THESIS

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Between fundamental and preclinical research in cancerology: evaluation of new CDK8/19 inhibitors

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List of Abbreviations

DNA : Desoxyribonucleic acid
RNA : Ribonucleic acid
RNAPII: RNA polymerase II
ATCC: American Type Culture Collection
ATP : Adenosine Triphosphate
AURKB: Aurora B kinase
CCD : Cell Cycle Division
CCNC: Cyclin-C
CDK : Cyclin-dependent kinase
CDK19: Cyclin-dependent kinase 19
CDK7 : Cyclin-dependent kinase 7
CDK8 : Cyclin-dependent kinase 8
CDK9 : Cyclin-dependent kinase 9
CDK8i: Cyclin-dependent kinase 8 inhibitor
CI_{50} : Concentration inhibitrice 50%
CPC : Chromosomal passenger Complex
CTD : C-terminal domain
CTRL : Control
DMSO: Dimethylsulfoxide
E2F1 : E2F transcription factor 1
EDTA : Ethylenediaminetetraacetic acid
ESFTs: Ewing's sarcoma family tumors
FBS : Fetal bovine serum
GAS : Interferon-Gamma-Activated-Sequence
GI50 : Growth inhibition 50%
H3 : Histone 3
INC : Notch intracellular
IFN-γ : Interferon γ
IgG : Immunoglobulin G
ISG : Interferon Stimulated Genes
Log : Logarithm
Med12: Mediator complex subunit 12
Med13: Mediator complex subunit 13
Mg2+ : Magnesium 2+
mARN: Messenger RNA
NaCl : Sodium chloride
NK : Natural killer
NSB : Non-signal bounding
p53 : Tumor suppressor p53
RIPA : Radioimmunoprecipitation assay buffer
RT : Room temperature
RPMI 1640: Medium developed at Roswell Park Memorial Institute
S : Serine
SDS PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Smad : SMA and Mad related proteins
STAT1: Signal transducer and activator, of transcription 1
TCF : T-cell factor
TFIIH: Transcription factor II Human
TOTB: Total bounding
Thr 3 : Threonine 3
Tris:Tri(hidroxymethyl)aminomethane
Z' : Z-prime

INTRODUCTION

The incidence of cancers increases over the life and it is related with the aging of human and animal populations. Therefore, the cancers have been placed in the category of pathologies known as "elderly subjects" (1). In reality, the multiplication of childhood cancers continues to grow in economically developed countries, which raises a lot of questions about their etiology (2). This multifactorial pathology affects many populations and its incidence worldwide is conditioned by the environment and lifestyle of the patients.

Through research, the discovery of new molecules and the implementation of new treatments are certainly effective, but still fall short of the final objective: patient survival and quality of life. Currently, the major challenge is to overcome the problems associated with the high toxicity of drugs inducing side effects, their lack of specificity and therefore resistance to chemotherapy treatments. To do this, it is essential that the therapies in development are as targeted as possible. The discovery of new ever more effective and specific treatments for tumor cells is therefore the main challenge for research teams.

Validation of the ability of a molecule to inhibit cancer proliferation involves several stages of testing, starting with the demonstration of activity at the cellular level. Various techniques organized according to a very precise methodology then make it possible to define the mechanisms of action and determine the genetic targets. One of the strategies used for the discovery of new antiproliferative compounds involves the investigation of the bioactive potential of a large number of chemical compounds (screening) in cancer cell lines. In this thesis, we will use: U2OS (osteosarcoma line), A673 and TC71 (Ewing sarcoma lines) cell lines.

The chemical compounds used in the experimental part of this thesis were synthesized in the Therapeutic Chemistry department of the CNIO (National Center of Investigations in Oncology in Madrid, Spain), then tested in the Biology Section of the Experimental Therapeutics Program department. Among the various compounds studied, the reference compound is a molecule derived from synthetic chemistry (ETP-827) selected from several analogous molecules, thanks to *in vitro* and *in vivo* tests previously carried out in the biology and animal experimentation.

Today, the goal for a compound with specific intrinsic activity is to precisely target the proliferation mechanisms of cancer cells. The current challenge for the various research laboratories is therefore to discover active ingredients capable of pharmaco-modulating genes that are essential for the survival and growth of the cancer cell. This thesis, based on

bibliographical and applied research, highlights certain genomic mechanisms involved in stopping the proliferation of cancer cells as well as their repercussions on biomarkers of interest.

Understanding the interest of the modulation of a target requires the study of its involvement in the cell cycle, and the most detailed analysis of its modulation. Different molecular biology techniques are then used successively to select a gene, then to study its regulation at the level of transcription and translation. These research steps carried out in the Biology and Chemistry laboratory of the CNIO's Experimental Therapeutics Program have led to structural improvements in several CDK8 inhibitors (CDK8i) represented by very specific small molecule targets. The synthesis of these compounds has resulted in CDK8i, the main CDK8 target also exerting an inhibitory action on the HASPIN gene. We will show throughout this thesis the advantage of this dual function of these new CDK8i.

The anti-tumor potential of the lead molecule "ETP-827" has been proven in colorectal cancer lines and confirmé by *in vivo* experiments in mouse models. The aim of the laboratory is now to show the efficacy of these molecules on other types of tumours. Thus, the experimental approach of this thesis aims, firstly, to demonstrate the sensitivity of cell lines derived from Osteosarcoma and Ewing's Sarcoma to these CDK8i, then to demonstrate the antiproliferative capacity of the ETP-827 molecule on these lines in order to constitute, eventually, *in vivo* models allowing to establish the proof of concept.

1. CONTEXT AND BIBLIOGRAPHIC REVIEW

1.1 THE CELL CYCLE

The process of cell division is defined by several phases, regulated by a large number of proteins intervening transiently in a defined order. It is a sequence of events during which the cell duplicates its contents and then divides in two. Classically, the cycle is divided into four phases: the G1 phase (from "Gap", interval), post-mitotic, a DNA synthesis S phase, a G2 phase and a mitotic M phase, itself subdivided into a series of morphological and biochemical steps. At the molecular level, the interphase comprises the G1, S, G2 phases and is the period during which cell growth and DNA replication take place in a coordinated manner in preparation for cell division. The cell therefore grows progressively throughout the interphase. This phenomenon of division allows the maintenance of the integrity of an organism in multicellular beings and the formation of a new organism in unicellular beings. (3).

1.1.1 Regulation and control of the cell cycle

For living eukaryotic organisms, it is essential that the different phases of the cell cycle are precisely coordinated. The phases follow a certain order, i.e. one phase must be correctly completed before the next phase starts. Errors in coordination can lead to chromosomal alterations; chromosomes or parts of chromosomes can be lost, rearranged or distributed unevenly between the two daughter cells. Normally, proteins such as CDK/cyclin complexes ensure quality control at each stage of the cycle and thus allow the cell cycle to progress. If an abnormality is detected, specific proteins block the complex at a control point and thus prevent the damaged cell from continuing its cycle. However, errors made by these verification systems can lead to the appearance of a cancerous cell. (4)

1.1.1.1 The cyclins and CDKs

One of the major discoveries in the field of cell cycle was the identification of a family of protein kinases, the cyclin-dependent kinases (CDKs). These serine/threonine kinases play an essential role in the initiation, control and harmonious succession of the different phases of the cycle. The nomenclature of these CDKs has recently been updated and there are currently 20 proteins in this family: from CDK1 to CDK20 (5). The activation of these CDKs requires the form of a complex between a catalytic subunit (CDK) and a regulatory subunit (cyclin). CDKs, depending on their involvement in cell division and transcription, can be divided into two groups (Figure 1). The first group includes CDKs which, by binding to multiple cyclins, can regulate the cell cycle. The second group includes CDKs that are activated by a single cyclin and involved in the regulation of genetic transcription. The number of CDKs has increased steadily during evolution; humans currently have 20 CDKs and 29 cyclins. (6).

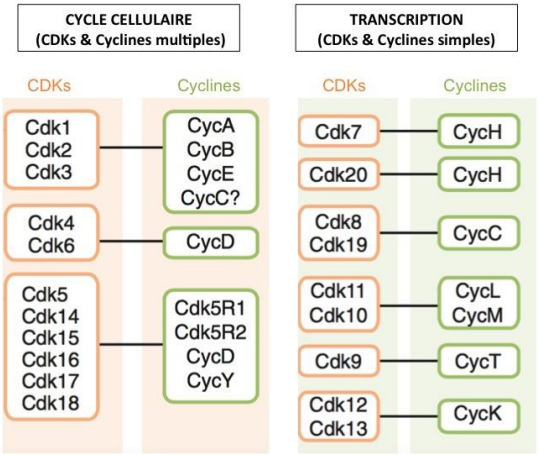

Figure 1. Interactions between Cyclins & CDKs at level of the cell cycle & transcription (5)(6)

The cyclin family includes about 30 proteins with a mass ranging from 35 to 90kDa. These proteins are structurally defined by the presence of a "cyclin box", a domain of about 100 amino acid residues forming a five-helix stack α. Most cyclins have two cyclin boxes, one box at the N-terminal end for binding to CDKs, and one at the C-terminal end necessary for cyclin folding. This cyclin box is found in other molecules such as retinoblastoma protein (Rb), TFIIB transcription factor and Cables (CDK5 and ABL1 enzyme substrate 1), which do not act as CDK activators. In general, cyclins have less sequence similarity than CDKs. The cyclin family contains approximately 29 proteins in humans, grouped into 16 subfamilies and three major groups: group I or "cyclin B" group (A-, B-, D-, E-, F-, G, J, I and O); group II or "cyclin Y" group - partner of the Cdk5 subfamily; and group III or "cyclin C" group: C-, H-, K-, L- and T- (main transcription CDKs partners). (6,7).

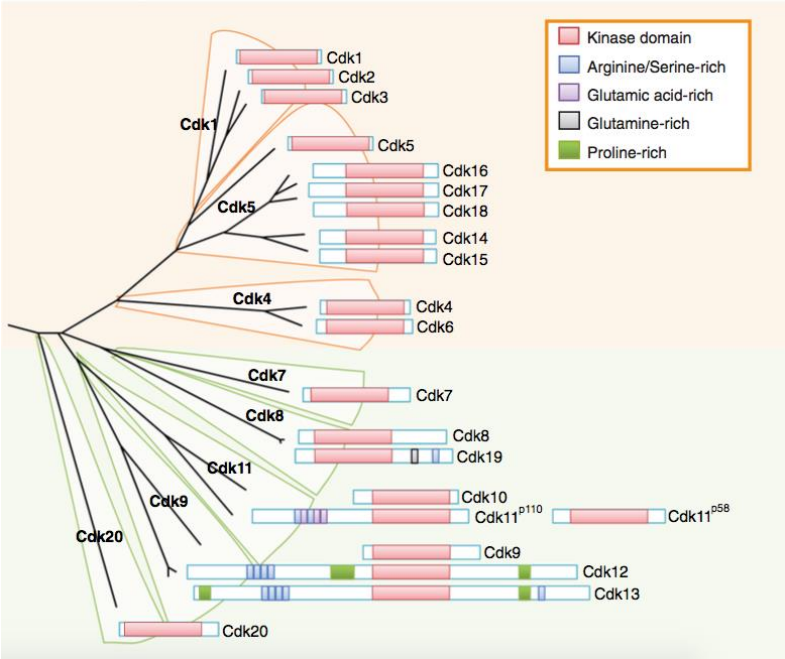

Figure 2. Evolution of the CDKs subfamilies in mammals (8).

The names of the different CDK subfamilies involved in the cell cycle (orange) or transcription (blue) are written in bold. The structural domains are represented for each protein kinase (see legend); this is for example the case for the conserved domain of protein kinases shown in red. Human cells contain 2 distinct genes, CdK11A and Cdk11B, each encoding a relatively long isoform, Cdk11p110, and a short protein, Cdk11p58 generated by an internal binding site of the ribosome. This phylogenetic tree is based on the comparison of the domains of human kinase (8,9).

Evolutionary studies suggest that CDKs divide into 8 subfamilies: CDK1, CDK4 and CDK5 (for CDKs related to the cell cycle) and CDK7, CDK8, CDK9, CDK11 and CDK20 (for CDKs related to genetic transcription) (6,10) (Figure 2). In both mammals and yeast, CDK1 is the only CDK essential for the cell cycle, while CDK2 and CDK3 are not

essential. (11,12). Unlike CDKs involved in the cell cycle, transcriptional CDKs are more conserved in terms of sequence and function. For example, CDK7, a subunit of the transcription factor TFIIH, is involved in initiating transcription by phosphorylation of the Ser5 residue of RNA polymerase II (RNAAPII) at the C-terminal domain of the promoter genes. CDK7 is also capable of phosphorylation and activation of other CDKs, acting as a CAK, CDK-activating kinase. CDK8 and CDK19 are enzymatic components of the Mediator Complex, involved in the regulation of ARNPII during transcription.

Unlike cell cycle-bound CDKs, the cyclin subunits of transcriptional CDKs do not exhibit significant oscillations during the cell cycle, so transcriptional CDKs are regulated by protein-protein interactions or other mechanisms. Transcriptional CDKs may have emerged after cell cycle-related CDKs and have diversified due to increased transcription complexity. (13).

1.1.1.2 Overview of the CDKs

Most CDKs function within the nucleus, some are attached to the cell membrane and few have cytoplasmic activity. (Figure 3).

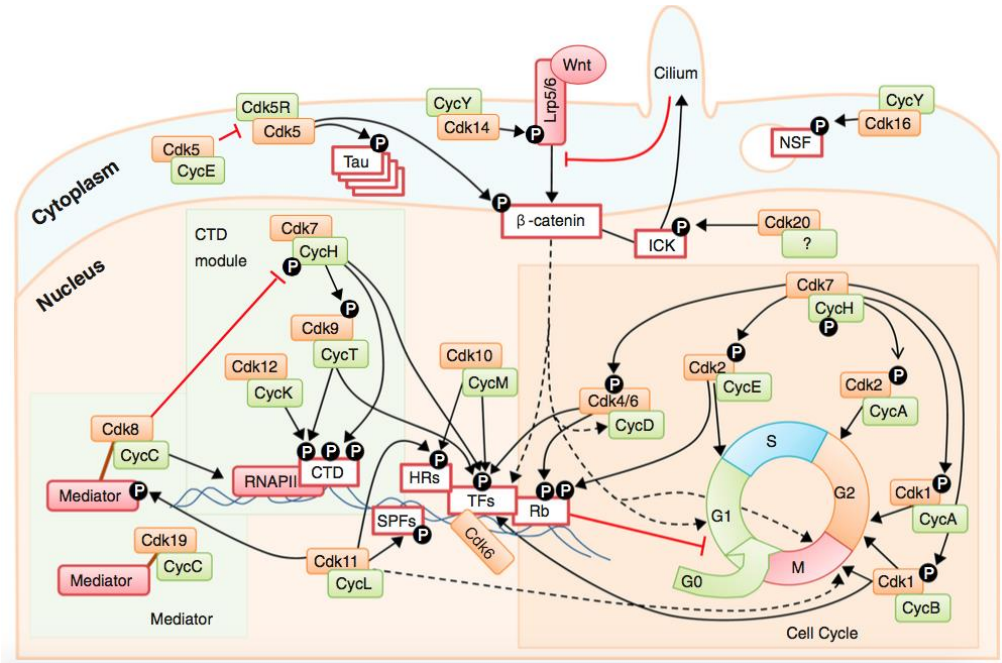

Figure 3. Overview of cyclin-dependent kinases (CDKs) & their functions within the cell (8). Each CDK (framed in orange) is presented in the form of a complex with its main partner (framed in green). For clarity, the author has depicted only a few substrates.

The CDKs involved in the cell cycle (CDK4, CDK6, CDK2 and CDK1) regulate the transition of the different phases of the cell division cycle. These activities are at least partially mediated by the control of several transcription factors (TFS) or regulatory

elements such as retinoblastoma protein (Rb). CDK10 and CDK11 also control transcription by phosphorylation of TFs, hormone receptors and associated regulators (HRs), or via splicing factors (SPFs). Cdk7, Cdk9 and Cdk12 directly phosphorylate the C-terminal domain (CTD) of RNA polymerase II (RNAAPII), thereby modulating the different phases of transcription. The Mediator complex is very specifically regulated by CDK8 or its paralogue CDK19. CDK7 functions as an activating kinase, such as CAK, by directly phosphorylating several of the aforementioned CDKs. Cdk5 displays various functions within the cell but is best known for its control of neuronal proteins such as Tau. Members of the CDK14 subfamily, such as CDK14 itself or CDK16, are activated at the membrane level by Y-cyclin and also participate in several signalling pathways, such as the Wnt pathway or signal transduction in the primary cilia. It is important to note that, for the sake of clarity, many interactions between CDKs and other cellular partners, substrates or processes have not been represented in Figure 3. For example, Cdk1 can bind to other cyclins and phosphorylate more than 100 substrates upon entry into mitosis, which are not shown in the figure.

1.2 THE CARCINOGENESIS

1.2.1 Definition of the cancer

The development of cancer is closely linked to cell proliferation. The organisms concerned by this pathology are multicellular eukaryotes with so-called "renewable" tissues. Indeed, the balance between the different cell populations and the continuous cell proliferation generates permanent risks of DNA alterations. (14). Cancer is a disease characterized by an uncontrolled proliferation of cells within normal body tissue. These cells are all derived from the same clone called the "cancer initiating cell", which has acquired certain characteristics that allow it to divide indefinitely and form metastases. Typically, there is a progression to a precancerous lesion and then to a malignant tumour. These changes result from interactions between the subject's own genetic factors and external agents called carcinogens that can be classified into several categories. Aging is another fundamental factor in the development of cancer. This is probably due to the accumulation of risks of specific cancers throughout life, combined with the fact that repair mechanisms generally tend to become less effective with age. (15)

1.2.2 General characteristics of the cancer cell

Tumour cells share common properties that differentiate them from "normal" cells. (16). First of all, tumour cells are independent of signals from the environment. Indeed, they are independent of proliferation signals, insensitive to antiproliferative signals and resistant to apoptosis. Second, these cells possess unlimited replication potential (loss of senescence), sustained angiogenesis capacity, and capacity for invasion and metastatic spread. These six fundamental characteristics have been complemented by the discovery of two new functions: the ability to deregulate energy metabolism and to avoid destruction mechanisms by immune cells.

1.2.3 Behavior of the cancer cells in vitro

Unlike normal cells, cancer cells can proliferate and therefore be cultured indefinitely. This immortality would be due, on the one hand, to the failure of cell cycle control systems, and on the other hand, to the presence of an enzyme specific to cancer cells: telomerase.

In vitro, the proliferation of normal human embryonic cells does not exceed an average of 50 divisions. At the end of each chromosome, at the level of all cells, are the telomeres, consisting of repeating TTAGGG patterns (Figure 4). These DNA fragments will shorten during each S-phase (replication). After a certain number of divisions (about 50), telomeres that have become too short will send a message inducing the cells to senesce. If the cells do not senesce, a final shortening of the telomeres will trigger a critical phase called "chromosomal chaos", fatal to the normal cell. During this phase, the DNA repair system (which includes P53 and Rb) is activated and leads to permanent inhibition of the cell cycle. Only two types of lines, germ lines and stem cells, can multiply without limit. Indeed, they express a particular protein: telomerase, which has reverse transcriptase activity, i.e. it is capable of de novo synthesis of the telomeric TTAGGG motifs constituting the telomeres, and therefore maintains the number of repeats allowing the cells to multiply. Cancer cells adapt and defeat systems that limit their number of cell cycles by restoring their destroyed telomeric DNA fragments at each cycle round.

Nearly 90% of them express telomerase, which allows them to escape senescence and crisis to acquire immortality. In addition, P53 and Rb proteins are inactivated in many cancer cells.

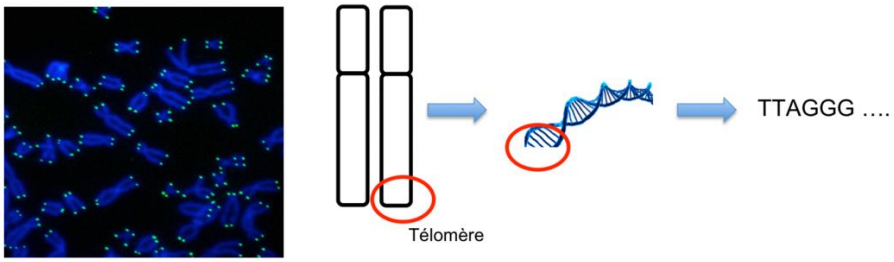

Figure 4. General mechanism of the enzymatic action of telomerase.
Human telomeres seen in fluorescence microscopy. Telomeres contain repetitive DNA sequences that cover the ends of chromosomes (T. de Lange, The Rockefeller University, 2014).

1.2.4 Therapeutic perspectives

Cancers have long been considered as pathologies whose initiation and evolution depended solely on the acquisition of genetic and epigenetic abnormalities by the affected cells. However, work carried out over the last ten years has shown that this is a restrictive view of these pathologies and has highlighted the crucial role played by the complex interactions between cancer cells and their microenvironment. Thus, multiple therapeutic perspectives have been developed in order to combat the development of cancer pathology at several levels (Figure 5).

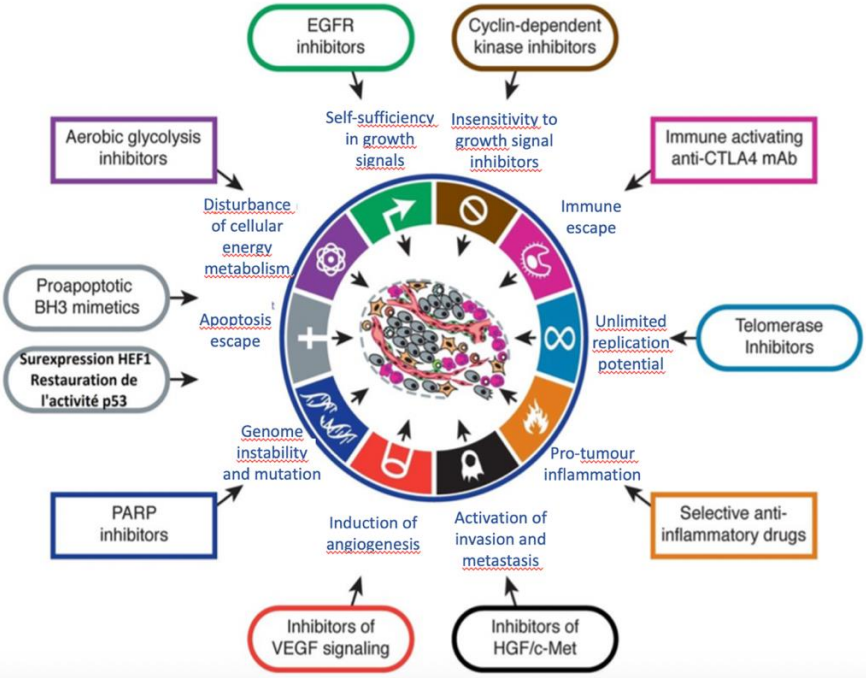

Figure 5. Targeted therapeutic perspectives based on the characteristics of tumorigenesis and the tumor microenvironment (adapted from Hanahan & Weinberg, 2011 and B. Rousseau, 2015) (17)

Medicines used in targeted therapy act by intervening at a specific level of tumor cell development and in particular, in the context of signal transduction. The so-called tyrosine kinase pathway is the best known to date. This pathway can be blocked by

monoclonal antibodies (Mab) or enzyme inhibitors (inib). These drugs can block the growth of cancer cells, starve the tumour by preventing angiogenesis or direct the body's immune responses against these cancer cells.

Finally, in order to develop a long-term "à la carte" therapy, it is essential to continue studying new molecular targets in all types of cancer. Indeed, the various oncogenes and tumour suppressor genes are modified within each tumour and therefore for each patient. Characterization of the genetic profile of tumors is a tool for identifying mutated signaling pathways in cancer cells and thus targeting the corresponding gene(s).

1.3 THE CDK8 INHIBITORS

The therapeutic goal for CDK inhibitors is to block the molecular pathways pushing tumor cells into the cell cycle, or to act at monitoring points. As cyclin/CDK complexes are overactivated in the majority of cancers, one of the principles of the development of CDK inhibitors is based on the fact that the proliferation and survival of tumor cells depend on the overactivity of these CDKs.

CDK8 is a CDK involved in transcription regulation. A fraction of CDK8 is associated with the Human Mediator Complex itself composed of 25-30 proteins arranged in several modules. This complex acts as a bridge between DNA-binding transcription factors and gene-specific transcription factors. It is a general transcription factor required for the regulation of RNA polymerase II (ARNPII) activity. (18). CDK8 and its paralogue CDK19 are Cyclin C-dependent enzymes (CCNC) which, in partnership with MED 12 and MED 13, form, as described above, a module of the Mediator Complex consisting of 4 sub-units called the "CDK8 Module". This module can be reversibly associated with the Mediator Complex and regulate the initiation of transcription and re-priming by controlling the RNAPII-Mediator interactions. In order to understand the specific functions of CDK8, it is important to distinguish between the activities of the "CDK8 module" and the kinase activities of CDK8 which are two major mechanisms involved in transcription repression.

First of all, the CDK8 module induces the conformational change of the Mediator complex, which physically disrupts the interaction with the Pol II RNA, and blocks the following transcription steps. Second, due to its kinase activity, CDK8 phosphoryl different substrates which include several general transcription factors (19) : ARNPII in the C-ter (Ser 2 and Ser 5), MED2 and MED13 domains, cyclin H, a subunit of the transcription initiation factor (TFIIH) and certain transactivators, such as Intracellular Notch (INC) and E2F1 . As a result, CDK8 has been described primarily as a

transcriptional repressor. However, current research is providing new information about the different roles of CDK8 in gene activation. Indeed, CDK8 can also positively regulate transcription: by direct phosphorylation of p53, SMAD proteins or by facilitating the assembly of the Pol II elongation complex. Moreover, CDK8 has been described as a co-activator in several signalling pathways with a major biomedical impact such as the Wnt/ß-catenin or TGFß (20). Furthermore, the involvement of CDK8 in the phosphorylation of STAT1 at the Serine 727 residue (SER727) makes this phosphoprotein a biomarker of choice for assessing CDK8 activity in vitro and in vivo. In sum, the various CDK8 activities highlight the dual function of this protein, which can act as an activator or co-repressor of transcription.

Overexpression of the CDK8 gene is associated with increased mortality in patients with melanocarcinoma, breast, colon, ovarian, gastric (21) and colorectal (22). CDK8 has been identified as an oncogene in colorectal cancers; it is located at the 13q12.13 level, a region where the copy number is amplified very regularly in this type of cancer. The transcription factor E2F1 is phosphorylated by CDK8 to dissipate its repression mediated by the ß-catenin/TCF signalling pathway. Indeed, E2F1 controls hundreds of genes involved in cell growth and proliferation as activators or repressors of the transcription (23). Thus, the link between carcinogenesis, kinase activity and CDK8 overexpression that are associated with high morbidity in colon cancer can be understood. (24). The function and role of CDK19, the analogue of CDK8 with an amino acid sequence very close to that of CDK8, is less well explored. Both CDK8 and CDK19 are associated with similar Mediator complexes. It has been proposed that CDK8 and CDK19 play opposite roles, but these observations may well be the result of compensatory overexpression of the paralogue (CDK19) in specific "knockdown" cells. The mutual exclusivity of CDK8 and CDK19 within the mediator complex as well as in cell expression models has raised important questions about the choice of preferential selectivity of the inhibitors under study. (25,26).

1.4 THE HASPIN GENE

Some of the Cdk8 inhibitors used in the second part of this thesis also have an effect on the HASPIN (Haploid germ cell-specific nuclear protein kinase) gene, a member of the serine/threonine kinase family. HASPIN is required at the time of mitosis during metaphase to ensure correct chromosome alignment. Thus, in order to regulate the course of mitosis, HASPIN phosphorylates histone H3 at the "threonine 3" residue (27), thus

recruiting the Aurora B kinase (AURKB) and the Passenger Chromosome Complex (CPC) to control microtubule assembly at the kinetochore level (Figure 6). The phosphorylation of Histone 3 at the level of Threonine 3 (P-H3T3) is a biomarker allowing the evaluation of HASPIN activity; this phosphoprotein will therefore be used as such in the experimental part of this thesis.

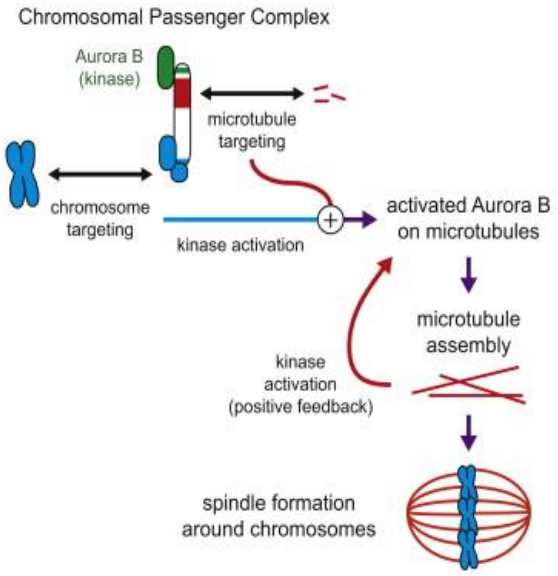

Figure 6. Detection of chromosomes and microtubules by the Chromosomal Passenger Complex (CPC) (28)

2. SCIENTIFIC OBJECTIVES OF THE INVESTIGATION: EVALUATION OF CDK8 INHIBITORS IN OSTEOSARCOMA AND EWING'S SARCOMA CELL LINES

Currently, cancer therapies, especially chemotherapy, are linked to many side effects. As described in the previous section (see 1.3. CDK8 inhibitors), Cdk8 inhibitors have emerged as a new class of small targeted molecular agents with great therapeutic potential based on *in vitro* and *in vivo* studies in various types of cancer. Research at the Experimental Therapeutics Laboratory of the CNIO in Madrid is focused on the development of these new compounds. The project underway for the various research teams is aimed at developing CDK8 inhibitors with a structure that would allow better behaviour in terms of selectivity in order to reduce the side effects linked to treatment. Initially, the aim was to obtain a small molecule with preferential selectivity for CDK8 over CDK19. However, recent studies have shown that the binding affinity for CDK8 and CDK19 is highly correlated. (29). As a result, Cycline C/CDK8 and CDK19 are considered to be a complex with a dual functionality that must be kept in mind during development.

The ultimate goal is to obtain a treatment completely based on small molecules, or at least to combine these agents with chemotherapy and/or radiotherapy with the aim of reducing the doses and side effects of the different therapies.

Given the encouraging research previously conducted in the biology laboratory on cell lines derived from colorectal cancers, we decided to work in this study on cell lines derived from Ewing's osteosarcomas and sarcomas, both of which are transcription-dependent cancers. Indeed, primary malignant bone tumors such as Ewing's osteosarcomas and sarcomas (ESFTs) are fatal diseases affecting children and young adults. Their complex etiologies leave room for the perpetual discovery of new mechanisms and thus new treatments. Overexpression of the transcription factor Runx2 has recently been implicated in the complex pathogenesis of osteosarcomas and is also correlated with the development of metastases and low response rate to chemotherapy. (30). With regard to ESTs, recent studies have shown that the native Ewing's Sarcoma Protein (EWS) and its partners are directly involved in the synthesis of mRNA and the splicing phenomenon in ESFTs. (31).

Previous work carried out in the laboratory has led to the discovery of new small molecule CDK8/CDK19 inhibitors that have shown antiproliferative activity on several cell lines; in particular on hematological and colon cancer cell lines. Here, the study described was designed to generate data on the ability to inhibit these new compounds on several target proteins. The objective is to evaluate the effect of these compounds on the proliferation (GI50) of several cell lines derived from Ewing's sarcoma and osteosarcoma. Then, to carry out treatments over a longer term with the most powerful compound (ETP-827) through "Colony Formation" and biomarker modulation assays.

3. MATERIALS AND METHODS

3.1 THE CELL LINES

3.1.1 The U2OS cell line

The U2OS cell line (Figure 7) established in 1964 is listed by the *American Type Culture Collection* (ATCC), number HTB-96. It is a line derived from a bone tumour collected from the tibia of a 15-year-old patient with osteosarcoma.

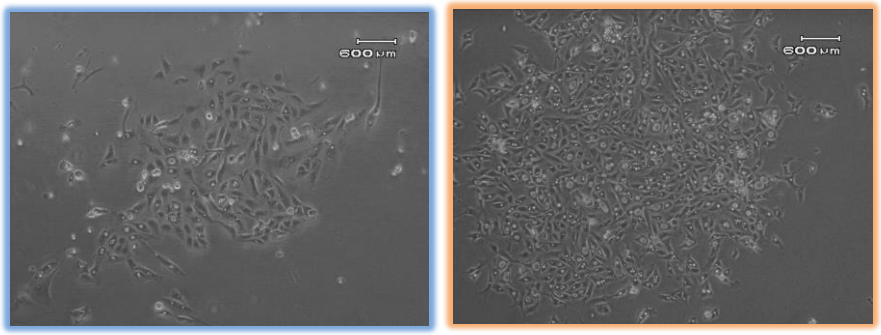

Figure 7. Microscopic observation of the growing U2OS line in RPMI medium supplemented with serum and antibiotics fixed on a solid support
In blue, the cell density is low (magnification x 40). In orange, the cell density is high (magnification x20).

3.1.2 The A673 cell line

The A673 cell line (Figure 8) established in 1973 is listed by the *American Type Culture Collection* (ATCC), number CRL-1598. It is a line derived from a solid muscle tumour collected from a 15-year-old Ewing's Sarcoma patient.

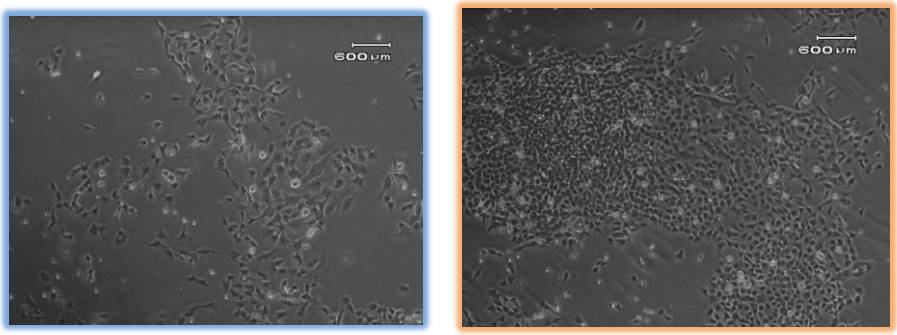

Figure 8. Microscopic observation of the growing line A673 in RPMI medium supplemented with serum and antibiotics fixed on a solid support
In blue, the cell density is low (magnification x 40). In orange, the cell density is high (magnification x20).

3.1.3 The TC71 cell line

The TC71 cell line (Figure 9) was established in 1981. It is a line derived from a bone tumour taken from the humerus of a 22-year-old patient with Ewing's Sarcoma.

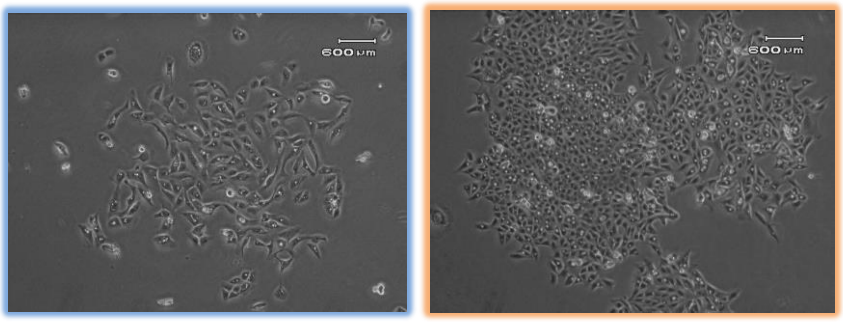

Figure 9. Microscopic observation of the growing TC71 line in RPMI medium supplemented with serum and antibiotics fixed on a solid support
In blue, the cell density is low (magnification x 40). In orange, the cell density is high (x20).

3.1.4 Maintenance of the cell lines

The three cell lines are grown in a Rosewel Park Memorial Institute (RPMI 1640, Sigma®) medium supplemented with 10% fetal bovine serum (FBS, Sigma®), 0.5% Fungizone (Gibco®) and 1% antibiotics (penicillin and streptomycin, (Gibco®). The cells are confined in flasks with filter caps (Falcon®). FBS is the most complex component because it contains growth factors, hormones, and everything else the blood can contain except for figurative elements, clotting factors, and complement. Cells in culture are incubated at 37°C under a controlled atmosphere with 5% CO2 and 95% humidity. These cells are adherent to the support and must therefore be detached with trypsin before being transplanted (approximately every 72 hours). Before each subculturing, the cells are rinsed with PBS (5mL) and then contacted with 2 mL tryspin for 5 min. The action of trypsin is stopped by adding 5-8mL of culture medium. The cell clusters are fractionated with a sterile syringe by aspiration/rejection and part of the cell suspension is transferred to a new flask. Dilution is then carried out so that there are fewer cells in the flask. In order to preserve the cell line and to have a stock of cells available for future experiments, the cells can be frozen in culture medium supplemented with 5% DMSO. Moreover, all experiments are performed with cultures that do not exceed 30-35 transplantatings.

3.2 METHODS

3.2.1 Cell viability assay

The antiproliferative activity is determined by evaluating the concentration of the compound inhibiting 50% of cell growth compared to a control grown under the same conditions in the absence of the compound studied. This simple and rapid test makes it possible to carry out a rapid selection of molecules or compounds having an activity capable of limiting or stopping the growth of cancer cells. This test is carried out in a 96-well microplate (Becton Dickinson Falcon Microplates) where 5000 cells are planted in each well in a volume of 200 µL. For each test, a stock solution of the test compound is prepared at a concentration of 10 mM in DMSO in order to facilitate its use. The final concentration of DMSO during the test must not exceed 1% (concentration which has no effect on cell growth).

From these stock solutions, several dilutions are made in a reference plate with a wide range of concentrations. The volume of solution taken from this plate (2µL) is then automatically transferred (Beckman Coulter, Biomek FX-96 Channel Disposable Tip

Pipetting Head) into the plate containing the cells and the culture medium (200µL) allowing the final dilution to be performed and thus obtaining the required final concentration of each compound. For each test, each concentration is carried out in duplicate. (Figure 10). On the microplate, four ranges of controls have been kept: two negative controls containing culture medium without cells or compounds (CTRL -; DMSO -); a positive control containing culture medium and cells (CTRL +); a control containing culture medium without cells or compounds but in the presence of DMSO (DMSO +).

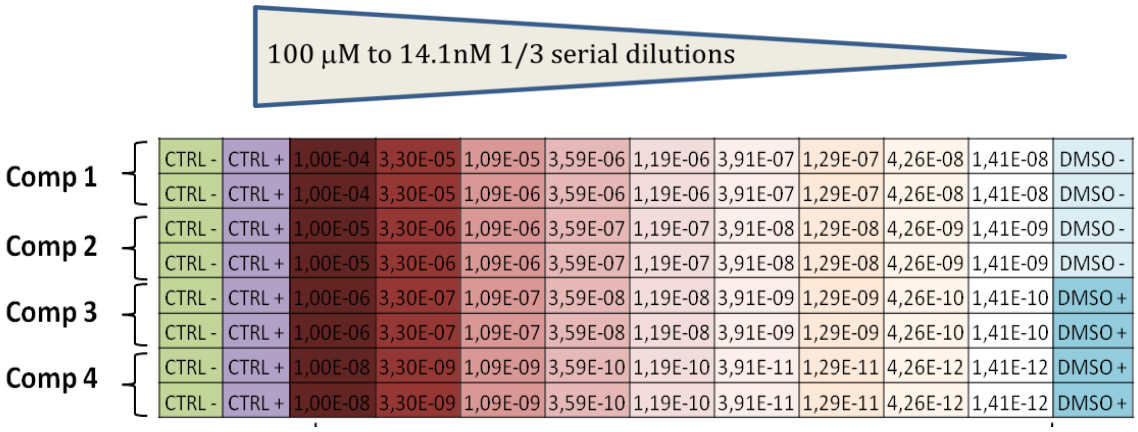

Figure 10. Cell proliferation assay

Day 1: The cells are planted in a 96-well plate (5000 cells / well); only the first column contains only culture medium. Day 2: After 24 hours, the compounds are added to the plate according to the scheme defined by the reference plate. The first column contains only culture medium, the second contains cells and culture medium without compounds, from the third to the ninth, the columns contain different concentrations of compounds ranging from 100 µM to 14.1 nM. The last column contains cells and culture medium with (DMSO +) or without (DMSO-) DMSO.

The cells are incubated with the various compounds for 72 h. After this period, the cell density is evaluated via the "CellTiter-Glo® Luminiscent Cell Viability Assay (Promega)", based on the generation of a luminescent signal proportional to the ATP rate, itself proportional to the number cells present in culture. As a result, 50 µL of CellTiter-Glo® Reagent is added automatically (Beckman FX 96 tip) to each well, then the luminescence is measured using the "multiparametric reader Victor II" (Perkin Elmer).

3.2.2 Methods of analysis of the viability assay

Growth Inhibition percentage: Raw Data are values obtained after luminescence read-out from wells with cells and compounds added. Mean Lower Bound is the average of NSB values, which corresponds to the background. Mean Upper Bound is the average of TOTB values, which corresponds to the maximum proliferation value in the presence of DMSO.

$$Growth\ Inhibition\ \% = 100 - \frac{RawData - MeanLowerBound}{MeanUpperBound - MeanLowerBound} * 100$$

Quality controls of the assay: In order to assess the quality of the assay, Z' allows the comparison of TOTB signal (upper bound, negative control) with the NSB signal (lower bound, positive control). Z' measures the dynamic range of the assay that is defined as the difference between the means of the negative and positive control; and the signal variability (by using standard deviation). An optimal assay should have a Z'=1, which means a large difference between positive and negative control, and a low dispersion of the values. To validate a proliferation assay: a Z' higher than 0,5 is accepted.

$$Z - prime = 1 - \frac{(3 * SDUpperBound) + (3 * SDLowerBound)}{MeanUpperBound - MeanLowerBound}$$

The Z-DMSO lets us know how the DMSO acted over the cells; it is calculated using CTRL values (without DMSO) against BLANK values (with DMSO). A low Z-DMSO value represents a small difference between the cells proliferation in presence and absence of DMSO. In order to control the edge effect, a ratio between TOTB and BLANK is calculated and should be situated between 0,7 and 1,3.

$$Z - DMSO = 1 - \frac{(3 * SDCTRL) + (3 * SDBLANK)}{CTRL - BLANK}$$

Growth Inhibition 50 (GI_{50}): It is the compound concentration that inhibits 50% of cellular proliferation. It provides a value of the inhibitory proliferation ability of the compounds. GI_{50} is calculated from a sigmoid curve defined by the raw data. The top value is set at 100 and the low value at 0. GI_{50} values have been calculated using ActivityBase from IDBS, and the figures were represented through GraphPad.

3.2.3 Clonogenic test: cell colony formation assays

The cells were seeded out into 6 wells plates with appropriate dilutions to form colonies in 1-3 weeks. Following the experiments, the compound ETP-827 was added to the medium at several concentrations. The medium was changed every three days and new compound was added. After between 8-14 days of incubation at 37°C depending on the cell line, the colonies formed were fixed with glutaraldehyde (6.0% v/v) and stained with crystal violet (0.5% w/v). A colony is defined to consist of at least 50 cells. The absorbance of each well was measured with a multiparametric reader Victor II) in order to

determine the compounds effectiveness. Only a fraction of seeded cells retains the capacity to produce colonies.

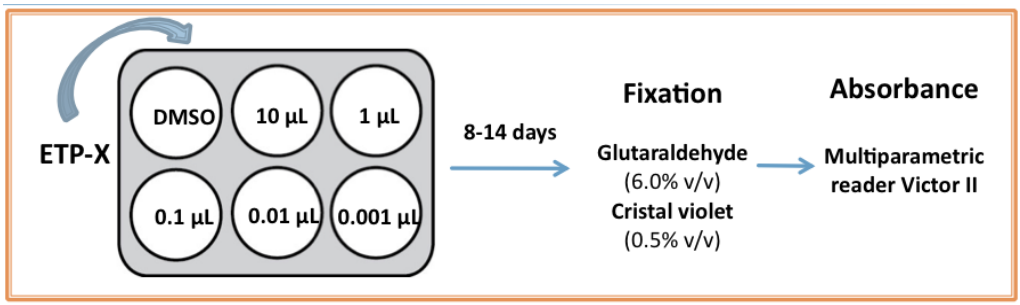

Figure 11. Clonogenic tests from the reference protocol of the CNIO biology laboratory
The waiting period between the application of the compound and the fixation of cells was adapted to the lines U2OS, A673 and TC71 and to the compound ETP-827.

3.2.4 Modulation of biomarkers of interest by Western Blot analysis (STAT-1 and H3)

The different cell lines were plated at density of 250 000 cells in 2 mL of growth media per well using 6 wells plates; and incubated for 24 hours at 37°C before adding the interferon and/or the compounds. Then, the cells were lysed with a RIPA buffer (Sigma-Aldrich) supplemented with proteases inhibitors (Complete Mini EDTA free, Roche), phosphatases inhibitors (Phospho Stop, Roche), Tris pH 7.5, NaCl and 1% IGEPAL CO-630 (Sigma). Proteins were separated by electrophoresis (90min at 150V) using 10% acrylamide/bisacrylamide SDSPAGE gels and transferred to nitrocellulose membranes (120 min at 200 mA). Subsequently, the membranes were blocked with the Odyssey Blocking Buffer (Bonsai) during 120 min at RT on the shaker and co-incubated overnight at 4 o C with appropriate primary antibodies. The primary antibodies used were: the anti-phospho STAT1(S727) (rabbit polyclonal antibody from Cell Signalling), anti STAT1 (mouse monoclonal antibody from BD Biosciences), anti-phosphoHistone H3 Thr3 (rabbit polyclonal antibody from Millipore), anti -Histone 3 (mouse polyclonal antibody from Millipore), and anti-ßactin (mouse monoclonal antibody from Sigma). They were washed and then incubated with the following secondary antibodies coupled to fluorescent molecules: Goat anti-Rabbit IgG (H+L) Alexa Fluor 680, (ThermoFisher Scientific) and Goat anti-mouse IgG (H+L) Dylight 800 (ThermoFisher Scientific). Proteins were visualized using an Odyssey infrared imaging system (Li-Cor Biosciences). The quantification of the phosphorylation was carried out against dividing the intensity of PSTAT1 or PH3 by the intensity of STAT1 and H3 respectively (as a loading control) and normalized against phosphorylation in cells treated with DMSO.

4. RESULTS

The aim of the study was to consider CDK8 as a potential therapeutic target in osteosarcoma and Ewing's sarcoma; we evaluated the antiproliferative capacity of a panel of CDK8 inhibitors on three cell lines from these two tumor types: U2OS, A673 and TC71. Before starting this study, a biochemical evaluation of the compounds was first performed in order to estimate their capacity to inhibit the primary target: CDK8/19 and secondary target: HASPIN (Table 1).

ETP-827, ETP-070 and ETP-055 are compounds from the CNIO Experimental Therapeutics Laboratory known as "ETP-CNIO". These three CDK8/19 inhibitors have more or less interesting IC50 values compared to CDK8 and CDK19 and also exert a slight action on the HASPIN gene. Senexin B and the compound "CCT-251545" are relatively well known and widely described in the literature as potential new CDK8 inhibitors. However, these compounds have no action on the HASPIN gene. (32,33). Finally, since CDK8/19 inhibitors from the CNIO also inhibit HASPIN with a lesser but present capacity, we chose a last CDK8 inhibitor (ETP-914), less potent against CDK8 compared to HASPIN, in order to decipher what the contribution of HASPIN inhibition could be in addition to CDK8 and CDK19 modulation.

Compounds	CDK8 IC_{50} (nM)	CDK19 IC_{50} (nM)	HASPIN IC_{50} (nM)	Selectivity HASPIN vs CDK8
ETP-827	0.50	1.41	47.5	95
CCT-251545	1.60	1.41	100000	62500
ETP-055	6.48	2.62	171	26,39
Senexin B	19.30	41.60	50000	2590
ETP-070	19.60	51.80	567	28,9
ETP-914	218.00	288.00	70	0,32

Table 1. Biochemical IC_{50} inhibition for CDK8, CDK19 and HASPIN
In order to determine their preferential target, the selectivity of each compound has been calculated in reference to the CDK8 IC50 values.

4.1 EVALUATION OF THE ANTIPROLIFERATIVE POTENTIAL OF A PANEL OF CDK8i OVER A SHORT PERIOD OF EXPOSURE (72h) VIA THE CELLULAR VIABILITY ASSAY.

We have carried out several proliferation trials, performing several treatments over a short period of time. The compounds were deposited on the cells for 72 hours in order to evaluate their impact on cell proliferation as a function of their selectivity and potency according to the targets (Figure 12).

Overall, ETP-827 appears to be the most potent and effective compound against each cell line. Indeed, its inhibition capacity is the highest at the lowest concentrations; on average: 6.6 +/+2.2 µM for U2OS, 1.5+/-0.8 µM for A673 and 5.87+/-0.08 µM for TC71. Conversely, ETP-070 and Senexin B have a relatively weak effect on cell proliferation. Indeed, most of the concentrations needed to reach the IC50 exceed 100 µM; the exception is the TC71 cell line which is surprisingly very sensitive to Senexin B. Both inhibitors are nearly two thousand times less potent in terms of CDK8 and CDK19 inhibition than ETP-827. Within the same concentration range, ETP-055 and ETP-914 appear to have a similar effect on the proliferation of all three cell lines. ETP-055 is a potent compound, capable of inhibiting CDK8 at nanomolar concentrations but also capable of effectively inhibiting HASPIN with a selectivity of 26.39 towards it. Conversely, ETP-914 is a less potent CDK8 inhibitor that more actively inhibits HASPIN (selectivity of 0.32 for HASPIN). In terms of GI50 values, these two compounds are relatively close in all 3 cell lines. It would therefore appear that inhibition of HASPIN promotes the antiproliferative activity of CDK8 inhibitors. Finally, compound CCT-251545 has the most heterogeneous behaviour for each cell line with more disparate values: between 15.6 +/- 7.2 µM for U2OS, 63 and more than 100 µM for A673 and 22.4 +/- 18 µM for TC71.

Although CCT-251545 is close to ETP- 827 in terms of its potential to inhibit CDK8 and CDK19 targets, unlike ETP-827, CCT-251545 has no inhibitory effect on HASPIN (selectivity greater than 62500). Therefore, the higher antiproliferative effect of ETP-827 compared to CCT-251545 is probably related to its strong CDK8/19 inhibition capacity but also to its inhibitory action towards HASPIN.

Depending on the sensitivity of the cell lines to the different compounds, we chose to continue the study with the most effective compound. Based on the results of the proliferation assays, ETP-827 was selected as the reference compound because of its potent inhibition of CDK8/19 targets as well as its inhibitory effect on HASPIN (IC50 = 47.5)

A)

	U2OS	**A673**	**TC71**
	GI_{50} (µM)	**GI_{50} (µM)**	**GI_{50} (µM)**
ETP-827	6.6 +/- 2.2	1.5 +/- 0.8	5.87 +/- 0.08
CCT-251545	15.6 +/- 7.3	81,5+/-26,1	22.4 +/- 18
ETP-055	32.8 +/- 1.5	29.2 +/- 1.9	41.2 +/- 3.7
(Senexin B)	> 100	82.9 +/- 1.3	0.67 +/- 0.53
ETP-070	71.85 +/- 22.9	>100	95,6+/- 6,15
ETP-914	20.65 +/- 3.0	20.7+/- 1.8	32.45 +/- 4.6

B)

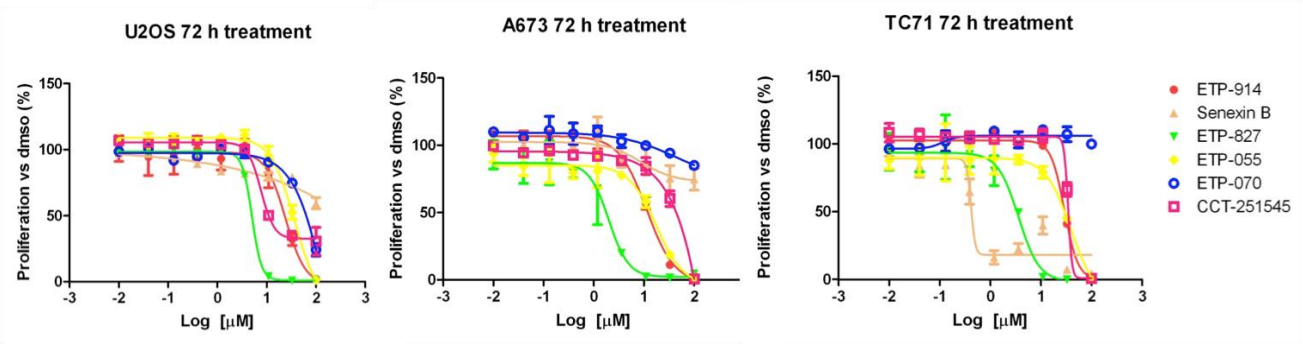

Figure 12. Antiproliferative activity of CDK8 inhibitors in U2OS, A673 and TC71 cell lines

A) GI 50 values of U20S, A673 and TC71 cell lines after 72h of treatment*. B) Representation of dose-response to CDK8 inhibitors for U2OS, A673 and TC71 cell lines adjusted to sigmoid curves that represent the compounds concentration (Log scale of µM concentrations) versus the proliferation percentage. All assays were performed in duplicate (Mean ± sd), and all the experiments were repeated at least two times

*Treatment corresponds to the addition of the several compounds (ETP-070, ETP-827, Senexin B, ETP-914, ETP-055, CCT-251545) at different concentrations (from 100µM to 14nM) during 72 hours.

4.2 EVALUATION OF THE ANTIPROLIFERATIVE POTENTIAL OF THE REFERENCE COMPOUND OVER A LONG PERIOD OF EXPOSURE (8,12 and14 days) VIA CLONOGENIC TESTS.

Colony formation tests or clonogenic tests were carried out in order to refine the results obtained with the proliferation tests. The cell lines were exposed to compound ETP-827 for an extended period of time ranging from 8 to 14 days versus 72 hours in the proliferation assays. This treatment performed over an extended period of time, simulating *in vivo* treatments in mice, allowed us to more accurately determine the GI_{50} value for each cell line. Given that no tests had previously been performed on this type of cell lines in the laboratory, we started the trials with the setting up of the experimental conditions (Figure 13). The purpose of these initial trials was to determine the appropriate number of cells to perform the following clonogenic tests. We started the first trial by inoculating 200, 500 and 1000 cells in triplicates in a 6-well plate for each cell line. After 9 days, the appearance and number of colonies obtained for the U2OS cell line were conclusive for a starting number of 1000 cells per well. Nevertheless, the number of colonies obtained for the A673 and TC71 cell lines after 10 and 15 days respectively was relatively low. In sum, we decided to start the clonogenic assays with 5000 cells per well for these two cell lines.

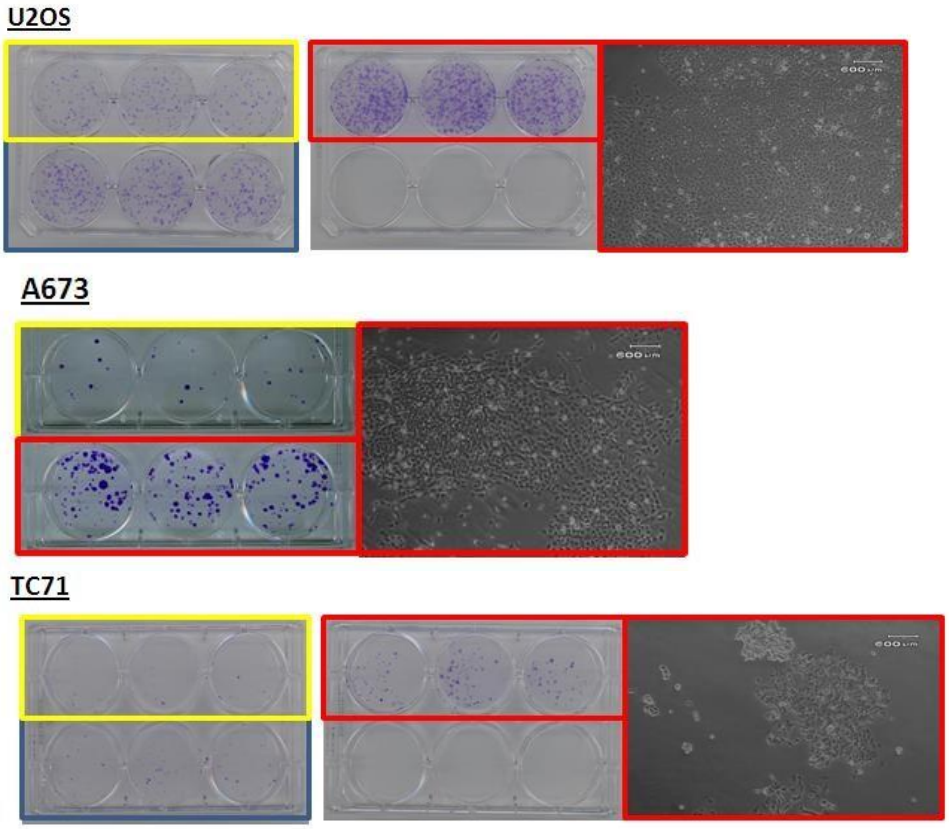

Figure 13. Setting up of experimental conditions

Photos of colonies of the U2OS, TC71 and A673 cell lines after 9,10 and 15 days respectively. 200 cells were planted in the yellow box against 500 in the blue box and 1000 in the red box. The photos in the red box correspond to the photography of a colony of each cell line when the trial started with 1000 cells (magnification x 40).

We used several clonogenic tests to compare the number and size of colonies remaining after administration of the reference compound to the control wells (with DMSO). The clonogenic test allowed us to measure the anti-proliferative effect of the compound ETP- 827 after at least 8 days of treatment. All assays were performed in duplicate for each cell line with the exception of line TC71 due to technical problems. Indeed, TC71 cells tend to clump easily, which prevents the proper distribution of colonies.

The medium was changed every three days and compound ETP- 827 was added to the wells after several dilutions: 10 µM, 1 µM, 0.1µM, and 0.01µM 0.001µM versus DMSO (control well). The results indicate that all three cell lines are more sensitive to ETP-827 when the exposure period is extended; the GI50 values for each line are in the nanomolar range of concentration.

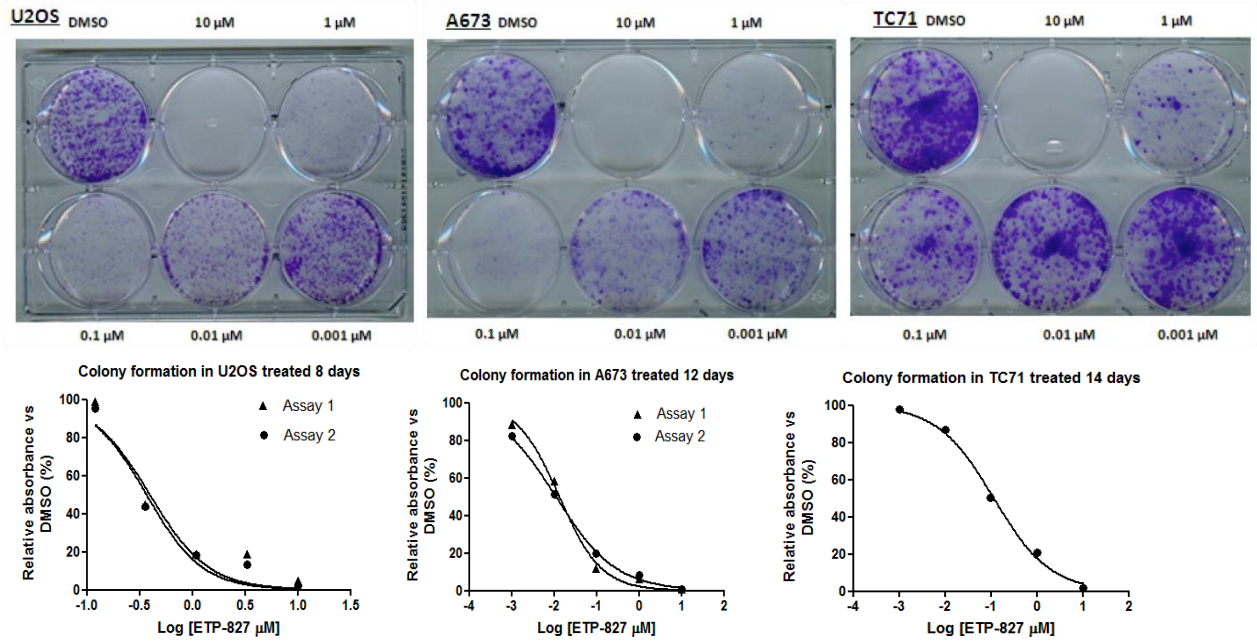

Figure 14. Effect of ETP-827 in colony formation on U2OS, A673 and TC71 cell lines.

A) Representative picture of clonogenic assay performed with the ETP-827 compound. 1000 cells per well were plated for U2OS and 5000 for A673 and TC71 cells lines. The assays were respectively stopped after 8, 12 and 14 days for U20S, A673 and TC71 cell lines.

B) Representation of dose-response to ETP-827 inhibitor. The curves represent the compounds concentration (Log scale of µM concentrations) versus the relative absorbance. All assays were performed in duplicate except for TC71 due to technical problems.

The U2OS cell line (Figure 14.A) was treated for 8 days according to the protocol described above (see 3.2.1) and the FTE-827 concentrations required to reach the GI50 are 0.397µM and 0.368µM for the first and second assays, respectively (Figure 14.B). Compared to the proliferation test, these values are approximately 10 times lower. For line A673 (Figure 14.A), line A673 was treated for 12 days and the GI50 values obtained indicate that this cell line is the most sensitive to ETP-827. Indeed, the values are 0.013µM and 0.011µM respectively for the first and second assays (i.e. 100 times lower than the proliferation assay) (Figure 14.B). Finally, cell line TC71 (Figure 14.A) was treated for 14 days. GI50 value is 0.116µM (Figure 14.B), which is 10 times lower than the proliferation test. These clonogenic tests demonstrated that cell lines are more sensitive to the compound when exposure period is extended (8 to 14 days). On average, concentrations were reduced by 10 to 100-fold compared to the cell proliferation assays (Table 2).

Cell lines	GI_{50} (µM) (Clonogenic assays)	GI_{50} (µM) (Proliferation assays)
U2OS	0.383 +/- 0.02	6.6 +/- 2.2
A673	0.012 +/-0,001	1.5 +/- 0.8
TC71	0.116	5.87 +/- 0.08

Table 2. Comparison between the GI_{50} values obtained with the proliferation tests and the clonogenic tests for the U2OS, A673 and TC71 cell lines

4.3 MODULATION OF THE BIOMARKERS OF CDK8 AND HASPIN ACTIVITY

In order to improve the data and the development process of compound ETP-827 on cell lines derived from Ewing's sarcoma osteosarcoma, we continued the manipulations with the analysis of biomarkers, which are indicators of target modulation. Phosphorylations of STAT1 on the Serine 727 residue and Histone 3 on the Threonine 3 residue were reported as biomarkers of CDK8 kinase activity (33) and HASPIN activity (27), respectively. The objective of this assay is to verify whether these biomarkers are modulated in the presence of the compound ETP-827 in order to control whether the compound can actually reach its intended targets in the cell. If the compound is capable of modulating biomarkers in cells, the estimated biochemical effect towards Cdk8 and HASPIN (Table 5) can be confirmed at the cellular level.

To do this, we first set up the experimental conditions to compare the difference between basal phosphorylation within the three cell lines (U2OS, A673 and TC71) and cytokine-induced phosphorylation on STAT1 (S727).

As described in the literature (34,35), STAT1 plays a key role in the induction of IFN-γ-mediated inflammation and foam cell transformation. The IFN-γ signaling pathway induces phosphorylation of two STAT1 residues: Tyr701 (Y701), which facilitates dimerization, nuclear translocation and DNA binding; and Serine 727 (S727), which allows STAT1 to reach its maximum transcription activity. (35). The target of interest is residue S727, so we performed a western blot in triplicate to assess whether there was sufficient target protein in each cell line used to continue manipulations without induction of phosphorylation (Figure 15). We therefore treated each cell line, U2OS, A673 and TC71 with IFN-γ (50 ng / ml) for 3 hours versus the DMSO-only control. The level of phosphorylated STAT-1 or P-STAT1 was analyzed relative to the STAT-1 level. The results of this preliminary trial indicate that the baseline phosphorylation level of STAT1 is sufficient to continue experiments with A673 and TC71 lines without IFN-γ induction. However, we should have more reservations about the baseline level of STAT1 phosphorylation in the U2OS cell line.

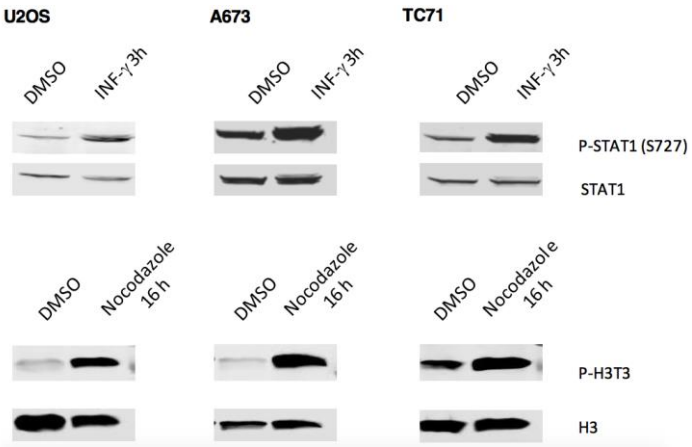

Figure 15. Comparison between basal phosphorylation and phosphorylation induced by IFN-γ on STAT1-S727 as well as basal phosphorylation and phosphorylation induced by Nocodazole on H3-Thr3 for lines U2OS, A673 and TC71.

For IFN-γ-induced STAT-1 S727 phosphorylation, the cell lines were stimulated 3 hours with IFN-γ (50ng/mL) to induce CDK8-mediated STAT1 S727 phosphorylation. For Nocodazole-induced H3-T3 phosphorylation, the cell lines were treated for 16 hours with Nocodazole (250ng/mL) in order to synchronize the cells in mitosis where HASPIN phosphorylates H3.

We performed same preliminary test to evaluate basal level of histone 3 phosphorylated at threonine 3 or P-H3-T3 residue level by comparing it with phosphorylation induced by Nocodazole for each cell line. Phosphorylated histone 3 is a mitosis regulator that recruits Aurora B kinase (AURKB) and passenger chromosome complex (CPC) at the kinetochores to ensure microtubule alignment during metaphase (Figure 16).

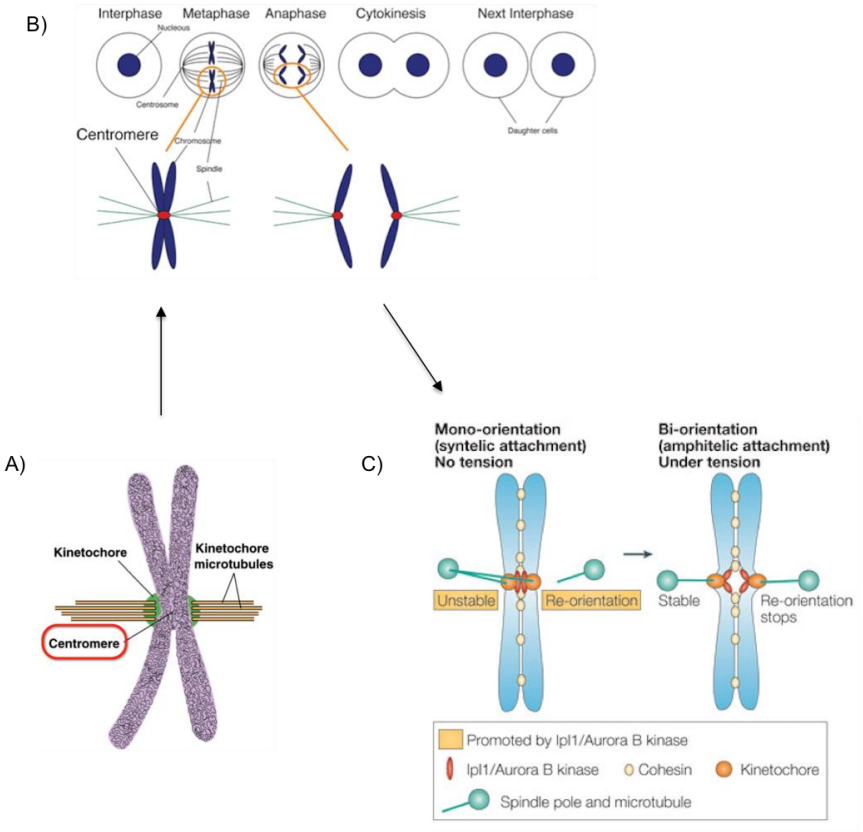

Figure 16. Cell division with the involvement of AURORA B.

A) Cell division and chromosomal segregation. The microtubules of the mitotic spindle bind to kinetochores located at the level of the centromere. **B) The different phases of the cell cycle focused on metaphase. C) Effects of Aurora B on microtubule alignment and orientation.**

To ensure cell synchronization, we used an antineoplastic agent, Nocododazole, which interferes with the polymerization of the microtubules of the synchronized cells in mitosis or HASPIN phosphoryl H3. The cells treated with Nocodazole can thus enter mitosis, but cannot form the mitotic spindle because it is impossible for the microtubules to polymerize. The lack of attachment of the microtubules to the kinetochore activates the spindle assembly control point, causing the cell to stop in prometaphase. We performed this assay in the same way as STAT1 (Figure 16) by treating the cells with 250 ng/ml Nocodazole for 16 h versus DMSO. The level of P-H3T3 was analysed in relation to the level of H3. The results of this preliminary trial demonstrated that the baseline levels of P-H3T3 are sufficient to continue the trials without the use of Nocodazole for all cell lines.
The first biomarker modulation assay was therefore not performed without induction of phosphorylation for either STAT-1 or H3 in any of the lines. As described above, the cells were treated for 1 hour with DMSO and several dilutions of the compound ETP-827 in order to obtain a dose-response: 10 µM, 2 µM, 0.4µM, and 0.08µM 0.016 µM. The cells were then lysed and subjected to Western-Blot analysis using antibodies to the target proteins (P- STAT1, STAT1, PH3 and H3). The U20S cell line shows a clear modulation of P-STAT1 at a concentration of 2 µM and 10 µM while modulation of P-H3 for the same cell line appears to be possible for a concentration lower than 0.016µM (Figure 40). The concentration of ETP-827 required to target HASPIN within the U2OS lineage is therefore lower than the concentration required to target CDK8.

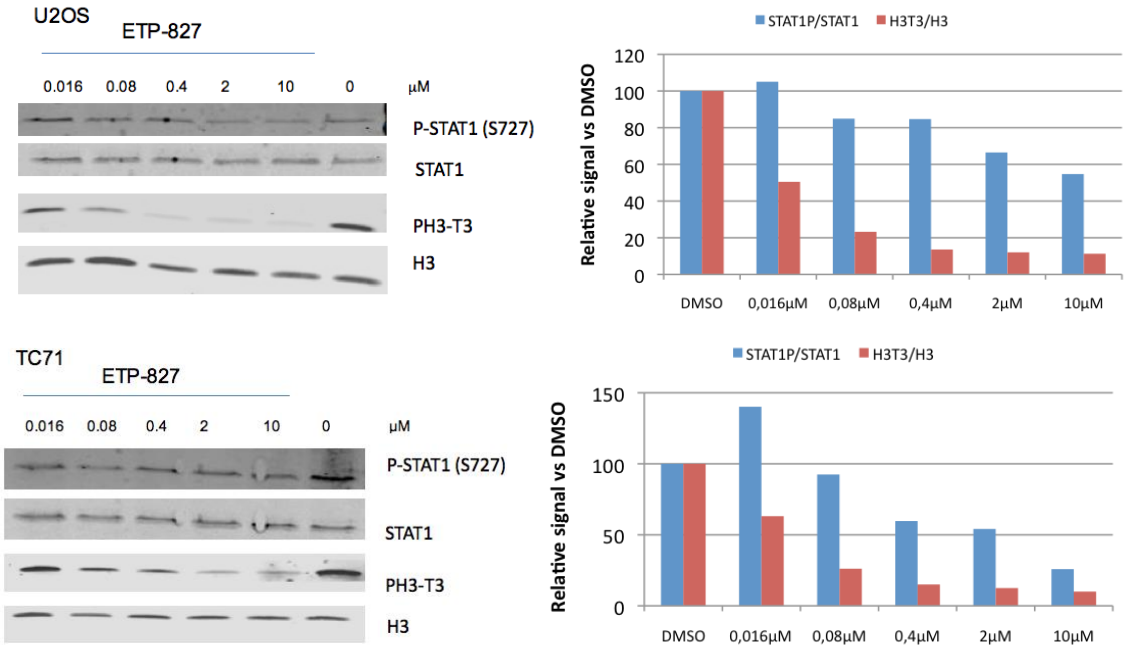

Figure 17. Biomarkers modulation by ETP-827
U2OS and TC71 cells lines were treated for 1h with ETP-827 at different doses. Cell extracts were analysed by immunoblotting with antibodies against phosphorylated STAT1 at S727 (P-STAT1 (S727) and against total STAT1 and phosphorylated H3 at Thr3 (P-H3-T3) and against total H3.

Concerning the TC71 line, the modulation of P-STAT1 is clear for an ETP-827 concentration higher than 0.04µM. On the other hand, modulation of P-H3 seems to be possible for a concentration lower than 0.016µM just like the U2OS cell line. These results allowed us to obtain information concerning the sensitivity of the U20S and TC71 cell lines to the reference compound. Both cell lines have an interesting modulation of their biomarkers during a one-hour exposure period. On the other hand, H3 modulation appears to be higher with low concentrations of FTE-827 for both cell lines. Finally, modulation of P-STAT1 is better in the TC71 lineage compared to U2OS at lower concentrations. As a result, TC71 appears to be more sensitive to the reference compound.

5. DISCUSSION

Cyclin-dependent kinases are proteins kinases whose activity depends on a regulatory subunit - a cyclin, which provides additional sequences required for the enzymatic activity. These CDKs (CDK1 to CDK20) can be grouped as, first, CDKs that bind multiple cyclins and can regulate the cell cycle and second, CDKs that are activated by a single cyclin and involved in the regulation of transcription (8). CDK8 and its closely related family member CDK19 associate with Cyclin C as part of the multi-subunit

Mediator complex which couples the action of transcription factors with the molecular machinery that carries out transcriptions. However, CDK8 has emerged as an important kinase to control not only transcription, by binding RNAPII via Mediator complex, but also cell cycle, by direct binding to some proteins in a Mediator complex-independent manner (25). CDK8 resides on a region of chromosome 13 known to undergo a gain of gene copy number in ~60% of colorectal cancers. Furthermore, it has been reported that CDK8 expression correlates with β-catenin activation in colon and gastric cancers and with increased mortality in colorectal, breast and ovarian cancers (21,22). Besides, CDK8 is overexpressed and essential for cell proliferation in melanoma (21).

These various results let thinking that CDK8 acts as a kinase-dependent driver oncogene. Moreover, CDK8 has an important role in the suppression of NK cell cytotoxicity and tumor surveillance that increase the interest on its downregulation by specific inhibitors as a possible immunotherapy strategy (35). It therefore becomes necessary to test new small-molecule inhibitors of CDK8 and CDK19 to further explore their roles in various biological and pathological contexts as well as their potential utility for the treatment of cancer and other human diseases. Besides, the combination of CDK8/19 inhibitors with other kind of inhibitory proteins could give more interesting results in terms of antiproliferative potency with lower compounds concentrations. The aim of this study and those, which will follow subsequently, is to develop a compound that causes less or no side effects compared to the current cancer chemotherapies.

To do so, the laboratory has been focus trying to develop high selective CDK8/19 inhibitors and after analyze its selectivity in a panel of more than 450 kinases we have found that the only off-target was HASPIN. Maybe the HASPIN inhibition on top of CDK8/19 inhibition could allow decreasing the concentration of inhibitor necessary to obtain a therapeutic effect. Further investigations have to be done in order to understand the mechanism of action.

As described before, HASPIN belongs to the Serine/Threonine protein kinase superfamily. It is a nuclear protein predominantly associated with chromosomes in mitosis. It phosphorylates histone H3 at threonine-3 (H3 P-Thr3) during mitosis, activating the CPC and providing a docking site for the Aurora B complex at centromeres. Aurora B functions to correct improper kinetochore-microtubule attachments and alert the spindle checkpoint to the presence of misaligned chromosomes (36).

It has been shown that HASPIN inhibitors decreased H3T3 phosphorylation, resulting in loss of centromeric Aurora B and reduced phosphorylation of centromere and kinetochore

Aurora B substrates. Consequently, metaphase chromosome alignment and spindle checkpoint signaling were compromised. Many reviews demonstrate that HASPIN is currently the subject of a number of drug discovery efforts (36,37). The future use of HASPIN inhibitors should provide new insight into the cellular functions of these kinases and highlight their mechanisms of action. Then, it could and help to determine more accurately their utility as cancer therapeutic targets.

To evaluate the potential of CDK8/19 inhibition as a therapeutic target in osteosarcoma and Ewing's sarcoma, we have selected 6 compounds, all of them dual CDK8/CDK19 inhibitors (except the ETP914 that it is more active inhibiting HASPIN) with different selectivities against HASPIN. Then, we have implemented proliferation assays in order to assess the antiproliferative potency of these compounds. The purpose was trying to identify how their different potency inhibiting CDK8, CDK19 and HASPIN can impact their antiproliferative potency.

Interestingly, we observed that Senexin B, a reported inhibitor of CDK8 and CDK19 (32), did not exhibit potent cell-based activity and have not yielded conclusive results on the three cell lines tested, showing unexpected potency on TC71 that we do not have an explanation. Given its biochemical activities, the results can be correlated to the fact that its potency inhibiting CDK8/19 is not enough and it doesn't have inhibition activity against HASPIN, if the duality could be relevant for the antiproliferative activity.

The CCT-251545, reported in the literature as potent and selective chemical probe for CDK18 and CDK19 (33), has a better antiproliferative effect on the three cell lines. Its selectivity against HASPIN is very high and is a more potent CDK8/19 inhibitor. Then, we can expeculate that a higher potency inhibiting CDK8 is necessary to retrieve better antiproliferative results. This is also the case for the CNIO's compounds: the ETP-055 and ETP-914, which have relatively similar inhibition potencies on the three cell lines but preferential selectivities for each target. Indeed, the ETP-055 is very potent against CDK8/19 and less potent against HASPIN and the ETP-0914 is more potent against HASPIN than CDK8/19. These results suggest that the inhibition of both CDK8/19 or HASPIN could impact in the proliferation of these three cell lines. Overall, the hit compound remains the ETP-827, with the higher effects on the cell lines at the lowest concentrations. This compound is the most potent inhibitor against CDK8/19 and HASPIN according to the biochemical IC 50 values. Then, probably this dual inhibition has more potent antiproliferative activity than inhibiting one of the targets alone. Besides, we can conclude that the Ewings' sarcoma cell line, A673, is the most sensitive to this compound.

It would be interesting to deepen the experiments for each compound. Thus, we could test the compounds efficacy during a prolonged exposure on the cell lines or their effects on the biomarkers modulations but due to lack of time we focused our study on the hit compound: ETP-827.

The *colony formation assay* is the *gold standard* for measuring the effects of cytotoxic agents on cancer cells *in vitro*. We performed this assay in order to measure the effect during a prolonged period of exposure of the ETP-827 compound on the U2OS, A673 and TC71 cell lines. The results obtained through the colony formation assays have confirmed those of the proliferation assays. The A673 cell line is the most sensitive to the ETP-827 followed first by TC71 and then by U2OS cell line. Otherwise, we have shown that the concentration required to inhibit 50% of the colony formation when performing this assay are 10 to 100 times lower than the concentration required to inhibit 50% of the cell proliferation during the proliferation assay. Hence, we can conclude that longer exposure to ETP-827 retrieves better inhibition of proliferation.

Finally, the biomarkers modulation study helped us to further investigate the impact of the ETP-827 compound on the cell lines. We performed this assay with P-STAT1(S727) and PH3-T3 as biomarkers of CDK8 and HASPIN activity in order to check inside the cell that the ETP-827 compound is able to reach these two targets.

The first purpose of the assay was to verify the P-STAT1(S727) modulation potency of the ETP-827 compound. In response to cytokines and growth factors, STAT family members are phosphorylated by the receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators (38). Thus, in response to IFN-γ, STAT1 forms homodimers or heterodimers with STAT3 that bind to the GAS, (Interferon-Gamma-ActivatedSequence) a promoter element that leads to an increased expression of ISG (Interferon Stimulated Genes). As reported in the literature and confirmed in our hands, IFN-γ-induced STAT1(S727) phosphorylation modulates the transcription and occurs after the STAT1 dimers bind to the DNA (34). Furthemore, a recent report showed that the basal phosphorylation on STAT1(S727) is mediated by CDK8 (35). We have confirmed that IFN-γ-induced STAT1(S727) phosphorylation in U2OS, TC71 and A673 cell lines, but as all of the cell lines even in the absence of the stimulus show high levels of PSTAT1(S727), we have decided to evaluate the inhibition of STAT1 phosphorylation in the absence of stimulus to better correlate the results with the antiproliferative activity were the cells grow in normal medium without IFN-γ.

Therefore, we have demonstrated a dose dependent reduction of P-STAT1(S727) levels in U2OS and TC71 cell lines, for the A673 cell line we can conclude anything due to technical problems. When the ETP-827 compound was tested at concentrations closed to its GI 50 (obtained in the proliferation assay), it has reduced P-STAT1(S727) within 60 min of treatment. Indeed, the modulation of P-STAT1(S727) was better for concentrations values closed to those obtained with the proliferation assay than those obtained with the colony formation assay. It should be interesting for further research to treat the cell lines during a prolonged period of time (4h, 24h). Thereby, we could verify if ETP-827 is able to reduce PSTAT1(S727) levels at concentrations consistent with those that we used for the colony assays in the same cell lines. Moreover, it has been reported in the literature that the phosphorylation of STAT1(S727) seems to be the most robust and reliable target engagement biomarker of CDK8 inhibition (33) although the correlation between STAT1(S727) phosphorylation and the sensitivity of hematological cell lines to CDK8 inhibitors has not been demonstrated. (39). Thus, further investigations should be done with the references and CNIO's compounds that we have tested before (Senexin B, CCT-251545, ETP-914, ETP-070, ETP055) in order to see if these compounds are potent against to CDK8 target in osteosarcoma and Ewing's sarcoma cell lines and determine how it correlates with its antiproliferative activity.

ETP-827 is a CDK8/19 inhibitor, also able to inhibit HASPIN according to the Biochemical IC 50 values. In order to confirm this data at cellular level, we performed a biomarker modulation assay using PH3-T3 as a selected biomarker of HASPIN activity. As described previously, HASPIN is a kinase that phosphorylates H3 on Threonine 3, which provides a signal for the Aurora B kinase, a component of the Chromosomal Passenger Complex, to localize to the centromere of mitotic chromosomes to kinetochores to regulate mitosis. To date, the PH3T3 is the only well-characterized HASPIN substrate.

Besides, apart from this well-characterized centromeric function of HASPIN activity, the broader cellular functions of the kinase and the phosphorylation events that control these remain essentially unknown. The biomarker assay was performed with the same concentrations used for the P-STAT1(S727) assay. Nonetheless, the ETP-827 has reduced phosphorylation at PH3-T3 within 60 min of treatment at lower concentration than those required to inhibit the P-STAT1(S727). Hence, we can conclude that the two cells lines are sensitive to the ETP-827 compound with concentrations closed to those used for the colony formation assay. Further investigations should be done during a prolonged period of time (4h, 24h) at lower concentrations in order to increase the data about the

ETP-827 compound PH3-T3 modulation ability. Besides, it will be important in future research to elucidate the HASPIN mechanism of action and contribution to proliferation.

To summarize, the experiments performed with the ETP-827 compound gaves us new informations about the dual inhibition potency against CDK8 and HASPIN. These results gave us several data through experiments on osteosarcoma and Ewing's sarcoma cell lines but further experiments with more cell lines are needed in order to validate it. Overall, the most sensitive cell line to the ETP-827 compound is the A673 cell lines, followed by TC71 and U20S cell lines for all experiments (even though biomarker modulation assay for A673 is missing and should be determined). Hence, we can conclude that the Ewing's sarcoma cell lines seem to be more sensitive than the osteosarcoma cell line to the compound. Besides, if we look more closely the results giving by the proliferation assay, we can note that second compound that has more or less the same effect on all the cell lines proliferation is the ETP-914, which is a potent HASPIN inhibitor with less inhibiting activity on CDK8. Hence, we can conclude that dual inhibition potency allowed decreasing the compound concentrations necessary to have an effect on cell proliferation. Further investigations have to be done with this compound in order to confirm that HASPIN inhibition potentiates the effect of CDK8 inhibition. Combinations assays with selective CDK8 and HASPIN inhibitors and comparison with each inhibitor alone could give us information about the relevance of these dual inhibition activities in one compound.

Although the effects of CDK8 at cellular level are roughly known, how the inhibition of its kinase activity could modulate its effects need to be study in more detail. On the other hand, the role of HASPIN is relatively unknown and using the inhibitors could give us additional information about other cellular function.

Moreover, the phosphorylation of CDK8 and HASPIN biomarkers is the source of several cellular mechanisms that could act on a wider field of pathologies (40). Indeed, the transcription factor STAT1 is important in natural killer (NK) cells, which provide immediate defense against tumor and virally infected cells. CDK8-inducted phosphorylation of STAT1 at serine 727 was shown to be suppressive for the NK cells activity. Putz and coworkers have demonstrated that STAT1-S727A-mutated NK cells displayed increased release of cytotoxic proteins such as granzyme B and perforin and in general higher cytotoxicity toward cancer cells (35). This work provides a rationale for further studies with CDK8 inhibitors also as cancer immunotherapy. Moreover, STAT1 has have been identified as a CDK8-dependent paracrine mediator that might account for the survival of cancer cells in tumor microenvironment. STAT1, upon phosphorylation

mediate paracrine signals into changes in gene expression. The attenuation of STAT1 (S727) site resulted in impaired RNAP II elongation and lowered gene expression of IFN-γ dependent genes. This work implicated CDK8 as a controller of STATs and selective regulator of IFN-γ target-gene expression.

In summary, the researches on the involvement of the P-STAT1(S727) CDK8 biomarker need to be deepening in order to elucidate with more accuracy its implication on immunity or cancer development role.

Concerning the biomarker of HASPIN activity, H3T3 is the only well-characterized HASPIN substrate. It has been related that the inactivation of HASPIN induces Aurora B centromeric delocalization, leading to a loss of phosphorylation in chromatin associated Aurora B substrates (41)(42). Then, further reasearch need to be establish due to the lack of information about the broader cellular functions of the kinase and the phosphorylation events that control it.

6. CONCLUSION, PERSPECTIVES

This whole thesis content has allowed us to respond to the various hypotheses stated at the outset. First of all, the bibliographical approach has enlightened us about the interest of kinase-dependent cyclin inhibitors at the cell cycle level, their impacts at the cellular scale and interactions with the different actors of the cellular machinery. The experimental part of this thesis allowed us to demonstrate the sensitivity of cell lines from two types of tumors that have not been candidates for studies of specific CDK8/19 inhibitors until now. Indeed, the cells were shown to be sensitive to both short- and long-term treatments. In addition, we have shown that the antiproliferative capacity of CDK8/19 inhibitors correlates with biochemical studies and is enhanced when there is an inhibitory action against HASPIN. A dual inhibitor of CDK8/19 and HASPIN, potent against both targets, appears to be effective on cell lines derived from osteosarcoma and Ewing's sarcoma. As a result, the research teams need to test these compounds on a wider range of cancer cell lines and to further explore their mechanisms of action. The long-term objective will be to find new therapeutic applications for CDKS inhibitors, and in particular, CDK8.

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SUMMARY

This thesis is a revised and reshaped version of the French version "Entre recherche fondamentale et préclinique en cancérologie : évaluation de nouveaux inhibiteurs de CDK8/19". The previous thesis was presented and publicly supported on October 6th, 2016 in the Faculty of Pharmacy of Nantes in France.

Between fundamental and preclinical research in cancerology: evaluation of new CDK8/19 inhibitors

Abstract: CDK8 is a cyclin-dependent kinase, that together with its partner Cyclin C, MED12 and MED13 are components of the multi-protein "Mediator complex". This complex is a pivot which couples the action of transcription factors with the molecular machinery that carries out transcriptions. The overexpression of CDK8 has been implicated in many cancers, especially in colorectal cancer where it has been identified as an oncogenic protein allowing the tumor to be maintained in a dedifferentiated form. Thus, there are considerable interests in developing drugs specifically targeting the CDK8 kinase activity. The "CDK8 inhibitors" have emerged as a novel class of small targeted molecular agents with great therapeutic potential in various types of cancer *in vitro* and *in vivo*. Within this thesis, new compounds were synthesized and previously tested in the laboratory of experimental therapeutics of the CNIO (National Center for Oncology Investigations) in Madrid

KEY WORDS:

CANCER, DIFFERENTIATION, BIOMARKERS MEDIATORS, CDK8 INHIBITORS, HUMAN THERAPEUTICS.