRKCE, PRKCZ, MTOR, CDK4, AR, PGR, PDCD1, BCL2, ESR1	6.15	7.39E-09
7	Response to organic substance	24	PRKCA, TUBA1A, CDK6, CD274, MAPK8, PIK3CA, MAPT, AR, PRKCD, ESR2, PRKCI, PRKCB, EGFR, PRKCE, DHFR, PRKCZ, MTOR, ATIC, CDK4, AR, PGR, BCL2, ESR1, AR	4.88	7.92E-09
8	Peptidyl-serine modification	10	PRKCA, MAPK8, PRKCD, PRKCI, PRKCB, PRKCE, TOP1, PRKCZ, MTOR, BCL2	26.06	1.03E-08
9	Cellular response to oxygen-containing compound	17	PRKCA, TUBA1A, CD274, MAPK8, PIK3CA, AR, PRKCD, ESR2, PRKCI, PRKCB, EGFR, PRKCE, PRKCZ, MTOR, CDK4, AR, ESR1	8.17	1.11E-08
10	Regulation of apoptotic process	19	PRKCA, ERBB3, CD274, MAPK8, PIK3CA, AR, PRKCD, ESR2, PRKCI, EGFR, PRKCE, PRKCZ, MTOR, CDK4, AR, PGR, PDCD1, BCL2, ESR1	6.32	2.65E-08

Figure 4.2.2 Pie chart representing molecular functions of rhythmic anti-breast cancer drug targets.

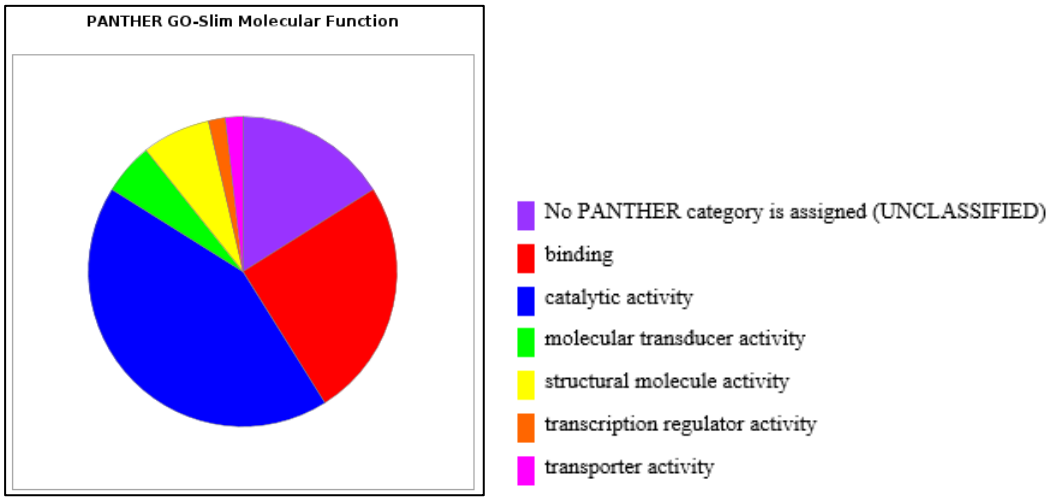


Table 4.2.2 Summary of molecular functions of rhythmic anti-breast cancer drug targets.

S.No.	Molecular function	Gene count	Genes	Fold enrichment	False Discovery Rate (FDR)
1	Calcium-dependent protein kinase C activity	6	PRKCA, PRKCD, PRKCI, PRKCB, PRKCE, PRKCZ	> 100	3.00E-09
2	Protein kinase C activity	6	PRKCA, PRKCD, PRKCI, PRKCB, PRKCE, PRKCZ	> 100	3.24E-09
3	Transferase activity, transferring phosphorus-containing groups	16	PRKCA, ERBB3, CDK6, MAPK8, PIK3CA, PRKCD, PRKCI, PRKCB, PARP2, EGFR, PRKCE, TOP1, PARP3, PRKCZ, MTOR, CDK4	9.32	3.44E-09
4	Purine nucleotide binding	21	PRKCA, AR, AR, ERBB3, TUBA1A, CDK6, MAPK8, PIK3CA, PRKCD, PRKCI, PRKCB, TUBG1, EGFR, PRKCE, TOP1, DHFR, PRKCZ, TUBD1, MTOR, TUBE1, CDK4	5.73	3.94E-09
5	Nucleotide binding	22	PRKCA, AR, AR, ERBB3, TUBA1A, CDK6, MAPK8, PIK3CA, AR, PRKCD, PRKCI, PRKCB, TUBG1, EGFR, PRKCE, TOP1, DHFR, PRKCZ, TUBD1, MTOR, TUBE1, CDK4	5.55	3.96E-09

6	Nucleoside phosphate binding	22	PRKCA, AR, AR, ERBB3, TUBA1A, CDK6, MAPK8, PIK3CA, AR, PRKCD, PRKCI, PRKCB, TUBG1, EGFR, PRKCE, TOP1, DHFR, PRKCZ, TUBD1, MTOR, TUBE1, CDK4	5.55	5.94E-09
7	Small molecule binding	23	PRKCA, AR, AR, ERBB3, TUBA1A, CDK6, MAPK8, PIK3CA, AR, PRKCD, PRKCI, PRKCB, TUBG1, EGFR, PRKCE, TOP1, DHFR, TYMS, PRKCZ, TUBD1, MTOR, TUBE1, CDK4	4.83	6.12E-09
8	Phosphotransferase activity, alcohol group as acceptor	14	PRKCA, ERBB3, CDK6, MAPK8, PIK3CA, PRKCD, PRKCI, PRKCB, EGFR, PRKCE, TOP1, PRKCZ, MTOR, CDK4	10.75	9.12E-09
9	Calcium-dependent protein serine/threonine kinase activity	6	PRKCA, PRKCD, PRKCI, PRKCB, PRKCE, PRKCZ	> 100	9.45E-09
10	Calcium-dependent protein kinase activity	6	PRKCA, PRKCD, PRKCI, PRKCB, PRKCE, PRKCZ	> 100	9.75E-09

Figure 4.2.3 Pie chart representing cellular component of rhythmic anti-breast cancer drug targets.

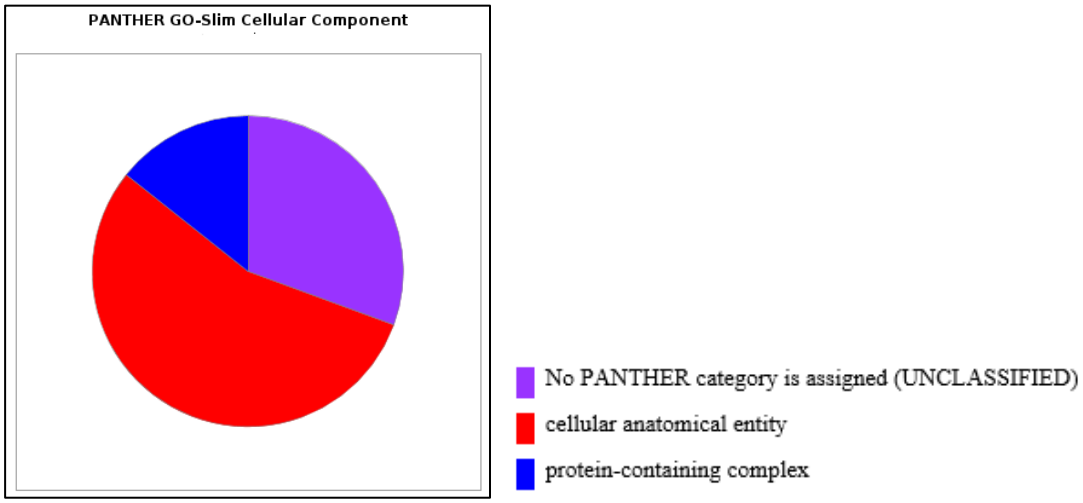


Table 4.2.3 Summary of cellular component of rhythmic anti-breast cancer drug targets.

S.No.	Cellular component	Gene count	Genes	Fold enrichment	False Discovery Rate (FDR)
1	Nucleus	33	PRKCA, EBP, ERBB3, CDK6, CD274, MAPK8, MAPT, AR, PRKCD, FUBP1, GGPS1, ESR2, PRKCI, PRKCB, PARP2, TUBG1, EGFR, PRKCE, TOP1, DHFR, TYMS, PARP3, PRKCZ, TUBD1, MTOR, FDPS, CDK4, AR, PGR, BCL2, ESR1, AR, NR1I2	2.44	1.87E-06
2	Cytosol	25	PRKCA, AR, AR, TUBA1A, CDK6, MAPK8, PIK3CA, MAPT, AR, PRKCD, GGPS1, PRKCI, PRKCB, PRKCE, DHFR, TYMS, PRKCZ, TUBD1, MTOR, ATIC, FDPS, CDK4, AR, PGR, BCL2	3.3	2.66E-06
3	Perinuclear region of cytoplasm	11	PRKCA, KCNH2, GGPS1, ESR2, EGFR, PRKCE, PRKCZ, CDK4, AR, BCL2, ESR1	8.22	2.51E-05
4	Cytosolic region	5	PRKCA, PRKCD, PRKCB, PRKCE, MTOR	53.44	2.86E-05
5	Cell junction	18	PRKCA, ERBB3, TUBA1A, MAPK8, PIK3CA, MAPT, PRKCD, FUBP1, PRKCI, PRKCB, EGFR, PRKCE, PRKCZ, MTOR, CDK4, AR, PGR, ESR1	4.15	3.76E-05
6	Mitochondrion	16	PRKCA, AR, AR, AR, MAPK8, PRKCD, ESR2, PRKCE, DHFR, TYMS, MTOR, ATIC, FDPS, PGR, BCL2, ESR1	4.38	5.02E-05
7	Synapse	15	PRKCA, ERBB3, TUBA1A, MAPK8, MAPT, PRKCD, FUBP1, PRKCI, PRKCB, EGFR, PRKCE, PRKCZ, MTOR, PGR, ESR1	4.79	5.10E-05
8	Intracellular membrane-bounded organelle	38	PRKCA, AR, AR, AR, EBP, ERBB3, TUBA1A, CDK6, CD274, MAPK8, MAPT, AR, PRKCD, FUBP1, GGPS1, ESR2, PRKCI, PRKCB, PARP2, TUBG1, EGFR, PRKCE, TOP1, DHFR, TYMS, PARP3, PRKCZ, TUBD1, MTOR, ATIC, FDPS, CDK4, AR, PGR, BCL2, ESR1, AR, NR1I2	1.73	5.68E-05

9	Intracellular anatomical structure	41	PRKCA, KCNH2, AR, AR, AR, EBP, ERBB3, TUBA1A, CDK6, CD274, MAPK8, PIK3CA, MAPT, AR, PRKCD, FUBP1, GGPS1, ESR2, PRKCI, PRKCB, PARP2, TUBG1, EGFR, PRKCE, TOP1, DHFR, TYMS, PARP3, PRKCZ, TUBD1, MTOR, ATIC, FDPS, TUBE1, CDK4, AR, PGR, BCL2, ESR1, AR, NR1I2	1.51	7.55E-05
10	Intracellular organelle	39	PRKCA, AR, AR, AR, EBP, ERBB3, TUBA1A, CDK6, CD274, MAPK8, MAPT, AR, PRKCD, FUBP1, GGPS1, ESR2, PRKCI, PRKCB, PARP2, TUBG1, EGFR, PRKCE, TOP1, DHFR, TYMS, PARP3, PRKCZ, TUBD1, MTOR, ATIC, FDPS, TUBE1, CDK4, AR, PGR, BCL2, ESR1, AR, NR1I2	1.62	7.76E-05

Figure 4.2.4 Pie chart representing protein class of rhythmic anti-breast cancer drug targets.

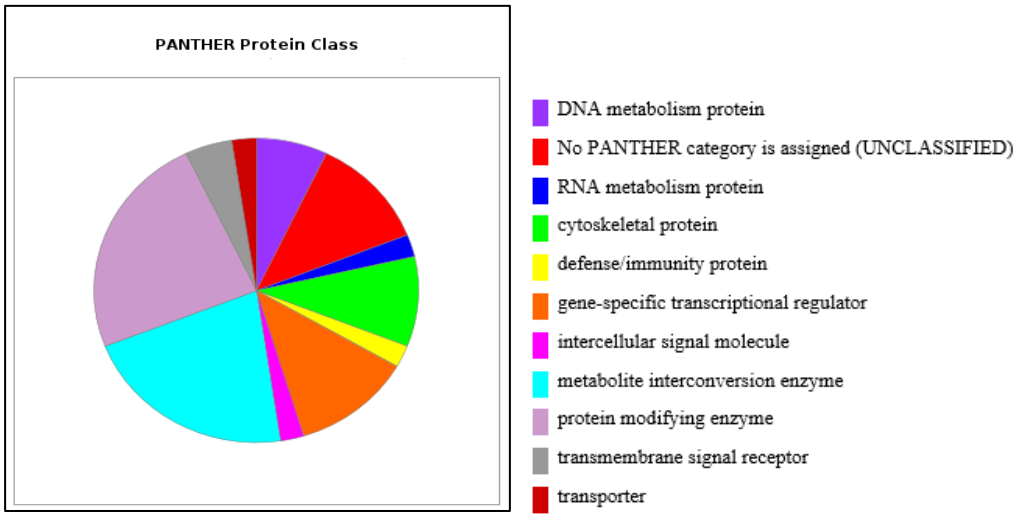
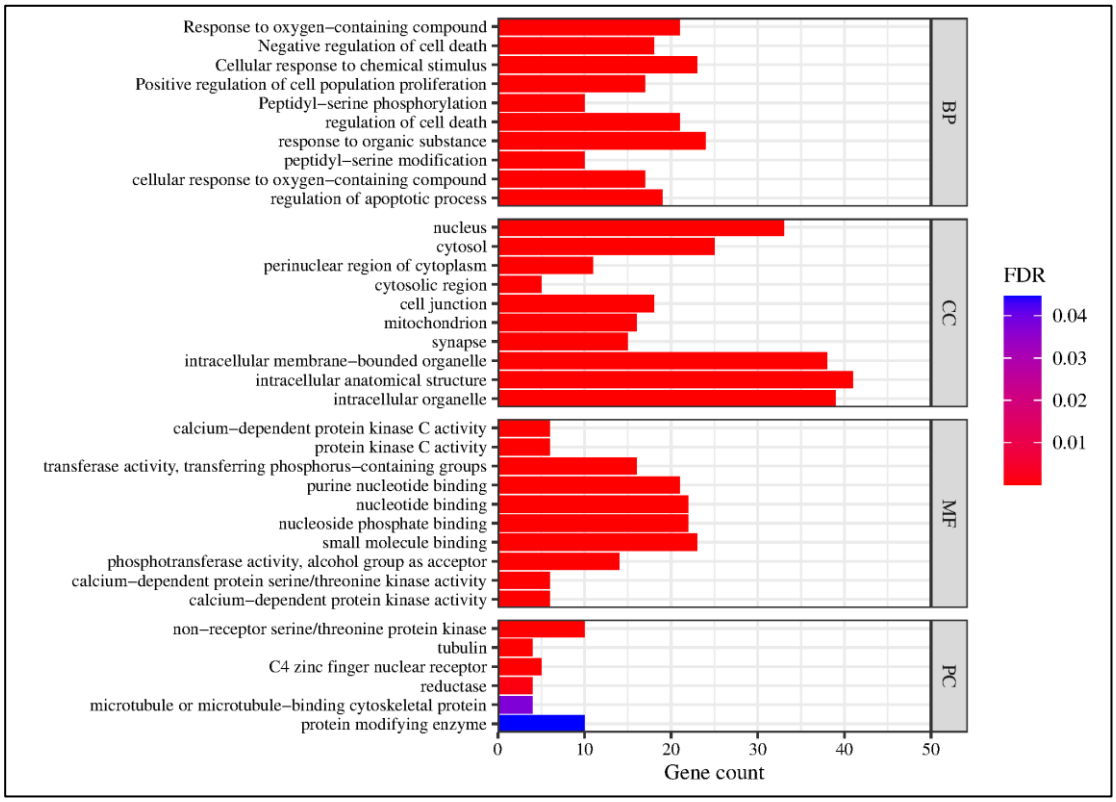


Table 4.2.4 Summary of protein class of rhythmic anti-breast cancer drug targets.

S.No.	PANTHER Protein Class	Gene count	Genes	Fold enrichment	False Discovery Rate (FDR)
1	Non-receptor serine/threonine protein kinase	10	PRKCA, CDK6, MAPK8, PRKCD, PRKCI, PRKCB, PRKCE, PRKCZ, MTOR, CDK4	14.63	2.69E-07
2	Tubulin	4	TUBA1A, TUBG1, TUBD1, TUBE1	> 100	6.45E-06
3	C4 zinc finger nuclear receptor	5	AR, ESR2, PGR, ESR1, NR1I2	51.35	6.88E-06
4	Reductase	4	AR, AR, DHFR, AR	24.94	1.16E-03
5	Microtubule or microtubule-binding cytoskeletal protein	4	TUBA1A, TUBG1, TUBD1, TUBE1	8.8	3.76E-02
6	Protein modifying enzyme	10	PRKCA, CDK6, MAPK8, PRKCD, PRKCI, PRKCB, PRKCE, PRKCZ, MTOR, CDK4	3.08	4.47E-02

Figure 4.2.5 Summary graph of gene ontologies and protein class.



4.3 KEGG pathway analysis using DAVID bioinformatics resource

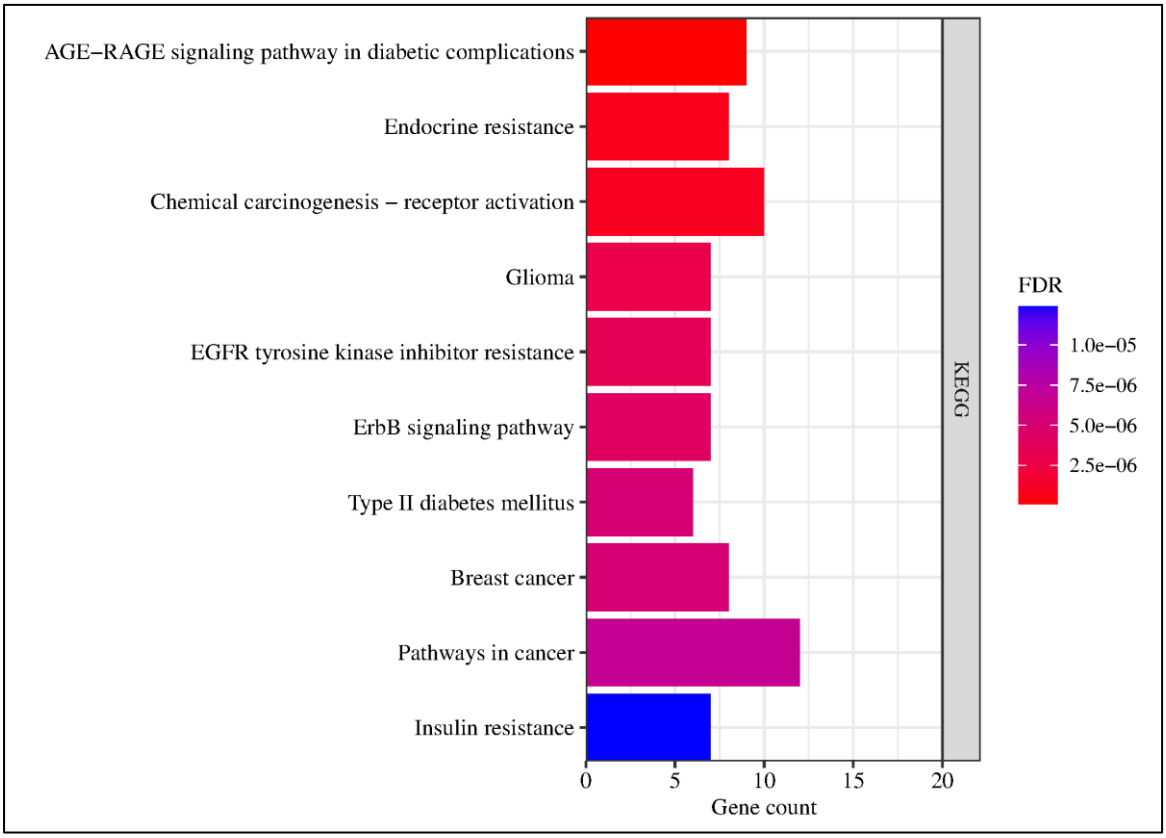
To further comprehend the understanding of mechanism of rhythmic drug targets in *Mus musculus* organism, KEGG pathway maps of genes were studied. The KEGG pathway maps describe how genes are engaged in numerous signalling pathways, metabolic pathways, organismal systems, and disrupted pathways linked to human disorders. The 37 rhythmic anti-breast cancer drug targets/official gene symbols were uploaded for analysis and only top 10 pathways were selected based on least False Discovery Rate (FDR). A table of the result (Table 4.3) was created containing information about top 10 pathways, name and number of genes in the uploaded list that correspond to the pathway, fold enrichment of the genes indicating the category is overrepresented (if greater than 1) or underrepresented (if less than 1) and minimum False Discovery Rate (FDR<0.1) as calculated by the Benjamini-Hochberg procedure. The KEGG pathways of rhythmic anti-breast cancer drug targets are summarized in Figure 4.3. This analysis identified the molecular interactions and networks related to cancer metabolic and signaling pathways. The disrupted pathways can used to identify the potential clock gene biomarker that can be targeted to rectify the biological disease along with insights for personalized medicine and optimal time prediction of the drug.

Table 4.3 Summary of functional pathways of rhythmic anti-breast cancer drug targets.

S.No.	Pathway	Gene count	Genes	Fold enrichment	False Discovery Rate (FDR)
1	AGE-RAGE signaling pathway in diabetic complications	9	MAPK8, PIK3CA, CDK4, PRKCB, PRKCE, PRKCD, BCL2, PRKCA, PRKCZ	25.90	8.27E-08
2	Endocrine resistance	8	MAPK8, PIK3CA, CDK4, BCL2, ESR1, EGFR, MTOR, ESR2	25.00	7.66E-07
3	Chemical carcinogenesis - receptor activation	10	AR, PIK3CA, PRKCB, BCL2, PGR, PRKCA, ESR1, EGFR, MTOR, ESR2	12.92	8.94E-07
4	Glioma	7	CDK6, PIK3CA, CDK4, PRKCB, PRKCA, EGFR, MTOR	27.50	2.92E-06
5	EGFR tyrosine kinase inhibitor resistance	7	PIK3CA, ERBB3, PRKCB, BCL2, PRKCA, EGFR, MTOR	25.76	3.46E-06
6	ErbB signaling pathway	7	MAPK8, PIK3CA, ERBB3, PRKCB, PRKCA, EGFR, MTOR	24.22	4.17E-06

7	Breast cancer	8	CDK6, PIK3CA, CDK4, PGR, ESR1, EGFR, MTOR, ESR2	15.82	5.14E-06
8	Type II diabetes mellitus	6	MAPK8, PIK3CA, PRKCE, PRKCD, PRKCZ, MTOR	36.33	5.14E-06
9	Pathways in cancer	12	AR, MAPK8, CDK6, PIK3CA, CDK4, PRKCB, BCL2, PRKCA, ESR1, EGFR, MTOR, ESR2	6.42	6.72E-06
10	Insulin resistance	7	MAPK8, PIK3CA, PRKCB, PRKCE, PRKCD, PRKCZ, MTOR	18.50	1.24E-05

Figure 4.3 Summary graph of KEGG pathways of rhythmic anti-breast cancer drug targets.



(All the pathways are arranged in ascending order of their False Discovery Rate (FDR)).

4.4 Molecular docking study of SR9009 with rhythmic anti-breast cancer drug targets

In this analysis, blind molecular docking of the top 10 rhythmic drug targets (Table 4.4.2.) out of 37 drug targets (selected on the basis of the minimum Q-value) was performed with SR9009 (PubChem CID: 57394020) (ligand) using AutoDock Vina, and the binding energies (ΔG) were compared with positive control, NR1D1 (REV-ERB α) (PDB ID: 8D8I) (Table 4.4.1). The free binding energy of PARP2 (PDB ID:7R59) was -9.77±0.03 kcal/mol while of the positive control, NR1D1 (REV-ERB α) (PDB ID: 8D8I) was -5.73±0.03 kcal/mol, when docked with SR9009. This demonstrated that SR9009 has better binding interaction with PARP2 drug target than the positive control, NR1D1(REV-ERB α). Minimum free energy of binding (ΔG) was chosen as a selective parameter to find one of the best binding affinities and pose for ligand molecule docked into the active site of each drug target. The top 10 docked complexes with SR9009 are shown in Figure 4.4.2. The SR9009-NR1D1 complex is shown in Figure 4.4.1. The docking analysis helped in the interpretation that SR9009 can interact with rhythmic anti-breast cancer drug targets with high binding affinity and subsequent good binding energies with the top 10 drug targets as compared to NR1D1 (REV-ERB α). The MTOR (PDB ID:4DRI) (-9.03-±0.07 kcal/mol) and PARP2 (PDB ID:7R59) (-9.77±0.03 kcal/mol) depicted strong binding interactions with SR9009. Further invitro interaction and clinical studies between SR9009 and PARP2 can be conducted to check the action of PARP-2 with SR9009 treatment in breast cancer cells.

Table 4.4.1 Summary of molecular docking study of SR9009 with positive control, NR1D1 (REV-ERB α).

		POSITIVE CONTROL								
CLOCK MODULATOR	**S.NO.**	**DRUG TARGET**	**PDB ID**	**ΔG 1**	**ΔG 2**	**ΔG 3**	**AVERAGE (μ)**	**STANDARD DEVIATION**	**STANDARD ERROR (SE)**	**ΔG= - (μ±SE) kcal/mol**
(Source: PubChem)		(Source: DRUGBANK)	(Source: RCSB PDB)							
SR-9009 (PubChemCID:57394020)	1	NR1D1 (REV-ERB α)	8D8I	-5.8	-5.7	-5.7	-5.73	0.06	0.03	-5.73±0.03

Figure 4.4.1 Molecular docked complex of positive control, NR1D1 (REV-ERB α) with SR9009.

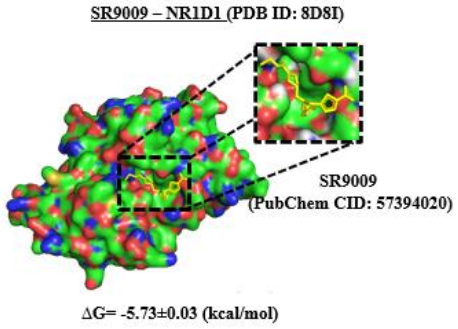
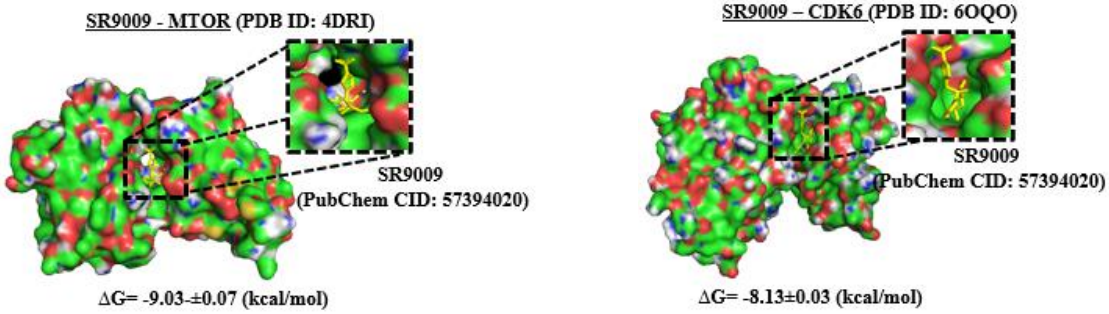


Table 4.4.2 Summary of molecular docking study of SR9009 with top 10 rhythmic anti-breast cancer drug targets.

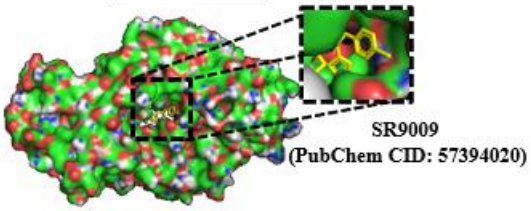
		RHYTHMIC ANTI-BREAST CANCER TARGETS								
CLOCK MODULATOR	S.NO.	DRUG TARGET	PDB ID	ΔG 1	ΔG 2	ΔG 3	AVERAGE (μ)	STANDARD DEVIATION	STANDARD ERROR (SE)	ΔG= - (μ±SE) kcal/mol
(Source: PubChem)		(Source: DRUGBANK)	(Source: RCSB PDB)							
SR-9009 (PubChemCID:57394020)	1	MTOR	4DRI	-9.1	-9.1	-8.9	-9.03	0.12	0.07	-9.03±0.07
	2	CDK6	6OQO	-8.2	-8.1	-8.1	-8.13	0.06	0.03	-8.13±0.03
	3	MAPT	5MO3	-6.8	-7.3	-7.1	-7.07	0.25	0.15	-7.07±0.15
	4	NR1I2	6P2B	-6.3	-6.1	-6	-6.13	0.15	0.09	-6.13±0.09
	5	TYMS	3ED7	-7.4	-7.5	-7.4	-7.43	0.06	0.03	-7.43±0.03
	6	TOP1	1K4T	-8.2	-8.6	-8.6	-8.47	0.23	0.13	-8.47±0.13
	7	ESR1	7UJO	-7.3	-7.1	-6.9	-7.10	0.20	0.12	-7.10±0.12
	8	PDCD1	5GGS	-7.4	-7.4	-7.4	-7.40	0.00	0.00	-7.40±0.00
	9	CD274	4Z18	-8.5	-8.6	-8.5	-8.53	0.06	0.03	-8.53±0.03
	10	PARP2	7R59	-9.8	-9.8	-9.7	-9.77	0.06	0.03	-9.77±0.03

(The top 10 rhythmic drug targets were selected based on the minimum Q-value).

Figure 4.4.2 SR9009-drug target docked complex: figure obtained after molecular docking interactions which represents ligand (SR9009) in yellow color while drug targets (protein) in green color.

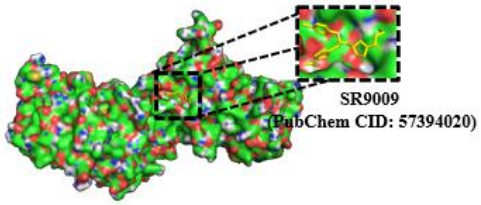


SR9009 – MAPT (PDB ID: 5MO3)



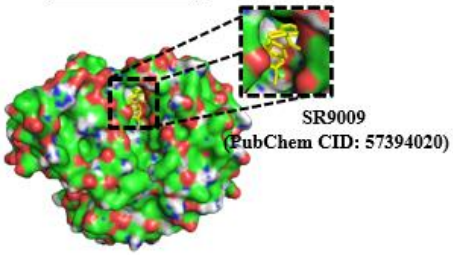
ΔG= -7.07±0.15 (kcal/mol)

SR9009 – NR1I2 (PDB ID: 6P2B)



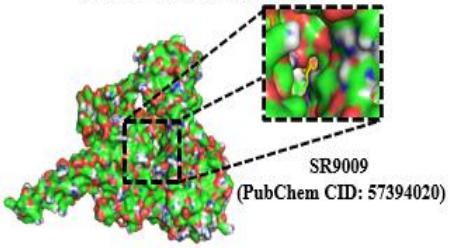
ΔG= -6.13±0.09 (kcal/mol)

SR9009 – TYMS (PDB ID: 3ED7)



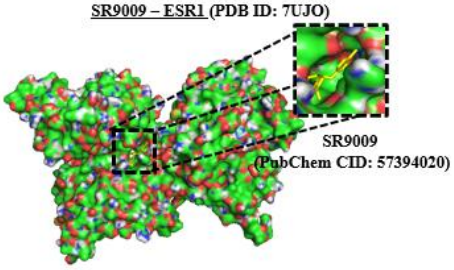
ΔG= -7.43±0.03 (kcal/mol)

SR9009 – TOP1 (PDB ID: 1K4T)



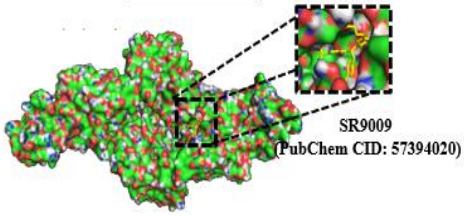
ΔG= -8.47±0.13 (kcal/mol)

SR9009 – ESR1 (PDB ID: 7UJO)



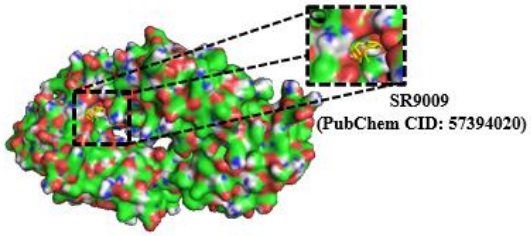
ΔG= -7.10±0.12 (kcal/mol)

SR9009 – PDCD1 (PDB ID: 5GGS)



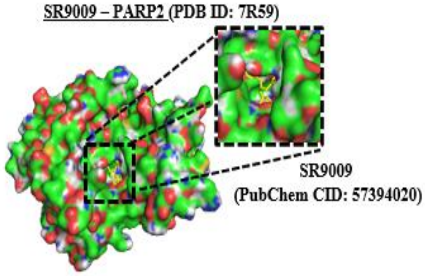
ΔG= -7.40±0.00 (kcal/mol)

SR9009 – CD274 (PDB ID: 4Z18)



ΔG= -8.53±0.03 (kcal/mol)

SR9009 – PARP2 (PDB ID: 7R59)



ΔG= -9.77±0.03 (kcal/mol)

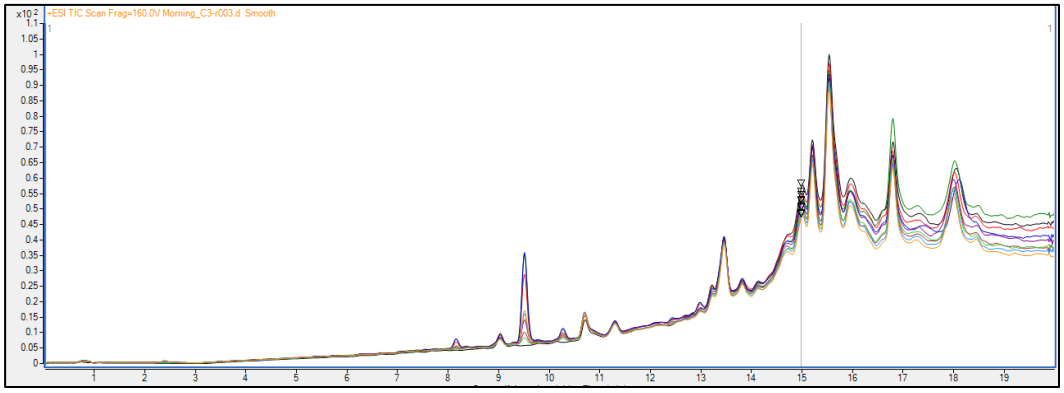
4.5 Metabolite profiling of 4T1 cells after SR9009 treatment

After treating 4T1 cells with SR9009 (20 μM) in morning and evening for 24 h, reversed-phase HPLC-Q-TOF/MS was performed in ESI^+ mode. Representative total ion chromatograms of endogenous cellular metabolites were obtained, as shown in Figure 4.5.1 which revealed significant differences between groups. The extracted data was analysed in the public mass spectrometry database to confirm the identities and chemical structures of the labeled metabolites. In this untargeted metabolomics study, 576 and 463 metabolites in morning and evening respectively were identified from the ESI+ data. Further, analysis of only SR9009 (morning vs evening) data in Mass Profiler Professional (MPP) software, resulted in 614 metabolites according to $p < 0.05$ and fold change >2. After removing the redundant and non-significant metabolites from the data table on comparison with HMDB, KEGG and METLIN database, only 42 metabolites were found to be differentially abundant (refer Table 4.5.1). Among these, L-arginino-succinate, (5Z,9Z)-nonadeca-5,9-dienoic acid, laudanosine, Umbelliferone, 15Z,18Z,21Z,24Z-triacontatetraenoic acid etc were upregulated in the morning whereas Octadecenoic acid, m-Cresol, N-docosanoyl-sphinganine (Cer(d18:0/22:0)), 5,10 methylenetetrahydrofolate etc were downregulated in the evening after SR9009 treatment in the respective morning and evening time-points. From these, 14 marker metabolites were of therapeutic significance and may play role in in breast cancer treatment (refer Table 4.5.2). These include L-arginino-succinate, (+)-costunolide, 5,10-methylenetetrahydrofolate, Umbelliferone, 2-(14,15-Epoxyeicosatrienoyl) Glycerol, 12,14-Pentacosadiynoic acid, lysophosphatidylcholine (LysoPC(16:0/0:0)), L-tryptophan, 20-hydroxyecdysone (Crustecdysone), N-docosanoyl-sphinganine (Cer(d18:0/22:0)), hypoxanthine, phosphatidylcholine (PC(O-16:1(9Z)/18:0)), m-Cresol, and Glutathione. Only 8 metabolites were found to be circadian metabolites from previous literature which are as follows: L-arginino-succinate (Lin R *et al.*, 2017), 5,10-methylenetetrahydrofolate (Wegner C, & Nau H, 1991), lysophosphatidylcholine (LysoPC(16:0/0:0)) (Chua ECP *et al.*, 2013), L-tryptophan (Davies SK *et al.*, 2014), N-docosanoyl-sphinganine (Cer(d18:0/22:0)) (Jang YS *et al.*, 2012), hypoxanthine (Chagoya de Sánchez V *et al.*, 1983), phosphatidylcholine (PC(O-16:1(9Z)/18:0)) (Pan X *et al.*, 2020), and Glutathione (Tuñón MJ *et al.*, 1992). The UPLC-Q-TOF/MS data were summarized using volcano plot (Figure 4.5.2) and principal component analysis (PCA) (Figure 4.5.3) of SR9009 (Morning vs Evening) treatment. To evaluate the effect of SR9009 on 4T1 cell

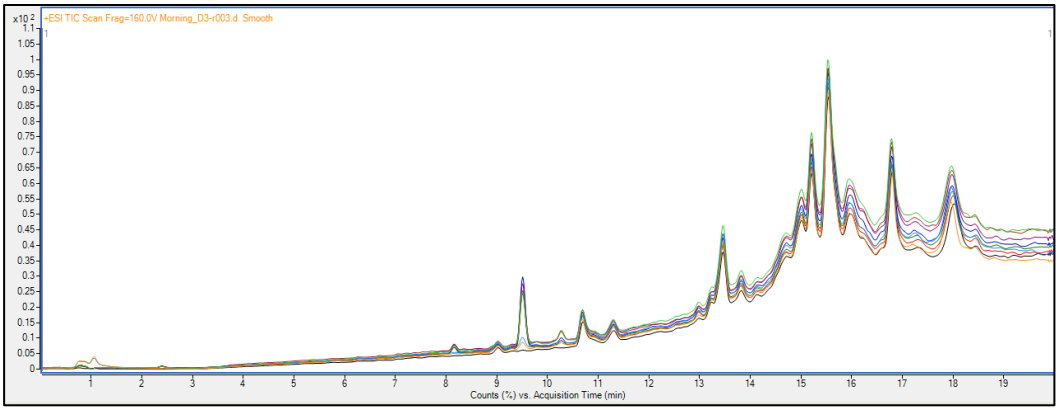
metabolism, the MetaboAnalyst 5.0 database was used for metabolic pathway enrichment analysis of the prominent metabolites listed in Table 4.5.1. Metabolic pathways with effect values of $p < 0.2$ were selected as potential critical pathways. The regulation of 4T1 cell metabolism by SR9009 involved 14 metabolic pathways (Figure 4.5.4). Among them, Alpha Linolenic Acid and Linoleic Acid Metabolism, Phospholipid Biosynthesis, and Glycerolipid Metabolism were considered as important metabolic pathways through which SR9009 effected 4T1 cells. The metabolic pathways with $p > 0.2$, are summarized in Table 4.5.3.

Figure 4.5.1 Representative total ion chromatogram (TIC) obtained for metabolite profiles in 4T1 cells after SR9009 treatment (ESI^+). (i) Morning: (A) control group; (B) 20 µM SR9009. (ii) Evening: (C) control group; (D) 20 µM SR9009.

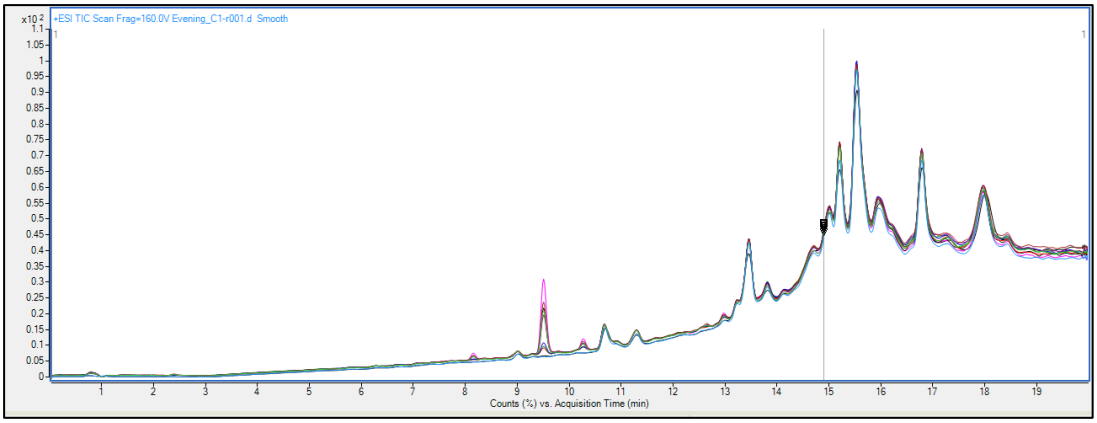
(i): (A)



(i): (B)



(ii): (C)



(ii): (D)

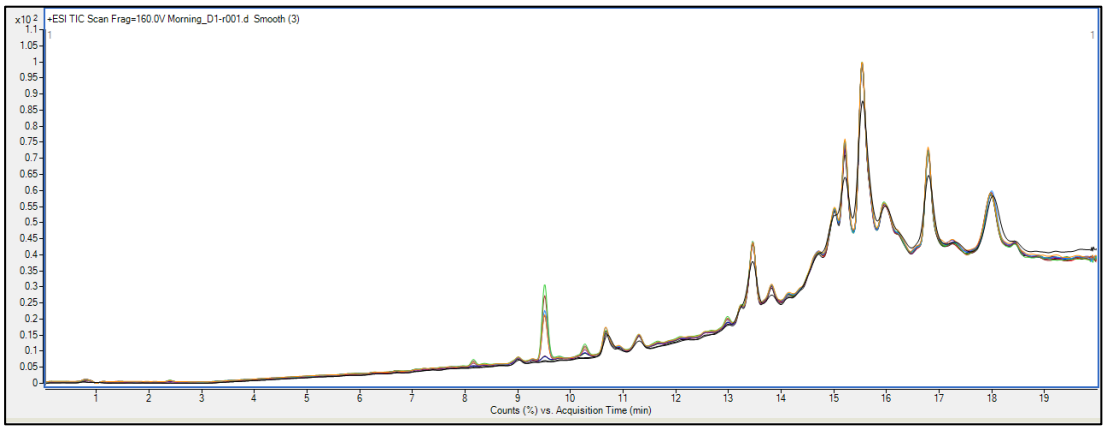


Table 4.5.1 Altered metabolites of 4T1 cells after SR9009 treatment in ElectroSpray Ionization positive (ESI^+) mode.

S.No.	Compound	Morning regulation	Evening regulation	p value	Log Fold change	Mass	Retention Time	HMDB ID	KEGG ID	PubChem ID	Formula
1	Geranic acid	down		1.21 E-25	-18.33	185.14	9.23	HMDB0036103		5275520	C10 H16 O2
2	2-benzylsuccinate		up	0.013	8.92	104.04	14.49	HMDB0012127	C09816		C11 H12 O4
3	L-arginino-succinate	up		4.15 E-23	17.48	290.12	7.09	HMDB00052	C03406		C10 H18 N4 O6

4	Sinapyl-alcohol	up		9.96 E-43	19.67	210.09	8.96	HMDB13070	C02325		C11 H14 O4
5	Methyl (2E,6Z)-dodecadienoate	down		5.69 E-07	-15.76	210.16	12.53	HMDB0031014			C13 H22 O2
6	cis-9-tetradecenoic acid	down		7.34 E-05	-13.39	226.19	11.88	HMDB0002000			C14 H26 O2
7	(+)-costunolide	down	up	7.44 E-05	-13.36	232.14	10.9	HMDB0036688	C09382		C15 H20 O2
8	Stearidonic acid	down		8.11 E-05	-14.00	276.21	13.29	HMDB0006547	C16300		C18H28O2
9	6-isopentyl-9-methyl-5-decenoic acid		up	7.44 E-05	13.62	254.23	14.32			5282681	C16 H30 O2
10	Monoacylglyceride (MG(0:0/16:0/0:0))	up		2.57 E-28	22.04	330.28	15.09	HMDB0011533			C19 H38 O4
11	Dehydroepiandrosterone	down		5.96 E-07	-15.43	288.21	13.32	HMDB0000077	C01227		C19 H28 O2
12	16-Heptadecynoic acid		up	4.45 E-30	16.22	288.21	13.58			5312626	C17 H30 O2
13	9,12-Octadecadiynoic acid		up	7.34 E-05	13.63	298.19	12.86	HMDB0247623			C18 H28 O2
14	(9S,13S)-10,11-dihydro-12-oxo-15-phytoenoic acid	down	up	3.01 E-27	18.89	294.22	13.93		C04780	5280729	C18 H30 O3
15	Octadecenoic acid	down	down	4.94 E-33	-19.04	282.25	15.57	HMDB0245277			C18 H34 O2

16	Oryzalide A	up		7.31 E-05	14.30	320.20	14.52	HMDB37591			C19 H28 O4
17	10,13-Nonadecadiynoic acid		up	5.54 E-31	17.36	290.22	13.77			5312636	C19 H30 O2
18	(5Z,9Z)-nonadeca-5,9-dienoic acid	up		7.35 E-05	13.68	311.28	13.66	HMDB0340927			C19 H34 O2
19	5,10-methylenetetrahydrofolate		down	0.0021	-10.43	479.15	14.37	HMDB01533	C00143		C20 H23 N7 O6
20	5,8,11-Eicosatriynoic acid	down		7.37 E-05	-13.61	300.21	14.01	HMDB0246870			C20 H28 O2
21	Umbelliferone	up		7.70 E-36	18.13	162.03	12.93	HMDB0029865	C09315		C9 H6 O3
22	8-[3]-ladderane-octanoic acid	down	down	0.0045	-10.45	304.24	14.93			10380342	C20 H32 O2
23	Laudanosine	up		6.06 E-07	17.01	357.20	12.64	HMDB30213	C09558		C21 H27 N O4
24	Phosphatidylcholine (PC(O-16:1(9Z)/18:0))	down		1.59 E-25	-18.49	745.60	15.36	HMDB0013412			C42 H84 N O7 P
25	2-(14,15-Epoxyeicosatrienoyl) Glycerol		down	7.39 E-05	-13.44	394.27	14.002	HMDB0013651			C23 H38 O5
26	12,14-Pentacosadiynoic acid	down		5.56 E-07	-19.44	402.35	14.48			9543642	C25 H42 O2
27	11,15,19-trimethyl-5Z,9Z-eicosadienoic acid	up		7.55 E-26	18.59	372.30	14.18			52929812	C23 H42 O2

28	Lysophosphatidylcholine (lysopc(16:0/0:0))		up	2.51 E-30	17.08	496.34	15.23	HMDB10382			C24 H50 N O7 P
29	5-(14-Nonadecenyl)-1,3-benzenediol	down		5.60 E-07	-19.81	374.32	14.73	HMDB0031677			C25 H42 O2
30	Smilagenone		down	3.34 E-36	-19.04	436.30	17.62	HMDB0036941			C27 H42 O3
31	L-tryptophan		up	0.0095	23.15	204.09	4.7	HMDB00929	C00078		C11 H12 N2 O2
32	24-Hydroxycalcitriol	down		7.44 E-05	-14.51	432.33	15.16	HMDB0006228			C27 H44 O4
33	20-hydroxyecdysone (Crustecdysone)		up	7.50 E-05	13.34	480.31	15.78	HMDB30180	C02633		C27 H44 O7
34	Chenodeoxycholylglutamic acid		up	0.014	7.91	538.36	13.58	HMDB0242405			C29 H47 N O7
35	15Z,18Z,21Z,24Z-triacontatetraenoic acid	up	up	5.21 E-34	17.97	222.20	14.42			44151232	C30 H52 O2
36	21Z,24Z,27Z,30Z-hexatriacontatetraenoic acid		up	7.40 E-05	12.49	528.49	15.37			52921805	C36 H64 O2
37	2-Hexaprenyl-3-methyl-5-hydroxy-6-methoxy-1,4-benzoquinol		up	4.58 E-37	18.39	578.43	15.72	HMDB0012146			C38 H58 O4
38	N-docosanoyl-sphinganine (Cer(d18:0/22:0))		down	0.0047	-8.91	623.62	15.23	HMDB11765			C40 H81 N O3
39	Cobalt-precorrin-8	down		7.32 E-05	-14.00	949.32	15.75			135926593	C45 H53 Co N4 O14

40	Hypoxanthine		up	0.0098	18.53	136.04	3.92	HMDB00157	C00262		C5 H4 N4 O
41	m-Cresol		down	0.0011	-11.08	108.06	14.48	HMDB02048	C01467		C7 H8 O
42	Glutathione	down	up	5.64 E-07	16.51	482.18	13.99	HMDB0000125			C10 H17 N3 O6 S

All the metabolites have been filtered out by applying statistical parameters: $p < 0.05$ and fold change >2

Circadian metabolites

Figure 4.5.2 Volcano plot of SR9009 (Morning vs Evening).

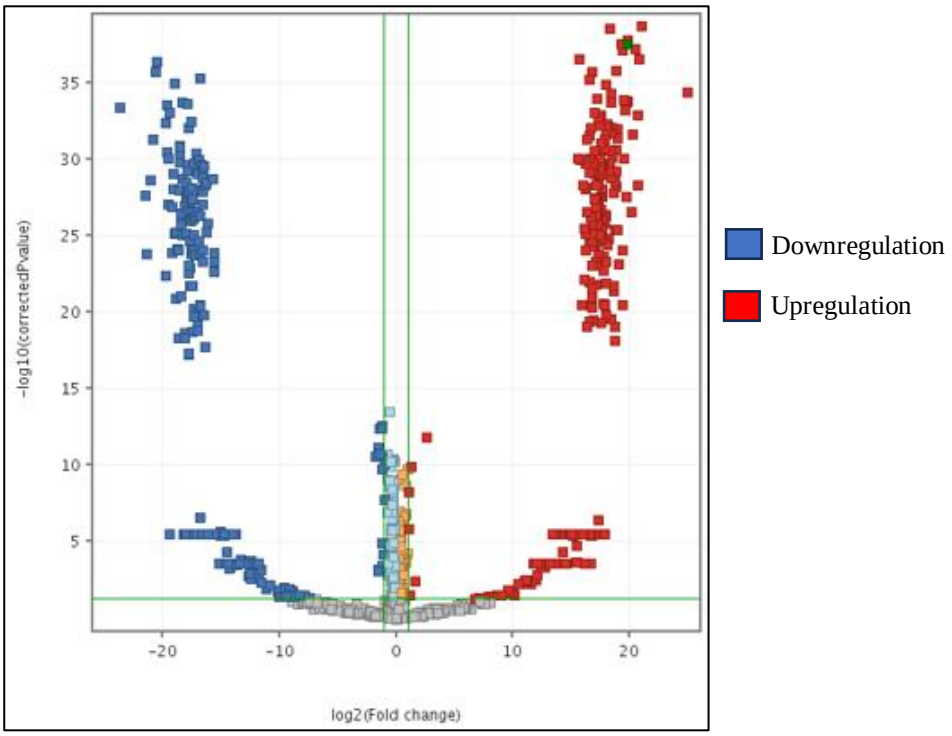


Figure 4.5.3 Principal component analysis (PCA) plot for endogenous metabolites of 4T1 cells in electrospray ionization-positive (ESI+) mode treated with SR9009 (Morning vs Evening).

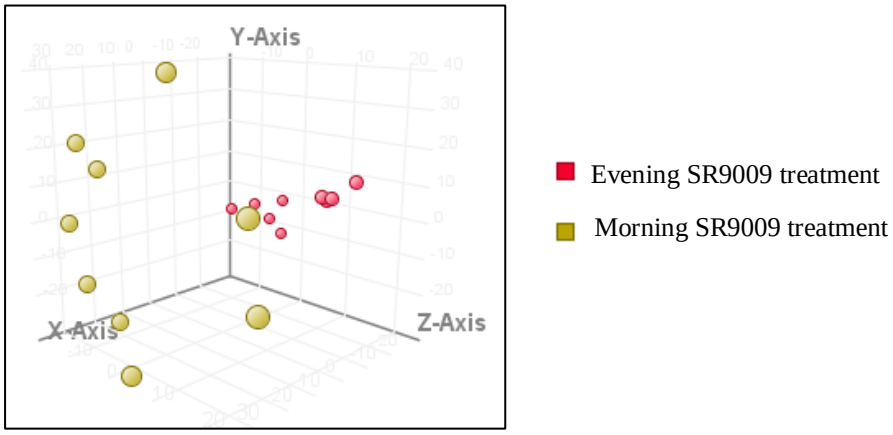


Table 4.5.2 Metabolites of therapeutic significance for breast cancer treatment obtained from SR9009 administration in 4T1 cells.

S.No.	Compound	Class	Function	Morning regulation	Evening regulation	Interpretation
1	L-arginino-succinate (Lin et al., 2017, PMID: 28985504)	Amino acid	Arginosuccinic acid is a basic amino acid. It is a precursor to fumarate in the citric acid cycle via argininosuccinate lyase (ASL). It is involved in urea cycle, arginine metabolism and is a direct activator of mTOR, a nutrient-sensing kinase strongly implicated in carcinogenesis.	up		Downregulation of ASL has been shown to inhibit the growth of breast cancer tumors in vitro and in vivo. Further experimental study with SR9009 can be conducted at different time-point to evaluate its anti-breast cancer treatment.
2	(+)-costunolide	Terpenoid	It is naturally occurring sesquiterpene lactones. It exerts anti-inflammatory, antioxidant, hair growth promoting, antidiabetic, antiallergic, and anticancer properties.	down	up	Administration of SR9009 at evening time point may be beneficial in breast cancer treatment.

3	5,10-methylenetetrahydrofolate (Wegner and Nau, 1991, PMID: 1810574)	Vitamin	It is an intermediate in glycine, serine and threonine metabolism and one carbon metabolism. It is involved in different metabolic pathways such as ammonia recycling, pyrimidine and folate metabolism. It is a substrate for Methylenetetrahydrofolate reductase (MTHFR), which is is reportedly involved in breast cancer. MTHFR converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate.This reaction is required for the multistep process that converts the amino acid homocysteine to methionine. The body uses methionine to make proteins and other important compounds.		down	Low or deficient folate status is associated with increased risk of many cancers. Mutations in the MTHFR ge ne may promote the development of breast cancer. Further experimental study with SR9009 can be conducted at different time-point to evaluate its anti-breast cancer treatment.
4	Umbelliferone	Coumarin	It is a primary metabolite that are metabolically or physiologically essential metabolites. It was found to exhibit significant anticancer effects via the induction of apoptosis, cell cycle arrest and DNA fragmentation in HepG2 cancer cells.	up		Administration of SR9009 at morning time point may be beneficial in breast cancer treatment.
5	2-(14,15-Epoxyeicosatrienoyl) Glycerol	Lipid	It is a cytochrome P450 metabolite of 2-arachidonoyl glycerol in the kidney. It is involved in fatty acid metabolism , cell signaling , lipid metabolism pathway, and lipid peroxidation. Elevated 14,15- epoxyeicosatrienoic acid are associated with aggressiveness of human breast cancer.		down	Administration of SR9009 at evening time point may be beneficial in breast cancer treatment.
6	12,14-Pentacosadiynoic acid	Lipid	It is a very long-chain fatty acid. It exhibits strong absorption in the UV-visible region, making it useful in various applications such as sensors, optoelectronics, and biotechnology. It has been reported to have anticancer properties and can induce apoptosis in cancer cells.	down		Further experimental study with SR9009 can be conducted at different time-point to evaluate its upregulation and anti-breast cancer treatment.

7	Lysophosphatidylcholine (LysoPC(16:0/0:0)) (Chua et al., 2013, PMID: 23946426)	Lipid	LysoPC(16:0) is a lysophospholipid (LyP). LPCs have a number of protective or anti-inflammatory effects. It is involved in phospholipid biosynthesis, cell signaling, apoptosis and lipid peroxidation. LysoPC inhibited lung cancer cell proliferation, by inducing mitochondrial dysfunction, altering lipid metabolisms, and increasing fatty acid oxidation.		up	Administration of SR9009 at evening time point may be beneficial in breast cancer treatment. Further experimental study with SR9009 can be conducted at different time-point to evaluate its therapeutic efficiency.
8	L-tryptophan (Davies et al., 2014, PMID: 25002497)	Amino acid	It is an essential and alpha-amino acid. It is involved in NAD, tryptophan and alanine metabolism and protein synthesis. It has carcinogenic and tumor promoter properties. It promotes tumor cell intrinsic malignant properties as well as restricts antitumour immunity.		up	Further experimental study with SR9009 can be conducted at different time-point to evaluate its downregulation and anti-breast cancer treatment.
9	20-hydroxyecdysone (Crustecdysone)	Lipid	They are bile acids, alcohols or derivatives bearing at least hydroxyl group and are thus considered to be a sterol. It induces tumor suppressive effect on breast cancer cell lines.It also dampened glycolysis and respiration, as well as reduced the metabolic potential of triple negative breast cancer cell lines.		up	Administration of SR9009 at evening time point may be beneficial in breast cancer treatment.
10	N-docosanoyl-sphinganine (Cer(d18:0/22:0)) (Jang et al., 2012, PMID: 21773940)	Lipid	It is a is a ceramide (Cer). It controls fundamental cellular processes such as cell division, differentiation, and cell death. Impairments associated with sphingolipid metabolism are associated with many common human diseases such as diabetes, various cancers, diseases of the cardiovascular and respiratory systems. The cancer cells maintain low levels of ceramides through different mechanisms, such as increased ceramide turnover or storage of ceramide in lipid droplets.		down	Increasing ceramide levels has proved to be important for activating cell death in cancer cells. Further experimental study with SR9009 can be conducted at different time-point to evaluate its upregulation and anti-breast cancer treatment.

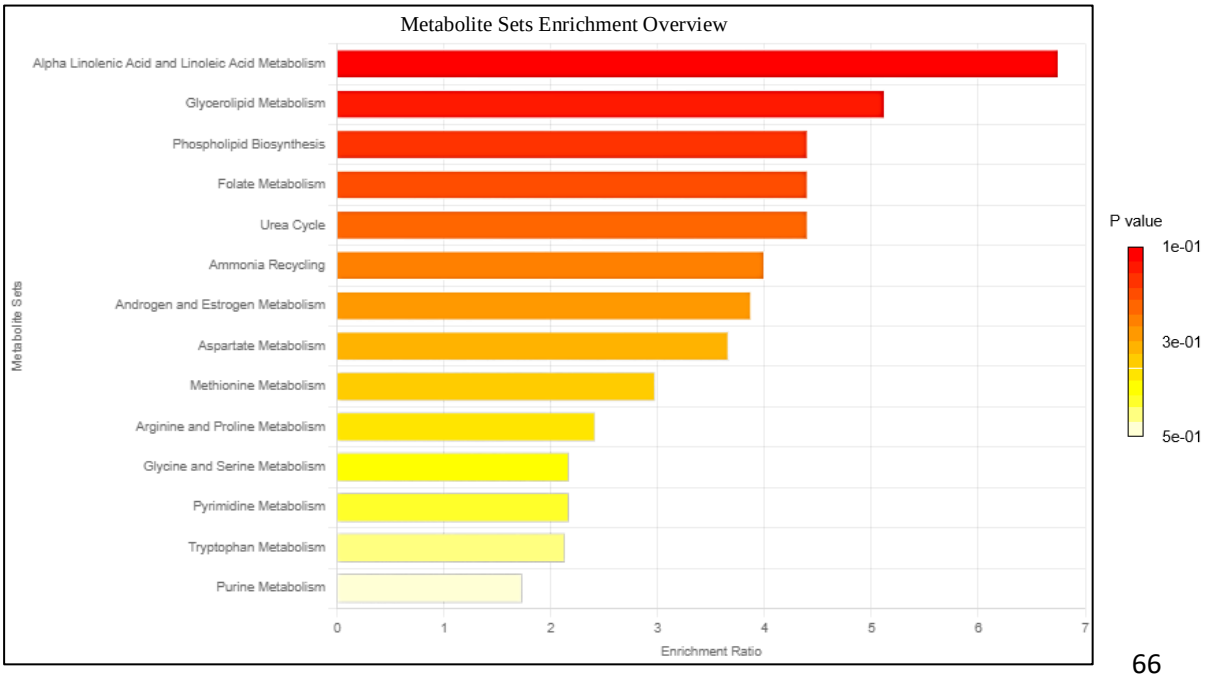
11	Hypoxanthine (de Sánchez et al., 1983, PMID: 6888162)	Purine	Hypoxanthine is a naturally occurring purine derivative and a reaction intermediate in the metabolism of adenosine and in the formation of nucleic acids by the nucleotide salvage pathway. Hypoxanthine is also involved in the metabolic disorder called the purine nucleoside phosphorylase deficiency. Hypoxanthine phosphoribosyl transferase 1 (HPRT1) is upregulated in breast cancer. HPRT catalyzes the conversion of hypoxanthine to inosine monophosphate, and guanine to guanosine monophosphate.		up	Further experimental study with SR9009 can be conducted at different time-point to evaluate its downregulation and anti-breast cancer treatment.
12	Phosphatidylcholine (PC(O-16:1(9Z)/18:0)) (Pan et al.,2020, PMID: 32705594)	Lipid	It is a glycerophospholipid in which a phosphorylcholine moiety occupies a glycerol substitution site. Increased phosphatidylcholine metabolism supports cancer cell accelerated growth by providing the major cellular membrane component.Phosphatidylcholine also generates lipid mediators that exert an intercellular crosstalk favoring cancer cell survival, proliferation, and immune modulation that culminate in resistance to therapy.	down		Administration of SR9009 at morning time point may be beneficial in breast cancer treatment.
13	m-Cresol	Phenol	m-Cresol is an isomer of p-cresol and o-cresol. It has a role as a human xenobiotic metabolite. Cresols are tumor promoters in rodents.		down	Administration of SR9009 at evening time point may be beneficial in breast cancer treatment.
14	Glutathione (Tuñón et al., 1992, PMID: 1380339)	Amino acid	Glutathione is a compound synthesized from cysteine, perhaps the most important member of the body's toxic waste disposal team. It plays role in red and white blood cell formation and throughout the immune system. The elevated GSH levels in tumor cells are associated with tumor progression and increased resistance to chemotherapeutic drugs.	down	up	Administration of SR9009 at morning time point may be beneficial in breast cancer treatment.

Circadian metabolites

Table 4.5.3 Statistical analysis results of the main metabolites in 4T1 cells.

S.No.	Pathway name	Total	Expected	Hits	Raw p
1	Alpha Linolenic Acid and Linoleic Acid Metabolism	19	0.15	1	1.40E-01
2	Glycerolipid Metabolism	25	0.20	1	1.80E-01
3	Phospholipid Biosynthesis	29	0.23	1	2.06E-01
4	Folate Metabolism	29	0.23	1	2.06E-01
5	Urea Cycle	29	0.23	1	2.06E-01
6	Ammonia Recycling	32	0.25	1	2.25E-01
7	Androgen and Estrogen Metabolism	33	0.26	1	2.31E-01
8	Aspartate Metabolism	35	0.27	1	2.44E-01
9	Methionine Metabolism	43	0.34	1	2.91E-01
10	Arginine and Proline Metabolism	53	0.41	1	3.47E-01
11	Glycine and Serine Metabolism	59	0.46	1	3.79E-01
12	Pyrimidine Metabolism	59	0.46	1	3.79E-01
13	Tryptophan Metabolism	60	0.47	1	3.84E-01
14	Purine Metabolism	74	0.58	1	4.52E-01

Figure 4.5.4 Metabolic pathway enrichment analysis of differential metabolites conducted based on the Small Molecule Pathway Database (SMPDB). Bar chart.



5. DISCUSSION

Circadian clocks are important time-tracking mechanisms that help organisms adjust to the right time of day and regulate a variety of physiological and cellular functions. Hepatocellular carcinoma, breast, colon, prostate, lung, and ovarian cancers have all been connected in some way with disrupted circadian rhythms. Also, cancer has been linked to specific circadian system components at the molecular level (Lin HH & Farkas ME, 2018). The finding that the circadian clock controls half of the mammalian protein-coding genome has clear medical implications. Numerous studies have shown that the circadian clock affects the way human heart disease and cancer treatment outcomes (Ruben MD *et al*., 2018). Many previous transcriptome profiling have showed circadian oscillations of many important drug targets (Zhang R *et al*., 2014). Some target genes' rhythmic expression is connected to the rhythmic activity of anticancer drugs for time-of-day specificity (Lee Y *et al*., 2021).

Thus, chronotherapy involves optimizing the timing of the cancer drugs to improve efficacy and reduce side effects. The concept behind drugging the clock is that the circadian rhythmic outputs keep the body in a state of balance by momentarily suppressing pathways and preventing prolonged activation that would be harmful. Several drug-like compounds have been developed against the clock components. One such compound focused in this study is SR9009, which acts as REV-ERBs-specific agonist. Previously, it has shown chemotherapeutic effects on human glioblastoma cells (Wagner PM *et al*., 2019). It has been implicated in therapeutic approach for first- or second-line small-cell lung cancer (SCLC) treatment. By directly repressing the autophagy gene Atg5, the anti-SCLC effect of SR9009 was achieved by REV-ERB dependent suppression of autophagy (Shen W *et al*., 2020). Moreover, SR9009 was reported to improve the metabolism of diet-induced obese mice markedly improving dyslipidaemia and hyperglycaemia (Solt LA *et al.*, 2012).

Hence elucidating the effect of SR9009 on metabolism in a 24-hour period may help in metabolite to biomarker discovery for treatment of metabolic diseases. Thus, present study evaluates the role of SR9009 on the mouse breast cancer metabolism in a 24-hour period to decipher the metabolite up or down regulation with possible therapeutic potential.

Firstly, the anti-breast cancer drug targets of Food and Drug Administration (FDA) approved breast cancer drugs were evaluated for circadian rhythmicity analysis. Then the resulting 37 rhythmic drug targets were subjected to functional gene and pathway analysis to understand the biological meaning of the targets in mammals. The result of this analysis revealed that rhythmic anti-breast cancer drug targets were involved in different biological pathways and processes such as AGE-RAGE signaling pathway in diabetic complications, type II diabetes mellitus, negative regulation of cell death etc in addition to breast cancer pathway. Among the rhythmic breast cancer targets, 22 targets were involved in different metabolic processes such as carbohydrate, lipid, and protein metabolism. This rhythmicity analysis data can be used to understand the optimum drug administration time of breast cancer drugs to increase the drug's therapeutic efficiency and reduce their toxic effects.

Next, SR9009 molecular docking interactions with rhythmic anti-breast cancer drug targets was performed taking REV-ERBα (NR1D1) as positive control. SR9009 is a known synthetic REV-ERB ligand for *in vivo* applications such as treatment of circadian-related metabolic diseases and sleep disorders (Cha HK *et al.*, 2018). The docking analysis resulted in strong binding interaction of SR9009 with PARP2 (PDB ID:7R59) (-9.77±0.03 kcal/mol) as compared to NR1D1 (REV-ERB α) (PDB ID: 8D8I) (-5.73±0.03 kcal/mol). This analysis suggested that SR9009 that target core clock component REV-ERB α (NR1D1), can also bind with rhythmic anti-breast cancer targets with high affinity. This computer-aided technique has emerged as a crucial instrument in drug design research. Through molecular docking simulation, the drug development process is accelerated by reducing the time required for the examination of the mutual interaction of drug and receptor, resulting in high accuracy and easy analysis. PARP stands for poly-ADP ribose polymerase. PARP2 has role in DNA repair processes. It has distinct and varied regulatory roles in cellular physiology, from proliferative signaling and inflammation to genomic stability and epigenetics. Studies have reported PARP-2 in many of the hallmarks of cancer (Ali SO *et al.*, 2016). Thus, pharmacological PARP-2 inhibition may produce a shift away from cancer cell glycolytic tumor metabolism towards an oxidative function along with increased lipid catabolism. Hence our docking study suggest strong interaction between SR9009 and PARP-2 but whether the interaction is agonist or antagonist to PARP-2 in breast cancer is yet to be known.

Further, to check the global metabolite profile and the modulations conducted by SR9009 in 24-hour period in the metabolites at the cellular level, LC/MS metabolomic analysis of mouse 4T1 breast tumor model was carried out with SR9009 treatment at two time-points i.e, morning and evening. This resulted in 42 differentially abundant metabolites, among which 14 metabolites were of therapeutic significance such as L-arginino-succinate, (+)-costunolide, 5,10-methylenetetrahydrofolate, lysophosphatidylcholine (LysoPC(16:0/0:0)), L-tryptophan, 20-hydroxyecdysone (Crustecdysone), N-docosanoyl-sphinganine (Cer(d18:0/22:0)), hypoxanthine, phosphatidylcholine (PC(O-16:1(9Z)/18:0)) etc. . From these, 8 circadian metabolites were identified from previous literature. The circadian clock's activity can be connected to the diurnal fluctuation of circadian body metabolites. These clock-dependent metabolites can be identified and measured by metabolomic studies. Circadian metabolites sustain cellular rhythmicity through pleiotropic processes (Dyar KA and Eckel-Mahan KL, 2017). The identified metabolites are involved in different pathways such as ammonia recycling, phospholipid biosynthesis, pyrimidine metabolism, tryptophan metabolism etc. Total fatty acid levels are significantly higher in malignant cells during the processes of proliferation, migration, and invasion (Xu S *et al*., 2021). Among the metabolites, lysophosphatidylcholine (LysoPC(16:0/0:0)) and phosphatidylcholine (PC(O-16:1(9Z)/18:0)) are produced via phospholipid biosynthesis. The lysophosphatidylcholines (LysoPCs) are characterised by their fatty acyl groups and contain a constant polar head, which can be produced by the oxidation of fatty acids or the hydrolysis of phosphatidylcholine by phospholipase A2. They can be utilised as biomarkers for pathophysiological research, therapy, and clinical diagnosis (Li X *et al*., 2023). In our study the lysophosphatidylcholine (LysoPC(16:0/0:0)) was upregulated in the evening whereas phosphatidylcholine (PC(O-16:1(9Z)/18:0)) was downregulated in the morning after SR9009 treatment. Consequently, lysophosphatidylcholine (LysoPC(16:0/0:0)) and phosphatidylcholine (PC(O-16:1(9Z)/18:0)) may be biomarkers for the diagnosis of breast cancer. Also, from the metabolite list it may be suggested that the evening treatment of SR9009 may help increase its therapeutic efficiency in breast cancer treatment.

6. CONCLUSION

Circadian medicine strives to improve diagnosis and therapy by using knowledge of 24-hour biological rhythms. Optimizing the time of drug administration preserves clinical benefit with optimal tolerability. Hence, time-dependent drug dosage aims to deliver treatment in harmony with “on-target” physiology without causing “off-target” toxicity. Technological developments have made metabolomics a more common "omics" method for researching metabolism, and extensive research have lately started in "circadian metabolomics" (i.e., examining the 24-h metabolome). Our metabolomic analysis reveal the effect of SR9009 on metabolite regulation in morning and evening at cellular level in breast cancer cell. Collectively, this data suggests that evening treatment of SR9009 may contribute to breast cancer treatment. Yet, to enhance the quality of SR9009 administration, further invitro experiments and clinical studies at different time-points need to be conducted for data validation at the cohort level.

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OTHER TECHNIQUES

Apart from the project wok, following techniques were learnt:

1. Protein extraction and BCA quantification in mouse 4T1 breast cancer cells.
2. Trypsinization and Desalting of proteins.
3. SDS-PAGE of proteins
4. Western Blotting
5. RNA Extraction from 4T1 cells
6. Primer design using SnapGene Viewer software