Study on biological clocks that underly various diseases

様々な病気をもたらす生物時計に関する研究

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Abstract

The circadian rhythm is an endogenous rhythm with a period of approximately 24 hours observed in almost all living organisms. The circadian clock regulates numerous physiological and behavioral processes including metabolism, hormone secretion, and sleep-wake cycles. The suprachiasmatic nucleus (SCN) is the central pacemaker of the circadian system, a bilateral structure located in the anterior part of the mammalian hypothalamus, that regulates peripheral circadian rhythms in the body. Importantly, most peripheral tissues and cells also host self-sustained circadian clocks. The mammalian circadian clock serves as a transcription-translation feedback loop. Circadian locomotor output cycles kaput (CLOCK) and brain and muscle Arnt-like protein-1 (BMAL1) activate *Cryptochrome* (*Cry*) genes and *Period* (*Per*), which then feedback and repress their transcription. There is increasing recognition of an essential link between circadian rhythm disorders and various diseases. Chronic disruption of the circadian clock is associated with many diseases, such as cardiovascular diseases, cancer, and immune and metabolic disorders. Therefore, it is crucial to develop circadian clock modulators to cure or prevent various diseases.

A drug repositioning approach is an effective approach for developing new therapeutic targets for the existing drugs. In general, the new drugs development, from drug discovery to market approval, is both costly and time-consuming. In contrast, the drug repositioning approach enables shorter drug development cycles, lower cost, higher efficiency, and minimal risk of side effects. Kampo medicine originated from Chinese medicine and gradually developed into a unique medical system in Japan. Kampo medicine has long been used to treat various diseases and is fully integrated into the modern medical system. The crude drugs, used in traditional Japanese Kampo medicine, are an excellent source of new chemical entities in drug discovery. To discover new circadian clock modulators, I focused on Japanese Kampo medicine.

In the present study, I screened 137 crude drug extracts in human U2OS cells stably expressing the clock reporter *Bmal1-dLuc* for circadian clock modulators. I initially examined the effect of crude drugs on the circadian clock by measuring luminescence oscillations under the control of clock gene promoters in U2OS cells. This analysis identified seventeen drugs that affected the biological clock oscillations, mainly in terms of period and phase, suggesting that approximately 12 % of Kampo medicine modulates the circadian rhythm. The effects of hit candidates were validated by examining the dose-dependent effects on the circadian clock. To further investigate if the impact of hit crude drugs were specific to reporter genes (*Bmal1-dLuc*) or cell types (U2OS cells), Allii Chinense Bulbus, Artemisiae Capillaris Flos, and Perillae Herba) were examined as representative hit crude drugs and confirmed in Rat-1 fibroblasts (*Per2-dluc*).

It is well known that all Kampo medicines are mixtures of multiple active ingredients. Therefore, active ingredients of Kampo medicines were examined in U2OS (*Bmal1-dLuc*). Interestingly, the most frequent targets for the hit active ingredients were AKT (Protein kinase B, PKB) and its related pathways. AKT-related diseases mainly include cancer, neurological diseases, diabetes, cardiovascular diseases, inflammation, and autoimmune diseases. Although the participation of *Akt* in the circadian clock has been reported in *Drosophila*, its involvement in the mammalian circadian system remains unclear. Therefore, I examined the effects of AKT inhibitor A-443654 and AKT activator SC79 in U2OS cells. The AKT activator SC79 shortened the circadian period and advanced the phase at the maximum dosage. In opposite, the AKT inhibitor A-443654 shortened the circadian period at lower doses and lengthened the period at the maximum dosage. Three different AKT isoforms exist in mammals, namely AKT1, AKT2, and AKT3. To further explore the role of AKT in the circadian clock, siRNAs targeting *AKT1*, *AKT2*, and *AKT3* sequences were obtained. The

effects of siRNA were examined, and the triple knockdown of *AKT1*, *AKT2*, and *AKT3* shortened the circadian period.

Chronic disruption of the circadian clock caused by shift work or travel across time zones has lasting impacts on human health. Drug repositioning has become a trendy and powerful approach owing to the high cost and time-consuming nature of developing new drugs. In the present study, I have identified circadian clock modulators from Japanese Kampo medicine. In addition, I have uncovered the involvement of the AKT signaling pathway in the regulation of the mammalian circadian system. Therefore, the drug repositioning approach utilizing Kampo medicines will contribute to the identification of potential therapeutic for chronic circadian disruption.

Keywords: circadian clock, circadian clock disorders, drug repositioning approach, AKT isoforms

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Chapter I

General Introduction

I-1 Mammalian circadian clock system

Organisms are constantly impacted by the daily and seasonal changes caused by the Earth's rotation and orbit around the sun, as a result, almost all living beings on the earth have evolved biological rhythms to adapt to the changes in the natural environment, such as the circadian and circannual rhythms. The word circadian is derived from the Latin term circa, which means “approximately,” and dies, which means “day”. Circadian rhythm refers to the behavioral, physiological, and molecular changes with a cycle length of approximately 24 hours (Yamanaka, 2020). The circadian rhythm is observed in almost all organisms such as fungi (Brody, 2019), plants (McClung, 2006), and animals (Peirson et al., 2018), including humans (Chang et al., 2012). The circadian clock enables us to prepare and adjust our physiology to cyclical changes in the environment.

In mammals, most of the physiological processes are governed by daily oscillations that are regulated by a circadian system with a complex hierarchy. The mammalian circadian system consists of input pathway, output pathway, and a central clock located in the hypothalamus's suprachiasmatic nucleus (SCN). The circadian rhythms are synchronized with the light-dark cycles through a non-visual pathway from the retina to the SCN (Lucas et al., 2001). The SCN serves as a pacemaker synchronizing essential timing information to the autonomic peripheral oscillators through neural (e.g., parasympathetic and sympathetic) and humoral outputs (e.g., glucocorticoids) (Dibner et al., 2010). Coordinated by the central pacemaker, the phases of the numerous clocks are synchronized to produce coherent physiological and behavioral responses (Husse et al., 2015). Almost all peripheral and non-SCN central tissues have cell-autonomous and self-sustained circadian oscillators that can

respond to signals coordinated by the SCN (Mohawk et al., 2012). These circadian clocks coordinate almost all physiological processes in mammals, including the sleep-wake cycle, body temperature rhythms, and pineal melatonin secretion (Borjigin et al., 2012; Morf & Schibler, 2013; Waterhouse et al., 2012).

Circadian clocks in cultured cells, tissues, and whole organisms show strikingly homogeneous properties at the cell-autonomous level. In some tissues (e.g., liver, lung, and kidney), rhythmic oscillations persist for more than 20 daily cycles *in vitro* (Cassone et al., 2004; Yoo. et al., 2002). The suppression of rhythmicity in peripheral cell types arises from intercellular asynchrony. In contrast, SCN explants show more robust rhythms due to tight coupling among neurons. Many cultured cell lines also exhibit robust circadian rhythmic oscillations (Menger et al., 2005). For example, rodent fibroblasts (e.g., Rat-1 fibroblasts (Welsh et al., 2004) and NIH/3T3 immortalized fibroblasts (O'Neill et al., 2008)) and human cancer cell lines (e.g., U2OS osteosarcoma cells (Hoffmann et al., 2014)), show strong circadian gene expression *in vitro.* Therefore, underlying mechanisms of circadian clock can be analyzed by cell-based assays.

I-2 Mammalian circadian clock genes

Adapting to the periodic pattern of light-dark cycles, organisms have evolved approximately 24 hours endogenous circadian timing systems (Panda et al., 2002). A transcription-translation feedback loop (TTFL), consisting of two negative feedback loops, works together to generate a robust 24 hours rhythm of gene expression with behavioral and physiological effects (Hastings et al., 2019). The core TTFL consists of positive regulatory arms (CLOCK and BMAL1) and negative regulatory arms (PER and CRY) (Takahashi, 2017). Transcriptional activators, circadian locomotor output cycles kaput (CLOCK), and brain and muscle Arnt-like protein-1 (BMAL1), function as a heterodimer at E/E'-box elements, and regulate the transcription of clock genes, including *Period* (*Per1, Per2,* and *Per3*) and *Cryptochrome* (*Cry1, Cry2*). PER and CRY proteins heterodimerize in the cytoplasm and translocate to the nucleus to interact with CLOCK:BMAL1, thereby inhibiting further transcriptional activation (Cho et al., 2012; Preitner et al., 2002). PER and CRY proteins are degraded via a ubiquitin-dependent pathway, and inhibition of CLOCK:BMAL1 is relieved and the cycle begins again within approximately 24 hours. In addition, the CLOCK:BMAL1 heterodimer induces a regulatory loop and activates the transcription of REV-ERBα and retinoic acid-related orphan nuclear receptors α (RORα) (Preitner et al., 2002). REV-ERBα and RORα then compete to bind to the retinoic acid-related orphan receptor response element (RORE) in the *Bmal1* promoter. Members of retinoic acid-related orphan nuclear receptors (RORα, RORβ, and RORγ) and REV-ERB (REV-EBα, REV-ERBβ) have been shown to regulate *Bmal1* through RORE. RORs activate transcription of *Bmal1*, while REV-ERBs repress the transcription process.

Real-time monitoring of the expression of circadian rhythms in cultured cells and tissue *in vivo*, *ex vivo*, and *in vitro* can be achieved by using circadian promoters to drive the expression of luciferase and/or fluorescent reporter genes (Sellix et al., 2010; Wilsbacher

et al., 2002; Izumo et al., 2003). Basic clock properties such as period, phase, and amplitude can be calculated. As mentioned above, many cells have an integrated circadian clock oscillation system, and the establishment of a stable circadian promoter-luciferase reporter gene system enables real-time monitoring of the dynamics of the circadian clock genes (Yamazaki et al., 2005). At the same time, the circadian promoter-luciferase reporter system serves as a screening tool to identify factors that affect the circadian function of cells. Hirota et al. used U2OS (*Bmal1-dLuc*) to identify a new potent cellular circadian regulator by high-throughput chemical screening (Hirota et al., 2010). Chen et al. used mouse fibroblast cells (*Per2-dLuc*) as well as peripheral tissue explant to screen small molecule drugs for possible targets of pharmacological intervention in circadian clockwork (Chen et al., 2012). Tamai et al. identified circadian clock modulators from the existing drug library (Tamai et al., 2018).

I-3 Circadian clock disorders

Over the past century, lifestyle shifts in response to technological advances have led to unprecedented changes in the duration and timing of light exposure (Dominoni et al., 2016). Chronic desynchronization of the external environment and internal rhythms has resulted in the breakdown of biological rhythms. Shift work is a typical case, where exposure to light at night disrupts the circadian system (Thorpy, 2011). Chronic night shift work brings more pressure on both physical and mental health, and it also has contributed to an increased incidence of certain cancers, cardiovascular, and metabolic diseases (Ramin et al., 2015; Wang et al., 2011). Night work, afternoon work, and even early morning work all contribute to the circadian rhythm sleep disorder, known as shift work sleep disorder (SWSD) (Schwartz & Roth, 2006). The SWSD is associated with persistent sleep deprivation. Carrier et al. proposed that the reduction of SWSD via caffeine during daytime sleep increased the effects of circadian arousal signals on sleep. Liira et al. also concluded that melatonin improves sleep duration after night shifts, but not in other sleep quality parameters. Although appropriate caffeine or melatonin interventions can ameliorate SWSD, it is not a fundamental solution to the SWSD (Carrier et al., 2007; Liira et al., 2014).

Traveling across time zones also disrupts circadian rhythms. When a person travel spanning multiple time zones, it takes several days for the circadian rhythms to be entrained into the new light-dark cycles (Choy & Salbu, 2011). The lag in synchronizing internal rhythms with contemporary light and non-light cues can lead to circadian rhythm dysregulation with decreased sleep quality, daytime fatigue, mood anxiety, and digestive disturbances (Sack, 2009). Remarkably, rapid entrainment of *Per1*, the core clock gene of the SCN, to the new time zone is achieved within a day, whereas it takes at least six days for other tissues to be entrained, including the lung, muscle, and liver (Wolff et al., 2013). Interestingly, traveling towards the east disrupted sleep and increased anxiety and depression more

significantly than towards the west due to the advancement of the circadian clock (Montange et al., 1981).

The feeding schedule is one of the main contributors to the phase shift of the peripheral circadian clock (Sasaki et al., 2016). An irregular diet is related to obesity, weight gain, and related metabolic disorders (Escobar et al., 2007). Individuals who skip breakfast, including those with the nocturnal eating syndrome (NES) (Allison et al., 2010), or dietary rhythm shifts may induce maladaptive alignment between endogenous rhythms and metabolically, eventually leading to a higher risk of metabolic syndrome (Wennberg et al., 2015; Wennberg et al., 2016).

Circadian rhythm disorders can be modulated by small molecules (Chen et al., 2018; Hatori & Hirota, 2022; Ruan et al., 2021), including compounds targeting clock proteins CRY1/2, REV-ERB, and ROR (Cha et al., 2019). For example, KL001 and its analogs are CRY stabilizers that can prevent the ubiquitin-dependent degradation of CRY, and thereby lengthen the circadian period (Hirota et al., 2012; Nangle et al., 2013). Furthermore, KL001 suppresses glucagon-induced gluconeogenesis in primary hepatocytes ahus embodying the possibility of clock-based diabetes therapy (Hirota et al., 2012). However, these compounds have not been proved to be safe yet.

I-4 Drug repositioning approach

Developing a new therapeutic product is a long, complex, and expensive process that typically takes at least 10 to 15 years and requires approximately $1.3 billion per successful product launch. The drug repositioning approach is to identify novel therapeutic potentials for the existing drugs. Three approaches are widely used for the drug repositioning process, including the computational approach, experimental biological approach, and hybrid approach (Jarada et al., 2020; Wilkinson & Pritchard, 2015). Drug repositioning has significant advantages over traditional drug discovery in terms of the shorter drug development cycle, lower cost, higher efficiency, and minimal risk of failure (Nosengo, 2016).

The COVID-19, which is caused by SARS-CoV-2, has created a global pandemic that has put tremendous pressure on global health systems. Developing a therapeutic drug within a minimum period is desired and necessary. Mei and Tan have re-evaluated the potential of various drugs on the market for treating COVID-19 and suggested the Gilead Pharmeceuticals' Veklury® (Remdesivir), a U.S. Food and Drug Administration (FDA) approved drug, as a new COVID-19 treatment (Mei & Tan, 2021). Thus, the drug repositioning approach is promising.

I-5 Kampo medicine

Kampo medicine is derived from ancient Chinese medicine. Japan started localization reform due to the materials acquisition (Nishimura et al. 2009). After years of development, Kampo medicine has formed its unique system in Japan. With the completion of "Kampo Medicine Practice" writing, modern Kampo medicine was launched in 1941 (Yasui, 2007). Kampo medicine has been used in clinical applications to treat multiple diseases (Masuzaki et al., 2021; Sakurai et al., 2009; Tahara et al., 2020). It offers effectiveness, safety, as well as social and economic advantages.

Scientists are gradually uncovering the molecular mechanisms for the action of Kampo medicine. For example, Jose et al. demonstrated that Tricaproin isolated from *Simarouba glauca* inhibited the growth of human colorectal cancer cell lines by targeting class 1 histone deacetylases (Jose et al., 2018). Hou et al. elucidated the role of nuclear transcription factor E2-related factor 2 (Nrf2) and hepatic transporter protein for Isoglycyrrhizin (ISL) (Hou et al., 2018). Cassia seed extract promotes GLUT4 translocation through activation of the PI3K-Akt-AS160 signaling pathway, which improves glucose metabolism in the skeletal muscle of diabetic rats (Zhang et al., 2018). Although significant advancements in clarifying the underlying mechanisms of multiple Kampo medicines have been achieved in the past decades, the mechanistic basis for a large number of others remains unknown. Therefore, a comprehensive investigation of the molecular mechanisms of Kampo medicines is warranted and will facilitate their applications.

Chapter II

Drug repositioning approach to identify circadian clock modulators from Japanese Kampo medicine

II-1. Introduction

The mammalian circadian clock engages a transcription translational feedback loop in which CLOCK and BMAL1 activate the *Cryptochrome* and *Period* genes, following feedback and repression on their own transcriptions (Koike et al., 2012; Takahashi, 2015). Circadian disruption is linked to multiple diseases, such as cerebrovascular and cardiovascular diseases (Alibhai et al., 2017), cancer (Sulli et al., 2018), immune system (Zhuang et al., 2017), and metabolic disorders (Hatori et al., 2012). The development of circadian clock modulators is expected to treat these diseases.

Kampo medicine was derived from Chinese medicine and gradually developed into a unique type of medical system in Japan. Kampo medicines have a long historical application to treat various diseases and have been fully integrated into the modern medical system (Motoo et al., 2009; Yu et al., 2006). However, only a few studies investigated the regulation of Kampo drugs of the circadian clock (Motohashi et al., 2017). To maximize the usage of original medicine, it is of great significance to screen Kampo medicines for circadian rhythm modulators (Tamai et al., 2018).

I used Kampo medicines to screen drugs that modulate the circadian clock. Candidates with changes in period and phase were further analyzed for the dose-dependent effect. It is well known that all Kampo medicines are a mixture of multiple active ingredients, therefore, the active ingredients of the effective Kampo medicines were examined in U2OS cells to examine their potential as circadian clock regulators.

II-2. Materials and Methods

II-2-1 Crude drugs

I selected 137 crude drugs (Table 1). These crude drugs are frequently used as ingredients in the traditional Japanese Kampo formulations. Crude drugs that met the grade standards of the Japanese Pharmacopoeia 17th Edition or non-pharmacopoeial crude drugs from Uchida Wakanyaku (Tokyo, Japan), Daiko Shoyaku (Nagoya, Japan), Tochimoto Tenkaido (Osaka, Japan), and Tsumura (Tokyo, Japan). The preparation of methanol extracts was described in a previous report (Tashiro et al., 2018). Five grams of each drug was sonicated in a volume of 100 mL of methanol for 30 min and then filtered. The residue was also extracted two times by the same method, and the three filtered liquids were combined, evaporated under reduced pressure, and finally lyophilized to obtain the final crude extract. All extracts were suspended in methanol (MeOH) at 100 mg/mL and kept at -20 °C. The origins of the hit crude drugs are presented in Table 3.

Table 1. List of 137 Kampo medicines.

Number	生薬名	漢名
1	アキョウ	阿膠
2	イレイセン	威霊仙
3	インチンコウ	茵陳蒿
4	ウイキョウ	茴香
5	ウヤク	烏薬
6	エンゴサク	延胡索
7	オウギ	黄耆
8	オウゴン	黄芩
9	オウバク	黄柏
10	オウレン	黄連
11	オンジ	遠志
12	ガイヨウ	艾葉
13	カシュウ	何首烏
14	カッコン	葛根
15	カロコン	栝楼根
16	カロニン	栝楼仁
17	カンキョウ	生姜
18	カンゾウ	甘草
19	キキョウ	桔梗根
20	キクカ	菊花
21	キジツ	枳実
22	キョウカツ	姜活
23	キョウニン	杏仁
24	クジン	苦参
25	ケイガイ	荊芥穂
26	ケイヒ	桂皮
27	ゲンノショウコ	現之証拠
28	コウカ	紅花
29	コウブシ	香附子
30	コウボク	厚朴
31	ゴシツ	牛膝
32	ゴシュユ	呉茱萸
33	ゴボウシ	牛蒡子
34	ゴミシ	五味子
35	サイコ	柴胡
36	サイシン	細辛
37	サンシシ	山梔子
38	サンシュユ	山茱萸
39	サンショウ	山椒
40	サンソウニン	酸棗仁
41	サンヤク	山薬
42	ジオウ（乾）	乾地黄
43	ジオウ（熟）	熟地黄
44	ジコッピ	地骨皮
45	シツリシ	蒺藜子
46	セキシャク	赤勺
47	ビャクシャク	白勺
48	ジャショウシ	蛇床子
49	シャゼンシ	車前子
50	ジュウヤク	十薬
51	シュクシャ	縮砂
52	ショウキョウ	生姜
53	ショウマ	升麻
54	シンイ	辛夷
55	センキュウ	川芎
56	センコツ	川骨
57	センタイ	蝉退
58	ソウジュツ	蒼朮
59	ソウハクヒ	桑白皮
60	ソヨウ	蘇葉
61	ダイオウ	大黄
62	タイソウ	大棗
63	タクシャ	沢瀉
64	チクジョ	竹茹
65	チクセツニンジン	竹節人参
66	チモ	知母
67	チョウジ	丁子
68	チョウトウコウ	釣藤鉤
69	チョレイ	猪苓
70	チンピ	陳皮

Number	生薬名	漢名
71	テンナンショウ	天南星
72	テンマ	天麻
73	テンモンドウ	天門冬
74	トウガシ	冬瓜子
75	トウキ	当帰
76	トウニン	桃仁
77	ドクカツ	独活
78	ニンジン	人参
79	ニンドウ	忍冬
80	バイモ	貝母
81	バクガ	麦芽
82	バクモンドウ	麦門冬
83	ハッカ	薄荷
84	ハマボウフウ	浜防風
85	ハンゲ	半夏
86	ビャクゴウ	百合
87	ビャクシ	白芷
88	ビャクジュツ	白朮
89	ビワヨウ	枇杷葉
90	ビンロウジ	檳榔子
91	ブクリョウ	茯苓
92	ブシ	附子
93	ボウイ	防已
94	ボウフウ	防風
95	ボクソク	樸樕
96	ボタンピ	牡丹皮
97	ボレイ	牡蠣
98	マオウ	麻黄
99	マシニン	麻子仁
100	モクツウ	木通
101	モッコウ	木香
102	ヨクイニン	薏苡仁
103	リュウガンニク	竜眼肉
104	リュウタン	竜胆
105	リョウキョウ	良姜

Number	生薬名	漢名
106	レンギョウ	連翹
107	レンニク	蓮肉
108	イカリソウ	淫羊藿
109	カキノハ	柿葉
110	キコク	枳殻
111	カロジツ	括楼実
112	キササゲ	梓実
113	ケツメイシ	决明子
114	キッピ	橘皮
115	ゴウカンヒ	合歓皮
116	コウジン	紅参
117	シソシ	紫蘇子
118	セキショウコン	石菖根
119	シンギク	神麹
120	ソボク	蘇木
121	タラノキ	楤根皮
122	ビワノハ	枇杷葉
123	ナンバンモウ	南蛮毛
124	トウジン	党参
125	アカメガシワ	赤目柏
126	イクリニン	郁李仁
127	ウバイ	烏梅
128	ゴレイシ	五霊脂
129	イチョウバ	銀杏葉
130	イズイ	玉竹
131	ウラジロガシ	萎蕤
132	エキナセア	紫馬簾菊
133	オウヒ	桜皮
134	カリン	木瓜
135	ガイハク	薤白
136	フェネグリード	胡廬巴
137	ゲンノショウ	中日老鸛草

All crude drugs in the table are registered in the Japanese Pharmacopoeia 17th Edition or the Japanese standards for non-Pharmacopoeial crude drugs, 2015.

II-2-2 Chemicals

In total, eight compounds were used (Table 2). I purchased six compounds from ChemFaces (Wuhan, China), they are Chrysoeriol (cat no. CFN98785), Galangin (cat no. CFN98918), Platycodin D (cat no. CFN98134), Kaempferide (cat no. CFN98782), Luteolin (cat no. CFN98784), and Luteoloside (cat no. CFN98565), respectively. In addition, Apigenin (cat no. 010-18914) was purchased from Fujifilm Wako Pure Chemicals (Osaka, Japan), and Amygdalin (cat no. A0443-1G) was obtained from Tokyo Chemical Industry (Tokyo, Japan).

Table 2. Summary of 8 chemicals.

Chemicals	CAS-NO.	Chemical structure	Company	Quality	State
Platycodin D	58479-68-8	$C_{57}H_{92}O_{28}$	Cayman chemical	1mg	powder
Luteolin	491-70-3	$C_{15}H_{10}O_6$	Cayman chemical	10mg	powder
Apigenin	520-36-5	$C_{15}H_{10}O_5$	Wako	5g	powder
Chrysoeriol	491-71-4	$C_{16}H_{12}O_6$	ChemFaces	5mg	powder
Luteoloside	5373-11-5	$C_{21}H_{20}O_{11}$	ChemFaces	10mg	powder
Galangin	548-83-4	$C_{15}H_{10}O_5$	ChemFaces	10mg	powder
Kaempferide	491-54-3	$C_{16}H_{12}O_6$	ChemFaces	10mg	powder
Amygdalin	29883-15-6	$C_{20}H_{27}NO_{11}$	Tokyo Chemical Industry	1g	powder

All chemicals were suspended in Dimethyl sulfoxide (DMSO) at 5 mM and kept at -20 °C.

II-2-3 Cell culture

Human U2OS cells and Rat-1 cells were from ATCC (American Type Culture Collection). Human U2OS cell lines stably express the circadian luciferase reporter gene Bmal1-dluc to monitor circadian rhythms in culture, obtaining rhythm parameters (period and phase) (Hirota et al., 2008). Similarly, Rat-1 fibroblasts stably express the circadian luciferase reporter gene *Per2-dLuc* to monitor circadian rhythms *in vitro* (Welsh et al., 2004). Cells were cultured in DMEM (Dulbecco's modified eagle medium; D2902, Sigma-Aldrich, St. Louis, MI, USA), with the requirement that three reagents, 2 mM L-glutamine, 10% fetal calf serum (FBS, FB-1290/500; Biosera, Rue Lacaille, Nuaillen, France), 100 µg/mL streptomycin and 100 U/mL penicillin (Pen Strep, 15070063, Thermo Fisher Scientific, Waltham, MA, USA).

II-2-4 Chemical screening

U2OS cells were seeded at a density of 4,000 cells/well in 384-well plates. Cells were incubated for two days until confluent. The crude drug extracts were diluted to 20 µg/mL and 200 µg/mL, respectively, in the assay medium for the luminescence assay. It was ensured that the final methanol concentration in the control and drug-treated samples remained consistent at 0.2%. Ingredients were analyzed for a variety of doses (0.025, 1.3, 2.5, 5.0, 10, 20, 40, and 80 µM). Luminescent assay media was a mixture of DMEM, 10 mM HEPES, 0.35 g/L sodium bicarbonate, 3.5 g/L D-glucose, 2% B27 (Life Technologies, Carlsbad, CA, USA), 100 U/mL penicillin, 100 µg/mL streptomycin, 100 nM dexamethasone (Sigma), and 0.1 mM luciferin (Fujifilm). Then diluted extracts were pipetted into triplicate wells of a 384-well plate containing U2OS cells (prepared above). The bioluminescence was monitored for one week using a Churitsu CL384 Series luminometer (Churitsu Electric Corporation, Nagoya, Japan). For time series analysis, the circadian period and phase were determined using NINJA SL00-01 software (Churitsu Electric Corporation), and the hit crude drugs were identified based on changes in the circadian period and phase.

II-2-5 Statistical analysis

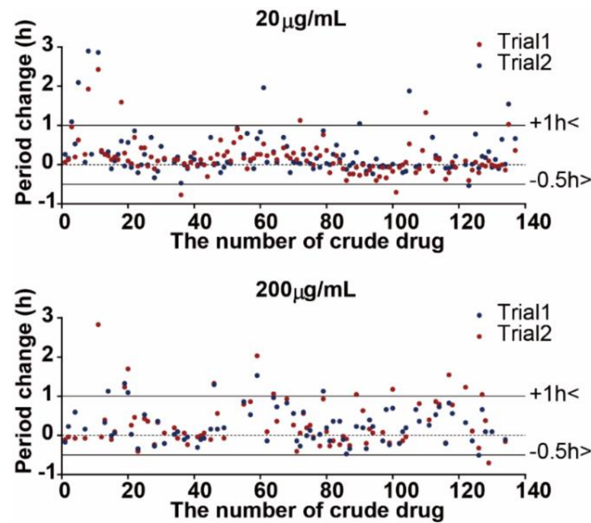
Statistical analysis was conducted using GraphPad Prism 9 (https://www.graphpad.com/scientific-software/prism/). The significance of differences was analyzed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons tests or Student's t-tests. Values are expressed as mean ± SD.

II -3. Results

II-3-1 Identification of circadian clock modulators from 137 crude extracts.

Kampo medicine is an ancient medicine still used to treat many diseases. The present study aimed to explore the possible causal link between Kampo medicine and modulations of the circadian clock. To examine the Kampo medicines' potential in circadian clock-related disorders, 137 Kampo medicines were screened in U2OS cells (*Bmal1-dLuc*) at high concertation of 200 µg/mL and low concertation of 20 µg/mL (Figure 1, Table 1), and Allium Chinense Bulbus was treatment in different concentrations (20 µg/mL and 2 µg/mL). To reduce false positives, I repeated the experiment 2 times (trial1 and trial 2). The raw drug that showed an effect on the period and/or phase in both trials was selected as a candidate for the next investigation. Two crude drugs (Allii Chinense Bulbus and Polygalae Radix) extracts of low concertation (Polygalae Radix is 2 µg/mL; Polygalae Radix is 20 µg/mL) and four crude drugs (Pueraiae Radix, Platycodi Radix, Paeoniae Radix, and Chrysanthemi Flos) extracts of 200 µg/mL were identified to show period lengthening effects by at least one hour, respectively (Figure 1, Table 3). Five crude drugs (Polygalae Radix, Artemisiae Capillaris Flos, Alpiniae Officinari Rhizoma, Perillae Herba, and Caryophylli Flos,) extracts of 20 µg/mL and eight crude drugs (Cicadae Periosrtacum, Chrysanthemi Flos, Uncariae Uncis cum Ramulus, Schizonepetae Spica, Lonicerae Folium cum Caulis, Mume Fructus Praeparatus, Acori Graminei Rhizoma, and Perillae Fruitus) extracts of 200 µg/mL were identified to show phase changing effects by at least two hours, respectively (Figure 1, Table 3). Table 3 summarizes the information on hit crude drugs. The serial number in the table corresponds to that in Figure 1. Interestingly, some candidates, such as Number 11 (Polygalae Radix), can dramatically change the period and phase simultaneously.

A



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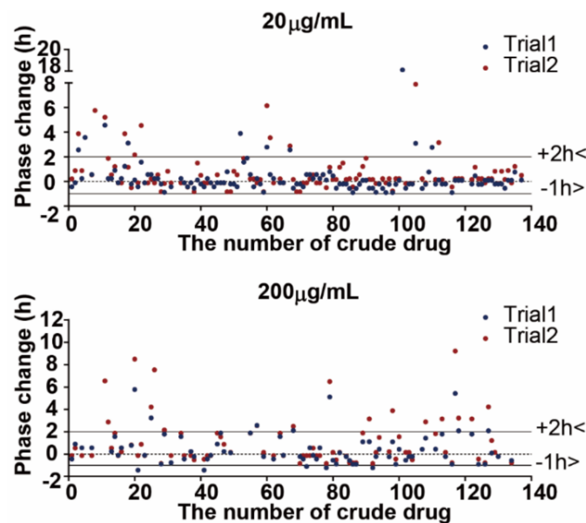


Figure 1. Identification of crude drug extracts that modulate the circadian clock.

Screening of crude drug extracts for circadian clock modulators. A total of 137 crude drug extracts were screened in U2OS cells (*Bmal1-dLuc*) at 20 μg/mL (top graph) and 200 μg/mL (bottom graph) for the circadian period (A) and phase (B) changes. (A) 2 independent trials identified 2 crude drugs at 20 μg/mL and 4 crude drugs at 200 μg/mL that show consistent period lengthening effects by one or more hours. (B) This screening also identified 5 crude drugs at 20 μg/mL and 8 crude drugs at 200 μg/mL that show consistent phase changing effects by two or more hours.

Table 3. Summary of 17 hit Kampo medicines.

Number	漢名	Kampo Name	Origin
3	茵陳蒿	Artemisiae Capillaris Flos	The dried capitulum of *Artemisia capillaris* Thunberg
11	遠志	Polygalae Radix	The dried root bark of *Polygala tenuifolia* Willdenow
14	葛根	Puerariae Radix	The dried root of *Pueraria montana* var. *lobata* (Willd.) Sanjappa & Pradeep
19	桔梗根	Platycodi Radix	The dried root of *Platycodon grandiflorum* (Jacques) A.De Candolle
20	菊花	Chrysanthemi Flos	The dried capitulum of *Chrysanmum morifolium* Ramatulle
25	荊芥穂	Schizonepetae Spica	The dried spike of *Nepeta tenuifolia* Bentham
46	赤勺	Paeoniae Radix	The dried root of *Paeonia lactiflora* Pallas
57	蝉退	Cicadae Periosrtacum	The dried slough of *Cryptotympana atrata (CA)*
60	蘇葉	Perillae Herba	The dried leaves and branch tios of *Perilla frutescens* Britton var. *crispa* W. Deane
67	丁子	Caryophylli Flos	The dried flowering bud of *Syzygium aromaticum* (Linné) Merrill et Perry
68	釣藤鉤	Uncariae Uncis cum Ramulus	The dried hook-bearing stem of *Uncaria rhynchophylla* Miquel ex Haviland
79	忍冬	Lonicerae Folium cum Caulis	The dried leaves and stems of *Lonicera japonica* Thunberg
105	良姜	Alpiniae Officinari Rhizoma	The dried rhizome of *Alpinia officinarum* Hance
118	紫蘇子	Perilla fructus	The dried fruit of *Perilla frutescens* (L.) Britt.
119	石菖根	Acori gramineus Rhizome	The dried root of *Acorus gramineus Aiton & Acorus tatarinowii Schott*
129	烏梅	Mume Fructus Praeparatus	The fruit of Ume *Prunus mume* Siebold et Zuccarini
137	薤白	Allium Chinense Bulbus	The bulbs of Rakkyo *Allium chinense* G.Don

All crude drugs are registered in the Japanese Pharmacopoeia 17th Edition or the Japanese standards for non-Pharmacopoeial crude drugs, 2015.

II-3-2 The dose-dependent effects of crude drugs on the circadian clock.

Seventeen candidates were selected based on the primary screening. To determine whether there is a dose-dependent effect on period and phase, candidate drugs were screened for the second time at multiple concentrations (5, 10, 25, 50, and 100 μg/mL), and Allium Chinense Bulbus was treatment in lower concentrations (0.5, 1, 2.5, 5, and 10 μg/mL).

The effects of seventeen candidates on luminescent traces (Figure 2， left graph), period (Figure 2, middle graph), and phase (Figure 2, right graph). As expected, most of the candidates exhibited a distinct dose-dependent pattern in terms of period and/or phase. Some crude drugs of them only show strong effects in the period, such as Perillae Fruitus, Chrysanthemi Flos, Uncariae Uncis cum Ramulus, and Schizonepetae Spica. Surprisingly, five crude drugs (Allii Chinense Bulbus, Polygalae Radix Artemisiae Capillaris Flos, Alpiniae Officinari Rhizoma, Perillae Herba, and Caryophylli Flos) extracts showed a dose-dependent pattern of strong alterations in period and phase. Some crude drugs only show strong effects in the period, such as Perillae Fruitus, Chrysanthemi Flos, Uncariae Uncis cum Ramulus, and Schizonepetae Spica.

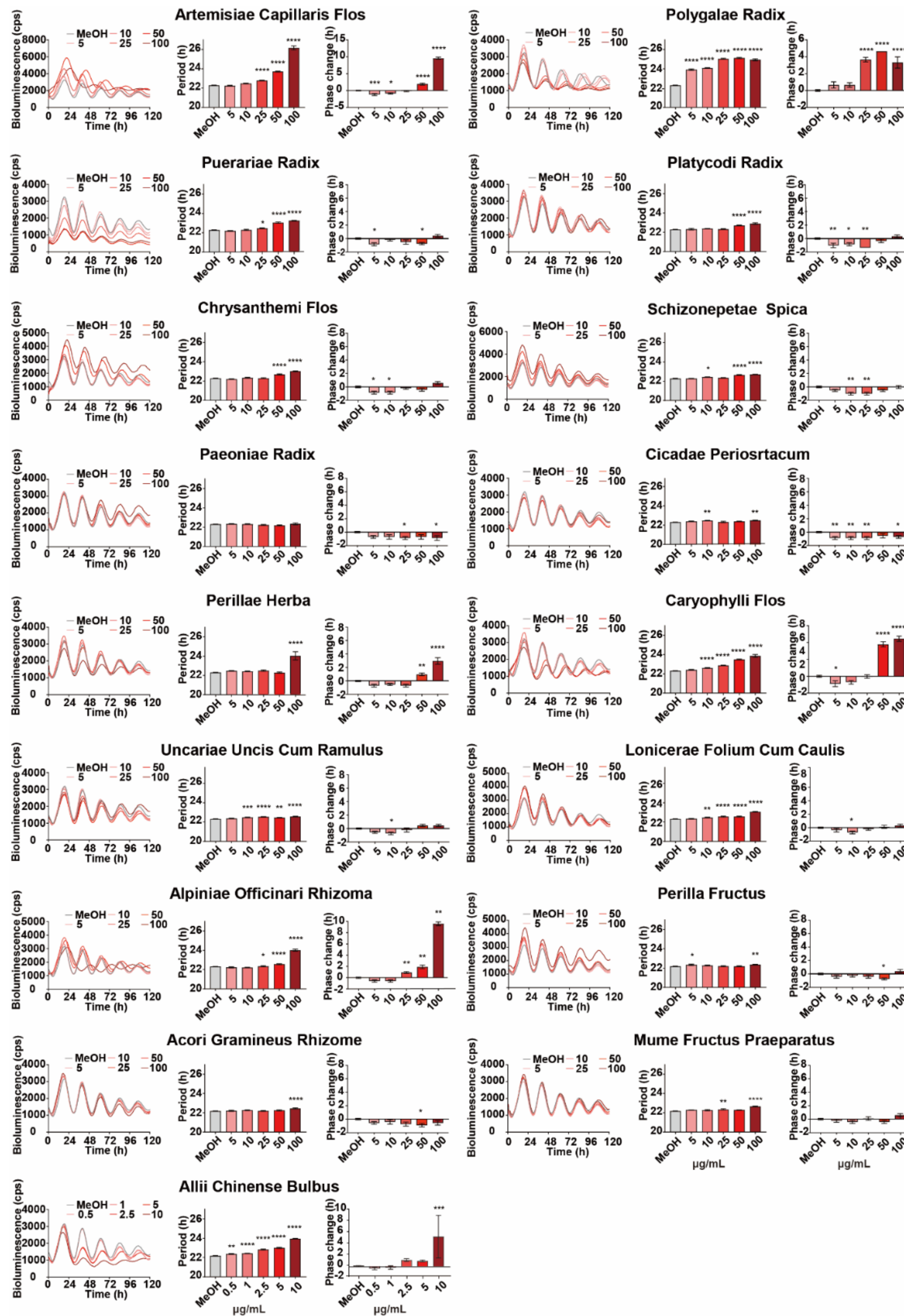


Figure 2. Seventeen hit drugs show dose-dependent effects both in period and phase.

Dose-dependent effect of seventeen hit crude drug extracts in U2OS cells expressing *Bmal1-dLuc*. Luminescent traces (left graph) and dose-dependent effects on the circadian period (middle graph) and phase (right graph) in U2OS cells. Values are averages of six replicates ± SD. Data were analyzed by one-way ANOVA, followed by Dunnett's multiple comparisons tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$).

II-3-3 Dose-dependent effects of three representative crude drugs on the circadian clock examined in Rat-1 *Per2-dLuc* fibroblasts.

To determine whether the effects of crude drugs on the circadian clock were reporter gene- or cell type- specific, the effects of three representatives hit crude drugs (Allii Chinense Bulbus, Artemisaie Capillaris Flos, and Perillae Herba) were examined in Rat-1 *Per2-dLuc* fibroblasts (Figure 3). The period lengthening and phase changing effects of Artemisiae Capillaris Flos and Perillae Herba were shown in Rat-1 cells in a dose-dependent partner (Figure 3). It is consistent with the results obtained from U2OS cells (Figure 2).

Allii Chinense Bulbus showed a dose-dependent effect of period lengthening and phase delay in U2OS cells (Figure 2). However, it was shown in rat-1 cells causing period shortening and phase advance in a dose-dependent pattern (Figure 3). This may be related to the fact that the crude drug extract is a mixture containing many active ingredients, and the expression levels of the drug targets may differ between cells.

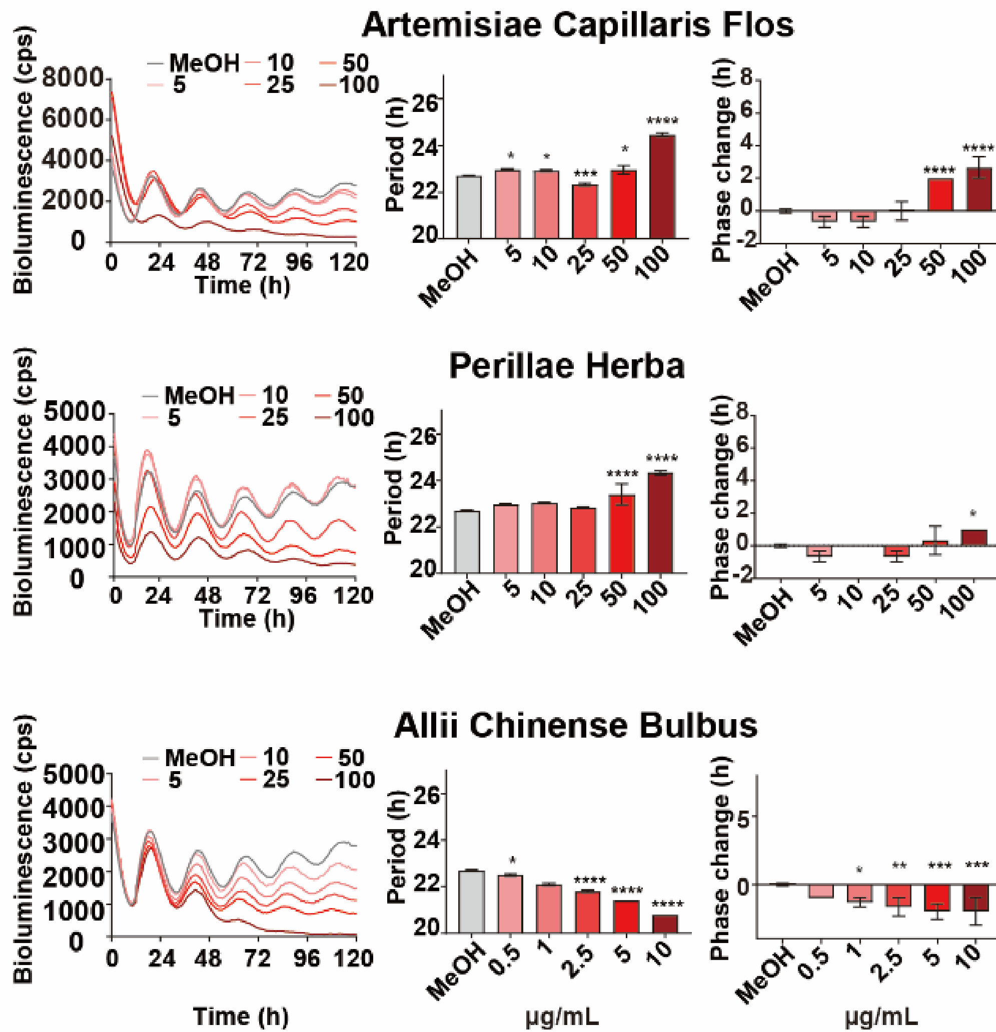


Figure 3. Effects of three crude drugs in Rat-1 *Per2-dLuc* fibroblasts.

Effect of three crude drug extracts (Allii Chinense Bulbus, Artemisaie Capillaris Flos, and Perillae Herba) on Rat-1 fibroblasts expressing *Per2-dLuc*. Luminescent traces (left graph) and dose-dependent effects of hit crude drugs on the period (middle graph) and phase (right graph) in Rat-1fibrablasts. Values are averages of six replicates ± SD. Data were analyzed by one-way ANOVA, followed by Dunnett's multiple comparisons tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$).

II-3-4 Ingredients of hit crude drugs modulate the circadian rhythm.

The above results demonstrated the regulatory effect of crude drugs on the circadian clock. It is well known that crude drugs are mixtures with many active ingredients. The principal components and potential targets are summarized in Table 4. In addition, the administration of crude lysates sometimes affects the experimental results due to the formation of macromolecular complexes and/or medium pH change. Therefore, validation using known active ingredients is important. Several interesting active ingredients were verified for their modulating effects on the circadian clock. As shown in Figure 4, the most active ingredients of crude drugs show a dose-dependent effect on the circadian clock. Among the eight chemicals, Kaempferide and Amygdalin showed the effects of Phase delayed. Other chemicals (Luteolin, Apigenin, Chrysoeriol, Luteoloside, Galangin, Platycodin D) showed a dose-dependent pattern of strong alterations in period and phase. The result provides direction for the development of circadian clock regulators and highlights the efficiency of the drug repositioning approach.

Table 4. Major constituents and suggested targets of hit Kampo medicines.

Kampo Name	CAS-NO.	Ingredient Name	Chemical Structure	Suggest Target	Reference
Artemisiae Capillaris Flos	495-74-9	Capillin	$C_{12}H_8O$	Mitochondrial pathway	Masuda et al., 2015
	56365-38-9	Capillarisin	$C_{16}H_{12}O_7$	MAPK /NF-κB signaling pathway	Kim et al., 2017
				Nrf2/HO-1 signaling	Kim et al., 2017
	120-08-1	Scoparone	$C_{11}H_{10}O_4$	TLR4/NF-κB signaling pathway	Liu et al., 2019
Polygalae Radix	20183-47-5	Tenuifolin	$C_{36}H_{56}O_{12}$	NF-κB pathway	Chen & Jia, 2020
	90-50-6	3,4,5-Trimethoxycinnamic acid	$C_{12}H_{14}O_5$	GABAaergic systems	Chen et al., 2016
	559-70-6	Beta-Amyrin	$C_{30}H_{50}O$	Cannabinoid receptors	Askari, 2018
Puerariae Radix	3681-99-0	Puerarin	$C_{21}H_{20}O_9$	Nrf2/HO-1 signaling pathway	Zhang & Li, 2019
				TGF-β1/Smad2 pathway	She et al., 2014
				AMPK-mTOR-ULK1 signaling pathway	Wang et al., 2018
	486-66-8	Daidzein	$C_{15}H_{10}O_4$	the mitochondrial monoamine oxidase-aldehyde dehydrogenase pathway	Rooke et al., 2000
				NF-κB pathway	Wei et al., 2019
				STAT3 signaling	Yang et al., 2019
	529-59-9	Genistin	$C_{21}H_{20}O_{10}$	P2X7/NF-κB pathway	Gu et al., 2016
				cAMP-dependent protein kinase pathway	Liu et al., 2006
Platycodi Radix	68051-23-0	Platyconic Acid A	$C_{57}H_{90}O_{29}$	SMAD and PPAR signaling pathway	Choi et al., 2019
	58479-68-8	Platycodin D	$C_{57}H_{92}O_{28}$	Hsp90/Cdc37 complex and mTOR signaling	Li et al., 2017
				AKT signaling	Li et al., 2016
				AMP-activated protein kinase	Kim et al., 2019
				NF-κB pathway	Zhang et al., 2017
	481-18-5	Spinasterol	$C_{29}H_{48}O$	COX /transient receptor potential vanilloid 1	Brusco et al., 2017
Chrysanthemi Flos	491-70-3	Luteolin	$C_{15}H_{10}O_6$	MEK-ERK and PI3K-Akt pathway	Meng et al., 2016; Lee et al., 2006
				Akt–GSK-3b–Cyclin D1 pathway	Ong et al., 2010
				p53 protein	Shi et al., 2007
	520-36-5	Apigenin	$C_{15}H_{10}O_5$	PI3K/Akt pathway	Bao et al., 2015
				mTOR/AMPK/ULK1 pathway	Zhang et al., 2019
				JAK2/STAT3 pathway	Liu et al., 2017
	491-71-4	Chrysoeriol	$C_{16}H_{12}O_6$	PI3K/Akt pathway	Limboonreung et al., 2019
				MAPK/ERK signaling pathway	Wei et al., 2019
				NF-κB and STAT3 pathway	Wu et al., 2020
	520-34-3	Diosmetin	$C_{16}H_{12}O_6$	PI3K/Akt/GSK-3β/Nrf2 pathway	Chen et al., 2019

Kampo Name	CAS-NO.	Ingredient Name	Chemical Structure	Suggest Target	Reference
Schizonepetae Spica	491-70-3	Luteolin	$C_{15}H_{10}O_6$	MEK-ERK and PI3K-Akt pathway	Meng et al., 2016; Lee et al., 2006
				Akt–GSK-3b–Cyclin D1 pathway	Ong et al., 2010
				p53 protein	Shi et al.,2007
	520-36-5	Apigenin	$C_{15}H_{10}O_5$	PI3K/Akt pathway	Bao et al., 2015
				mTOR/AMPK/ULK1 pathway	Zhang et al., 2019
				JAK2/STAT3 pathway	Liu et al., 2017
	5373-11-5	Luteoloside	$C_{21}H_{20}O_{11}$	PPARγ/Nrf2/NF-κB signaling pathway	Li et al., 2019
				ROS-mediated AKT/mTOR/p70S6K signaling pathway	Zhou et al., 2017
				MAPK and mTOR signaling pathway	Shao et al., 2018
	520-34-3	Diosmetin	$C_{16}H_{12}O_6$	PI3K/Akt/GSK-3β/Nrf2 pathway	Chen et al., 2019
Paeoniae Radix	23180-57-6	Paeoniflorin	$C_{23}H_{28}O_{11}$	LKB1/AMPK and AKT Pathways	Li et al., 2018
				Akt/GSK-3β pathway	Ma et al., 2018;Sun et al., 2017
				SOCS2/IRS-1 pathway	Sun et al., 2017
				IRAK1/NF-κB signaling pathway	Ji et al., 2018
				p38/MAPK pathway	Yu et al., 2017
				Ca^{2+}/CaMKII/CREB signaling pathway	Zhang et al., 2017
				RAGE/mTOR/autophagy pathway	Chen et al., 2017
				Akt/NF-κB signaling	Hu et al., 2018
	39011-90-0	Albiflorin	$C_{23}H_{28}O_{11}$	LOX-1/NF-κB pathway	Sun et al., 2018
				AMPK and PI3K/AKT pathway	Jeong et al., 2017
				Monoamine transporters	Jin et al., 2016
Perillae Herba	20283-92-5	Rosmarinic Acid	$C_{18}H_{16}O_8$	Viral epsilon RNA-polymerase	Tsukamoto et al., 2018
Caryophylli Flos	6753-98-6	Humulene	$C_{15}H_{24}$	Akt signaling	Chen et al., 2019
				NF-kB and the AP-1	Rogerio et al., 2009
Uncariae Uncis Cum Ramulus	154-23-4	(+)-Catechin	$C_{15}H_{14}O_6$	Melatonin receptors	Geng et al., 2018
	76-66-4	Rhynchophyl line	$C_{22}H_{28}N_2O_4$	BDNF/NF-κB pathway	Long et al., 2019
				PI3K/Akt/GSK3β/MEF2D signaling pathway	Hu et al., 2018
				MAPK/NF-κB signaling	Lai et al., 2019
	6859-01-4	Isorhynchop hylline	$C_{22}H_{28}N_2O_4$	Nrf2/MAPK pathway	Zhang et al., 2019
				PI3K/Akt/GSK-3β signaling pathway	Xian et al., 2019
Lonicerae Folium Cum Caulis	25694-72-8	Lonicerin	$C_{27}H_{30}O_{15}$	NF-κB signaling pathway	Liu et al., 2020

Kampo Name	CAS-NO.	Ingredient Name	Chemical Structure	Suggest Target	Reference
Alpiniae Officinari Rhizoma	480-19-3	Isorhamnetin	$C_{16}H_{12}O_7$	PI3K-AKT pathway	Gao et al., 2017
				P38/PPAR-α pathway	Lu et al., 2018
				TGF-β/Smad signaling	Yang et al., 2016
				TGF- β1/Smad3 and TGF- β 1/p38 MAPK pathway	Liu et al., 2019
				NF-κB,TLR4 pathway	Kim et al., 2019
				MEK1/PI3K	Kim et al., 2011
	548-83-4	Galangin	$C_{15}H_{10}O_5$	PPAR-γ signaling pathway	Choi et al., 2017
				Protein kinase-dependent NF-κB phosphorylation	Yang et al., 2018
				MEK1/2-ERK1/2 and PI3K-AKT pathway	Wang et al., 2019
				NF-κ B, PI3K/AKT and NLRP3 pathway	Lu et al., 2019; Fu et al., 2018
	491-54-3	Kaempferide	$C_{16}H_{12}O_6$	PI3K/Akt/GSK-3 β pathway	Wang et al.,, 2017
Perilla fructus	491-70-3	Luteolin	$C_{15}H_{10}O_6$	MEK-ERK and PI3K-Akt pathway	Meng et al., 2016; Lee et al., 2006
				Akt–GSK-3b–Cyclin D1 pathway	Ong et al., 2010
				p53 protein	Shi et al.,2007
	463-40-1	Linolenic Acid	$C_{18}H_{30}O_2$	NF-κB	Piermartiri et al., 2015
	5392-40-5	Citral	$C_{10}H_{16}O$	PPAR-γ	Shen et al., 2014
				TLR4/TLR2/Dectin-1/CB2 Cannabinoid Receptor/ ATP-sensitive K^+ channel	Goncalves et al., 2020
Acorus Gramineus Rhizome	5273-86-9	Beta-Asarone	$C_{12}H_{16}O_3$	Wnt/β-catenin signaling	Wang et al., 2018
				HnRNP A2/B1-mediated pathway	Li et al., 2018
				NF-κB signaling	Lv et al., 2019
				PERK/CHOP/Bcl-2/Beclin-1 pathway	Ning et al.,2019
Mume Fructus Praeparatus	29883-15-6	Amygdalin	$C_{20}H_{27}NO_{11}$	Akt-mTOR pathway/NF-κB /NLRP3 signaling pathway/TGFβ/CTGF pathway	Liczbiński & Bukowska, 2018
	508-02-1	Oleanic acid	$C_{30}H_{48}O_3$	PI3K/Akt/mTOR and ROS-dependent pathway	Shi et al., 2016
				MAPK/ERK signaling pathway	Guo et al., 2013
				NF-κB, p53 and p38 signalling pathway	Zhao et al., 2017
Allium Chinense Bulbus	556-27-4	Alliin	$C_6H_{11}NO_3S$	AMP-activated protein kinase-dependent pathway	Lu et al., 2018
				PPAR-γ	Wang et al., 2017
	2179-57-9	Diallyl disulphide	$C_6H_{10}S_2$	NF-κB-NFATc1 signal pathway	Yang et al., 2019
				PI3K/Akt/mTOR pathway	Yue et al., 2019
				CD44/PKM2/AMPK pathway	Xie et al., 2018
				EGFR/ERK/PKM2 pathway	Luo et al., 2015

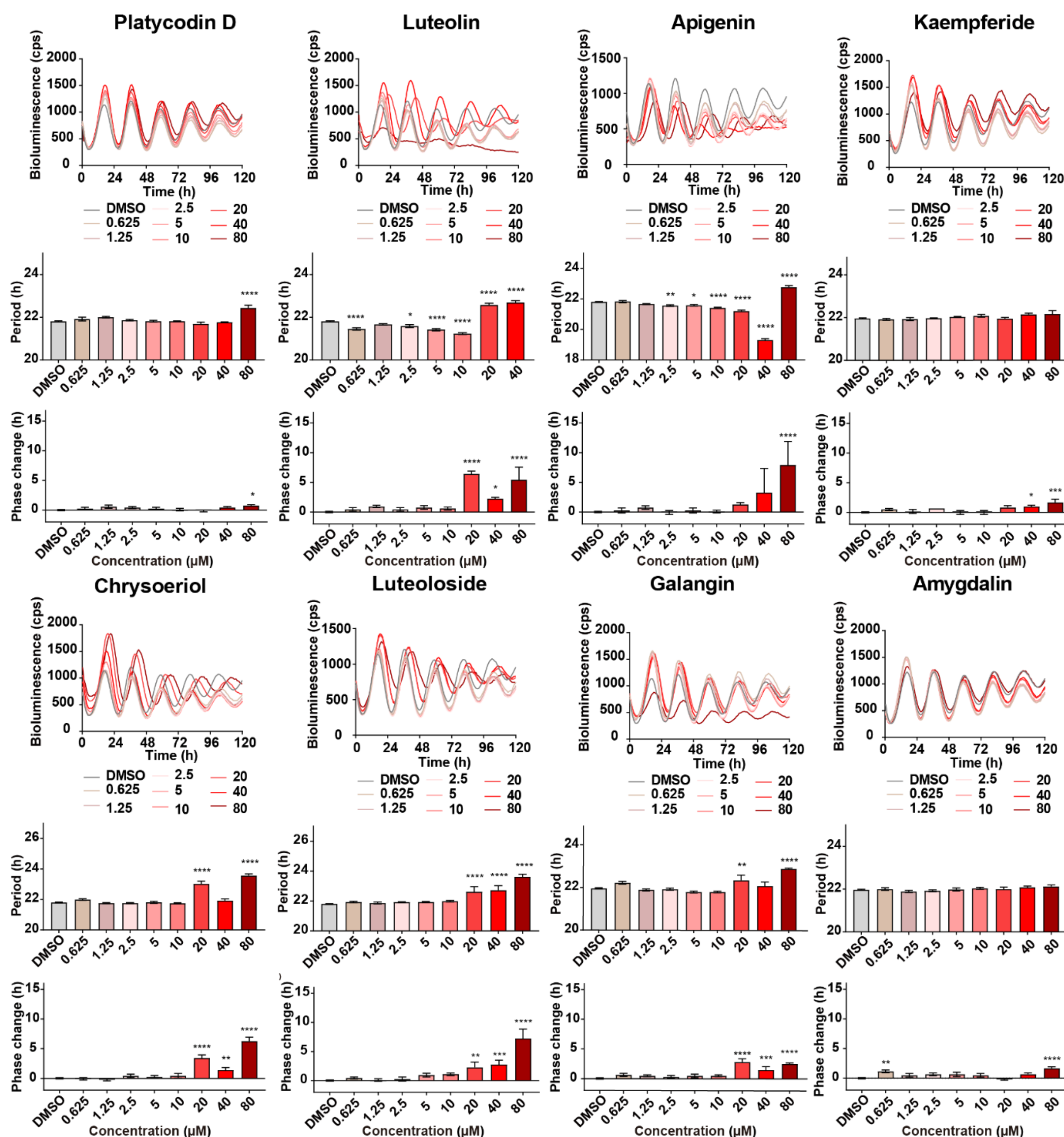


Figure 4. Ingredients of hit crude drugs modulate the circadian rhythm.

Luminescent traces (top graph) and dose-dependent effects of candidates on the period (middle graph) and phase (bottom graph) in U2OS cells. Values are averages of six replicates ± SD. Data were analyzed by one-way ANOVA, followed by Dunnett's multiple comparisons tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$).

II -4. Discussion

Chronic disruptions in the circadian clock due to shift work or jetlag can have long-term effects on human health, leading to an increased risk of many diseases (Sulli, et al., 2018). The development of small molecule modulators of the circadian clock is therefore of importance (Ribeiro et al., 2021). Identifying new therapeutical potentials for the existing drugs is known as the drug repositioning approach, which avoids the drawbacks of costly, time-consuming, and high failure rate in new drug development (Pushpakom et al., 2019). Crude drugs used in Japanese Kampo medicine are the main source of new chemical entities for drug discovery (Tashiro et al., 2018).

In this study, 137 crude drug extracts were screened. Among them, approximately 12% modulated circadian rhythms (Figures 1 and 2, Table 3). A previous study using the U.S. Food and Drug Administration (FDA)-approved drug library and the International Drug Collection (IDC) Library reported that approximately 5% of the available drugs altered circadian rhythms (Tamai et al., 2018). The hit rate of Kampo medicine was much higher than that of other existing drug libraries. This is probably because crude drug extracts are mixtures containing many active ingredients. Truly, the effects of the major ingredients are weaker than those of the original crude drugs (Figures 2 and 4).

To further investigate whether the effects of hit crude drugs were specific to reporter genes (*Bmal1-dLuc*) or cell types (U2OS cells), I examined three representative crude drugs (Allii Chinense Bulbus Artemisiae Capillaris Flos, and Perillae Herba,) in Rat-1 fibroblasts (*Per2-dLuc*). While the effects of Perilla Herba and Artemisiae Capillaris Flos extract were aligned with those observed in U2OS cells, Allii Chinense Bulbus extract showed the opposite period-shortening effect in Rat-1 cells (Figure 3). Differences in the effects of

crude drugs (i.e. shortened or prolonged periods) between different reporter genes and cell types may result from cell type specific functions of circadian clock genes and/or rhythmic dissociation of intracellular clock genes (Ramanathan et al., 2014). However, crude drug extracts are mixtures containing multiple active ingredients, and the expression levels of drug targets may also vary among different cells. This study demonstrates the circadian clock regulating effect of crude drugs at the cellular level. This study also demonstrates that the drug repositioning approach using Kampo medicines helps identify potential treatments for circadian rhythm disorders.

Chapter III

Involvement of AKT signaling pathway in circadian clock discovered by drug repositioning approach

III -1. Introduction

Lifestyle shifts have led to circadian rhythm disorders and increased risk of multiple diseases. Therefore, discovering small molecule modulators of the circadian clock is important (Dominoni et al., 2016). In a previous study, a screening of circadian clock modulators was conducted using Kampo medicines, and seventeen hit crude drugs were obtained (Figure 1, Table 3). These Kampo medicines affect the circadian clock in a dose-dependent manner (Figure 2). It is well known that all Kampo medicines are mixtures of multiple active ingredients. The composition of the active ingredients of seventeen hit crude drugs was analyzed and their effects on the circadian clock were further verified in U2OS cells (Figure 4).

The drug repositioning approach is an attractive endeavor because it can reduce the risks associated with the safety testing of new drugs and greatly reduce the time required to bring drugs into clinical application (Nosengo, 2016). It is widely used in new drug development as an effective method for developing existing drugs for new therapeutic purposes (Pushpakom et al., 2019; Xue et al., 2018). In addition, it is a popular tool for investigating the underlying molecular mechanisms. For instance, the targets of the major components of crude drugs are already known. The major components of these drug candidates targeted NF-κB, mTOR, or AKT signaling pathways (Table 4), which emphasizes that the drug repositioning approach is a valuable method to understand the underlying mechanisms of the circadian clock.

Whether Kampo medicines' effect on the circadian clock is mediated by the AKT pathway has not yet been reported. Therefore, the effects of AKT activator SC79 (Jo et

al., 2012), inhibitor A-443654 (Luo et al., 2005), and the triple knockdown of AKT1/2/3 using siRNA were analyzed in U2OS cells. I used Kampo medicines to explore the role of the AKT signaling pathway in the modulation of the circadian clock.

III -2. Materials and Methods

III -2-1 Cell culture

Human U2OS cells were obtained from ATCC (American Type Culture Collection). Human U2OS cells with a *Bmal1-dLuc* reporter gene were used. Cells were cultured in DMEM ((Dulbecco's modified eagle medium; D2902, Sigma-Aldrich, St. Louis, MI, USA), with the requirement that three reagents, 2 mM L-glutamine, 10% fetal calf serum (FBS, FB-1290/500; Biosera, Rue Lacaille, Nuaillen, France), 100 µg/mL streptomycin and 100 U/mL penicillin (Pen Strep, 15070063, Thermo Fisher Scientific, Waltham, MA, USA).

III -2-2 Chemicals

I purchased the AKT inhibitor, A-443654 (cat no. HY-10425), and the activator, SC79 (cat no. HY-18749) from MedChemExpress (Monmouth Junction, NJ, USA). All chemicals were suspended in Dimethyl sulfoxide (DMSO) at 5 mM, and kept at - 20 °C. Eight doses (0.025, 1.3, 2.5, 5.0, 10, 20, 40, and 80 µM) of AKT inhibitor A-443654 were analyzed, while AKT activator SC79 was analyzed at a various of doses (0.063, 0.13, 0.25, 0.5, 1.0, 2.0, 4.0, and 8.0 µg/mL). Luminescent assay media for recording was a mixture of DMEM, 10 mM HEPES, 0.35 g/L sodium bicarbonate, 3.5 g/L D-glucose, 2% B27(Life Technologies, Carlsbad, CA, USA), 100 U/mL penicillin, 100 µg/mL streptomycin, 100 nM dexamethasone (Sigma), and 0.1 mM luciferin (Fujifilm). Then diluted extracts were pipetted into triplicate wells of a 384-well plate containing U2OS cells (prepared above). The bioluminescence was monitored for one week using a Churitsu CL384 Series luminometer (Churitsu Electric Corporation, Nagoya, Japan). For time series analysis, the circadian period and phase were determined using NINJA SL00-01 software (Churitsu Electric Corporation).

III -2-3 siRNA-mediated knockdown

For gene knockdown analysis, small interfering RNA (siRNA) targeting the *AKT1*, *AKT2*, and *AKT3* sequences and non-targeting siRNA were obtained from Qiagen (FlexiTube GeneSolution siRNA, Hilden, Germany). Negative Control siRNA Use AllStars Negative Control siRNA (5 nmol) from QIAGEN (cat no.1027280, QiagenGermanyry). Dilute the siRNA to 2 μM siRNA stock and stock at -20 °C.

The experiment was performed with six replicates in every group. Generally, I introduced these siRNAs singly, or as a mixture of two or three siRNAs into U2OS cells using HiPerFect transfection reagent (cat no. 301704, Qiagen, Germany) protocol in 35 mm dishes. The day before transfection, 1.5 x 10^5 U2OS cells were seeded in 35 mm dishes and incubated in the incubator (37°C and 5% CO_2) until the cells were 80% confluent. For the single siRNA group (NC siRNA, AKT1 siRNA, AKT2 siRNA, AKT3 siRNA) dilute 50 ng of the corresponding siRNA in 100 μl of serum-free medium (2 μL 0f 2 μM siRNA stock). For the double siRNA group (AKT1/2 siRNA, AKT1/3 siRNA, AKT2/3 siRNA) dilute 50 ng plus 50 ng of the corresponding siRNA in 100 μl of serum-free medium (2 μL 0f 2 μM siRNA stock), and for the AKT1/2/3 siRNA group, add 50 ng of siAKT1, siAKT2 and siAKT3, respectively, for a total of 150 ng of siRNA. Then add 12 μL of HiPerFect transfection reagent to the diluted siRNA, vortex the mixture, and incubate for 10 min at room temperature (15-25°C) to allow for the formation of transfection complexes. Add the complexes drop by drop to the cells. Gently rotate the plate to ensure uniform distribution of the transfection complex. Incubate the cells (37°C and 5% CO_2) for 24 hours.

In the assay for siRNA Knockdown efficiency, remove the old culture medium after 24 hours, wash the cells with PBS and extract the RNA for backup. Bioluminescence was monitored in a LumiCycle 32 (Actimetrix, Wilmette, IL, USA) for one week.

III -2-4 Quantitative PCR

The knockdown effect was verified using quantitative PCR. Reverse transcription was performed on total RNA (200 ng) using ReverTra Ace (Toyobo, Osaka, Japan) and oligo-dT primers. The reaction mixture contained SYBR Premix Ex Taq II (Takara, Kusatsu, Japan), 0.4 μM gene-specific primers (Table 5), and 2 μL synthesized cDNA in a total volume of 20 μL. qPCR was performed on an Applied Biosystems QuantStudio 3 Real-Time PCR System (Foster City, CA, USA). PCR condition was as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. ΔCt was determined using GAPDH as a housekeeping gene. Relative expression was calculated using the ΔΔCt method by comparing gene-specific siRNA samples to the negative siRNA control.

Table 5. Primer sequences used for RT-qPCR.

Gene	Primer	Sequence
Human *AKT1*	Forward primer	ACTTTCGGCAAGGTGATCCT
	Reverse primer	CTTGGCCACGATGACTTCCT
Human *AKT2*	Forward primer	TGCCACCATGAATGAGGTGAAT
	Reverse primer	GGGCCTCTCCTTGTACCCAA
Human *AKT3*	Forward primer	ACTGGAGGCCAAGATACTTCCT
	Reverse primer	TTTTCCCCTCAGTACTTGCCA
Human *GAPDH*	Forward primer	TCAACGGATTTGGTCGTATTG
	Reverse primer	TGGGTGGAATCATATTGGAAC

III -2-5 Statistical analysis

Statistical analysis was conducted using GraphPad Prism 9 (https://www.graphpad.com/scientific-software/prism/). The significance of differences was analyzed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons tests or Student's t-tests. Values are expressed as mean ± SD.

III -3. Results

II-3-1 AKT is a major signaling hub among the potential targets of active ingredients.

Since crude drug extracts are mixtures containing multiple active ingredients, the modulation (period and/or phase) of eight ingredients of hit crude drugs on the circadian rhythm was confirmed in U2OS cells (Figure 4, Chapter II). To further understand the mechanistic basis for these crude drugs, I searched for the composition of these biologics based on previous literature and next summarized the known targets of these active ingredients in the PubMed database. Interestingly, AKT, NF-κB and mTOR were the most common targets of these active ingredients (Table 4). Notably, AKT is located at the center of the network, suggesting that its regulatory role in circadian rhythms deserves to be explored (Figure 5).

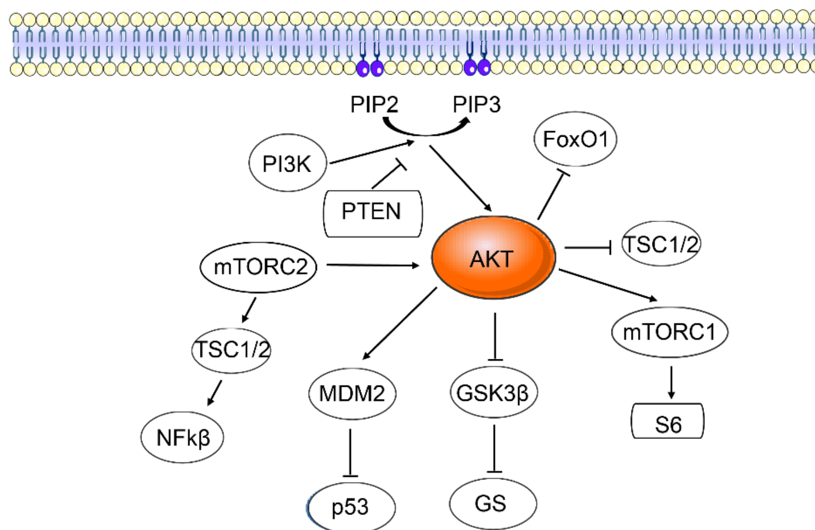


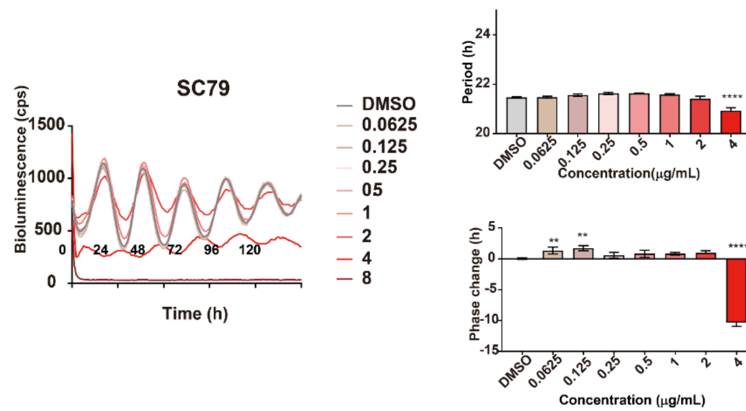
Figure 5. Schematic diagram of AKT pathway interaction.

More than half of the major components in crude drugs target AKT and related signaling pathways. AKT is known to be a major signaling hub.

II-3-2 Effects of AKT activator and inhibitor on circadian rhythm.

Akt signaling has been shown to affect the circadian period in *Drosophila* (Zheng & Sehgal, 2010). However, there are three isoforms of AKT in mammals. Among them, *Akt1-/-* mice show normal behavioral rhythms (Luciano et al., 2017). Here, I chose an inhibitor and activator that can target three AKT isoforms. SC79 specifically binds the PH structural domain of Akt, for enhancing Akt activity under various physiological and pathological conditions (Jo et al., 2012). A-443654 is a potent small molecule inhibitor of Akt serine/threonine kinase that induces Akt Ser-473 phosphorylation in all human U2OS (Han et al., 2007). The AKT activator SC79 shortened the circadian period and advanced the phase at the highest dose. Conversely, the AKT inhibitor A-443654 shortened the circadian period at lower doses and lengthened the period at the highest dose (Figure 6).

A



B

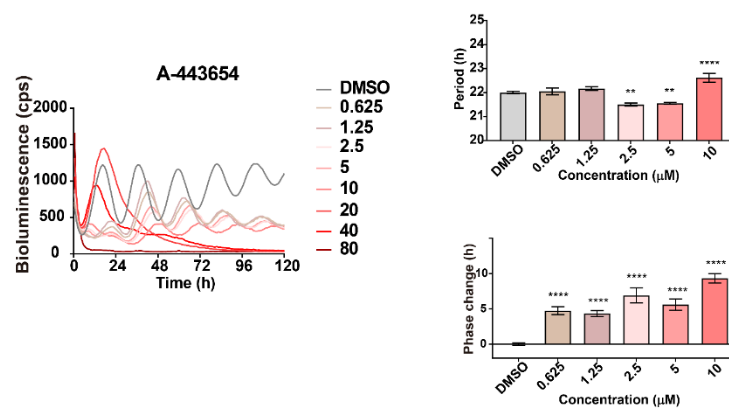
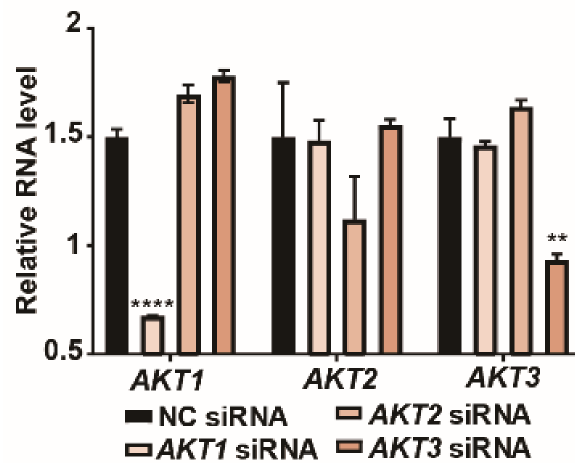


Figure 6. Effects of AKT activator SC79 and AKT inhibitor A-443654 at different concentrations. Effects of AKT activator SC79 (A) and AKT inhibitor A-443654 (B) at different concentrations. Dose-dependent effects of luminescence traces (left graph), period (top right graph,) and phase (bottom right graph) in U2OS cells. Values are the averages of six replicates ± SD. Data were analyzed by one-way ANOVA, followed by Dunnett's multiple comparisons tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$).

II-3-3 Effects of siRNA knockdown of three AKT isoforms.

There are three AKT isoforms in mammals. AKT activator SC79 and AKT inhibitor A-443654 indistinguishably target three isoforms. Therefore, the effects of siRNA knockdown on AKT isoforms were examined. The knockdown efficiency of siRNA targeting *AKT1, AKT2*, and *AKT3* sequences in U2OS cells were verified by RT-qPCR, The calculation of knockdown efficiency of siRNA is compared with the NC group of each group (Figure 7A). The knockdown of the single siAKT has resulted in a trend toward a shorter period. Notably, the circadian rhythm was significantly altered in the presence of triple knockdown AKT isoforms (Figure 7B). In summary, AKT signaling is engaged in the mammalian circadian clock (Figures 6 and 7).

A



B

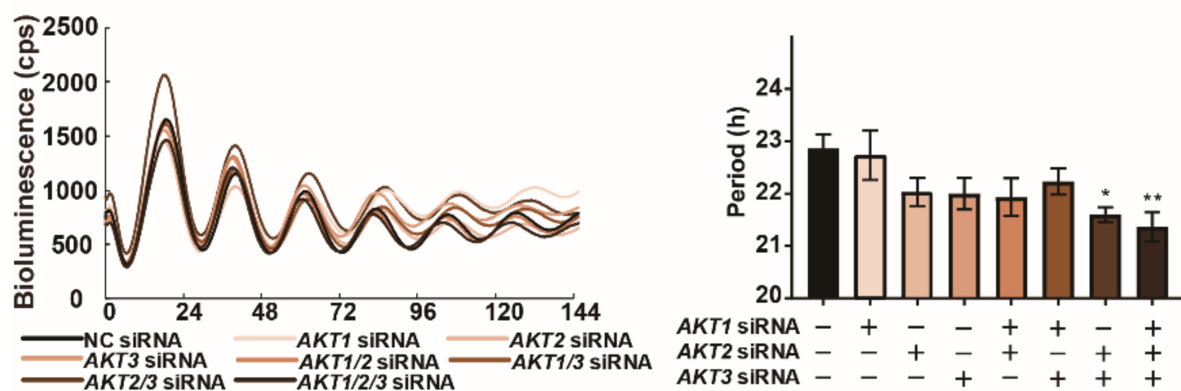


Figure 7. Effects of the siRNA knockdown of *AKT1/2/3*.

U2OS cells were transfected with negative control (NC) siRNA, *AKT1* siRNA, *AKT2* siRNA, or *AKT3* siRNA. (A) The knockdown efficiency of *AKT* was confirmed by RT-qPCR, Knockdown efficiency compared with NC siRNA group. (B) Luminescent traces (left graph) and effects on the circadian period (right graph) in U2OS cells. Values are the averages of six replicates ± SD. Data were analyzed by one-way ANOVA, followed by Dunnett's multiple comparisons tests (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, **** $p < 0.0001$).

III -4. Discussion

It is well known that all Kampo medicines are mixtures of multiple active ingredients. Therefore, active ingredients of Kampo medicines were examined in U2OS (*Bmal1-dLuc*). Interestingly, the most common targets for the hit active ingredients were AKT and its related pathways (Table 4). Moreover, AKT is located at the signaling hub (Figure 5).

AKT-related diseases mainly include cancer, neurological disorders, diabetes, cardiovascular and cerebrovascular diseases, and inflammatory and autoimmune diseases (Brown & Banerji, 2017; Jo et al., 2012; Koren et al., 2015; Wu & Mohan, 2009; Yu et al., 2015). Therefore, AKT is a valuable therapeutic target, and its inhibitors and activators have been used to develop drug treatment regimens. Under physiological conditions, phosphatidylinositol (3,4,5) - trisphosphate (PtdIns (3,4,5) P3, PIP3) is known to mediate plasma membrane translocation, activating AKT through phosphorylation (Jethwa et al., 2015). Jo et al. identified an AKT activator SC79, like PIP3, through a cell-based high-throughput chemical genetic screen. SC79 specifically binds to the Pleckstrin Homology (PH) domain of AKT to induce AKT conformation that favors phosphorylation (Jo et al., 2012). Han et al. identified a potent small-molecule inhibitor of AKT serine/threonine kinase, A-443654. A-443654 induces AKT Ser-473 phosphorylation in a time- and dose-dependent manner in Phosphatase and Tensin Homolog (PTEN) and Tuberous Sclerosis Complex 2 (TSC2)-deficient cells. This study also highlights that induction of AKT Ser-473 phosphorylation is dependent on Phosphatidylinositol 3-kinase (PI3K) and/or mTOR Complex 2 (mTORC2) (Han et al., 2007). As shown in Figure 6, the period and phase changes caused by AKT activators and inhibitors are opposite. The inhibitor of AKT, SC79, shortens the period and advances the phase, whereas the activator of AKT, A-443654, lengthens the period and delays the phase. It also provides evidence for the involvement of AKT in circadian clock regulation. Interestingly,

Akt signaling has been shown to affect circadian rhythms in *Drosophila* (Zheng & Sehgal, 2010).

However, three AKT isoforms exist in mammals, including *AKT1*, *AKT2*, and *AKT3* (Brodbeck et al., 1999; Jones et al., 1991; Konishi et al., 1995). *AKT1* is widely expressed in various tissues, whereas *AKT2* is mainly expressed in insulin-sensitive tissues and to a lesser extent in other tissues. *AKT3* is specific to the brain, lung, heart, kidney, testis, and skeletal muscle. Different genes encode the AKT isoforms, but the proteins share a high degree of structural homology (Kumar & Madison, 2005). The research on subcellular locations of AKT isoforms showed that AKT1 is in the cytoplasm, AKT2 in the mitochondria, and AKT3 in the nucleus (Santi & Lee, 2010). *Akt1-/-* mice were found to exhibit normal behavioral rhythms in the previous studies (Luciano et al., 2017). Therefore, a siRNA triple knockdown study of AKT isoforms was performed (Figure 6), and the results showed that *AKT* knockdown resulted in a shortened period, consistent with the effects of AKT inhibitors. Moreover, the triple knockdown superimposed the period shortening effect. The results indicate that AKT signaling is implicated in the mammalian circadian clock (Figure 6). It is worth mentioning that PI3K is one of the signaling molecules upstream of the AKT signaling pathway, which regulates feeding-mediated entrainment of the peripheral circadian clock. In short, the drug repositioning approach demonstrates the involvement of AKT signaling in the circadian clock.

Chapter IV

General Discussion

Circadian rhythms are cell-autonomous approximately 24 hours biological rhythms observed in nearly all organisms. Mammalian circadian clocks involve transcription-translation feedback loops (Honma, 2018). Recent studies have shown that the circadian clock can be disrupted by genetic factors or the living environment (Khan et al., 2018). It is also of note that chronic circadian clock disruptions caused by modern lifestyles are known to increase the risk of various diseases, such as diabetes, cardiovascular disease, and cancer.

The genetic basis of circadian behavior and molecular clocks has been revealed, and the identification of small molecule circadian clock regulators is beneficial for discovering treatments for circadian clock-related diseases (Chen et al., 2018). Therefore, it is crucial to develop circadian clock modulators. The drug repositioning approach is the process of identifying the new therapeutic potential for the existing drugs. The advantages of the drug repositioning approach compared with the traditional drug development method include shortened drug development cycles, lower cost, higher efficiency, and minimal risk of failure (Nosengo, 2016). Kampo medicine is used in clinical applications to treat a variety of diseases and is a library of natural sources of drugs. Recently, Kampo medicine is also used for the identification of circadian clock molecules（Motohashi et al. 2017).

In chapter II, the cell-based chemical screening was used to identify potential biomolecules in the Kampo drug library. I screened 137 crude drug extracts to identify the circadian clock using a human osteosarcoma U2OS cell line stably expressing the clock reporter gene of *Bmal1*. The primary screening identified seventeen drugs that affect circadian clock oscillations, mainly in terms of period and phase. A previous study using the FDA-approved drug library and the International Drug Collection (IDC) library reported that only approximately 5% of the available drugs in the library medicines altered circadian rhythms

(Tamai et al., 2018). The hit rate for our Kampo medicine library is much higher than that for the other drug libraries. This is probably because crude drug extracts are mixtures containing many active ingredients (Table 4). These results suggested that Kampo medicine is an excellent resource to discover new circadian clock modulators.

Notably, in seventeen Kampo medicines identified by U2OS cells, Polygalae Radix is shown to have the effect of period lengthening (Figure 2). Nevertheless, Haraguchi et al. reported that Polygalae Radix shortened the period length of the *Per2-dLuc* expression rhythm in the *in vitro* experiments (Haraguchi et al. 2022). Moreover, the period lengthening and phase changing effects of Allii Chinense Bulbus are different between U2OS *Baml1-dLuc* and Rat-1 *Per2-dLuc* fibroblasts (Figures 2 and 3). Such differences may result from cell type-specific functions of biological clock genes and/or rhythmic dissociation of intracellular clock genes (Ramanathan et al., 2014). It is also probable that since crude drug extracts are mixtures containing multiple active ingredients, the expression levels of drug targets may also differ among cells. In any case, my study and previous studies demonstrated the effects of Kampo medicine to modulate the circadian clock.

Interestingly, more than half of the active ingredients of the selected crude drugs targeted AKT and its related signaling pathways (Manning & Cantley, 2007). In chapter III, I focused on the link between the circadian clock and the AKT signaling pathway. I confirmed the effects of AKT activators SC79 and inhibitors A-443654. Mammals have three *AKT* isoforms. Therefore, I used siRNA to triple knockdown of *AKT1/2/3*. Triple knockdown of AKT1/2/3 shortened the circadian period. It is reported that enhanced Akt or target of rapamycin (TOR) activity lengthens the circadian period, whereas weakened Akt signaling shortens it in *Drosophila* (Zheng & Sehgal, 2010); Mammalian Target of Rapamycin (mTOR) heterozygous mice show lengthened circadian period of locomotor activity in both constant darkness and constant light (Ramanathan et al., 2018). This is consistent with the present

findings and supports the idea that the enhancement of AKT is positively correlated with the period length. These findings are beneficial to predict the potential of current drugs in the treatment of circadian clock disorders.

In the present study, I have demonstrated the involvement of AKT signaling in the mammalian circadian clock. However, precise mode of action of Kampo medicine on AKT signaling and hence circadian clock remains unclear. It is interesting to note that Dan et al. found that Insulin promotes postprandial Akt-mediated Ser42-phosphorylation and suppression of *Bmal1* transcriptional activity (Dan et al. 2016). Luciano et al. found that CLOCK is a substrate for AKT phosphorylation *in vitro* (Luciano et al. 2018). This indicates that the AKT signaling pathway may have several mechanisms to modulate the activity of the central components of the circadian clock. Therefore, it is worthy of further exploration of the regulation of the circadian clock by Kampo medicines via the AKT signaling pathway.

In conclusion, chronic disruption of the circadian clock due to shift work or travel across time zones has long-term effects on human health. In the present study, I have identified circadian clock modulators from Japanese Kampo medicine. In addition, the involvement of the AKT signaling pathway in the regulation of the mammalian circadian system is suggested. The drug repositioning approach is a powerful method to identify circadian clock regulatory molecules and explore the interaction between the circadian clock and the AKT signaling pathway.

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