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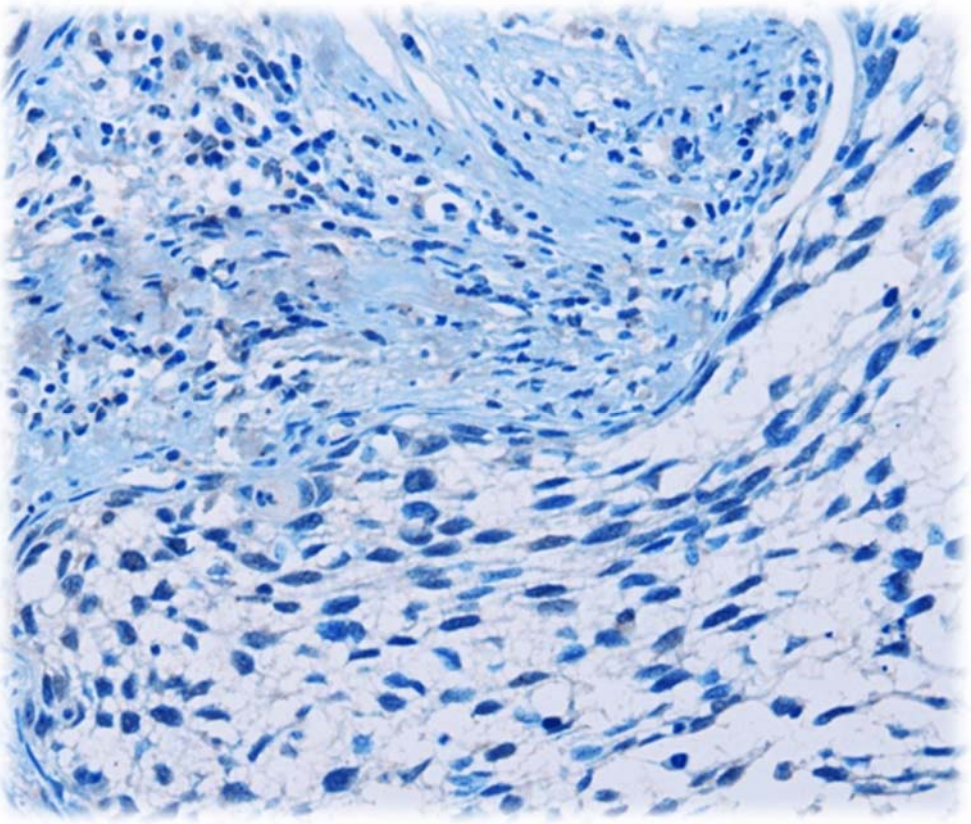
EARLY PHARMACOLOGICAL PROFILING OF CK1ε INHIBITORS



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Trabajo de Fin de Grado

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Grado en Biología

Julio 2020

La presente propuesta de investigación ha sido redactada por el alumno Javier Juliá Sánchez durante el curso académico 2019-2020 y bajo la dirección del Dr. José M. Padrón Carrillo. El trabajo forma parte de la línea de investigación *Diseño, descubrimiento y evaluación de fármacos anticancerígenos.*

La Laguna, a 7 de julio de 2020.

Fdo. José M. Padrón

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ABSTRACT

Breast cancer is one of the leading causes of premature death, increasing its incidence in modern times, making scientific research a fundamental tool for its treatment. Synthetic lethality approach shows a great potential for this goal. CK1ε protein is a promising target for its application. Its physiological role is directly involved in growth inhibition dependent on several metabolic pathways, such as the canonical and non-canonical Wnt pathways. However, the high similarity it has with its isoform, CK1δ, represents a high difficulty for the development of selective inhibitors. Here, we present a methodology suitable for the profiling of inhibitors, using selective (**PF-4800567** and **GSD0054**) and dual inhibitors (**IC261**, **PF-5006739** and **PF-5006739**) for comparative purpose. These inhibitors will be studied against several breast cancer cell lines at diverse doses. At the same time, the affinity of these compounds towards the CK1ε proteins over its δ isoform will also be evaluated. This work should prove useful for the profiling of future CK1ε inhibitors that can help in the development of better chemotherapeutic strategies in the fight against cancer.

RESUMEN

El cáncer de mama es una de las principales causas de muerte prematura, con una creciente incidencia en la sociedad moderna, haciendo de la investigación científica una importante herramienta para su tratamiento. La aplicación de la letalidad sintética muestra un gran potencial para alcanzar dicha meta. La proteína CK1ε es una diana prometedora para dicha aplicación. Su inhibición está directamente relacionada con inhibición del crecimiento en varias rutas metabólicas, como las vías Wnt canónica y no canónica. Sin embargo, la alta similaridad que tiene con su isoforma, CK1δ, representa un obstáculo para el desarrollo de inhibidores selectivos. Aquí presentamos una metodología apropiada para la descripción de inhibidores de CK1ε, utilizando los inhibidores selectivos (**PF-4800567** y **GSD0054**) y los duales (**IC261**, **PF-5006739** y **PF-5006739**) por razones comparativas. La actividad antiproliferativa de estos inhibidores se sobre varias líneas celulares de cáncer de mama a concentraciones distintas. Al mismo tiempo, se evaluará la afinidad de los inhibidores frente a la proteína respecto a su isoforma δ. Este trabajo debería resultar útil para la descripción de futuros inhibidores de CK1ε que ayuden al desarrollo de mejores estrategias quimioterapéuticas en la lucha contra el cáncer.

INTRODUCTION

Cancer impact

Cancer is most certainly the first or second leading cause of premature death, i.e. at ages 30-69, in 134 out of 183 countries. In 2016, 4.5 million people out of 15.2 million deaths (29.8%) died of cancer from non-communicable diseases worldwide[1]. More specifically, lung cancer is the most common cancer type worldwide in terms of both incidence and mortality, with 2.1 million new cases and 1.8 million deaths in 2018. On the other hand, breast cancer is the most commonly diagnosed cancer in women, having an alarmingly increasing rate of apparition (Fig. 1), with 2.1 million new cases in 2018, and the leading cause of cancer death in women globally, with 627.000 deaths in the same year[2]. Early age at menarche along with later age, first birth and lower parity are risk factors for breast cancer apparition, all having an increased presence in society. These data, along with the high incidence and mortality of other cancer types make research an important tool for providing welfare for people from both poor and developed countries alike.

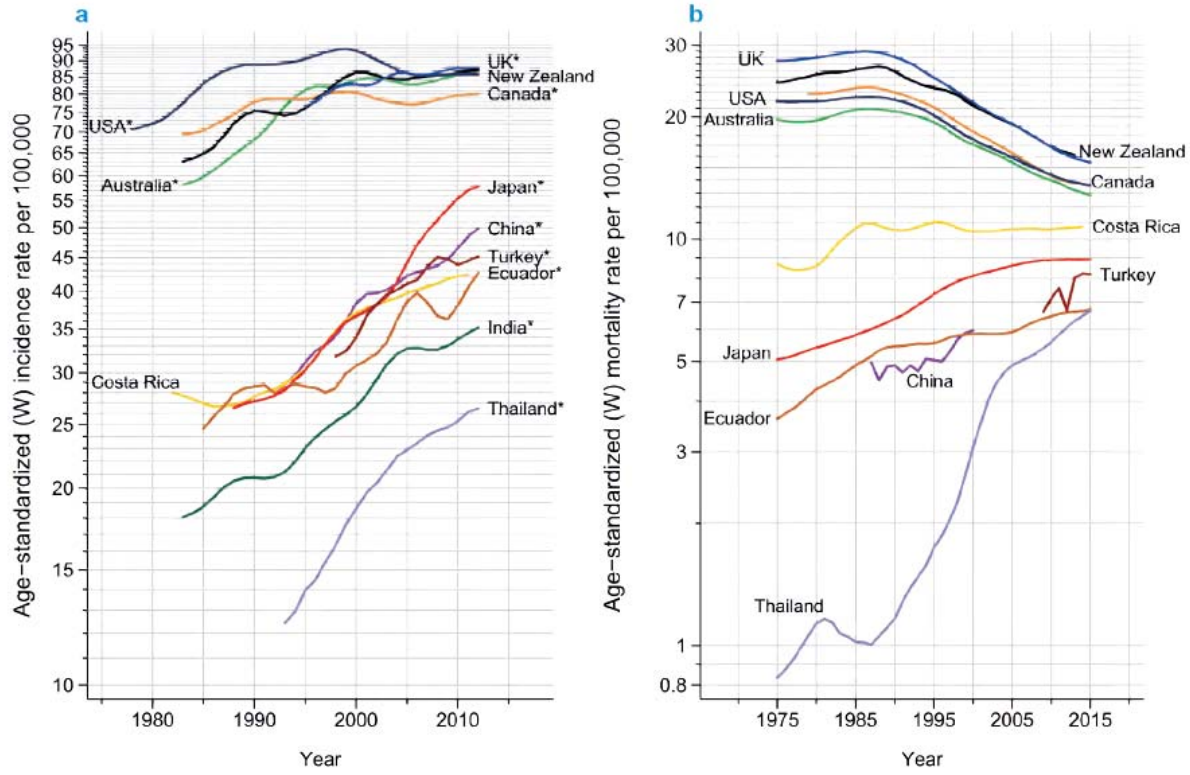

Figure 1. Breast cancer statistics by year between 1975-2015. (Left) incidence rate. (Right) mortality rate. Taken from Wild et al. (2020)

Emerging therapies

The development of anticancer selective therapeutic strategies has been always an elusive goal. However, recent approaches show a great potential for the achievement of this task. An example of this is the application of synthetic lethality. The concept of synthetic lethality consists in a combination of mutations in two or more separate genes that leads to cell death (Fig. 2). Considering that cancer emanates from genetic alterations, finding and inhibiting the synthetic lethal gene (or the corresponding translated protein) partner of an specific cancer-promoting mutation would make a better chemotherapeutic therapy than conventional ones, as non-cancer cells would remain unaffected, lacking the mutated partner present in cancer cells[3,4].

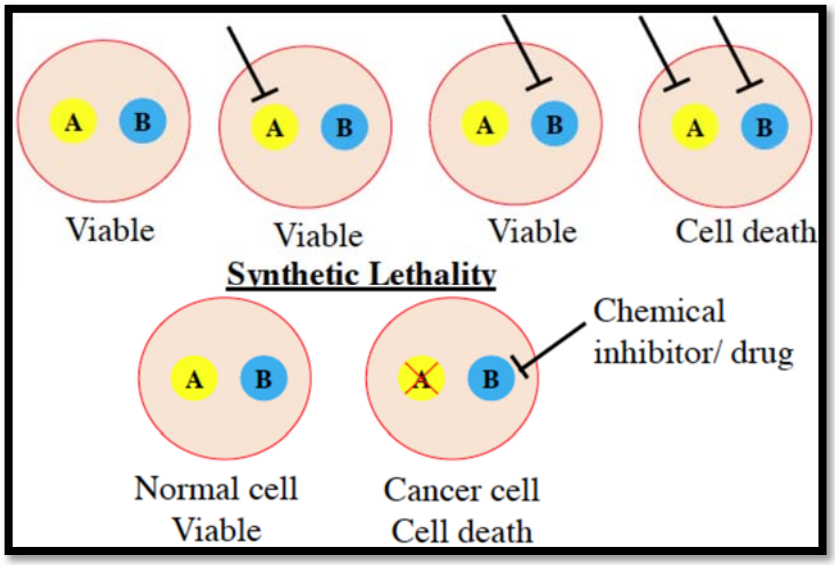

Figure 2. Schematic representation of synthetic lethality. Gene A is mutated in cancer cells. Exogenous inhibition by the use of chemical inhibitors, short hairpin RNAs or small interferent RNA of its synthethic lethal partner, B gene, will lead to selective cancer cell killing. Taken from Dar, A. et al. (2017)

CK1 family

CSNK1E is a synthetic lethal partner to many cancer mutations. It encodes the protein casein kinase 1 epsilon (CK1ε), a member of the casein kinase 1 family (CK1). The CK1 family of proteins forms a group within the superfamily of serine/threonine-specific protein kinases (STKs). STKs are enzymes that use the gamma phosphate of ATP or GTP to generate phosphate monoesters, preferentially on serine or threonine residues (N-terminally flanked by already phosphorylated amino acid residues or acidic amino acids, in the case of the CK1 family). The phosphorylation is a reversible process that plays a relevant role in intracellular

signal pathways[5–9]. This way, STKs regulate almost all cellular processes, nuclear and cytoplasmic, including cell growth and proliferation, metabolism, differentiation, apoptosis, DNA replication and repair[5,9–13].

The CK1 protein kinase family is evolutionary conserved and several casein kinase genes are present in mammals. There are seven isoforms described so far (α, β, γ1, γ2, γ3, δ and ε) in addition to their splice variants. CK1ε has three splice variants described in rat, differing in their kinase activity and tissue expression[13]. These isoforms show a high homology, e.g. CK1ε and CK1δ have a 98% homology in their highly conserved kinase domain and 53% in their C-terminal regulatory domain[14]. Both isoforms encode 416 amino acids, 285 of which belong to the kinase domain, while 123 belong to the carboxyl-terminal domain (residues 294-416)[11,14,15]. The high homology shared by these two proteins represents a challenge for CK1ε inhibitor development, which is arguably poor nowadays, having only five described, half of which target both isoforms, the so-called dual CK1ε/δ inhibitors.

CK1ε involvement in cancer

As aforementioned, CK1ε is a synthetic lethal partner of several genes and pathways. Next, the role of this protein in several oncogenic processes will be discussed, highlighting its relevance as a therapeutic target.

a. Role in the circadian clock

Circadian rhythms control key physiologic processes including sleep-wake cycles; glucose, lipid and drug metabolism; heart rate; stress and growth hormones, as well as basic cellular processes such as timing of the cell division cycle[16,17]. Moreover, rhythm genes can regulate the cell cycle, apoptosis, DNA damage repair and cancer-associated inflammation during cancer progression. Disruption of circadian rhythm causes physiologic stress and frequently links to night-shift work. In 2007, it was classified as a probable carcinogen supported by a positive relationship of developing breast cancer in several epidemiological studies focused on nurses and female flight attendant due to the night shifting nature of their jobs. Several studies using rodent models linked the effect of light at night, melatonin deprival and deregulation of circadian genes, such as PERIOD genes, with cancer related pathways[18,19]. Evidence was insufficient for addressing circadian disruption as source for tumor growth by itself, as a study on cryptochrome knockout mice proposed; being cryptochrome 1 and 2 core components of the circadian clock. However, inactivation of the mouse circadian period gene “mPER2” by a deletion in the PAS-B domain showed an

increased sensitivity towards γ radiation and tumor development, along with deregulation in the temporal expression of genes involved in cell cycle regulation and tumor suppression, such us cyclin D1, cyclin A, Mdm-2, Gadd45α and c-myc[20,21]. These results pointed in the direction of PER2 as an indirect regulator of tumor suppression. More recent studies confirm PER2 gene as antiproliferative, considering that its expression is lowered in a variety of tumors, but its overexpression results in decreased proliferation and promoted autophagy in oral squamous cell carcinoma (OSCC)[22]; Lewis lung carcinoma (LLC); mammary carcinoma (EMT6)[23]; Sarcoma S180[24], and k562 leukemia cell lines[25]. In fact, even intratumor delivery of the mPER2 gene into plasmids showed malignant growth inhibition by apoptosis and cell arrest[26]. Most importantly for this study, PER2 has been associated to the estrogen receptor (ER) in breast cancer, enhancing its degradation and, therefore, reducing proliferation in response to estrogen reception[27].

CK1ε displays a regulatory role in the circadian clock. It is responsible for causing a shortened free-running circadian period in golden hamsters in a gain-of-function mutation named "tau", consisting in a C→T transition resulting in an arginine-to-cysteine amino acid substitution at residue 178. This led to the aforementioned shortened circadian period, because of an increased phosphorylation rate and degradation by the ubiquitin/proteasome pathway of the circadian regulators PER1 and PER2. The inhibiting effect over BMAL1/CLOCK transcription factors heterodimers, whose uninhibited activity enhances PER2 transcription, but, at the same time, reduced its nuclear residence and stability[16,28–31]. The BMAL1/CLOCK heterodimer, whose transcription is inhibited by PER2, naturally enhance PER2 transcription in a negative feedback loop, as the increased PER2 transcription results in BMAL1/CLOCK transcription inhibition (Fig. 3). Therefore, overexpression of CK1ε, provokes PER2 reduced stability, probably being a reason for the accelerated growth rate. This is supported by the fact that CK1ε knockdown using short hairpin RNA provokes PER2 dependent growth arrest, caused by the PER2 overexpression, product of the cease of CK1ε phosphorylation of PER2, and consequently degradation by the proteasome[32]. The thought that alterations in CK1ε affect cancer expression by altering the circadian clock by itself, are indeed displaced by the fact that CK1ε inhibition alter minimally circadian period, being the circadian alteration due to the inhibition of CK1δ by the use of dual (CK1δ/ε) inhibitors[33].

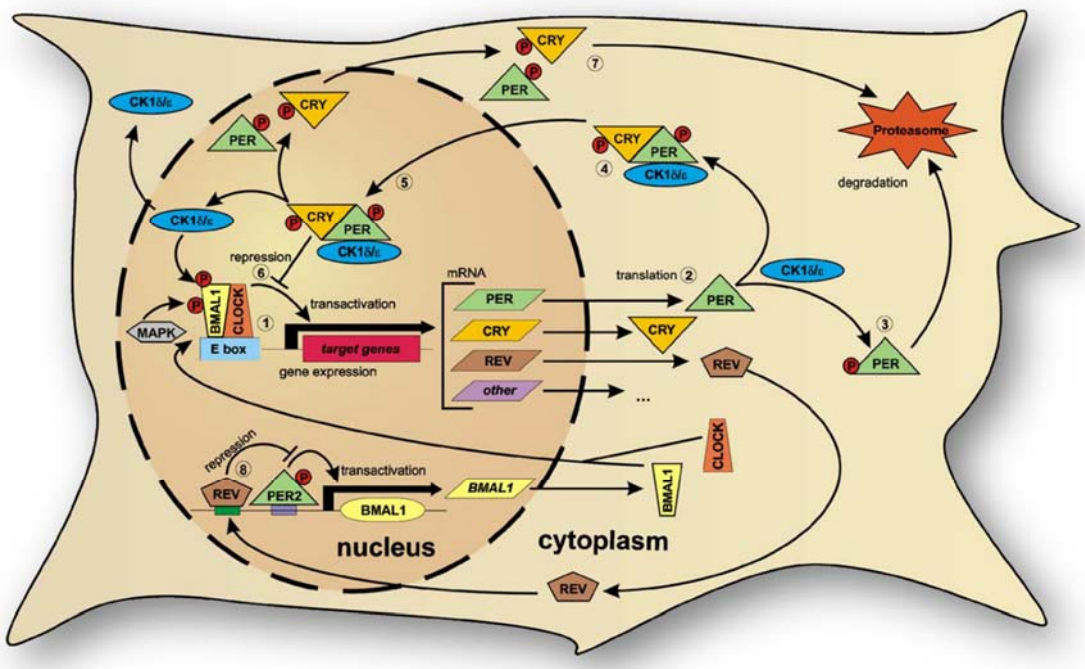

Figure 3. CK1 and the circadian rhythm. Proposed model for CK1δ/ε heterodimer in regulating the circadian clock. (1) BMAL1/CLOCK heterodimers bind to E-box containing target genes, amidst them PER1-3, CRY1 and 2 and REV-ERBα. (2) Transcripts are translocated to the cytoplasm, where they are translated into proteins. (3) Phosphorylation of cytoplasmatic PER by CK1δ/ε triggers their degradation by the proteasome. (4) Binding of CRY proteins to PER proteins inhibits PER-degradation and promotes translocation of CRY-PER-CK1δ/ε complex into the nucleus (5). (6) The CRY-PER- CK1δ/ε complex represses BMAL1/CLOCK dependent transcriptional activation. CRYs and PERs relocate into the cytoplasm and are degraded at the proteasome (7). (8) A second negative feedback loop is built by transcriptional repression of BMAL1 expression by REV-ERBα. These feedback (-) loops result in cyclic expression of E-box genes and BMAL1. Taken from Knippschild, U. et al. (2005)

This degradation-promoting effect of CK1ε over PER2, along with PER2 relation to decreased proliferation fuels our interest over CK1ε inhibition as a therapeutic target.

b. Canonical Wnt pathway

The Wnt/β-catenin signaling pathway, along with its intracellular effector, β-catenin, regulate many aspects of development, proliferation and oncogenic processes[34]. Mutations in the pathway cause cumulative up-regulation of β-catenin, underlying numerous human cancers. CK1ε phosphorylates and thereby regulates several components in the Wnt signaling cascade, including Dv1, LRP6, APC, Axin and catenin[35]. Moreover, as studies based on RNAi suggest, CK1ε is involved in this pathway as a positive regulator, being an essential regulator of β-catenin, whose activity is necessary for cancer cell lines that exhibit catenin aberrant up-regulation, such as some breast cancers[15]. Its inhibitors, along with knockdown by interference RNA dump β-catenin accumulation in nucleus and cytoplasm as a response

to the pathway, making *CSNK1E* to act as a synthetic lethal gene to β-catenin aberrant activation[36].

c. Non-canonical Wnt Pathway

CK1ε is involved in the non-canonical Wnt pathway. It is overexpressed in ovarian cancer cells while interacts with the mitochondrial proteins annexin A2, prohibitin and ANT2, instead of regulating β-catenin, even though it is overexpressed in these cells. In this case, CK1ε appears to regulate cell proliferation and migration. Its inhibition results in ATP downregulation, rending cancer cells susceptible to chemotherapy[37].

d. RNA translation

CK1ε is capable of increasing cell proliferation through regulation of mRNA translation. This regulation takes place by phosphorylation of the eukaryotic translation initiation factor 4E-BP1. This phosphorylation reduces 4E-BP1 binding to the other eukaryotic translation initiation factor, eIF4E, which increases mRNA translation. Inhibition of CK1ε reduces 4E-BP1 phosphorylation, which allows a higher rate of 4E-BP1 binding to eIF4E, inhibiting translation in breast cancer cells[38].

CK1ε inhibitors

Most CK1 inhibitors are small molecules target the ATP binding site, mimicking the ATP binding to the kinase in its active form. These are known as ATP competitive type-1 inhibitors[39]. The active form they bind to is characterized by an open conformation of the activation loop, typically 20 to 30 residues in length, beginning with a DFG motif (usually aspartate-phenylalanine-glycine)[40]. **IC261**, a known dual CK1δ/ε inhibitor, binds to the ATP binding pocket of CK1δ/ε[41].

Type-II kinase inhibitors bind to and stabilize the inactive form on the kinase, and their development leads to more specific and higher selective chemical compounds. They bound to the same area occupied by the type I compounds, but also extends to the hydrophobic interactions in the allosteric site. As result, type II compounds generally display a lower dissociation rate and weaker competition from cellular ATP (as they lock on the inactive form), which has a favorable impact on kinase inhibition[39]. The drawback is the higher molecular weight of type II compounds, which results in lower cellular penetration and ligand efficiency[42]. Examples are **PF-4800567** and **PF-670462**, a CK1ε specific and CK1δ/ε dual inhibitor, respectively[33,43].

Next, all reported CK1ε inhibitors are summarized below.

a. Selective inhibitors (Fig. 4)

PF-4800567[33]**:** First selective CK1ε inhibitor synthetized, having over 20-fold selectivity towards CK1ε over its δ isoform (CK1δ IC_{50} = 711 nM; CK1ε IC_{50} = 32 nM). However, high concentrations still could affect the latter. Furthermore, this inhibitor has a big drawback. It is that it does not inhibit cell proliferation at low concentrations (1 μM), and even at high concentrations, it does not cause cell death[44,45].

GSD0054[46]**:** Represents the first and only selective CK1ε with proven antiproliferative activity. However, this inhibitor has been recently synthetized and still requires further studies to fully comprehend its role and potential use as a therapeutic agent against cancer[47].

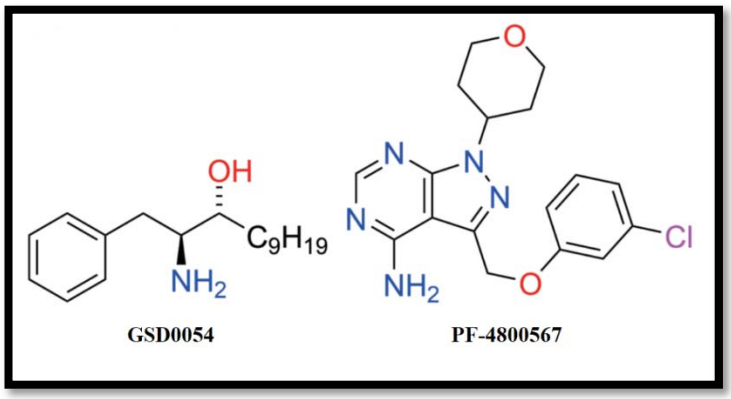

Figure 4. Chemical structure of selective CK1ε inhibitors.

b. Dual inhibitors (Fig. 5)

PF-670462[43]: This inhibitor has similar affinity towards both proteins (CK1δ IC_{50} = 14 nM; CK1ε IC_{50} = 7.7 nM), but like PF-4800567, it is unable to produce cell death[44].

IC261[41]: This inhibitor selectively suppresses human cancer cell growth, but it has less affinity towards CK1δ/ε than PF-670462 (CK1δ IC_{50} = 0.7-1.3 μM; CK1ε IC_{50} = 0.6-1.4 μM). Its potent antiproliferative activity is due to its high affinity to tubulin, which makes it an inhibitor of microtubule polymerization, inducing cell cycle arrest at prometaphase and stop cell division[44].

PF-5006739[48]: This inhibitor is studied in the development of drug addiction, reducing drug-seeking behaviors. It shows 4-fold affinity towards CK1δ (CK1δ IC_{50} = 3.9 nM; CK1ε IC_{50} = 17 nM), however, the affinity to CK1ε is higher than that of the selective PF-4800567.

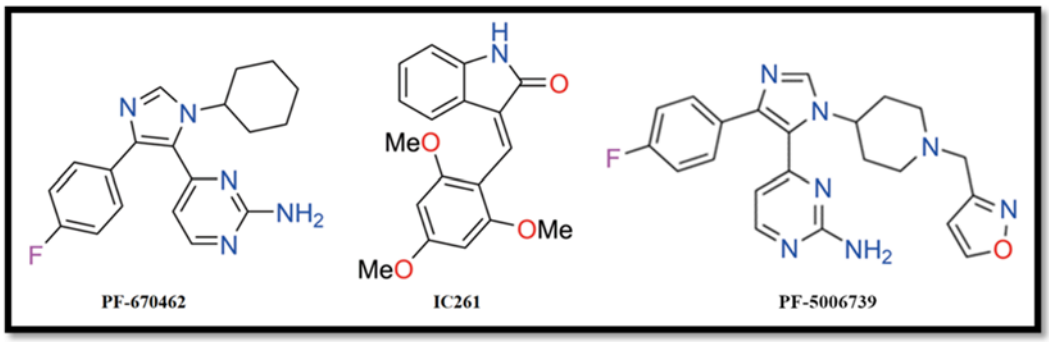

Figure 5. Chemical structure of dual CK1δ/ε inhibitors.

Cell lines

Here we show a brief description of the cell lines chosen for this work. The cell lines were selected according to their status of their β-catenin regulation, estrogen receptor (**ER**), progesterone receptor (**PR**) and HER2 receptor (**HER2**).

MCF7: This cell line derives from a metastatic breast cancer. It has been widely used in the studies of cancer response to hormones, as it possesses **ER** and **PR**[49]. Aside from it, this cell line shows high levels of β-catenin[15].

T-47D: This cell line derives from an invasive ductal breast carcinoma[50]. It presents **ER** and **PR** and is β-catenin positive, but it has been less studied[15].

MDA-MB-231: It is an epithelial, human breast cancer β-catenin positive cell line established for a metastatic mammary adenocarcinoma. It is one of the most commonly studied breast cancer cell lines. It is a triple negative breast cancer cell line, characterized by its aggressive behavior, diverse metastasis patterns and lack of targeted therapies[46,51]. It also shows high levels of β-catenin[15].

MDA-MB-453: This androgen responsive cell line derives from a metastatic breast carcinoma. Contrary to the others, this cell line barely shows levels of nuclear and intracellular β-catenin and it does have **HER2**[15,52].

In this work, we attempt to exploit the concept of synthetic lethality in order to find and describe new, more potent and selective inhibitors of the CK1ε protein, a promising and understudied target for chemotherapy, taking a step forward in cancer treatment.

HYPOTHESIS

CK1ε is a protein responsible for cell proliferation, playing a relevant role in cancer progression. CK1ε is overexpressed in diverse types of tumors. Given the fact that this protein is directly involved in cellular proliferation, its inhibition will result in a slowdown of tumor progression by the cease of said proliferative activity, making the research and application of CK1ε inhibitors a fruitful strategy in the treatment against cancer.

OBJECTIVES

The main purpose is to develop a procedure for the early pharmacological profiling of CK1ε inhibitors, to find a complementary strategy for cancer treatment. To achieve this goal, the specific objectives are considered:

1. Run a literature search to gather preexistent confirmed CK1ε inhibitors.
2. Generation of CK1ε knockdowns in order to find if the growth inhibition is due to off-target effects.
3. Evaluate CK1ε expression in wild and knockout cultures to validate the knockouts and the viability of the use of inhibitors).
4. Run cell line experiments looking for growth inhibition in response to CK1ε inhibition.
5. Perform CK1ε and CK1δ kinase assays in order to find the selectivity of the inhibitors.

MATERIALS AND METHODS

Reagents

Inhibitors **PF-4800567**, **PF-670462**, **PF-5006739** and **IC261** are commercially available, while **GSD0054** is available at our research group. CK1ε antibodies, CK1ε and CK1δ proteins will be acquired from commercial suppliers. If not possible, both proteins can be obtained through the cloning of the corresponding gene followed by purification of the expressed proteins. Vectastain Elite ABC Kit can be acquired from Vector laboratories. Kinase-Glo Plus Assay and CellTiter-Glo Assay might be acquired from Promega. Remaining reagents will be commercially acquired, but can be produced in the laboratory.

Cell lines and culture

Breast cancer cell lines **MCF7**, **MDA-MB-231**, **MDA-MB-453** an d **T-47D** (available at the American Type Culture Collection)**,** will be cultured in DMEM (Dulbecco's modified Eagle's medium) with 2 mM L-Glu, sodium pyruvate and 10% inactivated fetal bovine serum, 100 U/mL of penicillin G and 0.1 mg/mL of streptomycin at 37ºC, 95% humidified atmosphere of 5% CO_2[15,47]. Cells are maintained in 100 mm culture dishes in culture medium (5 mL) at low passage for two weeks[39].

Knockdown generation

Cells will be trypsinized and resuspended in medium. Cell number will be measured using a Moxi Z cell counter, allowing to dilute the suspension until a quantity of 10^4 cells per well is achieved. Then, cells will be seeded in a 96 well microtiter plate. 24 hours after the plating, the lentiviral stock will be thawed on ice, and hexadimethrine bromide will be added to the cells at a final concentration of 8 µg/mL. Next, 0.01 mL of the lentiviral solution containing 10^5 transduction units will be added to each well. 24 hours later, the medium containing viral particles shall be removed, replacing it with fresh, prewarmed medium. 24 hours later, the old medium will be discarded, replacing it with fresh medium containing additionally 10 µg/mL of puromycin. Resulting cells shall be used in the immunohistochemistry and antiproliferative assays.

Immunohistochemistry assay

Two fundamental groups will be tagged: shRNA (+) and shRNA (-), being the knockdown and wild groups, respectively. Inside each group, every cell line will be treated in three other subgroups, each one having a distinct antibody. The reaction may be visualized using a peroxidase-based Vectastain Elite ABC Kit with diaminobenzidine as a chromogen. Primary antibodies used will be monoclonal mouse anti-human CKIε. The steps will be taken as follows (Fig. 6).

Sections will be washed in PBS for 5 minutes. Then, they will be incubated for 20 minutes in blocking serum (contained in the kit). Excess serum must be tipped off. After it, Primary antibodies (anti-CK1ε) will be added, diluted with 2.5% blocking serum. Then again, sections will be washed in PBS for 5 minutes. Once finished, addition of the biotinylated secondary antibody (Reagent A, in the kit) will take place. Next, the avidin/biotinylated enzyme complex (Reagent B) will be added. After washing for the last time in buffer, sections will be incubated into a peroxidase solution for 10 minutes. Finally, preparations will be cleared in tap water. Few drops of diaminobenzidine will be added as a chromogen, and preparations shall be mounted for its observation at microscope.

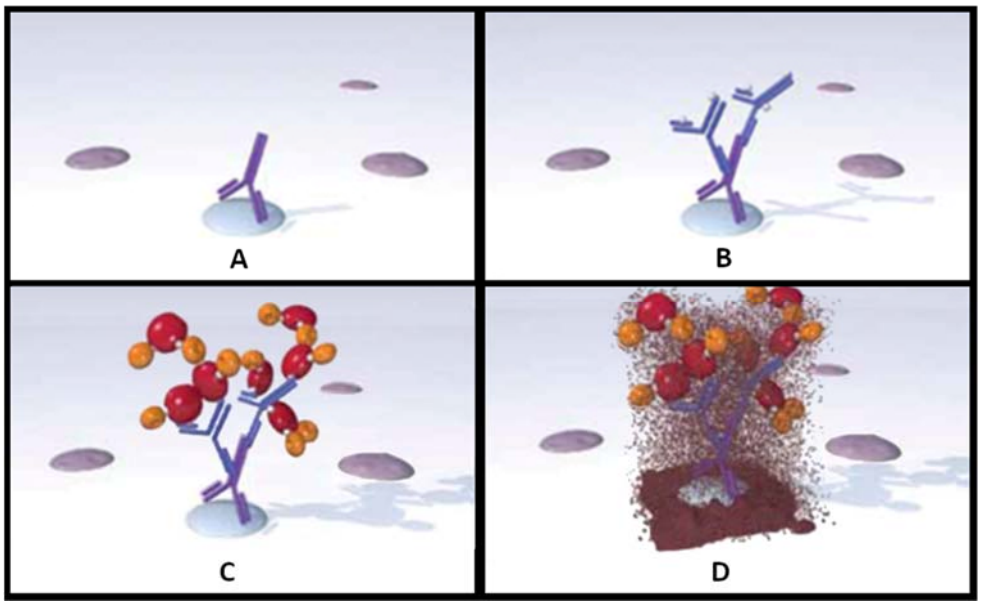

Figure 6. Depiction of the fundamental steps involving the immunochemistry assay. (A) The primary antibody binds to its target, CK1. (B) The secondary biotinylated antibody binds to the primary antibody. (C) The ABC complex bind to the secondary target, exploiting the affinity of avidin towards biotin. (D) The peroxidase binds to the avidin/biotinylated enzyme complex.

Antiproliferative assay

a. Drug addition

Non transducted shRNA (-) cells will be trypsinized and resuspended in medium. Then, said cells will be plated into a 96 well microtiter plate. Each plate will count with 10^4 cells, counted with the Moxi Z counter. 24 hours later, they will be treated each drug. Pure compounds will be dissolved in DMSO at 400 times the desired final maximum test concentration. Control cell samples will be exposed to an equivalent concentration of DMSO (0.25% v/v) as a negative control. All compounds will be tested at different doses (0.1-100µM) and exposure times (e.g. 24, 48, and 72 h), having a control (-) group with no drug.

b. Cell viability assay

Cell viability will be measured in both shRNA (+), drug-treated and control shRNA (-) groups using a CellTiter-Glo Assay, adding a single 0.1 mL reagent to cultured cells and using an orbital shaker for 2 minutes to homogeneously mix the reagent across the solution[15]. This will result in cell lysis and the generation of a luminescent signal proportional to the amount of ATP present through the luciferase reaction (Fig. 7). The amount of ATP is directly proportional to the number of cells present in culture[53]. To stabilize the luminescence signal, the plates will be incubated for 10 minutes before the reading. The resulting luminescent output can be measured on a plate reader.

Figure 7. The luciferase reaction. Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of Mg2+, using ATP and molecular oxygen as substrate.

c. ATP curve

At the same time, an ATP curve will be generated. For this purpose, ATP will be added to culture medium, having a final volume of 0.1 mL and 10^{-10} moles of ATP (1 µM ATP). Then, two serial tenfold dilutions will be prepared ranging from 1 µM to 10 nM ATP. All three concentrations will be added the same the same volume of CellTiter-Glo reagent, and shaken, incubated and read in a multiplate reader.

Kinase assays

The CK1ε and CK1δ kinase assays are to be performed in a 50 µL volume in buffer containing 40 mM Tris, pH 7.5, 20 mM $MgCl_2$, 1 mM DTT, 10 µM ATP, and 42 µM peptide substrate, PLSRTLpSVASLPGL[8]. The final enzyme concentrations should be around 2 nM. Assays will be run in a panel format in the presence of 0.1-10 µL of CK1 inhibitor, with 4% DMSO. Negative control groups will have no kinase concentration. The reactions shall then be incubated for 60 min at 25ºC for CK1δ and 85 min at 25°C for CK1ε, followed by detection with the use of 50 µL of Kinase-Glo Plus reagent[54]. The resulting luminescent output can be measured on a plate reader.

IMPLEMENTATION

We looked for scholarly literature using the Pubmed, Pubchem and Google Scholar search engines. The main search terms used were: “CK1ε”, “CK1ε inhibition”, “breast cancer line”, “Per2 antiproliferative”, “Wnt-β antiproliferative”. The article selection process was oriented to the search of reported inhibitors and sensible breast cancer cell lines.

A knockdown version of every cell line will be generated by transduction using a Lentiviral-vector solution. Lentivirus contain a plasmid which has a DNA that can be transcripted into CK1ε-targeting shRNA. Puromycin will be added, killing non-transducted cells. Three days after, once shRNA has accumulated in them, surviving cells will be plated for the cell line experiments. Then, we will study the effects of the different compounds on knockdown and wild-type cell lines.

To evaluate CK1ε on knockdown and wild lines we will apply anti CKIε antibodies to portions of every culture. This will be followed by the application secondary biotinylated antibody, followed by an avidin complex and peroxidase. Always washing in PBS between applications. Lastly, a chromogen, diaminobenzidine will be added. After this the preparations will be mounted for its observation under the microscope.

Several breast cancer cell lines will be cultured for two weeks. After so, them, along with CK1ε knockdown cells, will be in a known amount plated and treated with different doses of each compound. The antiproliferative effect of these drugs will be evaluated by measuring the amount of ATP of each plate. A cell lysate will be required for the release of the intracellular content, followed by the addition of exogenous luciferase and luciferin for the measurement of total ATP, through the resulting luminescent output. Antiproliferative potency will be measured in terms of GI_{50}. An ATP curve will also be calculated using known ATP concentrations in order to calculate the amount of ATP from the readings. This curve will also be used in the kinase assay.

We will test the selectivity of each inhibitor towards both the ε and δ kinases applying several doses of each inhibitor to a solution basically containing the kinase, ATP and a peptidic substrate for the kinase. The final ATP concentration will be measured using a luciferase reaction. Resulting luminescent output will be quantified and the selectivity will be expressed in terms of IC_{50} ratios.

EXPECTED IMPACT

The bibliographic search afforded a total five CK1ε inhibitors, three dual and two selective, all included in this study. The number of inhibitors described in the literature is relatively low. Learning their reported characteristics, such as the affinity for both isoforms, remains fundamental for the formulation of mechanistic hypothesis.

The expression of the transducted plasmid inside the cells will result in gene silencing. Its transcription will result into a pre-shRNA that will be cleaved by the RNA-induced silencing complex (RISC). RISC will load a fragment of the initial transcript that will use to "search" a complementary sequence, that will be the CK1ε mRNA, and cleave it, silencing gene expression and impeding CK1ε protein translation. For that reason, this assay will serve to detect potential off-target of the compounds, as its effects cannot on these cells be due to CK1ε inhibition, as it is missing in the cell. If this assay fails, it will be perceived on the next assay, while evaluating CK1ε expression. An alternative procedure for the *CSNK1E* gene will be the knock-out by the deletion of exon 4 using CRISPR/Cas-9 technology for its application and the NCBI gene database for its design.

To make sure CK1ε expression is negative in the knockdown groups and positive in the wild ones, this immunohistochemical assay will take place. The primary antibodies shall bind to present CK1ε, and they shall bind to the biotinylated secondary antibodies. As the third component, the ABC complex, contains avidin, it will bind to the secondary antibody, which contains biotin, having both of them a very high affinity. Then, the peroxidase will bind to the newly formed complex, and oxidize the diaminobenzidine, which will turn its color to brown. This assay is designed to confirm the validity of the knockdown assay and the cell lines used for the experiments, in order to find out if the CK1ε levels are the expected. An alternative for this trial is to introduce a reporter gene, such as the GFP next to the promoter sequence of the *CSNK1E* and red it through flow cytometry.

Cell line experiments should provide an initial impression of the antiproliferative potency of each drug. The amount of ATP has a lineal relation to the number of viable cells, allowing us to estimate it from the cell lysate. ATP measure has the same fundament as in the previous assay, depending on the light emission from a luciferase reaction. The difference in number amount between treated and untreated groups will allow to evaluate the antiproliferative potency in term of GI_{50}.

The expected outcome mainly depends on the tested inhibitor.

- **IC261**: We expect that this inhibitor shows a great antiproliferative effect in all cell lines even at low doses, attributed to its affinity to tubulin.
- **PF-670462** & **PF-5006739**: It is expected to find a low antiproliferative effect of these two compounds on high doses due to their similarly low affinity to CK1ε.
- **PF-4800567**: Should probe to have the worst antiproliferative effect of all 5 inhibitors, as it has the smallest affinity to the protein.
- **GSD0054**: As the only inhibitor with high affinity and no described off-target effects, we expect this compound to show high antiproliferative effect.

Except for **IC261**, whose antiproliferative activity derives from off-target effects, the results might be affected by the cell line status of β-catenin and **ER**.

- **MDA-MB-453**: We expect it to show the slightest decrease in proliferation, as it lacks the **ER** and has low levels of β-catenin.
- **MDA-MB-231**: As it lacks **ER** but is β-catenin positive, we expect this cell line to be the second most affected.
- **MCF7** and **T-47D**: Both possess **ER** and high β-catenin levels. We therefore expect them to be the most sensible cell lines to CK1ε inhibitors.

This work should provide an initial description for the five used inhibitors, in terms of potency and selectivity towards each cancer cell line, any of both isoforms and other possible important cell substrates.

Additionally, it could be used for the early profiling of new-synthetized CK1ε inhibitors, in order to find more potent and specific compounds that can be used to implement into new chemotherapeutic strategies in the fight against cancer.

The kinase assay will allow us to evaluate the affinity of each drug towards each isoform. It is so because inhibition effect will decrease the rate of phosphorylation of the kinase to the peptidic substrate. This reaction requires ATP, so higher inhibition will result in higher final levels of ATP, as the kinase activity is totally or partially impaired. Therefore, we can assess compound affinity to both isoforms through the measure of final ATP. ATP is also used by the luciferase reaction, emitting a luminescent output directly proportional to the amount of ATP. This luminescence will give us the appropriate information and, thus, allow us establish the selectivity of the studied inhibitors. We expect to find a positive correlation between the drug selectivity to the epsilon isoform and its antiproliferative activity.

CONCLUSIONS

Chemical CK1ε inhibition is an understudied field, with two selective inhibitors and only one of them exerting selective cell killing capacity. This, along with CK1ε potential in cancer treatment as a synthetic lethal gene makes it a promising target for the development of novel chemotherapeutic strategies. In this work, we attempt to exploit the concept of synthetic lethality in order to find and profile new, more potent and selective inhibitors of the CK1ε protein, providing support in the fight against cancer.

CONCLUSIONES

La inhibición química de CK1ε es un campo poco estudiado, teniendo dos inhibidores selectivos, de los cuales solo uno de ellos posee la capacidad de eliminar células de forma selectiva. Esto, junto con el potencial de CK1ε en el tratamiento del cáncer como gen letal sintético, lo hacen un objetivo prometedor para el desarrollo de nuevas estrategias quimioterapéuticas. En este trabajo, intentamos explotar el concepto de letalidad sintética para descubrir y describir inhibidores de la proteína CK1ε nuevos, más potentes y selectivos, aportando un apoyo en la lucha contra el cáncer.

REFERENCES

1. Forouzanfar, M. H. *et al.* Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet* **388**, 1659–1724 (2016).
2. Bray, F. *et al.* Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA. Cancer J. Clin.* **68**, 394—424 (2018).
3. McLornan, D. P., List, A. & Mufti, G. J. Applying synthetic lethality for the selective targeting of cancer. *New England Journal of Medicine* vol. 371 1725–1735 (2014).
4. Gupta, A., Ahmad, A., Dar, A. I. & Khan, R. Synthetic Lethality: From Research to Precision Cancer Nanomedicine. *Curr. Cancer Drug Targets* **18**, 337–346 (2018).
5. Capra, M. *et al.* Frequent alterations in the expression of serine/threonine kinases in human cancers. *Cancer Res.* **66**, 8147–8154 (2006).
6. Hanks, S. K. & Hunter, T. The Protein Kinase FactsBook. in *The Protein Kinase FactsBook* 7–47 (Academic Press, 1995). doi:10.1016/b978-012324719-3/50003-0.
7. Edelman, A. M., Blumenthal, D. K. & Krebs, E. G. *PROTEIN SERINE/THREONINE KINASES*. *Ann. Rev. Biochem* vol. 56 www.annualreviews.org (1987).
8. Flotow, H. *et al.* Phosphate groups as substrate determinants for casein kinase I action. *J. Biol. Chem.* **265**, 14264–14269 (1990).
9. Knippschild, U. *et al.* The CK1 family: Contribution to cellular stress response and its role in carcinogenesis. *Frontiers in Oncology* vol. 4 MAY 96 (2014).
10. Takano, A., Hoe, H. S., Isojima, Y. & Nagai, K. Analysis of the expression, localization and activity of rat casein kinase Iε-3. *Neuroreport* **15**, 1461–1464 (2004).
11. Schittek, B. & Sinnberg, T. Biological functions of casein kinase 1 isoforms and putative roles in tumorigenesis. *Molecular Cancer* vol. 13 1–14 (2014).
12. Utz, A. C. *et al.* Analysis of cell type-specific expression of CK1ε in various tissues of young adult BALB/c mice and in mammary tumors of SV40 T-Ag-transgenic mice. *J. Histochem. Cytochem.* **58**, 1–15 (2010).
13. Knippschild, U. *et al.* The casein kinase 1 family: Participation in multiple cellular processes in eukaryotes. *Cellular Signalling* vol. 17 675–689 (2005).
14. Fish, K. J., Cegielska, A., Getman, M. E., Landes, G. M. & Virshup, D. M. Isolation and characterization of human casein kinase Iϵ (CKI), a novel member of the CKI gene family. *J. Biol. Chem.* **270**, 14875–14883 (1995).
15. Kim, S. Y. *et al.* CK1ε is required for breast cancers dependent on β-catenin activity. *PLoS One* **5**, e8979 (2010).
16. Gallego, M., Eide, E. J., Woolf, M. F., Virshup, D. M. & Forger, D. B. An opposite role for tau in circadian rhythms revealed by mathematical modeling. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 10618–10623 (2006).
17. Zhou, J. *et al.* The aberrant expression of rhythm genes affects the genome instability and regulates the cancer immunity in pan-cancer. *Cancer Med.* **9**, 1818–1829 (2020).
18. Blair, A. *et al.* Painting, firefighting, and shiftwork. *IARC Monogr. Eval. Carcinog.*

Risks to Humans **98**, 9–38 (2010).
19. Wild, C. P., Weiderpass, E. & Stewart, B. W. *World Cancer report 2020: Cancer Research for Cancer Precention*. *Cancer Control* vol. 199 (2020).
20. Gauger, M. A. & Sancar, A. Cryptochrome, circadian cycle, cell cycle checkpoints, and cancer. *Cancer Res.* **65**, 6828–6834 (2005).
21. Fu, L., Pelicano, H., Liu, J., Huang, P. & Lee, C. C. The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo. *Cell* **111**, 41–50 (2002).
22. Liu, H., Gong, X. & Yang, K. Overexpression of the clock gene Per2 suppresses oral squamous cell carcinoma progression by activating autophagy via the PI3K/AKT/mTOR pathway. *J. Cancer* **11**, 3655–3666 (2020).
23. Hua, H. *et al.* Circadian gene mPer2 overexpression induces cancer cell apoptosis. *Cancer Sci.* **97**, 589–596 (2006).
24. Miyazaki, K., Wakabayashi, M., Hara, Y. & Ishida, N. Tumor growth suppression in vivo by overexpression of the circadian component, PER2. *Genes to Cells* **15**, 351–358 (2010).
25. Sun, C. M. *et al.* Per2 inhibits K562 leukemia cell growth in vitro and in vivo through cell cycle arrest and apoptosis induction. *Pathol. Oncol. Res.* **16**, 403–411 (2010).
26. Hua, H. *et al.* Inhibition of tumorigenesis by intratumoral delivery of the circadian gene mPer2 in C57BL/6 mice. *Cancer Gene Ther.* **14**, 815–818 (2007).
27. Gery, S., Virk, R. K., Chumakov, K., Yu, A. & Koeffler, H. P. The clock gene Per2 links the circadian system to the estrogen receptor. *Oncogene* **26**, 7916–7920 (2007).
28. Ralph, M. R. & Menaker, M. A mutation of the circadian system in golden hamsters. *Science (80-.).* **241**, 1225–1227 (1988).
29. Lowrey, P. L. *et al.* Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. *Science (80-.).* **288**, 483–491 (2000).
30. Eide, E. J. *et al.* Control of Mammalian Circadian Rhythm by CKI -Regulated Proteasome-Mediated PER2 Degradation. *Mol. Cell. Biol.* **25**, 2795–2807 (2005).
31. Reppert, S. M. & Weaver, D. R. Molecular Analysis of Mammalian Circadian Rhythms. *Annu. Rev. Physiol.* **63**, 647–676 (2001).
32. Seok, W. S. & Stockwell, B. R. Inhibition of casein kinase 1-epsilon induces cancer-cell-selective, PERIOD2-dependent growth arrest. *Genome Biol.* **9**, R92 (2008).
33. Walton, K. M. *et al.* Selective inhibition of casein kinase 1ε minimally alters circadian clock period. *J. Pharmacol. Exp. Ther.* **330**, 430–439 (2009).
34. Sakanaka, C., Leong, P., Xu, L., Harrison, S. D. & Williams, L. T. Casein kinase Iε in the Wnt pathway: Regulation of β-catenin function. *Proc. Natl. Acad. Sci. U. S. A.* **96**, 12548–12552 (1999).
35. Price, M. A. CKI, there's more than one: casein kinase I family members in Wnt and Hedgehog signaling. *Genes Dev.* **20**, 399–410 (2006).
36. Swiatek, W. *et al.* Regulation of Casein Kinase Iε Activity by Wnt Signaling. *J. Biol. Chem.* **279**, 13011–13017 (2004).
37. Rodriguez, N. *et al.* Casein kinase I epsilon interacts with mitochondrial proteins for the growth and survival of human ovarian cancer cells. *EMBO Mol. Med.* **4**, 952–963 (2012).

38. Shin, S., Wolgamott, L., Roux, P. P. & Yoon, S. O. Casein kinase 1ε promotes cell proliferation by regulating mRNA translation. *Cancer Res.* **74**, 201–211 (2014).
39. Zuccotto, F., Ardini, E., Casale, E. & Angiolini, M. Through the "Gatekeeper Door": Exploiting the Active Kinase Conformation. *J. Med. Chem.* **53**, 2681–2694 (2010).
40. Modi, V. & Dunbrack, R. L. Defining a new nomenclature for the structures of active and inactive kinases. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 6818–6827 (2019).
41. Mashhoon, N. *et al.* Crystal structure of a conformation-selective casein kinase-1 inhibitor. *J. Biol. Chem.* **275**, 20052–20060 (2000).
42. Angell, R. M. *et al.* Biphenyl amide p38 kinase inhibitors 4: DFG-in and DFG-out binding modes. *Bioorg. Med. Chem. Lett.* **18**, 4433–4437 (2008).
43. Badura, L. *et al.* An inhibitor of casein kinase Iε induces phase delays in circadian rhythms under free-running and entrained conditions. *J. Pharmacol. Exp. Ther.* **322**, 730–738 (2007).
44. Cheong, J. K. *et al.* IC261 induces cell cycle arrest and apoptosis of human cancer cells via CK1δ/ε and Wnt/B-catenin independent inhibition of mitotic spindle formation. *Oncogene* **30**, 2558–2569 (2011).
45. Puerta, A. *et al.* Facts and myths of selective casein kinase 1ε einhibition. *J. Mol. Clin. Med.* **1**, 195–200 (2018).
46. Silveira-Dorta, G., Sousa, I. J., Fernandes, M. X., Martín, V. S. & Padrón, J. M. Synthesis and identification of unprecedented selective inhibitors of CK1ε. *Eur. J. Med. Chem.* **96**, 308–317 (2015).
47. Lagunes, I. *et al.* Differential mechanism of action of the CK1ε inhibitor GSD0054. *J. Mol. Clin. Med.* **1**, 77–84 (2018).
48. Wager, T. T. *et al.* Casein Kinase 1δ/ε Inhibitor PF-5006739 Attenuates Opioid Drug-Seeking Behavior. *ACS Chem. Neurosci.* **5**, 1253–1265 (2014).
49. Levenson, A. S. & Jordan, V. C. MCF-7: the first hormone-responsive breast cancer cell line. *Cancer Res.* **57**, 3071–3078 (1997).
50. T-47D ATCC ® HTB-133™ Homo sapiens mammary gland; derived f. https://www.lgcstandards-atcc.org/Products/All/HTB-133.aspx?geo_country=es.
51. Anders, C. & Carey, L. Understanding and Treating Triple-Negative Breast Cancer. *Oncology (Williston Park).* **22**, 1233–9; discussion 1239 (2008).
52. Holliday, D. L. & Speirs, V. Choosing the right cell line for breast cancer research. *Breast Cancer Res.* **13**, 215 (2011).
53. Crouch, S. P. M., Kozlowski, R., Slater, K. J. & Fletcher, J. The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *J. Immunol. Methods* **160**, 81–88 (1993).
54. Mente, S. *et al.* Ligand-protein interactions of selective casein kinase 1δ inhibitors. *J. Med. Chem.* **56**, 6819–6828 (2013).