Survivin and Aurora B kinase, two targets in the search for anti - mitotic drugs; identification of a new class of Aurora kinase inhibitors.

Thi My-Nhung Hoang

Université Joseph Fourier-Grenoble 1

CHIMIE ET SCIENCE DU VIVANT

(Arrêtés ministériels du 5 juillet 1984 et du 30 mars 1992)

THÈSE

pour obtenir le titre de
Docteur de l'Université Joseph Fourier
Discipline Biologie

Présentée et soutenue publiquement par

HOANG Thi My Nhung

Le 31 Janvier 2008

SURVIVIN AND AURORA B KINASE, TWO TARGETS IN THE SEARCH FOR ANTI-MITOTIC DRUGS; IDENTIFICATION OF A NEW CLASS OF AURORA KINASE INHIBITORS.

COMPOSITION DU JURY

Véronique Baldin	**Rapportrice**
Claude Prigent	**Raporteur**
Dinh Duy Khang	**Examinateur**
Marylin Vantard	**Présidente**
Stéfan Dimitrov	**Examinateur**
Tran Cong Yen	**Directeur de thèse (VNU)**
Annie Molla	**Directrice de thèse (UJF)**

Thèse préparée au sein du Centre de Recherche INSERM
Mécanismes d'assemblage et de régulation de l'appareil génétique
Unité INSERM U823
Institut Albert Bonniot – Université Joseph Fourier – Grenoble 1

Acknowledgements

First of all, I would like to express my deepest gratitude to Dr. Annie Molla for her supervision, patient guidance, and encouragement from the beginning of my research in France as well as giving me excellent advice throughout this study. I also want to thank her for assistances in preparing and writing this thesis.

I would like to warmly thank to my thesis co-supervisor, Dr. Tran Cong Yen, for his invaluable support and enthusiasm to my graduate experience. I could never have embarked and started all of this without his prior teachings in biology and thus opened up unknown areas to me.

My special thanks to Dr. Stefan Dimitrov for giving me the opportunity to work on his equipment and for his assistances, suggestions in my research.

I gratefully thank Dr. Véronique Baldin and Dr. Claude Prigent for reviewing my thesis and for their constructive comments on this manuscript.

I would like to thank Dr. Marylin Vantard and Dr. Dinh Duy Khang for accepting to be members of the committee.

My stay and my PhD in France is financial supported by a Scholarship from Sandwich Programme, a project initiated and managed from Ministry of Education and Training of Vietnam and partly from French. I also thank to ARC organization which supported for the last year of my thesis.

It was a pleasure for me to work with all wonderful people in our lab here in IAB. I would like to give many thanks to the fellow graduated students before me, Gaelh, Marlene, Flore, and Fabienne Sirot, for sharing experiences and knowledge during the time they were here. My thanks are given to another Fabienne for "science" discussions and the pleasure working together in the lab. I would also acknowledgment Véro for helping me in protein extraction and purification technique, thank for her technical assistance. I am grateful to my colleagues: Andre, Thierry, Eric, Sophie, Fang, Jean, Amina…

I would like to thank to the members of INSERM U823 for their support and their comradeship. Thank Aude for her indispensable help dealing with administration and bureaucratic matter during my stay in IAB.

I am very grateful to members of platform, Alexei and Jack, whose expertise, understanding and patience in microscope are very helpful for my work.

I also want to thank Catherine Souchier for helps in using confocor microscope.

A great thank to my department, Faculty of Biology, Hanoi University of Science, Hanoi National University (VNU) for supporting and creating good conditions for me to do my PhD.

To my best French friend, Cedric Badowski, I gratefully acknowledge him for his kindness and useful advices during the lifetime I spent in France. He provides me unflinching encouragement and support in various ways. I have much benefited by advice and guidance from him who always kindly grants me his time even for answering some of my unintelligent question about microscope and molecular technique…. I am much indebted to him!

My years as a PhD student would not have been as much fun without my friends: Khoat, Nam, Dung, Hung, Vi Thuy, Thai, Chi, Lien…with whom I shared the unforgettable moments. Thanks!

It is pleasure to express my gratitude wholeheartedly to Annie's family for their kindness hospitality during my stay in Grenoble. Thank Thierry for his assistance on editing my thesis writing.

Finally, I take this opportunity to express my profound gratitude to my grand family: my beloved parent, parent in law, my brothers, sisters for their inseparable support and patience during my study in France.

Especially, I wish to express all my warmest thanks and deepest appreciations to my dear husband and my little daughter for their love and sacrifices for me. Without my two loves, I would not finish my thesis!!!

Grenoble, December 2007

Abstract

The chromosomal passenger complex (CPC) plays a key role in mitosis : controlling both chromosome segregation, spindle tension, anaphase onset and cytokinesis. The complex is composed of four proteins: INCENP, Aurora B kinase, Survivin and Borealin. Taking into account that Survivin is phosphorylated by Aurora B and has a pivotal role in the complex, we have studied the phosphomimetic mutant SurvivinT117E. Survivin phosphorylation is required for anaphase onset and the phospho-mutant is poorly linked to centromere. Moreover it exhibits a dominant negative function in cytokinesis, preventing abscission.

In a search for Aurora kinase inhibitors we have identified a new class of Aurora B kinase inhibitors that prevents Histone H3 phosphorylation, impairs mitotic spindle checkpoint. Moreover these molecules prevent tumor cell proliferation. These inhibitors are interesting tools for understanding CPC function and represent a new lead for the development of anti-cancer drugs.

Survivin and Aurora B kinase, which are expressed exclusively in mitosis, are thus two druggable targets for new anti-mitotic therapies.

Key words: Mitosis, chromosomal passenger complex, Aurora kinase, Survivin, mitotic checkpoint.

Résumé

Le complexe passager joue un rôle clé en mitose: contrôlant à la fois la ségrégation des chromosomes, la tension du fuseau, l'entrée en anaphase et la cytodirèse. Le complexe est composé de quatre protéines: INCENP, la kinase Aurora B, Survivine et Boréaline. Sachant que la protéine Survivine est phosphorylée par Aurora B et qu'elle a un role pivot au sein du complexe, nous avons étudié un mutant mimant sa phosphorylation: Survivine T117E. La phosphorylation de Survivine est nécessaire à la transition Métaphase/ Anaphase. Le mutant Survivine T117E est faiblement lié aux centromères en métaphase et agit comme un dominant négatif de la cytodirèse, empêchant la séparation des deux cellules filles.

Lors de la recherche d'inhibiteurs des Aurora kinases, nous avons identifié une nouvelle classe de molécules qui inhibent la phosphorylation de l'histone H3 et le point de contrôle du fuseau. Ces molécules préviennent la prolifération des cellules tumorales. Ces composés sont des outils intéressants pour étudier la fonction du complexe passager et représentent un nouveau motif moléculaire pour le développement de drogues anti-cancéreuses.

Survivine et Aurora B kinase dont l'expression est restreinte à la mitose sont deux cibles pour de nouvelles thérapies anti-mitotiques.

Mots clé : Mitose, complexe passager, Aurora kinase, Survivine, point de contrôle du fuseau.

ABBREVIATIONS

APC/C :	Anaphase promoting complex/Cyclosome
ATP:	Adenosine 5'-triphosphate
BIR:	Baculovirus IAP Repeat
CDE/CHR:	Cell cycle Dependent Element/ Cell cycle genes Homology Region;
Cdks:	Cyclin-dependent kinases
CENP-A:	Centromere protein A
CENP-E:	Centromere protein E
CIN:	Chromosomal INstability
CPC:	Chromosomal Passenger Complex
CPP:	Chromosomal Passenger Protein
DAPI:	4',6-diamidino-2-phenylindole
DMSO:	Dimethyl Sulfoxide
DNA:	Deoxyribo Nucleic Acid
DTT:	Dithiothreitol
EDTA:	Ethylene Diamine Tetraacetic Acid
FACS:	Fluorescence Activated Cell Sorting
FRAP:	Fluorescent Recovery After Photobleaching
GDP:	Guanosine Di-Phosphate
GFP:	Green Fluorescent Protein
GST:	Glutathione S-Transferase
GTP:	Guanosine Tri-Phosphate
HP1:	Heterochromatin Protein 1
HRP:	Horse Radish Peroxidase
IAPs:	Inhibitors of apoptosis proteins
INCENP:	INner CENtromere Protein
IPTG:	Isopropyl-ß-D-1-thiogalactopyranoside
kDa:	kilo Dalton
MCAK:	Mitotic Centromere Associated Kinesin
MCC:	Mitotic Checkpoint Complex
MPF :	M-phase promoting factor
MKLP1:	Mitotic Kinesin-Like Protein 1
OD_{600}:	Optical Density at 600 nm
PBS:	Phosphate Buffer Saline
PFA:	ParaFormAldehyde
PP1:	Protein Phosphatase 1
RNA:	Ribo Nucleic Acid
rpm:	round per minute
SAC:	Spindle Assembly Checkpoint
SDS:	Sodium dodecyl sulfate
siRNA:	silencing interference RNA
TD-60:	Telophase Disc 60 kDa
TEA:	Triethanolamine
v/v:	volume/volume
w/v:	weight/volume

List of Figures and Tables

Introduction

Figure 1: Overview of cell cycle. In eukaryotic cells, the cell cycle is characterized by four distinct phases (G1, S, G2 and M phase)
Figure 2 Steps in mitosis
Figure 3 Schematic diagram of Cytokinesis
Figure 4 The centrosome duplication cycle
Figure 5 Spindle assembly checkpoint signalling
Figure 6 Organization of the vertebrate kinetochore/centromere
Figure 7 Localization of passenger protein during mitosis
Figure 8 Three Aurora kinases in human
Figure 9 The model of kinetochore-microtubule attachments
Figure 10.1. A model of the mechanisms to correct syntelic chromosome mal-orientations during cell division
Figure 10.2. Aurora B contributes to the correction of merotelic attachments by promoting microtubule destabilization
Figure 11 Survivin is a dimer
Figure 12 Survivin variants
Figure 13 Post-transcriptional modifications of Survivin
Figure 14 A model for Survivin functions in genetic fidelity and spindle formation
Figure 15 INCENP and interacting proteins
Figure 16 Interaction between passenger proteins in the complex
Figure 17 Survivin , INCENP and Borealin form a ternary complex
Figure 18 Genomic instability leads to cancer
Figure 19 Aurora kinase inhibitors
Table 1 Mitotic spindle checkpoint components and complex
Table 2 Aurora B substrates in vertebrates and yeast
Table 3 Anti-Survivin strategies and interventions

Materials and Methods

Figure 20 Schema of the possible situations in a FRAP experiment
Table 4 Primary antibodies used in the experiments
Table 5 Secondary antibodies used in the experiments

Results

Figure 21 Phosphorylated Survivin has effect on the stable spindle assembly checkpoint
Figure 22 Phosphorylated Survivin impairs the localisation of MKLP1 at the spindle midzone/midbody
Figure 23 Purification of both the kinase domain of Aurora A and Aurora B kinase
Figure 24 High throughput screening results
Figure 25 Hit from the Villeurbanne library
Figure 26 Flavone compounds
Figure 27 The best hit found in the Curie-CNRS library
Figure 28 The level of Cyclin B1 decreases in the presence of benzopyrido[e]indole
Figure 29 Surivin-GFP and Aurora-GFP share the same behavior in the presence of Aurora kinase inhibitors
Figure 30 Localisation of Survivin and INCENP in the absence of Aurora B
Figure 31 Time-lapse experiments with HeLa cells expressing Survivin-GFP
Figure 32 Binary choice at the Metaphase/Anaphase transition
Table 6 Parameters of the kinase assay

Table of content

INTRODUCTION **5**

CHAPTER I: CELL CYCLE **7**
I.1. Phases in cell cycle. **7**
I.1.2. M phase **9**
I.1.3. Control of cell cycle **12**
I.1.4. Mitotic slippage **13**
I.2. Two important structures in cell division: Mitotic spindle and Centromeres **13**
I.2.1. Mitotic spindle **13**
I.2.2. Centromeres **19**

CHAPTER II: CHROMOSOMAL PASSENGER PROTEINS **23**
II.1. General localization of chromosomal passenger proteins **23**
II.2. Members of chromosomal passenger proteins **23**
II. 2. 1. Aurora B **24**
II.2.1.1. Aurora B: a member of Aurora kinases **24**
II.2.1.2. Aurora B regulation **26**
II.2.1.3. Aurora B substrates **27**
II.2.1.4. Aurora B function **28**
II.2.2. Survivin **32**
II.2.2.1. Survivin: a member of the IAP family **32**
II.2.2.2. Survivin isoforms **33**
II.2.2.3. Survivin regulation **34**
II.2.2.4. Role of Survivin in cell division **36**
II.2.2.5. Role of Survivin in cell death **38**
II.2.3. INCENP (Inner CENtromeric Protein) **39**
II.2.4. Borealin/Dasra B: a novel chromosomal passenger protein **40**
II.2.5. TD-60 telophase disc 60 ..... **40**
II.3. Chromosomal passenger complex CPC) **41**
II.3.1. Interaction between passenger proteins in the CPC **42**
II.3.1.1. The links between passenger proteins in the complex **42**
II.3.1.2. The interaction between passenger proteins is required for the right localization of the CPC **44**

CHAPTER III: CHROMOSOMAL PASSENGER PROTEINS AND CANCER **47**
III.1. Passenger proteins as possible targets for cancer therapy **47**
III.1.1. Expression of passenger proteins in cancer **47**
III.1.2. Passenger proteins and their role in aneuploidy promotion **48**
III.2. Chromosomal passenger proteins and cancer therapy **49**
III.2.1. Aurora-kinase inhibitors **49**
III.2.2. Survivin: a target for cancer therapy **50**

MATERIALS AND METHODS **53**

CHAPTER I: BIOCHEMISTRY **55**
I.1. Cells extracts **55**
I.2. Electrophoresis **55**
I.2.1. SDS-polyacrylamide gel electrophoresis **55**
I.2.2. Transfer of protein from SDS-polyacrylamidee gel to solid supports. **55**
I.2.3. Immunoblotting **56**

CHAPTER II: MOLECULAR BIOLOGY **57**
II.1. Agarose electrophoresis **57**
II.1. Competent cells **57**
II.2. Bacterial transformation **57**
II.3. DNA Miniprepations **57**

CHAPTER III: EXPRESSION OF PROTEIN IN E. coli AND PURIFICATION **59**
III.1. Protein over-expression **59**
III.2. Protein extraction **59**
III.3. Protein purification **59**
III.4. Storage of purified proteins **60**
III.5. Preparing sample for SDS-polyacrylamide gel Electrophoresis **60**

CHAPTER IV: PROTEIN KINASE ASSAY **61**
IV.1. Protein kinase assay **61**
IV.2. High throughput screening (HTS) **61**

CHAPTER V: CELL CULTURE **63**
V.1. Cell lines **63**
V.2. Conservation of cells **63**
V.3. Drugs used **63**
V.4. Synchronisation **64**
V.4.1 Double Thymidine block (early S-phase block) **64**
V.4.2. Thymidine-Nocodazole block (mitotic block) **64**
V.5. FACS (Fluorescence Activated Cell Sorting) **64**
V.7. Cell proliferation and viability **64**
V.7. Cell transfection **65**
V.7.1 Plasmids **65**
V.7.2 siRNA transfection **66**

CHAPTER VI: MICROSCOPIC TECHNIQUE **67**
VI.1. Immunofluorescence **67**
VI.2. Time – lapse experiments **68**
VI.3. FRAP (Fluorescent Recovery After Photobleaching) **68**

CHAPTER VII: BUFFER **69**
VII.1. Biochemistry **69**
VII.2. Immunofluoresence **69**
VII.3. Protein extraction and purification **70**

RESULTS **71**

CHAPTER I: ROLE OF SURVIVIN PHOSPHORYLATION BY AURORA B IN MITOSIS **73**
I.1. Role of Survivin T117E mutant in mitosis **75**
I.2. Consequence of Survivin T117E over-expression in mitosis **84**
I.2.1 Survivin^{SR}T117E-GFP mutant affects the spindle checkpoint **84**
I.2.2 Phosphorylated Survivin is required for the correct localization of MKLP1 ..... **84**

CHAPTER II: IDENTIFICATION OF AURORA KINASE INHIBITORS **87**
II.1. Kinase purification **89**
II.2. Setting up the protein-kinase assay **89**
II.3. High throughput screening **90**
II.4 Characterization of the hits **91**
II.4.1 Flavone inhibits Aurora kinase *in vitro* **91**
II.4.2 Benzo[e]pyridoindoles a new class of Aurora kinase inhibitors **93**
II.5 Aurora kinase inhibitors, interesting tools for studying the CPC **127**
II.4.2. The effect of compound on the level of Cyclin B proteins **127**
II.4.3. Aurora kinase activity is required for accurate localization of CPC on centromere **128**

DICUSSION & PERSPECTIVES **131**

Chapter I : Role of Survivin phosphorylation by Aurora B in mitosis **133**
Chapter II: Search for Aurora B kinase inhibitors **135**

Perspectives **139**

REFERENCES **141**

INTRODUCTION

CHAPTER I: CELL CYCLE

CHAPTER II: CHROMOSOMAL PASSENGER PROTEINS

CHAPTER III: CHROMOSOMAL PASSENGER PROTEINS AND CANCER

CHAPTER I: CELL CYCLE

Cell is the basic unit of all living organisms (Rudolf Virchow) and the name "cell cycle" was given by Howard and Pecl (1953). Definitely, cell cycle or cell-division cycle is an ordered set of events, culminating in cell growth and division into two daughter cells. It is a very important process by which a single cell fertilized egg develops into a mature organism and by which hair, skin, blood cells and some organs are renewed.

I.1. Phases in cell cycle.

The goal of cell cycle is to produce two genetically identical cells from one precursor cell. This requires the replication of each chromosomal DNA, the separation of the two set of chromosomes into the daughter cells and the physical division of cell to produce two identical daughter cells. Details of cell cycle vary from organism to organism and may occur at different times in an organism's life. We will concentrate the description mostly to superior eukaryotes.

In the cell cycle, there are different phases named: Go, G1, S, G2 and M phase (Figure 1). Go, G1, S and G2 phase are known as Interphase. The G1 phase stands for "GAP 1". The S phase stands for "Synthesis" because in this stage DNA replication occurs. The G2 phase stands for "GAP 2". The M phase itself is composed of two stages: mitosis (chromosomes separation) and cytokinesis (cytoplasmic division). We will first describe the different phases of the cycle and then, its regulation.

I.1.1. Interphase

During this period cells are engaged in metabolism and prepared for mitosis. Visually, this period is inactivity but in fact, on molecular level, it is quite an active time for the cells. In interphase, the cells replicate their nuclear DNA, produce protein and increase in size to ensure that after dividing, new created daughter cells are quite identical. Interphase is divided into three phases although these various stages are not morphologically distinguishable. Each phase of the interphase has a distinct set of specialized biochemical processes that prepare the cell for initiation of the cell division.

a. Go phase

The Go phase is a quiescent period in the cell cycle. In fact, when cells are unable to get through the G1 restriction point, they enter the Go phase. This usually occurs in response to a lack of growth factors or nutrients. During the Go phase, the cell cycle machinery is dismantled and cyclins and cyclin-dependent kinases disappear. Cells remain in the Go phase until there is a pressure for them to divide. For instance, epidermal

fibroblasts remain in the Go phase until stimulated by growth factors, which are generated in response to the wound healing process. Although many cells in the Go phase may die along with the organism, this does not mean that cells that enter the Go phase are destined to die. Go represents not simply the absence of signals for mitosis but also the active repression of the transcription of genes needed for mitosis. Cancer cells cannot enter Go and are destined to repeat the cell cycle indefinitely.

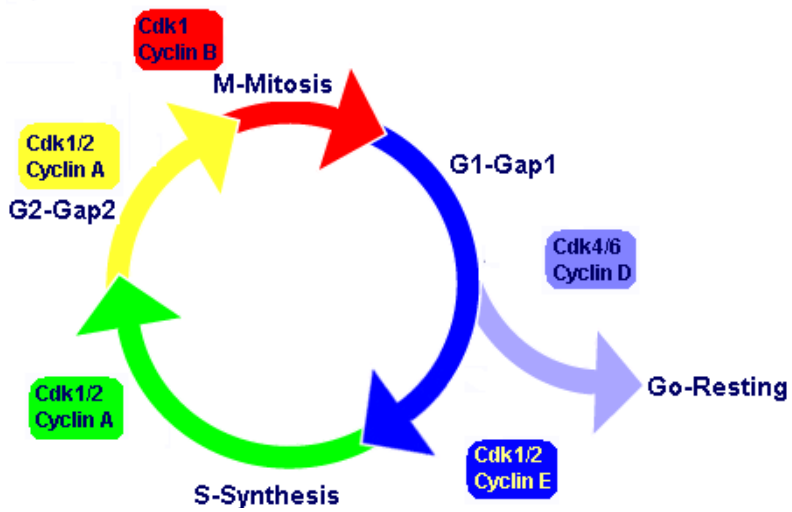

Figure 1. Overview of cell cycle. In eukaryotic cells, the cell cycle is characterized by four distinct phases (G1, S, G2 and M phase). In Go phase, cells enter a state of resting or quiescence. The regulatory molecules (cyclins and cyclin depend kinases –Cdk) which control the cell cycle are also indicated. (Modified from http://www.med.unibs.it/~marchesi/dna.html)

b. G1 phase

The G1 phase is the major period of cell growth. During this stage, cells produce RNA, synthesize proteins and increase in size. New organelles are also synthesized, so the cells need both structural proteins and enzymes. In this phase, there is an important activated cell cycle control mechanism (G1 checkpoint). At the restriction point, cells assess external and internal stimuli and decide whether to pause or to divide. A cell can pause in G1 and then, enter in Go phase. Committed cells go on S phase.

c. S phase

Following G1, cells enter S phase (short for **Synthesis phase**). During this phase, replication occurs. The complete DNA instructions of the cells have to be duplicated. So at the end of this stage, each chromosome has two identical DNA double helix molecules. The flawless operation of this cycle is important not only for the maintenance of cell viability but also, for preventing genetic instability that could potentially drive tumourigenesis (Blow and Dutta, 2005). DNA damage takes place often during this phase, and DNA repair is

initiated following the completion of replication. Incomplete DNA reparation may flag cell cycle checkpoint, which in turn, halts the progression in the cell cycle.

d. G2 phase

After successful completion of DNA synthesis, cells enter in the G2 phase. This is the final subphase of the cell cycle interphase. In this period, nucleus is still well defined and surrounded by the nuclear envelope; chromosomes are not clearly discerned in nucleus since they are still in the form of loosely packed chromatin fibres. During this phase, the cell will continue to grow and synthesize proteins. At the end of this gap there is another control checkpoint (G2 checkpoint) which determines whether the cells can initiate M phase or must extend the gap for further cell growth. G2 checkpoint helps to maintain genomic stability since it prevents cells from entering mitosis if DNA is damaged. It provides an opportunity for DNA repair and thus, stops the proliferation of damaged cells.

I.1.2. M phase

During M phase, separation of chromosomes plays a key role for producing two genetically identical daughter cells. In a typical somatic cell cycle, M phase comprises mitosis and cytokinesis. Mitosis is a complex and highly regulated process by which a cell separates its duplicated genome into two identical halves. The main purpose of mitosis is to segregate sister chromatids into two nascent cells, such that each daughter cell inherits one complete set of chromosomes (Nigg, 2001a). During cell division, an inaccurate separation of chromosomes can lead to an abnormal chromosome number and create genomic instability, a crucial step in the development of human cancer (Draviam *et al*., 2004). In cytokinesis cytoplasm, organelles and cell membrane are split equally into two daughter cells. This stage occurs in conjunction with mitosis.

a. Mitosis

- **Prophase**: prophase is a process in which chromatin condenses into a highly ordered structure called chromosome (Figure 2). Each chromosome which has been duplicated during an earlier S phase now contains two identical copies of itself, called *sister chromatids*. The sister chromatids attach together in a specialized region of the chromosome known as the centromere (see part I.3.2a). Chromosome condensation is accompanied by extensive phosphorylations of both histone and non-histone proteins. Phosphorylation of the core histone H3 at serine 10 is required for proper chromosome segregation in at least some organisms (Hans and Dimitrov, 2001; Nigg, 2001a).

- **Prometaphase**: this stage occurs immediately after prophase and sometime, is considered as a part of prophase. Early during this period, nuclear membrane breakdown,

microtubules radiate from centrosomes and invade the nuclear space. Then they quickly interact with the kinetochores on the chromosomes. This is called an opened mitosis and occurs in most multicellular organisms. Some protists, such as algae, undergo a variation called closed mitosis where the microtubules are able to penetrate an intact nuclear envelope (Ribeiro *et al*., 2002). During prometaphase, kinetochores are initially captured by a single microtubule that extends from a spindle pole and are then transported pole ward along the microtubule (Tanaka *et al*., 2005) (Figure 5). The kinetochore will provide the pulling force which is necessary to separate two chromatids. Throughout mitosis, microtubule-kinetochore interactions are highly dynamic (Nigg, 2001b).

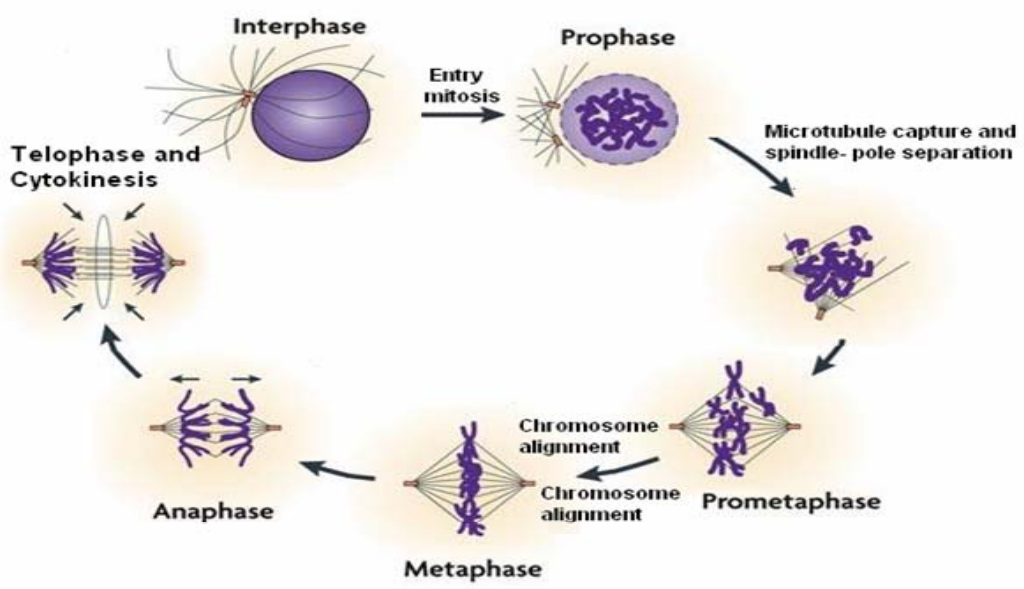

Figure 2. **Steps in mitosis**. Mitosis is a continual and dynamic process. The sequence events in mitosis are divided into five subphases: prophase, prometaphase, metaphase, anaphase and telophase. (Jackson et al., 2007).

- **Metaphase**: This phase is characterized by the highly condensed chromosomes alignment in the middle of the cell. In fact, early events in metaphase start with the later events of prometaphase when kinetochores attach microtubules. The centromeres of chromosomes convene themselves on the metaphase plate (or equatorial plate) - an imaginary line that is equidistant from the two centrosome poles (Figure 2). Unattached chromosomes apparently generate a signal that delays progression to anaphase until all sister chromatin are attached to the spindle apparatus. Such a signal is called the mitotic spindle checkpoint (see part I.2.1c). This process ensures the equal segregation of sister chromatids to the two daughter cells (Yu, 2006). In human cells, there is also a DNA decatenation-sensitive mitotic checkpoint which functions independently of DNA damage or spindle assembly checkpoint response (Skoufias *et al*., 2004).

- **Anaphase**: Anaphase onset starts only when the checkpoint is turned off. Within anaphase, two distinct processes occur. During early anaphase, the chromatids simultaneously separate and sister chromatids are pulled apart towards their respective centrosomes at the poles. In the late anaphase, the microtubules elongate and slide relatively to each other to drive the spindle poles further apart.

- **Telophase**: This is the last stage of mitosis. At telophase, chromosomes are re-condensed into chromatin and progressively nuclear membrane is reconstituted. At telophase, the non-kinetochore microtubules continue to lengthen, elongating the cell even more. Both sets of chromosomes, now surrounded by the new nucleus membrane, unfold back into chromatin. Each daughter nucleus has a complete copy of the genome of its parent cell.

b. Cytokinesis.

Cytokinesis is the final event in the cell cycle and whereby the cytoplasm of a single cell is divided to spawn two daughter cells. It usually occurs meanwhile the nuclear envelope is reforming, although they are distinct processes. The goal of cytokinesis is to physically separate a mother cell into two identical daughter cells.

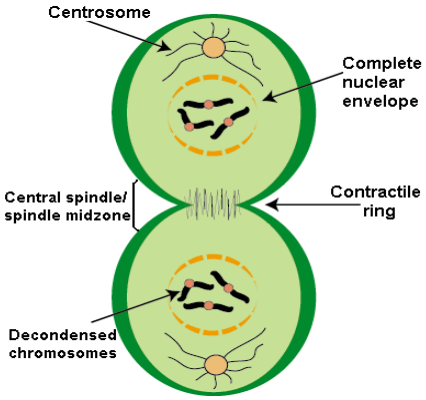

Figure 3. **Schematic diagram of Cytokinesis**. Major features and important organels are indicated. (Modified from http://www.sparknotes.com/biology/cellreproduction/mitosis/section3.rhtml)

In animal cells, a cleavage furrow develops where the metaphase plate used to be, pinching off the separated nuclei. This furrow contains actin, myosin, and other proteins that are organized into a contractile ring called the actomyosin ring (Figure 3). The ring then, ingresses generating a membrane barrier between the cytoplasmic content of each daughter. The ingressing furrow constricts components of the spindle midzone into a focused structure called the midbody (Guertin *et al*., 2002). Many signalling proteins are found in the midbody during cytokinesis, including GTPases, phosphatases, kinases and proteases-proteins as well as components of the DNA-damage or spindle-assembly checkpoints (Zeitlin and Sullivan, 2001). In fact, the final event in cytokinesis is the furrow "sealing" that generates two completely separate cells. Recently, it was shown that Rab 35, a GTPase

protein, is required for the final abscission by controlling Septin 2 and PiP2 subcellular distribution during cell division (Kouranti *et al*., 2006).

I.1.3. Control of cell cycle

Controls during the cell cycle are necessary to bring about an orderly progression through both S-phase and mitosis and to couple these two processes with cell growth (Nurse, 1997; Castro *et al*., 2005). How cell division (and thus, tissue growth) is controlled is very complex.

There are two main key classes of regulatory molecules that determine the cell cycle progression: the cyclins and cyclin-dependent kinases (Cdks) (Norbury and Nurse, 1991) (Figure 1). The most prominent mitotic kinase is the Cdk1-the founding member of cell cycle regulation. These molecules are major control switches within the cell cycle, causing the cell to move from G1 to S (G1 cyclin-Cdk complexes) such as Cyclin D/Cdk4; Cyclin D/Cdk 6; Cyclin E/Cdk2; or from G2 to M (Mitotic Cyclin-Cdk complexes) such as Cyclin B/Cdk1 (also knows as Cdc2). This complex which was first called M-phase promoting factor (MPF) is the key regulator on one hand of the G2-M transition of the cell cycle and on another hand of the APC/C activation (Brito and Rieder, 2006).

There are also some non-cyclin proteins involved in the regulation of the cell cycle. For example, p53 is a protein whose function is to block the cell cycle when DNA is damaged. If the damages are severe this protein can lead cells to apoptosis (cell death). To note mutations of p53 are commonly reported in transformed cells (May and May, 1999).

The nuclear protein $p21^{WAF1}$ (p21), $p27^{KIP1}$ (p27), and $p57^{KIP2}$ are universal Cdk inhibitors (Sherr, 1995; Sherr, 1996; Weinberg and Denning, 2002). By inhibiting Cdks, they interrupt the transition from G1 to S phase in the cell cycle. Expression of p21 is induced by wild-type p53 in response to DNA damage. This mechanism is necessary for the maintenance of genomic fidelity. Perturbation of p53 functions leads to the loss of the checkpoint control mediated by p21, thus causing DNA instability. Therefore, expression of p21 protein is expected to be low when normal functions of the p53 are lost. However, p21 protein accumulation may also be induced through p53 independent pathways (Amatya *et al*., 2001). p27 protein is another Cdk inhibitor, which inhibits cyclin complexes by a posttranslational degradation through ubiquitin-dependent proteasomal proteolysis. Thoroughly, cells are blocked at the entry into S phase (Schiffer *et al*., 1999).

Anaphase promoting complex (APC) promotes degradation of structural proteins associated with the chromosomal kinetochores. APC also targets the mitotic cyclins for degradation, ensuring telophase and cytokinesis progression. Recently, studies brought to

light additional mitotic kinases to the regulation of M phase progression, including members of the Polo, the Aurora and the NIMA (never in mitosis A) families (Nigg, 2001b).

I.1.4. Mitotic slippage

We have described previously that cells pausing in G1 can escape in Go. Arrested mitotic cells may also benefit of such a binary choice. In the presence of an active spindle assembly checkpoint (SAC), cells escape from mitotic arrest and enter the next G1 as multinucleated. This is called mitotic slippage (Blagosklonny, 2007; Brito and Rieder, 2006; Rieder and Maiato, 2004).

As we said above, the sequential activation and inactivation of cyclin-dependent protein kinases ensure the proper timing and order of cell cycle events. Activated Cyclin B/Cdk1 complex regulates cells entry into mitosis. The destruction of cyclin B is essential for proper exit from mitosis (Raff *et al*., 2002). When the spindle assembly checkpoint is not satisfied (for instance, drug-mitotic arrest such as Nocodazole, Taxol treatment) cells will delay exit from mitosis by preventing the APC complex. However, in the absence of transcription, cells may die in prolonged mitotic arrest, the SAC does not arrest cells permanently and therefore cells may eventually escape from mitotic arrest without dividing. They enter in G1 as multinucleated cells. These cells either do not reproduce anymore or die (Blagosklonny, 2007; Brito and Rieder, 2006; Rieder and Maiato, 2004).

I.2. Two important structures in cell division: Mitotic spindle and Centromeres

I.2.1. Mitotic spindle

The function of mitotic spindle is to segregate chromosomes during cell division. This structure contributes to the mitotic eukaryotic cytoskeleton. It consists of a bundle of microtubules joined at the ends but spread out in the middle. It is mostly ellipsoid in shape. At the pointed ends, known as spindle poles, microtubules are nucleated by the centrosomes. Forces that are generated by the attachment between microtubules to the kinetochores will pull chromosomes into alignment along the spindle midzone in order to build up the metaphase plate. Since the center of the spindle specifies the plate along which the cell will divide during cytokinesis, this ensures that each daughter cell will receive one of each chromatid.

a. Centrosomes.

The centrosome is the main microtubule-organizing centre (MTOC) of the cell and also regulates cell-cycle progression. It was discovered, in 1888, by Theodor Boveri and was described as the special organ of cell division.

Centrosomes are composed of two orthogonally arranged centrioles surrounded by an amorphous mass of pericentriolar material (PCM). Centrioles are very important in the cell division process. They organize the PCM which in turn, plays a role in the mitotic spindle assembly. Centrosomes are associated with the nuclear membrane during interphase. The centrosomes nucleate and organize microtubules, playing a role in mitosis. Their position is thought to dictate the organization of the cell microtubule network (Manneville and Etienne-Manneville, 2006).

During cell division the centrioles are duplicated, so a pair will be available for each daughter cell (Figure 4). Each new paired set of centrioles is composed of one original centriole. The centrosome itself replicates once and only once per cell cycle (Mazia, 1987). Two centrosomes form spindle poles and direct the formation of bipolar mitotic spindle. The presence of more than two centrosomes (chromosome amplification) severely disturbs mitosis process and cytokinesis via formation of more than two spindle poles. It will lead to increase the chromosome segregation errors (chromosome instability - CIN) (Deans *et al*., 2003; Fukasawa, 2005). Loss of certain tumour suppressor proteins leads to centrosome amplification, which in turn destabilizes chromosomes (Fukasawa, 2005). Moreover, loss of BRCA1 ubiquitination also results in centrosomes amplification (Sankaran and Parvin, 2006).

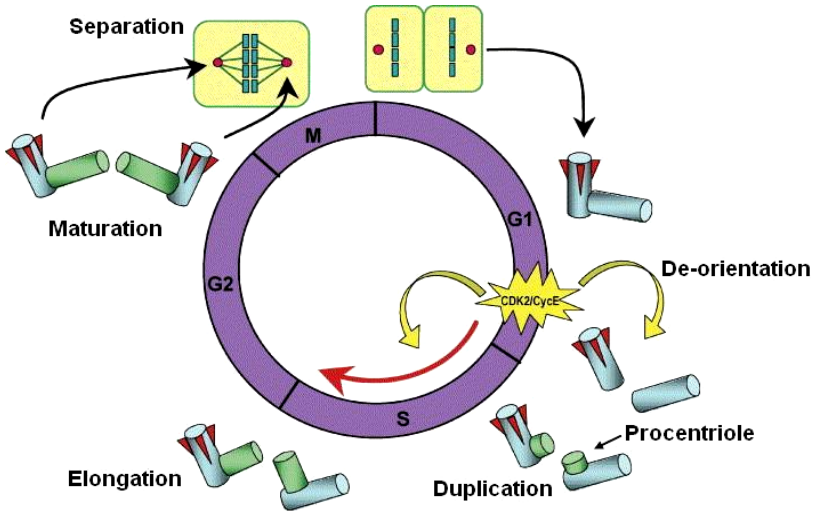

Figure 4. **The centrosome duplication cycle**.
Specific activation of Cdk2/cyclin E triggers initiation of centrosome duplication in late G1. Centrosome duplication begins with the physical separation of the paired centrioles, which is followed by formation of procentrioles near the proximal ends of each pre-existing centriole. During S and G2 phases, procentrioles elongate, and two centrosomes progressively recruit PCM. In late G2, the daughter centriole of the parental pair acquires appendages (shown as red wedges), and two identical centrosomes are generated. During mitosis, two duplicated centrosomes form spindle poles, and direct the formation of bipolar mitotic spindles. Upon cytokinesis, each daughter cell receives one centrosome. (Modified from Fukasawa, 2005).

The centrosome is a platform required for a multitude of cellular functions, including organization of signalling pathways, cellular responses to stress and cell cycle transition (Doxsey, 2005). Many regulators of the cell cycle associate with the centrosome, so cells that have lost their centrosomes arrest in the next cell cycle (Rieder *et al*., 2001). Recent researches suggest that centrosomes may have their own genome, as previously described for mitochondria and chloroplasts. There may be an interconnection between the functional network of DNA damage response and the dynamic structural organization of eukaryotic cells through centrosomes (Loffler *et al*., 2006).

b. Microtubules

Microtubules are cytoskeletal structures assembled from mostly α/β-tubulin heterodimers that play an essential role in many cellular processes, such as cell motility, organelle transport, maintenance of cell polarity, and cell division (Nogales, 2000). The tubulin dimers polymerize end to end in protofilaments. Another important feature of microtubule structure is polarity with α-tubulin at one end of the polymer (the minus end) and β-tubulin at the other (the plus end). The plus end explores the cellular space, switching rapidly between phases of growth and shrinkage, a behaviour called dynamic instability. (Moritz *et al*., 2001; Lansbergen and Akhmanova, 2006). Centrosome-nucleated microtubules probe the cytoplasm with their plus ends to search and capture chromosomes in prometaphase. γ-Tubulin, a protein related to α/β-tubulin, does not assemble into microtubules. In most cells, it is found at centrosomes. γ-Tubulin has been found in two main protein complexes: the γ-tubulin ring complex (γTuRC) and the γ-tubulin small complex (γTuSC) (Moritz *et al*., 2001).

Microtubules are intrinsically dynamic, in that they alternate abruptly and stochastically between two stages of growth and shortening, a phenomenon termed "dynamic instability" (Karsenti *et al*., 2006; Mitchison and Kirschner, 1984). These stages can generate force, in addition to motor proteins that move along the microtubule. The major microtubule motor proteins are kinesin, which generally moves towards the (+) end of the microtubule, and dynein, which generally moves towards the (-) end. Three microtubule motors – CENP-E, dynein and MCAK and several microtubule-binding proteins have been also localized at metazoan kinetochores (Garrett and Kapoor, 2003). An accurate control of microtubule dynamics is required for kinetochore tension generation and chromosome alignment during mitosis (Zhou *et al*., 2002). Yang Ge *et al*. have developed the single-fluorophore imaging in order to examine microtubule organization in the vertebrate meiotic spindle and they proposed that the mechanical integrity and force transmission across the spindle must be regulated by dynamic crosslinking of loosely connected microtubules (Yang *et al*., 2007). The microtubule dynamics can be altered by drugs. The taxan drug class (e.g.

paclitaxel or docetaxel) used in the treatment of cancer, blocks dynamic instability by stabilizing GDP-bound tubulin to the microtubules. Thus, there is no more depolymerization and the microtubule does not shrink back. Nocodazole, Colchicine and Vinblastine have the opposite effect, blocking the polymerization of tubulin into microtubules. Noscapin does not significantly promote or inhibit microtubule polymerization but alters the steady state dynamics of microtubule assembly (Zhou *et al*., 2002).

c. Mitotic spindle checkpoint.

The spindle checkpoint is a survey mechanism that delays anaphase onset until all chromosomes are correctly attached in a bipolar fashion to the mitotic spindle (May and Hardwick, 2006). It is an evolution conserved mechanism, which ensures that cells with misaligned chromosomes do not exit mitosis and therefore do not divide. The spindle assembly checkpoint prevents thus aneuploidy (Bharadwaj *et al*., 2004). It is an active signal produced by improperly attached kinetochores. As chromosome attachment to the spindle microtubules is a stochastic process, not all chromosomes achieve alignment at the spindle equator at the same time (Yu, 2006). Even a single unaligned chromosome can prevent the onset of anaphase (Rieder *et al*., 1995). When sister kinetochores are properly attached to opposite spindle poles, forces in the mitotic spindle generate tension at the kinetochores. The interdependence between tension and microtubules attachment makes it difficult to determine whether these signals are separable. But some recent evidences show that defects in tension act as the primary checkpoint signal (Pinsky and Biggins, 2005).

The spindle checkpoint inhibits the anaphase-promoting complex/cyclosome (APC/C) leading to the delay of anaphase onset. APC/C is a multiprotein E3 ubiquitin ligase that ubiquitynates a range of cell-cycle regulators, targeting them for degradation by the 26S proteasome (Castro *et al*., 2005; Yamano *et al*., 2004). Once all kinetochores have bipolar attachments, the checkpoint is switched off and APC/C is activated (May and Hardwick, 2006). Then, anaphase onset starts. The molecular basic of the dynamic signalling carried out by spindle checkpoint proteins is listed in Table 1. Many proteins are involved in either activation or silencing of checkpoint. Some play a role in detecting the signal which is generated as a result of the lack of microtubule occupancy and kinetochore tension (Chan and Yen, 2003); some regulate APC/C activities; and finally others are involved in silencing the checkpoint. Recent works in yeast, frogs and mammals have outlined the signalling of spindle assembly checkpoint (Figure 5). For example Mad2 has been proposed as a specific marker of unattached kinetochores, whereas Aurora B (in complex with the other passenger proteins) is required for sensing the spindle tension. The activation of spindle checkpoint starts by the recruitment of spindle checkpoint proteins. The kinases Mps1 and Bub1 act in concert with BubR1; and the CENP-E kinesin triggers the rapid recruitment of the Mad1–

Mad2 complex to the kinetochore (Tan *et al*., 2005). The mitotic arrest deficiency 2 (Mad2) inhibits APC/C through binding to its mitotic-specific activator - Cdc20. Binding of Mad2 to Cdc20 involves a large conformational change of Mad2 and requires the Mad1–Mad2 interaction *in vivo* (Yu, 2006). BubR1 functions synergistically with Mad2 in inhibiting Cdc20-APC activity (Tan *et al*., 2005; Zhou *et al*., 2002). MCC complex, which contains the BubR1–Mad2–Bub3–Cdc20 proteins, strongly inhibits APC/C activity, causing a delay in anaphase.

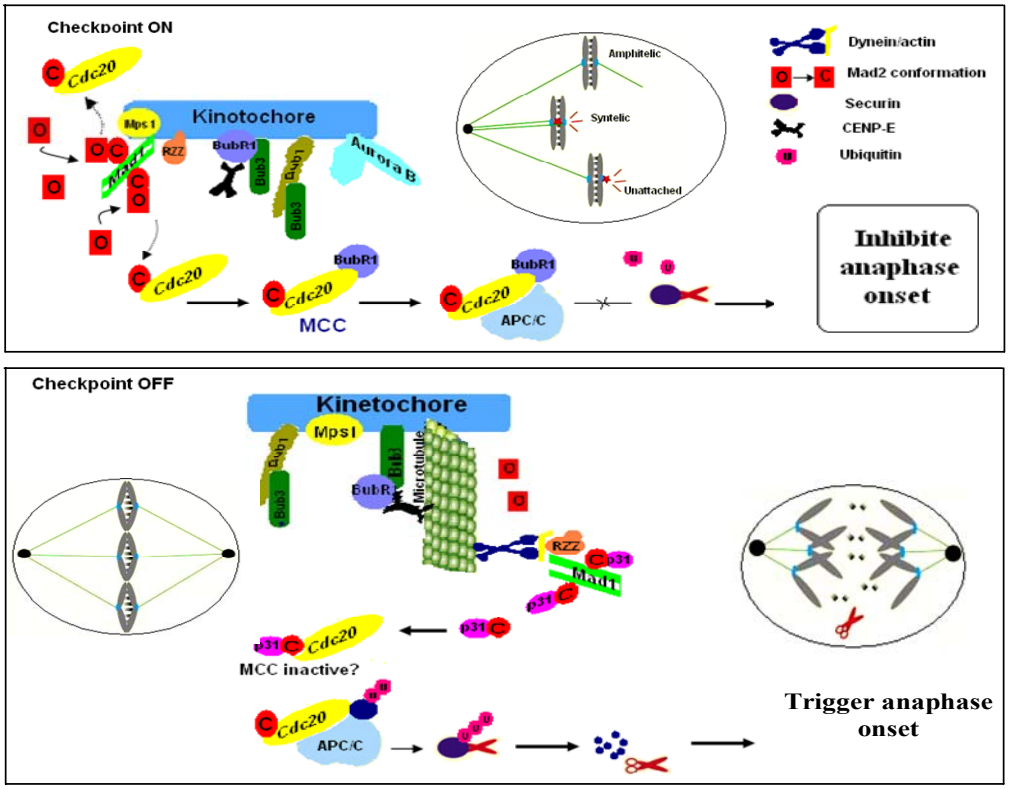

Figure 5: Spindle assembly checkpoint signalling (Modified from May and Hardwick, 2006).

As soon as all chromosomes are properly attached to the kinetochore microtubules and aligned at the metaphase plate, the spindle checkpoint is turned off. Mad1/Mad2 and BubR1 are transported away from the kinetochore along microtubules by dynein, preventing further inhibitory signalling (May and Hardwick, 2006). p31^{comet} (p31C) binds to 'closed' conformation of Mad2 involved in the checkpoint switch off. The binding of microtubules to CENP-E downregulates BubR1 kinase activity and results in the silencing of the checkpoint (Mao *et al*., 2003). Activated Cdc20-APC catalyzes the ubiquitination of securins, leading to their degradation through proteosome-mediated proteolysis. Degradation of securins in turn causes the release of separases. The free separases destroys cohesin (the molecular glue holding sister chromatids together), thus allowing chromatids to be pulled to opposite poles and finally anaphase starts.

Table 1: Mitotic spindle checkpoint components and complex (May and Hardwick, 2006).

Checkpoint components	Comment
Core checkpoint proteins	
Mad1	Binds to Mad2 and recruits it to kinetochore; also localises Mad2 to the nuclear periphery (NP) in interphase; required for checkpoint activation in response to tension; binds to Bub1 and Bub3 upon checkpoint activation in budding yeast.
Mad2	Binds to Bub1 and Cdc20; exist in two conformations ('closed' C-Mad2 on binding Mad1 and Cdc20, or 'open' O-Mad2 when unbound); interacts with Cdc20 and Bub1/Mad3 to form MCC which inhibits the APC; excess Mad2 inhibits the APC/C in many experimental systems.
BubR1/Mad3	Binds to Bub3; interacts with Mad2 and Cdc20 to form MCC; C-terminal kinase domain of BubR1is activated by CENP-E.
Bub1	Protein kinase; binds to Bub2; requirement for recruiting the other checkpoint proteins differs depending on systems; reported substrates include Bub3, Mad 2 and adenomatous polyposis coil; kinase activity is not required for the checkpoint arrest.
Bub3	Binds to Bub1 and MCC components; required for the Bub1 and BubR1 localization to the kinetochore.
Mps1/Mph1	Protein kinase; phosphorylates Mad1 *in vitro*; excess activates the checkpoint; required for the recruitment of Mad1; Mad2 and CENP-E to the kinetochore.
Other protein required for checkpoint function	
CENP-E	Kinesin family member; binds to BubR1; stimulates BubR1 activity; required for capture and stabilization of microtubules at the kinetochore; only found in higher eukaryotes.
RZZ complex	Complex of Rod, ZW10 and Zwilch, in higher eukaryotes only recruits dynein and Mad1/Mad2 to the kinetochore.
Cdc20	APC/C activator; by binding to Mad2 and BubR1, it forms the MCC, which inhibits APC/C activation.
Aurora B	Protein kinase; chromosomal passenger protein binds INCENP and Survivin; required for checkpoint in response to the lack of tension but not attachment.
P31^{comet}	Binds specifically to the 'closed' conformation of Mad2; excess disrupts checkpoint signalling; involved in checkpoint switch off.
Dynein/Dynactin	Minus-end-directed motor that transports Mad2 and BubR1 away from kinetochore; though to be required for switching off the checkpoint; dynactin complex recruits 'cargo' to dynein.
Downstream of the checkpoint	
APC/C	E3 ubiquitin ligase; target mitotic regulators for destruction by the proteasome; downstream effector of the checkpoint.
Securin	Binds and inhibits Separase
Separase	Protease; cleaves the cohesin subunit Scc1and breaks the cohesion ring
Cohesin	Protein complex of Scc1, Smc1and Smc3 that form a ring around sister chromatids, holding them together; cleavage of Scc1 is required for sister separation
The complexes	MCC: the mitotic checkpoint complex (MCC); Mad1-Mad2; Mad2-Cdc20; Bub3-Bub1; Bub3-BubR1; Mad2-Cdc20-BubR1-Bub3; BubR1-CENP-E; BubR1-Cdc20 Rod-ZW10-Zwilch; AuroraB-INCENP-Survivin-Borealin (chromosomal passenger complex)

I.2.2. Centromeres

a. Centromere

The short definition of centromere is the point where the two chromatids are linked and where the microtubules attach. It plays as a key role in sister chromatid adhesion, in the kinetochore formation, in the pairing of homologous chromosomes and is also involved in the control of gene expression. Centromeres are highly complex chromosomal substructures involved in essential aspects of chromosome transmission during cell division.

Centromere consists of large arrays of repetitive DNA (e.g. satellite DNA) where the sequence within individual repeat elements is similar but not identical. The complex array of DNA repeats found in human centromeres has suggested a subunit model for the assembly of mammalian centromeres (Zinkowski *et al*., 1991). In fission yeast, centromeric repeats are transcribed into small interference RNA precursors (pre-siRNA) which are processed by Dicer to direct the formation of heterochromatine (Djupedal *et al*., 2005; Hall *et al*., 2003). RNAi recruitment around centromeric chromatin maintains this specialized structure (Hall *et al*., 2003; Volpe *et al*., 2002). The centromeric DNA is normally in a heterochromatin state that is probably essential for its function. Centromeric function depends upon a specialized centromeric organization where distinct domains of CENP A and dimethyl K4 histone H3 are positioned on the near surface of the chromosomes (Greaves *et al*., 2007). But histone variants H2AZ and non histone proteins SMC3 seem also to participate to this peculiar centromere organization that may vary slightly from chromosome to chromosome (Greaves *et al*., 2007; Jansen *et al*., 2007). In all active centromeres, the normal histone H3 is replaced in part with CENP-A, a centromere-specific variant (Sullivan *et al*., 2001). The presence of CENP-A is believed to be important for the assembly of the kinetochore on the centromere and may play a role in the epigenetic inheritance of the centromere site. CENP-A participates to the early organization of centromeric chromatin structure during interphase (Regnier *et al*., 2005). Assembly of new CENP-A nucleosomes is restricted to a demonstrating coupling progression through mitosis and maturation of the next generation of centromere (Jansen *et al*., 2007).

Within the cell nucleus, centromeres undergo changes in their intranuclear localization during the cell cycle. They have been found to associate or colocalize with subnuclear structures, such as the nucleolus or nuclear bodies. Dynamic interactions between centromeres and other nuclear substructures may be important in controlling gene expression as well as centromere function. The structure-function relationships of centromere components and their dynamic interplay within the nucleus will provide insight

into centromere function and may help to understand mechanisms underlying genome instability (Figure 6).

b. Kinetochores

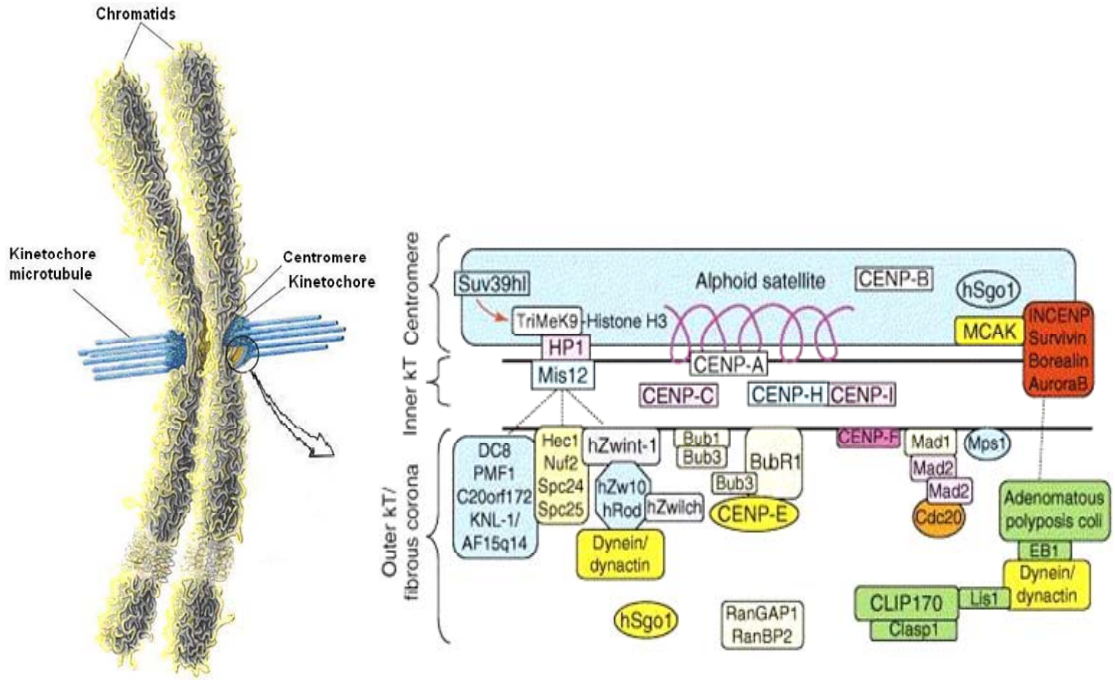

Figure 6. **Organization of the vertebrate kinetochore/centromere showing the locations of some of its component proteins and the relationship between them (**updated from Pluta *et al*., 1995,Rieder and Salmon, 1998), (modified from Chan and Yen, 2003). Red arrow indicates the enzymatic relationship between the trimethyl-lysine 9 modification of histone H3 and the suv39h1 methyltransferase. There are some complexes among these proteins. All protein complexes with their subunits are cartooned as a single shape. The yellow shapes indicate kinetochore MT-binding proteins. The green shapes indicate plus-end MT-binding proteins.

Kinetochores are large protein complexes which assemble on the centromere and link the chromosome to microtubule mitotic spindle during mitosis and meiosis. The kinetochore contains two regions: the **inner kinetochore**, which is tightly associated with the centromeric DNA and provides the foundation for building the kinetochore–microtubule interface and **the outer kinetochore** which interacts with microtubules. In budding yeast, more than 60 kinetochore components have been identified so far by genetic and biochemical approaches and these proteins organize into at least 14 different subcomplexes (McAinsh *et al*., 2003). In animal cells, there are presently more than 80 proteins that show either exclusive or partial localization at kinetochores during mitosis (Gassmann *et al*., 2007) (Figure 6). The kinetochore is a dynamic structure whose composition is cell cycle-dependent. Most kinetochore components assemble in late G2 and prophase whereas others appear after nuclear envelope breakdown leading to competent kinetochores which are able

to engage spindle microtubules (Chan and Yen, 2003). These transient mitotic components leave the kinetochore at different times during mitosis. In contrast, constitutive components, such as the inner kinetochore component CENP-A, are bound to chromatin throughout the cell cycle. They 'mark' the future kinetochore location on the decondensed interphasic chromatin (Maiato *et al*., 2004). Many of these proteins are now known to be directly involved either in microtubule attachment or in the quality control of the attachments (Chan and Yen, 2003).

Since the kinetochore is a complex proteinaceous structure that mediates and monitors the attachment of spindle microtubules to chromosomes, it is essential for chromosome alignment and segregation of sister chromatids. Kinetochores are initially captured in prometaphase by a single microtubule. After kinetochores being captured by microtubules, they are transported along the microtubules toward the spindle pole. This process is regulated by ATP-driven motor proteins of the Kinesin and Dynein families. Each sister kinetochore must eventually attach to K-fibers from opposite poles. This is known as sister kinetochore bi-orientation or amphitelic attachment (Gassmann *et al*., 2007). This process is very important for the correct segregation of genetic information into daughter cells (Tanaka *et al*., 2005).

CHAPTER II: CHROMOSOMAL PASSENGER PROTEINS

II.1. General localization of chromosomal passenger proteins

Chromosomal passenger proteins (CPP) were first described by Cooke *et al.* (1987). To date, five proteins were described: including INCENP (INner CENtromere Protein) (Cooke *et al*., 1987), Aurora B kinase (Bischoff *et al*., 1998), Survivin (Ambrosini *et al*., 1997), Borealin (Gassmann *et al*., 2004) and the fifth protein: Telophase disc 60 TD-60) (Andreassen *et al*., 1991; Martineau-Thuillier *et al*., 1998).

Chromosomal passenger proteins are mostly absent in interphase. They are present, in the nuclear, in late G2 and their expression peaks in G2/M. Chromosomal passenger proteins are characterized by a peculiar localization during mitosis (Figure 7). In prophase, passenger proteins associate along the length of the condensing chromosomes and gradually concentrate at centromeres. At prometaphase and metaphase, they accumulate in the inner centromere. At anaphase onset, they leave the chromosomes and are transferred to the central spindle in association with microtubules. Finally, passenger proteins are concentrated in the midbody during cytokinesis (Cooke *et al*., 1987; Vagnarelli and Earnshaw, 2004).

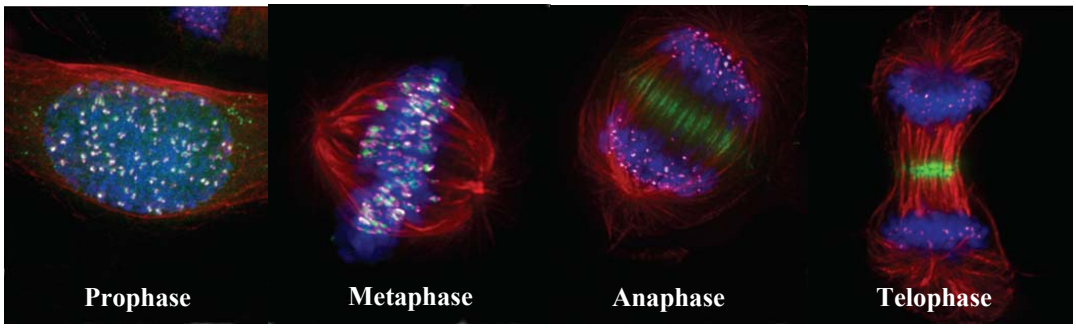

Figure 7: Localization of passenger protein during mitosis. Aurora B (green); kinetochore (pink); α-tubulin (red); DNA (blue). (Modified from Ruchaud *et al*., 2007a).

II.2. Members of chromosomal passenger proteins

Although the chromosomal passenger proteins play together a key role in many processes during mitosis and assemble in complex, they each have their own characteristics. Here we will introduce mostly Aurora B kinase and Survivin, then briefly INCENP, Borealin and TD-60.

II. 2. 1. Aurora B

II.2.1.1. Aurora B: a member of Aurora kinases

Aurora proteins are a family of serine/threonine kinases. This family is conserved form yeast to human. In yeast, there is only one *Aurora kinase* gene. In Drosophila melanogaster and Caenorhabditis elegans, two kinases are present whereas in mammals, this family comprises three members: Aurora A, B and C (Meraldi *et al*., 2004). Aurora kinases play a key role in many processes of cell division and cytokinesis, such as centrosome cycle, spindle assembly, chromosome condensation, microtubule–kinetochore attachment and the spindle checkpoint control, ….

The three mammalian Aurora kinases share similar structure like the catalytic domain flanked by a very short C-terminal tail. But they differ in the length and sequence of the N-terminal domain (Figure 8). Aurora A and B exhibit 70% identity in the carboxyl-terminal catalytic domain (Carmena and Earnshaw, 2003). Aurora kinases present differences in their expression patterns, subcellular localization, and timing of activities. However, all three Aurora kinases are overexpressed in many types of cancer. Aurora kinases are thus potential drug targets for cancer therapy (Carmena and Earnshaw, 2003; Giet *et al*., 2005).

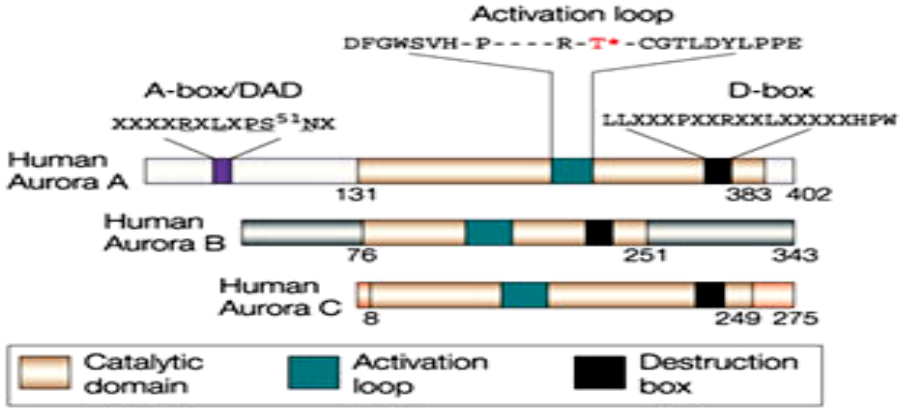

Figure 8: **Three Aurora kinases in human**. They share similar structures, as the catalytic domain, the activation-loop domain and the D-box (Destruction box). Aurora A encompases both the A-box and D-box-activating domains (DAD). These Aurora kinases differ in the length and sequence of their N-terminal domains (Carmena and Earnshaw, 2003). The numbers on the right indicate their respective size in amino acids.

II.2.1.1.1. Aurora A

The original *Aurora* allele was identified in a screen for Drosophila melanogaster mutant, and is now classified as Aurora A (also termed AIK, STK6, or STK15) (Carmena and Earnshaw, 2003; Udayakumar *et al*., 2006). Aurora A is expressed at a low level during

G1 and S phases, and peaks during G2/M phase (Katayama *et al*., 2003). It localizes on the duplicated centrosomes from the end of S phase to the beginning of G1. From metaphase to anaphase, Aurora A is located on the microtubules close to the spindle poles and then concentrates in the midbody during cytokinesis. The expression level of Aurora A falls down during mitotic exit and entry into G1 phase of the next cell cycle (Katayama *et al*., 2003). Aurora A is called the polar kinase due to its presence either on or close to centrosomes (Sugimoto *et al*., 2002).

Aurora-A kinase is necessary for centrosome separation and maturation as well as for spindle assembly (Brown *et al*., 2004; Prigent and Giet, 2003). Aurora-A is first activated, in late G2 phase, on the centrosome and Bora (Aurora Borealis), an Aurora A partner, may be responsible for such activation at mitosis onset (Hutterer *et al*., 2006). By using Clostridium difficile toxin B, Ando *et al.* have shown that Rho GTPases are also involved in centrosomal activation of Aurora-A during G2/M transition (Ando *et al*., 2007). Moreover, Aurora A is also activated by TPX2 on the mitotic spindle (Anderson *et al*., 2007; Bayliss *et al*., 2003; Giet *et al*., 2005), by protein phosphatase inhibitor-2 (I-2) (Satinover *et al*., 2004) and by Ajuba in G2 phase (Hirota *et al*., 2003; Prigent and Giet, 2003). Aurora A is degradated in late mitosis by Cdh1/Fizzy, a related form of the anaphase-promoting complex (APC) (Carmena and Earnshaw, 2003; Giet *et al*., 2005) and this process depends on both intact A- and D-boxes (Nguyen *et al*., 2005).

Mutations of either cysteines 8, 33 and 49 or of phenylalanine 31 in the N terminus of Aurora-A prevent its ubiquitination (Briassouli *et al*., 2006). Aurora A is in complex with PP2A (a protein phosphatase) at the cell poles during mitosis. PP2A dephosphorylates the serine 51 of Aurora A, a residue located in the A box, and thoroughly controls the degradation of Aurora A kinase (Horn *et al*., 2007).

The human *Aurora A* gene lies within the locus 20q13 that is amplified in many forms of cancer. In addition, Aurora A is overexpressed in several solid tumours (Barr and Gergely, 2007; Meraldi *et al*., 2004).

II.2.1.1.2. Aurora B

Aurora B has been identified in Drosophila, in yeast and mammalian cells. It is known as Ipl-1 (Chan and Botstein, 1993), Aurora 1 (Bischoff *et al*., 1998), Aik2 (Kimura *et al*., 1998), Ark2 (Shindo *et al*., 1998), and Aim-1 (Terada *et al*., 1998). The human *Aurora B* gene is located on 17p13.1, very close to p53, in a region often susceptible to allelic loss in tumours. Like Aurora A, Aurora B expression peaks at G2/M phase and maximum kinase activity is reached at transition during metaphase to anaphase (Katayama *et al*., 2003). Aurora B is a chromosomal passenger protein with a dynamic localization. The

domain kinase of Aurora B shares higher identity with Aurora C catalytic domain than with Aurora A domain. A small C-terminal sequence of Aurora B is responsible for its localization and function, but its N- terminal sequence seems not to be required. (Scrittori *et al*., 2005). The role and regulation of Aurora B will be described in details hereafter.

II.2.1.1.3. Aurora C

The unique member of Aurora kinases that has only been described in mammals is Aurora C. To date, little is known about this protein. It was found overexpressed in testis and certain tumour cell lines such as HepG2, HuH7, MDA-MB-453 and HeLa cells (Giet *et al*., 2005; Meraldi *et al*., 2004). The *Aurora C* gene is located on human chromosome 19 (locus 19q13) (Bernard *et al*., 1998). Aurora-B and Aurora-C exhibit a high degree of similarity (92% similarity and 85% identity) within their catalytic domain. Surprisingly, Aurora C was found to co-immunoprecipitate with Aurora B and the behaviour of Aurora-C, like Aurora-B, is typical of chromosomal passenger proteins (Chen *et al*., 2005; Li *et al*., 2004). Moreover, Li *et al.* have also indicated that Aurora-C interacts with INCENP and both cooperate for phosphorylating histone H3 (Chen *et al*., 2005; Sasai *et al*., 2004). Aurora C can also be associated with Aurora B and Survivin *in vivo* (Lens *et al*., 2006; Yan *et al*., 2005) and may even rescue Aurora-B-depleted cells (Giet *et al*., 2005; Sasai *et al*., 2004). Conversely, overexpression of an Aurora C kinase-deficient mutant disrupts the Aurora B-INCENP complex and induces polyploidy (Chen *et al*., 2005). Recently, Aurora C was known to be essential for male fertility in men (Dieterich *et al*., 2007), and mice (Kimmins *et al*., 2007).

II.2.1.2. Aurora B regulation

As in many other kinases, its phosphorylation state directly regulates its kinase activity. Aurora B as well as A is activated by phosphorylation (Walter *et al*., 2000; Murnion *et al*., 2001). In Xenopus leavis, it has been described that Aurora B is phosphorylated at multiple sites throughout the cell cycle. Aurora B seems to be involved in a kinase cascade since it was reported to be regulated by both the Raf kinase inhibitory protein (Eves *et al*., 2006), the checkpoint kinase Chk1 (Zachos *et al*., 2007) and the C. elegans Tousled like kinase (Han *et al*., 2005). Aurora B is also autophosphorylated at Thr 232. This phosphorylation at Thr 232 is an essential regulatory mechanism for Aurora B activation (Yasui *et al*., 2004). Protein phosphatase 1 (PP1), presents in kinetochores, is known to antagonize Aurora B activity (Hsu *et al*., 2000; Sugiyama *et al*., 2002; Trinkle-Mulcahy *et al*., 2003).

The regulation of Aurora B activity depends on its interactions with INCENP and Survivin (these interactions are described in more detail part III.2.1a). Chan and colleagues

(Kang *et al.*, 2001) published that the *in vitro* activity of the budding yeast Aurora B (Ipl1p) is stimulated 10- to 20-fold by the direct binding of the INCENP homolog (Sli15p). INCENP is a specific substrate of Aurora B kinase and in turn, acts as the targeting subunit that localizes Aurora B to kinetochores (Adams *et al*., 2000). The binding of INCENP to Aurora B increases Aurora B kinase activity towards other substrates (Kang *et al*., 2001). Aurora B activation is triggered by autophosphorylation after its association with INCENP (Bishop and Schumacher, 2002). All that explain why INCENP is called a "substrate activator" of Aurora B. Bolton showed that in Xenopus leavis, the addition of recombinant Survivin protein to Aurora B immunoprecipitations stimulates 10-fold the mitotic kinase activity (Bolton *et al*., 2002). Survivin binding could increase Aurora B kinase activity. All these data means that Aurora B kinase activity is stimulated by its association with INCENP and Survivin and is fully active within the whole passenger protein complex. (Andrews *et al*., 2003; Giet *et al*., 2005; Katayama *et al*., 2003).

Recent studies of Monaco *et al.* indicated that Aurora B physically and specially associates with PARP-1 (poly(ADP-ribose) polymerase 1. Moreover, Poly(ADP-ribosyl)ation of Aurora B by PARP-1 inhibits its kinase activity in response to DNA damage (Monaco *et al*., 2005).

Aurora B is degradated by the ubiquitin-proteasome pathway via APC/C. Nguyen *et al.* have indicated that this degradation depends on intact KEN and A-boxes (Nguyen *et al*., 2005). The conserved D-box at the COOH terminus is involved in the specific recognition of Aurora B by APC/C-Cdh1. Kinetics of the degradation of Aurora B by the APC/C-Cdh1 pathway suggest that this degradation may be coupled to the initiation, progression, or completion of cytokinesis (Stewart and Fang, 2005).

II.2.1.3. Aurora B substrates

Aurora B has an over-expanding number of mitotic substrates *in vitro*. These substrates are very diverse, consisting of passenger proteins, chromosome-associated proteins, and proteins involved in cytokinesis as well as microtubule associated proteins (Table2).

Table 2: Aurora B substrates in vertebrates and yeast.
Letters in italic indicate the substrates reported in yeast

Aurora B substrates	Phases in mitosis
Passenger proteins	
Survivin (Wheatley *et al*., 2004)	metaphase
INCENP (Bishop and Schumacher, 2002)	metaphase/anaphase
Borealin (Gassmann *et al*., 2004; Hayama *et al*., 2007)	non determined
Chromosome associated proteins	
Shugoshin (Sgo 1) (Resnick *et al*., 2006)	prophase
Histone H3 (Hsu *et al*., 2000)	G2/metaphase
Histone H2A (Brittle *et al*., 2007)	pro/metaphase
Ndc80 complexe (Cheeseman *et al*., 2002)	*metaphase*
Ndc10p (Biggins *et al*., 1999)	*metaphase*
Boi1 and Boi2 (Norden et al., 2006)	*metaphase*
REC-8 (Buonomo et al., 2000)	*metaphase/anaphase*
CENP-A (Zeitlin *et al*., 2001b)	metaphase
Topoisomerase II alpha (Morrison *et al*., 2002)	metaphase
BubR1 (Lampson and Kapoor, 2005)	metaphase
Tousled kinase TLK-1 (Han *et al*., 2005)	G2/metaphase
Cytoskeleton associated proteins	
Dam1p(Cheeseman et al., 2002)	*metaphase*
MCAK (Andrews *et al*., 2004)	metaphase
MgcRac1GAP (Minoshima *et al*., 2003)	metaphase
Stathmin(Gadea and Ruderman, 2006)	metaphase
MKLP1 (Andrews, 2005)	anaphase
MRLC (Andrews, 2005)	cytokinesis
GFAP (Andrews, 2005)	cytokinesis
Septin 1 (Qi *et al*., 2005)	cytokinesis
Desmin (Goto *et al*., 2003; Kawajiri *et al*., 2003)	cytokinesis
Vimentin (Kawajiri *et al*., 2003)	cytokinesis
Myosin II regulatory light chain (Murata-Hori *et al*., 2000)	cytokinesis

II.2.1.4. Aurora B function

II.2.1.4.1. Aurora B is involvde in chromosome condensation and cohesion

Phosphorylation of histone H3 on serine 10 (Ser-10) is one of the main epigenetic modification of chromatin in mitosis. There is a precise spatial and temporal correlation between this process and the initial stages of chromatin condensation (Hendzel *et al*., 1997; Nowak and Corces, 2004). In mammalian cell lines, a coincidence between chromosome condensation and phosphorylation of histone H3 on another residue, serine 28 (Ser-28), has also been documented (Goto *et al*., 2002). Aurora B has been demonstrated to be responsible for both histone H3 phosphorylation on Ser-10 and Ser-28 during mitosis (Carmena and Earnshaw, 2003; Goto *et al*., 2002). Actually, Aurora B-mediated histone H3 phosphorylation is required for the recruitment of condensin and the other chromosomal

proteins to chromosomes (Adams *et al*., 2001a). Depletion of Aurora B impairs the localization of condensin I on chromosomes and consequently, chromosome condensation (Carmena and Earnshaw, 2003; Lipp *et al*., 2007). Inhibition of Aurora B kinase activity maintains the arms cohesion. In fact, Aurora B and Haspin kinases cooperate to regulate sister chromatid cohesion during mitosis (Dai *et al*., 2006). Moreover, Aurora B and Plk1 are required for cohesin dissociation from chromosome arms, a step leading to sister chromatid separation in anaphase (Gimenez-Abian *et al*., 2004).

II.2.1.4.2. Aurora B in chromosome alignment, separation and segregation

The proper segregation of chromosomes to opposite poles of the cell during mitosis is crucial for maintenance of genetic integrity in eukaryotic cells. For proper chromosome segregation, sister kinetochores must attach to microtubules extending from opposite spindle poles (bi-orientation or amphitelic attachment – Figure 9) prior to anaphase onset. Aurora B kinase inhibition correlates with the mis-orientated chromosomes (Figure 10) due to either the increasing in the formation, or decreasing in the correction, of improper chromosome attachments to the spindle. Activation of Aurora B kinase selectively eliminates improper chromosome-spindle attachments (Kaitna *et al*., 2002; Lampson and Kapoor, 2005).

Recent research suggested that Aurora B with INCENP have crucial roles in promoting amphitelic kinetochore attachment to microtubules. The Aurora B-INCENP complex also triggers the re-orientation of kinetochore-spindle pole connection in a tension – dependent manner and thereby facilitates bi-orientation (Dewar H. *et al.*, 2004; Tanaka T. U., 2005). Because the syntelic attachment does not generate tension on kinetochore-to-pole connections, the complex promotes reorientation of these connections (probably by phosphorylation of kinetochore components). When amphitelic attachment is established, tension is applied on kinetochore-to-pole connections, and thereby the complex stops promoting their reorientation, leading to preferential selection of the amphitelic mode of attachment. In yeast, Sandall *et al.* proposed a model for tension-regulated Aurora B activation by the Survivin-INCENP. The linkage between centromeres and microtubules acts as a tension sensor that activates Aurora B in the vicinity of incorrect syntelic attachments. Active Aurora B phosphorylates multiple targets of core attachment to dissociate the centromere from the microtubule. Conversely biorientation (tension) silences Aurora B activation, stabilizing the correct configuration (Sandall *et al*., 2006).

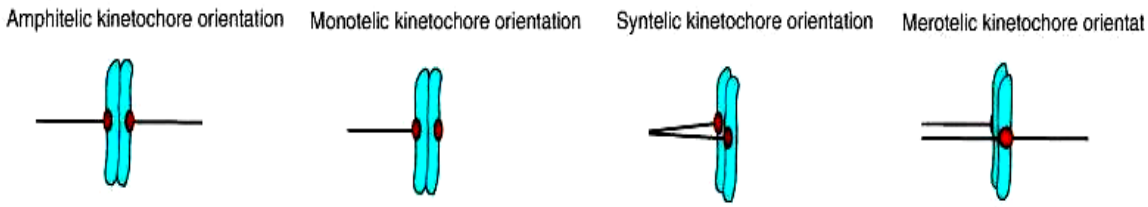

Figure II. 9: The model of kinetochore-microtubule attachments. Aurora B promotes the amphitelic kinetochore orientation. The mis-orientation, that consists in either monotelic or syntelic or merotelic kinetochore orientation, can lead to defects in chromosome segregation (Cimini and Degrassi, 2005).

It was recently shown that Aurora B is also required to correct syntelic attachments in mammalian cells (Hauf *et al*., 2003) (Figure 10.1). Aurora B kinase phosphorylates key substrates at the kinetochore and promotes the turnover of kinetochore microtubules (Cimini, 2007) (Figure 10.2). Therefore, Aurora B kinase ensures chromosome bi-orientation in a conserved manner from yeast to mammalian cells (Tanaka *et al*., 2005).

Aurora B is very important factor for the spindle assembly checkpoint. It activates the spindle checkpoint in response to a lack of kinetochore tension and maintains checkpoint signalling on (Hauf and Watanabe, 2004). The presence of massive amounts of lagging chromatin at anaphase/telophase is highly characteristic for the loss of either INCENP and/or Aurora B function(s). This lagging chromatin might represent a failure in chromosomes movement under the physical stress of anaphase onset (Adams *et al*., 2001a).

Since Aurora B is required both for the proper chromosome segregation by destabilizing incorrect kinetochore–microtubule interactions, it is also essential for maintaining the spindle assembly checkpoint in the absence of tension. The loss of spindle checkpoint response to tension is also found in cells treated with Aurora B inhibitors: ZM447439 or Hesperadin (Ditchfield *et al*., 2003; Hauf *et al*., 2003).

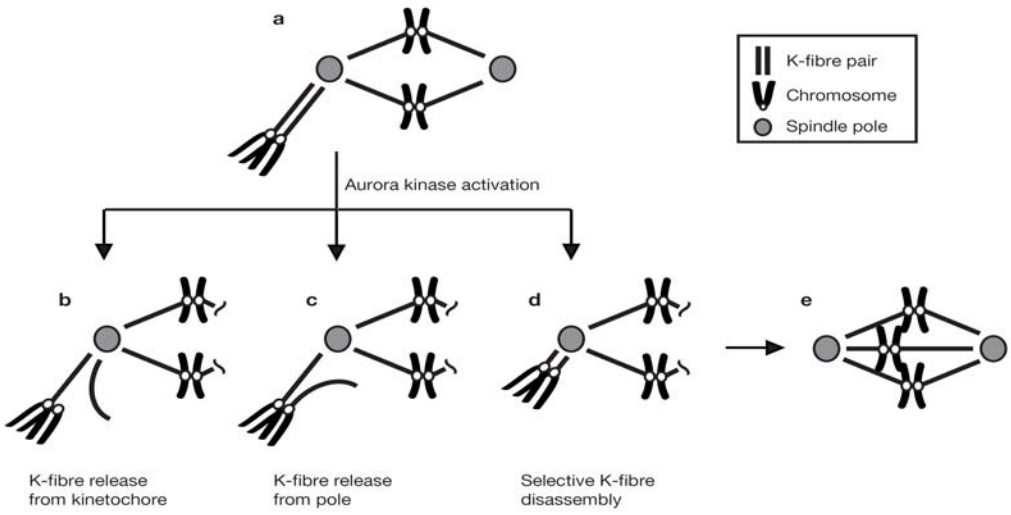

Figure10.1: A model of the mechanisms to correct syntelic chromosome mal-orientations during cell division (Lampson *et al*., 2004). (**a**) Syntelic mis-orientation arises during mitosis if K-fibres attach sister kinetochores to the same spindle pole. (**b**, **c**) Initial K-fibre release hypothesis: Aurora kinase activity may correct mis-orientation by K-fibre release from either the kinetochore (**b**) or the spindle pole (**c**). (**d**) Mis-orientated chromosomes move to the pole as K-fibres selectively disassemble. K-fibres initially remain attached to both kinetochores and the spindle pole. Lampson *et al* propose that capture by microtubule(s) from the opposite pole results in alignment at the metaphase plate (**e**).

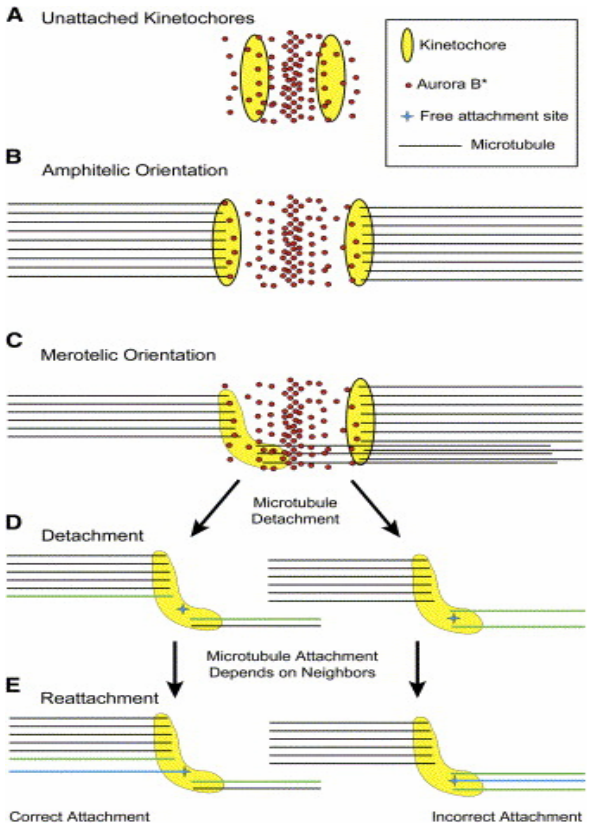

Figure10.2. Aurora B contributes to the correction of merotelic attachments by promoting microtubule destabilization
A destabilization gradient is produced by the kinase activity of Aurora B bound or recently dissociated from the inner centromere (Aurora B*).
For merotelic kinetochores (C), the attachment sites connected to the incorrect pole are positioned close to the region of high Aurora B*, which can thus induce microtubule destabilization and leave empty attachment sites (blue stars in [D]).
As diagrammed in (D) and (E), the probability of reattachment to the correct or incorrect pole depends on the nearest neighbors (microtubules highlighted in green). If microtubules on either side are to one pole, then it will be probable that the attachment will be to a microtubule (blue) from the same pole ([E], right). If nearby attachments are to opposite poles ([D], left), then the new attachment (blue microtubule) will be equally likely to be to one pole or the other (attachment to the correct pole is shown in the Figure) (Modified from Cimini, 2007).

II.2.1.4.3. Aurora B is essential for cytokinesis

Aurora B regulates and phosphorylates type-III intermediate filaments such as vimentin, GFAP and desmin. Aurora B phosphorylates specifically one site in desmin – a protein of the cleavage furrow that contributes to the destabilization of intermediated filaments, which are essential for the last stage of cytokinesis (Kawajiri *et al*., 2003). Mutation of the Aurora B phosphorylation site in desmin leads to defect in filament formation and segregation, and blocks the final stage of cytokinesis (Goto *et al*., 2003; Kawajiri *et al*., 2003). Aurora B also phosphorylates myosin II regulatory light chain. Inhibition of Aurora B causes the loss of myosin II localization and the disruption of the midzone organisation (Straight *et al*., 2003). Aurora B may coordinately regulate many aspects of cytokinesis, including choice of division plane via the regulation of cortical microtubule dynamics (Canman *et al*., 2003); furrow ingression by the regulation of acto-myosin force generation; stability of the spindle midzone; and possibly cell scission via regulation of the activity of small GTPases and intermediate filaments.

Cytokinesis must be coordinated with chromosome separation and segregation in order to ensure the maintenance of an intact genome. Therefore, it proceeds only after all chromosome arms are pulled out of the cleavage plane. In yeast, Aurora B seems to control and connect the completion of chromosome segregation to the onset of cytokinesis through the "NoCut" signal pathway which delays the completion of cytokinesis in cell with spindle-midzone defects (Norden *et al*., 2006).

In summary, Aurora B kinase plays several important functions in mitosis. It is involved in the condensation and cohesion of chromosomes. Aurora B is also a mitotic checkpoint kinase and is essential for cytokinesis. It has emerged as a critical coordinator of events on the cytokinesis pathway (Meraldi *et al*., 2004; Norden *et al*., 2006).

II.2.2. Survivin

II.2.2.1. Survivin: a member of the IAP family

Inhibitors of apoptosis proteins (IAPs) were originally identified as baculoviral products that could inhibit the defensive apoptotic response of infected insect cells. Members of this family are structurally characterized by the presence of 1-3 copies of a 70 amino acid zinc- finger fold motif called the Baculovirus IAP Repeat (BIR) (Altieri, 2006). Other structures were found in certain IAPs including a RING finger, an ubiquitin-conjugating domain and a nucleotide-binding P loop motif (Li *et al*., 1998). Some of IAPs are able to inhibit caspase-mediated apoptosis directly or indirectly (Altieri, 2003) .

Survivin is a unique member of the IAP gene family that has a single type II BIR domain in the N-terminal, an extended-COOH terminal α-helix, and the C-terminal region predicted as a coiled-coil domain (Li *et al*., 1998). Survivin is also different from the other members of this family because it lacks the typical RING finger domain that is essential for the anti-apoptotic activity of IAPs (Lens *et al*., 2006). Crystallography data predict that Survivin, in the absence of partners, behaves like a dimer (Chantalat *et al*., 2000) (Figure 11). Recent studies of Jeyaprakash *et al*. sugested that during interphase, Survivin may be present as a homodimer in the cytoplasm whereas it is in heterotrimeric complex during mitosis(Jeyaprakash *et al*., 2007). Both the BIR domain and the COOH-terminal α-helices contribute to the proper dynamic redistribution of Survivin during mitosis (Skoufias *et al*., 2000). The C-terminus of Survivin is necessary and sufficient for the targeting to the central spindle and midbody, whereas the presence of an additional BIR domain is needed to centromeric localization (Lens *et al*., 2006).

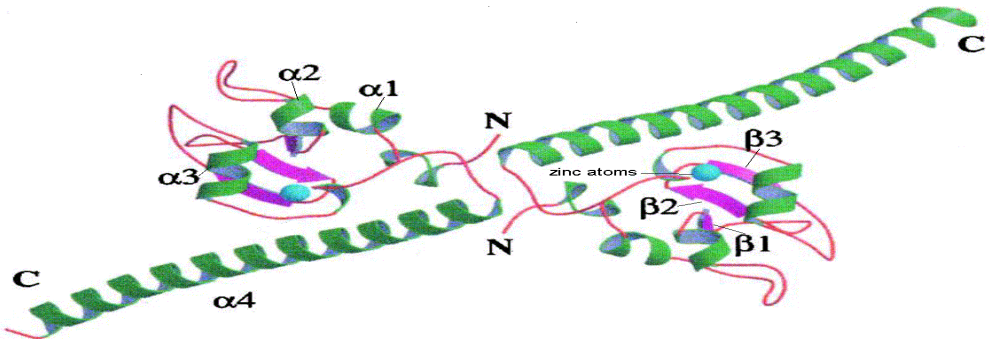

Figure 11: **Survivin is a dimer**. There are four helices (α1, α2, α3, α4) and three β-strands in a monomer Survivin. The interaction between two monomers locates mainly in the N-terminal region, the linker region and the N terminus of helix α4. The C-terminal helices contain hydrophobic clusters with the potential for protein–protein interactions. (Modified from Chantalat *et al*., 2000)

II.2.2.2. Survivin isoforms

The *survivin* gene locus gives rise to alternatively spliced transcripts, (Conway *et al*., 2000). Four splice variants of human Survivin have been described. They are named Survivin-2α, Survivin-2B, Survivin-Δ-Ex3 and Survivin-3B (Caldas *et al*., 2005) (Figure 12). Survivin-2B is generated by the insertion of an alternative exon, exon 2B. Survivin-Δ-Ex3 arises from skipping of exon 3. Survivin-3B results from the introduction of a novel exon 3B. The newest isoform discovered by Caldas H. *et al.* is Survivin-2α in which the α-helical coiled-coil domain of Survivin is lacking (Caldas *et al*., 2005).

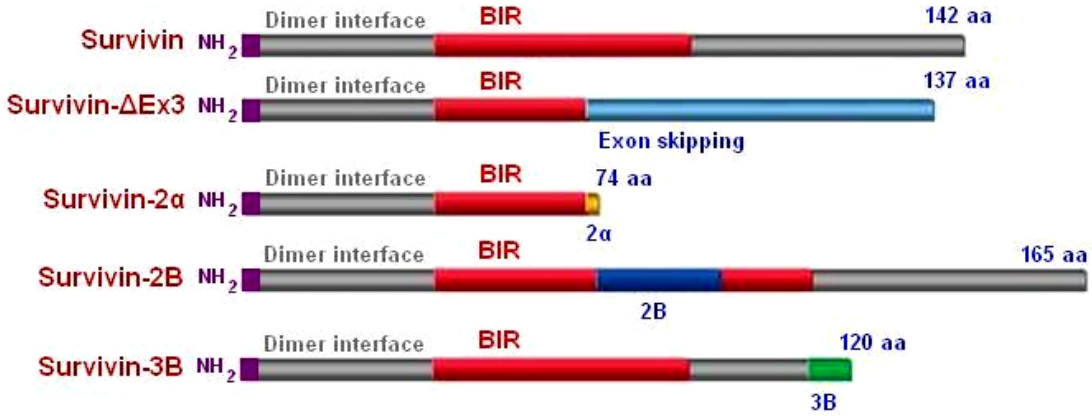

Figure 12: **Survivin variants**. All variants share common features at their N terminus that include the dimer interface and the BIR domain. The microtubule binding and nuclear export sites are located at the C-terminal of wild type Survivin. Organization of Survivin and its variants varies by either the insertion of an alternative exon (Suvivin-2B) or the removal of exon 3 (Survivin-Δ-Ex3). (Modified from Li and Ling, 2006).

Little is known about the different functions or specific expressions of the Survivin splice variants and how they may interact with the other proteins. Survivin-2α has the potential to attenuate the anti-apoptotic effect of Survivin (Caldas *et al*., 2005). Survivin-2B is proposed as a pro-apoptotic protein that sensitizes resistant leukaemia cells to chemotherapy in a p53-dependent process (Zhu *et al*., 2004). Survivin-Δ-Ex3 functions as an anti-apoptotic protein and is upregegulated in malignancies (Mahotka *et al*., 2002; Caldas *et al*., 2005). Survivin splice variants have differential subcellular localizations. Survivin and Survivin-2β localize to the cytoplasm while Survivin-Δ-Ex3 is mostly in the nucleus (Mahotka *et al*., 2002). Survivin splice variants can interact with Survivin wild type (Caldas *et al*., 2005) but there is no evidence that these isoforms play a role in mitosis (Noton *et al*., 2006).

II.2.2.3. Survivin regulation

II.2.2.3.1. The regulation of Survivin expression

Survivin is a relatively short-life protein with a half life of about 30 minutes. Survivin expression is cell cycle-regulated with the highest level in G2/M and the lowest in G1. Transcriptional mechanisms controlling Survivin gene expression involve CDE/CHR G1 receptor elements in the Survivin promoter (Altieri, 2003). Moreover, Jiang Y. *et al.* have indicated that Survivin is regulated by the RB/E2F family of proteins. Both pRB and p53 can interact with the Survivin promoter and repress Survivin transcription. It is hypothesized that multiple alterations within these pathways, such as deletions or mutation involving pRB, p16, p21 or amplifications of Cdk4, may be responsible for some of the downstream events regulating Survivin expression (Jiang *et al*., 2004). The stability of

Survivin is regulated by AIP (the Aryl hydrocarbon receptor-interacting protein). This protein interacts with Survivin at the C-terminal α-helical coiled-coil region (Kang and Altieri, 2006).

Survivin degradation is regulated by the ubiquitin-proteasome pathway in a cell cycle manner. Survivin level is kept low at interphase by this pathway (Altieri, 2003; Vong *et al*., 2005). Vong *et al.* have also shown that Survivin is ubiquitinated at several lysine residues (K48 and K63).

II.2.2.3.2. Survivin regulation in mitosis and cell death

Survivin behaviour at centromeres is also regulated by ubiquitin (Figure 13). Linkage of ubiquitin proteins via lysine 48 (K48) is involved in protein degradation, whereas ubiquitination via lysine 63 (K63) is important for regulating the localization of the protein. In mitotic cells, Survivin was found ubiquitinated at both K48 and K63. If K63 ubiquitination of Survivin is too low the protein cannot accumulate at the centromeres, in contrary, high ubiquitination makes Survivin stucky at centromeres. The correct balance between K63-linked ubiquitination promoted by p97-NP14-Ufd1, and deubiquitination by hFAM, ensures the accurate localization of Survivin in space and time at centromere and finally properly regulates chromosome spindle attachments (Earnshaw, 2005; Vong *et al*., 2005).

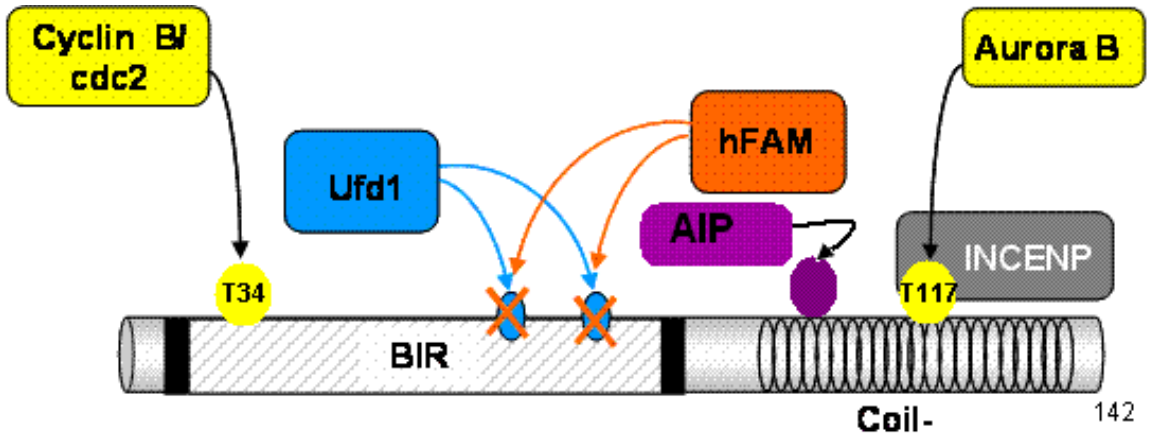

Figure 13: Post-transcriptional modifications of Survivin. Survivin is regulated by post-transcription modifications: the phosphorylation by the complex Cyclin B/Cdc2; the phosphorylation by Aurora-B kinase; and the cycle of ubiquitination/deubiquitination by Ufd1 and hFAM (Modified from Marlène Delacour-Larose's thesis).

Survivin is regulated by phosphorylations (Figure 13). It is a mitotic substrate of $p34^{cdc2}$-cyclin B1. It was found associated with the cyclin dependent kinase $p34^{cdc2}$ on the mitotic apparatus and is phosphorylated at Thr 34 *in vivo* and *in vitro* (O'Connor *et al*., 2000). Phosphoproteome analysis also predicts that Survivin is phosphorylated on Thr 34

(Nousiainen *et al*., 2006). This phosphorylation can increase the affinity of Survivin for active caspase-9 or, alternatively, stabilizes Survivin or Survivin-caspase-9 anti apoptotic complex at the midbody during cell division (O'Connor *et al*., 2000).

By using an *in vitro* kinase assay with recombinant GST-Aurora-B and GST-Survivin, Wheatley S. P. *et al.* have shown that Survivin is specifically phosphorylated by Aurora B at Threonine 117 in its carboxyl α-helical coil. By FRAP experiment, our laboratory has found that Survivin is mobile on centromere and immobile on microtubules. Moreover such mobility depends on Aurora kinase activity. Pointing thus that Survivin mobility may be due to its phosphorylation by Aurora B (Delacour-Larose *et al*., 2004).

II.2.2.4. Role of Survivin in cell division

II.2.2.4.1. Survivin: a mitotic regulator

The name "Survivin" reflects its essential role in cell cycle. In fact, in mouse, at embryonic day (E) 3.5, homozygous deletion of the Survivin gene resultes in a catastrophic defect of microtubule assembly, with the absence of mitotic spindle, the formation of multinucleated cells and then, one hundred percent embryonic lethality by E 4.5 (Altieri, 2003; Uren *et al*., 2000).

Survivin is a mitotic regulator, essential for cellular proliferation. Survivin-deficient cells were arrested in G1 after a defective cell division. This arrest occurs in a p53-dependent manner (Yang *et al*., 2004).

Survivin depletion causes an accumulation of prometaphase cells with defects in chromosome congression. Repression of Survivin sustains a prometaphasic block and delays mitosis. Cells can continue to divide after a stop in prometaphase, but are unable to align their chromosomes at the metaphasic plate, and consequently lagging chromatids are observed (Carvalho *et al*., 2003; Lens *et al*., 2003). Yang *et al.* have indicated that anaphase-lagging chromosomes in Survivin depleted cells are due to merotelic kinetochore attachment. So, Survivin is required for equal segregation of sister chromatids (Yang *et al*., 2004).

Survivin is also required for the proper functioning of the spindle checkpoint. It is essential for sustaining spindle checkpoint arrest in response to a lack of tension. Knocking down Survivin expression abrogates the mitotic arrest induced by taxol. In fact, Survivin is not required for the binding of BubR1- one of spindle checkpoint proteins (see part I.2.1c)- to kinetochores but for the maintenance of BuR1 at kinetochores during persistent activation of the checkpoint (Carvalho *et al*., 2003; Lens *et al*., 2003).

Survivin depletion also leads to failure in cytokinesis. This results in a gradual increase in the percentage of multinucleated or polyploidy cells, reflecting thus a defective checkpoint. Moreover, in budding yeast, mutants of Bir1p, the homologue of Survivin, affects Septin organization (Gillis *et al*., 2005; Thomas and Kaplan, 2007). Septins have been shown to form a diffusion barrier in anaphase. Such a barrier helps to ensure the proper localization of the membrane and cell wall synthesis machineries required for cytokinesis. Defects in Septin organization lead to the diffusion of these proteins away from the mother bud neck and therefore, to cytokinesis failure (Dobbelaere *et al*., 2000; Longtine and Bi, 2003). Bir1 is also required for the transportation of Ncd10p, a member of CBF3 complex, which is responsible for the splitting of the Septin ring during cytokinesis (Bouck and Bloom, 2005). All these phenotypes confirm that Survivin is a mitotic protein and acts as a regulator in mitosis.

II.2.2.4.2. Survivin functions in microtubule behaviours

Beltrami *et al.* have shown that a pool of Survivin associates with polymerized microtubules. Homozygous depletion of Survivin results in the nearly complete absence of mitotic spindle and the appearance of disorganized tubulin bundles (Okada *et al*., 2004; Uren *et al*., 2000). Depletion of Survivin by RNA interference increases the number of centrosome-nucleated microtubules and promotes microtubule instability by raising the incidence of mitotic catastrophe (Rosa *et al*., 2006). Conversely, overexpression of Survivin stabilizes microtubules and shortens metaphase pole-to-pole distance (Giodini *et al*., 2002). So, Survivin functions as a novel regulator of microtubule dynamics and microtubule nucleation throughout the cell cycle. Moreover, this regulation is independent on Aurora B kinase expression and activity (Altieri, 2006; Rosa *et al*., 2006).

Survivin may have crucial role in spindle formation (Figure 14). Alterie D. C. has proposed a model in which Survivin functions in genetic fidelity and spindle formation. In this model, Survivin regulates microtubule dynamics at kinetochores via chromosomal passenger complex and spindle formation via an independent pool of proteins not associated with passenger proteins.

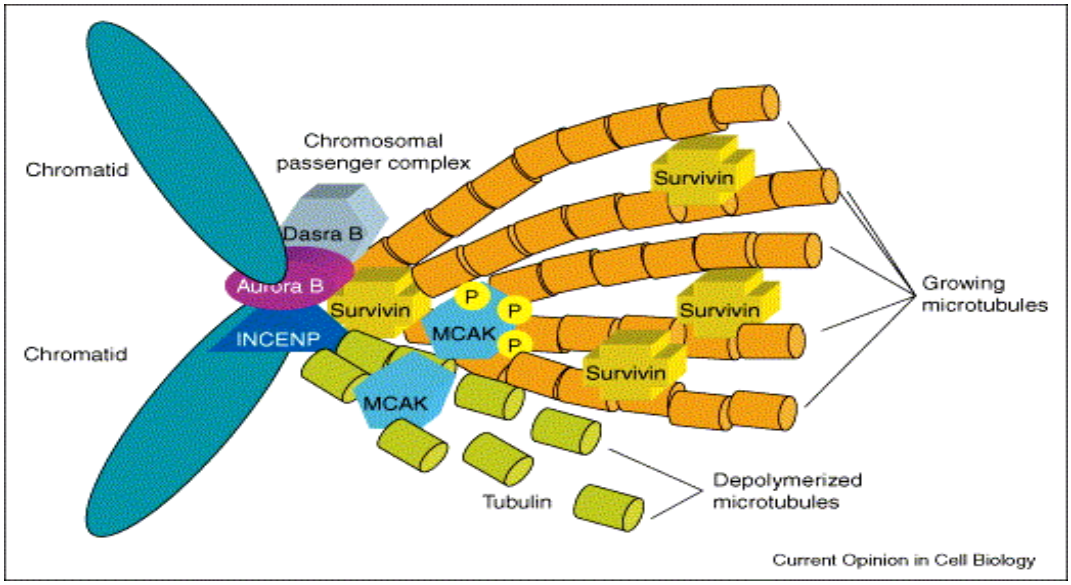

Figure 14: A model for Survivin functions in genetic fidelity and spindle formation. Survivin regulates microtubule dynamics at kinetochore via the chromosome passenger complex and spindle formation via an independent pool not associated with passenger proteins. Aurora B phosphorylation of MCAK prevents its microtubule destabilizing activity and promotes spindle formation (Altieri, 2006).

II.2.2.4.3. Survivin and its role in the chromosomal passenger complex

Survivin is a chromosomal passenger protein (Vagnarelli and Earnshaw, 2004). It is a member of chromosomal passenger complex (CPC), which plays a key role in cell division and cytokinesis. There is a strong interaction between these chromosome passenger proteins in the complex. Survivin interacts with the other passenger proteins to target the chromosomal passenger complex to centromere and midbody (Adams *et al*., 2001b). BIR domain within Survivin dictates centromere targeting in (pro)metaphase whereas the C-terminal coiled-coil domain targets to central spindle and midbody localization in anaphase and telophase (Lens *et al*., 2006). Survivin is also responsible for the association of the CPC with microtubules since it allow the localization of other passengers (Vader *et al*., 2006). Interaction between Survivin and Crm1 (export receptor *C*hromosome *r*egion *m*aintenance 1) is also required for the correct localization and function of the CPC (Knauer *et al*., 2006).

II.2.2.5. Role of Survivin in cell death

Survivin has been proposed to inhibit apoptosis during mitosis. Belonging to the IAP family Survivin has ability to block apoptosis induced by a variety of death signals such as Bax and Fas (Tamm *et al*., 1998). But its role in antagonizing apoptosis is not clear because Survivin do not bear structural motifs that in other IAPs mediate caspase binding. However, loss or interference with Survivin causes spontaneous cell death and enhances cell-death stimuli (Wheatley and McNeish, 2005). The anti-apoptotic function of Survivin has been

demonstrated *in vivo* using genetically engineered animals (Altieri, 2003). Down-regulation of survivin expression increases apoptosis (Chen *et al*., 2000). Recent studies imply that IAPs mechanism of action is only rarely involved in direct caspase inhibition (Altieri, 2006). Moreover, the cellular requirements of Survivin cytoprotection have been worked out in detail. Only Survivin which accumulate in mitochondria are enriched in response to cellular stress and rapidly discharged into cytosol after cell-death stimulation (Dohi *et al*., 2004). It means that Survivin inhibits apoptosis in a way which is independent and different from its role in mitosis (Altieri, 2006).

II.2.3. INCENP (Inner CENtromeric Protein)

The first discovered chromosomal passenger protein is INCENP. It was identified in a monoclonal antibody screen for novel components of the mitotic chromosome scaffold as a 130 kDa protein (Cooke *et al*., 1987; Vagnarelli and Earnshaw, 2004). Expression of various INCENP truncation constructs in culture cells has delineated specific domains of INCENP that are required for its association with microtubules, centromeres, and the central spindle (Bishop and Schumacher, 2002). Studies in higher eukaryotes have shown that the INCENP N-terminal domain contains an essential sequence required for both centromere targeting and the transfer from the chromosomes to the spindle at anaphase onset (Bishop and Schumacher, 2002; Ainsztein *et al*., 1998; Mackay *et al*., 1998). INCENP peculiar feature is a region predicted to form a coiled-coil structure. This domain is required for INCENP association with spindle microtubules (Mackay *et al*., 1993). The carboxyl terminus region of INCENP contains a highly conserved region known as IN-box (Ainsztein *et al*., 1998). Three residues (T892, S893, and S894) in this region correspond to possible phosphorylation sites by Aurora-B kinase**.** These sites are criticalt for INCENP function. Thirty-three phosphorylation sites were identified in INCENP by phosphoproteome analysis (Nousiainen *et al*., 2006).

INCENP binds HP1 (heterochromatin protein 1) by its N-terminal region. HP1, a chromosomal adapter protein, can bind to core nucleosomes containing methylated histone H3 (Lachner *et al*., 2001). So, HP1 may act as a "docking site" for INCENP on inactive chromatin (Adams *et al*., 2001a) (Figure 15).

INCENP is not only phosphorylated by Aurora B but also by Cdk 1; Thr 59 and Thr 388 are two major Cdk 1 phosphorylation sites on INCENP. These phosphorylations are involved in the recruitment of Plk1 (Polo-like kinase 1) to the kinetochore. The complex formation of Plk1 and INCENP is required for the metaphase-anaphase transition (Goto *et al*., 2006). INCENP is also necessary for the recruitment of MKLP1 (mitotic kinesin-like protein) to the spindle midzone/midbody, a crucial step for midbody formation and completion of cytokinesis (Zhu *et al*., 2005).

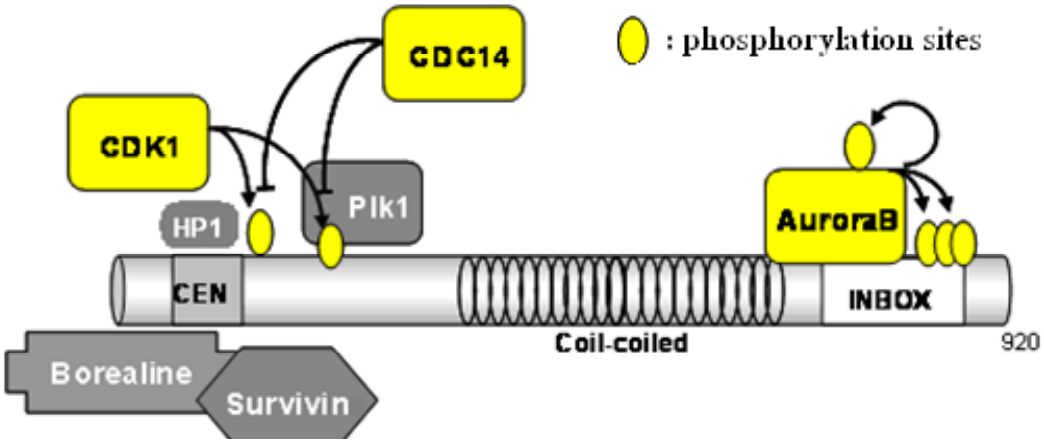

Figure15: INCENP and interacting proteins. INCENP interacts with HP1, Cdk1, Plk, Borealin and Survivin in its N-terminal region. Aurora B interacts in the carboxyl terminal region. This drawing also indicates the phosphorylation sites on INCENP that are phosphorylated by Aurora B (T893, S894, T895) and Cdk1 (Thr59 and Thr 388) (from Marlène Delacour-Larose's thesis).

II.2.4. Borealin/Dasra B: a novel chromosomal passenger protein

Borealin was discovered simultaneously in human cell lines and Xenopus extracts. During mitosis, it was found to share localization with the passenger proteins (Gassmann *et al*., 2004; Sampath *et al*., 2004). As Aurora B, INCENP and Survivin, Borealin level significantly increases in mitosis (Gassmann *et al*., 2004). Borealin, a 31 KDa protein, exhibits two external N and C terminus domains conserved among vertebrates.

Like Survivin, Borealin can oligomerize *in vitro* (Klein *et al*., 2006; Vader *et al*., 2006). Borealin binds to INCENP, Survivin and itself *in vitro*. The first ninety-two N-terminus amino acids are required for its interaction with Survivin (Chang *et al*., 2006). Recent data demonstrate that Borealin interacts with the N-terminal fragment of INCENP (from residues 1-58) (Klein *et al*., 2006) and that it is phosphorylated by Aurora B (Gassmann *et al*., 2004). So, Borealin and Survivin may act together for localizing INCENP and Aurora B (Lens *et al*., 2006) and for recruiting the chromosomal passenger proteins to the centromeres (Vader *et al*., 2006).

II.2.5. TD-60 telophase disc 60

TD-60 was first identified by a human autoimmune serum (JH serum) that contains prominent antibodies to a 60 kDa protein which behaves like the passenger proteins (Andreassen *et al*., 1991). At telophase, it is present at the spindle midzone also termed a "telophase disk". Therefore, the 60 KDa protein was named TD-60 (telophase disk-60 KDa) (Burke and Stukenberg, 2003).

TD-60 is a member of the RCC1 family. The sequence contains seven repeats that are homologous to the RCC1 family of guanine nucleotide exchange factors (GEF) (Burke and Stukenberg, 2003). TD-60 activates the small G protein Rac 1 which is involved in Stathmin activity in mitosis. Stathmin is a substrate of Aurora B (Gadea and Ruderman, 2006), so TD-60 may be either a regulator or an effector of Aurora B kinase. Stathmin is also a microtubule-destabilizing protein which regulates microtubule dynamics in the mitotic spindle. This suggests that TD-60 may be involved in microtubule regulation through Stathmin activity in prometaphase.

TD-60 has an important role in the integration between kinetochore and mitotic spindle. Its suppression activates the spindle assembly checkpoint and prevents progression to metaphase. Loss of TD-60 can affect the localization of passenger proteins in mitotic cells (Mollinari *et al*., 2003). TD-60 and INCENP are co-localized at any time, from G2 to the end of mitosis and they may cooperate in cleavage signalling during cytokinesis (Wheatley and Wang, 1996). Furthermore, Borealin is required for the proper localization of TD-60 in mitotic cells (Gassmann *et al*., 2004). All these data show that there is a strong functional link between TD-60 and the other passenger proteins. But does TD-60 form a complex with other passenger proteins? To date, there is still no evidence about the physical association between TD-60 and the other chromosomal passengers. The role of TD-60 in prometaphase progression suggests a function that may be at least partially independent on the other passenger proteins (Mollinari *et al*., 2003).

II.3. Chromosomal passenger complex CPC)

These chromosome passenger proteins share the same localization all over mitosis so their function may be interdependent. In fact, the passenger proteins are present in a complex, which includes at least INCENP, Aurora B, Survivin and Borealin. To date, Aurora B kinase is the only passenger protein with enzymatic activity. The binding of the non-enzymatic proteins with their enzymatic partner is essential for the function of the complex (Vader *et al*., 2006). Cells may have two chromosomal passenger complexes: in one hand INCENP, Aurora B, Borealin, Survivin holocomplex and in the other hand INCENP–Aurora B (Gassmann *et al*., 2004). However, in CPC depleted *Xenopus laevis* extracts, the co-translation of Aurora B, INCENP and Survivin failed to induce the correct localization of Aurora B to kinetochores. Moreover, in the cells depleted with one of passenger proteins by RNAi, there is a diffuse chromatin localization of the passenger proteins and a decrease of all the passenger proteins. Passenger protein depleted cells either stop in mitosis or become polyploid.

Hereafter we will describe the strong interactions between the passenger proteins among the complex and their functions in cell division.

II.3.1. Interaction between passenger proteins in the CPC

II.3.1.1. The links between passenger proteins in the complex

The first described interaction between chromosomal passenger proteins in the complex is the relationship between Aurora B and INCENP; they were found to be in complex in Xenopus egg extracts (Adams *et al*., 2000), in C. elegans (Kaitna *et al*., 2002). Such interactions are also confirmed by GST-pull down experiments (Adams *et al*., 2000). INCENP and Aurora B functions are tightly interlinked. Aurora B binds to INCENP carboxyl terminus and also phosphorylates INCENP in this region (Bolton *et al*., 2002; Honda *et al*., 2003). INCENP binding enhances Aurora B activity towards other substrates and stimulates the autophosphorylation of Aurora B at Thr232 (Carmena and Earnshaw, 2003). It means that INCENP has the ability to up-regulate Aurora B kinase activity (Figure 16). Both INCENP and Aurora-B kinase were found to be phosphorylated on a tyrosine residue. Although Tyr-73 in INCENP is not conserved among vertebrates, the Aurora-B residue (Tyr-12) is clearly conserved in mouse and *Xenopus laevis*. This finding suggests that the chromosomal passenger complex could be regulated by a tyrosine kinase or a dual-specificity kinase (Nousiainen *et al*., 2006).

Taking part into Aurora B-INCENP complex is Survivin. The ternary complex of INCENP, Aurora B and Survivin was first isolated from *Xenopus leavis* egg extracts (Bolton *et al*., 2002; Vagnarelli and Earnshaw, 2004). INCENP is described as a docking protein that allows Survivin and Aurora B interaction (Figure16).

In a two-hybrid assay as well as in pull-down experiments, it has been reported that *in vitro* Survivin and Aurora B interact directly. The proximal BIR domain of Survivin was responsible for Survivin binding to Aurora B. Survivin mutant (Surv-DD70, 71AA) disrupts the interaction of Survivin with Aurora B and causes multinucleation in HeLa cells. The binding of Survivin is required for full Aurora B activity (Cao *et al*., 2006; Honda *et al*., 2003). Aurora B kinase activity is thus regulated by both INCENP and Survivin binding (Bolton *et al*., 2002).

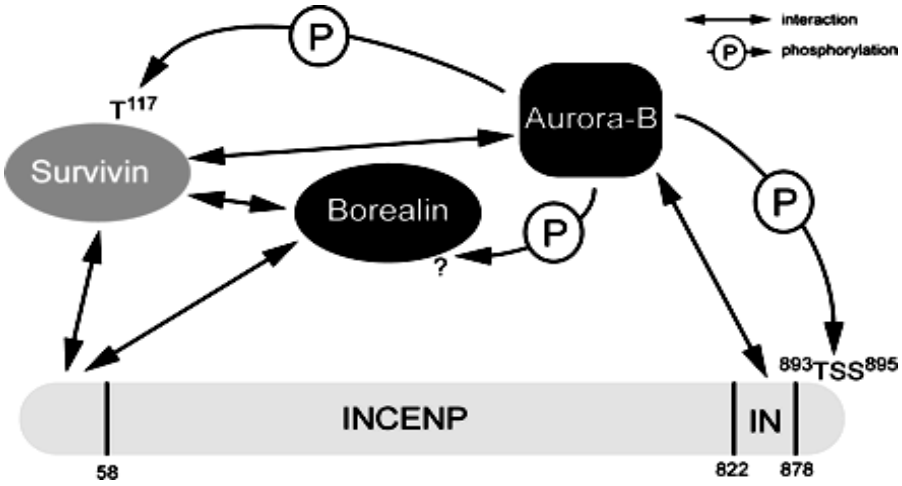

Figure 16: **Interaction between passenger proteins in the complex**. INCENP, a docking protein, interacts with all the other passenger proteins in different region of its sequence. INCENP, Survivin and Borealin are the substrates of Aurora B, the only enzymatic passenger protein known todate (Vader *et al*., 2006).

The last member of the complex is Borealin. In the precipitated immunocomplexes, The 1–92 N-terminal amino-acid residues of Borealin forms a complex with Survivin (Chang *et al*., 2006). Moreover, essentially all Survivin in mitotic cells is associated with Borealin (Vagnarelli and Earnshaw, 2004). Borealin also binds INCENP and itself, but neither Aurora B nor TD-60 (Gassmann *et al*., 2004). (Figure 16). Recent data showed that Borealin can interact with double-strand DNA *in vitro* (Klein *et al*., 2006).

Very recently, Jeyaprakash *et al* have reported that the minimal domains of INCENP, Survivin and Borealin can form a ternary complex. In this complex, Survivin is present as a monomer and its dimerization interface is occupied by Borealin. Jeyaprakash *et al*. have shown that the association of the minimal domain of these proteins occurs via a three α helix bundle, one helix from each protein (Jeyaprakash *et al*., 2007) (figure 17).

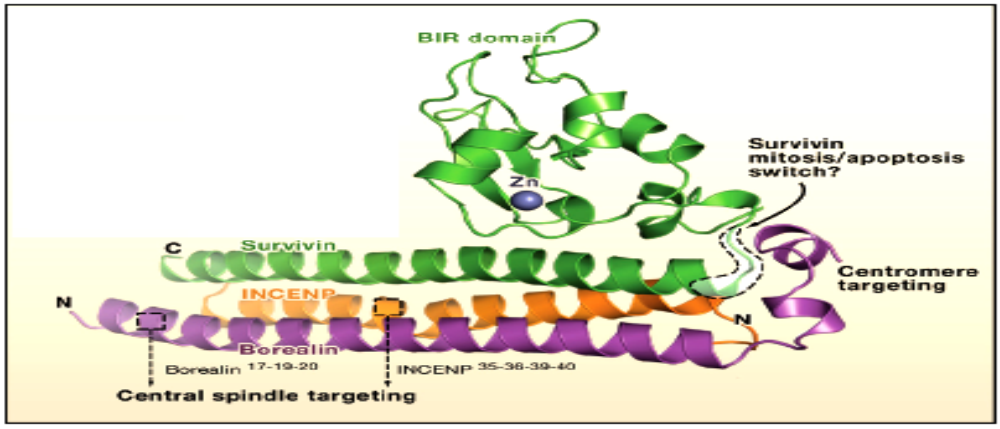

Figure 17. Survivin , INCENP and Borealin form a ternary complex. Survivin has a Zinc-binding globular domain (BIR domain) connected to the long C-terminal helix (C helix). Borealin and INCENP interact with the Survivin C helix to form a triple helical bundle. (Modified from Jeyaprakash *et al*., 2007; Ruchaud *et al*., 2007b).

II.3.1.2. The interaction between passenger proteins is required for the right localization of the CPC

The correct localization of each passenger is dependent on the correct function and localization of the others. Depletion, by RNA interference, of either Aurora B, INCENP, Survivin or Borealin impairs the localization of the entire passenger protein complex to centromeres and the central spindle and severely disturbes mitotic progression (Gassmann *et al*., 2004; Honda *et al*., 2003). In the absence of INCENP, Survivin cannot activate Aurora B kinase. INCENP is required for the proper targeting of Survivin to the centromeres and to the anaphase spindle, during mitosis. In cells in which INCENP localization is disrupted, Survivin adheres to the chromosomes and no longer concentrates at the centromeres or transfers to the anaphase spindle midzone. Suppressors of Survivin also lead to the identification roles for INCENP (Huang *et al*., 2005; Wheatley *et al*., 2001).

In Drosophila melanogaster, INCENP is required for the proper localization of Aurora B on the chromosomes, the central spindle, and the midbody during mitosis. Moreover Aurora B is also required for INCENP accumulation at centromeres and transfer to the spindle at anaphase (Adams *et al*., 2001b). Both Aurora B kinase activity and the formation of the Aurora B/INCENP/Survivin complex contribute to the localization of the CPC (Honda *et al*., 2003).

How is the CPC proteins targeted to the centromere and central spindle? Which member is at the center of the action? Until now these points are not clear. The complex may interact with its centromeric receptors as Ndc10 (Vader *et al*., 2006). Conversely

specific kinetochore components like CENP-A and Mis12 do not seem to be involved (Klein *et al*., 2006). However, CPC targeting to the centromere requires protein histone H2A.Z which can bind INCENP (Greaves *et al*., 2007). The passengers themselves play a key role for the centromeric targeting of the CPC. The NH_2-terminal domain of INCENP is needed for centromere localization of the CPC (Ainsztein *et al*., 1998). The Survivin BIR domain is the portion of the CPC that interacts with the centromere. Absence of a functional BIR domain of Survivin affects the centromere localization of the other passengers in the complex ((Lens *et al*., 2006; Vader *et al*., 2006). Mutated Borealins which can bind only INCENP (not Survivin) or only Survivin (not INCENP) fails to localize the CPC to either the centromere or the midbody (Jeyaprakash *et al*., 2007; Ruchaud *et al*., 2007b). Interestingly, the charge of the acidic cluster on INCENP near the distal end of the helical bundle and the basic cluster of Borealin are also involvde in the localization of the CPC. If these clusters are mutated to the opposite charge and the mutant are expressed in the cells in the absence of endogenous protein, the CPC can localize to centromeres but not to the central spindle or midbody (Jeyaprakash *et al*., 2007) (figure II.17).

The chromosomal passengers complex is required for a number of key functions during mitosis, including chromatin modifications, regulation of kinetochore-microtubule interactions, chromosome bi-orientation and stability of the bipolar spindle, mitotic checkpoint function, assembly of the central spindle and also cytokinesis. It is considered as a “conductor” of cell division (Ruchaud *et al*., 2007a). Defect of the CPC can lead to genetic instability and cancer.

CHAPTER III: CHROMOSOMAL PASSENGER PROTEINS AND CANCER

III.1. Passenger proteins as possible targets for cancer therapy

III.1.1. Expression of passenger proteins in cancer

It cannot be denied that chromosomal passenger proteins play very important roles in mitosis and cytokinesis. However, most of these proteins are found to be overexpressed in tumour cells.

Aurora A and B kinases are over-expressed in primary breast and colon tumours and in early oestrogen-induced tumour foci in the Syrian hamster kidney (Hontz *et al*., 2007). Their expression levels seem to rise or decline in parallel (Keen and Taylor, 2004). Overexpression of the centrosomal protein Aurora-A kinase is associated with poor prognosis in epithelial ovarian cancer patients (Landen *et al*., 2007). Moreover, the human Aurora A gene is located to the 20q13 amplicon, which is associated with a poor prognosis in breast cancer. Xenografts of mouse NIH-313 cells transfected with Aurora A kinase give rise to tumours in nude mice, suggesting that Aurora A may behave as an oncogene (Bischoff *et al*., 1998). Aurora B expression increase in correlation with advanced stages of colorectal cancer and its overexpression induces metastasis (Giet *et al*., 2005; Sorrentino *et al*., 2005). Aurora B overexpression might be a secondary event in p53-defective tumour cells that leads to malignancy (Giet *et al*., 2005; Ota *et al*., 2002).

Many studies indicated that Survivin plays also an important role in tumourigenesis, especially in colorectal tumour and in virus infection-induced carcinogenesis (Li and Ling, 2006). Survivin also presents sharp different expressions in cancer in comparison to normal tissue. It is undetectable in most terminally differentiated normal tissues but strongly expressed in embryonic and foetal organs (Altieri, 2003). Dramatic overexpression of Survivin was demonstrated in many tumours, such as lung, breast, colon, stomach, esophagus, pancreas, liver… (Fukuda and Pelus, 2006). Isoforms of Survivin are also found differently expressed in tumours (Caldas *et al*., 2005; Li and Ling, 2006; Yu *et al*., 2002).

Survivin expression in normal tissue is developmentally regulated and has been reported to be low in most terminally differentiated tissues. However, Survivin is found expressed in normal adult cells, particularly primitive hematopoietic cells, T lymphocytes polymorphonuclear neutrophils and vascular endothelial cells and may regulate proliferation and survival of these cells (Fukuda and Pelus, 2006).

Chang LJ. *et al.* have performed immunohistochemistry analysis using anti-Borealin specific antibodies on gastric cancer tissues. They have shown that Borealin expression is significantly correlated with Survivin and Ki67 overexpression in these tissues (Chang *et al*., 2006).

INCENP levels are also elevated in colorectal cancer cells. Mutations in the INCENP gene are unlikely to be the primary cause of tumourigenesis since the INCENP locus is not associated with any known genetically linked predisposition to disease. However, elevated levels of the protein could contribute to tumour progression either by stabilizing the Aurora-B kinase, or by targeting it to non-physiological, low affinity substrates. Alternatively, abnormalities of the INCENP/ Aurora system can lead to aneuploidies that contribute to tumour progression (Adams *et al*., 2001b).

III.1.2. Passenger proteins and their role in aneuploidy promotion

As said in chapter I, the main goal of mitosis is to equally segregate at least chromosomes and centrosomes into the two daughter cells. Multi fidelity-monitoring checkpoint systems and events have been involved in ensuring the correct temporal and spatial coordination of this process. Mitotic abnormalities can lead to genetic instability and cancer (Giet *et al*., 2005) (Figure 18).

Aneuploidy - alteration in the number of chromosomes – is consistently observed in all cancers. Aneuploidy, like defects in signalling pathways, is essential for tumourigenesis (Rajagopalan and Lengauer, 2004). A better understanding of the proteins involved in molecular mechanisms leading to aneuploidy holds promise for the development of cancer drugs that target these processes.

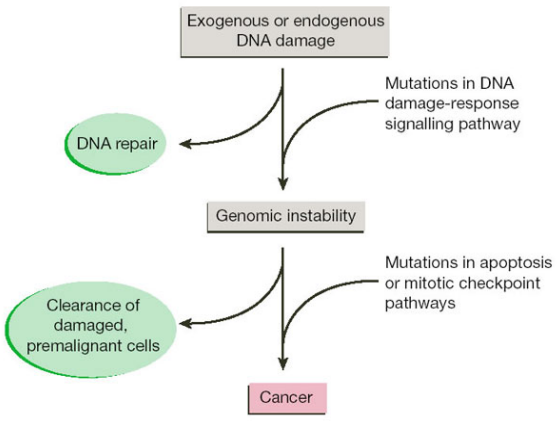

Figure 18: Genomic instability leads to cancer. DNA damage or abnormal mitosis and defective signalling pathways in cell-cycle can cause the genomic instability and lead to cancer development (Kastan and Bartek, 2004).

Since passenger proteins are involved in many key processes in mitosis, any defective function of one passenger protein can affect the accurate segregation of chromosomes or cytokinesis and causes the chromosomal instability (CIN). In turn, the

invocation of CIN works as the main cause of aneuploidy in cancer. The overexpression levels of some passenger proteins may provokes genetic instability underlying the tumourigenesis.

III.2. Chromosomal passenger proteins and cancer therapy

III.2.1. Aurora-kinase inhibitors

As a consequence of such relationships between passenger proteins and cancer, these proteins gain interest as targets for anticancer drugs. Due to the potentiality of kinase inhibitors in cancer therapy, lots of interests are focused on Aurora kinases.

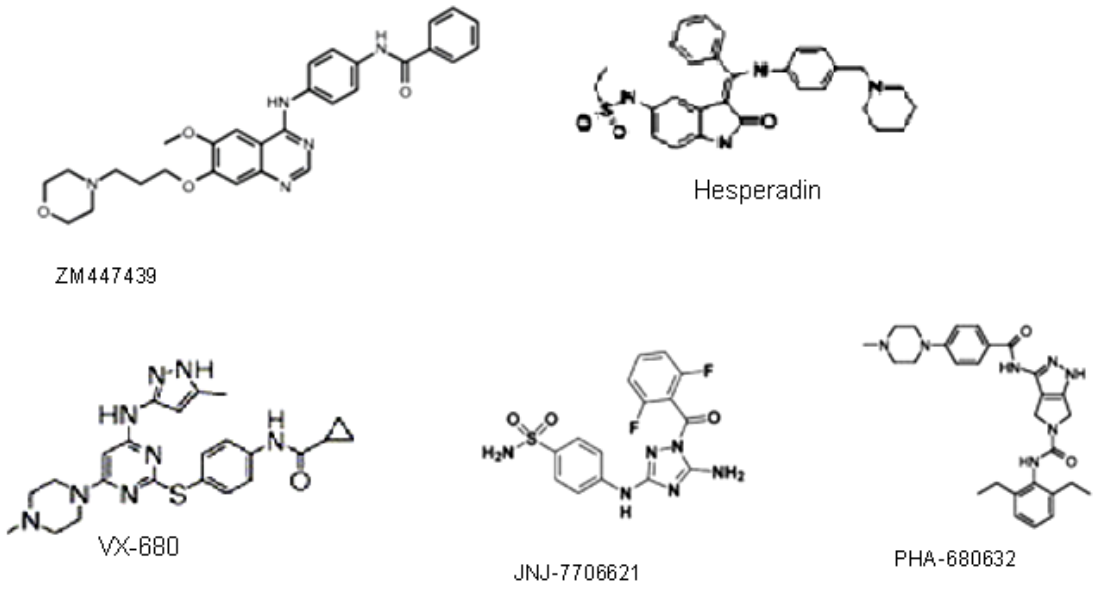

Figure 19 : Aurora kinase inhibitors
Five inhibitor molecules described in the literature are reported.

To date, there have been several Aurora kinases inhibitors which have been described. Among them, compounds that have played an important role in the validation of Aurora kinases as potent therapeutic targets are ZM447469 (Ditchfield *et al*., 2003); Hesperadin (Hauf *et al*., 2003); VX-680 (Harrington *et al*., 2004); JNJ-7706621 (Emanuel *et al*., 2005); and PHA-680632 (Soncini *et al*., 2006) (Figure 19). All these inhibitors exhibit similar effect on cells. They inhibit both the phosphorylation of histone H3 on serine 10 and cell division (Keen and Taylor, 2004; Soncini *et al*., 2006). In the presence of the inhibitors, chromosomes are oriented in syntelic manner. Cells treated with these inhibitors do not arrest in mitosis when exposed to taxol. ZM447469, Hesperadin and PHA-680632 can cause mislocalization of checkpoint protein, such as Bub1 and BubR1, to kinetochores (Ditchfield *et al*., 2003; Hauf *et al*., 2003; Soncini *et al*., 2006).

Cells treated with these inhibitors reflect an Aurora B kinase inhibition. Although ZM447469 and Hesperadin can potently inhibit both Aurora kinases *in vitro*, and presumably *in vivo*, the observed phenotype in cells treated with these drugs is not consistent with the phenotype reported for inhibition of Aurora A by genetic means (Keen and Taylor, 2004). Therefore, Girdler F *et al.*. have recently screened for molecules inducing a monophenotype consistent with Aurora A inhibition. They have described a novel ZM-related inhibitor, which is 20 times as potent against Aurora A compared with ZM447439, and induces a monopolar phenotype. This phenotype is consistent with repression of Aurora A (Girdler *et al*., 2006).

However, in terms of developing anticancer drugs, the objective is not only to inhibit Aurora kinases but also to selectively kill tumour cells. ZM447469 and VX-680 can selectively kill tumour cells *in vitro* but moreover, treatment with VX-680 induces tumour regression *in vivo* (Ditchfield *et al*., 2003; Harrington *et al*., 2004). PHA-680632 suppresses tumour growth in human tumour cell xenograft models and in syngeneic mouse and rat models (Soncini *et al*., 2006). Besides interesting effects in cancer, these inhibitors still have some disadvantages. VX680 is specific for Aurora kinases but also targets FLT-3, LcK and the mutant BcrAbl (T315I) kinases (Cheetham *et al*., 2007; Young *et al*., 2006). JNJ-7706621 is a novel cell cycle inhibitor that shows potent inhibition of several cyclin-dependent kinases (Cdk) and inhibits Aurora kinases (Emanuel *et al*., 2005). Furthermore, the activity of VX680 and ZM447439 are dependent on the p53 status (Ditchfield *et al*., 2003; Gizatullin *et al*., 2006). Although these Aurora kinase inhibitors are under clinical trials in cancer therapy, it is necessary to find and develop new Aurora inhibitors which can exhibit particular specificities. Moreover, mutations around kinase active sites appear frequently during tumour progression and justify developing several molecules against the same target.

III.2.2. Survivin: a target for cancer therapy

The prognostic relevance of Survivin in cancer has suggested that Survivin is an inducible resistant factor and may be involved in emergence of refractory phenotype to anti cancer therapies. Different strategies are devoted to target Survivin (Table 3). Some are based on the inhibition of its transcription or of its transduction (antisense, ribozym, siRNA, …) whereas others exploit dominant negative Survivin, or immunotherapy. Moreover, anti-Survivin therapies may not affect most nonproliferating adult tissues since Survivin is essentially expressed in proliferating tissues.

Table 3 : Anti-Survivin strategies and interventions (Fukuda and Pelus, 2006).

Disease model	Strategy	Effect	Toxicity
Breast cancer xenograft and i.p. disseminated breast cancers	Intratumoral infection of T34A survivin using adenovirus	Inhibition by 40% of established tumor growth and reduced i.p. tumor dissemination	No effect on normal proliferating cells *in vitro* and endothelial cells and no systemic toxicity
Breast cancer xenograft	Intratumoral infection of T34A survivin using adenovirus	Inhibition of preestablished tumors and triggering of apoptosis; ~60% reduction of tumor-derived blood vessels	
Breast cancer xenograft	Suppression of T34 survivin phosphorylation by flavopiridol	Inhibition of preestablished breast cancers and increased recipient mice survival	No systemic toxicity
Prostate cancer xenograft	Intratumoral infection of T34A survivin using adenovirus	Increased sensitivity to antiandrogen therapy	
Prostate cancer xenograft	Intratumoral injection of antisense survivin using adenovirus	Growth inhibition of tumors	No systemic toxicity
Glioma xenograft	Intratumoral injection of siRNA for survivin using adenovirus	Suppression of tumor growth	
Large-cell lymphoma	Intratumoral injection of antisense survivin or C84A survivin plasmid plus tumor-specific CTL	Growth inhibition of established tumors	
Rhabdomyosarcoma xenograft	Intratumoral injection of shRNA for survivin	>70% reduction in tumor growth	
Colon cancer xenograft	Intratumoral injection of C84A survivin using adeno-associated virus	Induction of apoptosis, inhibition of angiogenesis and tumor growth	No obvious cytotoxicity
Breast cancer xenograft, prostate cancer xenograft	Systemic administration of peptide blocking survivin and heat shock protein 90 interaction	Growth inhibition of tumors	No effect on normal cells *in vitro*; no overt systemic toxicity

These strategies, whose some are under clinical trials, seem not to be systematically toxic so they are extremely encouraging. More studies on Survivin will help to develop a selectively antagonizing Survivin strategy.

MATERIALS AND METHODS

CHAPTER I: BIOCHEMISTRY

CHAPTER II: MOLECULAR BIOLOGY

CHAPTER III: EXPRESSION OF E. coli AND PURIFICATION

CHAPTER IV: PROTEIN KINASE ASSAY

CHAPTER V: CELL CULTURE

CHAPTER VI: MICROSCOPIC TECHNIQUE

CHAPTER VII : BUFFER

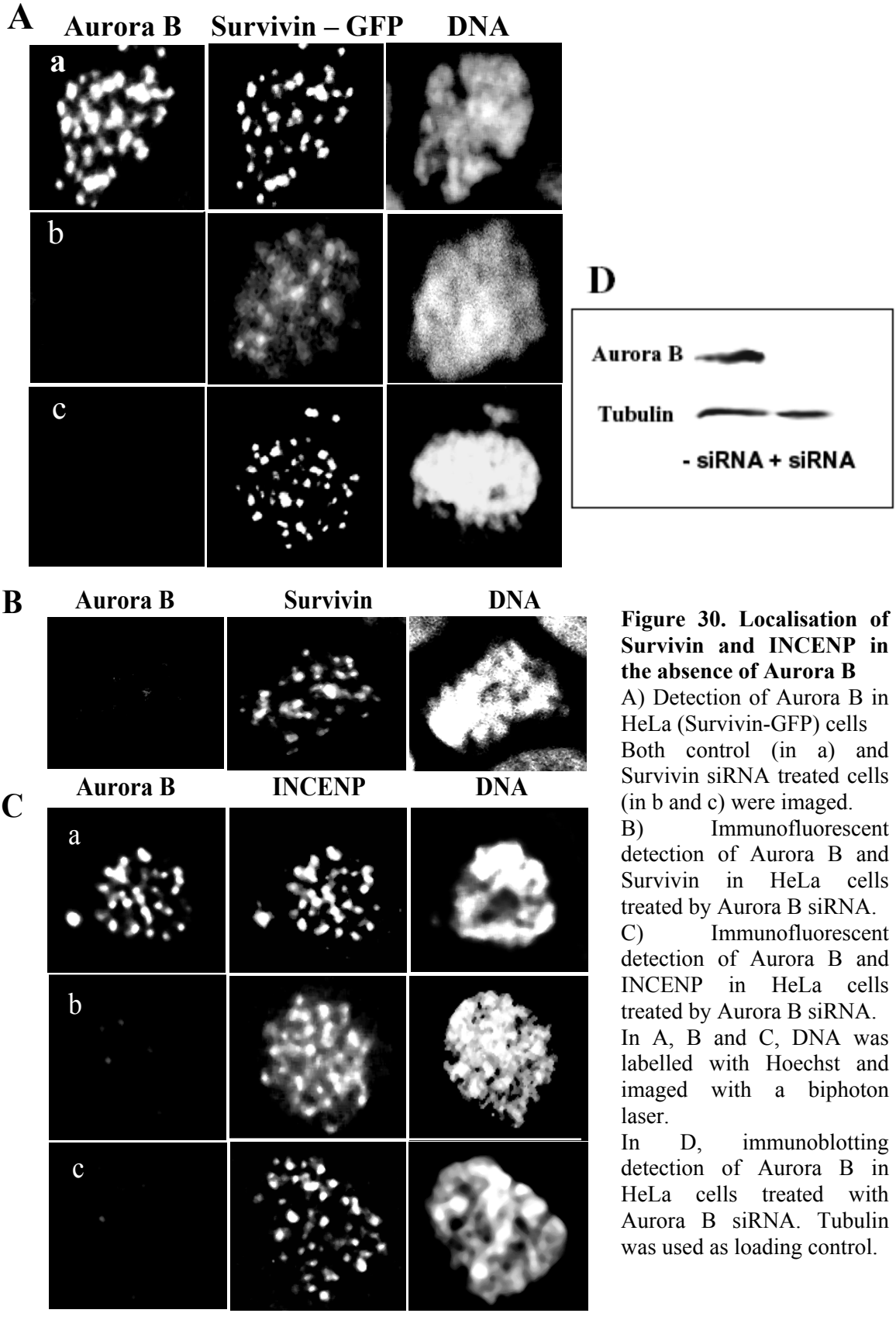

Figure 30. Localisation of Survivin and INCENP in the absence of Aurora B

A) Detection of Aurora B in HeLa (Survivin-GFP) cells Both control (in a) and Survivin siRNA treated cells (in b and c) were imaged.

B) Immunofluorescent detection of Aurora B and Survivin in HeLa cells treated by Aurora B siRNA.

C) Immunofluorescent detection of Aurora B and INCENP in HeLa cells treated by Aurora B siRNA.

In A, B and C, DNA was labelled with Hoechst and imaged with a biphoton laser.

In D, immunoblotting detection of Aurora B in HeLa cells treated with Aurora B siRNA. Tubulin was used as loading control.

CHAPTER I: BIOCHEMISTRY

I.1. Cells extracts

Cells were harvested and then, lysed in Laemmli sample buffer supplemented with 7M urea. Lysates were mixed carefully and boiled for 10 minutes at 95°C, then centrifuged at 10 000 rpm for 5 minutes and boiled again for 5 minutes.

I.2. Electrophoresis

I.2.1. SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis is carried out with a discontinuous system. From 30% acrylamide/bis-acrylamide stock solution (Sigma) we prepared separating gels of composition varying from 8 to 15 % acrylamide, depending on the range of proteins that we wished to separate. Pour the acrylamide solution into the gap between the glass plates; left sufficient space for the stacking gel. Using a pipette carefully overlaid the separating gel with deionized water. After polymerization is complete (30 minutes), pour off the overlay and wash the top of the gel several times with deionized water to remove unpolymerized acrylamide. Pour the stacking gel solution directly onto the surface of the polymerized separating gel. We used 5% stacking gel for Tris-glycine SDS-Polyacrylamide Gel Electrophoresis. Immediately insert a clean Teflon comb into the stacking gel solution, taking care not to catch bubbles under the teeth. Add more stacking gel solution in order to fill completely the spaces of the comb. After 30 minutes polymerization, load the sample into the bottom of the wells. Load an equal volume of 1X SDS gel-loading buffer into any unused wells. Set up current at 16 – 18 mA and run the gel.

Staining SDS-Polyacrylamide gels with coomassie brilliant blue: Gels were fixed with Methanol: glacial acetic acid and stained with Coomasie Brilliant Blue R250 for 1 hour. This solution was prepared by dissolving 0.25 g of coomassie blue in 90 ml methanol: water (1:1 v/v) and 10 ml of glacial acetic acid. Gels were destained by soaking them in the methanol: glacial acetic acid (4.5:1 v/v) on a slowly rocking platform for more than 10 hours. Change the destaining solution for 4-5 times.

I.2.2. Transfer of protein from SDS-polyacrylamidee gel to solid supports.

After electrophoresis, the gel and its attached nitrocellulose filter (GE Healthcare) were then sandwiched between six Whatman 3MM papers, two ropous pads. The sandwich was then placed in a cathode - anode plate, with the nitrocellulose filter on the cathode side. Note to roll out air bubbles between layers. This construction was run in transfer buffer. Apply 100V and the maximum current for a period of 1-2 hours depending on the range of

transferred proteins. Transfer of protein from the gel could be carried out at room temperature using ice for cooling the buffer.

I.2.3. Immunoblotting

After migration, proteins were transferred from the gel to a nitrocellulose sheet (GE Healthcare) for Western Blotting. The blocking membrane was saturated with 5% non fat milk in PBS for at least 1h. After a fast wash in PBS, the membrane was incubated overnight at 4°C in buffer I with primary antibodies. Then the membrane was washed three times (5 minutes per wash) in washing-buffer I, three times in washing-buffer II and incubated with secondary antibodies conjugated to horseradish peroxidase (HPR) in buffer II for 30 minutes at room temperature. Blots were developed using ECL-PLUS detection system according to manufacturer instruction (GE Healthcare).

CHAPTER II: MOLECULAR BIOLOGY

II.1. Agarose electrophoresis

We used agarose gels to separate and purify DNA fragments of 100-10 000 bp in length. For our experiments, it was convenient to use 6.5 x 9.0 x 1.0 cm minigels with 8-well combs, which hold 10 µl samples per well. Composition the gel varied from 0.8 to 1.6 % agarose in TAE buffer containing 0.4 µg/ml BET. Bands within the gels were visualized by UV trans-illumination and photos were taken with a camera.

II.1. Competent cells

Either top 10 or BL21 codon start ⊕ bacteria were grown on fresh LB plate. A single clone was inoculated in 5ml LB broth and bacteria were incubated overnight in the shaking machine at 37°C. Take 1ml and incubate with 200 ml to 500 ml sterile LB medium. Grow the bacteria on a shaker at 37°C until the OD_{550} reachs 0.3 to 0.5. Centrifuge in the Sorvall GSA rotor (250 ml centrifuge bottle) at 5,000 RPM for 10 minutes at 4°C. Gently resuspend the bacteria pellet on ice in 1/4 volume of ice-cold buffer 1 (see part VI). Centrifuge the cell suspension at 4,000 RPM in the Sorvall GSA rotor for 10 minutes. Resuspend the bacteria pellet on ice in 1/20 volume of ice cold buffer 2 (see part VI), keep this suspension on ice for at least 20 minutes. Centrifuge the cell suspension at 4,000 RPM in the GSA rotor for 10 minutes and resuspend the cell pellet in 1/50 volume of ice cold 15% glycerol (w/v). Dispense in 100 µL aliquots and freeze competent bacteria in liquid nitrogen and store at – 80 °C.

II.2. Bacterial transformation

Few nanograms (20-100) of plasmid were transferred into 100 µl of competent bacteria either Top 10 for the DNA amplification or BL21 colon start ⊕ for protein production. Competent bacteria were put on ice for 30 minutes then, perform a heat shock in a water bath at 42°C for 40-45 sec. 1 ml LB medium was added quickly into bacteria cells. Incubated at 37°C for 1 hour. Spread bacteria culture on the LB agar plate with the desired selection antibiotic. Inverted the plates and placed in the 37°C bacterial incubator overnight. Select an isolated bacterial clone and amplified it in LB medium overnight in the presence of antibiotic.

II.3. DNA Miniprepations

10 ml of culture overnight were used to isolate plasmid DNA following the protocol of Wizard® *Plus* SV Minipreparations DNA Purification System (Promega). Quantifying

DNA was performed by using a Bio Photometer machine (Ependorf). The quantity of DNA can be determined from its absorbance at 260 nm (1 OD_{260} corresponding to 50 μg/ml). The plasmid is analyzed by restriction and then sent for sequencing to genome express (Meylan). Midi-preparations were done with an Invitrogen kit under conditions suggested by the supplier.

CHAPTER III: EXPRESSION OF PROTEIN IN E. coli AND PURIFICATION

III.1. Protein over-expression

The Plasmids used for kinase productions were pET-M11(poly His-Aurora A kinase domain), pET-M11(poly His-Aurora A kinase) and pET-M11(poly His-Aurora B kinase). These constructions were prepared by Fabienne Hans and Fabienne Sirot. pET-M11 vector is a gift from Christoph Muller (EMBL). These plasmids were transferred in BL21 codon start ⊕ bacteria.

30 ml of overnight culture were mixed with 600 ml LB supplemented with glucose 2 % (w/v), ethanol 3 % (v/v), Chloramphenicol (34 µg/ml), and Kanamycin (100 µg/ml). Incubate the culture on shaking machine at 180 rpm, 37°C. Check the OD_{600} every hour until OD_{600} reached the value of 0.6. Using sterile technique, add IPTG to a final concentration of 1mM in order to induce the over-expression. The culture was incubated overnight on the shaking machine (250 rpm) at room temperature. When the OD_{600} reached the value of 1, the culture was centrifuged at 5000 rpm, 4°C for 15 minutes.

III.2. Protein extraction

Proteins are extracted from the bacteria for further purification. The bacterial pellet was resuspendd with 10 ml of lysis buffer containing 7 µl β-mercapto ethanol and a cocktail of anti protease (Roche Diagnostics). Agitation was performed overnight at 4°C.

We used sonication for lysing small quantities of cells. To prevent temperature raise, the suspension was kept on ice and sonicated by a number of short pulses (5-10 sec) with pauses (10-30 sec). The total time of sonication is about 2 minutes.

III.3. Protein purification

We purified proteins to homogeneity by Ni-affinity chromatography. After sonication, the suspension was centrifuged at 15 000 rpm, 4°C for 30 minutes. Take the supernatant and mix with Ni-NTA beads (Quiagen GmbH) under agitation at 4 °C for 1 hour. Proteins were purified on Ni-NTA resin in a column under conditions proposed by the supplier (Quiagen). Briefly, the cell lysate was slowly applied to the column. The column was washed by solutions 1 and 2 and then, proteins were eluted by the elution buffer. The presence of protein was checked by a dye reagent Bradford (Bio-Rad). To get a higher concentration of purified proteins, we used centricon system to concentrate proteins

(Millipore). All operations were carried out at 4°C or with sample placed on ice to minimize protein denaturation and proteolysis.

III.4. Storage of purified proteins

Proteins were preserved by adding 50 % (v/v) glycerol. The solution was kept in -80°C in small aliquots to avoid repeated freezing and thawing which may reduce the biological activity or affects the structure of the purified proteins.

III.5. Preparing sample for SDS-polyacrylamide gel Electrophoresis

We used electrophoresis to check the over-expression of proteins induced by IPTG 1 mM. Take 1 ml of bacteria culture before and after IPTG induction. Centrifuged them at maximum speed for 2 minutes. The pellets were resuspendd with the volumes of water and loading buffer counted as below: $V_{H20} = OD_{600} / 5$ (µl) and $V_{\text{Loading buffer}} = OD_{600} / 6$ (µl).

Heat the samples at 95°C for 5 minutes and centrifuge at 14 000 rpm for 3 minutes to pellet cell debris before loading on the gel. After electrophoresis, gels were stained with Coomassie brilliant blue.

CHAPTER IV: PROTEIN KINASE ASSAY

IV.1. Protein kinase assay

The reaction was performed in the presence of 20 mM KCl, 20 mM $MgCl_2$, 0.4 µM ATP, 0.4 mM DTT, 20 mM Tris (HCl), pH 7.5 and recombinant histone H3 was used as substrate. Mix carefully the mixture. The reaction started by the addition of the recombinant enzyme. After 1 hour of incubation at 37 °C the remaining ATP was monitored by addition of kinase-Glo™ (Promega, France) under the conditions suggested by the supplier. After ten minutes to allow the luminescent signal to stabilize, the fluorescence was recorded with Fluostar Optima (BMG Labtechnologies). Staurosporin (0.5 µM) was used as a negative control. Eight positive assays and eight negative assays were performed to determine the robustness of the test. The Z'-factor, a standard for evaluating HTS methods, is calculated using the formula: $Z' = 1 - [(3sp + 3sn) / (|\mu p - \mu n|)$

Where µp = mean of "positive control" (max ratio), µn = mean of "negative control" (min ratio), and sp and sn = the corresponding standard deviations (Zhang *et al*., 1999).

IV.2. High throughput screening (HTS)

We have screen 10 000 molecules belonging to either the Patrimonial French library (Curie Institute, Lyon, Grenoble) or to NCI library. The screening was performed by Caroline Barette on the HTS platform of CEA of Grenoble (Laurence Lafanéchère). The assay was performed in black 96-well plates (Greiner 651209) and started with the addition of the kinase domain of Aurora A. Molecules were in DMSO. The primary screening was performed in triplicate at the concentration of 15 µM (0.3 % DMSO) and the confirmation at 1.5 µM. The depletion of ATP was measured by a luminescent signal. Luminescence signal was recorded by Berthod Technologies Centro LB-960 machine. IC50 is defined as the concentration that leads to 50 % of inhibition.

CHAPTER V: CELL CULTURE

V.1. Cell lines

In the experiments, we used the following cell lines: HeLa, HeLa T-rex, H358, 3LL, HTC116 cell lines as well as A5 and S12 which are HeLa cells stably expressing Aurora GFP and Survivin GFP respectively.

HeLa cells: An epithelial cell line originally derived from human cervix.

T-REx HeLa cell line (INVITROGEN – R71407): T-REx™ HeLa cells stably express the tetracycline repressor protein.

H358 cell line (ATCC CRL5807): The human non-small lung adenocarcinoma of bronchioloalveolar origin.

3LL cell line (ATCC CRL1642): The mouse (*Mus musculus*) Lewis lung carcinoma.

HTC116 cell line (ATCC CCL247): The human colon colorectal carcinoma.

Cells were grown on Dulbecco's modified Eagle's 1 g/l of Glucose (Invitrogen) in tissue culture dish (Falcon). Media were supplemented with 10 % (v/v) foetal bovine serum (Invitrogen), penicillin-streptomycine 100 IU/ml (Invitrogen), L-glutamine 2mM (Invitrogen). Cells were grown in a humidified chamber in the presence of 5 % CO_2, at 37 °C. We used trypsine (0.5 g/l) to recover the cells.

V.2. Conservation of cells

Cells were trypsinized and centrifuged at 1500 rpm for 5 minutes. Resuspend the pellet with complete medium plus 10-20 % (v/v) DMSO and transferred into a special cryo-vial. Immediately put the vial into a passive freezer filled with isopropyl alcohol and place it at -80 °C. Frozen ampoules could be kept into the liquid nitrogen storage vessel.

V.3. Drugs used

Nocodazole (Sigma) prevents microtubule polymerisation. So, cells were blocked at G2/M border by incubating with 3 μM nocodazole (Sigma) for 15 hours.

Taxol (Sigma) prevents microtubule depolymerisation and thus, in its presence, cells stop in metaphase. Cells were incubated with 10 μM taxol (Sigma) for 8 hours or 330nM overnight. For western blotting analysis, mitotic cells were harvested by flushing.

VX-680 (Kavatechnology), an Aurora kinase inhibitor, was used at 300 nM or 1μM.

Tetracyclin (Invitrogen), used for induction of tet promoter, was used at 250 ng/ml overnight.

V.4. Synchronisation

In order to study mechanisms involved in cell cycle regulation, it is critical to synchronize the cells in the same phase of the cell cycle. To synchronize cells, the following methods were used.

V.4.1 Double Thymidine block (early S-phase block)

In this experiment, cells were synchronized at the G1/S border with a double thymidine block. Cells were growth in complete medium to get 25-30 % confluence at the time of synchronization. Expose cells to 2.5 mM thymidine (Euromedex) for 20 hours. Then, cells were washed two times with PBS and released into complete medium without thymidine for 10 hours. A second exposure to 2.5 mM Thymidine lasted about 18 hours.

V.4.2. Thymidine-Nocodazole block (mitotic block)

Perform the thymidine block has described before (S-phase block). Then, remove thymidine by PBS washing. Cells were induced in cell cycle by adding fresh complete DMEM for 10 h. After release, the mitotic arrest was obtained by addition of Nocodazole (100 ng/ml). If necessary, nocodazole was removed by PBS washing and fresh complete DMEM was added. Cells were recovered at varying times depending of the experiments

V.5. FACS (Fluorescence Activated Cell Sorting)

FACS is a method for separating cells that are phenotypically different from each other and for quantifying antigens. FACS also works well to access the cell cycle distribution of whole cell population.

Preparing cells for cell cycle analysis (Pi/FACS): Cells were synchronised and incubated with drugs. After harvesting from one 10 cm culture plate, cells were washed with cold PBS. Cells were then fixed by ice-cold 70 % ethanol for 1 hour and incubated with the mix of RNA (1 mg/ml) plus propidium iodide (50 µg/ml) in PBS for 30 minutes at 37 °C. Cell cycle profiles were determined by flow cytometric analysis.

V6. Spindle checkpoint status

Cells were incubated with 10 µM Taxol (Sigma) overnight and then either molecules to test or DMSO were added. The checkpoint status was determined either on fixed cells by counting the percentage of mitotic cells or by time-lapse experiment on living cells.

V.7. Cell proliferation and viability

Cell viability was accessed using Promega CellTiter 96® AQ to measure MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]. This assay is based on the determination of mitochondrial activity. Cells were

seeded in 96-wells plate, in normal growth conditions, and incubated with drugs for varying times. Then, add 20 µl CellTiter 96® Aqueous One Solution Reagent to 100 µl media in each well. Incubate the plate for 1-4 hours at 37 °C and then determined the OD at 490 nm on a Wallac microplate beta-counter. The OD_{490} reflects the quantity of living cells in each well.

V.7. Cell transfection

V.7.1 Plasmids

Plasmid construction: The full length Aurora B sequence was PCR-amplified from human foetal liver- Marathon ready cDNA. The Aurora B-GFP construct was built by cloning the DNA fragment containing the coding sequence of Aurora B into pEGFP-N1 vector (BD clontech). The human Survivin cDNA was subcloned into pEGFP-N1 vector (BD clontech) in order to produce Survivin fusion protein (Delacour-Larose *et al*., 2004).

Survivin^{SR} –GFP (SR for silent resistant) was obtained by mutagenesis of the parental Survivin-GFP. Mutations were introduced in Survivin by using the QuickChange Site-Directed Mutagenesis kit (Quiagen) under condition suggested by the manufacture. Survivin^{SR} –GFP, Survivin^{SR} –T117A-GFP and Survivin^{SR} –T117E-GFP were cloned in pcDNA5/TOplasmid (Invitrogen). Their expression was under the control of tetracycline promoter, the induction was performed by the addition of tetracycline (250 ng/ml), overnight.

Transfection: Cells were seed into tissue culture plates and incubated at 37 °C in a CO_2 incubator until reach at 60-70 % confluence. Remove old medium and add Opti-MEM (Invitrogen) onto the cells. For each transfection in either a six-well or a 35 mm tissue culture plate, dilute 3.2 µg DNA into 50 µl serum-free medium (solution A) and mix carefully. Dilute 2.5 µl lipofectamine reagent (Invitrogen) into 50 µl serum-free medium (solution B), mix carefully and wait for 5 minutes. With larger tissue culture plates, increase the amounts of all reagents proportionally to the surface area.

Combine the two solutions and mix gently from time to time, for 25 minutes. The diluted complex solution was then overlaid onto the cells. After 5 hours of incubation add completed medium without removing the transfection mixture. The medium was replaced 24 hours later.

For stable expression, instead of harvesting cultures 72 hours post-transfection, we amplified the transfected cells into selective medium (either geneticin (750 mg/ml) or hygromycin (200 mg/ml)) and cloned them by dilution. After several weeks, we checked for

the efficiency of and stability of the expression of the foreign sequence by immunofluorescence.

V.7.2 siRNA transfection

Single stranded Aurora B gene specific sense (GAAAGAGCCUGUCACCCCAUC) and antisens (AUGGGGUGACAGGCUCUUUCCG) RNA oligomers were synthesised using 2' –O-(tri-isopropyl)silyoxymethyl chemistry (Xeragon). Double stranded RNA was obtained following manufacturer's instructions. Double stranded RNAi for Survivin and INCENP suppressions were purchased from Eurogentec and they recognized the AAAGAACUGGCCCUUCUUGGA and AGGUUAUCCCGCAGAAAGUCU sequences respectively. Single stranded sense and antisense strands, used as controls and siRNA duplexes were transfected into cells using oligofectamine (Invitrogen) according to the manufacturer's protocols.

We used Oligofectamine^{TM} to transfect short interfering RNAs (siRNA) into the cells. One day before transfection, plate the cells in medium in order to get 50-60 % confluence at the time of transfection. Remove the old medium and add Opti-MEM. For either a six-well or a 35 mm tissue culture plate, dilute 25 µl of siRNA (100 µM) in 500 µl of Opti-MEM without serum; mix oligofectamine gently before use, then dilute 60 µl in 240 µl of Opti-MEM, mix gently. After 5 minutes incubation at room temperature, combine the diluted siRNA with the diluted Oligofectamine. Mix gently, time by time in 20 minutes at room temperature to allow the siRNA-Oligofectamine complexes to form. Add the complexes onto the cells drop by drop. Growth medium were replaced 24 hours post transfection.

CHAPTER VI: MICROSCOPIC TECHNIQUE

VI.1. Immunofluorescence

Cells grown on 14-mm-diameter glass coverslips (Knittel gläser) for 24 hours were fixed at 37 °C in 4% (w/w) paraformaldhehyde, 2% (w/w) sucrose and then permeabilized with 0.2% Triton X-100 in PBS for 5 min. Free binding sites were blocked with 0.5 mg/ml BSA and specific antibodies were then, incubated for at least 30 min in buffer I. Unbound antibodies were removed by washing with TPBS and specific staining was revealed with fluorescence conjugated antibodies. DNA was visualized with 0.1 μM Hoechst 33342 (Sigma). After being washed three times in TPBS, coverlips were mounted on slides with fluorescent mounting medium (Dakocytomation). Images were collected with a ZEISS 510 Laser Scanning Confocal apparatus with a 63x immersion oil objective. Argon2 was excited at 488-nm, Helium-Neon was at 543-nm and 643 nm whereas biphoton Titanium (Tsunami) was excited at 720 nm.

Table 4 : Primary antibodies used in the experiments

Specific antibodies	**Company**	**References**	***Species***	**IF dilution**	**WB dilution**
α - Tubulin	SIGMA	T5168	Mouse IgG_1	1/500	1/5000
β - Tubulin	Abcam	ab6046	Rabbit	1/200	1/500
Histone H3 P S10	Upstate	06-570	Rabbit	1/1000	1/1000
Aurora B	BD Biosciences	Monoclonal	Mouse IgG_1	1/50	1/250
Survivin	Abcam	Ab469	Rabbit	1/300	1/1000
MKLP1	Santa Crus Biotech	sc-867	Rabbit	1/100	
Cyclin B1	Epitomics	1495-1	Human	1/100	1/5000
Centromere	Immunovision	HCT-0100	Human	1/2000	
INCENP	A gift from Nigg E. A.		Rabbit	1/300	1/1000

Table 5: Secondary antibodies used in the experiments

Secondary antibodies	**Company**	**References**	***Species***	**IF/WB**
Anti-mouse Alexa 488	Invitrogen	NA 931	Goat	1/500
Anti-mouse Alexa 546	Invitrogen	NA 934	Goat	1/500
Anti-rabbit Alexa 488	Invitrogen	NA 11034	Goat	1/500
Anti-rabbit Alexa 546	Invitrogen	NA 11034	Goat	1/500
Anti-human IgG	Gene Tex	GTX 2957	Goat	1/200
Anti-rabbit IgG (H+L) hilyte fluor™ 555-labeled	Anaspec	28172-H555	Goat	1/500
Anti-rabbit IgG (H+L) hilyte fluor™ 488-labeled	Anaspec	28172-H488	Goat	1/500
Anti-rabbit IgG (H+L) hilyte fluor™ 647-labeled	Anaspec	28172-H647	Goat	1/500
Anti-mouse IgG Peroxidase	GE Healthcare	NA 931	Sheep	1/5000
Anti-rabbit IgG Peroxidase	GE Healthcare	NA 934	Donkey	1/5000

VI.2. Time – lapse experiments

Cells were grown on Lab-Tek chambered coverglass (Nalge Nunc International) for at least 24 hours. For imaging, cells were maintained at 37°C, 5% CO_2 on a temperature controlled stage in complete medium. Time lapse experiments, photobleaching and four-dimensional confocal microscopy are performed on a ZEISS LSM510 system using a PlanApochromat 40x water immersion objective. GFP was excited with a 488-nm Argon2 laser (power varying from 0.1 to 1%).

VI.3. FRAP (Fluorescent Recovery After Photobleaching)

One application of FRAP technique is to measure the ability of a molecule to move around over time. An outlined region is bleached with a full power laser and recovery is monitored repetitively during approximately 4 minutes (time suitable with mitosis phases). For rapid scanning, the registered region is restricted to a circle ROI of 40 mm of diameter. Fluorescence intensities are quantified either with a home-made software or Metamorph software and bleaching due to acquisition is corrected. It is less than 10% in all experiments. Arbitrarily, the intensity of the region prior to bleaching is set at 1 while that of background is set at 0. Relative intensities are represented as function of time.

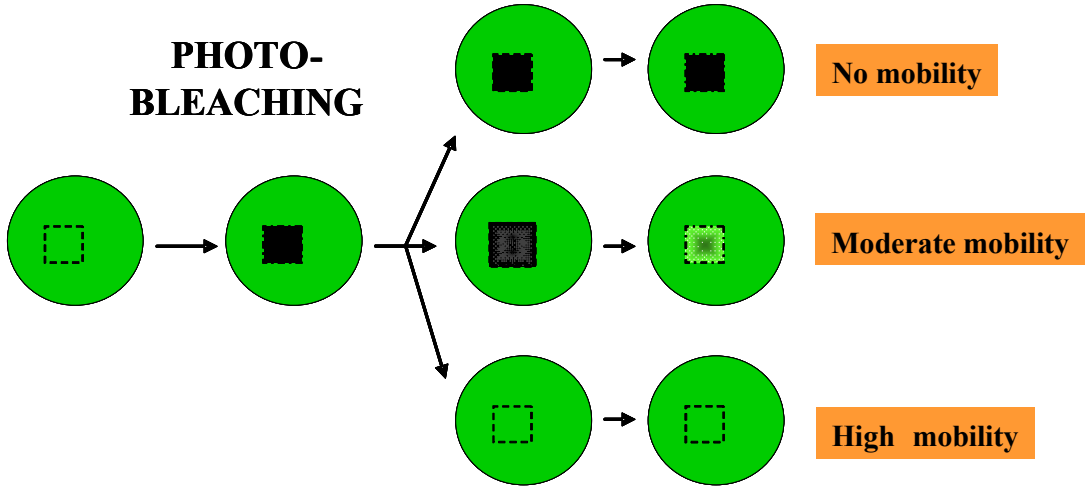

Figure 20. Schema of the possible situations in a FRAP (Fluorescent Recovery After Photobleaching) experiment
The square is the bleached fluorescent region and it appears black after photobleaching. Then the experiment consists in analyzing the fluorescent recovery in this area. Three situations can occur: either the protein is fully mobile and is recruited in the bleach area immediately or it is fully immobile and the bleached area will not be fluorescent anymore or in case of moderate mobility the area will progressively recover fluorescent proteins.

CHAPTER VII: BUFFER

VII.1. Biochemistry

Cells extract

Laemmli sample buffer 6X contains 10.28 % (v/v) SDS, 36% (v/v) glycerol, 5% (w/v) β-mercaptoethanol, 0.012% (w/v) bromophenol blue and 0.35M Tris (HCl), pH 6.6.

Electrophoressi and Western Blotting

1. Resolving gel 15 % resolving gel - 5 ml: H_20 (1.1 ml), 30 % acrylamidee/bis acrylamidee (2.5 ml), 1.5 M Tris pH 8.8 (1.3 ml), 10 % SDS (50 µl), 10 % APS (50 µl), TEMED (2 µl).

- 10 % resolving gel - 5 ml: H_20 (1.9 ml), 30 % acrylamidee/bis acrylamidee (1.7 ml), 1.5 M Tris pH 8.8 (1.3 ml), 10 % SDS (50 µl), 10 % APS (50 µl), TEMED (2 µl).
- 8 % resolving gel – 5 ml: H_20 (2.3 ml), 30 % acrylamidee/bis acrylamidee (1.3 ml), 1.5M Tris pH 8.8 (1.3 ml), 10 % SDS (50 µl), 10 % APS (50 µl), TEMED (3 µl).

2. 5 % Stacking gel - 3ml: H_20 (2.1 ml), 30 % acrylamidee/bis acrylamidee (0.5 ml), 1.5 M Tris pH 8.8 (0.38 ml), 10 % SDS (30 µl), 10 % APS (30 µl), TEMED (3 µl).
3. Migration 10X: 30g Tris base, 144g Glycine,10 % SDS (50ml), qsp H_2O to 1000 ml
4. Washing Buffer I: PBS, 0.4 M NaCl, 0.5 % (v/v) Triton X-100
5. Washing buffer II: PBS, 0.4 M NaCl.
6. Buffer I : PBS, 2 % (v/v) Tween-20, 2 % (v/v) goat serum
7. Buffer II: PBS, 2 % (v/v) Tween-20, 1 % (w/v) non fat powder milk.

VII.2. Immunofluoresence

1. Buffer I: PBS supplemented with 10 % (v/v) bovine serum albumin, 0.2 % (v/v) Tween-20 and 0.02 % (w/v) NaN3.
2. Permeabilization: PBS, 0.2 % (v/v) Triton X-100.
3. Saturation: BSA 0.5 mg/ml in PBS.
4. Washing buffer (TPBS): PBS, 0.2 % (v/v,) Tween 20.

VII.3. Protein extraction and purification

1. Lysis buffer : 500 mM NaCl, 10 mM Imidazole, 10 % (v/v) glycerol, 2.5 mM β-mercaptoethanol,100 mM Tris (HCl), pH 8.
2. Wash 1: 1 M NaCl, 20 mM Imidazole, 10 % (v/v) glycerol, 50 mM Tris (HCl), pH 8.
3. Wash 2: 250 mM NaCl, 20 mM Imidazole, 10 % (v/v) glycerol, 50 mM Tris (HCl), pH 8.
4. Elution buffer: 250 mM NaCl, 250 mM Imidazole, 50 mM Tris (HCl), pH 8.
5. Competent Buffer 1 : 30 mM potassium acetate (KOAc), 100 mM Kcl, 10 mM CaCl2, 50 mM MnCl2, 15 % glycerol, pH 5.8

   Competent buffer 2: 10 mM pipes, 10 mM KCl, 75 mM CaCl2, 15 % glycerol.

RESULTS

The chromosomal passenger proteins complex plays a central role in cell division. The functions of the four passenger proteins of the complex: INCENP, Survivin, Borealin, and Aurora B are tightly connected. As described in Introduction, this complex insures accurate mitosis, therefore its members may be very relevant targets towards mitosis abortion. In this line, these proteins are proposed as druggable candidates in cancer therapy.

The aim of our project is to get better insight into the functions of the passenger protein complex. The challenge is to propose new strategies for preventing CPC functions. Previous work, in the laboratory, points that Survivin may play a pivotal role in the complex; actually it is a mobile protein on centromeres while Aurora B is fully immobile (Delacour *et al*., 2004). We have pursued our investigations on Survivin behaviour in mitosis. Meanwhile we have started to search for kinase inhibitors in large chemical libraries.

The different part of the work will be organised as follow:

CHAPTER I : Role of Survivin phosphorylation by Aurora B in mitosis

CHAPTER II : Identification of Aurora kinase inhibitors

CHAPTER I: ROLE OF SURVIVIN PHOSPHORYLATION BY AURORA B IN MITOSIS

I.1. Role of Survivin T117E mutant in mitosis

I.2. Consequence of Survivin T117E over-expression in mitosis

I.2.1. Survivin^{SR}T117E-GFP mutant affects the spindle checkpoint

I.2.2. Phosphorylated Survivin is required for the correct localization of MKLP1

I.1. Role of Survivin T117E mutant in mitosis

The behaviour of Aurora B kinase and Survivin was studied by FRAP (Fluorescent Recovery After Photobleaching) in the different phases of mitosis. We have found that both proteins are fully immobile at metaphase and telophase. However Survivin conversely to Aurora B is mobile on centromere and its mobility seems to depend on the activity of Aurora B (Delacour-Larose *et al*., 2004).

We have extended this study to the other passenger members: INCENP and Borealin. Both proteins fused to GFP are expressed in HeLa cells and FRAP experiments are conducted on live cells. INCENP and Borealin behave mostly like Aurora B kinase, being fully immobile in mitosis. Therefore Survivin is a very peculiar member of the CPC.

Taking into account that on the one hand Survivin is phosphorylated by Aurora B at Threonine 117 and on the other hand that its mobility may be controlled by Aurora kinase, we have decided to study the mutants Survivin T117A and Survivin T117E. The questions we have decided to ask are: If the phosphorylation of Survivin is perturbed, what happen to the localization and the mobility of Survivin? And how are the mitotic cells in this case?

Structural data predict that Survivin may behave as a homodimer so we have decided to study the mutants in the absence of endogenous phosphorylatable Survivin. We have thus applied the pseudogenetic technique previously developed for Aurora kinase (Scrittori *et al*., 2005). We have expressed the Survivin non-phosphorylable T117A and the phosphomimetic T117E mutants that are resistant to siRNA (Silent Resistant) meanwhile the endogenous protein expression is prevented by siRNA transfection.

By using time-lapse experiments, we have shown that either in the presence or in the absence of endogenous Survivin, Survivin^{SR}T117A-GFP mutant is successfully recruited to centromere. Next, we have checked whether SurvivinT117A can rescue Survivin function in the cells depleted of endogenous Survivin. Using silence resistant Survivin-GFP, we have found that in the absence of endogenous Survivin, cells expressing Survivin^{SR}T117A-GFP are arrested in prometaphase meanwhile, in control cells expressing Survivin^{SR}-GFP, mitosis proceeds normally. The mobility of the non-phosphorylable Survivin^{SR}T117A-GFP mutant is investigated by FRAP experiments. This experience is conducted in cells expressing either Survivin^{SR}WT-GFP or Survivin^{SR}T117A-GFP both in control and siRNA conditions. While the fluorescence recovery for Survivin^{SR}T117A-GFP is similar to Survivin^{SR}WT-GFP in the presence of endogenous Survivin, there is a sharp decrease in the absence of endogenous Survivin. It means that Survivin^{SR}T117A-GFP is less mobile at centromere in comparison to Survivin^{SR}WT-GFP. These results supports that the

phosphorylation of Survivin is not only necessary for anaphase onset but is essential for its mobility at centromere.

Is Survivin phosphorylated in all phases during mitosis? In order to satisfy this question, we have carried out experiments with phosphomimetic Survivin^{SR}T117E-GFP. Several attempts have been conducted to obtain HeLa cells stably expressing Survivin^{SR}T117E-GFP. Although the technique is improved in our hands and the expression low in the absence of tetracycline, we have never succeeded in establishing such cells. This may suggest that this mutant is a dominant negative that prevents mitosis. In previous published data, Wheatley *et al*. have shown that on paraformaldehyde fixed cells, Survivin^{SR}T117E-GFP is dispersed throughout the cytoplasm and is excluded from chromosome while the other passenger proteins and moreover endogenous Survivin is still localized normally. On HeLa cells transiently expressing Survivin^{SR}T117E-GFP we have made the same observations. However, surprisingly, on living Survivin^{SR}T117E-GFP cells, we have found Survivin^{SR}T117E-GFP still localized at centromere from prophase to metaphase. Then it diffuses to whole chromosomes and disappears from chromatin at anaphase onset. Survivin^{SR}T117E-GFP is then diffuse in the cytoplasm and is neither present in the central mitotic spindle nor in the midbody. Why are there such differences between fixed and alive cells expressing Survivin^{SR}T117E-GFP? It may mean that Survivin^{SR}T117E-GFP is loosely linked to centromere. If so its mobility may be very high. Throughout FRAP experiment, we have found that the kinetic of fluorescence recovery of Survivin^{SR}T117E-GFP is much more rapid compared to Survivin^{SR}WT-GFP. Or in the other way, phosphomimetic Survivin is highly mobile at centromere.

Moreover, in the cell population expressing Survivin^{SR}T117E-GFP, we have observed an increase of polyploidy. This result may explain why Survivin^{SR}T117E-GFP behaves as a dominant negative mutant in cells. Based on the fact that polyploid cells may come from perturbation in either mitosis or cytokinesis, we have followed live cells expressing Survivin^{SR}T117E-GFP by time-lapse microscopy. In fact Survivin^{SR}T117E-GFP cells are unable to cleave properly.

All the above results were recently accepted for publication in Cell Cycle. Marlène Delacour-Larose has performed the former FRAP experiences as well as the characterization of the Survivin^{SR}T117A-GFP mutant whereas I have realized the description of the Survivin^{SR}T117E-GFP mutant. Additional data on this mutant will be described hereafter.

[Cell Cycle 6:15, e1-e7, EPUB Ahead of Print: http://www.landesbioscience.com/journals/cc/abstract.php?id=4482; 1 August 2007]; ©2007 Landes Bioscience

Report

Role of Survivin Phosphorylation by Aurora B in Mitosis

Marlène Delacour-Larose†
My-Nhung Hoang Thi†
Stefan Dimitrov
Annie Molla*

INSERM U823; Institut Albert Bonniot; La Tronche, France

†These authors contributed equally.

*Correspondence to: Annie Molla; INSERM U823; Institut Albert Bonniot; Domaine de la Merci; 38 706 La Tronche, Cedex France; Tel.: 33.476.54.95.74/ 33.476.54.94.70; Fax: 33.476.54.95.95; Email: Annie.molla@ujf-grenoble.fr

Original manuscript submitted: 04/18/07
Manuscript accepted: 05/21/07

This manuscript has been published online, prior to printing for Cell Cycle, Volume 6, Issue 15. Definitive page numbers have not been assigned. The current citation is: Cell Cycle 2007; 6(15):
http://www.landesbioscience.com/journals/cc/abstract.php?id=4482
Once the issue is complete and page numbers have been assigned, the citation will change accordingly.

KEY WORDS

mitosis, passenger protein complex, Aurora B kinase, survivin phosphorylation, survivin mutants

ABBREVIATIONS

CPC	chromosomal passenger protein complex
IF	immuno fluorescence

ACKNOWLEDGEMENTS

This work was supported by grants from INSERM, Rhone-Alpes Canceropole CLARA (EpiProNetwork) (S.D.) and la Ligue Nationale Contre le Cancer (S.D.) (Laboratoire Labellisé). S.D. also acknowledges the support from the Agence Nationale de la Recherche (NT05-3_42614). M.N.H.T is granted by Vietnam/French sandwich program. We appreciate the help of our colleagues from the IAB microscopy plateform.

ABSTRACT

The chromosomal protein passenger complex, a key mitotic regulator, consists of at least four proteins, INCENP, Aurora B, Survivin and Borealin. Survivin, in contrast to the other members of the chromosomal protein passenger complex (CPC), is mobile at metaphase. This protein is also phosphorylated by Aurora B at Threonine 117. In this work we have studied the role of the phosphorylation of Survivin in mitosis by using non phosphorylable T117A and phosphomimic T117E silent resistant Survivin mutants, inducible cell lines expressing these mutants and a combination of siRNA, time-lapse microscopy and FRAP analysis. Time lapse microscopy and FRAP analysis show that Survivin T117A mutant is very stably associated with centromeres and its expression induces a prometaphasic arrest in endogenous survivin depleted cells. In addition, Survivin T117A was unable to rescue the phenotypes of the endogenous survivin depleted cells. Expressed in these cells, the phosphomimic Survivin T117E mutant exhibits a very weak interaction with the centromeres and behaves as a dominant negative mutant inducing severe mitotic defects. Our data suggest that the Aurora B generated phosphorylation/dephosphorylation cycle of Survivin is required for proper proceeding of mitosis.

INTRODUCTION

The chromosomal passenger protein complex (CPC) plays key roles in mitotic events.[1] In early mitosis, CPC promotes chromosome alignment and bi-orientation by correcting mis-attachments of microtubules to the kinetochores.[2,3] CPC is also responsible for the phosphorylation of Histone H3 and in turn, for the displacement of HP1 (Heterochromatin protein 1) from condensed chromatin.[4,5] Furthermore, CPC is an up-stream actor of the mitotic spindle control, orchestrating mitotic spindle assembly and cytokinesis.[6]

The Chromosomal passenger complex is composed of at least four proteins: INCENP, Survivin, Aurora B kinase and Borealin.[7] Among the members of CPC, Aurora B plays a key role, since it is the only passenger protein, which exhibits enzymatic activity.[8] INCENP, the first identified member of CPC, binds to Aurora B through its C-terminus and stimulates its kinase activity.[2] Survivin also directly interacts with Aurora B and regulates the activity of the kinase.[9]

The passenger proteins show specific localization pattern during mitosis. At metaphase they are localized at the inner centromeres and, as mitosis proceeds, they are transferred to the central spindle at anaphase and finally to the midbody at cytokinesis.[1] Depletion of any one of the passenger proteins resulted in very similar mitotic defects, including redistribution of the other members of the CPC, perturbations in mitotic progression, kinetochore/spindle misattachments and the generation of polyploid cells.[1,2]

Survivin and Aurora B exhibit distinct dynamics during mitosis.[10] Both Survivin and Aurora B are immobile at telophase and cell cleavage, and Survivin, but not Aurora B, is highly mobile at prometaphase and metaphase. The mobility of Survivin is dependent on the presence of Aurora B, since the ablation of the kinase by siRNA treatment results in a dramatic decrease of the mobility of Survivin.[10,11] The reported data show, that Survivin, in contrast to Aurora B, is weakly associated with centromeric chromatin at prometaphase and metaphase.[10] Detailed studies on the mobility of the other passenger proteins during mitosis are not available in the literature. In addition, Survivin is phosphorylated at Threonine 117 by Aurora B. The role of this phosphorylation of Survivin is unknown.[12]

In this work we have studied the function of the Threonie 117 phosphorylation of Survivin during mitosis by expressing Threonine 117 nonphosphorylable or phosphomimic silent resistant Survivin mutants in endogenous Survivin depleted cells. Our

immunofluorescence, time-lapse microscopy and FRAP data evidence that the Aurora B dependent phosphorylation/dephosphorylation cycle of Survivin is required for proper proceeding of mitosis.

MATERIAL AND METHODS

SiRNA experiments and reagents. Double stranded RNAi for Survivin suppressions were purchased from Eurogentec. They recognized the AAAGAACUGGCCCUUCUUGGA sequence. Single stranded sense and antisense strands, used as controls and siRNA duplexes were transfected into cells using oligofectamine (Invitrogen) according to the manufacturer's protocols.

VX-680 was purchased from Kava technology, Inc, Blasticidin from Invitrogen and Nocodazole from Sigma.

Cell culture, transfection, immunofluorescence microscopy. Hela T-Rex^{TM} cells were grown on Dulbecco's modified Eagle's (BioWhittaker, Europe) supplemented with 10% fetal bovine serum (BioWhittaker, Europe) and blasticidin (5 mg/mL). Hela were grown under similar conditions but blasticidin was omitted.

Survivin silent resistant cDNA and Hela cells stably expressing Survivin-GFP or Aurora B-GFP were already described and characterised in Delacour et al.[10] Borealin was amplified from testis Marathon library (Clontech) and then cloned in pEGFP-N1 (Clontech) in order to express the fusion Borealin-GFP. GFP-INCENP was a gift from Takeshi Urano (Japan). Mutations were introduced in Survivin and Aurora B by using the QuickChange Site-Directed Mutagenesis kit (Quiagen) under conditions suggested by the manufacturer. Survivin^{SR}-GFP, Survivin^{SR}-T117A-GFP and Survivin^{SR}-T117E-GFP were cloned in pcDNA5/TO plasmid (invitrogen). Their expression was under the control of the tetracycline promoter, the induction was performed by the addition of tetracyclin (250 ng/ml), overnight. All plasmids were transfected into cells using lipofectamin (Invitrogen) under conditions suggested by the manufacturer. 48H after transfection cells were transferred in selecting medium (either geneticin (750 mg/ml) or hygromycin (200 mg/ml)).

For immunofluorescence experiments, cells grown on glass coverslips for 24 hours were fixed, for 15 min, at 37°C, in a solution of PBS, 4% paraformaldehyde and 2% sucrose. Cells were permeabilised in PBS containing 0.2% Triton X-100 for 10 minutes. Free binding sites were blocked with 0.5 mg/ml BSA and specific antibodies were then incubated for at least 30 min in PBS supplemented with 10% bovine serum, 0.2% Tween-20 and 0.02% NaN_3. Aurora B was detected by mouse monoclonal AIM-1 (1/100, transduction Laboratories). Unbound antibodies were removed by washing with PBS, 0.2% Tween-20 and specific staining was revealed with Hylite Fluor^{TM} 546-conjugated secondary antibodies (Euromedex, France). DNA was visualised with 0.1 mM Hoechst 33342 (Sigma). Images were collected with a Zeiss 510 laser scanning confocal apparatus with a 63X oil immersion objective.

Ex vivo microscopy. Ex vivo experiments were conducted on cells grown on Lab-Tek chambered coverglass (Nalge Nunc International) and maintained under standard culture conditions. RNAi were transfected since 48 hours. Images were acquired on a Zeiss LSM510 system using a Planapochromat 40 X water immersion objective. GFP was excited with a 488 nm Argon 2 laser (power varying from 0.1 to 2%). FRAP ("Fluorescent Recovery After Photobleaching") were conducted as already (described in ref. 10). Briefly, an outlined region was bleached with a full power laser and recovery was monitored repetitively during approximately 4 minutes. For rapid scanning, the registered region was restricted to a circle ROI of 40 mm of diameter.

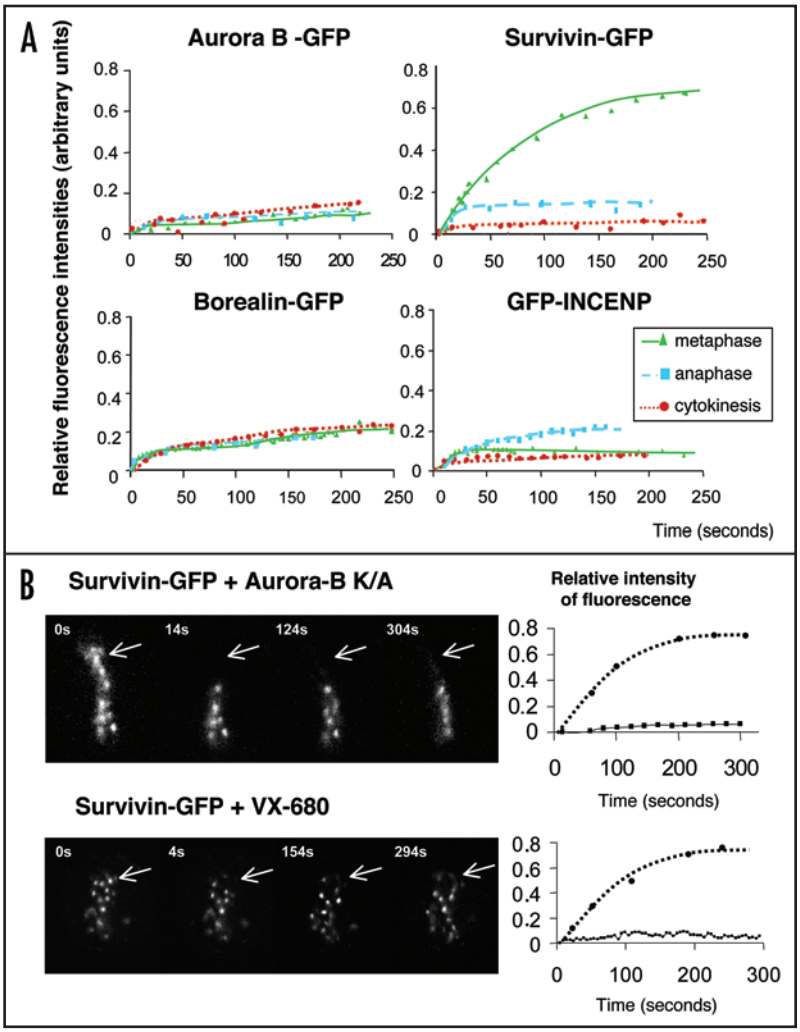

Figure 1. FRAP analysis of the passenger proteins mobility during mitosis. (A) HeLa cells were stably transfected with either Aurora B-GFP or Survivin-GFP or transitory transfected with either Borealin-GFP or GFP-INCENP. Either few centromeres at metaphase or a restricted area of the midzone at anaphase and cytokinesis were photobleached. The recovery of fluorescence was measured on the sequence of images acquired at the indicated intervals postbleaching. Note that Survivin, in contrast to the remaining passenger proteins, is highly mobile at metaphase. (B) The mobility of Survivin is dependent on an active Aurora B kinase. HeLa cells expressing stably Survivin-GFP were either transfected with an inactive Aurora B kinase (Aurora B K109A) or treated overnight with the specific Aurora B kinase inhibitor, VX-680 at a 300 nM concentration. Few centromeres, indicated by an arrow, were photobleached. The kinetics of recovery of fluorescence are shown in the right part of the figure.

RESULTS

Survivin exhibits distinct behavior at metaphase. We have recently reported that Aurora B and Survivin showed distinct mobility at mitosis: both proteins were found immobile on microtubules

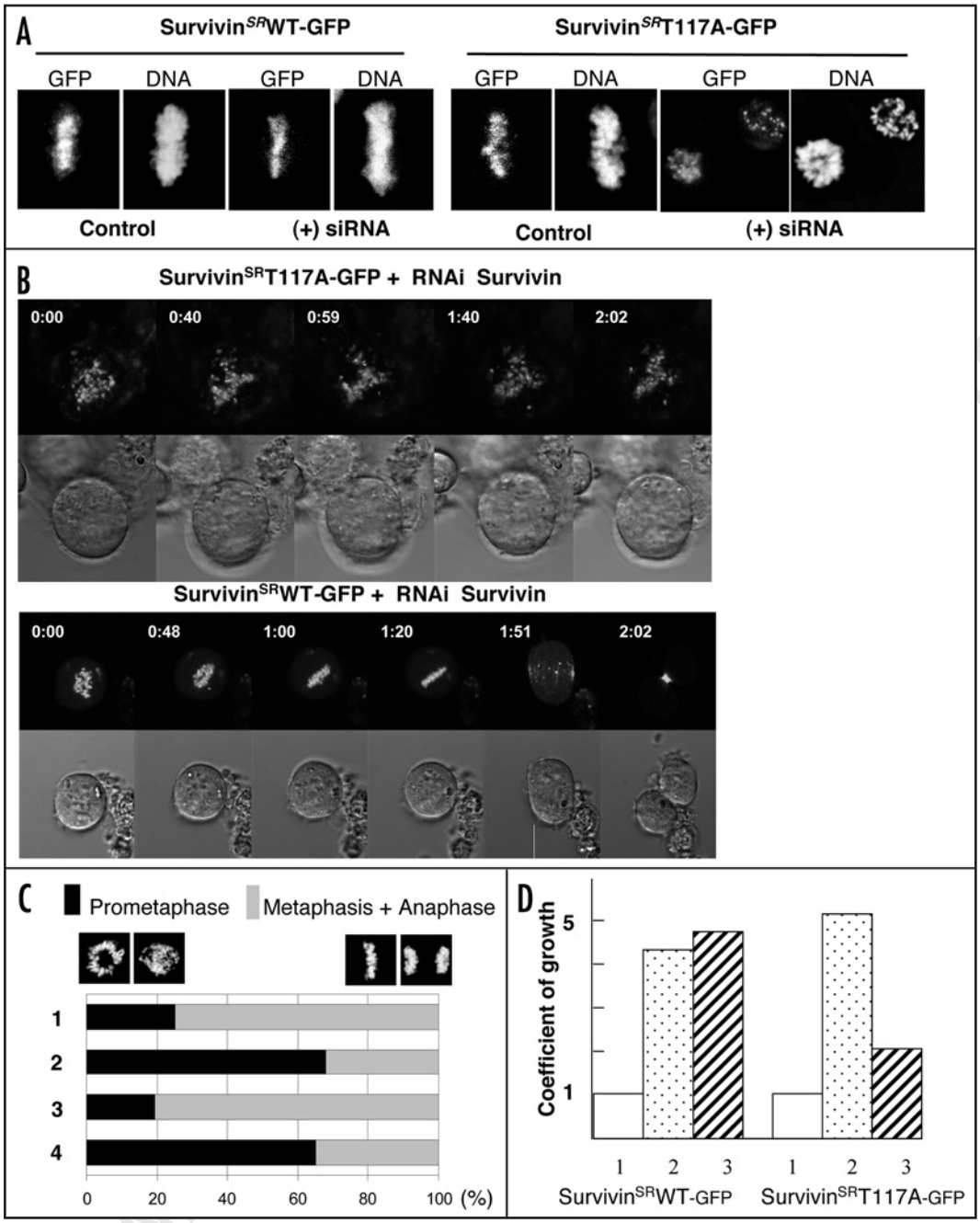

Figure 2. Ectopically expressed Survivin T117A mutant did not restore wild type Survivin function. (A) HeLa T-rex cells containing stably integrated tetracyclin inducible expression vectors for either silent resistant wild type Survivin (Survivin^{SR}WT-GFP, left panel) or silent resistant mutant Survivin (Survivin^{SR}T117A-GFP, right panel) were treated with Survivin siRNA for 48 hours and the expression of the exogenous Survivin proteins was induced by addition of tetracycline. 16 hours after the tetracycline addition, cells were analysed by microscopy. DNA was stained with Hoechst 33342. Note that the siRNA treated cells expressing Survivin^{SR}T117A-GFP were arrested at prometaphase. (B) GFP-fluorescence and transmission time-lapses imaging of endogenous Survivin depleted cells expressing either Survivin^{SR}T117A-GFP (top) or Survivin^{SR}WT-GFP (bottom). Note that the cells expressing Survivin^{SR}WT-GFP proceed normally in mitosis, while the Survivin^{SR}T117A-GFP are arrested at prometaphase. (C) Quantifications of the data. 1, control HeLa T-rex cells; 2, Survivin siRNA treated HeLa T-rex cells; 3, Survivin siRNA treated HeLa T-rex cells expressing Survivin^{SR}WT-GFP; 4, Survivin siRNA treated HeLa T-rex cells expressing Survivin^{SR}T117A-GFP. The data represent the average of three independent experiments. 100 mitotic cells were scored in each experiment. The percentage of prometaphase (black) and this of the sum of metaphase and anaphase (grey) cells are shown. (D) The expression of Survivin^{SR}T117A-GFP in endogenous Survivin-depleted cells affects cell growth. The same number of cells treated as described in (A) was seeded and the growth rate was estimated 84 hours post seeding. Punctuated rectangles (2) and striated rectangles (3) represent the coefficient of growth (the fold increase of the number of initially seeded cells) for control and Survivin siRNA treated cells, expressing either Survivin^{SR}WT-GFP (left) or Survivin^{SR}T117A-GFP (right), respectively. The initial amount of cells was set as one and presented as white rectangles (1).

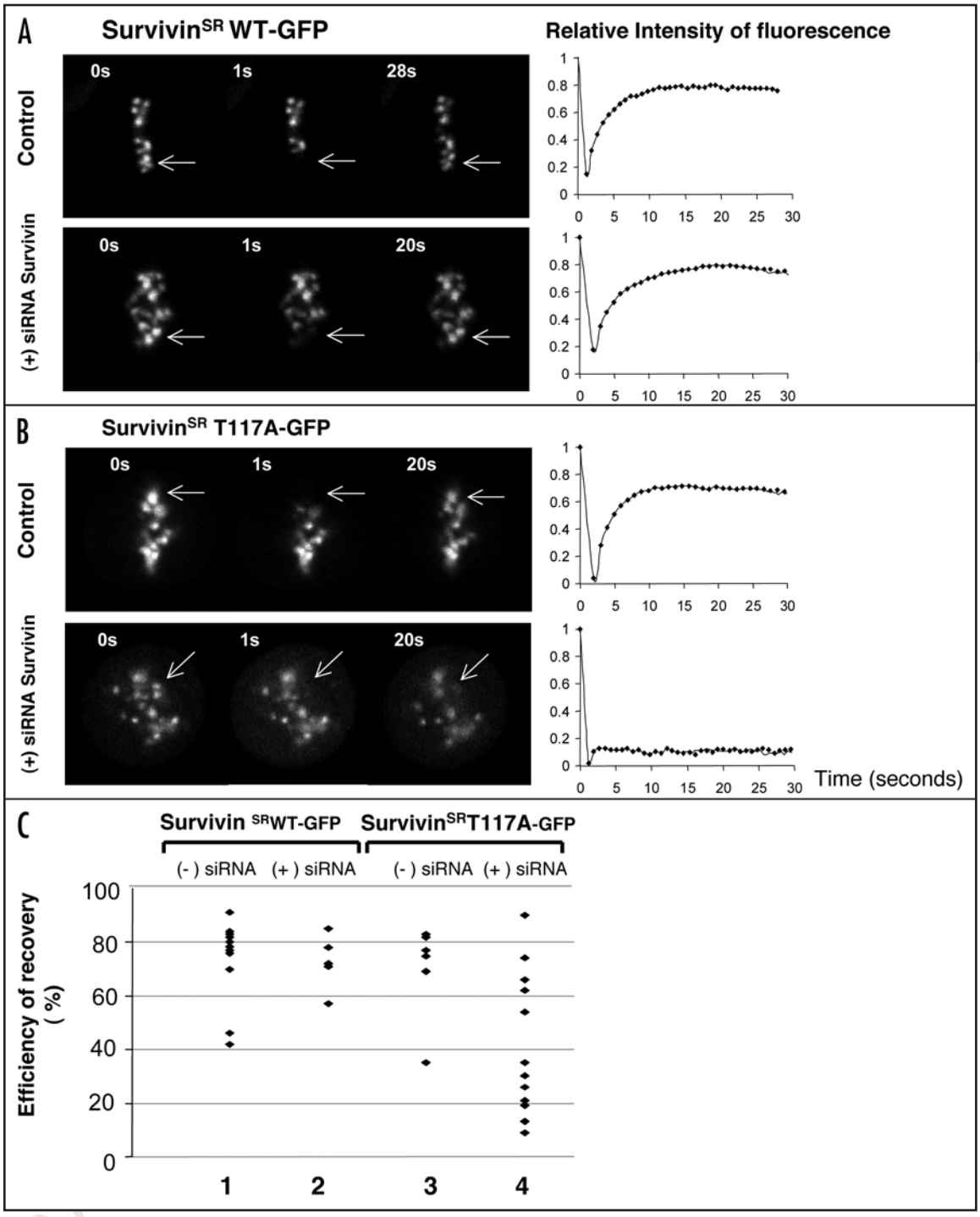

Figure 3. The substitution of threonine 117 for an alanine residue results in a strong decrease of the mobility of the mutant Survivin^{SR}T117A-GFP at metaphase. The expression of either Survivin^{SR}WT-GFP (A) or Survivin^{SR}T117A-GFP (B) was induced by addition of tetracyclin in either control or Survivin siRNA treated HeLa T-rex cells. The efficiency of expression of both mutatnts was very similar as judged by Western Blotting (data not shown). Few centromeres (indicated by an arrow) of the mitotic cells were photobleached and the recovery of fluorescence was measured at different times postbleaching. The quantification of the FRAP data is shown at the right of each panel. To note is that the kinetics of fluorescence recovery for Survivin^{SR}T117A-GFP in the siRNA treated cells was decreased to different extent (see panel C) and an extreme example of a quasi-total lost of mobility of Survivin^{SR}T117A-GFP is shown. C) Summary of the variation of the efficiency of fluorescence recovery (the saturation level of fluorescence recovery measured at 15 seconds postbleaching) in the different FRAP experiments for Survivin^{SR}WT-GFP and Survivin^{SR}T117A-GFP in control and Survivin siRNA treated cells. 1 and 2, the efficiency of Survivin^{SR}WT-GFP fluorescence recovery in control and siRNA treated cells, respectively; 3 and 4, same as 1 and 2, but for the efficiency of Survivin^{SR}T117A-GFP fluorescence recovery. Each diamond accounts for one cell. Note that Survivin^{SR}T117A-GFP exhibits very low mobility in a large number of individual Survivin siRNA treated cells.

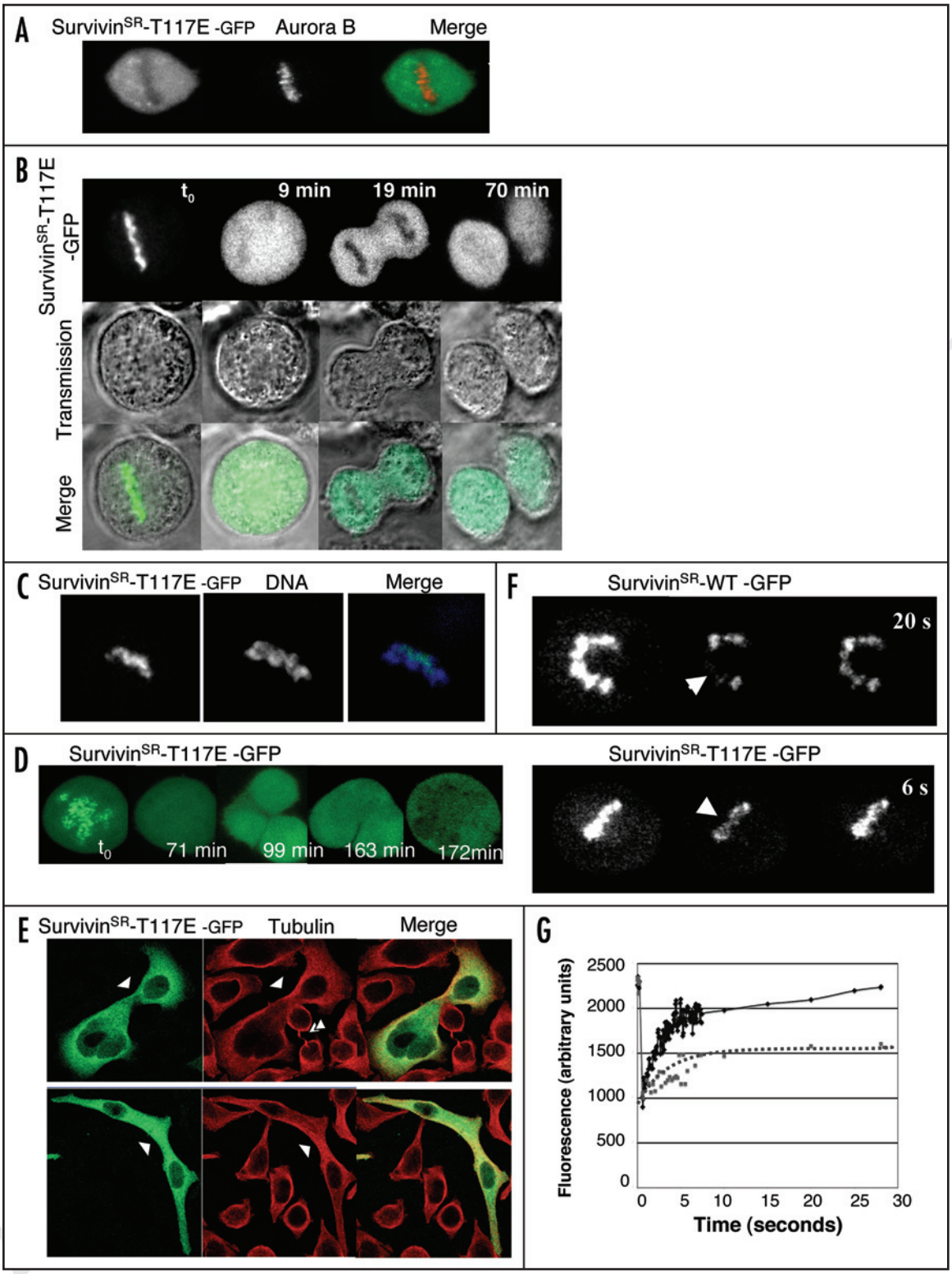

Figure 4. For legend, see page e6

and Survivin, but not Aurora B, was found mobile at centromeres at both prometaphase and metaphase.[10] The arising question was if the two other members of the CPC, INCENP and Borealin, behaved like Survivin or like Aurora B. To address this question we have carried out FRAP studies by using HeLa cell lines expressing GFP-fusions of either INCENP or Borealin or Aurora B or Survivin. All these GFP-fusions showed the typical localization pattern of the passenger proteins during mitosis. They localized at the centromeres at metaphaphase and were transferred to the central spindle and the midbody as mitosis proceeds[10] and data not shown. In agreement with our previous data, we observed that Aurora B-GFP was immobile during all mitotic phases, while Survivin-GFP

Figure 4. Localization and mobility of Survivin^{SR}T117E-GFP mutant. HeLa T-rex cells were transiently transfected with Survivin^{SR}T117E-GFP expression vector and the expression of Survivin^{SR}T117E-GFP was induced by tetracyclin. (A) Survivin^{SR}T117E-GFP loose its centromeric localization in paraformaldehyde fixed cells. HeLa T-rex cells expressing Survivin^{SR}T117E-GFP were fixed with parafolmaldehyde and the localization of Survivin^{SR}T117E-GFP was visualized by the fluorescence of its GFP. Aurora B was detected by immunofluorescence by using specific antibodies. DNA was stained with Hoechst 33242. Typical example of a metaphasic cell is shown. (B) GFP-fluorescence and transmission time lapse imaging of Survivin^{SR}T117E-GFP. The merge of both signals is also presented. Note that Survivin^{SR}T117E-GFP is localized at the chromosomes in metaphase, but is not transferred to the central spindle and the midbody as mitosis proceeds. (C) Survivin^{SR}T117E-GFP is associated with mitotic chromosomes. Survivin^{SR}T117E-GFP was visualized by the GFP-fluorescence, while DNA was stained with Hoechst 33242. Hoechst 33242 was added for 10 min in the culture medium. Following a quick wash with culture medium, the cells were immediately imaged at 488 nm (GFP) and 720 nm (Hoechst). The merge of both signals is also shown. (D) Expression of Survivin^{SR}T117E-GFP results in a complex cell phenotype. A Hela T-rex cell expressing Survivin^{SR}T117E-GFP was continuously imaged. This time-lapse reveals the formation of a polyploidy cell within 3 hours. (E) Immunofluorescence microscopy of fixed cells expressing Survivin^{SR}T117E-GFP. Cells were fixed and submitted to an IF. Green, Survivin^{SR}T117E-GFP; red, tubulin, detected with a specific anti-tubulin antibody. Single arrowheads point abnormal connection between cells, whereas double arrowhead point a normal mid-body in non transfected cells. (F) Survivin^{SR}T117E-GFP is highly mobile at metaphase. HeLa T-rex cells were transiently transfected with either Survivin^{SR}WT-GFP or Survivin^{SR}T117E-GFP expression vectors and the protein expression was induced by tetracyclin. Few centromeres were bleached and fluorescence recovery was imaged. (G) FRAP quantifications. FRAP were performed as in Figure 1 except that a rapid scanning of fluorescence recovery was performed by imaging a Region Of Interest (ROI) including the bleached region. Times between two scanning was 60 ms. The squares (dotted line) represented the chimera Survivin^{SR}-GFP and the diamonds (continuous line) the Survivin^{SR}T117E-GFP.

was mobile at metaphase, but not at anaphase and cytokinesis (Fig. 1A, the upper two panels and in ref. 10). Both Borealin-GFP and GFP-INCENP behaved like Aurora B-GFP, i.e., they exhibited a very restricted mobility during all phases of mitosis (Fig. 1A, the two lower panels). We conclude that Survivin shows a distinct behavior compared to the other CPC members, being the only CPC member mobile at metaphase.

The presence of Aurora B was required for the higher mobility of Survivin at metaphase.[10] To further test if the mobility of Survivin depended on the enzymatic activity of Aurora B, we have either expressed the dominant negative dead Aurora B K106A mutant[13] in stable Survivin-GFP cell lines or treated these cells with VX-680, a specific inhibitor for Aurora kinases. These procedures led to abolishment of the activity of Aurora B, as judged by the lack of phosphorylation of histone H3 at mitosis[13,14] and data not shown). Then we have carried out FRAP experiments. The data show very clearly that in both cases Survivin-GFP became very stably associated with centromeres at metaphase (Fig. 1B). Therefore, the enzymatic activity of Aurora B is essential for maintaining the higher mobility of Survivin at metaphase.

Survivin T117A mutant was unable to rescue survivin function in cells in which survivin has been ablated by siRNA. Aurora B phosphorylates Survivin at Threonine 117.[12] Since the enzymatic activity of Aurora B is required for the mitotic behavior of Survivin, this suggests that the phosphorylation of Survivin by Aurora B would affect the mitotic properties of Survivin and consequently the fate of the cells. To address this we have performed a series of experiments by using a non phosphorylable Survivin T117A-GFP mutant in which threonine 117 was substituted for alanine. In order to study the behavior of this mutant in the absence of endogenous Survivin we have taken advantage of the pseudogenetic approach described previously.[10] Briefly, the endogenous Survivin was ablated by siRNA treatment in HeLa T-rex cell lines containing stably integrated expression vector for either silent resistant SurvivinT117A-GFP mutant (Survivin^{SR}T117A-GFP) or silent resistant wild type Survivin (Survivin^{SR}WT-GFP). Both chimera were under the control of the tetracycline operator and the protein expression was induced by tetracycline (we have used HeLa T-rex cells since we expected this to allow a quick induction of the Survivin^{SR}T117A-GFP by tetracycline addition and the study of its behavior in relatively safe conditions for the cell, see below). Indeed, treatment with tetracycline resulted in a rapid induction of the expression of both proteins as judged by GFP fluorescence visualization and Western blotting (Fig. 2 and data not shown).

Both Survivin^{SR}WT-GFP and Survivin^{SR}T117A-GFP were recruited to centromeres in the presence of endogenous Survivin (Fig. 2A and B), a result in agreement with the previously reported data.[12] The centromeric localization of both proteins was not affected by the absence of endogenous Survivin (Fig. 2A and B). The suppression of the expression of the endogenous Survivin resulted, as previously reported,[2] in a prometaphase arrest (Fig. 2A–C). The ectopic expression of Survivin^{SR}WT-GFP, but not Survivin^{SR}T117A-GFP, was sufficient, however, to restore the proper proceeding of mitosis (Fig. 2C). In agreement with this, the growth rate of cells, in which the expression of endogenous Survivin was compromised by siRNA treatment, was rescued by the expression of Survivin^{SR}WT-GFP (Fig. 2D). Conversely, no growth rate cell rescue was observed following Survivin^{SR}T117A-GFP expression (Fig. 2D). Consequently, the substitution of the phosphorylable threonine 117 residue for alanine resulted in a nonfunctional Survivin mutant, suggesting that the phosphorylation of threonine 117 by Aurora B is essential for the function of Survivin at mitosis.

Survivin T117A mutant remains stably associated with centromeres at metaphase. Since Survivin was found highly mobile on centromeres, we next asked if the T117A mutation, which creates a nonphosphorylatable mutant, affects Survivin mobility. FRAP experiments were conducted on both control and Survivin siRNA treated HeLa T-rex, in which the expression of either Survivin^{SR}WT-GFP or Survivin^{SR}T117A-GFP was induced by tetracycline. The absence of endogenous Survivin did not affect the recovery of fluorescence of Survivin^{SR}WT-GFP (compare the upper and lower panels of Figure 3A; to note is that these recovery kinetics are more rapid than those for Survivin-GFP presented on Figure 1A; we attribute this to the higher expression of Survivin^{SR}WT-GFP induced by tetracycline in the HeLa T-rex cells). The picture was, however, completely different for Survivin^{SR}T117A-GFP. Indeed, while in the presence of endogenous Survivin, the fluorescence recovery curves for Survivin^{SR}T117A-GFP were very similar to those for Survivin^{SR}WT-GFP (compare Fig. 3A with the upper panel of Fig. 3B), the absence of endogenous Survivin resulted in an impressive decrease in the mobility of Survivin^{SR}T117A-GFP (Fig. 3B, lower panel and Fig. 3C). It is noteworthy that we have observed upon knock in of endogenous Survivin some variations in the mobility of Survivin^{SR}T117A-GFP within the individual cells (Fig. 3C). We attribute these differences mainly to different efficiencies of endogenous Survivin suppression by the siRNA treatment.

Therefore, the substitution of threonine 117 for alanine creates a mutant Survivin protein, which remains stably associated with centro-

meres at metaphase. This, in turn, suggests that the phosphorylation of Survivin might be essential for its increased mobility at metaphase. To test this we have used a Survivin^{SR}T117E-GFP mutant, which mimics the phosphorylated Survivin at threonine 117.

Localisation and behavior of Survivin T117E mutant. In spite of several attempts, we were unable to establish HeLa T-rex cell lines containing stably integrated tetracycline inducible expression vector for Survivin^{SR}T117E-GFP. All experiments were conducted on transiently transfected HeLa T-rex cells with the expression vector for Survivin^{SR}T117E-GFP and compared to HeLa T-rex cells expressing Survivin^{SR}WT-GFP under the same conditions.

Initially, we have carried out experiments on paraformaldehyde fixed cells that expressed Survivin^{SR}T117E-GFP and we have codetected Survivin^{SR}T117E-GFP and the other passenger proteins. Although Aurora B and INCENP decorated the centromeres, Survivin^{SR}T117E-GFP, in agreement with the previously reported data,[12] was found excluded from chromatin in metaphasic cells (Fig. 4A). However, when live cells were imaged, Survivin^{SR}T117E-GFP was found localized as endogenous Survivin on centromeres (Fig. 4B, t_0). The time-lapse studies performed on these cells revealed that Survivin^{SR}T117E-GFP was released from chromatin at the anaphase onset (Fig. 4B, 9min). In late anaphase, Survivin^{SR}T117E-GFP was present in the whole cytoplasm (Fig. 4B, 19 min) and no midbodies were decorated by Survivin^{SR}T117E-GFP at cell cleavage (Fig. 4B, 70 minutes). We further confirmed the early mitotic chromosomal localization of Survivin^{SR}T117E-GFP by adding Hoechst to the culture medium. GFP and DNA were immediately imaged. Survivin^{SR}T117E-GFP was detected on the centromeres in all imaged metaphasic cells (Fig. 4C).

Such a discrepancy between the observations in fixed and alive cells indicated that although localized at the centromeres, Survivin^{SR}T117E-GFP was poorly bound and dissociated easily during cell fixation. To further show that Survivin^{SR}T117E-GFP was loosely associated with centromeres we have carried out FRAP experiments (Fig. 4F and G). As seen, the kinetics of fluorescence recovery of Survivin^{SR}T117E-GFP was much more rapid compared to Survivin^{SR}WT-GFP and the saturation level of the fluorescence recovery was also higher (Fig. 4G). Taken collectively these data strongly suggest that the phosphorylation of Survivin at threonine 117 by Aurora B results in perturbations of its interactions with the centromeres at metaphase.

Moreover, Survivin^{SR}T117E-GFP behaved as a dominant negative mutant since its expression led to very severe cell phenotypes, mainly reflecting cytokinesis defects (Fig. 4D and E). Cells, expressing Survivin^{SR}T117E-GFP, were unable to cleave properly and polyploid cells were observed by both IF (Fig. 4E) and time-lapse microscopy (Fig. 4D).

DISCUSSION

In this work we have studied the role of the phosphorylation of Survivin at threonine 117 by Aurora B. First, we showed that Survivin exhibited unique property among the remaining passenger proteins: it was loosely associated with centromeres at both prometh-aphase and metaphase. We further presented evidence that the enzymatic activity of Aurora B was required for the generation of this peculiar property of Survivin. To test whether the phosphorylation status of Survivin itself was important for the mobility of Survivin at mitosis, we have expressed either a nonphosphorylable silent resistant Survivin (Survivin^{SR}T117A-GFP) or a phosphomimic silent resistant Survivin^{SR}T117E-GFP mutant, in both control and Survivin siRNA treated cells. We found that the substitution of threonine for alanine at a position 117 resulted in a marked decrease in the mobility of Survivin in cells in which the expression of endogenous Survivin was suppressed by the siRNA treatment, but not in control cells. This evidences that the nonphosphorylable Survivin, in contrast to the wild type one, associated in a stable manner with the centromeres at metaphase. We attributed the lack of change in the mobility of Survivin^{SR}T117A-GFP observed in the presence of endogenous Survivin in control (non-siRNA) treated cells to the dimeric structure of Survivin.[15,16] Survivin could form a "heterotypic dimer", consisting of one wild type protein and one Survivin^{SR}T117A-GFP mutant, which might be sufficient to preserve the mobility of the protein at the centromeres at metaphase.

The phosphomimic Survivin^{SR}T117E-GFP mutant, in contrast to the nonphosphorylatable mutant Survivin^{SR}T117A-GFP, exhibited a much higher mobility when compared to the wild type Survivin^{SR}-GFP in control, non siRNA treated cells. This suggests that the structure of the "heterotypic" Survivin^{SR}T117E-GFP-endogenous Survivin dimer would be strongly perturbed, affecting in turn its association with the centromeres at metaphase.

Interestingly, the expression of each Survivin mutant, either the nonphosphorylable or the phosphorylable one, had deleterious consequences for the cell. In the case of Survivin^{SR}T117A-GFP, the knock in of the endogenous protein was required for anaphase onset, whereas Survivin^{SR}T117E-GFP behaved as a dominant negative and its expression even in the presence of endogenous Survivin led to strong mitotic defects and polyploidy.

These data taken together could shed light on the role of the phosphorylation of Survivin at mitosis. We hypothesize that endogenous Survivin is subjected to a phosphorylation/dephosphorylation cycling at Threonine 117 and this dynamic equilibrium of Survivin phosphorylation determined by Aurora B would be required for its function at mitosis. The CPC complex is a "sensor" for the proper attachment of the microtubules to the kinetochores and it is implicated in the corrections of the improper microtubules-kinetochores attachment. The reported data suggest that the suppression of the enzymatic activity of Aurora B, which appeared to happen at improper attached kinetochores and thus, in the absence of tension, is associated with the degradation of the K-mitotic microtubule formed fibers and the subsequent correction of the attachment. In addition, it is well established that the presence of Survivin is essential for a functional CPC complex.[17] Our data suggest that Survivin phosphorylation would release it from the centromeres and the remaining incomplete CPC complex would be no longer functional. A signal for Survivin phosphorylation could be associated with the lack of tension at improper attached kinetochores. The generation of nonfunctional CPC complex (resulting from the release of the phosphorylated Survivin) and in particular, from a nonfunctional Aurora B, would lead to corrections of the attachment according to the above-described scenario. Once the phosphorylated Survivin is released from the kinetochores, it would be dephosphorylated and then it would be able to associate again with the centromeres and to fulfill its function. This model is in good agreement with our data and explains why the presence of only the nonphosphorylable Survivin^{SR}T117A-GFP or the phosphomimic Survivin^{SR}T117E-GFP affects severely the fate of the cells.

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I.2. Consequence of Survivin T117E over-expression in mitosis

Survivin^{SR}T117E-GFP behaves as a dominant negative mutant as shown in the manuscript and its over-expression induces the appearance of polyploidy cells. We have checked whether Survivin T117E may affect CPC functions like the control of the spindle checkpoint and the recruitment of cytokinesis players.

I.2.1 Survivin^{SR}T117E-GFP mutant affects the spindle checkpoint

Cells are arrested in mitosis by Taxol and the percentage of mitotic cells is counted 15 hours later (Figure 21). We have noted a significant decrease in the percentage of mitotic cells in the cell population expressing SurvivinT117E-GFP in comparison to the control population. The mitotic cells decrease from 28.1 % in the control to only 8.2 % in the Survivin^{SR}T117E-GFP cell population (Figure 21). This suggests that cells expressing Survivin^{SR}T117E-GFP can escape from mitotic arrest or thoroughly that the spindle checkpoint is disrupted.

I.2.2 Phosphorylated Survivin is required for the correct localization of MKLP1

The kinesin MKLP1 co-localizes with passenger proteins at the spindle midzone in anaphase and concentrates in the central of midbody. In telophase, it has a distal localization in the midbody, being present at the border of the two daughter cells. It is one of the constituents of the centralspindlin whose recruitment to the midbody matrix involved Aurora kinase activity. In cell expressing Survivin^{SR}T117E-GFP, MKLP1 has a more diffuse localisation (Figure 22). Therefore the phosphomimetic mutant perturbs the localisation of the kinesin. This mis-localisation may partly explain the dominant negative phenotype observed with Survivin^{SR}T117E-GFP in cytokinesis and may perhaps be due to the mis-function of the CPC. However this last point remain an open question.

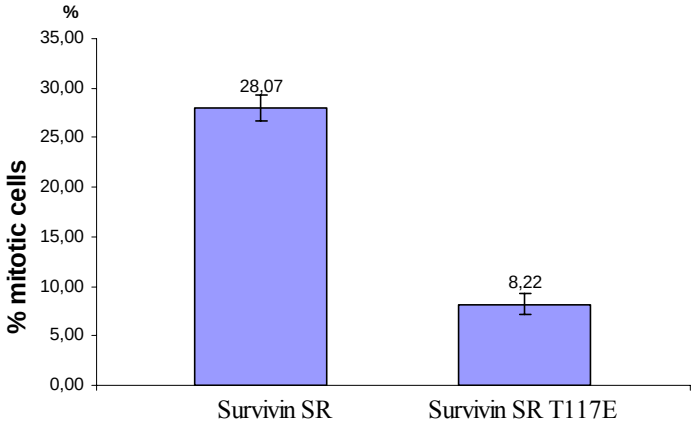


Figure 21: Phosphorylated Survivin has effect on the stable spindle assembly checkpoint. Cells expressing Survivin^{SR}T117E-GFP escape from mitotic arrest induced by Taxol. There is a decrease in the number of mitotic cells in the cells population transfected with Survivin^{SR}T117E-GFP in comparison to the controls.

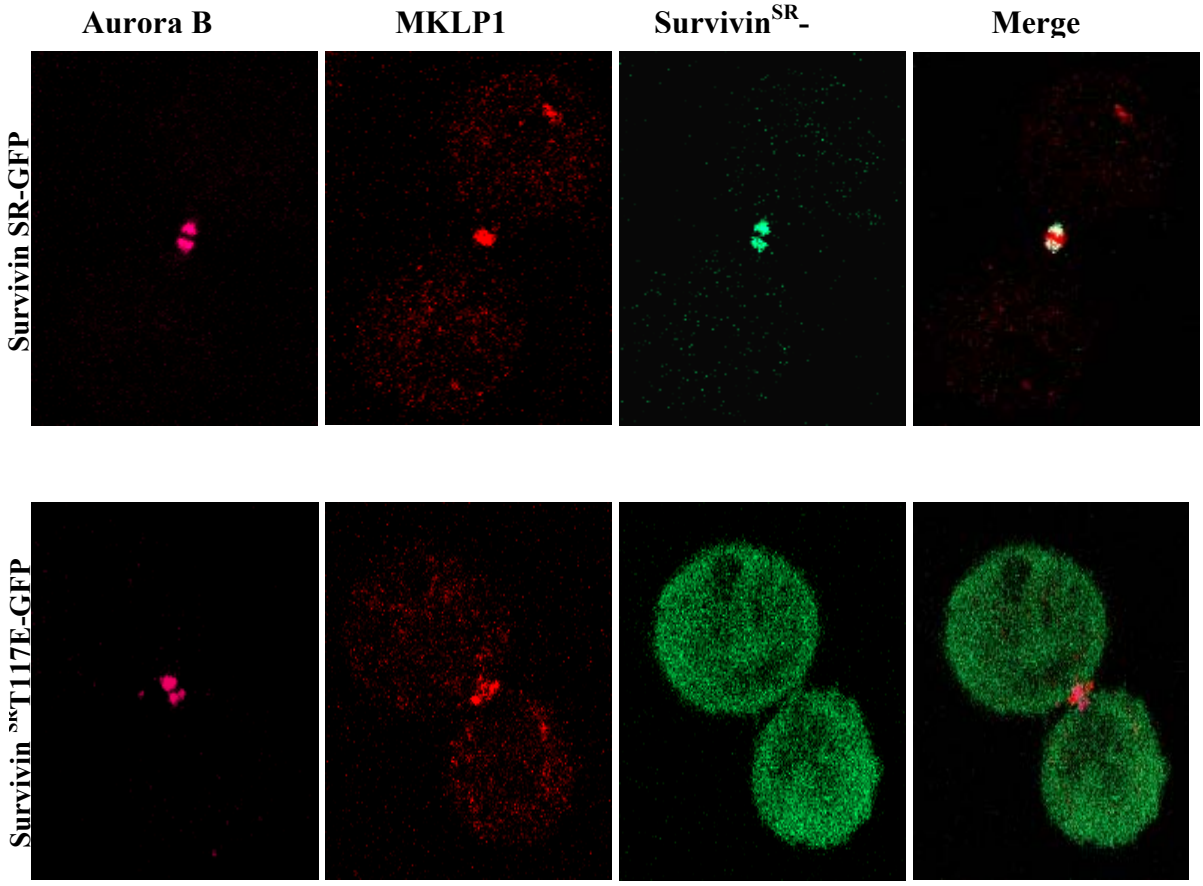


Figure 22: **Phosphorylated Survivin impairs the localisation of MKLP at the spindle midzone/midbody**. In cytokinesis, MKLP1 normally concentrates at the central middle of midbody while Aurora B and Survivin decorate the end border of midbody In the cells expressing SurvivinSTT117E-GFP, MKLP1is not well localization at midbody. Merge represents the three markers: Survivin in green, Aurora B in far red and MKLP1 in red)

Among the CPC, Aurora B and Survivin have their own features. Aurora B is the only enzymatic unit whereas Survivin is the only mobile passenger protein at centromere. They are in a close interaction since Survivin is phosphorylated at Thr117 by Aurora B kinase. Phosphorylation of Survivin at this residue is necessary for anaphase onset meanwhile its dephosphorylation is essential for the organization of midbody. The identification of a cytokinesis dominant negative Survivin contributes to the idea that Survivin may be considered as a target for cancer therapy.

CHAPTER II: IDENTIFICATION OF AURORA KINASE INHIBITORS

II.1. Kinase purification

II.2. Setting-up the protein kinase assay

II.3. High throughput screening

II.4 Characterization of the hits

II.4.1 Flavone inhibits Aurora kinase *in vitro*

II.4.2 Benzo[e]pyridoindoles a new class of Aurora kinase inhibitors

II.5 Aurora kinase inhibitors, interesting tools for studying the CPC

Aurora kinases play key roles in cell division and are thus potential targets for cancer therapy. Lots of efforts are devoted to the identification of molecules that may inhibit their functions; these inhibitors may gain interest in clinical purpose. We took advantage of the possible access to the CEA high throughput screening facilities (Laurence Lafanéchère, Grenoble) for searching Aurora kinase inhibitors in the French patrimonial library.

First, we have purified active kinases and then, set up a kinase assay suitable for high throughput screening. Last we have characterized the hits.

II.1. Kinase purification

In the laboratory, Fabienne Hans and Fabienne Sirot have established vectors that lead to the bacterial expression of Aurora kinases fused to a poly-histidine tag. Aurora A kinase domain and whole Aurora B kinase expressed on E. coli were purified with the QIA*express* protein purification system. The quality of the purified proteins was checked by SDS-PAGE electrophoresis and a typical purification is presented in Figure 23. The purified kinase is quite homogenous; The purity of the preparation is superior to 95 %.

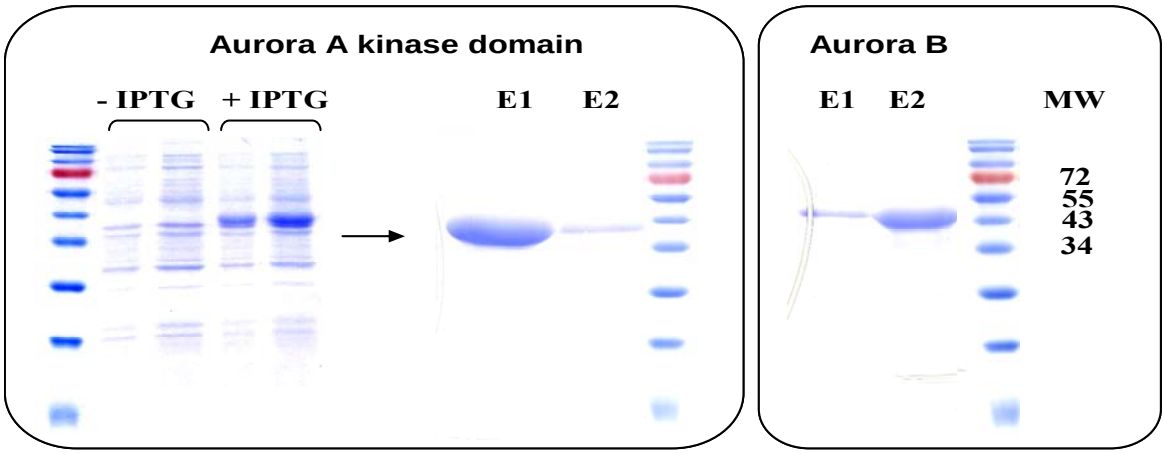


Figure 23. Purification of both the kinase domain of Aurora A and Aurora B kinase. SDS poly-acrylamide gel electrophoresis stained by Coomassie blue. On the left are shown bacterial lysates; Bacteria were either grown in the presence or the absence of IPTG. The samples eluted from the column are on the right (E1: the first elution. E2: the second elution). MW are in KDa.

II.2. Setting up the protein-kinase assay

The protein kinase assay is based on the luminescent determination of the amount of ATP remaining after the reaction and is set up with both recombinant enzymes and

substrate. The protein-kinases were described previously and the recombinant Histone H3 produced in E. coli was purified by Monique Charra.

In order to optimize the conditions for the assay, we have carried out testing under varying experimental conditions. The activity is found dependent on both the presence of Histone H3 and the kinase and, is inhibited by Staurosporine (Table 6). The variations of pH (from 6.5 to 8.5) have minor effect on the kinase domain activity.

The best final concentration for Aurora A domain kinase is 2.4 ng/µl and ATP should be used at the concentration of 0.4 µM. A statistical Z factor of 0.77 was determined, indicating an excellent quality assay. The test is thus robust enough for high-throughput screening of kinase inhibitors.

Table 6: Parameters of the kinase assay

Histone H3	+	+	+	-
Kinase	+	+	-	+
ATP	+	+	+	+
Staurosporin	-	+	-	-
Kinase Activity (%)	**100**	**9**	**0**	**0**

II.3. High throughput screening

The high throughput screening was performed on the CEA plateform developed by Laurence Lafanéchère. The screening was realized by Caroline Barette in interaction with Annie Molla.

We have tested around 10 300 molecules from the library of Curie Institute, Lyon, Grenoble and NIH (USA). The visualization of the results is shown in Figure 24.

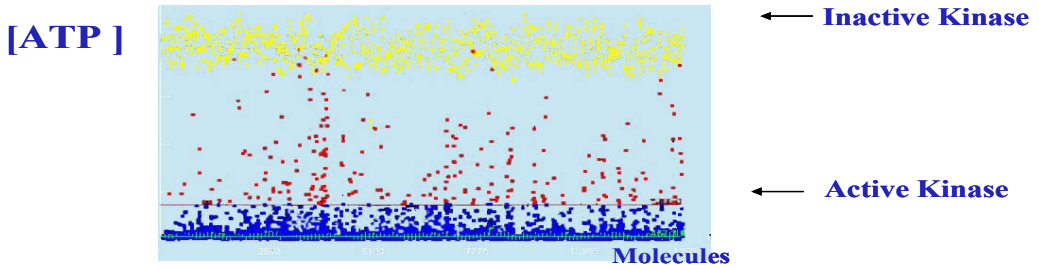


Figure 24: High throughput screening schema. The activity of Aurora kinase is determined based on the concentration of remaining ATP after the reaction between recombinant Aurora kinase and Histone H3. The highest concentration of ATP reflects the lowest Aurora kinase activity. Each point represents one tested molecule.

The first screening was performed at the concentration of 15 µM and positive hits were selected as follow: molecules that inhibit Aurora kinase by more than 50 %. Among them the hits also found in a previous screen devoted to the search of Casein kinase II inhibitors are not considered anymore. The positive molecules are confirmed at the concentration of 1.5 µM. Finally seven molecules inhibit Aurora kinase domain by more than 50 % at 1.5 µM. Two of these molecules belong to NIH library, one is from Villeurbanne (Lyon 1 University) and the others are from the proprietary Institut Curie-CNRS library. Molecules from Villeurbanne and Institut Curie will be described in detail.

II.4 Characterization of the hits

II.4.1 Flavone inhibits Aurora kinase *in vitro*

The positive hit belongs to the flavonoid class of molecules and exhibits an IC50 of 1.5 µM towards Aurora A kinase domain.

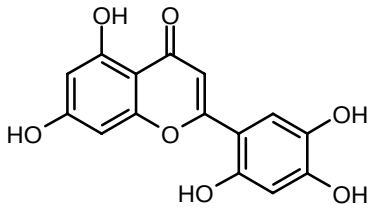
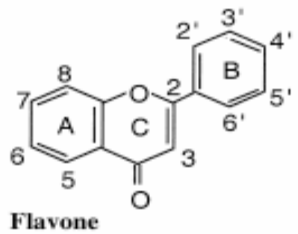


Figure 25 : Hit from the Villeurbanne library

Bernard Marquet (University Lyon 1) has provided us several similar compounds. Around 50 molecules of the flavanoid family were tested manually. The results of the few molecules are reported in Figure 26-a. These data strongly suggest that hydroxyles at positions 5, 7 and 4' may be important for Aurora kinase inhibition and prompt us to test two commercially available compounds Luteolin (C08) and Apigenin (DO6). The molecules are shown in Figure 26-b. As reported in Figure 26-a both compounds inhibit significantly Aurora kinase domain. Full inhibition was observed at 15 µM and inhibitions of 50 % and 33 % were found, at 1.5 µM, for C08 and D06 respectively. Therefore, in the second generation of compounds, we have determined an active scaffold for Aurora kinase inhibition but none of the compounds exhibits higher activity than the primary hit (ID 47, Figure 26-a). Although the reported inhibitions are modest we have tested the active molecules (ID 46, ID47, CO8 and D06) in HeLa cells at 1 µM and 5 µM. Aurora kinase are responsible for the phosphorylation of histone H3 in mitosis and we have access to a specific anti-phospho H3 histone. Immunofluorescences are thus conducted on control and

treated cells. We have not observed any modification of the level of histone H3 phosphorylation in mitosis and any significant effect on the cells (data not shown).

In conclusion although some flavone compounds are efficient toward Aurora kinase *in vitro* they do not target Aurora kinase in cell culture.



Flavone

ID	Activity % 15 µM/ 1.5 µM	5	6	7	8	2	2'	3 '	4'	5'	6'
27	**44**	OH		OH	OH						
28	**6**	OH	OH								
26	0	OH							OCH3		
21	0	OH		OH		OH A			OCH2Φ		
20	0	OH		OH		OH A		OCH3			
47	**96/44**	OH		OH		OH A		OH	OH	OH	
46	**96/53**	OH		OH				OH	OH	OH	
29	0	OH			OCH3						
30	**27**	OH			OH						
1	**19**	OH	OMe	OH	CH3	COCΦS					
22	0	OH		Ocyclo(OH)		OH A					
23	0	OH		OH	CH3	OH A					
24	0	OH		OH	CH3	OH A	OMe		OMe	OMe	
33	**33**	OH		OH				OMe	OH	OMe	
34	**9**	OH		OH				OMe	OMe	OMe	
35	**26**	OH	CH3	OH				OMe		OMe	
36	**49**	OH	CH3	OH					OMe		
37	0	OH		OH				OMe	OMe		
38	0	OH	CH3	OH	CH3						
44	0	OH		OMe							
48	**21**	OH	CH3	OMe	CH3					OMe	OMe
49	0	OCOMe		OCOMe						NH2	
C08	**100/50**	OH		OH				OH	OH		
D06	**100/33**	OH		OH					OH		

Figure 26-a

The molecule skeleton is represented in the upper part. A number is attributed to each carbon as well as a capital letter to each cycle. In the table the molecules are identified by a code. Inhibition activities are expressed in percent. They were determined at 15 µM and eventually at 1.5 µM. Efficient compounds are underlined in bold.

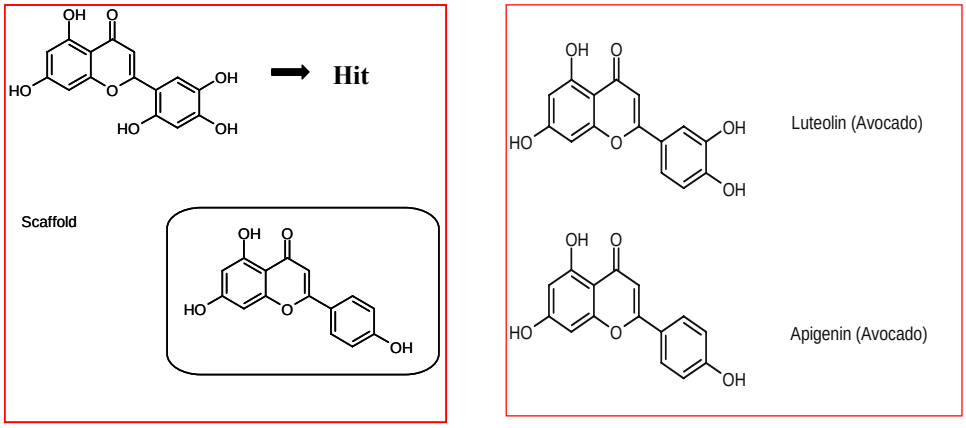


Figure 26-b. Flavone compounds
On the left panel are reported both the hit found by high throughput screening and the scaffold deduced from the test of the second generation of compounds. On the right panel two commercially available compounds fitting with the deduced scaffold.

II.4.2 Benzo[e]pyridoindoles a new class of Aurora kinase inhibitors

The molecules identified in the Curie-CNRS library belong to the benzo[e]pyridoindoles. The Institut Curie-CNRS library contains 6560 compounds, mostly mono- to penta-herocyclic molecules. Among them 40 were found to inhibit Aurora kinase by more than 50 % at a concentration of 15 µM. Of the fourteen most active molecules identified 43 % belong to the benzo[e]pyridoindole family (Figure 27).

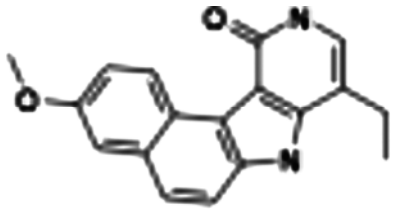


Figure 27. The best hit found in the Curie-CNRS library

Hit 1 exhibits an ethyl chain on the pyridin cycle that is turned to a methyl in hit 2. Both hits belong to the benzo[e]pyridoindole family molecules.

Other hits from this library are not considered pertinent since they are bi- and tricyclic aromatic compounds bearing reactive (aldehyde) functionality. The efficiency of the two best hits is analyzed both *in vitro* towards purified Aurora kinase and *ex vivo* in cell cultures.

Most of the data are gathered in a manuscript submitted for publication at Chem. Biol. Additional data will be presented at the end of the chapter.

Hit 1 inhibits Aurora A kinase domain by 50% at 320 nM compared to 660 nM for hit 2. The effect of hit 1 on the whole Aurora A and B kinases is also defined with IC50 of 450 nM and 600 nM respectively. *In vitro* the best hits present a moderate activity towards Aurora kinases but what about their efficiency in cell culture? We have examined their effects on downstream signalling. Histone H3 is a direct downstream target for Aurora kinases, especially for Aurora B kinase. Phosphorylation of a highly conserved residue Ser 10 in histone H3 is though to be crucial for entry in mitosis. We have found that compounds 1 and 2 suppress by 86 % and 53 % the phosphorylation of histone H3 respectively. These results are obtained by both Immunofluorescence and Western blotting. Therefore these molecules seem to diffuse in the cells and to target Aurora kinase in the cell environment.

We have also noticed that in the cell population treated with these compounds, there is an impressive increase in polyploid cells or irregular nucleus cells. Aurora kinase, especially Aurora B, is involved for maintaining the mitotic spindle checkpoint on, so we have checked the effect of compound 1 on mitotic arrest. In fact, there are less mitotic cells in the population treated overnight with both taxol and compound 1 in comparison to control cells. This result is confirmed by time-lapse experiment. After 2 hours incubation with compound 1, cells escape the spindle checkpoint induced by Taxol and exit from mitosis with duplicated DNA and lobed nuclei. Similar effects are also observed with VX-680, known as the reference Aurora kinase inhibitor molecule (Bain *et al.*, 2007).

Such a mitotic exit without cytokinesis is qualified of mitotic slippage and was described in details recently. Therefore these compounds induce mitotic slippage rapidly upon addition to the cell medium. Two conclusions may be withdrawn. First benzo[e]pyridoindole inhibits Aurora kinase *ex vivo* since they prevent Histone H3 phosphorylation and impairs the spindle checkpoint. Second these molecules diffused rapidly in the cells and may be interesting compounds for inducing mitotic arrest and inhibiting cell proliferation.

We have examined the effects of the hits on tumour cells growth both in 2D and 3D cultures. First we have checked the toxicity of compound 1 on quiescent cells. It is reported that H358 cells can go to a quiescent period when deprived of serum for at least 48 hours. We have incubated quiescent H358 cells with the compounds for 24 hours. At 250 nM, compounds 1 and 2 have only mild effect on H358 and around 75 % of the H358 cells are still alive at 1 μM. It means that these compounds are poorly toxic on non-proliferating cells. Results are very encouraging since toxicities are similar to those observed for VX-680, a compound presently under clinical trials.

We have tested the two best hits on four cell lines (HeLa, LL/2, H358 and HCT-116). The IC 50 values for compound 1varies from 960 nM to 145 nM. We have compared the efficiency of the selected molecules to VX-680, the reference Aurora inhibitor. Compound 1 is more potent in HeLa cells than VX-680 (IC50 of 960 nM and 1455 nM respectively). Their efficiency is quite similar in H358 cells (145 nM for hit 1 compared to 90 nM for VX-680). Conversely hit 1 is ten fold less efficient in HCT116 cells. Interestingly enough Compound 1 prevents HCT116 proliferation even when they are grown as spheroid and only a mild lost of efficiency is noted in the 3D culture compare to monolayer cells.

In conclusion the hits identified by high throughput screening, benzo[e]pyridoindoles 1 and 2 are Aurora kinase inhibitors *in vitro* and *ex vivo*. However *ex vivo*, they targets mostly Aurora B kinase. They inhibit histone H3 phosphorylation and inactivate mitotic spindle checkpoint. In the presence of the compounds, cells stop in mitosis even in the absence of any mitotic arrest drug but afterwards they quickly exit from mitosis without cytokinesis and abscission. Benzo[e]pyridoindoles 1 and 2 are efficient *ex vivo* for preventing cell proliferation and are thus an interesting starting scaffold for the development of Aurora kinase inhibitors. These results are described in a manuscript under review process in Chem. Biol.

Benzo[e]pyridoindoles inhibit Aurora kinases

and affect Aurora B localization

Thi My-Nhung Hoang 1,5, Annie Valette2, Caroline Barette3, Chi Hung Nguyen4, Laurence Lafanéchère3, David S. Grierson4, Stéfan Dimitrov1, Annie Molla*1

1: INSERM: U823 Institut Albert Bonniot, Université Joseph Fourier, 38 706 La Tronche, France

2: CNRS: 5088 Université Paul Sabatier 31032 Toulouse Cedex, France

3: CMBA, IRTSV, CEA 38054 Grenoble Cedex, France

4: UMR 176 CNRS-Institut Curie, Insitut Curie, Bat 110 Centre Universitaire, 91405 Orsay, France

5: University of Ha Noi, Ha Noi Vietnam

Corresponding author: * Annie MOLLA; Email: annie.molla@ujf-grenoble.fr

Tel 33476549474

Fax 33476549595

Running title: Aurora kinase inhibitor

Key words: Mitosis, Aurora kinase, small-molecule inhibitors.

Summary

Aurora kinases are serine/threonine protein kinases that play a key role in mitosis. A challenge is to discover Aurora kinase inhibitors with antiproliferative activity. By high throughput screening of a chemical library it was found that benzo[e]pyridoindole derivatives inhibited Aurora kinase *in vitro*. The two most potent compounds 1 and 2 prevented, *ex vivo*, the phosphorylation of Histone H3, induced mitosis exit and inactivated the spindle checkpoint, known phenomena observed upon Aurora B inactivation. These two compounds were also shown to affect the centromeric localization of Aurora B, as in the presence of the inhibitor the enzyme was rapidly delocalized on the whole chromosomes. In addition, compounds 1 and 2 inhibit the growth of HCT-116 multicellular tumour spheroids. Benzo[e]pyridoindoles 1 and 2 are represent an interesting new avenue for the development of Aurora kinase inhibitors.

Introduction

Aurora kinases are a family of serine/threonine protein kinases that play a key role in mitosis progression {Carmena, 2003 #17} {Adams, 2001 #19}. Aurora A is implicated in centrosome duplication, whereas Aurora B is important for chromosome condensation and kinetochore functions {Ditchfield, 2003 #26} {Ditchfield, 2005 #24}. Aurora B is also involved in spindle checkpoint activation and in cytokinesis completion {Lens, 2003 #3} {Adams, 2001 #19}. Aurora A and B are over-expressed in many cancers, including primary colon and breast cancer {Sen, 1997 #21} {Carmena, 2003 #17}. Furthermore, the human Aurora A gene is localised to the 20q13 amplicon, which is associated with a poor prognosis in breast cancer {Sen, 1997 #21}. Xenografts of mouse NIH-3T3 cells transfected with Aurora A give rise to tumours in nude mice, suggesting that Aurora A may behave as an oncogene {Bischoff, 1998 #20}. Under similar conditions, over-expression of Aurora B induces metastasis {Giet, 2005 #32}. In light of these observations, Aurora kinases have emerged as druggable targets for cancer therapy and thus, identification of small molecule type Aurora kinase inhibitors is of particular interest {Keen, 2004 #25}{Girdler, 2006 #23}{Jackson, 2007 #33}. Several Aurora A and B inhibitors, including ZM477439, hesperadine and VX-680 have recently been described {Hauf, 2003 #5} {Gadea, 2005 #13} {Gizatullin, 2006 #7} {Fancelli, 2006 #10} {Soncini, 2006 #11} {Hoar, 2007 #37}. VX-680 suppresses tumour growth *in vivo* and is presently undergoing evaluation in clinical trials {Harrington, 2004 #9}.

As described herein the high throughput screening of a proprietary Institut Curie-CNRS small molecule library of 6560 compounds in both *in vitro* and *ex vivo* assays led to the identification of benzopyridoindoles as new inhibitors of Aurora kinases. We have performed a structure affinity investigation and the hits tested *ex vivo* in HeLa cells were found to inhibit Aurora B kinase. Moreover the best hit affects significantly tumour cell grown in either 2D or 3D cultures whereas it was mildly toxic towards quiescent cells. Benzo[e]pyridoindoles are thus proposed as a lead for the development of Aurora kinase inhibitors.

Material and Method

Biological material :

Recombinant histone H3 and Aurora kinases (Aurora B and the kinase domain of Aurora A) were expressed in E. coli and purified to homogeneity. Recombinant Aurora A was purchased from Biomol GmbH (Euromedex, France). VX-680 was purchased from Kava technology Inc and Taxol from Sigma.

Protein kinase assay:

The reaction was performed in the presence of 20 mM KCl, 20 mM MgCl2, 0.4 μM ATP, 0.4 mM DTT, 20 mM Tris (HCl), pH 7.5 and recombinant histone H3 was used as substrate. The reaction started by the addition of the recombinant enzyme. After 1 hour of incubation at 37 °C the remaining ATP was monitored by addition of kinase-GloTM (Promega, France) under the conditions suggested by the supplier. Ten minutes later the fluorescence was recorded with a Fluostar Optima (BMG Labtechnologies). Staurosporin (0.5 μM) was used as negative control.

High throughput screening :

The assay was performed in black 96-well plates (Greiner 651209) and started with the addition of the kinase domain. The Z-factor of the assay was estimated to be 0.77 {Zhang, 1999 #6}. Molecules were in DMSO. The primary screening was performed in triplicate at the concentration of 15 μM (0.3 % DMSO) and the confirmation at 1.5

μM. IC50 was defined as the concentration that leads to 50 % of inhibition. The purity of the molecule was superior to 90 % and their concentrations ranged from 20 nM to 1.2 μM. Compound 1 and 2 synthesis are described in {Nguyen, 1992 #40} and {Nguyen, 1990 #41} respectively.

Cell culture

HeLa, HCT-116, Hek-293 and LL/2 were grown on Dulbecco's modified Eagle's (Biowhittaker, Europe). H358 cells were propagated in RPMI. Media were supplemented with 10% foetal bovine serum (Biowhittaker, Europe).

HeLa (Aurora B –GFP) were already described elsewhere {Delacour-Larose M, 2004 #1}. Hek-293 cells were transfected with a plasmid expreesing the fusion Histone H2A – GFP and a clonal fluorescent cell was selected and amplified.

For preventing cell proliferation H358 cells were washed extensively in serum free medium and seed in the absence of serum. Two days afterwards compounds were added and culture pursued for 24 additional hours.

The multicellular tumour spheroid (MTS) model:

We have adapted the hanging-drop method {Del Duca, 2004 #14} to produce HCT116 spheroids of similar diameters. 500 cells are suspended on the lid of an agar coated 24-petri dishes containing culture media. 48h later the spheroids were transferred to the culture medium. Spheroid volumes were measured before drug treatment (D_0). HCT116 spheroid were treated or not with various concentrations of the three compounds, for 5 days. The size of each spheroid was determined by

measuring 2 orthogonal diameters (d1 and d2) using an inverted microscope. The volume was calculated according to the formula: $V=4/3\pi r3$ where $r=1/2\sqrt{d1xd2}$. Spheroid growth was appreciated by the variations of volume.

Immunofluorescence :

Cells grown on glass coverslips for 24 hours were fixed at 37°C in 4% paraformaldhehyde, 2% sucrose and then permeabilized in PBS containing 0.2% Triton X-100 for 15 min. Free binding sites were blocked with 0.5 mg/ml BSA and specific antibodies were then incubated for at least 30 min in PBS supplemented with 10% foetal bovine serum, 0.2% Tween-20 and 0.02% NaN3. Phosphorylated histone H3 was detected by a polyclonal rabbit antibody from Upstate. Aurora B was detected by mouse monoclonal AIM-1 (1/100, transduction Laboratories) and Survivin with a rabbit polyclonal (1/300, Abcam). Unbound antibodies were removed by washing with PBS, 0.2 % Tween-20 and specific staining was revealed with either Hylite FluorTM 546- or 488-conjugated secondary antibodies (Euromedex, France). DNA was visualised with 0.1 mM Hoechst 33342 (Sigma). Images were collected with a ZEISS 510 Laser Scanning Confocal apparatus with a 63x immersion oil objective. Slices of 0.5 micron are shown.

Cells seeded on glass-coverslips were arrested in mitosis, for either 8 or 15 hours, by the addition of Taxol (33 nM).

Ex vivo microscopy

Ex vivo experiments were conducted on cells grown on Lab-Tek chambered coverglass (Nalge Nunc International) and maintained under standard culture conditions. Images were acquired on a Zeiss LSM510 system using a Planapochromat 40 X water immersion objective. GFP was excited with a 488 nm Argon 2 laser (power varying from 0.1 to 2%). Confocal slices are shown.

Results

To identify new small molecule Aurora kinase inhibitors an Aurora kinase assay was developed based on the phosphorylation of recombinant histone H3 by a purified active kinase under non-saturating ATP concentrations. Recombinant Aurora A kinase domain was used. The assay was suitable for high throughput screening as assessed by a statistical Z' factor of 0.77. In the screen of the Institut Curie-CNRS proprietary library (http://chimiotheque-nationale.enscm.fr), which contains 6560 distinct mono- to penta-heterocyclic compounds, forty molecules were found to inhibit Aurora kinase by more than 50 % at a concentration of 15 µM.

Of the fourteen most active compounds identified 43% belong to the benzo[e]pyridoindole family (Figure 1). Non-pertinent "hits", corresponded to bi and tricyclic aromatic compounds bearing reactive (aldehyde) functionality.

For the most potent molecules **1** and **2**, an OMe group is present at the 3-position of the benzo (A-ring). However, as the activities for compounds **3** and **5** demonstrate, this functionality is not a requirement for activity, and can further be moved to the 4-position. The presence of an alkyl chain at position-8 is a very distinguishing feature in these molecules, as the absence of this substituent resulted

in a clear loss in activity (compare **1** with **4**). Replacement of the pyridinone carbonyl oxygen by a hydrogen (**7**) or a chlorine atom (**6**) also resulted in a loss in activity. Finally, the double modification in which R1 and R2 are changed, as in **8**, lead to almost complete loss in activity as measured at 1.5 μM.

To gain some knowledge concerning the selectivity of benzo[e]pyridoindoles **1-8,** an *in vitro* screen was carried out against Casein Kinase II (C. Cochet, Grenoble, personal communication). In this screen compound **6** was found active. The other indole compounds displayed essentially no affinity for CKII.

An IC50 of 320 nM was determined for the most potent hit **1** against the kinase domain of Aurora A compared to 660 nM for compound **2**. The IC50 for compound **1** towards the whole Aurora A and B kinases were also determined and reported in Figure 1; IC50 of 450 nM for Aurora A and 600 nM for Aurora B respectively.

The Aurora kinase inhibition efficiency of these two compounds was also studied *ex vivo* in HeLa cells in culture (Figure 2). The entry in mitosis correlates with a burst in H3 phosphorylation, which lasts till the telophase. The Aurora B kinase activity was assessed by monitoring the phosphorylation of histone H3 at serine 10 by Immunofluorescence and Western blotting (Figure 2 A and B). Treatment of the cells with **1** and **2** decreased significantly H3 phosphorylation (Figure 2A-C). Under standard conditions, the bulk of the mitotic cells were positive, whereas only 14 % and 47 % of them scored positive in the presence of compound **1** (2 μM) and compound **2** (2 μM) respectively compared to 5 % for VX-680 (300

nM), a known Aurora B kinase inhibitor (Figure 2 C). The inhibition of Histone H3 phosphorylation suggested an inhibition of Aurora B kinase in these cells.

Treatment of HeLa cells with either compound **1** or **2** resulted in severe morphological perturbations of the HeLa cell nuclei (Figures 2B, 3A and 3B). Cells acquired enlarged lobed nuclei. Quantification showed that 20.7 ± 1.5 % of the cells exhibited irregular nuclei in the presence of **1** compared to around 1% in the control and to around 23.0 ± 3.5 % in the presence of VX-680. Compound **2** created similar defaults, the percentage of irregular nuclei being 15.7 ± 2.0 % (Figure 2 B). FACS on HeLa cells incubated for 48 h in the presence of two concentrations of compound **1** confirmed the polyploidisation of the cells. Mitotic cells (or 4N) represent around 12.5 % of the population in the control whereas they increase to around 30 % in the presence of compound 1 (Figure 3C). Moreover enlarged nuclei (Figure 3A) correlate with a significant increase of ploidy, since 58 % of the cells being pluriploid in the presence of compound **1** (700 nM, Figure 3C). These phenotypes (lobed nuclei and polyploid cells) reflect an inactivation of Aurora-B {Carmena, 2003 #17}.

We took advantage of the formely established HeLa cells expressing naturally fluorescent functional Aurora B kinase (HeLa (AuroraB-GFP) cells) to investigate the behaviour of the kinase upon inhibitor treatment. As shown in figure 4 A these cells underwent normally through mitosis. Early in mitosis Aurora B-GFP appeared as punctuated fluorescent points, in fact it decorated centromeres. These fluorescent centromeres progressively aligned (9 min); upon anaphase onset, fluorescence was

transferred to microtubules (42 min) and finally concentrated in the midbody, during cytokinesis (56 and 129 min). In the presence of low concentration of compound **1** (500 nM) Aurora B-GFP was present normally on centromere (Figure 4A) but the centromeres failed to properly aligned (see 60 min) and finally disaligned whereas fluorescent progressively decreased (130 min). At such a concentration compound **1** prevent centromere alignment. At a higher concentration (1 μM) we observed fluorescent centromeres (Figure 4A, see T0 (lines 3 and 4)) but rapidly fluorescence became more diffused and seemed to fused on the whole chromatin (see times 40 min line 3 and 60 min line 4). At last fluorescence appeared more diffuse and it looked like a fluorescent lobed nucleus (see times 160 line 3 and 100 min line 4). The same behaviour was observed when HeLa (AuroraB-GFP) cells were incubated with VX-680, a potent Aurora B kinase inhibitor (Figure 4A, line 5). An enlarged view of the leakage of Aurora B-GFP on chromosomes is shown in Figure 4A, line 3. In view to understand the behaviour of the cells in the presence of the inhibitors we have decorated chromatin with a fluorescent histone GFP-H2A. In control cells chromosomes aligned on the metaphasic plate and then were separated in two equal batches that progressively formed interphasic chromatin (Figure 4B and data not shown). In the presence of compound **1** (1 μM) chromatin normally assembled into mitotic chromosomes (Figure 4B, T0) but two individual lots of chromosomes were not formed (Figure 4B). Instead, non regular chromatin masses are detected, similar to these observed upon siRNA ablation of Aurora B {Vader, 2006 #38}. These data suggest that in the presence of compound **1** or VX-680, cells are arrested at mitosis,

mitotic chromosomes are unable to segregate and cell division failed. Finally mitotic slippage occurred {Blagosklonny, 2007 #39}.

The depletion of Aurora B kinase or its inactivation allowed taxol treated cells to escape the mitotic checkpoint {Gadea, 2005 #13}. If the identified hits inactivated Aurora B, mitotic slippage should be also observed. With this in mind, we incubated taxol-treated cells with compound **1** (2 μM) and measured the number of cells arrested in metaphase. We found that the percentage of mitotic cells decreased from 37.7 ± 1.2 % in the control to 19.2 ± 1.3 % when **1** is present (Figure 5A). In an additional experiment we have arrested cells overnight in Taxol (33 nM) and then added varying compound **1** concentrations for two hours (Figure 5B). While 68 % of the cells were in metaphase in the control this percentage decreased to less than 10 % in the presence of at least 1 μM compound **1**. The percentage of lobed nuclei was negligible in the control or in the 500 nM C1 treated cells. This percentage increased, however, to 61 % at a 1 μM C1 concentration and saturated at around 70 % at higher concentrations. In the presence of 1.5 μM C1 most of the cells have escaped the spindle checkpoint within two hours of treatment, suggesting a rapid diffusion of compound **1** within the cells. We took advantage of cells expressing GFP- H2A to investigate the behaviour of the cells escaping from mitotic arrest (Figure 5C). Under the microscope, we have pointed mitotic cells and followed them upon addition of compound **1** (1μM). Individual chromosomes failed to segregate into two distinct lots, cells loose their round shape, chromosomes loose their rodlike shape, decondensed in interphase like chromatin structures. In all followed cells (more than

30 in three independent experiments), the interphase type chromatin was always connected by bridges (see Figure 5C, 46 min). These cells exhibited 4 N ploidy and a polylobe nucleus. The localisation of Aurora B kinase was also investigated. Again few minutes after compound 1 addition, the nice punctuated centromeric localisation was lost and Aurora B-GFP spread all over the whole chromatin. In order to test if the observed loss of centromeric localization was not associated with the presence of GFP in the fusion Aurora B-GFP, we detected endogenous Aurora B by a specific antibody in HeLa cells (not expressing the fusion Aurora B-GFP) treated with compound **1** at a concentration of 1μM (Figure 5E). Again in the control cells we observed a punctuated labelling and no background around whereas in the treated cells the antibody detected both centromeric Aurora B but had also a cloudy distribution. Survivin, as expected, was found to co-localised with Aurora B in these cells in which Aurora B kinase was inhibited (Figure 5E).

Finally we have investigated the viability of tumour cell lines upon compounds **1** or **2** treatment and compared with the effect of VX-680 on these cell lines. First we have tested the toxicity of the molecules towards H358, a bronchioalveolar carcinoma cell line (Figure 6). These cells may survive as quiescent cells for several days and are thus suitable for testing the toxicity of Aurora kinase inhibitors on non-proliferating cells. As shown in Figure 6 A, around 90 % of the H358 cells are still alive in the presence of 250 nM of both compounds and this percentage decreases to around 75 % at 1μM concentration. These data show that these compounds exhibit an interesting feature, i.e. a relatively weak effect on the

viability of non-proliferative cells, which makes them valuable tools for the selective elimination of proliferative cancer cells.

Then four cell lines (HeLa, LL/2, H358 and HCT-116) were on grown as adherent cells and the concentration that inhibits growth by two was determined and reported in Figure 6B. The following IC50: 960 nM , 655 nM, 145 nM and 717 nM were determined towards HeLa, LL/2, H358 and HCT-116 cells respectively. Compound **1** was found to be more potent than VX-680 in HeLa cells otherwise it was found to be between 1.6 to 8.9 less potent than the known inhibitor in the other cell lines. Compound **1** was found to alter cell viability more efficiency than compound **2** (figure 6B). We have investigated the effects of benzopyridoindoles **1** and **2** on the HCT-116 carcinoma cells growing as MTS by measuring spheroid volumes. After treatment for 5 days the IC50 for HC-T116 spheroid growth inhibition for compounds **1** and **2** were 1066 nM and 983 nM, respectively (Figure 6 B and C). For comparison, the potent Aurora kinase inhibitor VX-680 displays an antiproliferative effect on HCT-116 MTS, with an IC50 of 108 nM (Figure 6 B and C).

Discussion

Aurora kinases have emerged as druggable targets for cancer therapy and in this line, the discovery of Aurora kinase inhibitors is an important challenge {Jackson, 2007 #33}. In the screen of a library containing 6560 mono- to penta-heterocyclic compounds, forty molecules were found to inhibit significantly the domain kinase of Aurora A. The most active compounds belonged to the

benzo[e]pyridoindole family. In these active benzopyridoindoles, the benzo[e] ring fusion gives them a "cresent moon" shape. This geometry seems to offer the best complimentary shape to the Aurora kinase ATP site, as amongst two hundred other tetracyclic indole compounds in the library screened, including the related benzo[g] and benzo[f]pyridoindoles and linear pyrido[4,3-b]carbazoles (ellipticine analogues), only two of them displayed weak activity at 1.5 μM. Molecules were tested towards the domain kinase of Aurora A but bearing in mind that enzymatic domains of kinases A and B share 85 % of homology {Carmena, 2003 #17}, we might expect to reveal molecules inhibiting both enzymes. Indeed, the best hit **1** was shown *in vitro* to be a potent Aurora A and B inhibitor (IC50 450 nM and 600 nM respectively).

Several different families of Aurora A and B inhibitors have now been identified, as well as the structures of Aurora A, Aurora A:TPX2, and in particular, the ternary complex of Aurora B:INCENP with the inhibitor hesperadin. {Cheetham, 2002 #36} {Bayliss, 2003 #43} {Sessa, 2005 #4} It thus becomes possible to relate structure to kinase inhibition activity in terms of the interactions giving rise to binding in the ATP site. Hesperadin, a member of the SU-family of indolinone type kinase inhibitors shares in common with compounds **1** and **2** the presence of a cyclic amide functionality {Hauf, 2003 #5}. It is interesting to note that upon superimposing these molecules via their amide functionalities (in two dimensions) the three phenyl rings in hesperadin are also aligned in space such that they form a "disconnected" crescent moon shape. This observation may be meaningful in terms

of how compounds **1** and **2** interact with Aurora kinases. Relevant also are the results of a recent "fragment approach" study to design new Aurora kinase inhibitors {Warner, 2006 #29}. It was found that the quinazoline ring, a frequently encountered scaffold in kinase inhibitors, including ZM447439 (an Aurora kinase inhibitor) {Mortlock, 2005 #34}, could be replaced by a tricyclic pyrimido[4,5-b]indole system; it was possible to interject a pyrrole ring between the benzo- and pyrimidine-rings which comprise the quinazoline motif and maintain activity. This observation also brings to light further aspects of the nature of the binding of **1** and **2** which can be used in future efforts to optimize the potency (and selectivity) of the benzo[e]pyridoindole type Aurora kinase inhibitors.

The two best hits (compounds **1** and **2**) prevent histone H3 phosphorylation in mitotic cells, revealing their potency towards Aurora B. Moreover, benzo[e]pyridoindole **1** or **2** were found to prevent chromosome alignment and segregation as well as anaphase onset. To note is that we have never detected monopolar phenotype, a known feature of Aurora A kinase inhibition. Treatment of HeLa cells with either hit **1** or **2** increases significantly the ploidy of the cells and induces mitotic slippage. This result correlates with an inhibition of the Aurora B kinase activity upon treatment of the cells with benzo[e]pyridoindole. The two other broad inhibitors of Aurora kinases VX-680 and Hesperadin were also reported to target selectively Aurora B *ex vivo* {Hauf, 2003 #5} {Sessa, 2005 #4} and they also override the spindle checkpoint. These data are important, since they imply that the molecules interact with Aurora B kinase even insight the passenger protein complex

{Adams, 2001 #19}. Moreover these compounds as well as VX-680 were found to delocalise metaphasic Aurora B kinase. Taking into account this result and the mislocalisation observed also upon either RNA invalidation of passenger proteins or expression of a dominant negative kinase suggested that the kinase activity might be involved in its localisation {Vader, 2006 #38} {Delacour-Larose M, 2007 #44}. In fact the docking of the kinase on the centromere is still unknown.

When comparing the efficiencies of benzo[e]pyridoindole **1** or **2** and VX-680 we found that both were poorly toxic on quiescent cells but prevented efficiently tumour cell proliferation. The potency of the three compounds was maximal towards H353 cells. These cells, established from a very aggressive lung non small tumour, bear a homologous deletion of P53. This result enforces thus, the suggestion of Gizatullin *et al* {Gizatullin, 2006 #7} who proposed that the integrity of the P53 dependent postmitotic checkpoint governs the response to Aurora kinase inhibitors.

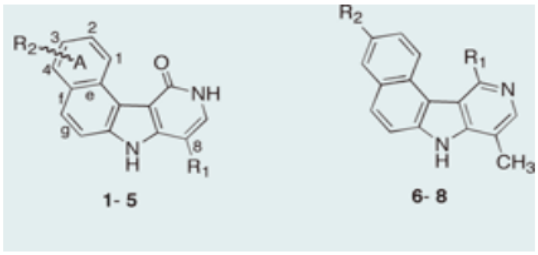
The multicellular tumour spheroid (MTS) model represents an intermediate level of complexity between cell growing as *in vitro* monolayers and solid tumours in animals {Sutherland, 1988 #16}. At a micromolar concentration, benzopyridoindoles **1** and **2** decreased by two the spheroid volumes of HCT116 carcinoma cells growing as MTS. The mild difference of IC50 in monolayer cultured cells compared to the spheroid model suggests a good biodisponibility of these compounds and its absence of multicellular resistance {Mellor, 2005 #15}.

Significance

In summary, benzo[e]pyridoindoles **1** and **2** were identified by high

throughput screening of a chemical library as Aurora kinase inhibitors. These compounds were found to affect *ex vivo* Aurora B kinases at hundred nanomolar concentrations. They are also efficient towards multicellular tumour spheroids in the micro-molar range whereas poorly toxic on quiescent cells. *Ex vivo* they are comparable in potency to VX-680, the reference molecule in the Aurora kinase field, which is currently undergoing clinical evaluation. These benzo[e]pyridoindoles, by their small size and their specific effects, represent thus an interesting starting scaffold for the development of new leads, or for improving known motifs.

Figure 1



Compound	R_1	R_2	Inhibition (in %)	
			15 μM	1.5 μM
1	Et	3-OMe	94	70
2	Me	3-OMe	90.3	57
3	Me	H	87.7	53
4	H	3-OMe	85.4	45
5	Me	4-OMe	79.6	37.8
6	Cl	OMe	76.7	31.9
7	H	OMe	47.4	18.5
8	H	$O(CH_2)_4COOH$	43.3	5.6

IC 50 (in nM)

Compound	Domain Kinase of Aurora A	Aurora A	Aurora B
1	320	450	600

Figure 1 : Aurora kinase hits. The chemical structure of the inhibitory hits is presented and preferential conformations drawn. Eight active compounds were described and their efficiency towards Aurora kinase domain was indicated at each concentration tested (15 μM and 1.5 μM). Data were collected from high throughput screening. The efficiency of the most potent hit, compound **1**, was evaluated by determining the IC50, i.e. the concentration that inhibits 50 % of the activity, towards the domain kinase of Aurora A and the Aurora A and B kinases. These determinations were performed by serial dilutions of compound **1**.

Figure 2

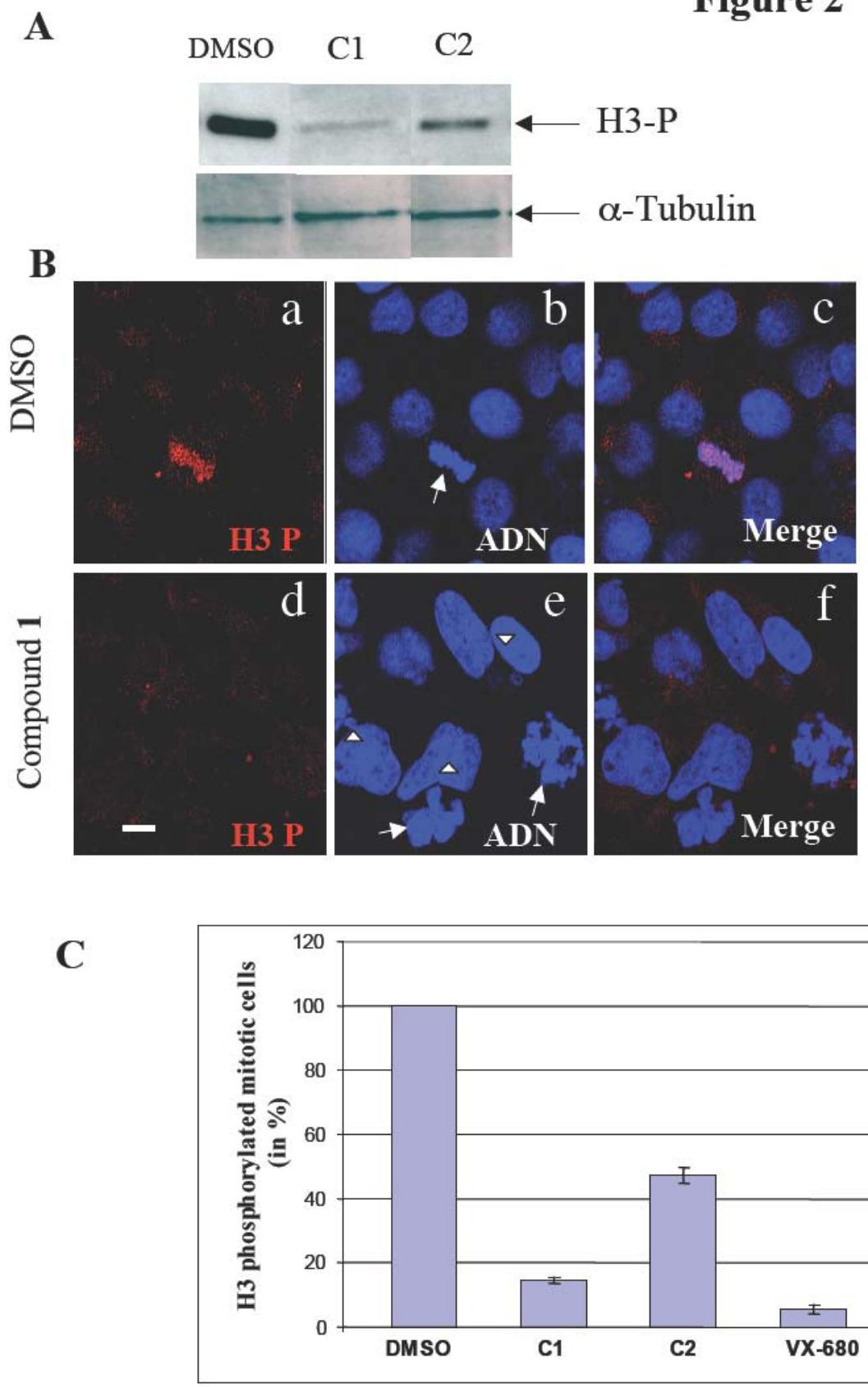

A
DMSO
C1
C2
H3-P
α-Tubulin
B
DMSO
Compound 1
a
b
c
d
e
f
H3 P
ADN
Merge
H3 P
ADN
Merge
C
H3 phosphorylated mitotic cells (in %)
120
100
80
60
40
20
0
DMSO
C1
C2
VX-680

Figure 2 : Compounds 1 and 2 prevent H3 phosphorylation

Hela cells were incubated overnight with Nocodazole (50 nM) and either DMSO or the molecules Compound **1** and Compound **2** at the concentration of 2 μM. Cells were then either collected and lysed for immunoblotting or fixed with paraformaldehyde for immunofluorescence experiments.

In A, histone H3 phosphorylation was revealed by a specific antibody on cell lysates separated on SDS-PAGE gels. Tubulin was used as a control of quantity.

In B, Immunofluorescences were performed on cells incubated overnight with either DMSO or Compound **1** (2 μM). H3 phosphorylation was visualised with a specific antibody in red (photos a and d) and DNA was stained by Hoechst 33 342 (b and e). Merge are shown in c and f; Full arrows indicate mitotic cells whereas arrow-head points abnormal nuclei. The bar scale represents 5 μM.

C: quantification of the percentage of H3 positive mitosis. Immunofluorescences were performed on cells incubated overnight with either DMSO or Compound **1** (2 μM) or Compound **2** (2 μM) or VX-680 (300 nM). 100 mitosis were analysed in each experiments and data are the mean of two independent experiences.

Compound **1** prevents significantly the phosphorylation of Histone H3 as shown both by immunoblotting and immunofluorescence. Compound **2** although less efficient than Compound **1** decreases H3 phosphorylation in around 60% of the cells. Moreover nuclei appeared irrugular in the presence of Compound **1**.

Figure 3

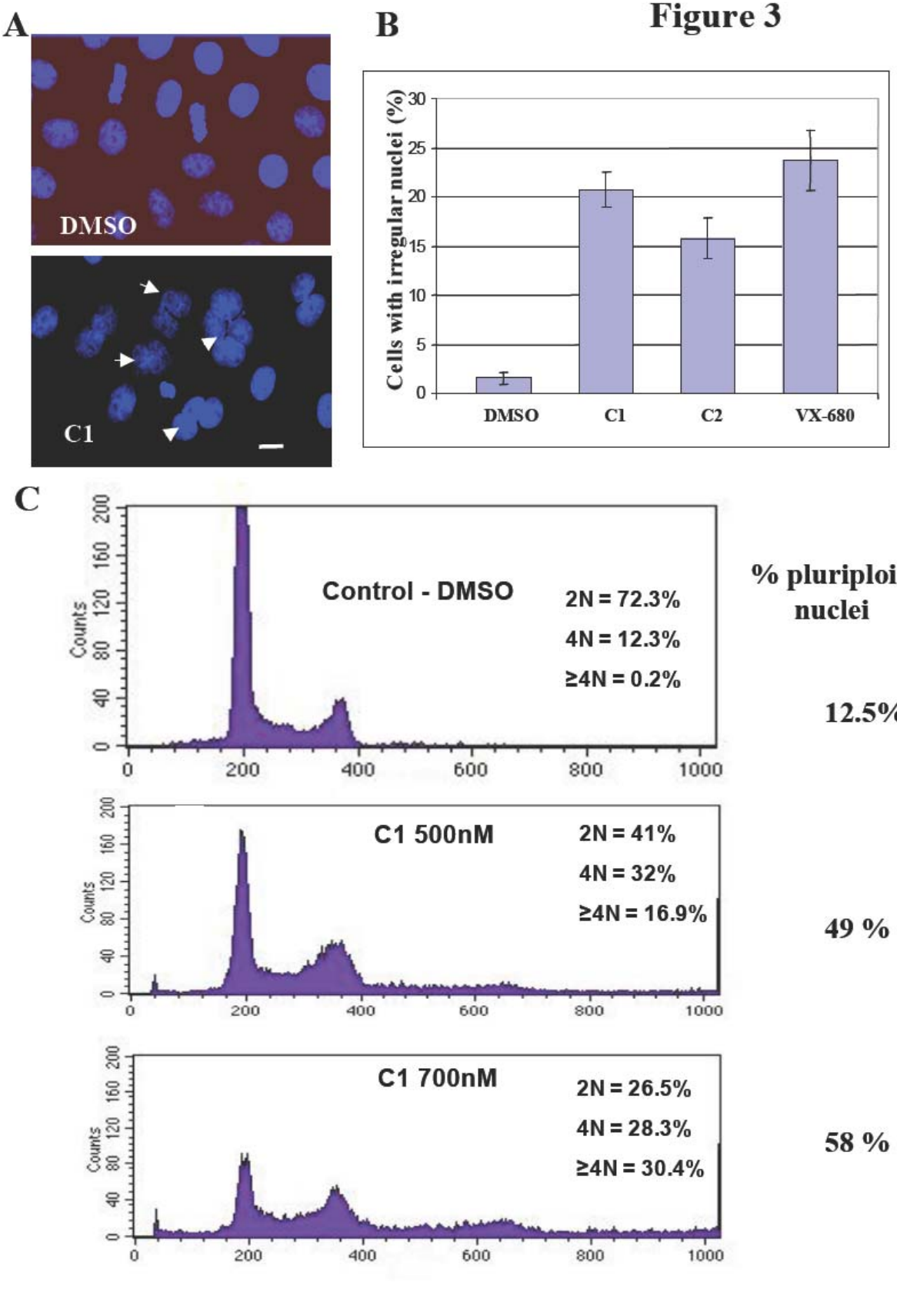

A
DMSO
C1
B
Cells with irregular nuclei (%)
30
25
20
15
10
5
0
DMSO
C1
C2
VX-680
C
Counts
Control - DMSO
2N = 72.3%
4N = 12.3%
≥4N = 0.2%
% pluriploid nuclei
12.5%
C1 500nM
2N = 41%
4N = 32%
≥4N = 16.9%
49 %
C1 700nM
2N = 26.5%
4N = 28.3%
≥4N = 30.4%
58 %

Figure 3: Effect of C1 on the cell cycle

HeLa cells were incubated overnight in the presence of either Compound **1** (2 µM) or Compound **2** (2 µM) or VX680 (300 nM) or DMSO. Nuclei were stained by Hoechst 33 342. In A, typical fields of Hela cells incubated in the presence of DMSO and C1 are represented. Arrows points irregular nuclei. The bar scale represents 5 µM.

In B, quantification of the experiment, cells were either incubated overnight with DMSO or Compound **1** (2 µM) or Compound **2** (2 µM) or VX-680 (300 nM). The percentage of irregular nuclei (lobed and polyploidy cells) was determined in two independent experiences; 100 cells analysed per experiment.

In C, FACS experiments were realized on Hela cells incubated with compound **1** (500 nM and 700 nM) for 48 hours and compared to a control in the presence of DMSO. DNA was stained with propidium iodine and samples analysed with a Beckton-Dickinson analyzer. The distribution of the cells is represented by diagrams and the percentage of pluriploid cells (cells with a ploidy superior to 2N) is indicated on the right part. Compound **1** induced severe morphological perturbations of the Hela cell nuclei, leading to polyploidisation.

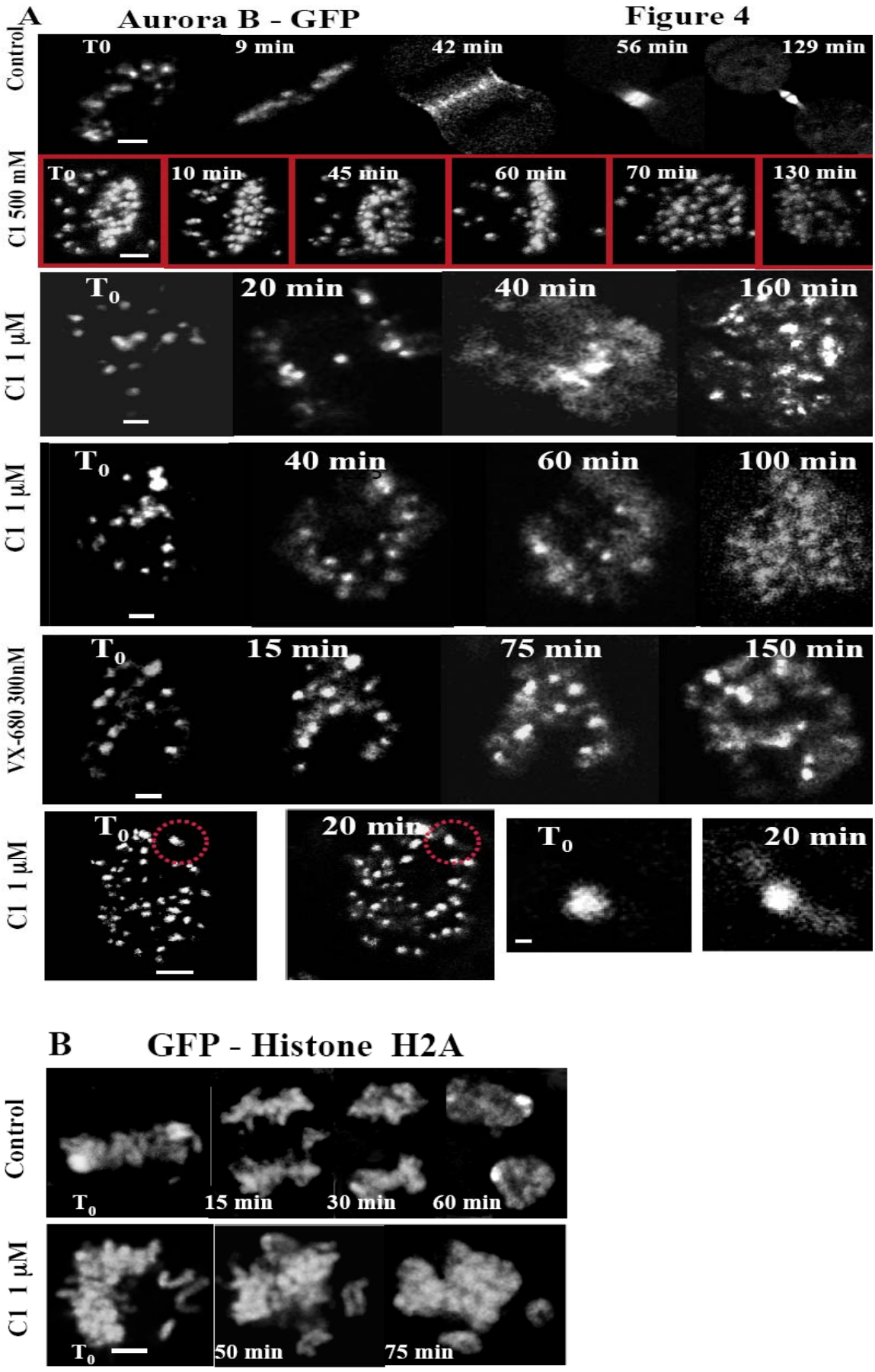

A
Aurora B - GFP
Figure 4
Control
T0
9 min
42 min
56 min
129 min
C1 500 mM
T0
10 min
45 min
60 min
70 min
130 min
C1 1 µM
T0
20 min
40 min
160 min
C1 1 µM
T0
40 min
60 min
100 min
VX-680 300nM
T0
15 min
75 min
150 min
C1 1 µM
T0
20 min
T0
20 min
B
GFP - Histone H2A
Control
T0
15 min
30 min
60 min
C1 1 µM
T0
50 min
75 min

Figure 4: Effect of Compound 1 *ex vivo*;

Localisation of Aurora B in the presence of compound 1

In A, we have established a cell line that stably expressed a functional naturally fluorescent Aurora B kinase: Hela (Aurora B – GFP) cells. These cells were continuously imaged under varying drug treatment (control, Compound **1** 500 nM, Compound **1** 1μM and VX-680 300 nM). Each line represents the same cell treated as indicated on the left and elapsed times from the starting point are indicated on each photo, Under standard conditions, these cells went normally through mitosis (Part A, control). When they were incubated in the presence of Compound **1** (500 nM) fluorescent centromeres aligned slowly and a clear metaphasic stop is observed. When cells were incubated with a higher concentration of Compound **1** (1μM) or with VX-680 (300 nM) they went out of mitosis without completing cytokinesis. Note the flux of Aurora B on chromatin that is shown on enlarged view (line6). The circle areas are represented on the right. The bar scale represents 5 μM.

In B, we have established a cell line that stably expressed a naturally fluorescent histone : Hek293 (Histone GFP – H2A) cells. These cells were continuously imaged under Compound **1** treatment (1μM) and may be compared to a control in the presence of DMSO. One cell is represented at three different times. Again a recondensation of chromatin is observed in the presence of Compound **1**.

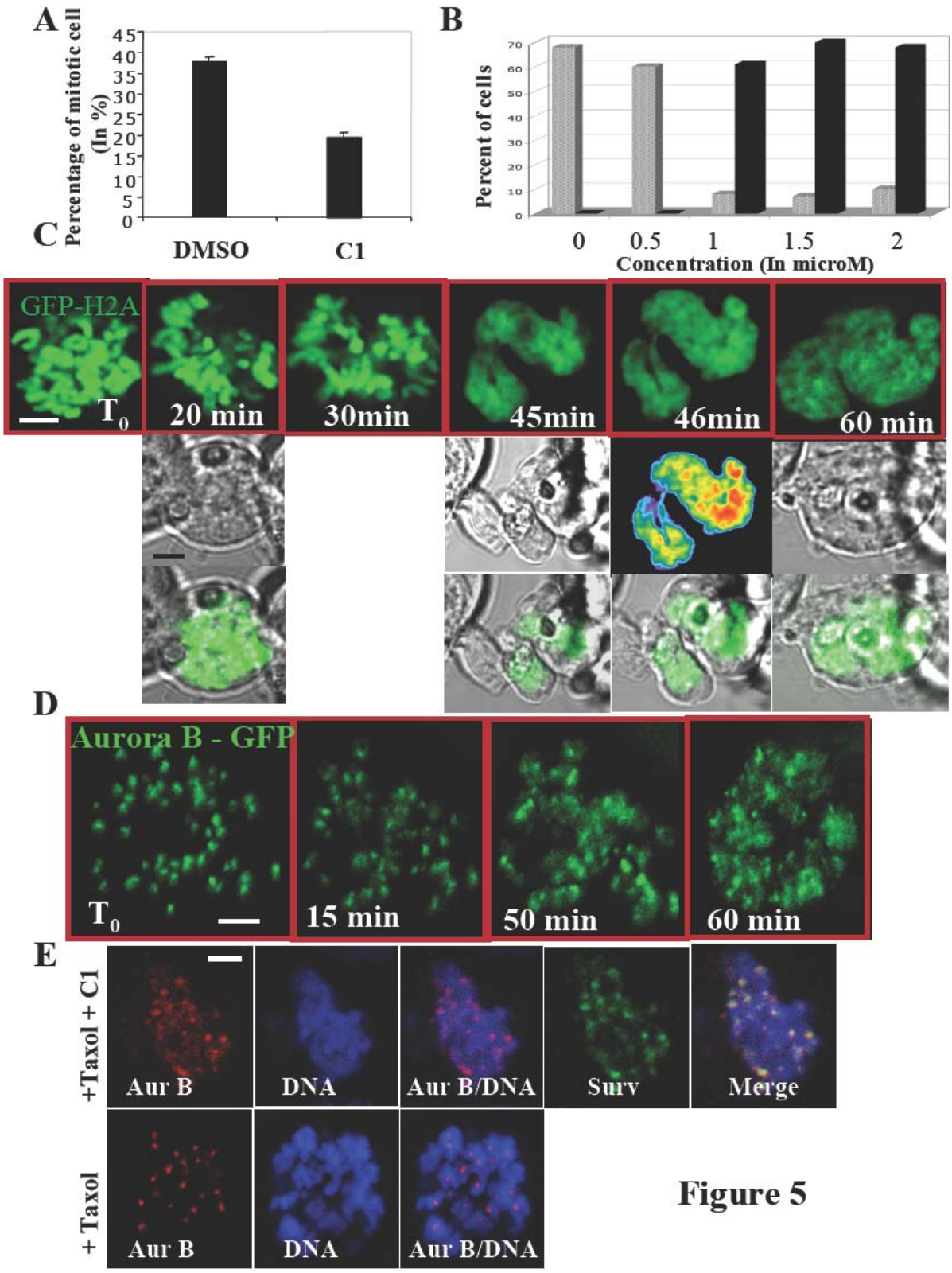

A
Percentage of mitotic cell (In %)
45
40
35
30
25
20
15
10
5
0
DMSO
C1
B
Percent of cells
70
60
50
40
30
20
10
0
0
0.5
1
1.5
2
Concentration (In microM)
C
GFP-H2A
T0
20 min
30min
45min
46min
60 min
D
Aurora B - GFP
T0
15 min
50 min
60 min
E
+Taxol + C1
Aur B
DNA
Aur B/DNA
Surv
Merge
+ Taxol
Aur B
DNA
Aur B/DNA

Figure 5

Figure 5 : Effect of Compound 1 on the spindle checkpoint

In A, Hela cells were incubated for 8 hours with either DMSO or the molecules Compound **1** at the concentration of 2 μM in the presence of Taxol (33 nM). Cells were then fixed and DNA was stained by Hoechst 33342. About 100 cells were analysed under UV light and mitotic cells were scored. Data are the mean of two independent experiments.

In B cells were arrested in mitosis by overnight incubation in Taxol (33 nM) then, varying concentrations of Compound **1** were added and incubated with the cells for two hours. Cells were then fixed. Mitosis and polyploid cells were scored. Around 100 nuclei were analysed in two different experiments. Mitosis are represented by punctated rectangles whereas polyploid cells (or polylobed nuclei) were drawn in black. Note the decrease of mitotic cells induced by increasing Compound **1** concentrations. Such a decrease is correlated with the appearance of irregular nuclei.

In C, Hek293 (GFP – Histone H2A) cells were incubated overnight with Taxol and, Compound **1** (1 μM) was added at T_0. Then, the cells were continuously imaged. Representative photos are shown and the elapse time indicated. The upper line represents the fluorescent GFP signal, the intermediate line the transmission view and the merge is shown in the lower line. For the time 46 min, the transmission image was replaced by a fluorescent image turned to false colours. Note, on this view, the connection between both nuclei parts. The bar scale represents 5 μM.

In D, Hela (Aurora B – GFP) cells were incubated overnight with Taxol (33 nM) and Compound **1** (1 μM) was added at T_0. Then, the cells were continuously imaged.

Representative photos are shown and the elapse time indicated. Aurora-GFP fused rapidly from centromeres.

In E, Hela cells were incubated with Taxol overnight and either Compound **1** (1 μM) or DMSO was added for 2 hours. Cells were then fixed and immunofluorescences were performed with anti Aurora B and Anti Survivin antibodies. Aurora B is labeled in red and Survivin in green by Hylite Fluor^{TM} -546 and -488 second antibodies respectively. DNA was stained by Hoechst 33342 and represented in blue. Merge views [Aurora B / DNA] and [Aurora B / Survivin / DNA] are represented.

A

Figure 6

Compound	Viability	
	250 nM	1 µM
VX-680	89 % ± 4 %	76.8 % ± 5.9 %
C1	95 % ± 7 %	73.1 % ± 2.5 %
C2	93 % ± 5 %	81.1% ± 8.6 %

B

Composé	2D cultures IC50 (nM)				Spheroid IC50 (nM)
	HeLa	LL/2	H358	HCT-116	HCT-116
VX-680	1 455 ± 4	225 ± 70	90 ± 10	80 ± 10	108 ± 10
C1	960 ± 140	655 ± 50	145 ± 25	717 ± 19	1066 ± 208
C2	1 160 ± 150	800	570	800	983 ± 256

C

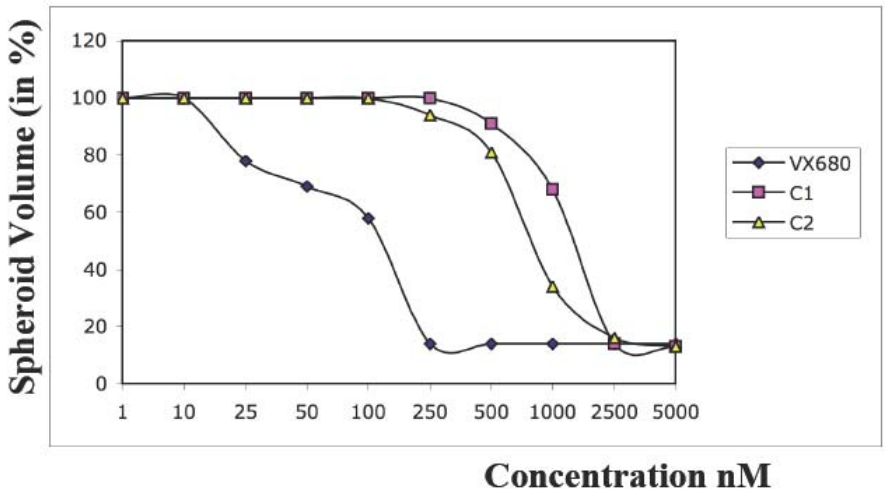


Figure 6 : Effect of Compound 1, Compound 2 and VX680 compounds on cell growth in both 2D and 3D culture conditions.

Cell growth and viability was tested under standard conditions in 96 well culture plates with MTT (Promega) cell counting.

In part A, H358 cells grown for 48 h in the absence of serum were incubated with varying concentrations of compounds VX-680, Compound **1** and Compound **2** for 24 h. The viability was then evaluated, reported in % by comparison with the control in the presence of DMSO. The viability of quiescent H358 cells was indicated at two drug concentrations (250 nM and 1 μM).

Cells were incubated with the three compounds (concentrations varying from 10 nM to 2 μM) and three independent experiments were conducted. IC50 for the four cell lines are reported in the Table. Part B.

We have produce HCT-116 spheroids of similar diameters and cell number. After a 48h period time required for cell aggregation the spheroids were transferred to the culture medium. Spheroid volumes were measured before drug treatment (day 0). HCT-116 spheroid were treated or not with various concentrations of the three compounds, for 5 additional days. Spheroid growth was appreciated by the variations of volume of each spheroid between day 0 and day 5 of treatment and the mean volume was compared to the size of untreated spheroids and then expressed in percent. The IC50 (mean +/- SD) of Compound **1**, Compound **2** and VX-680 compounds for the inhibition of HCT-116 spheroid growth determined in three independent experiments are reported in the Table part B.

In part C, the curve represents one representative HCT-116 spheroid experiment for each compound.

Acknowledgments

The authors thank Fabienne Sirot, Céline Frangia. N.H. is supported by a Vietnam/ French program and is a recipient of an ARC fellowship. This work was supported by INSERM, La Ligue Nationale contre le Cancer (Equipe labelisée), ANR (Project R05075CC), ARECA and CLARA (EpiProNetwork).

Microscopy was performed on the IAB platform.

II.5 Aurora kinase inhibitors, interesting tools for st udying the CPC

II.4.2. The effect of compound on t he level of Cyclin B proteins

Aurora kinase inhibitors (VX-680 as well as benzo[e]pyridoindoles) impairs the spindle checkpoint and induces a mitotic exist without chromosome segregation and complete cytokinesis. Similar cell behaviour was reported recently for cell escaping mitosis after long Taxol mitotic arrest and was qualified of mitotic slippage (Brito DA. *et al.*, 2006). During mitosis exist these authors have noted the destruction of cyclin B. These results prompt us to follow the level of cyclin B upon inhibition of Aurora B kinase. By both Western blotting and immunofluorescence, we have found that the expression of cyclin B decreases significantly after 2 hours incubation with kinase inhibitors (Figure 28). It seems that when Aurora B kinase is inhibited, the level of cyclin B decreases rapidly and is part of the mitotic slippage.

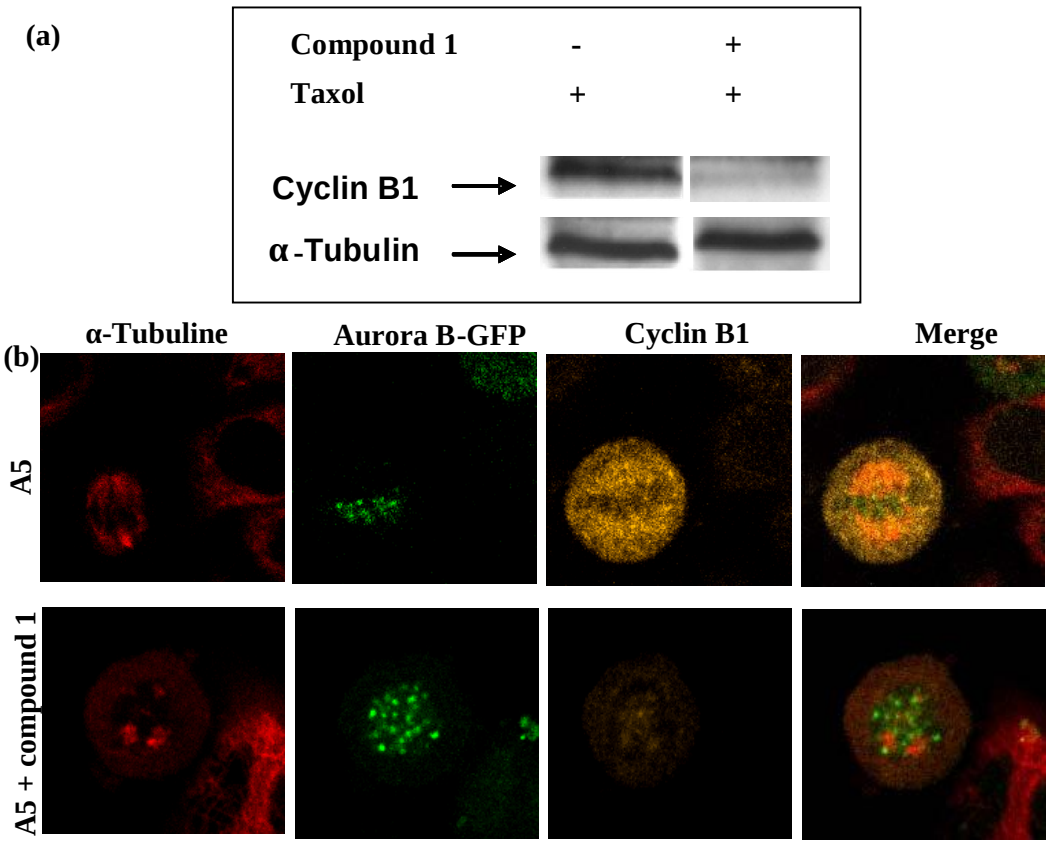


Figure 28 : The level of Cyclin B1 decreases in the presence of benzopyrido[e]indole
Cells are incubated overnight with taxol then, either benzopyrido[e]indole (compound 1) or DMSO (control) is added for two hours. Only non-spread cells are harvested and Western blots are performed in (a). The results are confirmed by immunofluorescence (b). We can see clearly that cyclin B1 expression in treated cells is far less than in the control. Both control and treated cells are imaged under the same conditions. Merge is the addition of all the signals (tubulin in red, Aurora B in green and Cyclin B in far red). A5: HeLa cells expressing Aurora B – GFP.

II.4.3. Aurora kinase activity is required for accurate localization of CPC on centromere

As shown by Time-lapse experiments with cells expressing Aurora B-GFP (Hoang *et al*., Figure 4), the localisation of Aurora kinase is affected by benzo[e]pyridoindole treatment. In control cells Aurora B kinase appears as punctuated fluorescent points decorating centromeres. Quickly after addition of benzo[e]pyridoindole the fluorescence fused from centromere to the whole chromatin. At last the fluorescence appears more diffused and its pattern looks like a lobed nucleus.

In order to checked whether this is a general effect of Aurora kinase inhibitors we checked the effect of VX-680 in HeLa expressing either Aurora B-GFP or Survivin-GFP (Figure 29).

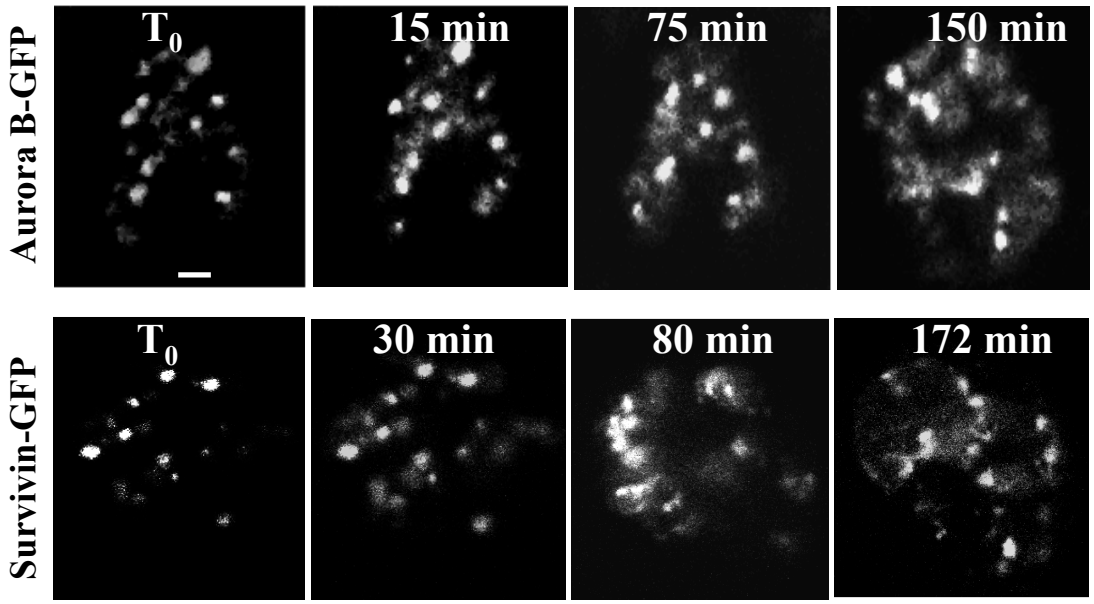


Figure 29. Survivin-GFP and Aurora-GFP share the same behavior in the presence of Aurora kinase inhibitors. When cells are incubated with the drug, they go out of mitosis without completing cytokinesis. The flux of both Aurora B-GFP (upper part) and Survivin-GFP (lower part) on chromatin are showed. Elapsed times are indicated on each photo.

The inhibition of Aurora kinase activity prevents the accurate localization of the kinase on centromere. Interestingly enough we have repetitively noted that upon siRNA treatment the other passenger proteins are either diffuse on the whole chromatin or punctuated as normal. For example when Aurora B is ablated by siRNA, Survivin and INCENP are detected either on centromere or on the whole chromatin.

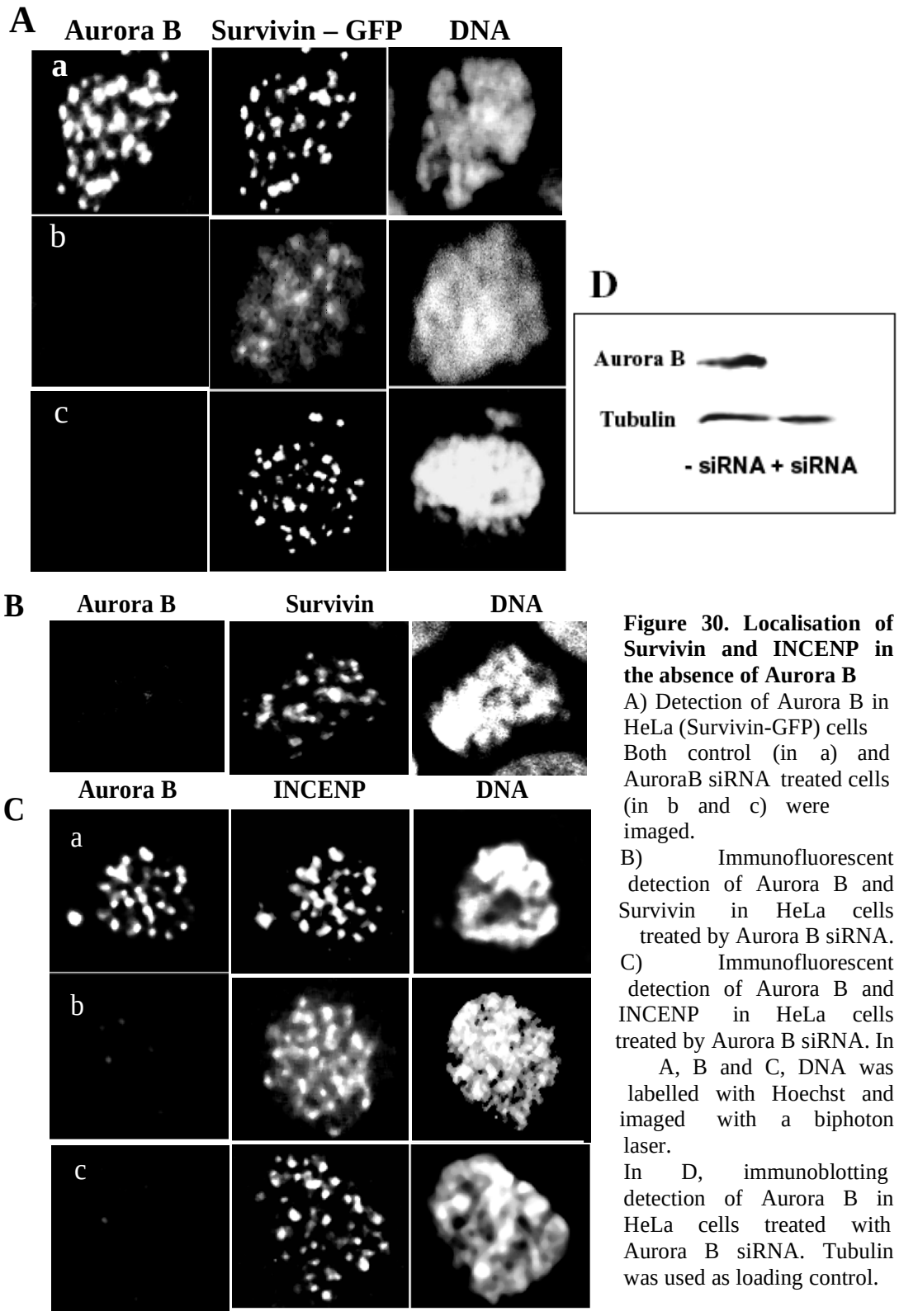


Figure 30. Localisation of Survivin and INCENP in the absence of Aurora B

A) Detection of Aurora B in HeLa (Survivin-GFP) cells Both control (in a) and AuroraB siRNA treated cells (in b and c) were imaged.

B) Immunofluorescent detection of Aurora B and Survivin in HeLa cells treated by Aurora B siRNA.

C) Immunofluorescent detection of Aurora B and INCENP in HeLa cells treated by Aurora B siRNA. In A, B and C, DNA was labelled with Hoechst and imaged with a biphoton laser.

In D, immunoblotting detection of Aurora B in HeLa cells treated with Aurora B siRNA. Tubulin was used as loading control.

Moreover by time-lapse on HeLa Survivin-GFP cells treated with Aurora B siRNA Marlène Delacour-Larose has observed that passenger proteins are normally localized on centromere but upon mitotic arrest the fluorescence fused on the whole chromatin and finally disappeared (Figure 31).

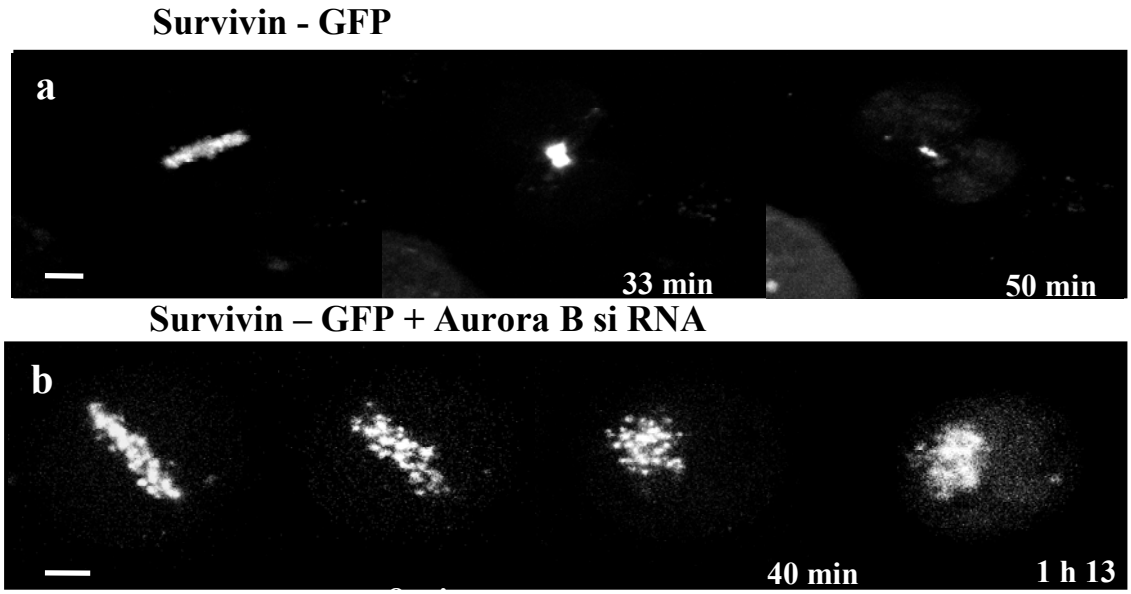


Figure 31 Time-lapse experiments with HeLa cells expressing Survivin-GFP
Time-lapse on HeLa (Survivin-GFP) cells in control conditions (a) or in the absence of Aurora B (b). Elapsed times are indicated on each photo. (This data were obtained by Marlène Delacour-Larose)

The targeting of CPC proteins to centromere seems to be independent of the partners but these data clearly indicate that the maintenance of the CPC on centromere requires a full Aurora B kinase activity.

Aurora kinase inhibitors are potential drugs for preventing cell proliferation but are also interesting tools for studying CPC function.

DICUSSION & PERSPECTIVES

CHAPTER I:

Role of Survivin phosphorylation by Aurora B in mitosis

CHAPTER II:

Search for Aurora B kinase inhibitors

Chapter I : Role of Survivin phosphorylation by Aurora B in mitosis

Survivin is a very important member of the CPC constituting the central core of the complex (Jeyaprakash *et al*., 2007)**.** Survivin is also a peculiar protein since it is mobile at the centromere whereas the other CPC members are tightly bound. Knowing on the one hand that the phosphorylation of Survivin is important for its mobility and on the other hand that it is phosphorylated at Thr117 by Aurora B kinase during mitosis, we have decided to study the behaviour of Thr117 mutants.

At the time we have started the experiments it was described that Survivin may be present mostly as a dimer (Chantalat *et al*., 2000). We have therefore decided to study the behaviour of the mutants in the absence of endogenous proteins, applying thus the pseudogenetic technique developed in our team (Scrittori *et al*., 2005). Moreover since we wonder whether the mutant may be toxic in the cells we have used tetracycline inducible expression vectors. Our data suggest that the non-phosphorylatable Survivin^{SR}T117A does not allow the metaphase/anaphase transition and is fully immobile on centromere. Meanwhile we were publishing this work, Wheatley and collaborators (Wheatley *et al*., 2007) have published a manuscript describing the same phospho-mutants. In their hand, the T117A mutant behaves as endogenous Survivin even in the absence of endogenous Survivin, supporting cell proliferation and anaphase onset. Till now, we have no explanation for this discrepancy except if the behaviour of the CPC varies in different cell lines as Wheatley *et al* has suggested in their manuscript. In our hand, in the presence of endogenous Survivin, the T117A mutant does not induce any perturbations, even as far as mobility is concerned. Our interpretation is that it may constitute heterodimer fully active. Recently it was proposed that the oligomer Survivin/INCENP/Borealin encompasses a monomeric Survivin ruling out the dimeric structure. However this structure is obtained with fragment proteins and is not compatible with the observed mobility of Survivin on centromere (Bourhis *et al*., 2007; Delacour-Larose *et al*., 2004). For fitting all the data, more experiments are needed and especially the determination of the precise step in which the CPC adopts such a conformation in the cells.

The studies of the phosphomimetic Survivin^{SR}T117E mutant are more consensual. Both groups described a protein with low affinity for centromere and high mobility. By using a specific T117-phospho-antibody Wheatley *et al* suggested that the phosphoprotein is localized in the central midbody surrounded by non-phospho-Survivin. They have reported

that a low level of phospho-protein is localized in the mid-body and this detection was possible only in cell over-expressing the mutant. Survivin T117E –GFP mutant is well localized till anaphase onset but it is never transferred to the microtubules and has then no specific localisation in the cell. This may suggest that the phosphorylation of Survivin is required for anaphase onset and shortly after Survivin is dephosphorylated and transferred to microtubules.

The important point we have raised with the phosphomimetic Survivin^{SR}T117E mutant is its dominant negative effect in cytokinesis. This mutant may impair CPC functions since it turns the spindle checkpoint off and prevents abscission of the two daughter cells. We have shown that, at telophase, it delocalizes MKLP-1. MKLP-1 bundles and stabilizes the spindle midzone/midbody interdigitating microtubules. It has been shown to play an important role in cytokinesis, in animal cells. This protein is not only an essential factor for midzone formation but is also required for midbody formation and completion of cytokinesis. MKLP-1 has strong relationship with the CPC, since its recruitment to the spindle midzone/mibody requires both INCENP and its phosphorylation by Aurora B kinase. The subsequent questions are: Is the effect of phosphoT117E-Survivin on MLKP-1 delocalization direct and does this mutant inhibit Aurora kinase activity?

The dominant negative effect on cytokinesis as well as the impairment of the spindle checkpoint are crucial in terms of therapeutic strategies. Survivin expression is tightly regulated in the cells, probably through degradation process. Consequently Survivin is present quite exclusively in cycling cells and may be proposed as a druggable target for mitotic therapies. Other Survivin mutants have also been reported to be dominant negative in cytokinesis. For example Survivin mutant (Surv-DD70, 71AA) disrupts the interaction of Survivin with Aurora B and causes multinucleation in HeLa cells (Cao *et al*., 2006). The nonphosphorylatable Survivin Thr^{34}→Ala also prevents phosphorylation of endogenous Survivin, resulting in apoptosis of various cancer cell types (O'Connor *et al*., 2000), and thus suppresses tumour growth *in vivo* (Grossman *et al*., 2001). The T117E is an additional motif that may be exploited for impairing cytokinesis in tumour cells.

Chapter II: Search for Aurora B kinase inhibitors

High throughput screening (HTS) is a very powerful method to gain access to active molecules. HTS allows a researcher to quickly conduct millions of biochemical, genetic or pharmacological tests. The screening of the 10 000 molecules towards Aurora kinase has lasted only 7 days. The advantage of using an *in vitro* test is the robustness of the test and the speed of the screening. The drawback is of course the possibility that the hits may be not efficient *ex vivo.*

Among the seven hits identified as Aurora kinase inhibitors, Flavone corresponds to these efficient inhibitor molecules that have no activity *ex vivo*. The *in vitro* assays with the flavone hit were very encouraging since IC_{50} determined were smaller than those reported in the literature for other kinases like Pi3 kinase or kinase C (1.5 μM in comparison to several μM) (Gamet-Payrastre *et al*., 1999). Unfortunately this molecule is inefficient in the cells probably due to the simultaneous presence of many targets. Hopefully among the hits those from the Curie-CNRS library have satisfied all the criteria. These molecules belong to the benzo[e]pyridoindole family.

The best hit exhibits IC50 of 450 nM and 600 nM toward the whole Aurora A and B kinase, respectively. All the assays *ex vivo* have revealed that this molecule exhibits mostly its activity towards Aurora B kinase. It prevents Histone H3 phosphorylation, impairs the spindle checkpoint and induces polyploidy cells whereas it has no significant effect on bipolar spindle assembly or chromosome duplication. Although in vitro benzo[e]pyridoindoles are broad Aurora kinase inhibitors, they target *ex vivo* mostly Aurora B kinase. Other inhibitors described in the literature exhibit the same targeting of Aurora B kinase rather than Aurora A kinase (Keen and Taylor, 2004; Mountzios *et al*., 2007). However, in the cells, Aurora A is an earlier player in the Aurora kinase family. This may suggest that other kinases may replace Aurora A when it is inactivated (Yang *et al*., 2005).

Interestingly enough benzo[e]pyridoindoles are efficient toward tumour cells cultured in 2D but also in 3D. The efficiency of the compound varies from cell to cells. It seems more efficient in cell with compromised p53 checkpoint. VX-680 is the reference inhibitor for Aurora kinase. In all the assays, we have compared the efficiency of the hit with VX-680 activity. Although VX-680 was also reported to be more efficient towards

P53- cells, the comparison of the two inhibitors reveals that the differences between p53 +/- are higher with benzo[e]pyridoindole than with VX-680. This may be an important point since on one hand results from clinical trial indicates side effect with VX-680 in skin and oesophagus and on the other hand tumour cells have often a compromise P53 checkpoint. However to strengthen this point, the effect of benzo[e]pyridoindole has to be investigated in more cells lines.

The inhibitions observed with HCT116 spheroid are very encouraging. The multicellular tumour spheroid (MTS) model represents an intermediate level of complexity between cell growing as *in vitro* monolayers and solid tumours in experimental animals (Sutherland, 1988). Recent studies have highlighted that MTS seems to be the appropriate model to study multicellular-mediated drug resistance (Mellor *et al*., 2005).

Of course several Aurora kinase inhibitors have already been described in the literature (Mountzios *et al*., 2007). Although benzo[e]pyridoindole represents a new Aurora inhibitor motif, the crescent moon shape scaffold identified in this molecule seems to be also present in Hesperadin. Since small compounds get generally better through clinical trials than complex molecules (Rees and Howard, 1999) benzopyridoindoles are proposed as attractive lead towards the development of Aurora kinase inhibitors. The chemists from Institut Curie are presently synthesizing the second generation of compounds. We hope improving the efficiency of the hit and that the next generation of molecule will be suitable for clinical trials. Presently several molecules are already under clinical trials (Mountzios *et al*., 2007). However, new molecules are still attractive since their potency may be specific to one pathology or to certain acquired resistances and some compounds may be more efficient to synergize ongoing treatments.

Moreover, Aurora kinase inhibitors represent useful tools for describing the CPC functions. We found that upon inhibition, Aurora B kinase flues from the centromere to the whole chromatin and all the CPC proteins are delocalized simultaneously. Similar results were obtained when one passenger protein is ablated by siRNA. This is observed when either Aurora B or survivin or INCENP is deleted. In fact, in this situation again, Aurora B kinase is not fully active since both Survivin and INCENP were reported to activate the kinase. This may suggest that passenger proteins are recruited individually on the centromere and a full active complex is required for the maintenance on the centromere.

In fact Anaphase onset is the consequence of a maximal Aurora B kinase activity on the centromere. When Aurora B kinase is lowered the chromosomes do not completely aligned on the metaphasic plate and the CPC cannot be transferred on microtubules. The CPC is no longer maintained on centromere, passengers fuse on chromatin and cells escape mitosis without cytokinesis; it is mitotic slippage. Interestingly enough, during anaphase onset and mitotic slippage the signalling are quite similar. In both situations, Cyclin B degradation is observed and the Cyclin B/Cdk1 complex is activated. At metaphase, the cell has a binary choice it may either pursue in mitosis or escape from mitosis as shown in Figure 32

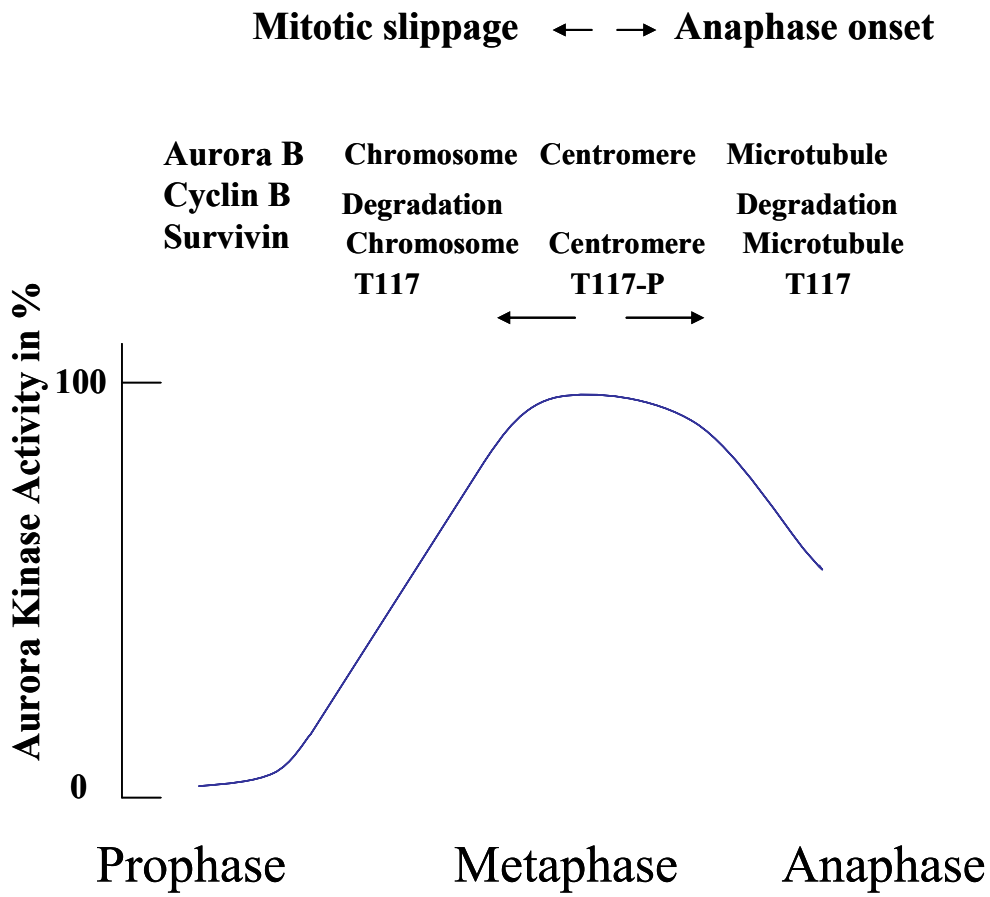


Figure 32: Binary choice at the Metaphase/Anaphase transition.
Maximal activity of Aurora kinase is required for the transition and for the maintenance of the passenger proteins on centromere. A sustained activity allows the alignment of all the chromosomes on the metaphasic plate and the transfer of the passenger proteins on microtubules. Therefore anaphase onset is induced and the spindle checkpoint is turned off meanwhile Cyclin B is degradated.Lower activities induce the flux of passenger proteins on chromatin and turn off mitotic spindle checkpoint inducing Cyclin B degradation. This is speculative drawing gathering all the data but that has to be strengthened with additional experiments

Perspectives

VX-680 and benzo[e]pyridoindoles are useful tools for understanding the CPC function. We will describe in more details the signalling downstream Aurora kinase inhibition. We plan to get insight in kinetic data and the major challenge is to set up an assay that allows the precise determination of the kinase activity on centromere. Presently the current test follows the phosphorylation of Histone H3, but Histone H3 is absent from centromere and moreover it is phosphorylated early in mitosis.

An interesting question is also to understand the mechanism supporting CPC maintenance on centromere. One may predict a conformational change induced by an Aurora kinase substrate but till now no data are available for ascertain this hypothesis. The role of the phospho-Survivine has to be evaluated.

This study confirm that Survivin is very peculiar protein among CPC and consequently that its role has to deeply investigated. The following directions may be followed in the future:

It is reported that ubiquitination of Survivin via lysine 63 is important for regulating its localization on centromere. Since we have defined that the mobile protein is phosphorylated on Thr117, we will study the connections between Lys63 ubiquitination and Thr117 phosphorylation of Survivin in the centromeric targeting.

Survivin T117E affects cytokinesis. It perturbs the localization of MKLP1. In yeast, Bir1p, the homologue of Survivin also affects Septin recruitment. Septins are also important proteins for abscission. The role of Survivin in cytokinesis has to be investigated in details and all the consequences of the dominant negative mutant have to be described carefully. Survivin are interesting druggable targets. One strategy may be based on the expression of minimal dominant negative peptides or short proteins. Then efforts have to be devoted to the vectorization of the active motif.

In future work, we will complete the characterization of the identified Aurora kinase inhibitors. A kinase profiling is planed to gain access to the specificity of the molecules. The differences observed with the different cell lines and especially when compared to VX-680 efficiency prompt us to test more cell lines. The hypothesis proposing that

benzo[e]pyridoindole efficiency may be different in cells with P53 compromise checkpoint will be evaluated. Normal HCT116 cells will be compared to HCT116 P53- cells. *In vivo* assays will also start soon in nude mice. The collaboration established with the Institut Curie will continue. Chemists are synthesizing the second generation of compounds. The new molecules have to be tested *in vitro* and *ex vivo* with the assays described in the manuscript.

In conclusion Survivin and Aurora B kinase are two potential members of the CPC that may be efficiently targeted for preventing cell proliferation.

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Abstract

The chromosomal passenger complex (CPC) plays a key role in mitosis : controlling both chromosome segregation, spindle tension, anaphase onset and cytokinesis. The complex is composed of four proteins : INCENP, Aurora B kinase, Survivin and Borealin. Taking into account that Survivin is phosphorylated by Aurora B and has a pivotal role in the complex, we have studied the phosphomimetic mutant SurvivinT117E. Survivin phosphorylation is required for anaphase onset and the phospho-mutant is poorly linked to centromere. Moreover it exhibits a dominant negative function in cytokinesis, preventing abscission.

In a search for Aurora kinase inhibitors we have identified a new class of Aurora B kinase inhibitors that prevents Histone H3 phosphorylation, impairs mitotic spindle checkpoint. Moreover these molecules prevent tumor cell proliferation. These inhibitors are interesting tools for understanding CPC function and represent a new lead for the development of anti-cancer drugs.

Survivin and Aurora B kinase, which are expressed exclusively in mitosis, are thus two druggable targets for new anti-mitotic therapies.

Key words: Mitosis, chromosomal passenger complex, Aurora kinase, Survivin, mitotic checkpoint.

Résumé

Le complexe passager joue un rôle clé en mitose: contrôlant à la fois la ségrégation des chromosomes, la tension du fuseau, l'entrée en anaphase et la cytodirèse. Le complexe est composé de quatre protéines: INCENP, la kinase Aurora B, Survivine et Boréaline. Sachant que la protéine Survivine est phosphorylée par Aurora B et qu'elle a un role pivot au sein du complexe, nous avons étudié un mutant mimant sa phosphorylation: Survivine T117E. La phosphorylation de Survivine est nécessaire à la transition Métaphase/ Anaphase. Le mutant Survivine T117E est faiblement lié aux centromères en métaphase et agit comme un dominant négatif de la cytodirèse, empêchant la séparation des deux cellules filles.

Lors de la recherche d'inhibiteurs des Aurora kinases, nous avons identifié une nouvelle classe de molécules qui inhibent la phosphorylation de l'histone H3 et le point de contrôle du fuseau. Ces molécules préviennent la prolifération des cellules tumorales. Ces composés sont des outils intéressants pour étudier la fonction du complexe passager et représentent un nouveau motif moléculaire pour le développement de drogues anti-cancéreuses.

Survivine et Aurora B kinase dont l'expression est restreinte à la mitose sont deux cibles pour de nouvelles thérapies anti-mitotiques.

Mots clé : Mitose, complexe passager, Aurora kinase, Survivine, point de contrôle du fuseau.

[Cell Cycle 8:5, 1-8; 1 March 2009]; ©2009 Landes Bioscience

Report

Benzo[e]pyridoindoles, novel inhibitors of the aurora kinases

Thi My-Nhung Hoang,[1,5] Bertrand Favier,[2] Annie Valette,[3] Caroline Barette,[4] Chi Hung Nguyen,[5] Laurence Lafanechère,[4] David S. Grierson,[5] Stéfan Dimitrov[1] and Annie Molla[1,*]

[1]INSERM: U823 Institut Albert Bonniot; Université Joseph Fourier; La Tronche, France; [2]GREPI/TIMC-IMAG CNRS: UMR 5525—Université Joseph Fourier; Grenoble, France; [3]CNRS: 5088 Université Paul Sabatier; Toulouse, France; [4]CEA, DSV, iRTSV, CNRS, UMR 5168; Centre de Criblage pour molécules Bio-Actives; Grenoble, France; [5]UMR 176 CNRS-Institut Curie; Institut Curie; Orsay, France; [6]Faculty of Biology; Hanoi University of Sciences; VNU, Vietnam

Key words: mitosis, aurora kinase, small-molecule inhibitors, chromosomal passenger complex, pyridoindoles, mitotic slippage

Aurora kinases are serine/threonine protein kinases that are involved in cancer development and are important targets for cancer therapy. By high throughput screening of a chemical library we found that benzo[e]pyridoindole derivatives inhibited Aurora kinase. The most potent compound (compound 1) was found to be an ATP competitive inhibitor, which inhibited in vitro Aurora kinases at the nanomolar range. It prevented, ex vivo, the phosphorylation of Histone H3, induced mitosis exit without chromosome segregation, known phenomena observed upon Aurora B inactivation. This compound was also shown to affect the localization of Aurora B, since in the presence of the inhibitor the enzyme was delocalized on the whole chromosomes and remained associated with the chromatin of newly formed nuclei.

In addition, compound 1 inhibited the growth of different cell lines derived from different carcinoma. Its IC_{50} for H358 NSCLC (Non Small Cancer Lung Cells), the most sensitive cell line, was 145 nM. Furthermore compound 1 was found to be efficient towards multicellular tumor spheroid growth. It exhibited minimal toxicity in mice while it had some potency towards aggressive NSCLC tumors. Benzo[e]pyridoindoles represent thus a potential new lead for the development of Aurora kinase inhibitors.

Introduction

Aurora kinases are a family of serine/threonine protein kinases that play a key role in mitosis progression.[1,2] Aurora A is found to be associated with both centrosomes and microtubules and it is required for centrosome duplication, entry into mitosis, formation of bipolar spindle and mitotic checkpoint.[3-5] Aurora B exhibits typical passenger protein behavior during mitosis. Initially, the kinase associates with centromeres, and as mitosis proceeds, it relocates to the central spindle and the midbody. Aurora B is essential for chromosome condensation, kinetochore functions, spindle checkpoint activation and cytokinesis completion.[2,6-8]

Aurora A and B are overexpressed in many cancers, including primary colon and breast cancer.[1,9] Furthermore, the human *Aurora A* gene is localized to the 20q13 amplicon, which is associated with a poor prognosis in breast cancer.[9] Xenografts of mouse NIH-3T3 cells overexpressing Aurora A give rise to tumors in nude mice, suggesting that Aurora A behaves as an oncogene.[10] Under similar conditions, overexpression of Aurora B may induce metastasis.[11] In the light of these observations, Aurora kinases have emerged as druggable targets for cancer therapy and thus, identification of Aurora kinase small molecule inhibitors is of particular interest.[12-14]

Several Aurora A and B inhibitors, including ZM477439, Hesperadin, VX-680 (MK-0457), MLN8054, PHA-739358 have been described.[15-20] Most of them have been included in clinical trials. VX-680, considered as the Aurora reference inhibitor, suppresses tumor growth in vivo and encouraging results were reported for three patients with refractory Chronic Myeloid Leukemia[21,22] perhaps partly through the inhibition of the T315I mutant BCR-ABL.[23]

The in vitro high throughput screening of the proprietary Institut Curie-CNRS small molecule library of 6560 compounds allowed the identification of benzo[e]pyridoindoles as new inhibitors of Aurora kinases. Ex vivo assays in HeLa cells showed that the identified best hit (compound 1, C1) inhibited the activity of Aurora B and mimicked the phenotype obtained after siRNA suppression of Aurora B expression. Experiments with several other tumor cell lines demonstrated that the C1 significantly affected tumor cell growth in either two- or three-dimensional cultures. C1 when used in the treatment of mice bearing H358 tumors with C1 suppressed tumor cell growth. Benzo[e]pyridoindoles are thus proposed as leads for the development of Aurora kinase inhibitors.

Results

High throughput screening for identification of aurora kinase inhibitors. We have developed an Aurora kinase assay for the identification of small molecule inhibitors of Aurora kinases. The assay is based on the phosphorylation of recombinant histone H3 by a purified recombinant Aurora A kinase domain under non-saturating ATP concentrations (see Materials and Methods for detail). The calculated statistical Z' factor was 0.77 making the assay suitable for high throughput screening. In the screen of the Institut Curie-CNRS proprietary library (http://chimiotheque-nationale.enscm.fr), which contains 6560 distinct mono- to penta-heterocyclic compounds, forty molecules were found to inhibit Aurora kinase by more than 50% when assayed at a concentration of 15 mM.

*Correspondence to: Annie Molla; AUTHOR: please complete mailing address; Tel.: 33476549474; Fax: 33476549595; Email: annie.molla@ujf-grenoble.fr

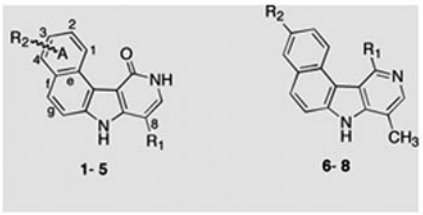
Submitted: 01/06/09; Accepted: 01/18/09

Previously published online as a *Cell Cycle* E-publication: http://www.landesbioscience.com/journals/cc/article/7879

Of the fourteen most active compounds identified, six belong to the benzo[e]pyridoindole family (Fig. 1). Non-pertinent "hits" corresponding to bi- and tricyclic aromatic compounds bearing reactive (aldehyde) functionality were also found. Because of their high chemical reactivity such compounds were considered as false positive and discarded.

The most potent molecules **1** and **2** exhibit an OMe group at the 3-position of the benzo (A-ring). However, the observed high activities of compounds **3** and **5** allow the conclusion that this position of the OMe group is not a requirement for activity, and can be further moved to the 4-position (Fig. 1). The presence of an alkyl chain at position **8** is, however, crucial for the inhibitory effect of these molecules, since its absence resulted in a clear loss in activity (compare **1** with 4). Replacement of the pyridinone carbonyl oxygen by a hydrogen (7) or a chlorine atom (**6**) resulted also in a loss of activity. Finally, the double modification in which R1 and R2 were changed, as in **8**, led to almost complete loss of activity as measured at 1.5 mM.

In an independent study (C. Cochet, personal communication) compounds 1–8 were evaluated in vitro for their activity against Casein Kinase II (CKII). In this screen compound 6 was found active. The other indole compounds displayed essentially no affinity for CKII. With this in mind, we have further focused on the characterization of the biological activity of benzo[e]pyridoindole 1 (C1), the compound showing the strongest inhibitory effect (Fig. 1), and we have compared its activity with that of the reference Aurora kinase inhibitor VX-680.[22]



Compound	R_1	R_2	Inhibition (in %) 15 μM	1.5 μM
1	Et	3-OMe	94	70
2	Me	3-OMe	90.3	57
3	Me	H	87.7	53
4	H	3-OMe	85.4	45
5	Me	4-OMe	79.6	37.8
6	Cl	OMe	76.7	31.9
7	H	OMe	47.4	18.5
8	H	$O(CH_2)_4COOH$	43.3	5.6

Figure 1. Novel Aurora kinase inhibitors identified by high throughput screening. The chemical structure and the preferential conformations of the identified eight most potent hits are presented. The inhibitory efficiency (percent of inhibition) towards the kinase domain of Aurora A for each hit at 15 μm and 1.5 μM are also shown.

The specificity of the molecule C1 (at 1 μM concentration) towards a panel of 36 different kinases was analyzed (Fig. 2). Most of the kinases tested were not affected by the presence of C1 (Fig. 2A). C1 targets essentially Aurora A, MELK and FGFR1 kinases. These kinases were inhibited by 91, 96 and 92%, respectively (Fig. 2A). C1 had also some inhibitory activity towards CHK1, MEK1, RSK1, RSK2 but did not target the cell cycle kinases CDK and PLK (Fig. 2A). Among the Aurora kinase family, C1 exhibited an IC_{50} of 61 nM, 31 nM and 124 nM for the Aurora kinases A, B and C, respectively (Fig. 2B). These IC_{50} were similar to those determined in the same assay for VX-680, but the selectivity of the two inhibitors was slightly different. Indeed, VX-680 was more potent towards Aurora A, while the highest inhibitory effect of C1 was towards Aurora B. C1 inhibited both CHK1 (IC_{50} = 262 nM) and MELK (IC_{50} = 42 nM), whereas VX-680 was poorly efficient towards these kinases. Both inhibitors exhibited thus different selectivity (Fig. 2B), although both of them are ATP competitive Aurora inhibitors (Fig. 2C and ref. 17).

Effect of C1 on HeLa cells. The Aurora kinase inhibition efficiency of C1 was also studied ex vivo in HeLa cells in culture (Figs. 3 and 4). To directly demonstrate the inhibitory effect of C1 we have studied the phosphorylation status of histone H3 by Western blotting (histone H3 is one of the favored substrate of Aurora B and the entry in mitosis correlates with a burst in histone 3 phosphorylation). (Figs. 3A and 4A). Treatment of the cells with C1 led to a significant decrease of H3 phosphorylation in mitotic HeLa cells (Fig. 3A). Moreover treatment of HeLa cells with compound 1 resulted in severe morphological perturbations of the HeLa cell nuclei (Fig. 3B and C). In particular, cells acquired enlarged lobed nuclei. Quantification showed that treatment with either C1 (2 mM) or VX-680 (300 nM) resulted in the generation of a comparable number of cells with such irregular nuclei (21% for C1 and 23% for VX-680, Fig. 3C). Note that under the same conditions, only ~1% of the control non-drug treated cells exhibited irregular nuclei (Fig. 3C).

Treatment with C1 led also to cell polyploidy (Fig. 3D). Mitotic (or 4N) cells represent about 12.5% of the population in the control, whereas they increase to 30% in the presence of compound 1 (Fig. 3D). Moreover the drug-dependent generation of enlarged nuclei (Fig. 3B) correlates with a significant increase of ploidy (58% of the cells are polyploid in the presence of 700 nM compound 1, Fig. 3D). These phenotypes (lobed nuclei and polyploid cells) were associated with an inactivation of Aurora-B,[1] suggesting that C1 is an efficient inhibitor of the Aurora B kinase activity.

Following an overnight paclitaxel treatment, the bulk mitotic cells tested positive for phospho-histone H3 (~37% of the total cell population), whereas only 3% of the total cell population was scored positive in the presence of compound 1 (2 mM) as compared to 1% for VX-680 (300 nM), the reference Aurora B kinase inhibitor (Fig. 4A and B). Note that upon C1 treatment the 4N cells number increased ~3-fold (Fig. 3D), but nonetheless a very strong inhibition of H3 phosphorylation was observed in the drug treated cells (Fig. 4A–C). The depletion of Aurora B kinase or its inactivation allowed paclitaxel treated cells to escape mitosis (mitotic slippage).[16] If C1 is really responsible for inactivating Aurora B, mitotic slippage should also be observed. With this in mind, we treated cells overnight with paclitaxel (33 nM), then added increasing C1 concentrations and pursued the incubation for two hours (Fig. 4C). The percentage

A

Kinase	% Enzyme activity	Kinase	% Enzyme activity
ABL1	53	FMS	85
ABL1 (T315I)	29	GSK3β	93
AKT2/PKBβ	100	LCK	39
AKT3	118	**MEK1**	20
Aurora A	9	**MELK**	5
CAM KI	91	MST1/STK4	24
Casein kinase II	100	MST2/STK3	23
CDK1/CyclinB	46	NEK2	55
CDK2/CyclinA	68	NEK6	93
CDK4/CyclinD1	63	P38α/MAPK14	95
CHK1	16	PAK2	92
c-MET	97	PKA	66
c-SRC	22	PKCα	88
EGFR	85	PLK1	93
ERK1	90	PLK2	72
FGFR1	8	**RSK1**	17
FLT1	85	**RSK2**	18

B

Kinase	IC50 (C1) nM	IC50 (VX-680) nM
Aurora A	61	13
Aurora B	31	37
Aurora C	124	117
CHK1	262	Sup 1000
MELK	42	410

C

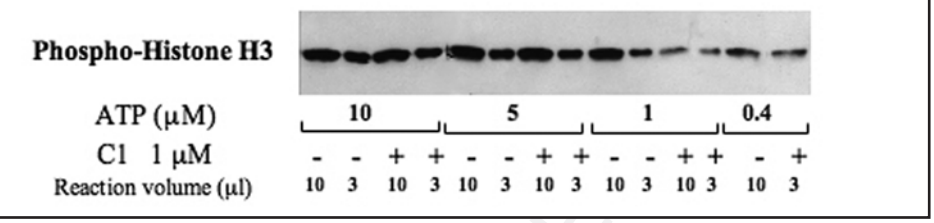


Figure 2. Kinase inhibition selectivity of C1. (A) C1 kinase profiling. 36 recombinant kinases were used in the study. The percentage of the remaining kinase activity measured upon treatment with 1 mM C1 in a solution containing 1 mM ATP is presented. Kinases, inhibited by more than 80%, are indicated in bold. (B) IC_{50} values for the five best kinase targets of C1. For comparison the IC_{50} for VX-680 for the same kinases is also shown. The measurements of IC_{50} for both compounds were carried out under identical conditions. (C) Western blot analysis of the effect of C1 on the phosphorylation of histone H3 by Aurora A domain kinase. A kinase assay was carried out either in absence (-) or in the presence (+) of C1 (1 mM) and at decreasing concentrations of ATP. Each reaction contained the same amount of histone H3. After completion of the reaction, 10 and 3 ml of the reaction mixtures containing either 10, or 5 or 1 mM ATP were run on an SDS gel (for the reaction carried out in the presence of 0.4 mM ATP, an aliquot of only 10 ml was run). After blotting, the phosphorylation of histone H3 was revealed by a specific antibody against the phosphorylated histone H3.

of lobed nuclei was negligible in the control and in the 0.5 mM C1 treated cells. The increase of C1 concentration up to 1 mM dramatically affected the cells. Indeed, while 37% of the cells were in metaphase in the control, this percentage decreased to less than 5% in the presence of 1 mM C1. At 0.5 mM C1 essentially no polyploid cells and no cells with irregular nuclei were observed. At 1 mM C1 a drastic increase of such "defective" cells was detected, i.e., ~22% of the total cell population exhibited such morphological defects (Fig. 4C). Together, these data show that cells treated with both paclitaxel and C1 exhibited mitotic slippage and therefore, reinforce the conclusion that C1 is an inhibitor of the Aurora B kinase activity.

The absence of Aurora B or the inhibition of its activity affected chromosome segregation[15] and thus, treatment with C1 was expected to have the same effect, which should result in the strong polyploidy already observed (Fig. 3). To study this in real time, we have used time-lapse microscopy and stable cell lines expressing GFP-H2A, cells which allow visualization of chromatin DNA (Fig. 4D and E). The control cells (without drug treatment) showed a typical mitotic behavior with properly aligned chromosomes at metaphase, which, as mitosis proceeded, were segregated and gave finally rise to the nuclei of the two daughter cells (Fig. 4D). The treatment with 1 mM C1 inhibited the chromosome alignment and the formation of metaphasic plate, chromosomes failed to segregate, and with time they loose their rodlike shape, decondensed and formed interphase like structure (Fig. 4E), In all analyzed cells (more than 30 in three separate experiments), the interphase type chromatin was always connected by bridges (see Fig. 4E, 46 min). These data demonstrate that upon treatment with C1 cells exit from mitosis without segregating chromosomes.

Interference with the function of the protein passenger complex resulted in a distribution of its members on the entire chromosome.[2] Treatment with C1 inhibited the enzymatic activity of Aurora B, and thus one should expect similar effects in the presence of C1. To test this we have followed in real time by time-lapse microscopy the mitotic behavior of Aurora B-GFP in stable HeLa cell lines in the presence or absence of C1 (Fig. 5). Aurora B-GFP, in the control cells, had a typical passenger protein localization, decorating the centromeres at metaphase and then transferring to the central spindle and the midbody as mitosis proceeded (Fig. 5A). As expected for an Aurora B inhibitor, C1 prevented centromere alignment on the metaphasic plate (Fig. 5A and C1 500 nM). Treatment with 1 mM C1 resulted in the partial dissociation of the GFP-tagged Aurora kinase from the centromeres (40 minutes point, Fig. 5A) and with time, Aurora B-GFP decorated also the entire chromatin (100 minutes, Fig. 5A). A similar behavior of Aurora B-GFP was observed upon treatment with VX-680, the effect being observed with a concentration of 300 nM (Fig. 5A). Immunofluorescense microscopy showed that the treatment with C1 of paclitaxel arrested mitotic cells resulted in a partial redistribution of the endogenous Aurora B on whole chromosomes (Fig. 5B), a result in agreement with the time-lapse microscopy data.

In summary, these data illustrate in real time that the treatment of the cells with C1 affected similarly to VX-680 the behavior of Aurora B during the cell cycle, but with 2–3 fold smaller efficiency (1 mM of C1 was necessary to observe effect close to those found with 300 nM VX-680, see Fig. 5).

Effect of C1 treatment on tumor cell line viability. The viability of tumor cell lines upon C1 treatment was investigated and compared with the effect of VX-680. Four distinct cell lines (HeLa, LL/2, H358 and HCT-116) were grown as adherent cells and the concentration that inhibits growth by two (IC_{50}) was determined (Fig. 6A). Compound 1 was found to be more potent than VX-680 to inhibit growth of HeLa cells. In the other cell lines studied it was found to be between 1.6 to 8.9 less potent than the VX-680 (Fig. 6A). Hematopoietic and lung cancer cells behaved mostly like HCT116 cells, i.e., the effect of C1 was 6–7 fold less than this of VX-680 (data not shown). We have also investigated the effects of C1 on the HCT-116 carcinoma cells growing as MCTS by measuring the volumes of the spheroids. After treatment for 5 days the IC_{50} for HC-t1116 spheroid growth inhibition was 1,066 nM for compounds 1, while it was of 108 nM for VX-680 (Fig. 6A).

Effect of C1 on tumor growth in mice. The efficiency of compound 1 was also studied in mice bearing H358 tumors. Mice bearing H358 tumors were injected with either C1 (5 mg/kg) and with the vehicle or vehicle only. The concentration of C1 was limited by its solubility in aqueous buffer. Mice were healthy after repetitive injections of the compound and grew normally. The only anomaly observed was related to the kidneys, which were slightly less colored. Blood analysis indicated neutropenia in the mice repetitively treated with C1 (average 1 x 10^6 neutrophil/l) compared to the control mice (average 2.6 x 10^6 neutrophil/l).

In the control group, tumors grew progressively and except for one, their growing index (i.e., the ratio of the tumor volume measured at the indicated time (V_D) to the initial (days 7 and 8) volume of the tumor (V_0)) was ~3.5 at the end of the experiment (day 51, Fig. 6B). Upon injection of C1, the tumor growth index was found to be very dispersed. Three tumors were still decreased in size at day 30, while the others behaved as control tumors. At day 37 only two tumors have regressed, while four had an index of 1 and one was growing. At day 44 two tumors increased in size (index 5.3 and 2.8) whereas two were still under control (index inferior at 1). At the end of the experiment (day 51) one tumor in the treated group has an index smaller than 1 (index 0.2). Conversely one tumor has an index superior to 6 suggesting that repetitive injections induced resistance to compound 1. Consequently the differences (mean tumor index and dispersion) between treated and control tumors were not statistically significant (Student-t and Fisher-F test). Importantly, tumors with a decreased size (growth index below 1) were always found in the treated group after 30 days. We also found that the level of H3-phosphorylation was lower in the tumors of the C1 treated mice compared to those of the untreated control mice (Fig. 6C). Similar results were obtained in a second separate experiment (data not shown).

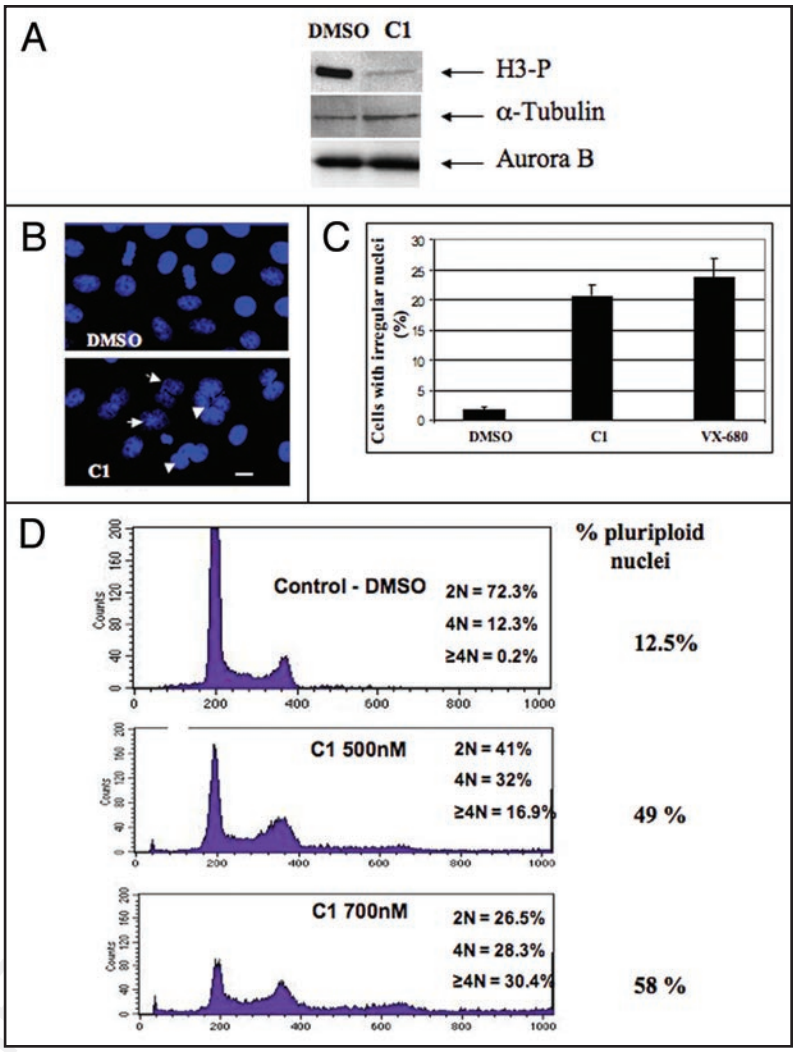


Figure 3. Effect of C1 in HeLa cells. (A) Western blot analysis of histone H3 phosphorylation. HeLa cells were incubated overnight with Nocodazole (50 nM) in the presence of either DMSO or C1 (2 mM). Cells were then collected and lyzed. Identical amounts of the lyzed cell samples were run on 18% acrylamide gel containing SDS. After transfer, the blot was revealed using an antibody against phosphorylated histone H3. The same membrane was also revealed using antibodies against α-tubulin and Aurora B for estimation of both the amount of loaded proteins and of mitotic cells, respectively. (B) Treatment with C1 resulted in perturbation of the structure of the cell nuclei. HeLa cells were incubated overnight in the presence of either C1 (2 mM) or DMSO (1%). Nuclei were stained with Hoechst 33 342 and visualized by fluorescence microscopy. Arrows indicate irregular nuclei. Similar perturbations in the nuclear structure were obtained upon treatment with 300 nM VX-680 (not shown). Bar, 5 microns. (C) Quantification of the data presented in (B). The percentage of irregular (lobed and polyploid) nuclei was determined in two independent experiments; 100 cells were analyzed per experiment. (D) FACS analysis shows that treatment with C1 results in a dramatic increase of the amount of polyploid cells. The experiments were performed with control HeLa cells (incubated only in the presence of 1% DMSO) and HeLa cells incubated with C1 (at either 500 nM or 700 nM) for 48 hours. DNA was stained with propidium iodine and the samples were analyzed by using a Beckton-Dickinson FACS analyzer. The percentage of polyploid cells (cells with a ploidy ≥2N) is indicated on the right part of the figure.

Discussion

By screening a library containing 6560 mono- to penta-heterocyclic compounds, we have identified forty molecules able to significantly inhibit Aurora A kinase. The most active compounds

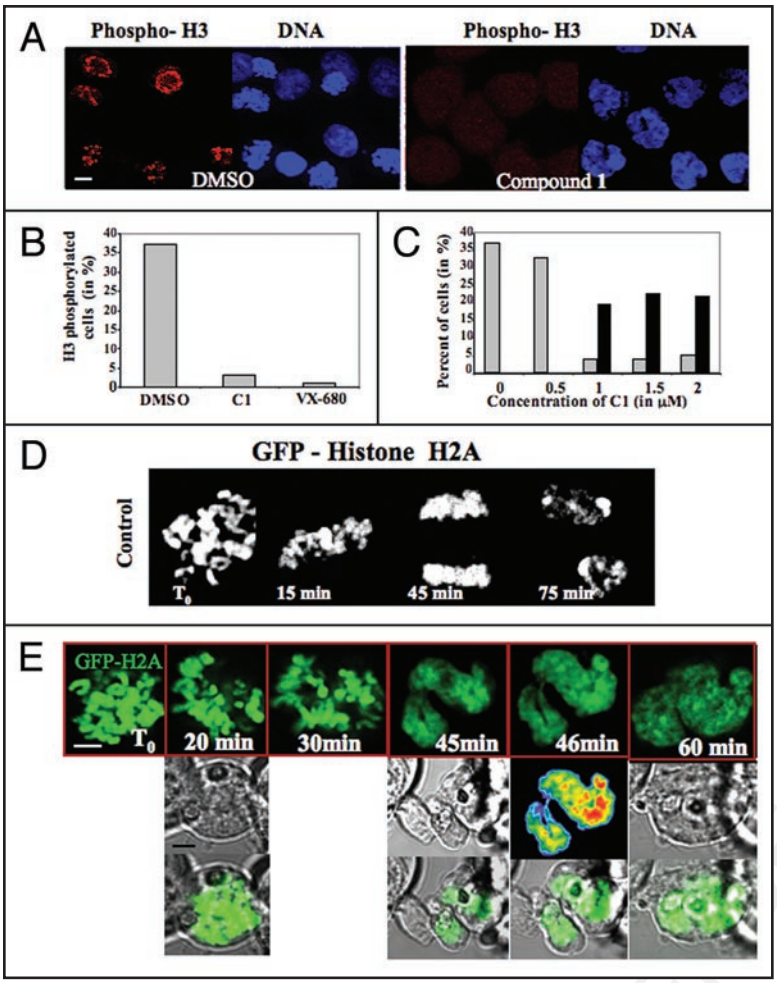


Figure 4. Effects of C1 on mitotic cells. (A) Visualization of the phosphorylation status of histone H3 in paclitaxel (33 nM) treated cells in the presence of either DMSO or C1. H3 phosphorylation (red) was visualized by a specific antibody and DNA (blue) was stained by Hoechst 33342. Similar results were obtained with VX-680 (not shown). The bar, 5 μm. (B) Quantification of the data presented in (A). 500 cells were analyzed in each experiment and the data of two independent experiments are shown. (C) Treatment with C1 induces mitotic slippage. HeLa cells were arrested in mitosis by overnight incubation in paclitaxel (33 nM). The cells were then incubated with the indicated concentrations of C1 for two additional hours and fixed. Mitotic and polyploid cells were scored. ~100 nuclei were analyzed in two different experiments; grey, mitotic cells; black, cells with polylobed nuclei. (D) Time-lapse microscopy of a stable mitotic HEK-293 cell expressing GFP-H2A histone fusion. (E) Time-lapse microscopy of C1 treated mitotic HEK-293 cells stably expressing GFP-H2A. C1 (1 μM final concentration) was added to the overnight paclitaxel (33 nM) treated cells and then the behavior of the cell was continuously imaged. Representative photos, made at the indicated time points, are shown. The first, second and third rows present the fluorescent GFP signal, the transmission signal and the merge signal, respectively. The bar, 5 μm.

belong to the benzo[e]pyridoindole family. These active benzopyridoindoles exhibit a "crescent moon" shape determined by the benzo[e] ring fusion. This geometry seems to offer the best complimentary shape to the Aurora kinase ATP site, since only two amongst the two hundred tetracyclic indole compounds screened (including the related benzo[g] and benzo[f]pyridoindoles and linear pyrido[4,3-b]carbazoles (ellipticine analogues)) displayed some weak inhibitory activity at 1.5 mM. The best hit (C1) was shown to be a potent in vitro, inhibitor of the three different Aurora kinases with IC_{50} of 61 nM, 31 nM and 124 nM for Aurora A, B and C, respectively. The profiling results reveal that C1 also efficiently inhibits also two other kinases, FGFR and MELK. MELK, maternal embryonic leucine zipper kinase, exhibits maximal activity during mitosis but its physiological substrates are unknown.[24]

Several different families of Aurora kinase inhibitors have now been identified and the structural basis for their inhibition selectivity was described.[25] For example, the tight association of either VX-680 or hesperadin with the hydrophobic pocket in the active site determined the high selectivity of the inhibitors.[26-28] Note that C1, as hesperadin (a member of the SU-family of indolinone type kinase inhibitors), exhibits a cyclic amide functionality.[15] Two-dimensional superimposition of C1 and hesperadin via their amide functionalities showed that the three phenyl rings in hesperadin form a "disconnected" crescent moon shape type structure, suggesting that C1 interacts with Aurora kinases in a manner similar to that of hesperadin, i.e., it may form a tight complex with the hydrophobic pocket of the kinase active site.

C1 prevents histone H3 phosphorylation in mitotic cells. This strongly indicates that in vivo the main target of C1 would be Aurora B. In agreement with this, the C1 treated cells did not exhibit monopolar phenotype, which is typically observed upon efficient inhibition of Aurora A. In addition, C1 impaired chromosome alignment and segregation as well as anaphase onset. It increased significantly the ploidy of the cells and induced mitotic slippage. All these effects were observed upon ablation of Aurora B,[2,6] further suggesting that *ex* vivo the main target of C1 is this kinase. The two other strong inhibitors of Aurora kinases, VX-680 and hesperadin, were also reported to selectively target Aurora B ex vivo and to override the spindle checkpoint.[15,28] We have also shown that both C1 and VX-680 were able to partly delocalize Aurora B kinase all over the chromosomes.

Both C1 and VX-680 efficiently prevented tumor cell proliferation. Although C1 and VX-680 have comparable in vitro inhibition efficiency towards Aurora kinases, the C1 antiproliferative activity was found to be between 1.6 and 8.9 folds weaker in the different cell lines studied compared to that of VX-680. The multicellular tumor spheroid (MCTS) model represents an intermediate level of complexity between cell growing as in vitro monolayers and solid tumors in animals. At a micromolar concentration, C1 decreased by two the spheroid volumes of HCT116 carcinoma cells growing as MCTS. The mild difference of IC_{50} in monolayer cultured cells compared to the spheroid model suggests a good biodisponibility of these compounds and its absence of multicellular resistance.

Furthermore, the effect of C1 was maximal (and comparable to that of VX-680) in H358 cell line, a cell line established from a very aggressive NSCLC tumor (Non Small Cancer Lung Cell). Patients bearing this type of tumors showed very bad prognoses and only a few relatively potent therapeutic agents against this type of tumors are available.[29] With this in mind, we have further studied the in vivo effect of C1 on mice bearing H358 tumors. Repetitive injections of C1 were well tolerated by the animals and affected tumor growth at least for one and a half month. Note that only in few cases a decrease

of the initial volume of the tumors was detected by the repetitive injections with C1 during the whole time course of the two independent experiments. This suggests that tumors escaped and became resistant to the compound upon repetitive injections.

In summary, our studies in mice revealed encouraging results with noticeable effect on a resistant model of tumors, and with few toxic effects observed so far. Benzopyridoindoles might thus be viewed as new leads for the development of Aurora kinase inhibitors that would have interesting potentials in NSCLC treatment. Combination of therapies, especially those acting via different mechanisms are proposed for improving the survival of patients with NSCLC; Aurora inhibitors, and among them Benzo[e]pyridoindole derivatives, may be included in these treatments.

Materials and Methods

Recombinant proteins. Recombinant Histone H3 and kinase domain of Aurora A were expressed in *E. coli* and purified to homogeneity. VX-680 was purchased from Kava technology Inc., while paclitaxel and nocodazole were from Sigma.

Protein kinase assay. The protein kinase assay was performed in 20 mM Tris-HCl, 20 mM KCl, 20 mM $MgCl_2$,0.4 mM ATP, 0.4 mM DTT, pH 7.5. Recombinant histone H3 was used as substrate. The reaction started by the addition of the recombinant enzyme. After 1 hour of incubation at 37°C the remaining ATP was monitored by addition of kinase-Glo™ (Promega, France) under the conditions suggested by the supplier. Ten minutes later the fluorescence was recorded with a Fluostar Optima (BMG Labtechnologies). Staurosporine (0.5 mM) was used as positive control.

High throughput screening. The assay was performed as described above in black 96-well plates and started with the addition of the Aurora A kinase domain. The Z-factor of the assay was estimated to be 0.77.[30] The primary screening was performed in triplicate at compound concentration of 15 mM (the compounds were dissolved in 0.3% DMSO) and the selected hits were tested again at a concentration of 1.5 mM. IC_{50} was defined as the compound concentration that leads to 50% of inhibition.

Kinase profiling and IC_{50} determination. Assays were performed on the RBC's Discovery Dot nanoliter screening platform (Reaction Biology Corp, USA), which combines the advantages of both radioisotopes and microarrays. A kinase profiling was performed with 36 recombinant kinases. Compound 1 was tested in duplicate, at the final concentration of 1 μM. Staurosporine was used as internal control. ATP concentration was 1 μM for all reactions. The purity of the molecule 1 was superior to 95% and its synthesis is described in.[31]

Compound 1, VX-680 and Staurosporine were tested against five selected kinases in a 10-dose serial dilution starting at 2 mM; ATP concentration was 1 μM for all reactions. Assays were run in duplicate. IC_{50} was defined as the compound concentration that leads to 50% of inhibition.

Cell culture. HeLa, HCT-116, Hek-293 and LL/2 were grown on Dulbecco's modified Eagle's medium (Biowhittaker, Europe). H358 cells were propagated in RPMI. Media were supplemented with 10% fetal bovine serum (Biowhittaker, Europe).

HeLa (Aurora B-GFP) stable cell lines were already described elsewhere.[32] HEK-293 cells were transfected with a plasmid expressing the fusion histone H2A—GFP and then a clonal fluorescent cell was selected and amplified.

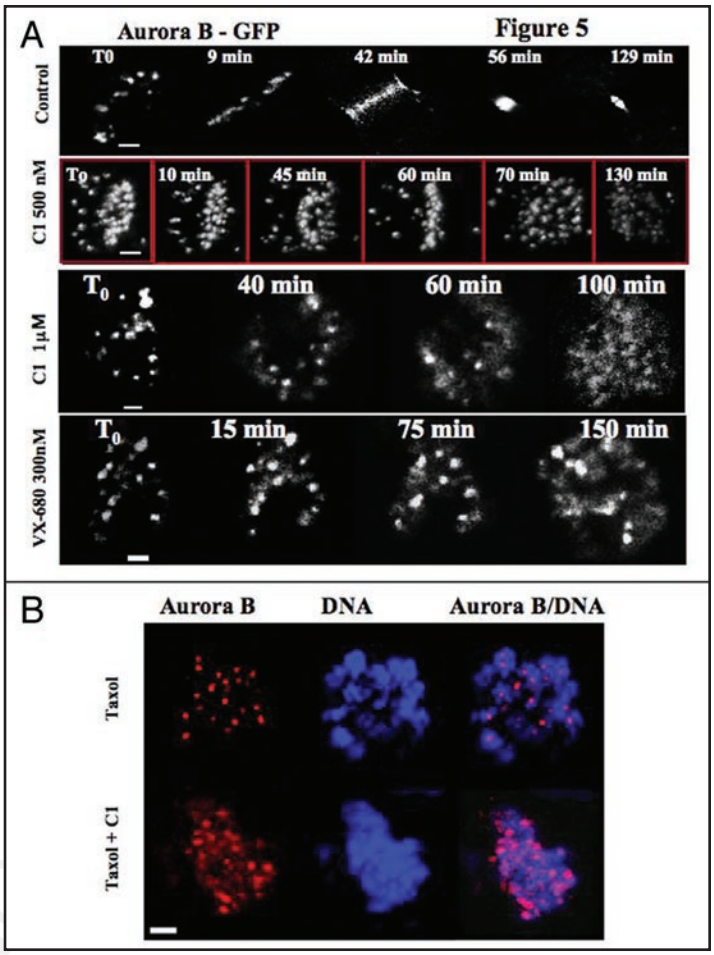


Figure 5. C1 induces the redistribution of Aurora B. (A) Time lapse microscopy of a mitotic stable HeLa cells expressing Aurora B-GFP fusion in the absence (control) or the presence of either C1 (at 500 nM or 1 μM) or VX-680 (300 nM). The compounds were added to the cell culture and then the behavior of the cells was continuously imaged. Representative photos, made at the times indicated, are presented. Note that both C1 and VX-680 delayed mitosis onset. In addition, the treatment with 1 μM C1 as well as with VX-680 resulted in a partial delocalization of Aurora B-GFP on the entire chromosomes. (B) C1 treatment induces also the redistribution of endogenous Aurora B. Overnight paclitaxel (33 nM) treated HeLa cells were incubated for two hours with C1 (1 μM), fixed and analyzed by immunofluorescence microscopy. The localization of Aurora B (red) was detected by anti-Aurora B antibody. DNA (blue) was stained by Hoechst 33342. Note that the strictly punctuated pattern (exclusively centromeric localization) of Aurora B in the control (paxitaxel) cells is no longer observed in the C1 treated cells. Aurora B being partly diffused on chromatin.

Cell proliferation assays were conducted in 96 well cultures plates. Assays were run in triplicate. Serial dilution of compounds started at 2 mM and viability was estimated, at day 5, by addition of MTT cell counting (Promega).

The multicellular tumor spheroid (MTS) model. We have adapted the hanging-drop method[33] to produce HCT116 spheroids of similar diameters. 500 cells were suspended on the lid of an agar coated 24-petri dishes containing culture media. 48 h later the spheroids were transferred to the culture medium. Spheroid volumes were measured before drug treatment (D_0). HCT116 spheroids were treated for 5 days with different concentrations of the compounds. Control HCT116 spheroids were grown under the same conditions,

A

Compound	2D cultures IC50 (nM) HeLa	LL/2	H358	HCT-116	Spheroid IC50 (nM) HCT-116
VX-680	1 455 ± 4	225 ± 70	90 ± 10	80 ± 10	108 ± 10
C1	960 ± 140	655 ± 50	145 ± 25	717 ± 19	1066 ± 208

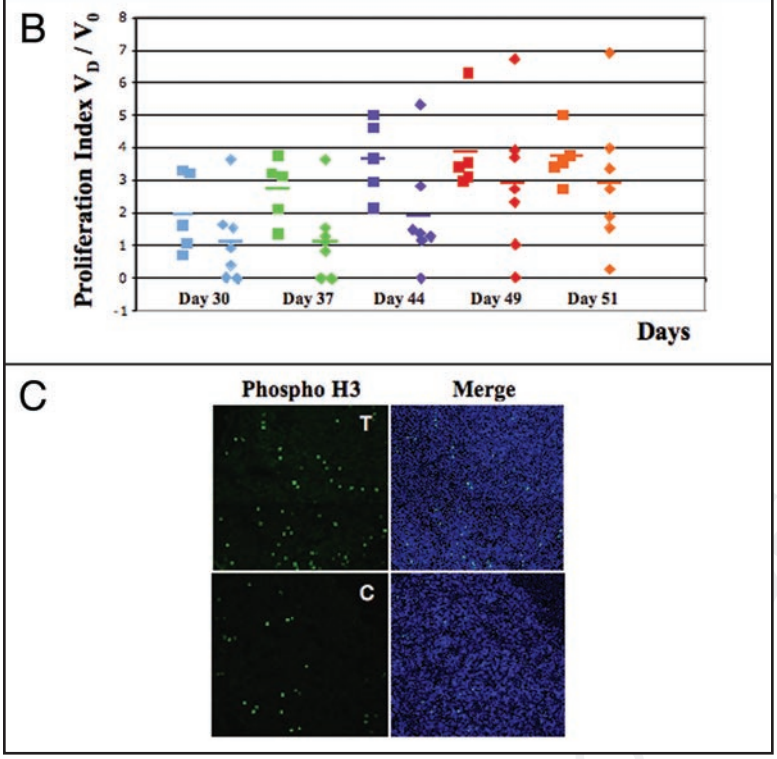


Figure 6. Effects of C1 on cell viability in two-dimensional and three-dimensional culture conditions; Effect on mice bearing H358 tumors. (A) C1 and VX-680 IC_{50} for HeLa, LL/2, H358 and HCT-116 cells in culture (two-dimensional conditions) and for HCT-116 spheroids (3D conditions). Cell growth and viability were tested under standard conditions in 96 well culture plates with MTT (Promega) cell assay. The average of three independent experiments is shown. Note that similar IC_{50} were determined for C1 and VX-680 towards H358 cells. (B) Proliferation indexes of H358 tumor growth. Mice were injected with 2 x 10^6 cells. Tumor dimensions were measured at days 7, 8, 30, 37, 44, 49 post-injection and the proliferation index (the ratio V_d/V_0, where V_d is the volume of the tumor at the respective day, and V_0 is the average volume of the tumor calculated at day 7 and 8). Square: control mice; diamond: treated mice. The data for day 30 (D30) post-injection are in blue, D37 data are in green, D44 data are in violet, D49 data are in red and D51 in orange. Each point presents the proliferation index of one mouse tumor and the horizontal bars represent the average index of the series. (C) Histone H3 phosphorylation is decreased in C1 treated tumors. At day 51 post-injection the control and the C1 treated H358 tumor bearing cell mice were sacrificed and a small part of the tumors were immediately frozen. The phosphorylation status of histone H3 in frozen sections of the tumors of the control (C) or C1 treated mice (T) was visualized by using a specific anti-phosphorylated histone H3 antibody (in green). DNA (blue) was stained by Hoechst 33342. Merge (DNA plus phospo—histone H3) is shown.

but without drug treatment. The size of each spheroid was determined by measuring 2 orthogonal diameters (d_1 and d_2) using an inverted microscope. The volume was calculated according to the formula: $V = 4/3\pi r^3$ where $r = 1/2 \sqrt{d_1} \times d_2$. Spheroid growth was calculated by measuring the variations in volume.

Ex vivo microscopy. Ex vivo experiments were conducted on cells grown on Lab-Tek chambered coverglass (Nalge Nunc International) and maintained under standard culture conditions as described in.[32] Images were acquired on a Zeiss LSM510 system using a Planapochromat 40x water immersion objective. GFP was excited with a 488 nm Argon 2 laser (power varying from 0.1 to 2%). Confocal slices are shown.

Immunofluorescence. Cells grown for 24 hours on glass coverslips were fixed at 37°C in 4% formaldhehyde, 2% sucrose and then immunofluorescences were then performed as described in.[34] Phosphorylated Histone H3 was detected by a polyclonal rabbit antibody (Upstate). Aurora B was detected by using mouse monoclonal AIM-1 (1/100, Transduction Laboratories). DNA was visualized with 0.1 mM Hoechst 33342 (Sigma). Images were collected with a ZEISS 510 Laser Scanning Confocal microscope with a 63x immersion oil objective. Slices of 0.5 micron are shown.

Cells seeded on glass-coverslips were arrested in mitosis by the addition of paclitaxel (33 nM). Immunohistochemistry was performed on 8 nm thick frozen sections as describe above.

In vivo experiments. In vivo experiments were conducted on four-weeks old female Swiss nude mice (Iffa Credo, Marcy l'Etoile, France). After one week of adaptation in the animal facility (French agreement number A38-516-01), the mice were inoculated subcutaneously with 2 x 10^6 exponentially growing H358 cells mixed with growth factor free matrigel (1/1, BD). Tumors were established at seven days post-injection. Then the mice from each cage were randomly divided into two groups, which allowed the equalization of the mean tumor size of each group. Tumor volumes were determined by measuring two perpendicular diameters using a clipper and then calculated as follows: $V = d_1^2 \times d_2/2$, where d_1 and d_2 are the smallest and the largest diameters, respectively. One mice group (7 animals) received the treatment (compound 1, 100 mg per 20 g mouse in vehicle buffer (PEG 300/PBS/DMSO; 5/4/1) intraperitoneally, whereas the control group (6 mice) was injected with vehicle only. Mice were injected four times a week (Monday, Tuesday, Thursday and Friday). Once a week, mice were weighed and the volumes of the tumors were measured. Only one animal (from the control group) displayed a weight loss and was sacrificed. All other mice received 23 injections, then sacrificed and autopsied. For further analysis, blood was sampled and tumors were dissected and weighed. A piece of tumor was also embedded in OCT compound and frozen for immuno-histochemistry experiments. A second similar separate experiment that included five animals for each group was also carried out.

Acknowledgements

The authors thank Fabienne Sirot for help in the initial phase of this work and Céline Frangia for her skilful technical assistance. We thank Dr. Dimitrios Shkoufias for reading the manuscript and suggestions. Thanks to the ChemAxon company (http://www.chemaxon.com), which has allowed the free use of the MarvinView package. T.H. was supported by a Vietnam/French program and was a recipient of an ARC fellowship. This work was supported by INSERM, ANR (Project R05075CC), ARECA and Rhônes-Alpes Canceropole CLARA (EpiProNetwork). S.D.

acknowledges La Ligue Nationale contre le Cancer (Equipe labelisée La Ligue).

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