

MSC



FCUP
ICBAS
2021

U. PORTO

Targeting mitosis for cancer therapy: improving the efficacy
of the second-generation antimitotics and identifying new
antimitotics

Pedro Henrique Alves Novais



U. PORTO



U. PORTO



Targeting mitosis for cancer therapy: improving the efficacy of the second-generation antimitotics and identifying new antimitotics

Pedro Henrique Alves Novais

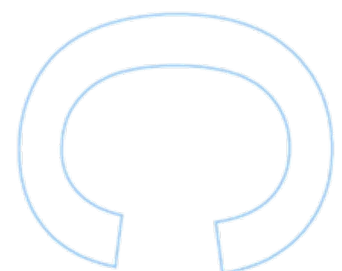
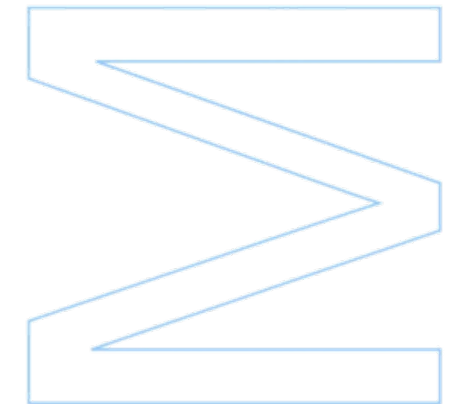
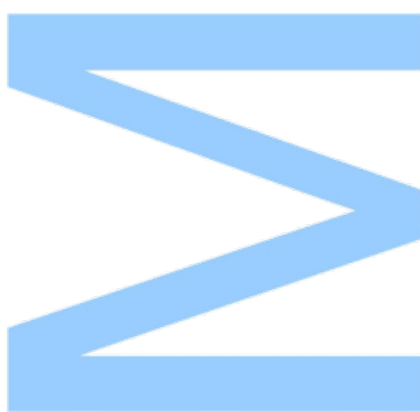
Master's thesis in Biochemistry presented to
Faculty of Sciences and
Abel Salazar's Institute of Biomedical Sciences
University of Porto

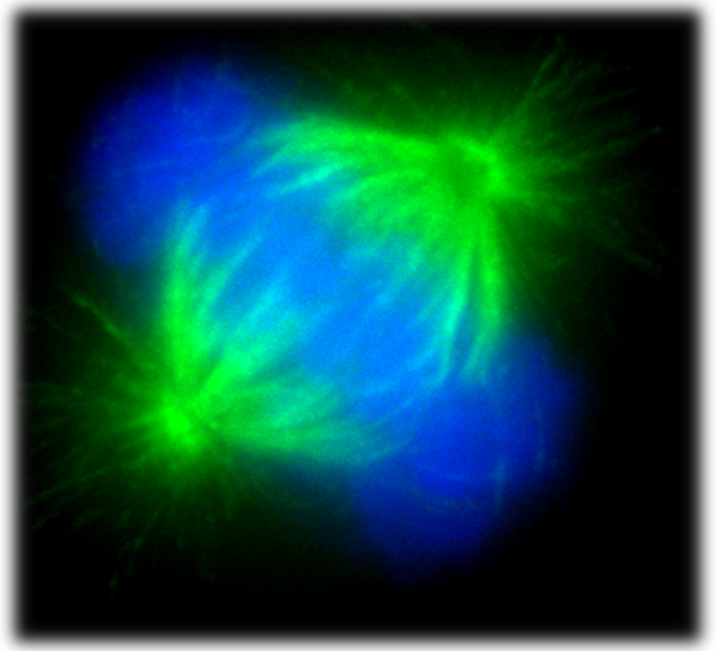
2021

U. PORTO



U. PORTO





Targeting mitosis for cancer therapy: improving the efficacy of the second-generation antimitotics and identifying new antimitotics

Pedro Henrique Alves Novais

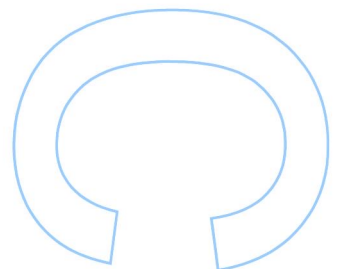
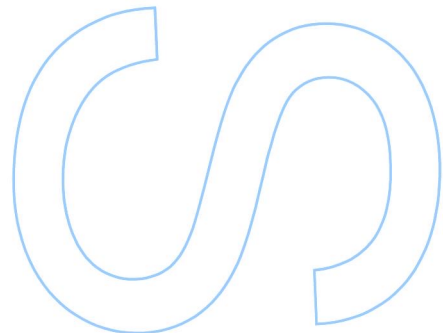
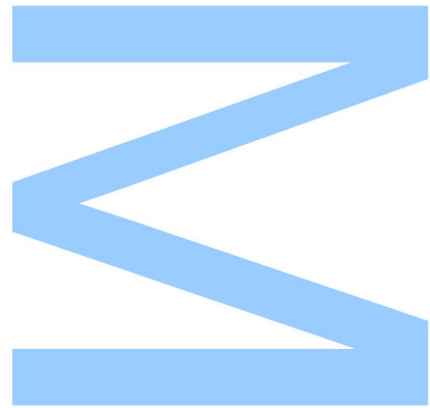
Master's Degree in Biochemistry
Chemistry and Biochemistry Department
2021

Supervisor

Hassan Bousbaa, Associate Professor, University Institute of Health Sciences, CESPU

Co-Supervisor

Maria Isabel Amorim, Assistant Professor, Faculty of Sciences of University of Porto





U. PORTO

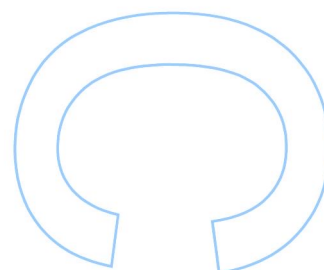
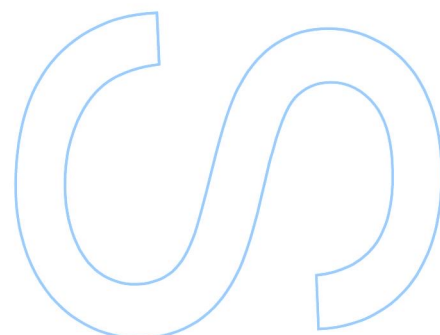
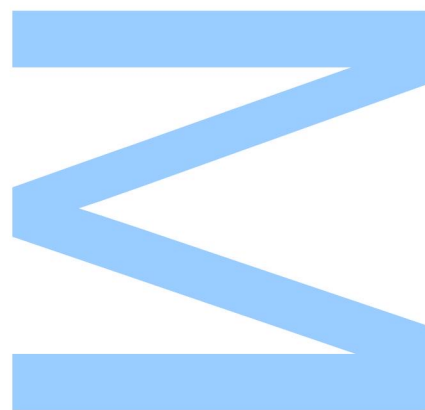


U. PORTO
FC FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Todas as correções determinadas
pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, ____/____/____



Acknowledgements/Agradecimentos

Terminada mais uma etapa do meu percurso académico, quero agradecer a todas pessoas que me apoiaram quer a nível pessoal como profissional, e que tornaram possível a realização desta dissertação. Gostaria de deixar o meu profundo agradecimento:

Ao Professor Dr. Hassan Bousbaa, meu orientador, pela de oportunidade realizar este projeto que me possibilitou emergir no mundo da investigação científica. Por todo o conhecimento partilhado que muito contribuiu para o meu crescimento profissional, por toda a disponibilidade, motivação, suporte e boa disposição demonstrada ao longo do último ano, terei gratidão eterna.

À Professora Dra. Maria Isabel Amorim, minha coorientadora, por ter aceitado participar neste projeto e por toda a generosidade, disponibilidade e tempo dedicado a este projeto.

A todos os membros da equipa Cancer Research Ana Henriques, Bárbara Pinto, João Silva, Mafalda Duarte, pela hospitalidade e simpatia que facilitou a minha integração na equipa, por toda a ajuda e conhecimento que partilharam, e principalmente por toda a amizade, conselhos e os bons momentos partilhados. O trabalho em equipa é a base para o sucesso e vocês são parte integrante do trabalho realizado neste projeto.

Um agradecimento especial à Dra. Patrícia Silva, por todo conhecimento transmitido, por todos os conselhos e suporte que sempre demonstrou e por todas as horas que se dedicou a mim e a este projeto. Guardarei todas as conversas e bons momentos partilhados, sem dúvida uma das pessoas que mais contribuiu para o meu crescimento científico e uma amiga que ganhei para resto da vida.

A toda a minha família. Aos meus pais, obrigado por fazerem de mim o homem que sou hoje. Ao Carlos, Lígia, Sr. Vítor, D. Glória, Tiago e Sara por todo apoio e incentivo que deram ao longo deste último ano.

À Rita por toda a força que me deste e paciência que tiveste durante esta etapa. Obrigado por me apoiares e compreenderes o meu desejo em seguir esta carreira desafiante e por me ajudares a enfrentar o futuro sem medo. Obrigado por seres o meu porto de abrigo e por me fazeres cada vez mais feliz a cada dia que passa.

Ao IINFACTS, CESPU pelo acolhimento, por disponibilizarem todas as infraestruturas necessárias à realização desta tese e pelo financiamento.

Abstract

Based on the uncontrolled proliferation of cancer cells, anticancer drugs have been developed to block the cell cycle, particularly at mitosis. Antimitotic agents rely on spindle assembly checkpoint activation which leads to prolonged mitotic arrest, expected to induce subsequent cell death. Classical antimitotic drugs have been widely used in cancer therapy, however, due to some limitations associated with these drugs, novel antimitotic molecules have been developed, known as second-generation antimitotics (SGAs). SGAs inhibit the activity of mitotic proteins, namely the kinases Mps1, Plk1, Aurora kinases A and B, and the motor proteins Eg-5 and CENP-E, that play crucial roles in different processes during mitosis. Globally, SGAs have demonstrated poor efficacy in clinical trials. In the first aim of this thesis, we proposed a strategy to improve the efficacy of the SGA BI2536, a Plk1 inhibitor, through its combination with Navitoclax, an apoptosis potentiator, in the non-small cell lung cancer cell line A549. We showed that Navitoclax precipitated the death of cells arrested in mitosis by BI2536, which reduced the risk of mitotic slippage, one of the reasons for therapeutical failure of SGAs. We found that Navitoclax and BI2536 synergize to induce tumor cell death by apoptosis.

Additionally, the second aim of this thesis focused on the identification of new antimitotics. A total of four molecules were analyzed: three tetracyclic thioxanthene derivatives, namely TX2.1a, TX2.17, and TX2.19, and a diarylpentanoid, the BP-M345. Among the tetracyclic thioxanthene derivatives, TX2.1a emerged as a hit compound with the lowest GI₅₀ in the tested cell lines, which led us to further investigate whether it harbored antimitotic properties. We observed that TX2.1a did not interfere with mitosis progression. Yet, we found that it exhibited fluorescence activity, making it a potential theranostic drug candidate. Relatively to the diarylpentanoid BP-M345, we found that it exhibited a potent antitumor activity. We further unveil that it exerted its cytotoxic activity acting as an antimitotic agent. We showed that BP-M345 induced persistent chromosome congression defects which led to chronic activation of spindle assembly checkpoint resulting in mitotic arrest that culminated into cell death by apoptosis, mostly in mitosis.

The work presented contributed to the development of new strategies to target mitosis for cancer therapy by improving the efficacy of the SGA BI2536 combining it with the apoptosis potentiator Navitoclax, and by providing a new antimitotic agent, the BP-M345, with promising antitumor activity.

Resumo

Devido à proliferação descontrolada das células tumorais, foram desenvolvidos ao longo dos últimos anos vários fármacos anticancerígenos que visam bloquear a progressão da mitose. Estes agentes antimetabólicos dependem da ativação crónica do *checkpoint* mitótico para induzir a paragem das células em mitose, o que leva à ativação do programa de morte celular (apoptose). Os antimetabólicos clássicos, têm sido amplamente utilizados na terapia de vários tipos de cancro, no entanto devido a limitações da sua eficácia e a mecanismos de resistência desenvolvidos pelas células tumorais, novos antimetabólicos foram desenvolvidos. Coletivamente, estas moléculas são designadas de antimetabólicos de segunda geração (SGA), e ao contrário dos antimetabólicos clássicos que têm como alvo as subunidades β -tubulina, estas moléculas visam inibir proteínas que desempenham papéis cruciais em diferentes processos durante a mitose, nomeadamente as cinases Mps1, Plk1, Aurora A e B e as proteínas motoras Eg-5 e CENP-E. No entanto, os SGAs demonstraram uma fraca eficácia em ensaios clínicos. No primeiro objetivo deste estudo, propusemo-nos a desenvolver uma estratégia para melhorar a eficácia do SGA BI2536, um inibidor da Plk1, através da sua combinação com Navitoclax, um composto potenciador da apoptose. A eficácia desta combinação foi analisada na linha celular de cancro do pulmão de não pequenas células, A549. Na análise do destino celular após tratamento com dois compostos, observamos que após paragem das células em mitose, provocado pelo BI2536, o Navitoclax acelerou a morte celular destas células. Como consequência, diminuiu o risco de as células saírem prematuramente da mitose sem se dividir, um fenómeno conhecido como *slippage*, que é apontado como um dos motivos para o insucesso terapêutico dos SGAs. Adicionalmente, verificámos também que o BI2536 e Navitoclax apresentaram uma ação sinérgica na indução da morte celular por apoptose.

No segundo objetivo deste estudo focamo-nos em identificar novas moléculas com atividade antimetabólica. Um total de quatro moléculas foram analisadas: três derivados de tioxanteno, denominados de TX2.1a, TX2.17 e TX2.19, e um diarilpentanóide designado de BP-M345. Entre os tioxantenos, o composto TX2.1a emergiu como a molécula com atividade antitumoral mais promissora apresentando o GI_{50} mais baixo nas linhagens celulares testadas, o que nos levou a investigar se ele possuía atividade antimetabólica. Verificámos que o composto TX2.1a não interferia com a progressão da mitose, no entanto, observámos que o composto TX2.1a apresentava atividade fluorescente, o que em conjunto com a sua atividade antitumoral, torna-o um potencial candidato a fármaco teranóstico. Em relação ao diarilpentanóide BP-M345, descobrimos que exercia uma potente atividade antitumoral. Verificámos ainda que

exerceu a sua atividade citotóxica atuando como agente antimitótico. Mostramos que BP-M345 induziu defeitos no alinhamento dos cromossomas que levou à ativação crónica do *checkpoint* mitótico, resultando na paragem das células em mitose culminando na morte celular por apoptose, maioritariamente durante a mitose.

Globalmente, este trabalho contribuiu para o desenvolvimento de novas estratégias anticancerígenas que visam impedir a progressão da mitose, melhorando a eficácia do SGA BI2536 combinando-o com o potenciador de apoptose Navitoclax, e descobrindo um novo agente antimitótico, o BP-M345, que demonstrou uma atividade antitumoral promissora.

Keywords: antimitotics, spindle assembly checkpoint, anticancer therapy, BH3-mimetics, mitosis, apoptosis, diarylpentanoid, tetracyclic thioxanthene

Contents

Acknowledgements/Agradecimentos	i
Abstract	iii
Resumo	iv
Contents	vi
List of Tables	ix
List of Figures	x
List of Abbreviations	xi
Thesis Outline	xv
Chapter I	1
General Introduction	1
1. Cancer overview	2
2. Cell cycle	4
2.1 Overview	4
2.2 Regulation and control	5
3. Mitosis	8
3.1 General description	8
3.2 Chromosome bi-orientation and congression	9
3.3 Spindle assembly checkpoint	12
4. Apoptosis	14
4.1 Pathways	15
4.2 Targeting apoptosis for cancer therapy: BH3 mimetics	17
5. Targeting mitosis for cancer therapy	19
5.1 Classical antimitotic drugs	19
5.2 Second-generation antimitotics	20
Chapter II:	26
Motivations and aims	26
Chapter III	32
Targeting the anti-apoptotic pathway sensitizes lung cancer cells to Plk1 inhibition	32
1. Introduction	33

2. Material and methods	33
2.1. Compounds	33
2.2. Cell lines and culture conditions	33
2.3 RNA isolation and quantitative real-time PCR	34
2.4 Mitotic index determination	34
2.5 Flow cytometry analysis	34
2.6 TUNEL assay	35
2.7 Live-Cell Imaging	35
2.8 Statistical analysis	36
3. Results	36
3.1 Plk1 is overexpressed in non-small cell lung cancer cell lines	36
3.2 PLK1 inhibition by BI2536 arrests cells in mitosis	37
3.3 Inhibition of an anti-apoptotic pathway by Navitoclax accelerates death of cells arrested in mitosis by BI2536	38
3.4 The combination of Navitoclax and BI2536 enhances cancer cell death over individual treatments	40
4. Discussion	43
Chapter IV	44
Identifying new antimitotics	44
GROUP I	44
Tetracyclic thioxanthene derivatives: Study on fluorescence and antitumor activity	44
1. Introduction	45
2. Material and methods	45
2.1 Compounds	45
2.2 Cell lines and culture conditions	45
2.3 Sulforhodamine B (SRB) assay	46
2.4 Phase contrast microscopy	46
2.5 Fluorescence microscopy	46
2.6 Flow Cytometry Analysis	47
3. Results	47
3.1 Tetracyclic thioxanthenes exhibit tumor growth inhibitory activity	47
3.2 Tetracyclic thioxanthene TX2.1a does not interfere with mitosis	48
3.3 Tetracyclic thioxanthene TX2.1a exhibit fluorescence activity	49
4. Discussion	50

GROUP II	52
The diarylpentanoid BP-M345 is a potent antitumor agent with antimitotic activity	52
1. Introduction	53
2. Material and Methods	53
2.1 Compound	53
2.2 Cell lines and culture conditions	53
2.3 Sulforhodamine B (SRB) assay	53
2.4 Mitotic index determination	54
2.5 Flow Cytometry	54
2.6 Indirect Immunofluorescence	55
2.6.1 MG-132 Proteasome Inhibitor Assay	55
2.6.2 Cold treatment assay	55
2.7 Colony Formation Assay	55
2.8 Live-Cell Imaging	56
2.9 Image Acquisition and Processing	56
2.10 Statistical Analysis	56
3. Results	57
3.1 BP-M345 exhibits a potent tumor anti-growth activity	57
3.2 BP-M345 arrests tumor cells in mitosis	57
3.3. BP-M345 disturbs mitotic spindle morphology and harms chromosome congression	59
3.4 BP-M345 interferes with the stability of kinetochore-microtubule attachments and promotes SAC activation	60
3.5 Fate of BP-M345-treated cells arrested in mitosis	62
3.6 BP-M345 impairs long-term tumor cell proliferation	65
4. Discussion	66
Chapter V	67
General Discussion and Future Perspectives	67
Chapter VI	71
References	71
Appendix	86

List of Tables

Table 1- IC ₅₀ values of BI2526 and Navitoclax in A549 cells after 48 of exposure	29
Table 2- Cell growth inhibitory activity of tetracyclic thioxanthenes	47
Table 3- Selectivity index of tetracyclic thioxanthenes	48
Table 4- Tumor cell growth inhibitory activity of compound BP-M345	57
Table 5- Selectivity index of compound BP-M345	57

List of Figures

Fig. 1- Representation of the eukaryotic cell cycle. step of the cell cycle. _____	5
Fig.2- Cell cycle regulation by Cdk-cyclin complexes. _____	7
Fig. 3- Representative immunofluorescence images showing the cell morphology at each mitotic phase. _____	9
Fig. 4- Forces that drive chromosome congression and kinetochore-microtubule mis-attachments. _____	11
Fig. 5- Spindle assembly checkpoint. _____	14
Fig. 6- Apoptotic pathways. _____	17
Fig. 7- Cell fates after treatment with MTAs. _____	20
Fig. 8- Targeting mitosis for cancer treatment. _____	25
Fig. 9- Model explaining cell fate variation after treatment with antimitotic agents. ____	27
Fig. 10- Structure of Plk1 inhibitor BI2536 and the BH3-mimetic Navitoclax. _____	28
Fig. 11- Structure of tetracyclic thioxanthenes TX2.1a, TX2.17, and TX2.19. _____	30
Fig. 12- Structure of the diarylpentanoid BP-M345. _____	30
Fig. 13- Plk1 is overexpressed in NSCLC cell lines. _____	37
Fig. 14- Treatment with BI2536 at low concentration induces mitotic arrest. _____	38
Fig. 15- Navitoclax enhances cell death in mitosis after mitotic arrest induced by BI2536. _____	40
Fig. 16- Combination of Navitoclax and BI2536 potentiates cancer cell death by apoptosis. _____	43
Fig. 17- TX2.1a does not interfere with mitosis. _____	49
Fig. 18- Cancer cells treated with TX2.1a emitted fluorescence in the green channel. 50	
Fig. 19- Treatment with BP-M345 induces mitotic arrest of tumor cells. _____	59
Fig. 20- BP-M345 disturbs mitotic spindle morphology. _____	60
Fig. 21- BP-M345 interferes with the stability of kinetochore-microtubule attachments. _____	61
Fig. 22- Treatment with BP-M345 leads to spindle assembly checkpoint activation. _	62
Fig. 23- BP-M345 promotes tumor cell death, mostly in mitosis. _____	63
Fig. 24- Tumor cells treated with BP-M345 undergo apoptotic cell death. _____	64
Fig. 25- BP-M345 inhibits long-term tumor cell proliferation. _____	65

List of Abbreviations

°C — Degree Celsius

A375-C5 — Human Malignant Melanoma IL-1 Insensitive

A549 — Human Lung Adenocarcinoma Epithelial Cells

AAS — Alive After Slippage

aMT — Astral Microtubule

APAF1 — Apoptotic Peptidase Activating Factor 1

APC/C — Anaphase Promoting Complex/Cyclosome

ATM— Ataxia-telangiectasia Mutated

ATP — Adenosine Triphosphate

ATR— Telangiectasia Mutated and Rad3 Related

BAD — BCL2-Associated Agonist of Cell Death

BCL-2 — B-Cell Lymphoma 2

BCL-W — BCL-2-like Protein 2

BCL-XL — B-cell Lymphoma Extra Large

BCR-ABL — Breakpoint Cluster Region-Abelson

BFL1 — BCL-2-related Protein A 1

BH — BCL-2 Homology Domains

BID — BH3 Interacting-domain Death Agonist

BIM — BCL-2-like Protein 11

BMF — BCL-2-modifying Factor

BNIP3 — BCL2/Adenovirus E1B 19 kDa Protein-interacting Protein 3

Bub1 — Budding Uninhibited by Benomyl 1

Bub3 — Budding Uninhibited by Benomyl 3

BubR1 — Bub1-related 1

c-FLIP — FLICE-like Inhibitory Protein

Cdc20 — Cell Division Cycle 20

Cdk — Cyclin-dependent Kinase

cDNA — Complementary DNA

CENP-E — Centromeric Protein E

CIP/KIP — Cyclin-dependent Kinase-interacting Protein/Kinase Inhibitory Proteins

CKI — Cyclin-dependent Kinase Inhibitors

cm² — Square Centimeter

CO₂ — Carbon Dioxide

CREST — Raynaud's Phenomenon, Esophageal Dysmotility, Sclerodactyly, and Telangiectasias

DAPI — 4',6-diamidino-2-phenylindole

DIC — Differential Interference Contrast

DiM — Death in Mitosis

DISC — Death-inducing Signaling Complex

DMEM — Dulbecco's Modified Eagle's Medium

DMSO — Dimethyl Sulfoxide

DNA — Deoxyribonucleic Acid

E2F — E2 Factor

FADD — Fas-associated Protein with Death Domain

FasR — Fas Receptor

FBS — Fetal Bovine Serum

FITC — Fluorescein Isothiocyanate

G0 — Gap 0 phase

G1 — Gap 1 phase

G2 — Gap 2 phase

GAPDH — Glyceraldehyde 3-phosphate dehydrogenase

GI₅₀ — Half-maximal Growth Inhibition

h — Hours

HCT116 — Colorectal Carcinoma Cells

HPAEpiC — Normal Pulmonary Alveolar Epithelial Cells

HRK — BCL2 interacting protein

IAP — Inhibitor of Apoptosis

IC₅₀ — Half-maximal Inhibitory Concentration

ICAD — Caspase-activated DNase Inhibitor

iMT — Interpolar Microtubule

INK4 — Inhibitor of Cyclin-dependent Kinase 4

K-fiber — Kinetochore Fiber

kMT — Kinetochore Microtubule

Mad1 — Mitotic Arrest Deficient 1

Mad2 — Mitotic Arrest Deficient 2

MCC — Mitotic Checkpoint Complex

MCF-7 — Human Breast Cancer Cell Line

MCL1 — Induced Myeloid Leukemia Cell Differentiation Protein

MDM2 — Mouse Double Minute 2

MELT — Methionine-Glutamate-Leucine-Threonine Motif

MI — Mitotic Index

min — Minutes

mL — Milliliters

mM — Milimolar

MOMP — Mitochondrial Outer Membrane Permeabilization

MPF— Mitosis-promoting Factor

Mps1 — Monopolar Spindle 1

mRNA — Messenger RNA

MTA — Microtubule-targeting Agent

MTOC — Microtubule-organizer Center

NCI-H460 — Non-small Cell Lung Cancer Cells

NEBD — Nuclear Envelope Breakdown

nm — Nanometer

nM — Nanomolar

NOXA — Phorbol-12-Myristate-13-Acetate-Induced Protein 1

NSCLC — Non-small Cell Lung Cancer

OMM — Outer Mitochondrial Membrane

PBS — Phosphate-buffered Saline

PCM — Pericentriolar Material

PI — Propidium Iodide

Plk1 — Polo-like Kinase 1

PMD — Post mitotic Death

PP1 — Protein Phosphatase 1

PP2A-B56 — Protein Phosphatase 2A-B56 isoform

PSD — Post Slippage Death

PUMA — p53 Upregulated Modulator of Apoptosis

qRT-PCR — Quantitative Real Time-Polymerase Chain Reaction

RB — Retinoblastoma

RNA — Ribonucleic Acid

RPMI — Roswell Park Memorial Institute

RZZ — Rod-Zwilch-Zw10

S — Synthesis Phase

SAC — Spindle Assembly Checkpoint

SD — Standard Deviation

sec — Seconds

SGA — Second-generation Antimitotic

siRNA — Small Interfering Ribonucleic Acid

SMAC — Second Mitochondrial Activator of Caspases

SRB — Sulforhodamine B

tBID — Truncated BID

TdT — Deoxynucleotidyl Transferase

TNF — Tumor Necrosis Factor

TNFR1 — TNF Receptor 1

TRAILR — TNF-related Apoptosis-inducing Ligand Receptor

TRIP13 — Thyroid Hormone Receptor Interactor 13

TUNEL — Terminal Deoxynucleotidyl Transferase-mediated Nick end Labeling

γ -TuRC — γ -Tubulin Ring Complex

μ g — Microgram

μ m — Micrometer

μ M — Micromolar

The work presented in this thesis was conducted at:

Cancer Research Group

IINFACTS – Institute of Research and Advanced Training in Health Sciences and
Technologies | iinfacts.cespu.pt

CESPU – Cooperativa de Ensino Superior e Universitário

Rua Central de Gandra, 1317

4585-116 Gandra PRD, Portugal



FINANCIAL SUPPORT

This work was funded by CESPU - Cooperativa de Ensino Superior Politécnico e
Universitário Crl [grant number AntiMitoSphere_APSFCT_IINFACTS_2021 and
ActivCHIRAL_PI2RL_IINFACTS_2021].

Thesis Outline

This thesis is organized into **six chapters** and **one appendix**

Chapter I provides a general introduction of the current literature. An overview of the cell cycle, its regulation and control with particular emphasis in mitosis. A brief review of apoptosis pathways and of the clinical approach to target apoptosis for cancer therapy focusing on BH3-mimetic molecules. Lastly, targeting mitosis for cancer therapy was focused, providing an overview of the current approaches and the second-generation antimitotics.

Chapter II describes the motivation and aims of this project as well as its specific objectives.

Chapters III and IV are related to the experimental work and are structured according to two main aims: improving the efficacy of the second-generation antimitotic BI2536 (**Chapter III**) and identifying new molecules with antimitotic properties (**Chapter IV**), which is divided into two groups, tetracyclic thioxanthene derivatives: study on fluorescence and antitumor activity (**Group I**) and the diarylpentanoid BP-M345 is a potent antitumor agent with antimitotic activity (**Group II**).

Chapter V provides overall conclusions and perspectives for future research.

Chapter VI comprises a list of all cited references is available.

Appendix contains the legends of the movies that were annexed to this thesis.

The information presented in the chapters I, III and IV was published in international peer review scientific journals and presented or accepted for presentation at congresses, according to the following:

Novais, P., Silva, P., Amorim, I., & Bousbaa, H. (2021). Second-Generation Antimitotics in Cancer Clinical Trials. *Pharmaceutics*, 13(7), 1011. <https://doi.org/10.3390/pharmaceutics13071011> (published paper)

Durães F, Silva PMA, **Novais P**, Amorim I, Gales L, Esteves CIC, Guieu S, Bousbaa H, Pinto M, Sousa E. Tetracyclic Thioxanthene Derivatives: Studies on Fluorescence and Antitumor Activity. *Molecules*. 2021 May 31;26(11):3315. doi: 10.3390/molecules26113315. PMID: 34073048; PMCID: PMC8198043. (published paper)

F. Durães, P. M. A. Silva, **P. Novais**, C. I. C. Esteves, S. Guieu, H. Bousbaa, M. Pinto, E. Sousa. Antitumor activity and fluorescence of tetracyclic thioxanthenes: new theranostic agents?. 26th EFMC International Symposium in Medicinal Chemistry, August 29 - September 2, 2021 (published poster)

Pedro Novais, Joana Moreira, Patrícia M. A. Silva, Isabel Amorim, Madalena Pinto, Honorina Cidade, and Hassan Bousbaa. Explorando a atividade antitumoral do composto BP-M345, in 4ª Reunião Internacional Rede Académica das Ciências da Saúde da Lusofonia (RACS). (accepted for oral communication in November 2021)

Chapter I

General Introduction

The information presented in this section was partially based in the following published paper:

Novais, P., Silva, P., Amorim, I., & Bousbaa, H. (2021). Second-Generation Antimitotics in Cancer Clinical Trials. *Pharmaceutics*, 13(7), 1011.
<https://doi.org/10.3390/pharmaceutics13071011>

1. Cancer overview

Cancer comprises a group of diseases that can start in any organ or tissue and consists of abnormal cells which proliferate uncontrollably and can invade adjoining tissues and spread to other organs. Despite the increased knowledge of cancer biology and the huge advances in cancer therapy, its incidence and mortality are rapidly growing worldwide. According to World Health Organization, there were approximately 19.2 million new cancer cases and 9.9 million deaths during 2020 (*Global Cancer Observatory*, 2020). Thereby, understanding the molecular mechanisms of tumorigenesis and cancer progression is extremely important to develop new anti-cancer therapies and to improve the existing ones.

Tumorigenesis is a stepwise process in which normal cells suffer consecutive genetic and/or epigenetic alterations that gradually transforms them into tumor cells. The successive accumulation of these alterations originates tumor cells with increasingly aggressive phenotypes driving tumor progression. There are two groups of genes that are frequently mutated in tumor cells and are the main contributors to cell transformation: proto-oncogenes and tumor suppressors genes. Proto-oncogenes are normal genes that when mutated (gain-of-function mutations) or with their expression altered become oncogenes and stimulate cell proliferation and survival (Lee & Muller, 2010). On the other hand, tumor suppressor genes prevent tumor development and are usually divided into gatekeepers that regulate cell growth by either inhibiting cell cycle progression or inducing apoptosis, and caretakers that are involved in the maintenance of genome integrity (Fanale et al., 2017). Mutations on these genes usually lead to its loss of function allowing tumor progression (Lee & Muller, 2010).

The remarkable progress of cancer biology research allowed the establishment of several tumor cell characteristics that are acquired during the transformation process, known as hallmarks of cancer (Hanahan & Weinberg, 2011). Genomic instability is one of the features observed in tumor cells and is related to the loss of function of caretaker genes which leads to the increase of mutation rate. Since tumorigenesis depends in large part on successive alterations in genomes, genomic instability is considered an enabling characteristic that is causally associated with the acquisition of all other hallmarks (Hanahan & Weinberg, 2011). To guarantee a high proliferation rate, tumor cells sustain the proliferative signaling through different mechanisms like synthesizing growth factors to which they are responsive, or through mutations on transcellular and intracellular traducers of growth pathways (Hanahan & Weinberg, 2011). Furthermore, tumor cells can also evade antigrowth suppressors, like retinoblastoma (Rb) (described below)

permitting persistent cell proliferation (Hanahan & Weinberg, 2011; Schaal et al., 2014). Another important feature of tumor cells that ensures tumor growth is their limitless replicative potential, which has been associated with the reactivation of telomerase in tumor cells (Bernardes de Jesus & Blasco, 2013; Hanahan & Weinberg, 2011). Additionally, tumor cells have the ability to escape apoptosis by several mechanisms like disrupting the balance between pro-apoptotic and anti-apoptotic proteins, reducing caspase function, or impairing the function of p53 (Pistritto et al., 2016). Another feature of tumor cells is that conversely to normal cells, which under aerobic conditions produce most of ATP through oxidative phosphorylation, tumor cells favored glycolysis to generate energy even in presence of oxygen. This phenomenon is known as the Warburg effect or aerobic glycolysis (Vaupel et al., 2019). Importantly, the tumor microenvironment encompasses different cell types besides tumor cells, like endothelial and inflammatory immune cells, as well as cancer-associated fibroblasts, that establish complex interactions with each other and with tumor cells to promote tumor progression (Hanahan & Weinberg, 2011). For instance, immune cells by producing several biomolecules like growth, survival, and proangiogenic factors, or extracellular matrix-modifying enzymes, generate a more tumor-permissive microenvironment and facilitates angiogenesis, invasion, and metastasis (Greten & Grivnickov, 2019; Hanahan & Weinberg, 2011). Furthermore, tumor cells can escape destruction either from innate or adaptative immune cells. All these characteristics cooperate with each other to drive tumor progression.

This increased knowledge of cancer biology has been allowing the development of new therapies targeting specific proteins that are mis-regulated in tumor cells through small molecule inhibitors, antibodies, and siRNAs (Baudino, 2015; Hanahan & Weinberg, 2011; Singh et al., 2018). Targeted therapies are more precise than classic chemotherapeutic agents and generally display fewer side effects. One of the most successful examples of targeted therapy is imatinib a small inhibitor of the abnormal kinase BCR-ABL which is responsible for promoting chronic myeloid leukemia (Claudiani & Apperley, 2018). Even so, classic chemotherapeutic agents continue to be widely used in the clinic. Classic chemotherapeutic drugs target rapidly dividing cells like tumor cells. However, they also affect other cells with high rates of division such as cells from bone marrow, the digestive tract, and hair follicles leading to their side effects (Baudino, 2015). Most of the chemotherapeutic drugs induce DNA damage or impair its synthesizes, thereby preventing cell division and inducing cell death. Other drugs, known as antimitotics, block the cell cycle at mitosis which leads to cell death by apoptosis.

2. Cell cycle

The cell cycle is a tightly regulated process in which a parental cell gives rise to two genetically identical daughter cells. It is an essential process to the maintenance of adult tissues allowing cell renewal. The eukaryotic cell cycle is divided into two main phases: the interphase and the M phase (Figure 1). The interphase is a time of synthesis and growth which represents the largest phase of the cycle contemplating the consecutive phases Gap 1 (G1), synthesis phase (S), and Gap 2 (G2). The M phase is the final step of the cell cycle during which the chromosomes are equally segregated (mitosis), followed by cytoplasmic division (cytokinesis) (Schafer, 1998).

2.1 Overview

During G1 the cell prepares for the next division. It is marked by a pronounced biosynthetic activity, primarily of proteins required for the following S phase, as well as the synthesis of other macromolecules and organelles, increasing the cell size. In late G1, the cell decides whether to commit or not to a new cycle based on nutrients and growth factors supply to overcome the major cell cycle control, known as restriction point. The restriction point is primarily regulated by extracellular growth factors and determinates the fate of the cell. If the amount of growth factors is satisfactory, the cell overcomes the restriction point and irreversibly commits to a new division. On the other hand, in the absence of a proper growth factor stimulus, the cell is delayed in G1 or returns to the quiescent state G0, a non-proliferating state but metabolically active (Figure 1). Some cells remain in G0 for their entire lifespan, while others can reenter the cell cycle in response to proper stimulus and continue dividing. During the S phase, the DNA is replicated, and each sister chromatid remains linked to each other by cohesin complexes. The centrosome is also duplicated in the S phase to assemble the mitotic spindle during the early stages of mitosis. During G2 the cell continues to grow and synthesize macromolecules in preparation for mitosis. In the M phase, the genetic material and the cytoplasm are divided, rising two daughter cells (Schafer, 1998).

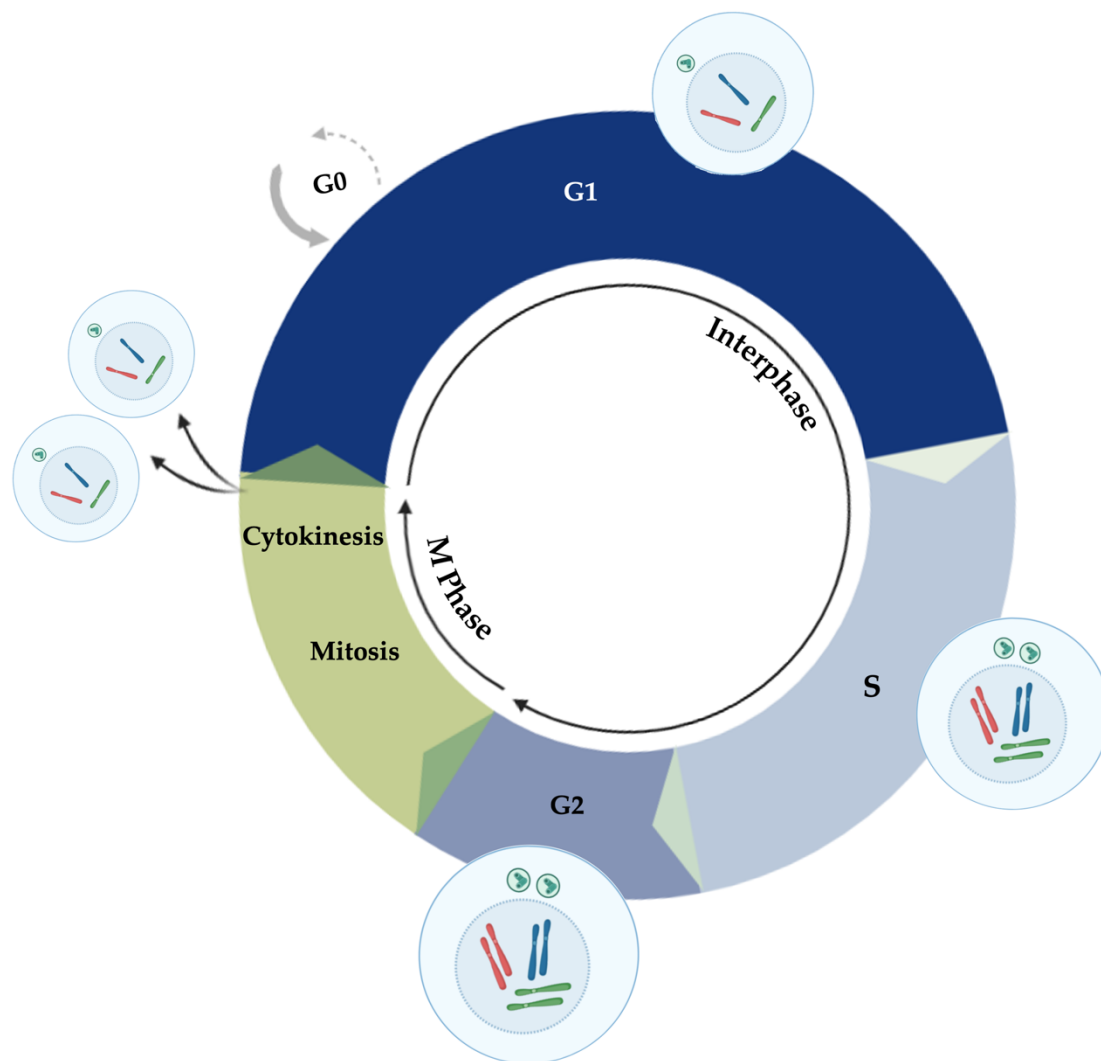


Fig. 1- Representation of the eukaryotic cell cycle. Interphase encompasses three phases G1, S, and G2. G1 is marked by cell growth and high biosynthetic activity; During the S phase the DNA and centrosome are replicated; At G2 the cell continues to grow and synthesizes macromolecules preparing itself for mitosis. Cells in the absence of a proper growth factor stimulus can exit the cycle, becoming quiescent, a non-proliferating state known as Gap 0 (G0). The M phase comprises mitosis when chromosomes are segregated and cytokinesis which represents the division of the cytoplasm and the final step of the cell cycle. Created in BioRender.

2.2 Regulation and control

The regulation of the cell cycle depends on the crosstalk between several proteins that control the timing of each event and ensure the unidirectionality of the cell cycle preventing for instance that the cell division occurs before the duplication of DNA. In the center of this orchestrated network are a family of serine/threonine kinases known as Cyclin-dependent kinases (Cdks 1, 2, 4, and 6) and its regulatory partners called

cyclins (A, B, D, and E) (Roskoski, 2019). Cdks phosphorylate several proteins essential for cell cycle progression, changing its activity or its interaction with other proteins. The concentration levels of Cdks are roughly constant throughout the cell cycle, and its activation primarily depends on binding to cyclins. In turn, cyclins are synthesized and degraded according to the cell cycle stage, resulting in the formation of specific Cdk-cyclin complexes at different phases (Figure 2). Cdks could be inhibited by some proteins, known as Cdk inhibitors (CKIs), namely the INK4 family (p16, p15, p18, and p19) which inhibit the complexes Cdk4/6-cyclin D, and the CIP/KIP family (p21, p27, and p57) capable of inhibiting all Cdks-cyclin complexes (Figure 2) (L. Ding et al., 2020).

Given the oscillatory levels of cyclin concentration throughout the cell cycle, they could be classified into four classes: G1 cyclins (D); G1/S cyclins (E); S cyclins (A); and G2/M cyclins (A and B). The control of cyclin levels relies on cyclin gene expression and its degradation by the ubiquitin-proteolytic system. Transcription of cyclin D starts in early G1 upon growth factor stimulus, allowing Cdk4/6-cyclin D activation. The activated Cdk4/6-cyclin D complexes phosphorylate the protein Rb which is the key component of the network that controls the restriction point (Blagosklonny & Pardee, 2002) (Figure 2). Briefly, the Rb when unphosphorylated binds to the transcriptional factor E2F which is responsible to activate the transcription of many genes whose products are essential for the following S phase, including cyclin E. Phosphorylation of Rb by Cdk4/6-cyclin D complex releases E2F which starts to induce cyclin E transcription. In turn, cyclin E binds to Cdk2 and this complex collaborates with Cdk4/6-cyclin D to complete Rb phosphorylation allowing the cell to overcome the restriction point and enter in the S phase (Blagosklonny & Pardee, 2002). Cyclin A levels increase at the onset of S phase and decline in early mitosis. Initially, cyclin A binds to Cdk2 to promote DNA synthesis and completion of S phase. Later in G2, cyclin A binds to Cdk1 and this complex cooperates with Cdk1-cyclin B to induced the mitotic entry (Johnson & Walker, 1999). Cyclin B levels peak at late G2 and its association with Cdk1 forms the major force that drives mitotic entry, known as Mitosis-Promoting Factor (MPF). Later in mitosis, cyclin B is degraded triggering mitotic exit (described below).

To ensure that the genetic material is equally segregated to the two daughter cells and with no damaged DNA, there are control mechanisms throughout the cell cycle called checkpoints (Figure 2). The G1/S, intra-S, and G2/M checkpoints respond to damage in DNA and delay the cell cycle until the errors are corrected. The central players of DNA damaged response are the kinases ATM and ATR that play as DNA damage sensors (Smith et al., 2020). Both induce the phosphorylation of the tumor suppressor

p53, reducing the affinity to its negative regulator, MDM2, a ubiquitin ligase that targets p53 for degradation. P53 plays an important role in DNA damage checkpoints since it induces the transcription of several genes including the CKI p21 and genes involved in DNA damage repair (Roos & Kaina, 2013; Smith et al., 2020). If DNA damage cannot be repaired, p53 is also capable of triggering apoptosis or cell senescence. Additionally, there is also a checkpoint during mitosis, known as spindle assembly checkpoint (SAC) which ensures that the chromosomes are equally segregated to the daughter cells (described below).

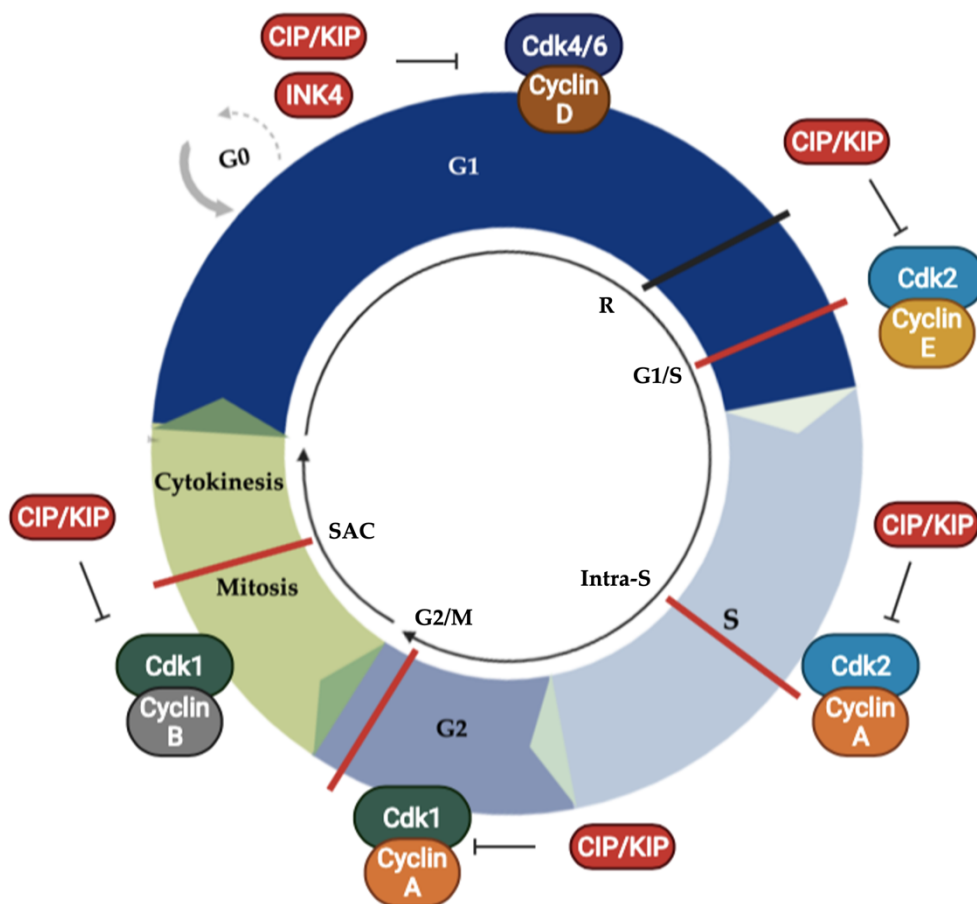


Fig.2- Cell cycle regulation by Cdk-cyclin complexes. Cdk4/6-cyclin D and Cdk2-cyclin E cooperate to overcome the restriction point (R). Cdk2-cyclin A promotes DNA synthesis, and it is required for S phase completion. Cdk2-cyclin A facilitates G2/M transition together with Cdk1-cyclin A which is the major force that drives mitotic entry. During interphase, the DNA damage checkpoints (G1/S, Intra-S, and G2/M) promote cell cycle arrest until the damaged DNA is repaired. In mitosis, the spindle assembly checkpoint (SAC) ensures the fidelity of chromosome segregation. The Cdk inhibitors (CKI) inhibit Cdk-cyclin complexes to delay the cell cycle. CKI comprises two families: INK4 (p16, p15, p18, and p19) which inhibit the complexes Cdk4/6-cyclin D, and the CIP/KIP family (p21, p27, and p57) capable of inhibit all Cdk-cyclin complexes. Created in BioRender.

3. Mitosis

Mitosis is the phase of the cell cycle when the genetic material is divided and it is followed by the division of the cytoplasm (cytokinesis), giving rise two independent daughter cells. Mitosis is a highly dynamic cell cycle phase during which the cells pass through several structural changes. Based on these structural changes, mitosis is divided into five sequential phases: prophase, prometaphase, metaphase, anaphase, and telophase (Figure 3).

3.1 General description

At the onset of prophase, the filaments of chromosomal DNA start to condense, and the centrosomes, the main microtubule-organizer center (MTOC) of animal cells, separate and start to migrate towards the opposite sites of the cell to initiate the bipolar microtubule array for the mitotic spindle assembly (Figure 3). The beginning of prometaphase is marked by the nuclear envelope breakdown (NEBD). At this phase, the chromosomes become accessible to the spindle microtubules allowing their dynamic interaction. Microtubules interact with chromosomes by attaching to kinetochores, a specialized protein complex localized on centromeric DNA of each sister chromatid, and then start to align at the spindle equator, a process known as chromosome congression. The metaphase is achieved when all chromosomes are bipolarly attached to spindle microtubules and aligned at metaphase plate (McIntosh et al., 2012). At this phase, the SAC which ensures the fidelity of chromosome segregation is turned off, allowing the transition to anaphase. During anaphase, the two sister chromatids are segregated (Figure 3). Anaphase is commonly described in two phases: anaphase A and anaphase B. Anaphase A is promoted by the shrinkage of microtubules that are attached to kinetochores (kinetochore microtubules), while anaphase B is driven by the elongation of microtubules that rise from each pole and interact at the cell middle zone (interpolar microtubules) pushing the spindle poles to opposite sides (Vukušić et al., 2019). During telophase, the filaments of chromosomal DNA start to decondense, and the nuclear envelope reassembles and packages the chromosomes and the other nuclear components, forming two daughter nuclei. The interpolar microtubules and the *de novo* assembled microtubules mediated by augmin complexes build the central spindle which provides the template for midbody assembly, a target platform for abscission factors required at cytokinesis. At late telophase, an actin-myosin contractile ring attached to the cell membrane is assembled at the equatorial cortex. Following its assembly, the contraction of this ring, at cytokinesis, leads to ingression of the attached plasma

membrane, which divides the cytoplasm, emerging two daughter cells, a process known as abscission (Fededa & Gerlich, 2012) (Figure 3).

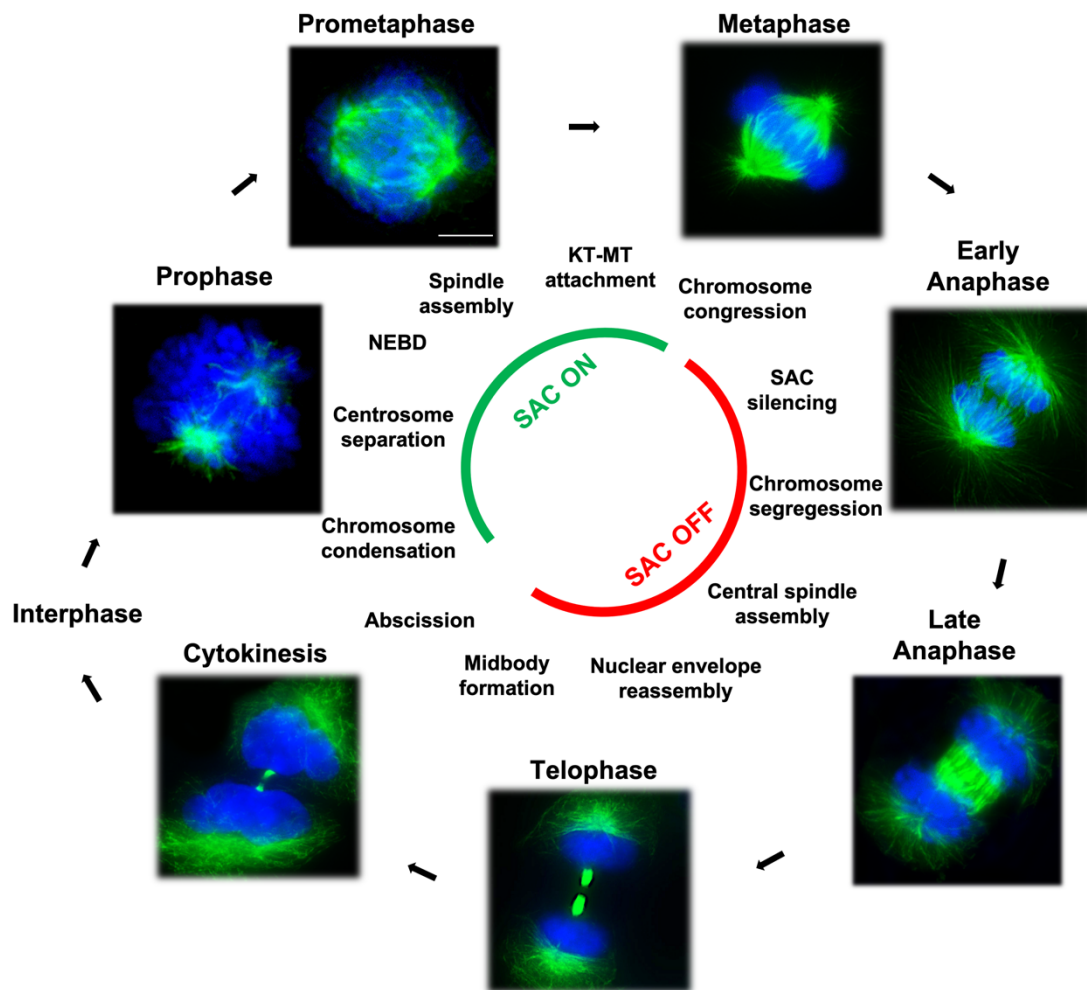


Fig. 3- Representative immunofluorescence images showing the cell morphology at each mitotic phase. Cells were stained with an anti- α -tubulin antibody to visualize spindle microtubules (green) and the DNA was stained with DAPI (blue). Description of the main cellular events during mitosis are shown. The progression throughout mitosis is monitored by the spindle assembly checkpoint activity (SAC ON and SAC OFF) that ensures the fidelity of chromosome segregation. Bar, 5 μ m.

3.2 Chromosome bi-orientation and congression

Microtubules are hollow cylindrical polymers formed by $\alpha\beta$ -tubulin heterodimers and are highly dynamic capable of polymerizing (growing) or depolymerizing (shrinking), a behavior known as dynamic instability. The transition of a polymerizing state to depolymerization is called catastrophe and the reverse is known as rescue. These processes occur in both microtubule ends however, the minus-end grows slowly and suffer catastrophe less frequently than the plus-end (Akhmanova & Steinmetz, 2015).

Microtubules rise from centrosomes which consists of a pair of centrioles surrounded by pericentriolar material (PCM). PCM is mainly composed by γ -tubulin, which assembles into γ -tubulin ring complexes (γ -TuRC) that serve as nucleation template for microtubule formation (Pimenta-Marques & Bettencourt-Dias, 2020). Based on the orientation of spindle microtubules plus-end, they are divided into three classes: astral microtubules (aMT) with the plus-end connecting the cell cortex; interpolar microtubules (iMT) which emanates from centrosomes toward the cell center connecting with the iMT from the other centrosome at the spindle midzone; and kinetochore microtubules (kMT) which attach kinetochores through their plus-end (Sharp et al., 2000). Furthermore, kinetochores attach to more than one kMT, creating a unique bundle known as kinetochore fiber (K-fiber).

Kinetochore-microtubule attachments are essential for chromosome congression and further segregation without errors. After NEBD, the chromosomes become accessible to microtubules which start with multiple cycles of shrinkage and growth in order to capture the dispersed chromosomes throughout kinetochores, a process known as “search and capture” (Kirschner & Mitchison, 1986). Initially, kinetochores interact with the lateral surface of microtubule (lateral attachment), which facilitates their capture by microtubules once the area of contact is much larger than the microtubule plus-end (K. Tanaka et al., 2005). The chromosomes positioned outside the spindle initially migrate to the spindle pole, driven by the minus end-directed motor protein dynein which slides along the aMTs (Barisic et al., 2014; C L Rieder & Alexander, 1990) (Figure 4). Once at spindle poles chromosomes start to move towards the cell equator promoted by the plus end-directed motor protein CENP-E, sliding either through iMT or K-fibers (Barisic et al., 2015; Kapoor et al., 2006) (Figure 4). Once within the spindle, the lateral attachment is converted to mature end-on attachment, in which the kinetochores attach to the microtubule plus-end (Cheeseman et al., 2006). When both sister kinetochores are end-on attached to K-fibers from opposite spindle poles, a condition known as amphitelic attachment, the chromosomes become bi-oriented. Once bi-oriented, diffuse gradients, that control K-fibers dynamics and length, ensure the complete alignment of chromosomes at the spindle equator (Auckland & McAnish, 2015; Maiato et al., 2017). Additionally, there is also a force on chromosome arms, known as polar ejection force, that eject the chromosome arms away from the spindle poles and is driven by the chromokinesins Kid and Kif4 (Wandke et al., 2012; Yajima et al., 2003) (Figure 4). All these forces operate in parallel to drive chromosome congression.

Amphitelic attachment (bi-orientation) is required for correct chromosome segregation at anaphase. However, other connections could occur namely monotelic, syntelic, and merotelic attachments. Monotelic attachment, which is frequently observed at the beginning of prometaphase and occurs when one sister kinetochore is unattached, and the other is attached to microtubules. The condition when both sister kinetochores are attached to microtubules from the same spindle pole is known as syntelic attachment. Merotelic attachment occurs when a sister kinetochore is simultaneously attached to microtubules from both spindle poles (T. U. Tanaka, 2010) (Figure 4). These errors are usually corrected by the kinase Aurora B. Aurora B, which is present at centromeres, acts as a tension sensor and destabilizes these mis-attachments by phosphorylating several components of kinetochores that mediate kinetochore-microtubule attachment. (D. Liu & Lampson, 2009; Welburn et al., 2010).

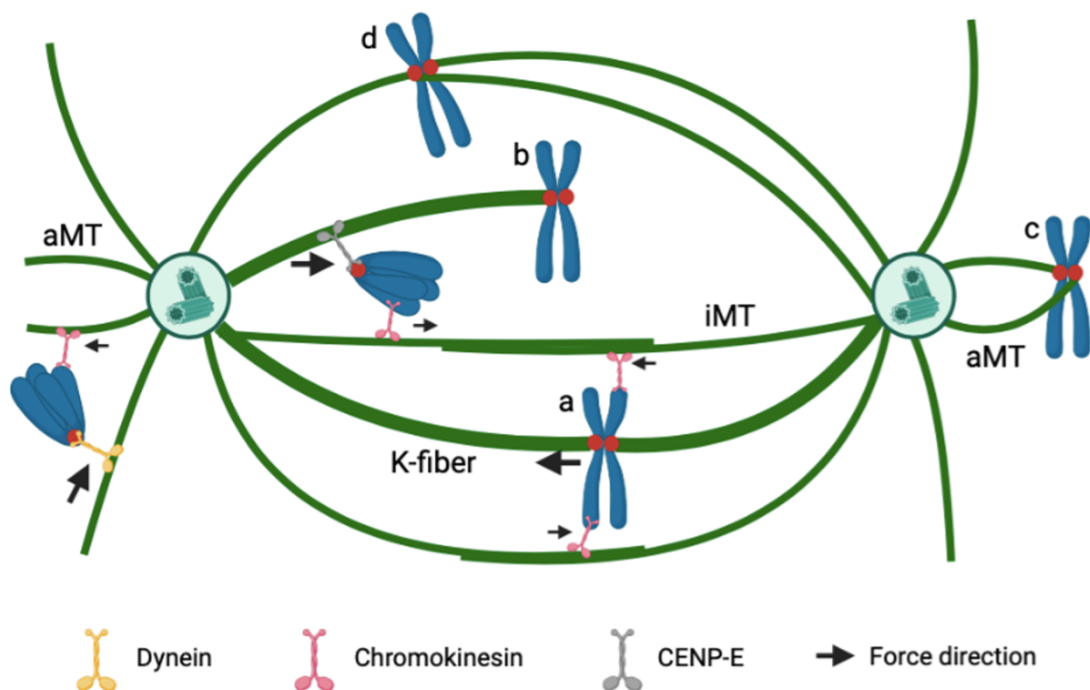


Fig. 4- Forces that drive chromosome congression and kinetochore-microtubule mis-attachments. Mitotic spindle encompasses three types of microtubules: astral (aMT), interpolar (iMT), and the kinetochore-fibers (K-fiber). The chromosomes laterally attached to microtubules and positioned outside the spindle are transported by dynein to the spindle poles, while chromosomes within the spindle are moved by CENP-E towards the spindle equator. Then, the lateral kinetochore-microtubule attachment is converted to end-on attachment in both sister kinetochores, resulting in chromosome bi-orientation. Once bi-orientated, the chromosomes align at the spindle equator moved by the pulling forces generated by the K-fibers. The chromokinesins apply a force on chromosome arms ejecting them away from the pole. Other kinetochore-microtubule attachments than amphitelic attachment (a) could occur: Monotelic (b), when one sister kinetochore is end-on attached and the other is unattached; Syntelic (c), when both kinetochores are attached to

microtubules from the same spindle pole; Merotelic (d), when the kinetochore (left) is simultaneously attached to microtubules from opposite spindle poles. Adapted from (Maiato et al., 2017).

3.3 Spindle assembly checkpoint

For a correct cell division, the genetic material must be equally segregated to the two daughter cells. As mentioned above, chromosome bi-orientation and alignment at the spindle equator are required for correct chromosome segregation. Therefore, the cell must delay the onset of anaphase until all chromosomes are aligned and correctly attached to K-fibers from opposite spindle poles, which is guaranteed by the spindle assembly checkpoint (SAC). The SAC operates like a surveillance mechanism that monitors kinetochore-microtubule attachments and acts through the assembly of the mitotic checkpoint complex (MCC) that ultimately inhibits the downstream SAC target, the anaphase promoting complex/cyclosome (APC/C) (Henriques et al., 2019) (Figure 5). A single unattached kinetochore is capable of activating the SAC signaling (Conly L Rieder et al., 1994, 1995).

The kinase Mps1 has been suggested to be the key regulator of SAC signaling (X. Liu & Winey, 2012b; Pachis & Kops, 2018; Stucke et al., 2002). Mps1 is recruited early in mitosis to unattached kinetochores, which is promoted by Aurora B and Cdk1-cyclin B (Hayward et al., 2019; Nijenhuis et al., 2013; Zhu et al., 2013). Once at kinetochores, Mps1 phosphorylates the outer kinetochore protein Kln1 at specific Methionine-Glutamate-Leucine-Threonine (MELT) motifs, generating docking sites for the complexes Bub1-Bub3 and Bub1-BubR1 (Primorac et al., 2013; Vleugel et al., 2013; Zhang et al., 2014). Further, phosphorylation of Bub1 by Mps1 establishes its interaction with Mad1, recruiting the complex Mad1-Mad2 to kinetochores (Ji et al., 2017; Moyle et al., 2014; Zhang et al., 2017). Mad1 can be also recruited to kinetochores in an alternative pathway by the RZZ complex (Allan et al., 2020). The protein Mad2 could adopt two conformations, open (O-Mad2) or closed (C-Mad2). When bound to Mad1, Mad2 adopts the closed conformation, forming the complex Mad1-C-Mad2. This complex catalyzes the conversion of novel O-Mad2 to C-Mad2, which directly binds to Cdc20, a co-activator of APC/C. Mps1 was also reported to promote the recruitment of O-Mad2 to Mad1-C-Mad2 complex. Furthermore, the phosphorylated Mad1, by Mps1, can directly interact with Cdc20 (Hewitt et al., 2010; Ji et al., 2017). The complex Cdc20-C-Mad2 further interacts with Bub3 and BubR1, completing the MCC assembly (Sudakin et al., 2001). Therefore, Mps1 facilitates the assembly of the MCC by promoting the spatial proximity of MCC subunits at unattached kinetochores. The MCC ultimately inhibits the

APC/C, a ubiquitin ligase, which is responsible to target securin and cyclin B for proteasomal degradation. Securin is the inhibitor of the protease separase which cleaves the cohesin complexes allowing the segregation of sister chromatids (anaphase onset). Cyclin B degradation, and consequent Cdk1 inactivation, leads to the mitotic exit. Thereby, inhibition of APC/C stabilizes securin and cyclin B, preventing the onset of anaphase and mitotic exit, respectively, until all kinetochores are correctly attached to microtubules. Once all chromosomes are bi-oriented and aligned at the spindle equator, the SAC must be silenced. SAC silence implies the interruption of MCC assembly and the disassembly of the existing ones. The motor protein dynein is responsible for the transport of SAC proteins from kinetochores towards the spindle pole, a process known as stripping, preventing the formation of more MCCs (Famulski et al., 2011; Silva et al., 2014). Furthermore, the phosphatases PP1 and PP2A-B56 counteracts the activity of the kinases Mps1 and Aurora B promoting the disassociation of the SAC proteins from kinetochores (Espert et al., 2014; Nijenhuis et al., 2014; Qian et al., 2017). The disassembly of existing MCCs is ensured by two proteins, the p31^{comet} which binds to Mad2, and the TRIP13, an AAA + family ATPase. These proteins cooperate to convert C-Mad2 to O-Mad2 which leads to MCC disassembly releasing the Cdc20 that binds and activates APC/C, which further ubiquitinates cyclin B and securin (M. Yang et al., 2007; Q. Ye et al., 2015).

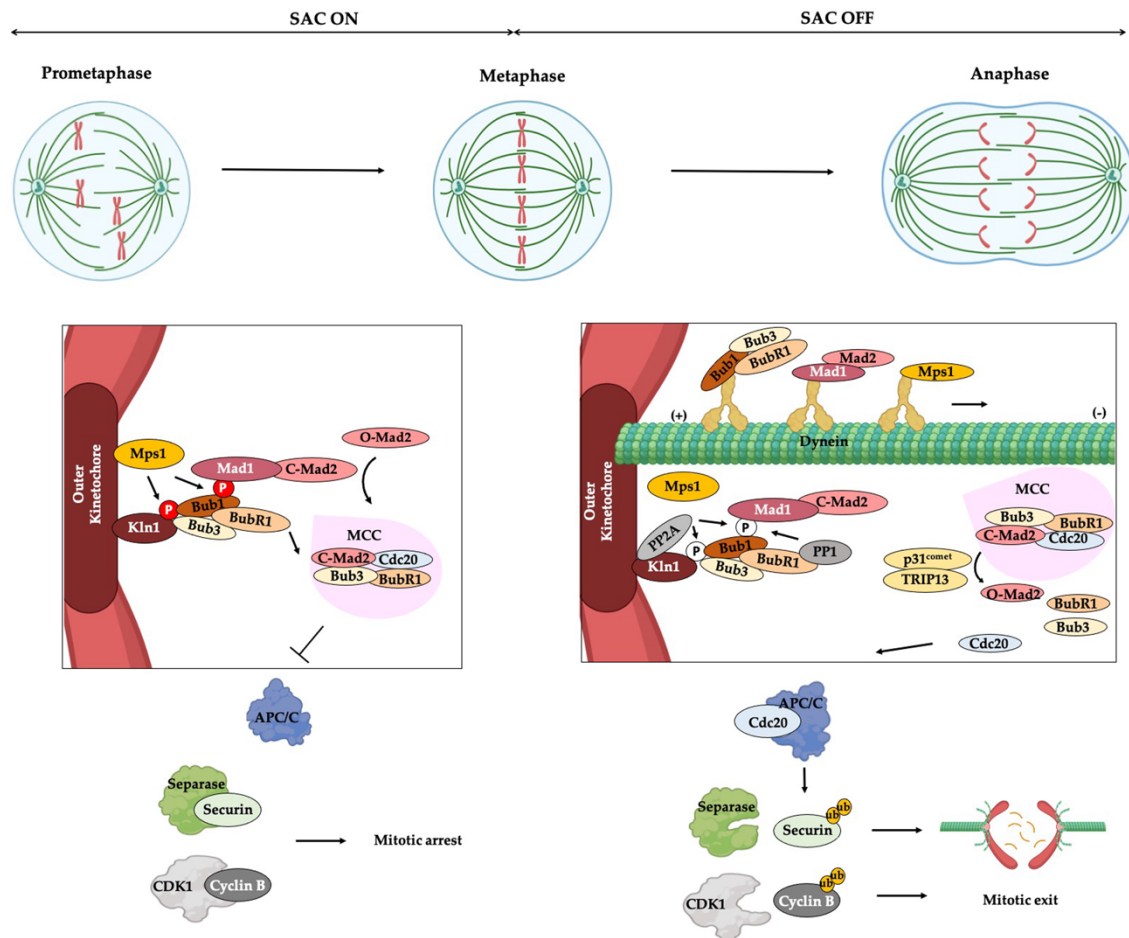


Fig. 5- Spindle assembly checkpoint. During prometaphase (SAC ON), Mps1 is recruited to unattached kinetochores and promotes the recruitment of the remaining SAC components. The complex Mad1-C-Mad2 converts the open Mad2 (O-Mad2) to close Mad2 (C-Mad2) which further associates with Bub3, BubR1 and Cdc20 to assemble the mitotic checkpoint complex (MCC). The MCC inhibits the ubiquitin ligase APC/C, preventing the proteasomal degradation of cyclin B and securin, resulting in mitotic arrest. When all kinetochores are correctly attached to microtubules (SAC OFF), the motor protein dynein transport the SAC components towards the spindle pole, and the phosphatases PP1 and PP2A-B56 dephosphorylates the Kln1 and Bub1, both processes promote the silencing of the SAC by preventing the formation of new MCCs. The proteins p31comet and TRIP13 cooperates to convert the C-Mad2, present in the MCC, to O-Mad2, promoting the disassembly of the MCC, releasing the APC/C co-activator Cdc20. Once activated, the APC/C target the securin and cyclin B for proteasomal degradation, leading to the cleavage of cohesin complexes by separase, and mitotic exit induced by Cdk1-cyclinB inactivation. Adapted from (Novais et al., 2021).

4. Apoptosis

Apoptosis, also known as programmed cell death, is a vital mechanism for multicellular organisms, participating in several events such as development, differentiation, defense against infections, and maintenance of homeostasis (Fuchs & Steller, 2011; Galluzzi et al., 2016; Jorgensen et al., 2017). Apoptosis is a complex process, which is regulated by various proteins, and differs from other types of cell death

based on its morphological and biochemical features. Morphological changes of apoptotic cells consist of nuclear condensation and fragmentation, cell shrinkage, membrane blebbing, and loss of adhesion to extracellular matrices or to neighbor cells, while biochemical alterations encompass chromosomal DNA cleavage, phosphatidylserine externalization, and activation of a family of cysteine proteases known as caspases that are the central effectors of the apoptotic machinery (Ouyang et al., 2012). Caspases are expressed in cells as inactive zymogens, known as procaspases, and are activated by cleavage. The caspases are classified into two groups, based on their position in the apoptotic signaling pathway: initiator caspases (8, 9, and 10), and the effector caspases (3, 6, and 7) (Julien & Wells, 2017).

4.1 Pathways

There are two well characterized apoptotic pathways: the extrinsic pathway, which involves cytoplasmatic receptors, and the mitochondrial-mediated intrinsic pathway (Figure 6). Both mechanisms culminate in the same execution pathway by activating the effector caspases. The extrinsic pathway is initiated by extracellular perturbations and is driven by death receptors, like the Fas receptor (FasR), tumor necrosis factor (TNF) receptor 1 (TNFR1), and the TNF-related apoptosis-inducing ligand receptor 1 and 2 (TRAILR1, TRAILR2), that upon binding to the respective ligands they trimerize. These receptors have a death domain at the intracellular tail, which is responsible to recruit several proteins, to assemble the death-inducing signaling complex (DISC), which differs in its composition depending on the type of receptor. A central component of DISC is the Fas-associated protein with death domain (FADD) which is responsible to recruit procaspase 8, finalizing the assembly of DISC (Dickens et al., 2012). Caspase 10 can also be recruited by FADD but its function seems to be primarily in the apoptosis of lymphoid cells (Fan et al., 2005). The recruitment of several molecules of procaspase 8 by FADD, facilitates its homodimerization followed by autoproteolytic cleavage leading to their activation (Tummers & Green, 2017). Activation of caspase 8 is negatively regulated by the protein c-FLIP which is a component of DISC (Irmeler et al., 1997). The caspase 8 cleavages and activates the caspase 7 and caspase 3 which in turn is able to activate caspase 6 (Fan et al., 2005; Tummers & Green, 2017). The effector caspases 3, 6, and 7 are responsible for cell demolition and the consequent morphological and biochemical changes of apoptotic cells by cleaving several substrates including, among others, ICAD leading to DNA fragmentation, actin and α -fodrin resulting in cell shrinkage and apoptotic bodies formation, and nuclear lamins promoting nuclear fragmentation (Fan et al., 2005; Nuñez et al., 1998).

The intrinsic pathway is triggered by stress resulting from a variety of stimulus like DNA damaged, chemotherapy, radiation, mitotic defects, among others. The critical step of this pathway is the mitochondrial outer membrane permeabilization (MOMP), which is regulated by pro-apoptotic and anti-apoptotic members of the BCL-2 family, that share some BCL-2 homology domains (BH1, BH2, BH3, BH4) (Tait & Green, 2010). MOMP is promoted by the pro-apoptotic effector proteins BAX, BAK, and BOK that encompass the domains BH1, BH2, and BH3. After a stimulus, they oligomerize and inserts into the outer mitochondrial membrane, creating pores along the membrane that leads to the releasing of apoptogenic factors present in the mitochondrial intermembrane space to cytosol, such as the cytochrome c, and the second mitochondrial activator of caspases (SMAC) (Tait & Green, 2010). Activity of BAX and BAK is regulated by the other Bcl-2 family members. They are antagonized by the anti-apoptotic members BCL-2, BCL-XL, BCL-W, MCL-1, and BFL-1 that contain all BH domains. The anti-apoptotic proteins exert their inhibitory activity either by directly binding to BAK and BAX preventing their oligomerization or by sequestration of the other pro-apoptotic BCL-2 family members, known as BH3-only protein activators (Cheng et al., 2001; Tait & Green, 2010). The BH3-only proteins are activated transcriptionally or post-translationally after a harmful stimulus operating as transducers of stress signaling (Tait & Green, 2010). They are usually divided into two groups: activators, such as BID, BIM, NOXA, and PUMA that can directly activate BAX and BAK; and sensitizers like BAD, BMF, BNIP3, and HRK that sequester the anti-apoptotic proteins. BH3-only activators can also inhibit the anti-apoptotic proteins (Chen et al., 2015; Czabotar et al., 2014; Du et al., 2011; Kim et al., 2006). Once at cytosol, the cytochrome c binds to apoptotic peptidase activating factor 1 (APAF1) and procaspase 9 to form the complex known as apoptosome, which is responsible to activate caspase 9 (Li et al., 1997). Caspase 9 in turn, activates caspase 3 and 7 (Fan et al., 2005). Cytosolic SMAC is responsible to associate and inhibit the inhibitor of apoptosis (IAP) family proteins, which can physically block caspases (Fan et al., 2005). Despite both pathways are usually described by separated molecular mechanisms that culminate in the same execution pathway, there is a crosstalk point between the pathways ensured by the caspase 8, which is activated through the extrinsic pathway and can cleave the BCL-2 family member BID, generating a truncated BID (tBID) which is capable of activating BAX and BAK (Tummers & Green, 2017). Several other proteins have been reported to participate in apoptosis, primarily by regulating the activity of anti-apoptotic and pro-apoptotic proteins. For instance, after DNA damage, p53 promotes the transcription of the pro-apoptotic proteins BAX, NOXA, and PUMA.

Furthermore, cytosolic p53 can also antagonize the inhibitory activity of BCL-2 and BCL-XL promoting the oligomerization of BAK and BAX at mitochondria (Amaral et al., 2010).

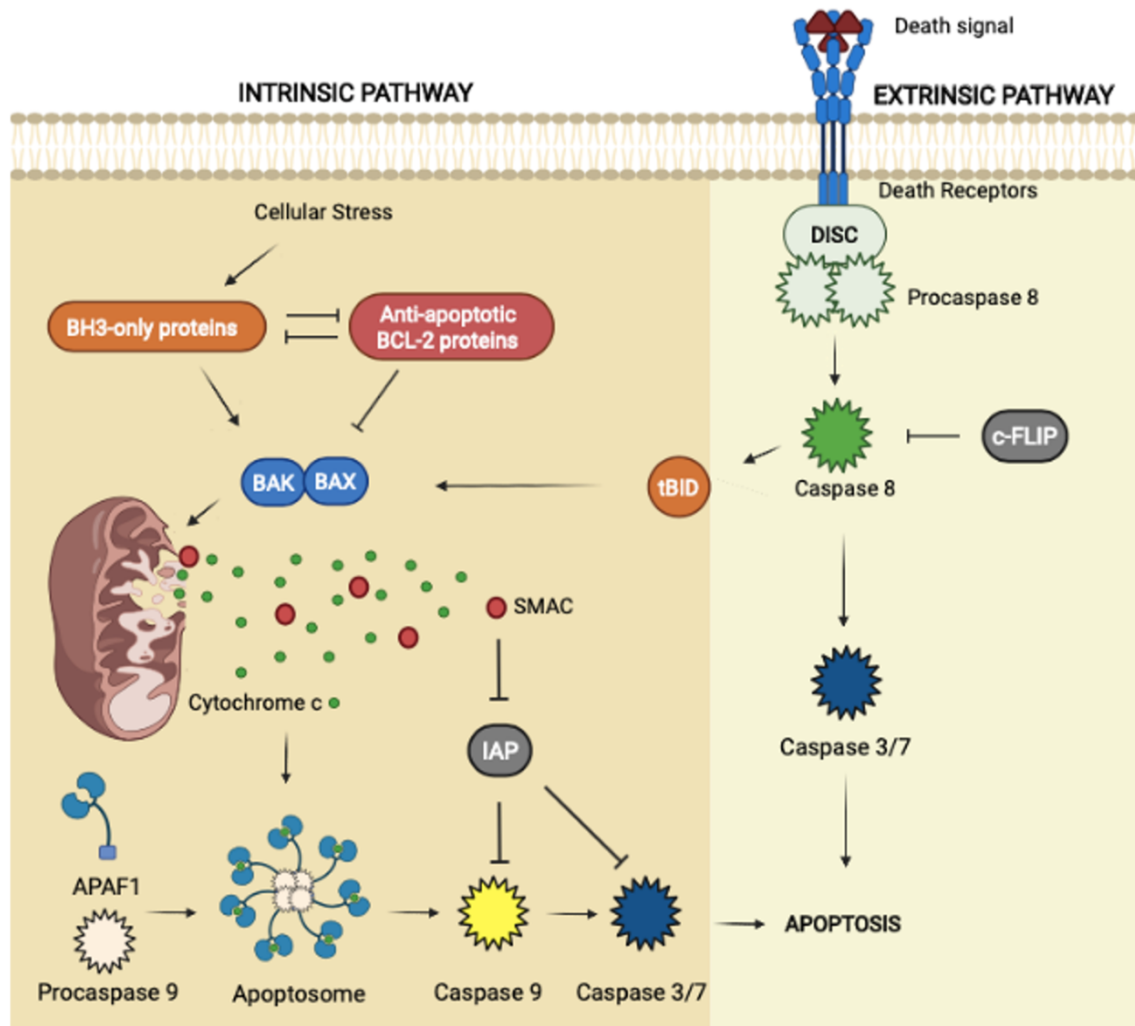


Fig. 6- Apoptotic pathways. Intrinsic pathway is triggered by stressful stimulus leading to activation of BAX and BAK that are responsible of permeabilize the outer mitochondrial membrane, resulting in the releasing of cytochrome c and SMAC to cytosol. Cytochrome c further associates with APAF1 and procaspase 9 that oligomerize to form the apoptosome which responsible to activate caspase 9. In turn caspase 9 activates caspase 3 and 7 responsible for cell demolition. SMAC inhibit the proteins of IAP family that in turn can inhibit caspases. Extrinsic pathway is driven by death receptors that after binding to their extracellular ligand, recruit procaspase 8 to their intracellular tail mediated by the protein complex DISC, resulting in the activation of caspase 8. Caspase 8, in turn activates the effector caspases 3 and 7, and cleavages the pro-apoptotic protein BID, which further activates BAK and BAX. c-FLIP is responsible to inhibit caspase 8. Created in BioRender.

4.2 Targeting apoptosis for cancer therapy: BH3 mimetics

Apoptosis is a fundamental mechanism to eliminate damaged cells, and its dysregulation is implicated in the development of various diseases, particularly cancer.

In fact, cancer cells usually have the apoptotic machinery altered, and the evasion of apoptosis is considered a hallmark of cancer (Hanahan & Weinberg, 2011). Reduced caspase function, mutations on p53, impaired death receptors signaling, and unbalanced levels of pro-apoptotic and anti-apoptotic proteins are the major mechanisms used by cancer cells to evade apoptosis (Carneiro & El-Deiry, 2020a). Therefore, block of apoptosis may promote tumorigenesis by keeping cells with damaged DNA alive, allowing them to proliferate and further accumulate more neoplastic features. Furthermore, several chemotherapeutic agents, including antimitotics, depend on apoptosis to exert their cytotoxic activity, thus, dysregulation of apoptosis has been also associated with drug resistance (Czabotar et al., 2014). For instance, the expression of the pro-apoptotic protein BIM and the anti-apoptotic proteins BCL-2, BCL-XL and MCL-1 determinates the response of tumors to paclitaxel (X. Shi et al., 2017; T. T. Tan et al., 2005). In this regard, several cancer therapies that target molecules of the apoptotic machinery have been developed.

One of these strategies comprises a group of molecules, known as BH3-mimetics, that are able to inhibit the anti-apoptotic BCL-2 family members. Various compounds have been developed and a few of them entered in clinical trials (Carneiro & El-Deiry, 2020b). Moreover, the BCL-2 selective inhibitor venetoclax was already approved by FDA for the treatment of patients with chronic lymphocytic leukemia (Stilgenbauer et al., 2018). Navitoclax, also known as ABT-263, is a BH3-mimetic capable of inhibiting both BCL-2 and BCL-XL (Tse et al., 2008). Pre-clinical studies of Navitoclax demonstrated its antitumor activity against several tumor cell lines that prompted Navitoclax to enter clinical trials (Mohamad Anuar et al., 2020). At least twelve phase I/II clinical trials were completed with Navitoclax as monotherapy or in combination with other anticancer agents in patients with hematologic and solid tumors (U.S National Library of Medicine, 2021). Overall, these studies indicate that combinational treatments with Navitoclax, especially with drugs that indirectly induce the activation of apoptosis, like antimitotics, should be the best strategy to enhance its antitumor activity. Furthermore, the clinical trial outcomes showed that Navitoclax provokes severe thrombocytopenia, thus combinational treatments could permit to reduce its dose, which could minimize its side effects (Mohamad Anuar et al., 2020).

5. Targeting mitosis for cancer therapy

Based on the uncontrolled proliferation of cancer, anticancer drugs have been developed to block the cell cycle, particularly at mitosis. Antimitotic agents rely on SAC activation which leads to mitotic arrest, expecting to induce subsequent cell death. Classical antimitotic drugs have been widely used in cancer therapy, however, due to some limitations associated with these drugs, novel molecules with different action mechanisms have been developed to disrupt the normal progression of mitosis, known as second-generation antimitotics (SGAs).

5.1 Classical antimitotic drugs

Classical antimitotic drugs target microtubule dynamics, for this reason there are commonly called microtubule-targeting agents (MTAs). MTAs are typically divided into two groups, based on their action mechanism: microtubule destabilizers and stabilizers (Jordan & Wilson, 2004). Microtubule destabilizers consists of vinca alkaloids such as vinblastine and vincristine that bind to β -tubulin subunits inducing microtubule depolymerization. On the other hand, microtubule stabilizers including epothilones and taxanes like paclitaxel and docetaxel, bind to β -tubulin subunits resulting in the stabilization of microtubules and increased polymerization (Perez, 2009). Both classes impair a functional mitotic spindle, leading to SAC activation and subsequent mitotic arrest (Matson & Stukenberg, 2011). Following prolonged mitotic arrest cells respond to MTAs by different ways: die in mitosis by apoptosis, divide unequally producing aneuploid daughter cells or exit mitosis without dividing, a process known as slippage, which results from a constant and slow degradation of cyclin B even when SAC is on, originating tetraploid cells (Brito & Rieder, 2006; Gascoigne & Taylor, 2009) (Figure 7). In turn, slipped cells can follow three possible fates: become senescent, undergo post mitotic death, or continue dividing (Gascoigne & Taylor, 2009; Nakayama & Inoue, 2016). Therefore, mitotic slippage, together with efflux pumps, mutations in tubulin genes, and deficient apoptotic signaling, represent the main reasons for the therapeutic failure of MTAs (Dumontet & Jordan, 2010). Additionally, MTAs treatment is frequently associated with neurological and myeloid toxicity due to the disruption of microtubule dynamics (Dumontet & Jordan, 2010).

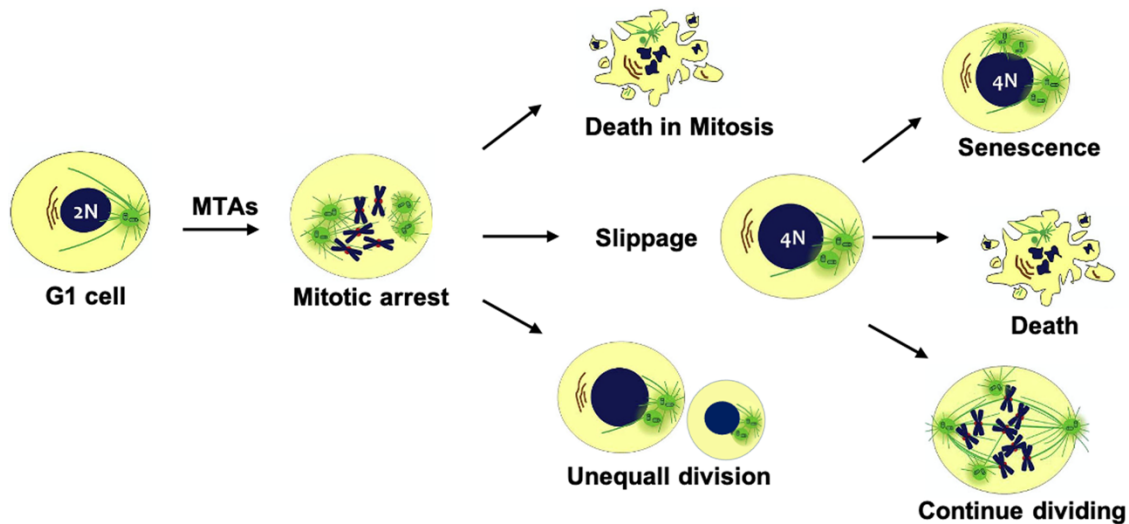


Fig. 7- Cell fates after treatment with MTAs. After mitotic arrest imposed by MTAs, cells could die in mitosis, divide unequally, or exit mitosis without divide (slippage). After slippage, cells could die, become senescent, or continue dividing. Adapted from (Henriques et al., 2019).

5.2 Second-generation antimetabolites

Due to the aforementioned limitations of MTAs, alternative approaches to block mitosis than directly targeting microtubules were developed. These new strategies consist of inhibiting the activity of mitotic proteins, especially the kinases Mps1, Plk1, Aurora A and B, and the motor proteins Eg-5 and CENP-E, that play crucial roles in different process during mitosis such as mitotic entry, spindle assembly, chromosome congression, or SAC regulation (Figure 8). The inhibition of these proteins is made through small molecules or small interfering RNAs (siRNAs), known as the second-generation antimetabolites (Novais et al., 2021). From a SAC response point of view, SGAs could be divided into two groups with different mechanisms whereby they kill cancer cells: mitotic blockers and mitotic drivers. Similar with MTAs, the mitotic blockers induce SAC activation leading to prolonged mitotic arrest followed by apoptosis. This group includes inhibitors of the proteins Eg-5, Plk1, Aurora A, and CENP-E. In contrast, mitotic drivers which consist of Mps1 and Aurora B inhibitors, induce SAC silencing provoking a premature mitotic exit with extensive chromosome missegregation, resulting in chromosome aberrations that are incompatible with the cell viability of daughter cells.

As mentioned above, CENP-E is a plus end-directed motor protein that plays a crucial role in cytokinesis, chromosome congression and alignment, and in SAC signaling through modulation of BubR1 function (Mao et al., 2005; Ohashi et al., 2016; Yu et al., 2019). Inhibition of CENP-E leads to defects on chromosome alignment that

results in chronic activation of SAC and consequent mitotic arrest (Kung et al., 2014; Ohashi et al., 2015; Tanudji et al., 2004). Some CENP-E inhibitors have been tested in pre-clinical studies, but only one small molecule, GSK923295, has reached clinical trials. GSK923295 was generally well tolerated but demonstrated a modest efficacy (Chung et al., 2012).

Eg-5 kinesin is a plus end-directed motor protein that plays a critical role in bipolar spindle assembly (Blangy et al., 1995; Sawin et al., 1992). The inhibition of Eg-5 results in monopolar spindles, which leads to SAC activation and mitotic arrest, and has demonstrated antitumor activity in human xenografts models (M. Liu et al., 2008; Weil et al., 2002; X. S. Ye et al., 2015). Together with its overexpression in several tumors, makes Eg-5 an attractive target for cancer therapy (S. Ding et al., 2011; C. Liu et al., 2017; L. Liu et al., 2016; M. Liu et al., 2010). Conversely to the kinesin CENP-E, ten Eg-5 inhibitors have reached clinical trials: 4SC-205, ARRY-520 (Filanesib), AZD4877, MK-0731, SB-715992 (Ispinesib), LY2523355 (Litronesib), SB-743921, EMD 534085, ARQ 621, and ALN-VSP02. Forty-five phase I/II trials against solid and hematological tumors, in monotherapy or in combinational treatments, have been completed or terminated. The most promising efficacy results were achieved with Filanesib in multiple myeloma patients, especially when combined with the proteasome inhibitors Bortezomib and Carfilzomib. It is expected that Filanesib will enter phase III trials.

Mps1 is a dual-specificity protein kinase phosphorylating serine/threonine and tyrosine residues (Lauzé et al., 1995). As already mentioned, Mps1 is recruited early in mitosis to unattached kinetochores, where it is responsible for SAC activation through the recruitment of several SAC components to kinetochores and subsequent MCC formation. It has also been involved in DNA damage checkpoint response, chromosome alignment, meiosis, cytokinesis, centrosome duplication, and error-correction of kinetochore–microtubule attachment (X. Liu & Winey, 2012a; Maciejowski et al., 2017; H. Yang et al., 2019). Mps1 is overexpressed in various tumors, correlating with poor prognosis (Xie et al., 2017). The inhibition of Mps1 activity compromises the SAC, which leads to premature mitotic exit, resulting in massive aberrant chromosome segregation and subsequent cell death (Jemaà et al., 2013; Kwiatkowski et al., 2010). Furthermore, inhibition of Mps1 has been shown to sensitize cancer cells to paclitaxel (Janssen et al., 2009). Hence, Mps1 became an attractive target for cancer therapy, and a variety of small molecules that inhibit Mps1 have been developed. So far, five Mps1 inhibitors have been approved to begin clinical trials in combination with paclitaxel, namely, BOS172722, BAY1217389, BAY1161909, CFI-402257 and S81694. Six phase I/II

studies have been undertaken: two have been completed, one has been terminated, two are recruiting participants and another one is ongoing. It is too early to draw a conclusion on the clinical efficacy and safety of these combinations, as only the outcomes of BAY 1161909 have been published to date. Nevertheless, in this trial, the combinational treatment was generally well tolerated, and partial responses were reported (Lorusso et al., 2018). Inhibition of Mps1 also demonstrated a synergic effect with cisplatin in malignant mesothelioma cisplatin-resistant cell lines *in vitro*. Therefore, it will be interesting to evaluate the clinical efficacy of Mps1 inhibitors combined with anti-cancer drugs other than taxanes, in particular with platinum-based agents (Szymiczek et al., 2017).

Aurora kinases A, B, and C are a family of serine/threonine kinases that play critical functions during mitosis. Aurora A is implicated in centrosome maturation and separation, cytokinesis, and bipolar spindle assembly, whereas Aurora B is involved in chromosome condensation and alignment, kinetochore–microtubule attachments, SAC activation, and cytokinesis (Bolanos-Garcia, 2005; Willems et al., 2018). All Aurora family members are overexpressed in several tumors and have been related to improving the survival and proliferation of tumor cells (Willems et al., 2018). The inhibition of Aurora A and Aurora B results in cell death through different mechanisms. Aurora B inhibition leads to defects in kinetochore–microtubule attachments that override the SAC, resulting in extensive aneuploidy and cell death (Girdler et al., 2006; Hauf et al., 2003). In contrast, inhibition of Aurora A provokes a shortened and disorganized microtubule spindle, inducing SAC activation and subsequent mitotic arrest, followed by apoptosis (Glaser et al., 2012; Kaestner et al., 2009). For these reasons, Aurora kinases were seen as attractive targets for cancer therapy. Therefore, several small molecules with broad-spectrum inhibition activity against Aurora kinases, known as pan-Aurora inhibitors, have entered clinical studies, including VX-680 (Tozasertib), CYC116, PHA739358 (Danusertib), SNS-314 Mesylate, PF-03814735, AS703569 (Cenisertib), TAK-901, ABT-348 (Ilorasertib), AMG-900, GSK1070916 (NIM-900), AT9283, and BI-847325. Additionally, various selective inhibitors for each kinase have also been developed and some of them entered clinical trials, such as the Aurora A inhibitors MLN8237 (Alisertib), LY3295668 (Erbumine), TAS-119, ENMD-2076, MK-5108/VX-689, KW-2449, and MLN8054; and the Aurora B inhibitors Chiauranib, AZD1152 (Barasertib), BI-811283, and BI-831266. In summary, considering all Aurora inhibitors, either those with broad-spectrum or with selective activity, the efficacy outcomes were better in hematological tumors than in solid tumors. There is a substantial debate as to whether it is more efficient to inhibit Aurora A, Aurora B, or both simultaneously. A pre-clinical study in pancreatic

cancer cells has pointed to Aurora A as a better target than Aurora B (Warner et al., 2006). However, another pre-clinical study has demonstrated that colon cancer cells were more sensitive to Aurora B inhibition compared to Aurora A (Girdler et al., 2006). In fact, the molecule with better efficacy outcomes in trials was the Aurora A inhibitor Alisertib, but both strategies have demonstrated antitumor activity, especially against hematological tumors. Perhaps, some tumors may be more sensitive to inhibition of one of the two kinases, but further studies are required to address this question.

Plk1 is a cell cycle-regulating serine/threonine kinase implicated in centrosome maturation and separation, mitotic entry, spindle assembly, kinetochore–microtubule attachment, the SAC, DNA damage checkpoint activation, and cytokinesis (Bruinsma et al., 2012; Colicino & Hehnly, 2018; Combes et al., 2017). Knockdown or inhibition through small molecules of Plk1 leads to monopolar spindle formation, G2/M arrest, and polyploidy, which ultimately leads to cell death (Pajtler et al., 2016; Ueda et al., 2019; Wang et al., 2020). Plk1 is overexpressed in several tumors, correlated with poor prognosis, and has been associated with resistance to chemotherapeutics such as Doxorubicin, Gemcitabine, and Paclitaxel (Gutteridge et al., 2016; Z. Liu et al., 2016). Taken together, these facts led to the development of several small molecules that target Plk1 for cancer therapy. Nine Plk1 inhibitors have already entered clinical trials: CYC 140, GSK461364, TAK-960, NMS-1286937 (Onvansertib), BI 6727 (Volasertib), BI 2536, Rigosertib, MK-1496, and lipid nanoparticles carrying the siRNA, TKM-080301. Seventy-nine phase I/II clinical trials with solid and hematological tumor patients have been initiated: fifty-two completed, five ongoing, fifteen terminated/withdrawn, and seven are recruiting participants. Additionally, three phase III studies with metastatic pancreatic adenocarcinoma and myelodysplastic syndrome (MDS) patients have been completed, and two studies with acute myeloid leukemia (AML) and MDS patients are ongoing. BI2536 is a potent ATP-competitive Plk1 inhibitor developed by Boehringer Ingelheim (Lénárt et al., 2007). Several clinical trials have been undertaken with this compound. Overall, the most common AEs were neutropenia and leukopenia. Partial responses were reported in non-small-cell lung cancer patients as monotherapy (2.1%, NCT00376623) and in combination with pemetrexed (5.2%, NCT02211833). Partial responses were also reported in pancreatic cancer patients (2.3%, NCT00710710) as a monotherapy (Ellis et al., 2013; Mross et al., 2012; Sebastian et al., 2010). Nevertheless, the greatest efficacy was observed in acute myeloid leukemia patients (NCT00701766), in which two complete remissions (3.7%) and three partial responses (5.5%) were reported, and in non-Hodgkin's lymphoma patients (NCT00243087), also as

monotherapy, in which three complete remissions (17.7%) and one partial response (5.9%) were achieved (Müller-Tidow et al., 2013; Vose et al., 2013).

Despite several inhibitors of these proteins entered clinical trials, until now, no small molecule was approved for clinical use. Either blocking mitosis with mitotic blockers or inducing premature mitotic exit with mitotic drivers, seem to be reasonable approaches to kill cancer cells. However, the clinical efficacy of SGAs was generally modest, and there are some concerns either with mitotic drivers or mitotic blockers. Mitotic drivers can fuel genomic instability. In case of the incomplete inhibition of mitotic driver targets, which is more likely to occur in vivo than in vitro, chromosome segregation errors may be generated below the threshold required to kill cancer cells, which, theoretically, increases genomic instability, thereby fueling malignancy. On the other hand, there is profound variability in terms of cell fates following the prolonged mitotic arrest of cancer cells treated with mitotic blockers, as it happens with MTAs. Therefore, mitotic slippage also represents a resistance mechanism against mitotic blockers. In this regard further research is required to improve the clinical efficacy of SGA by developing new molecules and combinational treatments with synergistic effects.

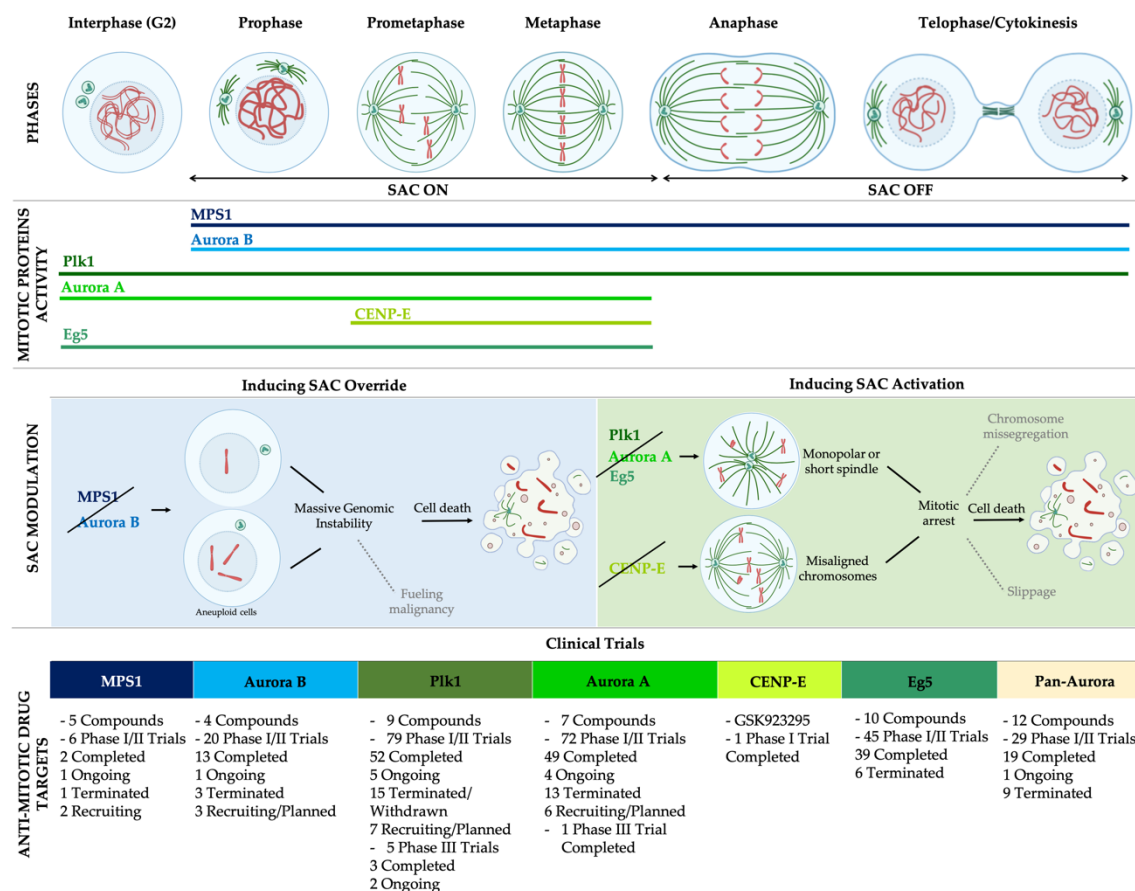


Fig. 8- Targeting mitosis for cancer treatment. Representation of the activity of Mps1, Plk1, Aurora A and B, CENP-E, and Eg-5 throughout mitosis and the cell fate upon their inhibition. Overview of the current clinical trials state of the inhibitors of each protein. Adapted from (Novais et al., 2021)

Chapter II:

Motivations and aims

The present study aims to provide new strategies to target mitosis for cancer therapy and comprises two specific objectives:

- i) improving the efficacy of the second-generation antimitotic BI2536
- ii) identifying new molecules with antimitotic properties

Targeting mitosis to kill cancer cells has been one of the most successful strategies in the past few years (Perez, 2009). Even so, antimitotic agents have been facing some limitations that impair their clinical efficacy. Antimitotic agents such as MTAs and the SGAs mitotic blockers, induce mitotic arrest which is expected to lead to cell death by apoptosis. However, cancer cells display a profound cell fate variation after exposure to antimitotic agents (Gascoigne & Taylor, 2008). The ability of cancer cells to undergo slippage, thereby escaping apoptosis has been pointed as a mechanism of resistance to antimitotics (Sinha et al., 2019). Slippage occurs due to the slow degradation of cyclin B even when SAC is on, triggering mitotic exit. What dictates whether cell die in mitosis or undergo slippage is the rate of cyclin B degradation *versus* the accumulation of the apoptotic signal. If cyclin B levels reach the mitotic exit threshold before the levels of death signals reach the threshold to trigger apoptosis, slippage occurs. On the other hand, if death signals reach the threshold to trigger cell death before the cyclin B levels fall below the mitotic exit threshold, cells die in mitosis (Figure 9).

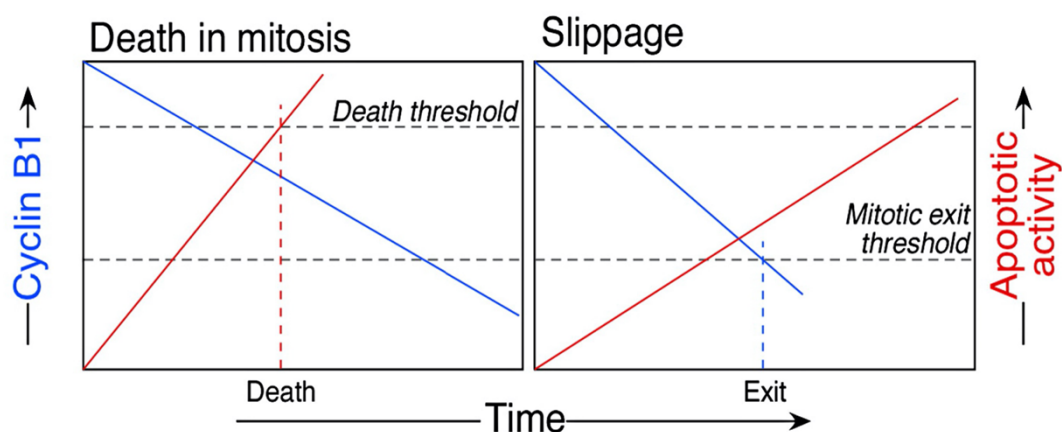


Fig. 9- Model explaining cell fate variation after treatment with antimitotic agents. Relationship between cyclin B degradation and apoptotic activity determines whether cells die in mitosis or undergo slippage. In cells that die in mitosis (left), the levels of death signals reach the death threshold before the cyclin B levels fall below the threshold to trigger mitotic exit, resulting in cell death. In cells that undergo slippage (right), the rate of cyclin B degradation is faster than the accumulation of death signals, consequently cyclin B levels reach the threshold to trigger mitotic exit before the death signals reach the death threshold, resulting in slippage. Adapted from (Gascoigne & Taylor, 2008)

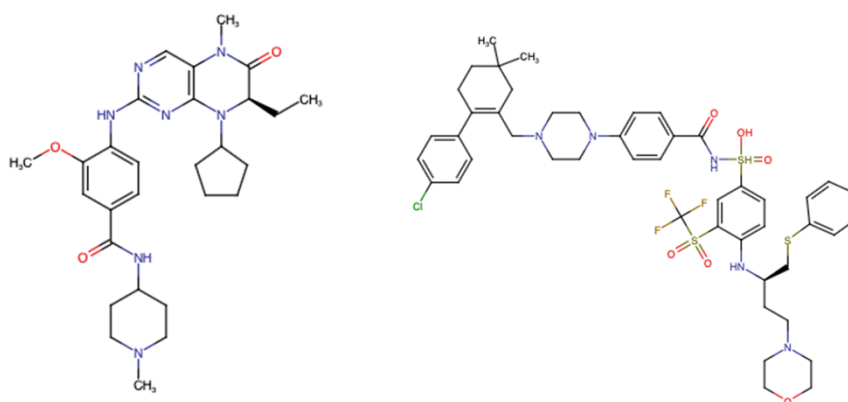


Fig. 10- Structure of Plk1 inhibitor BI2536 and the BH3-mimetic Navitoclax

Table 1- IC₅₀ values of BI2526 and Navitoclax in A549 cells after 48 of exposure

Compound	IC ₅₀ (nM)
BI2536	100.2± 17.3
Navitoclax	657.3± 40.1

Results are expressed as mean ± SD of three independent experiments.

The MTAs, such as taxanes and vinca alkaloids, are the current antimitotics used in the clinic (Perez, 2009). However, MTAs have limited effectiveness in several cancer due to hematopoietic and neurologic toxicities, owing to the role of microtubules in non-tumor cells. Additionally, resistance mechanisms have been developed by cancer cells such as upregulation of efflux pumps that lowers their intracellular concentration, and tubulin mutations that affect MTA binding sites (Perez, 2009). Therefore, discovery of new antimitotics that can overcome these disadvantages are required. Accordingly, the second aim of this thesis focused on identification of new antimitotic agents (Chapter IV). Two different groups of molecules were analyzed.

First, three tetracyclic thioxanthenes derivates were evaluated, TX2.1a, TX2.19, and TX2.17 (Group I) (Figure 11). Recently, tetracyclic thioxanthenes were described to exhibit potential antitumor activity (Hedidi et al., 2017; Mokhtari Brikci-Nigassa et al., 2020). The three tetracyclic thioxanthenes of this study were previously synthesized based on a thioxanthone which emerged as hit compound with antitumor properties, highlighting the potential of these molecules (Barbosa et al., 2016; Lima et al., 2016). Thus, to evaluate the potential antitumor activity of these tetracyclic thioxanthenes derivates, we accessed the growth inhibitory activity of the analyzed compounds in cancer cells, by a Sulforhodamine B (SRB) assay. The compounds with promising growth inhibitory activity were selected to further investigate whether they possessed antimitotic activity.

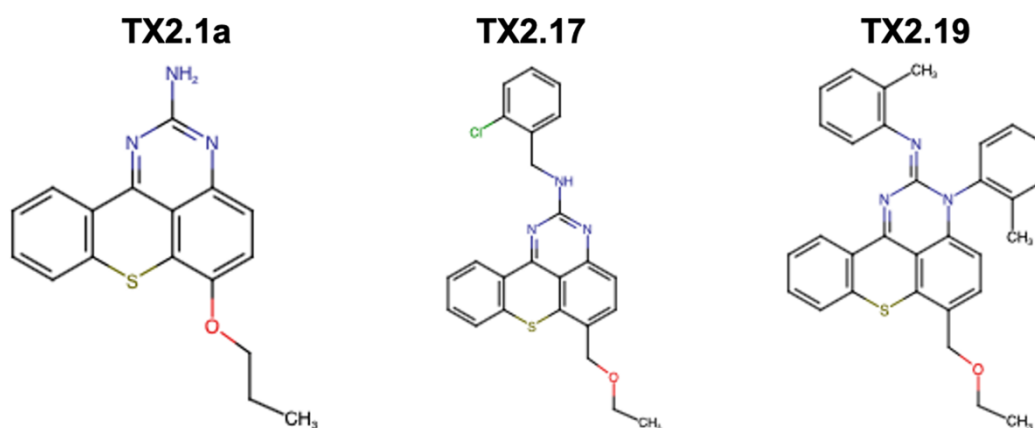


Fig. 11- Structure of tetracyclic thioxanthenes TX2.1a, TX2.17, and TX2.19.

The second molecule evaluated was a diarylpentanoid, BP-M345 (Group II) (Figure 12). Diarylpentanoids have been reported to exhibit antitumor activity by interfering with multiple biologic process, including cell cycle (Moreira et al., 2021; Paulraj et al., 2019). A recent study explored the activity of BP-M345 on activation of the p53 pathway through inhibition of p53 interaction with MDM2. Despite BP-M345 has demonstrated growth inhibitory activity in colon tumor cells HCT116, the results showed that BP-345 did not inhibit the interaction of p53 with MDM2 (Moreira et al., 2021). Thus, we investigated whether BP-M345 exerts its antitumor activity, by acting as an antimetabolite agent.

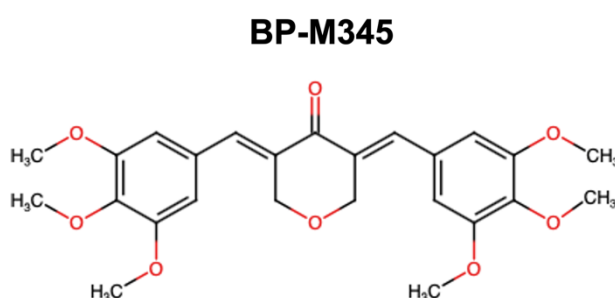


Fig. 12- Structure of the diarylpentanoid BP-M345.

Chapter III

Targeting the anti-apoptotic pathway sensitizes lung cancer cells to PIK1 inhibition

1. Introduction

Cancer cells display a profound cell fate variation after exposure to antimitotic agents (Gascoigne & Taylor, 2008). The ability of cancer cells to undergo slippage, thereby escaping apoptosis has been pointed as a mechanism of resistance to antimitotics (Sinha et al., 2019). Slippage occurs due to the slow degradation of cyclin B even when SAC is on, triggering mitotic exit. What dictates whether cell die in mitosis or undergo slippage is the rate of cyclin B degradation *versus* the accumulation of the apoptotic signal. The combination of apoptosis potentiators, like BH3-mimetics, with SGA is a reasonable strategy to improve their efficacy, once after a mitotic arrest imposed by SGA, the apoptosis potentiator may induce a faster accumulation of apoptotic signals, resulting in cell death and consequently decreasing the risk of slippage. Here we evaluate the cell fate of the human lung adenocarcinoma epithelial cells A549, after treatment with the Plk1 inhibitor BI2536, an SGA mitotic blocker in combination with the apoptosis potentiator Navitoclax, a BH3-mimetic.

2. Material and methods

2.1. Compounds

BI2536 and Navitoclax (MedChem Express) were reconstituted in sterile dimethyl sulfoxide (DMSO, Sigma-Aldrich Co. Ltd.) to a stock concentration of 10 mM. To preserve compound activity, several aliquots were prepared and stored at -20 °C. In the day of experiments, all compounds were diluted in fresh culture medium at desired concentrations.

2.2. Cell lines and culture conditions

NCI-H460 (non-small cell lung cancer) cell line was obtained from European Collection of Cell Culture was grown in RPMI-1640 culture medium (Roswell Park Memorial Institute, Biochrom) supplemented with 5% heat-inactivated fetal bovine serum (FBS, Biochrom). A549 (Human Lung Adenocarcinoma Epithelial Cells) was obtained from American Type Culture Collection, and HPAEpiC (Human Pulmonary Alveolar Epithelial Cells) was obtained from ScienCell Research Laboratories, both cell lines were grown in DMEM medium (Dulbecco's Modified Eagle's, Biochrom), supplemented with 5% heat-inactivated FBS and 1% of non-essential amino acids (Sigma-Aldrich Co. Ltd). Cell lines were cultured in 25 cm² VWR cell culture flasks with complete respective growth culture medium and maintained in a humidified incubator at 37 °C with 5% CO₂ (Hera Cell, Heraeus).

2.3 RNA isolation and quantitative real-time PCR

Total RNA was extracted using the PureZOL™ RNA Isolation Reagent (Bio-Rad Laboratories, Inc. Hercules), according to the manufacturer's instructions and quantified through spectrophotometry (NanoDrop 2000, Thermo Scientific). cDNA synthesis was performed using the iScript™ cDNA Synthesis Kit (Bio-Rad), following supplier's instructions and was amplified using iQ™ SYBR Green Supermix Kit (Bio-Rad) on iQ Thermal Cycler (Bio-Rad), according to the following program: initial denaturing step at 95.0°C for 3 min; 40 cycles at 94.0°C for 20 sec; 54.0°C for 30 sec and 72.0°C for 30 sec. The following primers were used at final concentration of 10 µM: Plk1: forward 5'-CCCCTCACAGTCCTCAATAA-3' and reverse 5'-TGTCCGAATAGTCCACCC-3'; GAPDH: forward 5'-ACAGTCAGCCGCATCTTC-3' and reverse 5'-GCCCAATACGACCAAATCC-3'; Actin: forward 5'-AATCTGGCACCACACCTTCTA-3' and reverse 5'-ATAGCACAGCCTGGATAGCAA-3'. Experiments were performed in triplicate for each data point. Data were acquired with CFX Manager™ Software (version 1.0, Bio-Rad) and the results were analyzed according Δ CT and normalized against Actin and GAPDH expression levels, which was used as control template. A fold value of mRNA level $\geq$ or $\leq$ 1.5 relatively to that of normal cells was considered as over- or subexpression, respectively.

2.4 Mitotic index determination

A total of 9.0×10^4 A549 tumor cells were seeded, with complete culture medium, in 6-well plates, for 24h. Cells were then treated for 24h, with 15 nM of BI2536, 220 nM of Navitoclax, with the combination of both, and with 1 µM of Nocodazole (Sigma-Aldrich Co. Ltd.) used as a positive control. DMSO, up to 0.25% concentration, were included as compound solvent control. Cells were then observed by phase contrast microscopy, using Zeiss Primo Vert microscope (Carl Zeiss) and a Nikon TE 2000-U microscope (Nikon) with a 10× objective were used. The Nikon microscope used a DXM1200F digital camera, with Nikon ACT-1 software (Melville). Then at least 3000 cells were accounted for the determination of the mitotic index (MI), from random phase contrast microscope fields. MI was calculated using the following formula: $MI (\%) = (\text{number of mitotic cells} / \text{total number of cells}) \times 100$.

2.5 Flow cytometry analysis

A total of 9.0×10^4 A549 tumor cells were seeded, with complete culture medium, in 6-well plates, for 24h. Cells were then treated for 48h with 15 nM of BI2536, 220 nM of Navitoclax, and with the combination of both. DMSO, up to 0.25% concentration, were

included as compound solvent control. Then A549 floating and adherent cells were collected and processed with the “Annexin V-FITC Apoptosis Detection Kit” (eBioscience) according to manufacturer’s instructions. Fluorescence was assessed by BD Accuri™ C6 Plus Flow cytometer (BD Biosciences) and data was analyzed with BD Accuri™ C6 Plus software, version 1.0.27.1 (www.bdbiosciences.com), at least 20.000 events per sample were collected.

2.6 TUNEL assay

A total of 9.0×10^4 of A549 cells were grown on poly-L-lysine-coated coverslips, for 24h. Then cells were treated with 15 nM of BI2536, 220 nM of Navitoclax, with the combination of both. DMSO, up to 0.25% concentration, were included as compound solvent control. After treatment, cells were immediately fixed in 4% paraformaldehyde (w/v, Sigma-Aldrich Co. Ltd) in PBS for 10 min, followed by PBS wash and permeabilization with 0.2% (v/v) Triton X-100 (Sigma-Aldrich Co. Ltd.) in PBS for 5 min. The TUNEL labeling was performed using DeadEnd Fluorometric TUNEL System (Promega), according to the manufacturer’s instructions. DNA was stained with 2 µg/ml of 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Co. Ltd) in Vectashield mounting medium. For image acquisition, an Axio Observer Z.1 SD microscope (Carl Zeiss) was used, coupled to an AxioCam MR3, and with the Plan Apochromatic 63×/NA 1.4 objective. The deconvolution was performed with the software AxioVision Release 4.8.2 SPC and the images were processed using ImageJ version 1.51. The level of apoptosis was established by counting TUNEL-positive cells in a total of approximately 500 cells in 10 random fields under fluorescence microscope. The apoptotic index was calculated as the percentage of positively TUNEL-stained cells over the cells.

2.7 Live-Cell Imaging

For live-cell imaging experiments, a total of 9.0×10^4 A549 cells were seeded onto LabTek II chambered cover glass (Nunc) containing complete culture medium, for 24 h at 37 °C with 5% CO₂. Cells were then treated with 15 nM of BI2536, 220 nM of Navitoclax, or with the combination of both. Images were captured at 5 min intervals up to 48h under differential interference contrast (DIC) optics, with a 63× objective on an Axio Observer Z.1 SD inverted microscope, equipped with an incubation chamber with the temperature set to 37 °C and an atmosphere of 5% CO₂. Movies were generated from the time-lapse images using ImageJ software version 1.51. Cell fate was followed since the first mitosis. Dead cells were classified into death in mitosis (DiM), post mitotic death (PMD) when death occurred after complete cell division, or post slippage death

(PSD) when death occurred after cells exited mitosis without dividing. Cells that exit mitosis without dividing and survive were classified as post slippage survival (PSS).

2.8 Statistical analysis

Statistical analysis was performed using One-way ANOVA with Tukey's multiple comparisons test or unpaired t-test in the GraphPad Prism version 6. Data are presented as the mean $\pm$ standard deviation (SD) of three independent experiments and the level of statistical significance was established considering the probabilities of ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

3. Results

3.1 Plk1 is overexpressed in non-small cell lung cancer cell lines

To gain insight into the relevance of Plk1 as a potential target for therapy of non-small cell lung cancer, we first analyzed its expression in the non-small cell lung cancer cell lines A549 and NCI-H460. Analysis of Plk1 mRNA levels by qRT-PCR demonstrated that it was overexpressed in both cancer cell lines compared with mRNA levels in the non-tumor cell line HPAEpiC (Figure 13). There was a 3.3 ± 0.3 and 3.4 ± 0.6 fold increase in Plk1 expression in A549 and NCI-H460 cell lines, respectively, compared to the non-tumor cell line. These results were in concordance with previous studies that reported that Plk1 is overexpressed in NSCLC. These results highlight the relevance of targeting Plk1 for therapy of NSCLC (Wolf et al., 1997; Zeng et al., 2020).

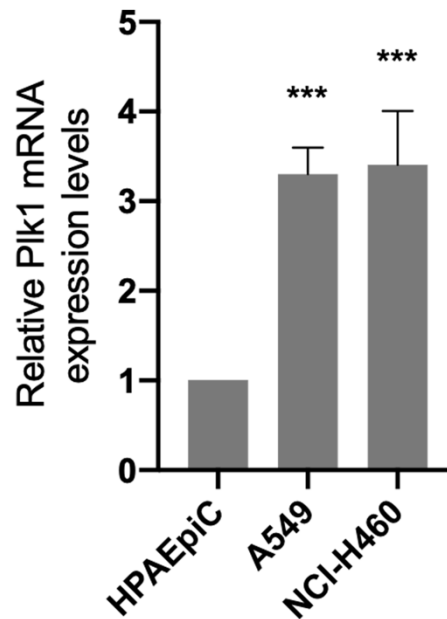


Fig. 13- Plk1 is overexpressed in NSCLC cell lines. Relative expression of Plk1 mRNA as determined by RT-PCR in NCI-H460 and A549 cell lines, comparatively to non-tumor HPAEpic showing overexpression of Plk1 in tumor cell lines with statistical relevance of *** $p < 0.001$ by unpaired t-test.

3.2 PLK1 inhibition by BI2536 arrests cells in mitosis

We started to analyze A549 cells by phase contrast microscopy after 24h treatment with 15 nM of BI2536 (6-fold less IC_{50}), 220 nM of Navitoclax (4-fold less IC_{50}), and combination of both, to verify whether BI2536 was effectively affecting progression of mitosis at this concentration. Nocodazole (an antimitotic agent) and DMSO (compound solvent) were included as control. We found an accumulation of bright and rounded cells reminiscent of cells arrested in mitosis after treatment with BI2536 either alone or in combination with Navitoclax (Figure 14 A). Then, we calculated the mitotic index (MI) which was increased in both treatment conditions ($75.4 \pm 8.0\%$ for BI2536 alone, and $74.3 \pm 6.8\%$ for combinational treatment) compared to untreated ($17.1 \pm 0.2\%$) and DMSO-treated cells ($16.9 \pm 1.0\%$) (Figure 14 B). Thus, these results indicated that BI2536 was effectively blocking cells in mitosis.

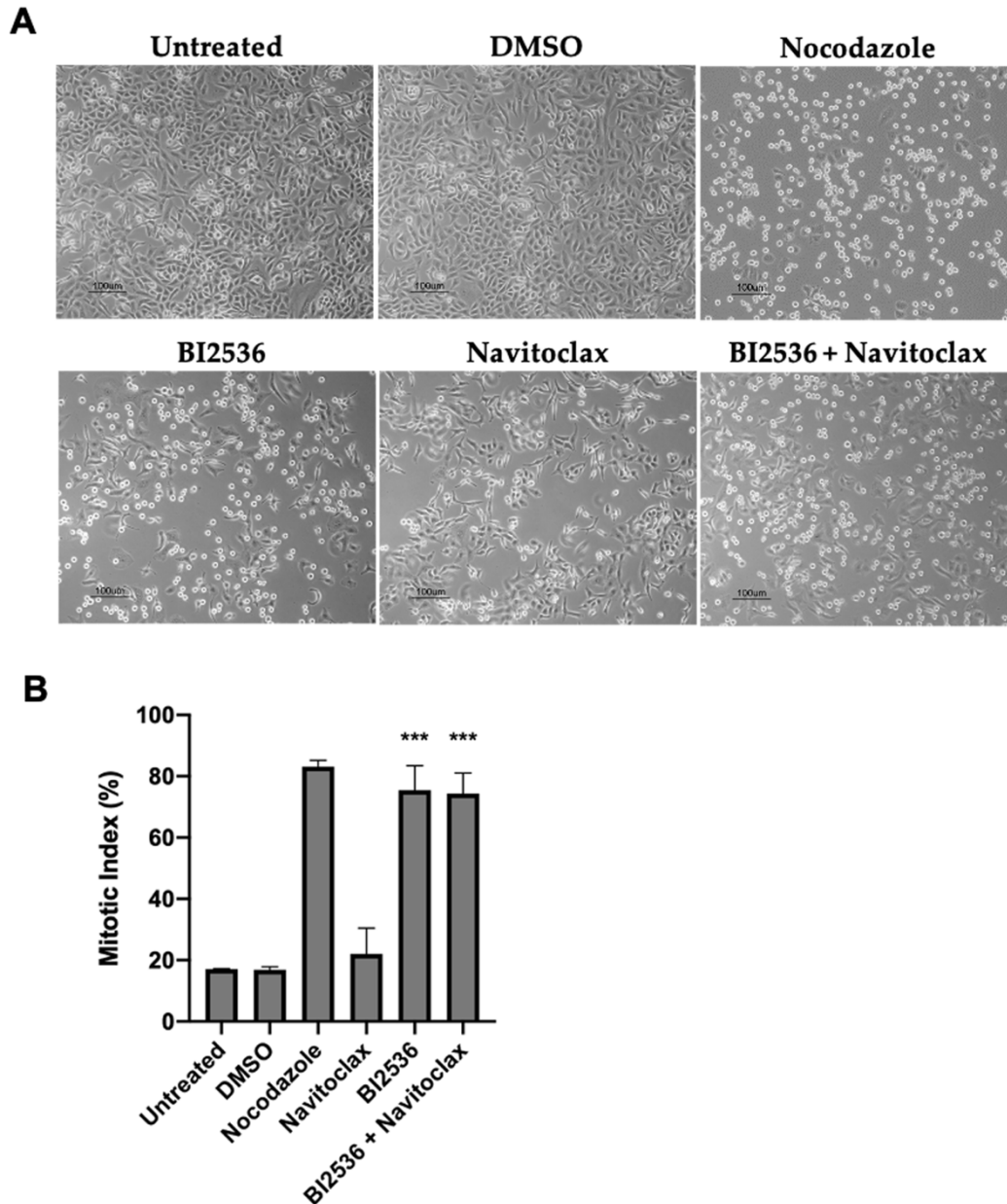


Fig. 14- Treatment with BI2536 at low concentration induces mitotic arrest. **(A)** Representative phase contrast microscopy images of untreated, BI2536-, and Navitoclax-treated cells, for 24 hours, showing accumulation of rounded and bright cells (mitotic cells). Cells treated with DMSO (compound solvent) and Nocodazole (antimitotic agent) were used as controls. **(B)** Mitotic index of data shown in A with statistical relevance of *** $p < 0.001$ by One-way ANOVA with Tukey's multiple comparisons test.

3.3 Inhibition of an anti-apoptotic pathway by Navitoclax accelerates death of cells arrested in mitosis by BI2536

As mentioned before, different cell fates are observed after treatment with antimitotic agents. To access the effect of the combinational treatment on cell fate, we

performed live-cell imaging using time-lapse differential interference contrast (DIC) microscopy (Figure 15 A). A549 tumor cells were treated with 15 nM of BI2536 and 220 nM of Navitoclax alone or in combination for 48h. Cell fate was followed since the first mitosis. As expected, BI2536-treated cells arrested in mitosis for several hours (918.2 ± 263.5 min) (Figure 15 B), while untreated and Navitoclax-treated cells undertook mitosis for about 30 min (30.5 ± 7.3 min and 36.0 ± 14.1 min) (Figure 15 B, Movie 1 and Movie 2, respectively). Curiously, cells treated with the combinational treatment arrested in mitosis during a shorter period than BI2536-treated only cells (558.2 ± 232.0 min) (Figure 15 B). Focusing on cell fate, Navitoclax-treated cells were able to divide normally, suggesting that at this concentration Navitoclax alone is not toxic to tumor cells (Figure 15 C and Movie 2). Different fates were observed in BI2536-treated cells, 21.1% of cells died in mitosis (DiM), which is the desired fate of cells treated with antimitotics (Figure 15 C and Movie 3). However, 78.9% of BI2536-treated cells exited mitosis without dividing, of which 53.3% died after slippage (post slippage death, PSD) and 46.7% have survived (post slippage survival, PSS) during the recorded time (Figure 15 C, Movie 5 and Movie 4, respectively). Interestingly, when treated with both compounds, 76.5% of cells died in mitosis (Figure 15 C and Movie 6). 5.9% of cells were able to complete mitosis but died in the following interphase (post mitotic death, PMD) (Figure 15 C). Additionally, percentage of cells that exited mitosis without dividing (17.6%) was significantly reduced compared to treatment with BI2536 alone (78.9%), of which 66.7% died after slippage and 33.3% have survived (Figure 15 C). Thus, the observation that cells treated with both compounds arrested in mitosis for a shorter period than BI2536-treated cells, is suggestive of an acceleration of cell death in mitosis potentiated by Navitoclax. Taken together, these results are indicative that inhibiting the anti-apoptotic pathway by Navitoclax, facilitates a faster accumulation of pro-apoptotic signals, resulting in an accelerated death in mitosis.

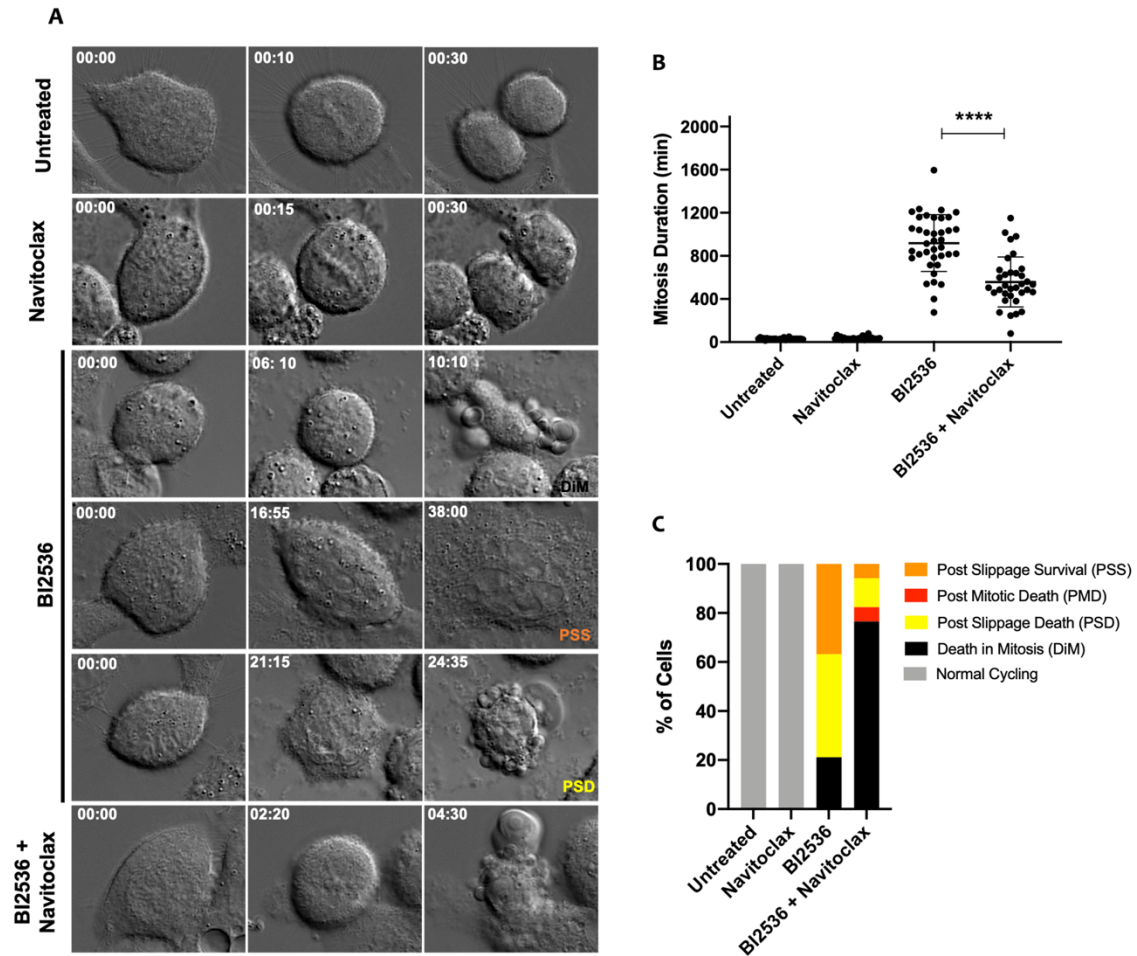


Fig. 15- Navitoclax enhances cell death in mitosis after mitotic arrest induced by BI2536. **(A)** Representative time-lapse sequences of untreated, individual, or combinatorial treated cells. Untreated and Navitoclax-treated cells undertake mitosis for about 30 min. BI2536-treated cells arrest in mitosis during several hours (918.2 ± 263.5 min) and die in mitosis (top) or undergo slippage followed by death (bottom) or stay alive (middle). Combinational treated cell arrest in mitosis for less time than BI2526-treated cells (558.3 ± 232.0 min) once Navitoclax accelerates cell death in mitosis. Numbers indicate times in 00 h:00 min. **(B)**. Quantification of mitosis duration over different treatments as determined by time-lapse microscopy with statistical relevance of **** $p < 0.0001$ by one-way ANOVA with Tukey's multiple comparisons test. Each spot represents one cell. **(C)**. Quantification of cell fate after treatment. Percentage of cells undergoing death in mitosis (DiM), post mitotic death (PMD), post slippage death (PSD), cells that remain alive after slippage (post slippage survival, PSS), and cells with normal cycling, over the total number of cells are represented.

3.4 The combination of Navitoclax and BI2536 enhances cancer cell death over individual treatments

Once after a prolonged arrest in mitosis induced by antimitotic agents, cells are expected to die by apoptosis, and as Navitoclax is an apoptotic potentiator, we explored whether the cell death observed in time-lapse assay was mediated by apoptosis. For this, A549 cells were treated with 15 nM of BI2536 and 220 nM of Navitoclax alone and

in combination for 48h. First, cells were observed by phase contrast microscopy. We found an accumulation of cells with characteristics that closely resembled apoptotic morphological changes (loss of adherence and shrinkage) when treated with BI2536 and with the combinational treatment (Figure 16 A). Thus, we next performed an Annexin V/PI flow cytometry assay (Figure 16 B). Annexin V is capable of binding to phosphatidylserine that is exposed on the plasma membrane outer leaflet of apoptotic cells. On the other hand, propidium iodide (PI) is a DNA intercalant fluorescent agent, which is not membrane-permeable, making it useful to differentiate necrotic, apoptotic, and healthy cells based on membrane integrity. The percentage of apoptotic cells after treatment with Navitoclax was residual ($5.4 \pm 2.4\%$), as well as in untreated ($1.6 \pm 0.7\%$) and DMSO-treated cells ($2.3 \pm 0.6\%$), in agreement with the phase contrast microscopy analysis (Figure 16 C). On the other hand, it was observed an increase of apoptotic cells after treatment with BI2536 alone ($29.8 \pm 2.7\%$) compared to untreated and DMSO-treated cells. After combinational treatment, the percentage of apoptotic cells was significantly higher ($44.9 \pm 4.3\%$) than with BI2536 alone (Figure 16 C). To confirm these results, we further performed a TUNEL assay. TUNEL assay allows detecting DNA fragmentation, a process that occurs in apoptotic cells (Figure 16 D). This assay relies on the activity of the terminal deoxynucleotidyl transferase (TdT) that is able to catalyze the attachment of deoxynucleotides (linked to a fluorochrome) to 3'-hydroxyl terminal of DNA breaks. Thus, the level of apoptosis was established by counting TUNEL-positive cells over the total number of cells for each condition. As observed in the previous results, Navitoclax alone did not induce a significant increase of apoptosis ($2.3 \pm 0.7\%$) compared to untreated ($0.2 \pm 0.3\%$) and DMSO-treated cells ($0.4 \pm 0.2\%$) (Figure 16 E). While in BI2536-treated cells it was observed an increase of apoptotic cells ($14.5 \pm 6.6\%$), and when treated with both compounds, the extension of apoptosis was even higher ($56.9 \pm 10.1\%$) (Figure 16 E). These results indicate that inhibition of the anti-apoptotic pathway by Navitoclax sensitizes tumor cells to the Plk1 inhibitor BI2536. Furthermore, since the extension of apoptosis in both assays was significantly higher in the combinatorial treatment than the sum of the effect of the individual treatments, this suggests that BI2536 and Navitoclax had a synergistic effect on promoting cancer cell death by apoptosis.

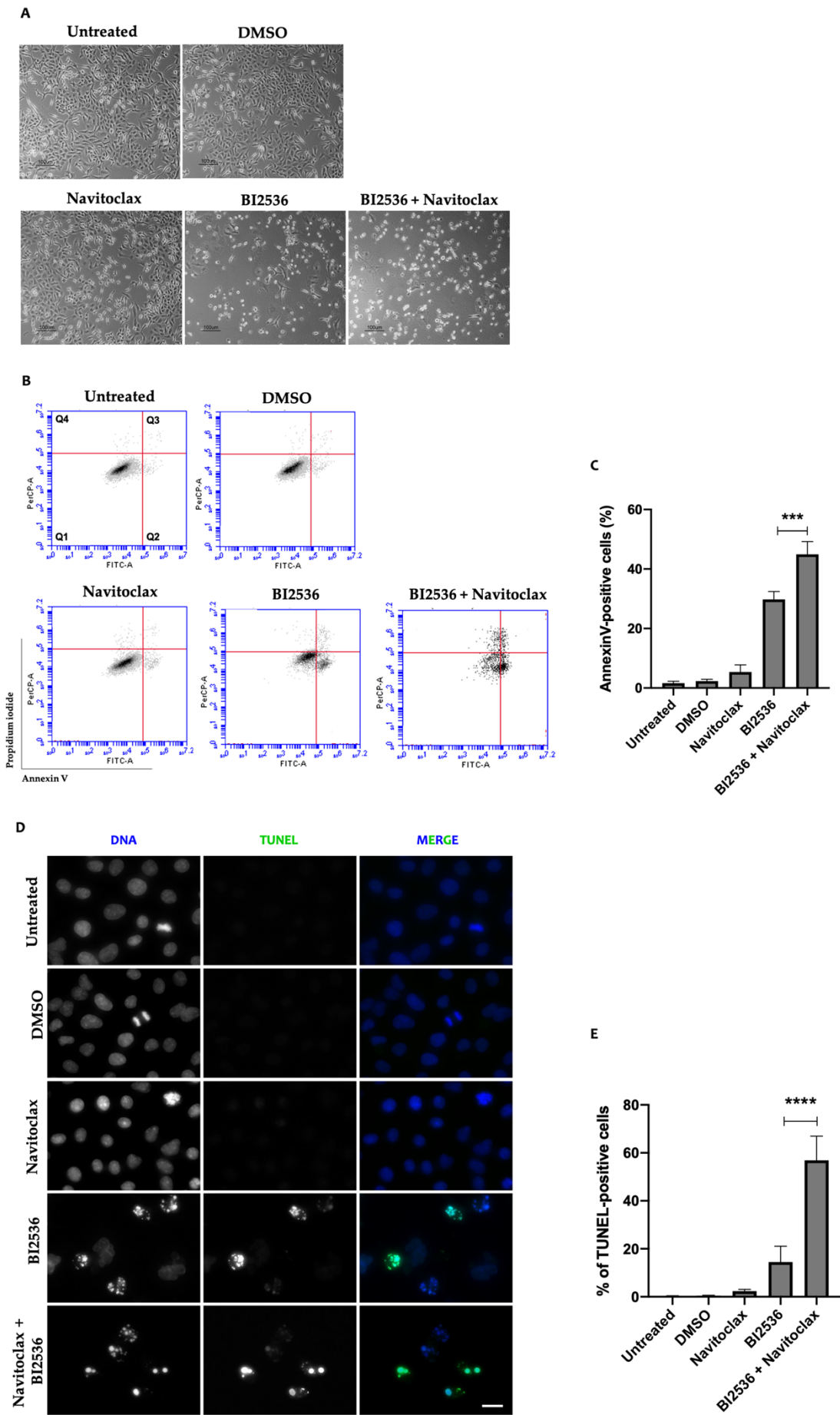


Fig. 16- Combination of Navitoclax and BI2536 potentiates cancer cell death by apoptosis. **(A)** Representative phase contrast microscopy images after treatment with BI2536 and Navitoclax for 48h showing accumulation of death cells when treated with BI2536 alone and in combination with Navitoclax. DMSO was included as negative control. Flow cytometry analysis of apoptosis by Annexin V/PI co-staining, 48 hours treatment. **(B)** Representative cytogram and **(C)**. Quantification of Annexin V-positive cells are shown with statistical relevance of *** $p < 0.001$ by one-way ANOVA with Tukey's multiple comparisons test. The quadrants Q were defined as Q1 = live cells (Annexin V- and PI-negative), Q2 = early stage of apoptosis (Annexin V-positive/PI-negative), Q3 = late stage of apoptosis (Annexin V- and PI-positive) and Q4 = necrosis (Annexin V-negative/PI-positive). **(D)** Representative images of apoptotic cells are shown after 48 hours of individual or Navitoclax and BI2536 co-treatment by TUNEL assay to detect DNA fragmentation (green). DNA (blue) was stained with DAPI. Bar, 5 μm . **(E)** Quantification of TUNEL-positive cells. **** $p < 0.0001$, by one-way ANOVA with Tukey's multiple comparisons test. The error bars represent mean $\pm$ SD.

4. Discussion

Inhibition of Plk1 has been extensively explored as a therapeutic strategy against cancer. We showed that Plk1 is overexpressed in NSCLC cell lines highlighting the relevance of Plk1 as drug target for cancer therapy. Several Plk1 inhibitors entered clinical trials and generally demonstrated poor efficacy (Novais et al., 2021). Mitotic slippage has been pointed as one of the reasons for the therapeutical failure of mitotic blockers, including Plk1 inhibitors (De Martino et al., 2018; Raab et al., 2015). In this study, we demonstrate that Navitoclax can influence cancer cell death and slippage, after mitotic arrest imposed by the Plk1 inhibitor BI2536. Since what dictates whether cells die in mitosis or undergo slippage in response to antimitotic agents is the relationship between the rate of degradation of cyclin B and the amount of death signals, this study suggests that after mitotic arrest, Navitoclax by inhibiting the anti-apoptotic proteins BCL-2 and BCL-XL, favors the accumulation of death signals, thereby, accelerating cell death in mitosis by apoptosis, and consequently preventing slippage. These results are in agreement with previous studies in which Navitoclax potentiated cell death after mitotic arrest imposed by antimitotic agents namely Eg-5 inhibitors and paclitaxel (J. Shi et al., 2011; N. Tan et al., 2011). Importantly, we showed that BI2536 and Navitoclax synergizes to induce tumor cell death by apoptosis, at concentrations lower than their respective IC_{50} previously determined by our group. Thus, this provides an opportunity to reduce the dose of both compounds in clinical trials which is expected to minimize their hematologic toxicity. For this, further *in vivo* studies are required to explore this combinational treatment. In sum, we demonstrate that Navitoclax by inhibiting the anti-apoptotic pathway sensitizes lung cancer cells to the second-generation antimitotic BI2536, providing a promising combinational treatment for cancer therapy.

Chapter IV

Identifying new antimetabolites

GROUP I

Tetracyclic thioxanthene derivatives: Study on fluorescence and antitumor activity

The information presented in this section was based in the following published paper:

Durães F, Silva PMA, **Novais P**, Amorim I, Gales L, Esteves CIC, Guieu S, Bousbaa H, Pinto M, Sousa E. Tetracyclic Thioxanthene Derivatives: Studies on Fluorescence and Antitumor Activity. *Molecules*. 2021 May 31;26(11):3315. doi: 10.3390/molecules26113315. PMID: 34073048; PMCID: PMC8198043.

1. Introduction

The MTAs are the current antimitotics used in the clinic (Perez, 2009). However, MTAs have limited effectiveness in several cancer due to their side effects and various resistance mechanisms (Perez, 2009). Therefore, discovery of new antimitotics that can overcome these disadvantages are required. Recently, tetracyclic thioxanthenes were described to exhibit potential antitumor activity (Hedidi et al., 2017; Mokhtari Brikci-Nigassa et al., 2020). In this study three tetracyclic thioxanthenes derivatives were evaluated, TX2.1a, TX2.19, and TX2.17. The three tetracyclic thioxanthenes of this study were previously synthesized based on a thioxanthone which emerged as hit compound with antitumor properties, highlighting the potential of these molecules (Barbosa et al., 2016; Lima et al., 2016). Thus, to evaluate the potential antitumor activity of these molecules, we accessed their growth inhibitory activity in cancer cells, by a Sulforhodamine B (SRB) assay. The compounds with promising growth inhibitory activity were selected to further investigate whether they possessed antimitotic activity.

2. Material and methods

2.1 Compounds

The tetracyclic thioxanthenes TX2.1a, TX2.19, and TX2.17, were provided by Faculty of Pharmacy, U. Porto. All compounds were reconstituted in sterile dimethyl sulfoxide (DMSO, Sigma-Aldrich Co. Ltd.) to a stock concentration of 60 mM. To preserve compound activity, several aliquots were prepared and stored at -20 °C. In the day of experiments, all compounds were diluted in fresh culture medium at desired concentrations.

2.2 Cell lines and culture conditions

A375-C5 (melanoma), MCF-7 (breast adenocarcinoma) and NCI-H460 (non-small cell lung cancer) cells were obtained from European Collection of Cell Culture were grown in RPMI-1640 culture medium (Roswell Park Memorial Institute, Biochrom) supplemented with 5% heat-inactivated fetal bovine serum (FBS, Biochrom). HPAEpiC (Human Pulmonary Alveolar Epithelial Cells) was obtained from ScienCell Research Laboratories and grown in DMEM medium (Dulbecco's Modified Eagle's, Biochrom) supplemented with 5% heat-inactivated FBS and 1% of non-essential amino acids (Sigma-Aldrich Co. Ltd.). Cell lines were cultured in 25 cm² VWR cell culture flasks with complete respective growth culture medium and maintained in a humidified incubator at 37 °C with 5% CO₂ (Hera Cell, Heraeus).

2.3 Sulforhodamine B (SRB) assay

A total of 5.5×10^4 NCI-H460, A375-C5, and MCF-7 tumor cells, and 6×10^4 of HPAEpiC non-tumor lung cell lines were seeded, with complete culture medium, in 96-well plates, and incubated for 24h at 37 °C and 5% of CO₂. The cells were then treated with two-fold dilutions of TX2.1a, TX2.19, and TX2.17 ranging from 0 to 75 µM and doxorubicin, used as a positive control, ranging from 0 to 0.07 µM. After 48h, cells were fixed with 50% (m/v) trichloroacetic acid (Merck Millipore), for 1h at 4 °C, and then washed with distilled water and left to dry. Then, cells were stained with Sulforhodamine B (SRB, Sigma-Aldrich Co. Ltd.), for 30 min at room temperature, and washed 5 times with 1% (v/v) acetic acid (Merck Millipore) and left to dry. SRB complexes were then solubilized with 10 mM Tris buffer (Sigma-Aldrich Co. Ltd.) for 30 min, and the absorbance was measured at 515 nm in a microplate reader (Biotek Synergy 2, BioTek Instruments, Inc.). A dose-response curve was obtained for each cell line treated with the compounds, and the concentration that caused cell growth inhibition of 50% (GI₅₀) was determined. The non-tumor cells HPAEpiC were used to assess the degree of selectivity of the compounds, as the ratio of GI₅₀ in non-tumor cells over GI₅₀ in cancer cells.

2.4 Phase contrast microscopy

A total of 9.0×10^4 NCI-H460 were seeded, with complete culture medium, in 6-well plates, for 24h. Cells were then treated for 24h, with 5.66 µM and 11,32 µM of compound TX2.1a, and with 1 µM of Nocodazole (Sigma-Aldrich Co. Ltd.) used as a positive control. DMSO, up to 0.25% concentration, were included as compound solvent control. Cells were then observed by phase contrast microscopy, using Zeiss Primo Vert microscope (Carl Zeiss) and a Nikon TE 2000-U microscope (Nikon) with a 10× objective were used. The Nikon microscope used a DXM1200F digital camera, with Nikon ACT-1 software (Melville).

2.5 Fluorescence microscopy

To analyze compound-associated intracellular fluorescence, a total of 9.0×10^4 cells were seeded on coverslips, allowing attachment for 24h, and were treated with 5.66 µM of compound TX2.1a. After 48h, cells were fixed with fresh 2% (w/v) paraformaldehyde (Sigma-Aldrich Co. Ltd) in PBS for 12 min, followed by 3 times wash in PBS for 5 min and then permeabilized with 0.5% (v/v) Triton X-100 (Sigma-Aldrich Co. Ltd.) diluted in PBS for 7 min. DNA was stained with 2 µg/ml 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Co. Ltd) diluted in Vectashield mounting medium (Vector, H-1000). Data analysis and image acquisition were performed using an Axio Observer Z.1 SD

microscope (Carl Zeiss), coupled to an AxioCam MR3, and with the Plan Apochromatic 63x/NA 1.4 objective. Fluorescence images were processed using ImageJ version 1.51

2.6 Flow Cytometry Analysis

For flow cytometry analysis, cells exposed to 5.66 μM of TX2.1a for 48 h were harvested, washed twice in PBS and the mean fluorescence intensity (MFI) of FITC-A was recorded in BD Accuri™ C6 Plus Flow cytometer (BD Biosciences). Cells treated with 1 μM of Rhodamine-123 (Sigma-Aldrich Co. Ltd.) were used as positive control. A total of 20.000 events per sample were collected. Data were analyzed with BD Accuri™ C6 Plus software, version 1.0.27.1

3. Results

3.1 Tetracyclic thioxanthenes exhibit tumor growth inhibitory activity

Firstly, all compounds were tested for their capability of inhibiting the growth of human tumor cell lines by Sulforhodamine B (SRB) assay which allowed to determine the GI_{50} , which represents the concentration that causes 50% cell growth inhibition. SRB assay was performed in three human cancer cell lines: A375-C5 (malignant melanoma), MCF-7 (breast adenocarcinoma), and NCI-H460 (non-small cell lung cancer). Among three thioxanthenes, TX2.1a has emerged as hit compound presenting the lowest GI_{50} ranging from 5 to 7 μM in the tested cell lines, while TX2.19 and TX2.17 harbored a GI_{50} with a range from 8 to 11 μM and 16 to 23 μM , respectively (Table 2).

Table 2- Cell growth inhibitory activity of tetracyclic thioxanthenes

	GI_{50} (μM)		
	A375-C5	MCF-7	NCI-H460
TX2.1a	6.48 $\pm$ 0.88	6.37 $\pm$ 0.49	5.66 $\pm$ 0.89
TX2.19	22.88 $\pm$ 3.75	18.85 $\pm$ 1.42	16.97 $\pm$ 3.12
TX2.17	8.02 $\pm$ 3.59	9.04 $\pm$ 0.89	10.64 $\pm$ 0.31
Doxorubicin	0.016 $\pm$ 0.005	0.018 $\pm$ 0.003	0.024 $\pm$ 0.006

The antitumor activity is presented as the concentrations that cause 50% cell growth inhibition (GI_{50}) after a continuous exposure for 48 h and represents mean $\pm$ SD from at least three independent experiments. Doxorubicin was used as positive control for the SRB assay.

We also performed cell toxicity study of the compounds using non-tumor Human Pulmonary Alveolar Epithelial Cells (HPAEPiC). We found that the GI_{50} of the four compounds in the non-tumor was higher than that in cancer cell lines under study (Table 3). The compounds exhibited a high degree of selectivity, as determined by selectivity

index calculation, suggesting that they are not toxic to healthy cells, at least at concentrations that are toxic to cancer cells.

Table 3- Selectivity index of tetracyclic thioxanthenes

	HPAEPiC	Selectivity Index		
	GI ₅₀ (μM)	A375-C5	MCF-7	NCI-H460
TX2.1a	26.75± 1.20	4.13	4.19	4.73
TX2.19	>150	-	-	-
TX2.17	127.9± 1.56	5.59	6.79	7.54
Doxorubicin	0.054± 0.005	3.38	3.00	2.25

Results are expressed as mean ± SD of three independent experiments.

3.2 Tetracyclic thioxanthene TX2.1a does not interfere with mitosis

Because it presented the lowest GI₅₀, the compound TX2.1a was selected to further investigate whether it harbored antimitotic activity. NCI-H460 tumor cells were selected for further studies once was the cell line in which TX2.1a presented the lowest GI₅₀. Cells were treated with 5.66 μM or 11,32 μM of TX2.1a (one and two-fold GI₅₀, respectively), and after 24h treatment, were analyzed by phase contrast microscopy (Figure 17). Nocodazole (antimitotic agent) was used as positive control and DMSO as compound solvent control. In cells treated with nocodazole, it was observed an accumulation of bright and rounded cells, reminiscent of mitotic cells, as expected. However, we did not notice an accumulation of mitotic cells after treatment with TX2.1a at both concentrations. We also analyzed TX2.1a-treated cells by fluorescence microscopy upon staining of DNA with DAPI (Figure 17). Based on chromatin morphology and condensation, we observed that most of the TX2.1a-treated cells were in interphase, and few cells in mitosis. Therefore, as we did not notice an accumulation of mitotic cells, TX2.1a does not exert its growth inhibitory activity by inhibiting mitosis.

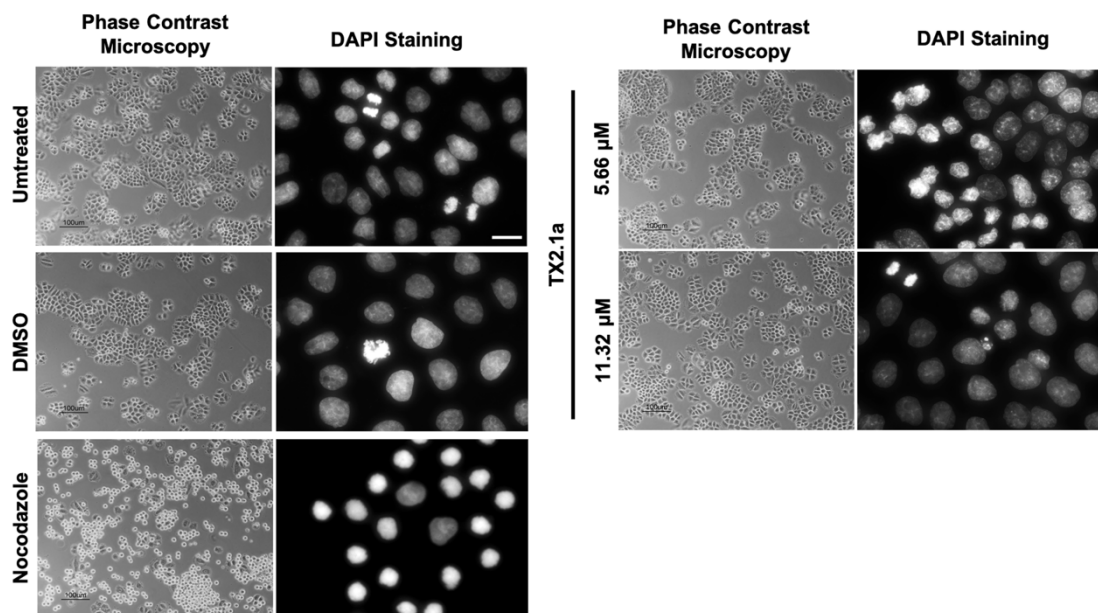


Fig. 17- TX2.1a does not interfere with mitosis. Representative phase contrast microscopy (Left) and DAPI staining (Right) images of untreated, DMSO-, nocodazole- and TX2.1a-treated cells, after 24 hours exposure to TX2.1a showing no accumulation of mitotic cells. Bar, 5 µm.

3.3 Tetracyclic thioxanthene TX2.1a exhibit fluorescence activity

Thioxanthene derivatives have been shown to harbor photochemical properties (Ataci et al., 2018, 2020). In this regard, a fluorescence microscopy analysis was undertaken to check the brightness of the TX2.1a in culture cells. The analysis revealed that compound TX2.1a emitted a bright intracellular fluorescence in the green channel, when cells were excited with 470 nm light (Figure 18 A). The fluorescence staining accumulated within intracellular round-shaped structures that closely resemble endosome/lysosome structures, which led us to suggest that the compound may accumulate in those structures. However, this was not confirmed by the colocalization study using the lysosome marker LAMP-1 antibody (data not shown). The fluorescence staining remained associated with the cells until cell death (Figure 18 A). The fluorescence property of the compound TX2.1a was confirmed when treated cells were analyzed by flow cytometry (Figure 18 B). A peak of fluorescent cells was obtained in the green channel, near the peak of Rhodamine 123, used as positive control. Moreover, the fluorescence was stable after keeping the cell preparations in the refrigerator for more than two weeks. Additional studies are needed to elucidate the exact subcellular localization of compound TX2.1a.

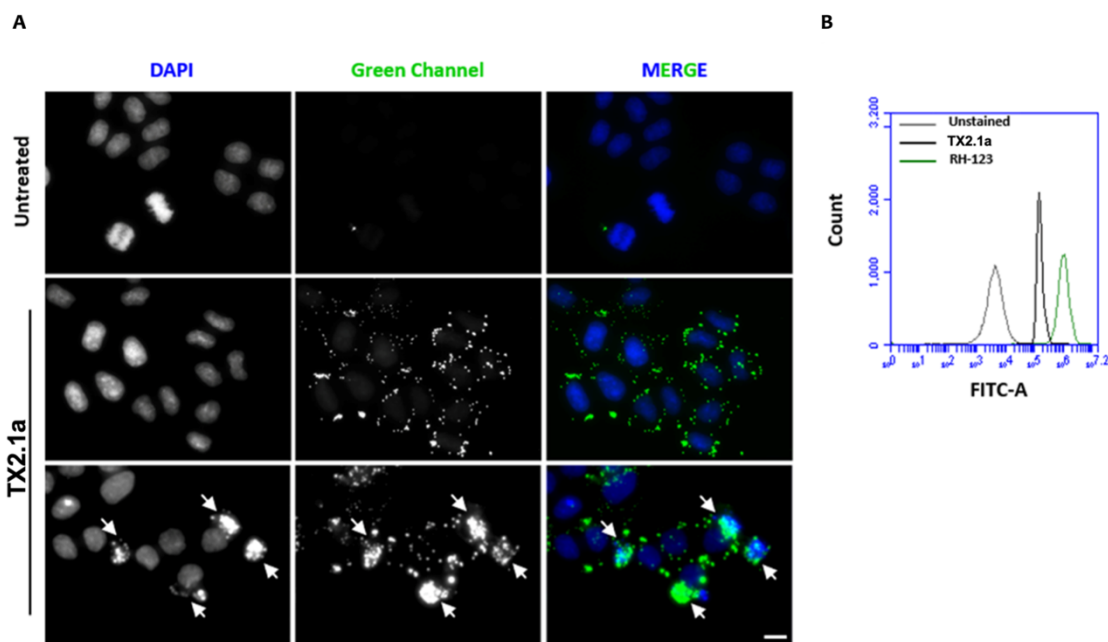


Fig. 18- Cancer cells treated with TX2.1a emitted fluorescence in the green channel. **(A)** Representative images of untreated (top) and TX2.1a-treated cells (middle and bottom), showing intracellular green fluorescence as emitted by the compound. Middle: representative microscopic field showing (still) living cells treated with the compound: intact nuclei can be seen after DAPI staining of DNA (blue); in the green channel, fluorescence staining of the compound is seen in the cytoplasm, excluding the nuclei. Bottom: representative microscopic field showing treated cells undergoing cell death (white arrows): micronuclei can be seen after DAPI staining (blue), and a bright fluorescence is seen due to chromatin over-condensation and shrinking of dead cells. Bar, 5 μ m. **(B)** Representative flow cytometry histogram of cell count versus FITC-A intensity in untreated (unstained) and TX2.1a-treated cells. Rhodamine-123, green fluorescent dye, was used as positive control.

4. Discussion

In this study we reported that three newly synthesized tetracyclic thioxanthene derivatives harbors potent tumor growth inhibitory activity *in vitro*, with a high degree of selectivity to tumor cells. Among all molecules, TX2.1a emerged as promising compound with the lowest GI₅₀. Despite TX2.1a does not exert its inhibitory activity as an antimitotic agent, we found that it harbors fluorescence characteristic. These two characteristics (antitumor and fluorescence activity) make TX2.1a a potential theranostic cancer drug candidate. Theranostic, the combination of therapy and diagnostic, has been gathering importance, particularly in cancer, through the application of molecular imaging towards the identification of cancer targets and the optimization of therapy (Penet et al., 2012). Importantly, the fluorescence of TX2.1a was stable for more than two weeks, highlighting its potential use as a stable theranostic agent. We showed that TX2.1a accumulates

within subcellular structures, therefore, it could be used to target the respective subcellular structure, as a plausible therapeutic intervention to potentiate cancer cell death. For this, future work is needed to unveil the subcellular localization of the compound and its relevance for potential application to cancer therapy.

Chapter IV

Identifying new antimitotics

GROUP II

The diarylpentanoid BP-M345 is a potent antitumor agent with
antimitotic activity

1. Introduction

In addition to the tetracyclic thioxanthene derivatives, we also evaluate a diarylpentanoid, BP-M345. Diarylpentanoids have been reported to exhibit antitumor activity by interfering with multiple biologic process, including cell cycle (Moreira et al., 2021; Paulraj et al., 2019). A recent study explored the activity of BP-M345 on activation of the p53 pathway through inhibition of p53 interaction with MDM2. Despite BP-M345 has demonstrated growth inhibitory activity in colon tumor cells HCT116, the results showed that BP-345 did not inhibit the interaction of p53 with MDM2 (Moreira et al., 2021). Thus, we investigated whether BP-M345 exerts its antitumor activity, by acting as an antimitotic agent.

2. Material and Methods

2.1 Compound

The diarylpentanoid BP-M345 was provided by Faculty of Pharmacy, U. Porto. BP-M345 was reconstituted in sterile dimethyl sulfoxide (DMSO, Sigma-Aldrich Co. Ltd) to a stock concentration of 60 mM. To preserve compound activity, several aliquots were prepared and stored at -20 °C. In the day of experiments, all compounds were diluted in fresh culture medium at desired concentrations.

2.2 Cell lines and culture conditions

A375-C5 (melanoma), MCF-7 (breast adenocarcinoma) and NCI-H460 (non-small cell lung cancer) cells were obtained from European Collection of Cell Culture and were grown in RPMI-1640 culture medium (Roswell Park Memorial Institute, Biochrom) supplemented with 5% heat-inactivated fetal bovine serum (FBS, Biochrom). HPAEpiC (Human Pulmonary Alveolar Epithelial Cells) was obtained from ScienCell Research Laboratories and grown in DMEM medium (Dulbecco's Modified Eagle's, Biochrom) supplemented with 5% heat-inactivated FBS and 1% of non-essential amino acids (Sigma-Aldrich Co. Ltd.). Cell lines were cultured in 25 cm² VWR cell culture flasks with complete respective growth culture medium and maintained in a humidified incubator at 37 °C with 5% CO₂ (Hera Cell, Heraeus).

2.3 Sulforhodamine B (SRB) assay

A total of 5.5 x 10⁴ NCI-H460, A375-C5, and MCF-7 tumor cells, and 6.0 x 10⁴ of HPAEpiC non-tumor lung cell lines were seeded, with complete culture medium, in 96-well plates, and incubated for 24h at 37 °C and 5% of CO₂. The cells were then treated with two-fold dilutions of BP-M345 ranging from 0 to 1.17 μM and doxorubicin, used as

a positive control, ranging from 0 to 0.07 μM . After 48h, cells were fixed with 50% (m/v) trichloroacetic acid (Merck Millipore), for 1h at 4 °C, and then washed with distilled water and left to dry. Then, cells were stained with Sulforhodamine B (SRB, Sigma-Aldrich Co. Ltd.), for 30 min at room temperature, and washed 5 times with 1% (v/v) acetic acid (Merck Millipore) and left to dry. SRB complexes were then solubilized with 10 mM Tris buffer (Sigma-Aldrich Co. Ltd.) for 30 min, and the absorbance was measured at 515 nm in a microplate reader (Biotek Synergy 2, BioTek Instruments, Inc.). A dose-response curve was obtained for each cell line treated with the compounds, and the concentration that caused cell growth inhibition of 50% (GI_{50}) was determined. The non-tumor cells HPAEpiC were used to assess the degree of selectivity of the compounds, as the ratio of GI_{50} in non-tumor cells over GI_{50} in cancer cells.

2.4 Mitotic index determination

A total of 9.0×10^4 NCI-H460 tumor cells were seeded, with complete culture medium, in 6-well plates, for 24h. Cells were then treated for 16h, with 0.74 μM of BP-M345 and with 1 μM of Nocodazole (Sigma-Aldrich Co. Ltd.) used as a positive control. DMSO, up to 0.25% concentration, were included as compound solvent control. Cells were then observed by phase contrast microscopy, using Zeiss Primo Vert microscope (Carl Zeiss, and a Nikon TE 2000-U microscope (Nikon) with a 10 $\times$ objective were used. The Nikon microscope used a DXM1200F digital camera, with Nikon ACT-1 software (Melville) Then at least 3000 cells were accounted for the determination of the mitotic index (MI), from random phase-contrast microscope fields. MI was calculated using the following formula: $\text{MI} (\%) = (\text{number of mitotic cells} / \text{total number of cells}) \times 100$.

2.5 Flow Cytometry

For cell cycle analysis, after 16h treatment with 0.74 μM of BP-M345 and with 1 μM of Nocodazole used as a positive control, NCI-H460 cells were harvested, washed twice in PBS and fixed in ice-cold 70% ethanol (PanReac AppliChem) at 4 °C for 30 min. DMSO, up to 0.25% concentration, were included as compound solvent control. Then, cells were treated with 5 $\mu\text{g/mL}$ of propidium iodide and 100 $\mu\text{g/mL}$ of RNase in PBS for 30 min and analyzed in the flow cytometer.

For apoptosis detection, after 24h treatment with 0.74 μM of BP-M345, floating and adherent cells were collected and processed with the “Annexin V-FITC Apoptosis Detection Kit” (eBioscience) according to manufacturer’s instructions. Fluorescence was assessed by BD Accuri™ C6 Plus Flow cytometer (BD Biosciences) and data was analysed with BD Accuri™ C6 Plus software, version 1.0.27.1

(www.bdbiosciences.com). DMSO, up to 0.25% concentration, were included as compound solvent control. For cell cycle and apoptosis analysis, at least 20.000 events per sample were collected.

2.6 Indirect Immunofluorescence

NCI-H460 cells were grown on poly-L-lysine-coated coverslips, for 24h. Then cells were treated with 0.74 μM of BP-M345 and after 16h were fixed with methanol (PanReac AppliChem) for 10 min at -20°C and washed 3 times with PBS for 5 min. After washing in PBS, cells were blocked with 10% FBS in 0.05% Tween-20 (Sigma-Aldrich Co. Ltd.) in PBS (PBST) for 30 min at room temperature. Cells were then incubated with primary antibodies diluted in 5% FBS in PBST, for 1h. The following primary antibodies were used: human anti-CREST (1:4000, gift from E. Bronze-da-Rocha) mouse anti- α -tubulin (1:2500, Sigma-Aldrich Co. Ltd.) mouse anti-BubR1 (1:200, Milipore Chemicon). Cells were then washed 3 times with PBST for 5 min, and incubated, for 1h with Alexa Fluor 488 and 568 conjugated secondary antibodies (Molecular Probes), diluted at 1:1500. DNA was stained with 2 $\mu\text{g/mL}$ 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Co. Ltd.) diluted in Vectashield mounting medium (Vector, H-1000,).

2.6.1 MG-132 Proteasome Inhibitor Assay

Cells were treated with 0.74 μM of BP-M345, for 16h, followed by addition of 10 μM of the proteasomal inhibitor MG-132 (Sigma-Aldrich Co. Ltd.) for 1 h. Then was performed the immunofluorescence using an antibody mouse anti- α -tubulin (1:2500, Sigma-Aldrich Co. Ltd.). Untreated and DMSO-treated cells were included as controls. The number of metaphases with full alignment and metaphases with misaligned chromosomes was quantified in 10 random microscope fields.

2.6.2 Cold treatment assay

NCI-H460 cells were prepared as in the MG-132 assay and then subjected at 4°C for 5 min followed by immunofluorescence using the follows antibodies: human anti-CREST (1:4000, gift from E. Bronzeda-Rocha) mouse anti- α -tubulin (1:2500, Sigma-Aldrich Co. Ltd). The numbers of attached and free kinetochores were counted from, at least, 6 random cells.

2.7 Colony Formation Assay

NCI-H460 cells were resuspended in culture medium and seeded at 500 cells/well onto 6-well plates, allowed to attach for 24h Then, cells were treated with 0.185 μM of BP-M345. Untreated and DMSO-treated cells were included as controls. 48h later, cells were

washed with PBS and maintained for 5 days in complete culture medium without drugs. Following 10 min methanol fixation, colonies were stained with 0.05% crystal violet for 20 min and were counted to determine the efficiency of clonal formation (percentage of grown colonies from 500 seeded cells). Only colonies with more than 50 cells were accounted for.

2.8 Live-Cell Imaging

For live-cell imaging experiments, 9.0×10^4 NCI-H460 cells were seeded onto LabTek II chambered cover glass (Nunc) containing RPMI, for 24 h at 37 °C with 5% CO₂. Cells were then treated with 0.74 μM of BP-M345 and images were captured at 5 min intervals up to 48 h under differential interference contrast (DIC) optics, with a 63× objective on an Axio Observer Z.1 SD inverted microscope, equipped with an incubation chamber with the temperature set to 37 °C and an atmosphere of 5% CO₂. Movies were generated from the time-lapse images using ImageJ software version 1.51. Cell fate was followed since the first mitosis. Dead cells were classified into death in mitosis (DiM) or post mitotic death (PMD) when death occurred during or following cell division, respectively.

2.9 Image Acquisition and Processing

For phase contrast microscopy, a Zeiss Primo Vert microscope (Carl Zeiss) and a Nikon TE 2000-U microscope (Nikon) with a 10× objective were used. The Nikon microscope used a DXM1200F digital camera, with Nikon ACT-1 software (Melville). For experiments where image acquisition was performed using fluorescence, an Axio Observer Z.1 SD microscope (Carl Zeiss) was used, coupled to an AxioCam MR3, and with the Plan Apochromatic 63×/NA 1.4 objective. The deconvolution was performed with the software AxioVision Release 4.8.2 SPC and the images were processed using ImageJ VERSION 1.51.

2.10 Statistical Analysis

Statistical analysis was performed using an unpaired Student's t test or two-way ANOVA with Tukey's multiple comparisons test in the GraphPad Prism version 6. Data are presented as the mean ± standard deviation (SD) of three independent experiments and the level of statistical significance was established considering the probabilities of * p < 0.05, ** p < 0.01, *** p < 0.001 and **** p < 0.0001.

3. Results

3.1 BP-M345 exhibits a potent tumor anti-growth activity

In order to evaluate the inhibitory activity of BP-M345 on tumor cell growth, it was performed a Sulforhodamine B (SRB) assay to determine the GI_{50} of the compound in three human cancer cell lines: A375-C5 (malignant melanoma), MCF-7 (breast adenocarcinoma), and NCI-H460 (non-small cell lung cancer). BP-M345 demonstrated a potent growth inhibitory activity against the three human cancer cell lines tested with a GI_{50} range from 0.24 μ M to 0.45 μ M (Table 4).

Table 4- Tumor cell growth inhibitory activity of compound BP-M345

	GI_{50} (μ M)		
	A375-C5	MCF-7	NCI-H460
BP-M345	0.24 $\pm$ 0.01	0.45 $\pm$ 0.06	0.37 $\pm$ 0.01
Doxorubicin	0.030 $\pm$ 0.04	0.028 $\pm$ 0.01	0.028 $\pm$ 0.01

GI_{50} represents the concentration that causes 50% cell growth inhibition at 48h. Doxorubicin was used as positive control. The results are expressed as mean $\pm$ standard deviation from three independent experiments.

Additionally, the GI_{50} of BP-M345 in the non-tumor cells HPAEpiC was 1.07 $\pm$ 0.16 μ M, at least two-fold higher than in the tumor cells, suggesting that BP-M345 is less toxic to non-tumor cells, at least at concentrations that is toxic to tumor cells. Importantly, from selectivity index calculation (Table 5), we found a notable degree of selectivity of compound BP-M345 in all cell lines studied. Interestingly, the selectivity index of BP-M345 demonstrated a better degree of selectivity than Doxorubicin. These results pointed to BP-M345 as a promising and selective anticancer agent that led us to further investigate the mechanism behind their cytotoxicity

Table 5- Selectivity index of compound BP-M345

	HPAEpiC	Selectivity Index		
	GI_{50} (μ M)	A375-C5	MCF-7	NCI-H460
BP-M345	1.07 $\pm$ 0.16	4.45	2.38	2.89
Doxorubicin	0.054 $\pm$ 0.012	1.81	1.91	1.95

Doxorubicin was used as positive control.

3.2 BP-M345 arrests tumor cells in mitosis

To unveil the cytotoxic mechanism of BP-M345, we firstly analyzed by phase contrast microscopy, NCI-H460 tumor cells (which was the cell line selected to perform

the further studies due to its suitability for quantitative evaluation of morphological changes) treated with BP-M345 for 16h (Figure 19 A). Untreated cells and cells treated with Dimethyl sulfoxide (DMSO), a compound solvent, were used as control. We found an accumulation of bright and rounded cells, reminiscent of mitotic cells, under BP-M345 treatment, that mirrored nocodazole-treated cells, an antimitotic agent, used here as a positive control (Figure 19 A). Then, we calculated the mitotic index (MI) in which was observed a significant increase of MI in BP-M345-treated cells ($29.8 \pm 5.6\%$) compared to the untreated ($5.3 \pm 1.8\%$) or DMSO-treated ($5.7 \pm 1.7\%$) cells (Figure 19 B). We further investigate the cell cycle distribution by flow cytometry. Cells were treated for 16h with BP-M345 and then stained with propidium iodide, which allowed to distinguish the cell cycle phases (G0/G1, S, G2/M,) based on DNA content. We noticed that the percentage of cells in the G2/M phase was significantly higher upon treatment with BP-M345 ($35.2 \pm 7.0\%$) compared to untreated ($22.75 \pm 1.5\%$) or DMSO-treated cells ($24.56 \pm 1.4\%$), thereby corroborating the results above obtained by contrast microscopy. Taken together, it was suggestive that the compound BP-M345 was inducing mitotic arrest, thereby, exerting its anti-growth activity by acting as an antimitotic agent.

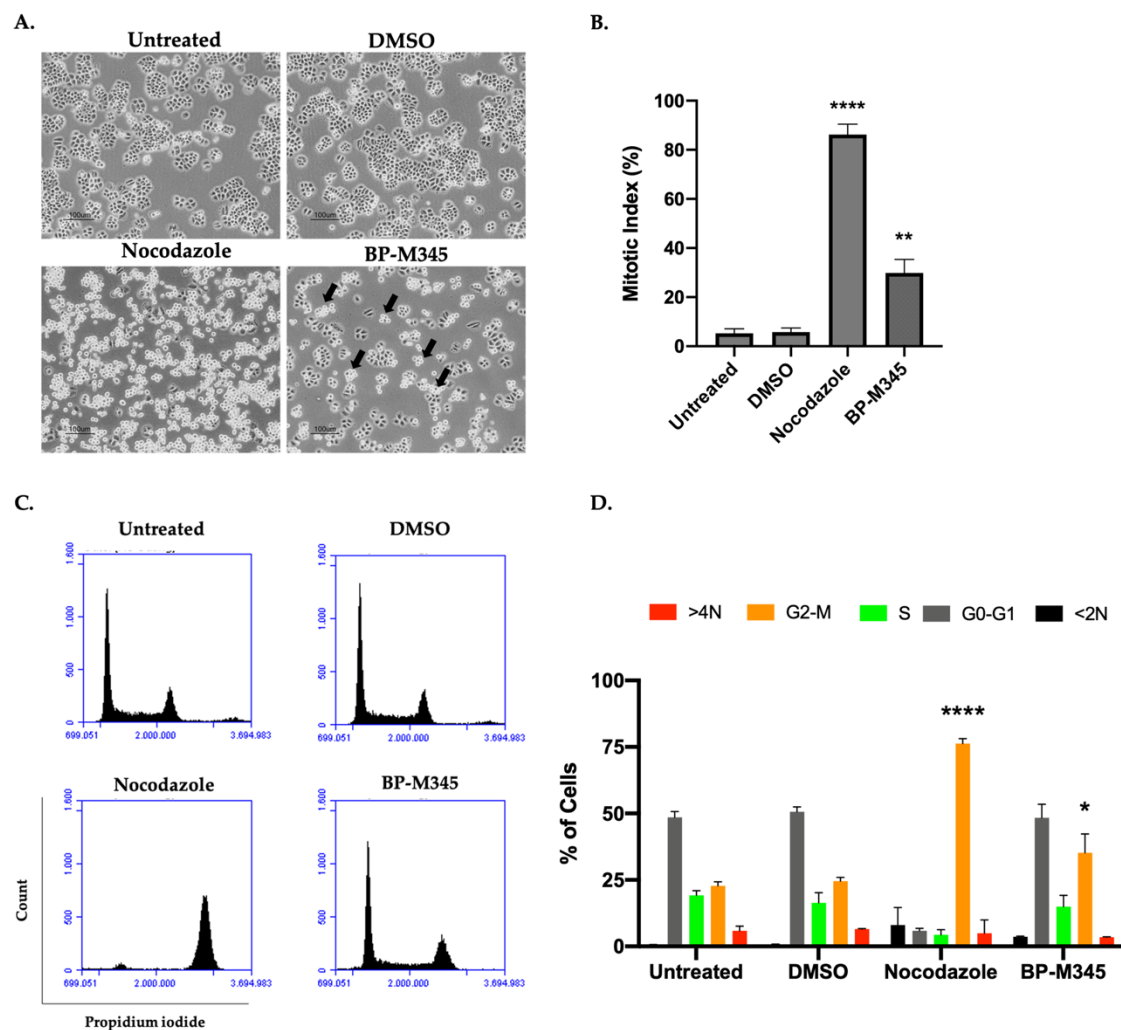


Fig. 19- Treatment with BP-M345 induces mitotic arrest of tumor cells. **(A)** Representative phase contrast microscopy images of untreated and BP-M345-treated tumor cells, for 16 hours, showing accumulation of rounded and bright cells (mitotic cells). Cells treated with DMSO (compound solvent) and Nocodazole (antimitotic agent) were used as controls. Bar, 100 μ m. **(B)** Mitotic index of data shown in A with statistical relevance of ** $p < 0.01$ and **** $p < 0.0001$ by unpaired t-test. **(C)** Representative flow cytometry histogram for cell cycle distribution using propidium iodide staining, after exposure of cells to BP-M345, for 16 hours. **(D)** Bar graphic showing the percentage of cell cycle stages (G0/G1, S G2/M) and <2N and 4N demonstrating an increase of cells in G2/M phase with statistical relevance of * $p < 0.05$ and **** $p < 0.0001$ by unpaired t-test.

3.3. BP-M345 disturbs mitotic spindle morphology and harms chromosome congression

To access the mechanism behind the mitotic arrest of BP-M345-treated cells, we performed an immunofluorescence assay using DAPI to visualize DNA and an anti- α -tubulin antibody to visualize the mitotic spindle morphology. After 16h treatment, we observed that most of BP-M345-treated cells exhibited various misaligned chromosomes and mitotic spindles with abnormal morphology, including spindles with decreased microtubule density, and some monopolar spindles, contrasting with the robust and bipolar spindle of control cells (Figure 20 A). To find out whether the misaligned chromosomes observed were a transitory or an irreversible phenotype, we quantified the number of full metaphases and metaphases with misaligned chromosomes in the presence or absence of the proteasome inhibitor MG-132. The MG-132 blocks the metaphase to anaphase transition by preventing the proteasomal degradation of cyclin B and securin (Logarinho et al., 2008; Logarinho & Bousbaa, 2008). Thus, by treating the cells with MG-132, cells have more time to align all chromosomes in the equatorial region before anaphase onset. We observed that in untreated and DMSO-treated cells the percentage of metaphases with all chromosomes aligned was significantly increased after addition of MG-132 (52.2 % to 78.5 % and 49.7% to 81.8%, respectively) (Figure 20 B). While in BP-M345-treated cells, the large majority of cells continued to exhibit misaligned chromosomes even after addition of MG-132 (96.7 % and 95.5 %, before and after addition of MG-132, respectively), suggesting that chromosome misalignment was a permanent defect that could not be corrected over time (Figure 20 B). Taken together these results indicated that the compound BP-M345 was disturbing the mitotic spindle and inducing irreversible defects in chromosome congression.

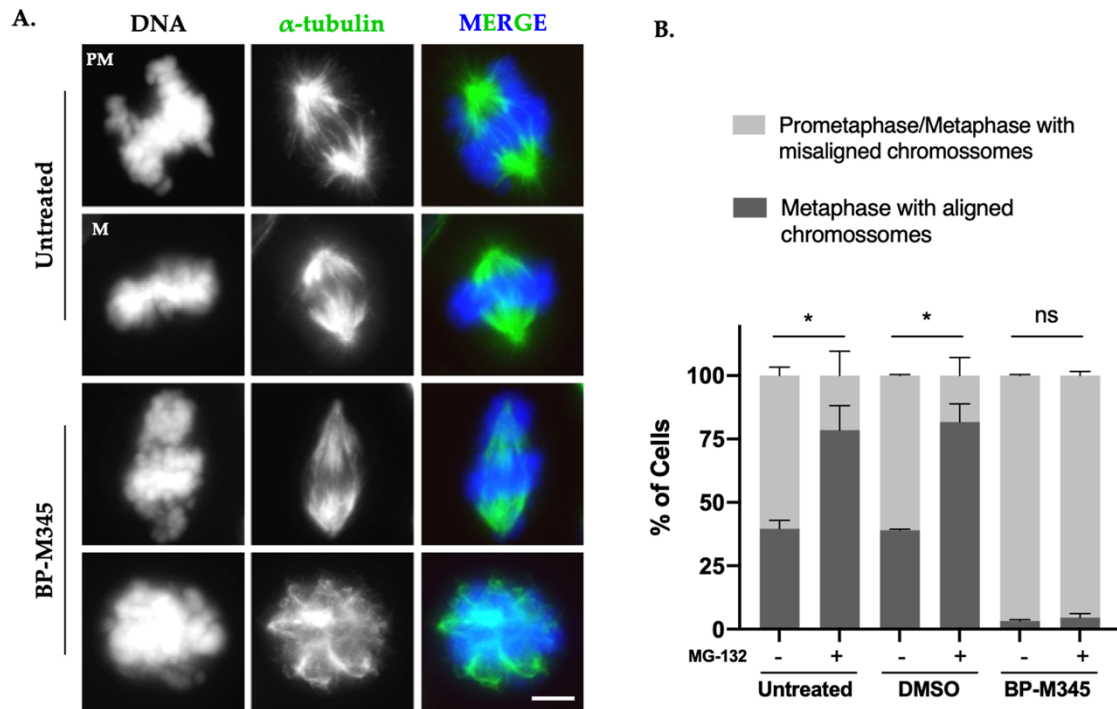


Fig. 20- BP-M345 disturbs mitotic spindle morphology. **(A)** Immunofluorescence images of untreated and BP-M345-treated cells, showing abnormal spindle configuration and misaligned chromosomes after the treatment, compared to bipolar spindle from untreated cells, in prometaphase (PM) and metaphase (M). Microtubules (green) were stained with anti- α -tubulin antibody and DNA (blue) with DAPI. Bar, 5 μ m. **(B)** Graphical representation of metaphases with misaligned vs aligned chromosomes, in absence (-) or presence (+) of proteasome inhibitor MG-132 at indicated conditions with statistical relevance of * $p < 0.05$ by two-way ANOVA with Tukey's multiple comparisons test.

3.4 BP-M345 interferes with the stability of kinetochore-microtubule attachments and promotes SAC activation

As robust and functional kinetochore-microtubule (KT-MT) attachments are required for chromosome congression and alignment, and because BP-M345 was inducing persistent misaligned chromosomes, we wondered if it was interfering with the stability of the KT-MT attachments. To evaluate this hypothesis, we performed a classic cold treatment assay. Unstable or weakened KT-MT attachments are disassembled when exposed to low temperature for a short time period, while stable and robust attachments (K-fibers) are resistant to this condition (Conly L. Rieder, 1981). Thus, we subjected the cells to 4 °C for 5 minutes under MG-132 treatment and then we performed an immunofluorescence assay using a human CREST (Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasias) antibody to localize kinetochores, and an anti- α -tubulin antibody to visualize the spindle microtubules. In untreated and DMSO-treated cells, almost all KTs remain attached to MTs (98.6 ± 0.4 %

and 98.2 ± 0.6 %, respectively) (Figure 21 A,B), while BP-M345-treated cells showed only few cold-stable KT-MT attachments (39.2 ± 8.1 %) but several free kinetochores (60.8 ± 8.1 %) (Figure 21 A,B). This result suggested that the BP-M345 weakened KT-MT attachments compromising chromosome congression, that results in the aforementioned persistent chromosome misalignment phenotype.

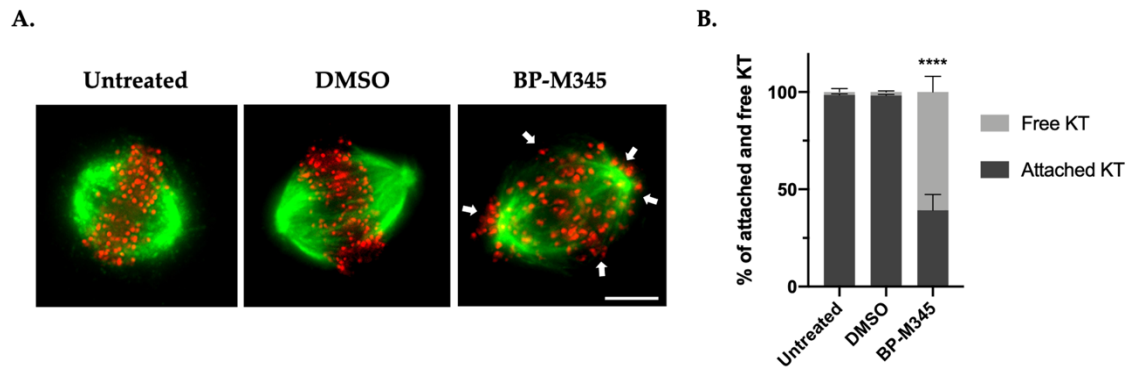


Fig. 21- BP-M345 interferes with the stability of kinetochore-microtubule attachments. **(A)** Representative immunofluorescence images after cold treatment assay, showing several unattached kinetochores (free red spots pointed by the white arrowheads) in cells with BP-M345 treatment, whereas most kinetochores were attached (Red spots with attached green fibers) in untreated cells. Microtubules (green) were stained with anti- α -tubulin antibody and the kinetochores (red) with anti-CREST antibody. Bar, 5 μ m **(B)** Quantification of cold-stable microtubules (as percentage of attached kinetochores per cell) after treatment with BP-M345 with statistical relevance of **** $p < 0.001$ by two-way ANOVA with Tukey's multiple comparisons test.

We then investigated whether BP-M345 was promoting the activation of SAC to sustain the observed mitotic arrest. The SAC is activated when there are unattached or improperly attached kinetochores, leading to mitotic checkpoint complex (MCC) assembly that ultimately inhibits the anaphase-promoting complex/cyclosome (APC/C) preventing metaphase to anaphase transition. The kinase BubR1 is a component of MCC and it is present at kinetochores when SAC is on. When kinetochores become correctly attached to microtubules and aligned at the metaphase plate, the BubR1 leaves the kinetochores during the process of MCC disassembly and SAC silencing. Thus, the BubR1 localization at kinetochores is a strong indicator that SAC is activated. In this context, we performed an immunofluorescence assay using antibodies against BubR1 and kinetochores (CREST), in untreated and BP-M345-treated cells. In untreated cells at metaphase, BubR1 localization at kinetochores was drastically reduced, as judged by the weak staining, indicating SAC inactivation (Figure 22). In contrast in BP-M345-treated cells, a strong and bright BubR1 staining was detected in all kinetochores, demonstrating that the compound was promoting SAC activation. (Figure 22) Taken together these results suggested that BP-M345 compromises the stability of KT-MT

attachments and chromosome congression resulting in a chronic activation of the SAC which induces cell arrest at mitosis.

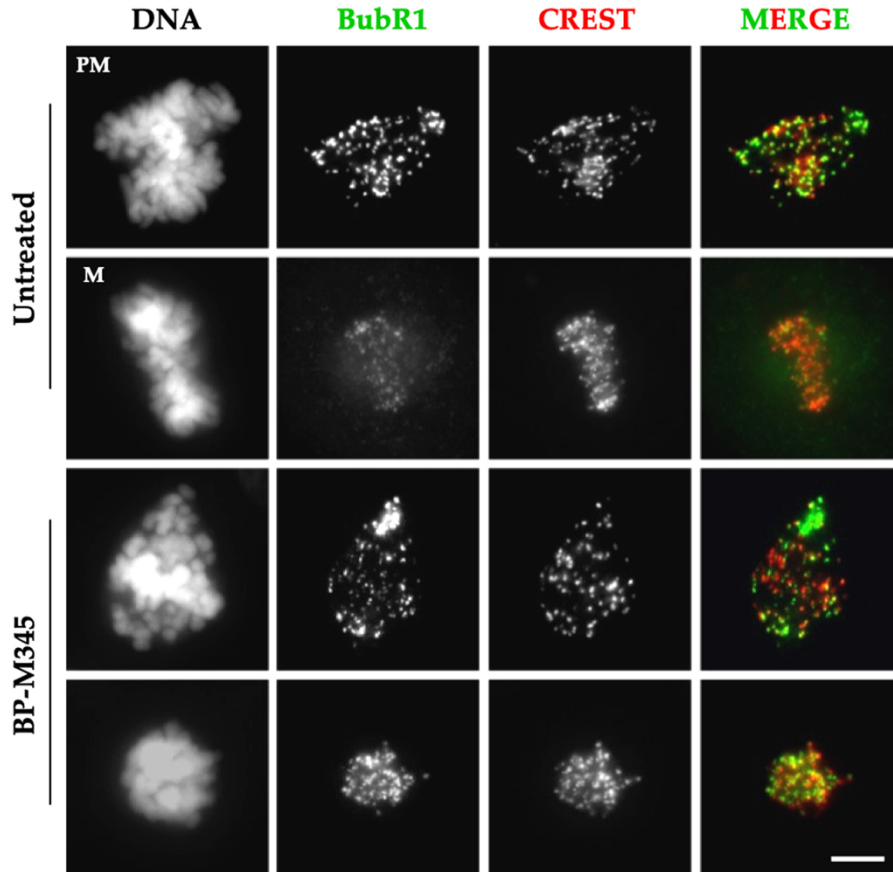


Fig. 22- Treatment with BP-M345 leads to spindle assembly checkpoint activation. Immunofluorescence staining using antibodies against BubR1 (green) and CREST (red), in untreated and BP-M345-treated cells. DNA was stained with DAPI. In untreated cells, BubR1 strongly localizes at kinetochores, in prometaphase (PM) and clearly decreases in metaphase (M) consistent with their normal localization pattern. In BP-M345-treated cells (bottom panel), BubR1 is present in all mitotic cells, indicating mitotic checkpoint activation. Bar, 5 μ m.

3.5 Fate of BP-M345-treated cells arrested in mitosis

In order to investigate the fate of cells that were arrested in mitosis by BP-M345, we performed a live-cell imaging using time lapse differential interference contrast (DIC) microscopy (Figure 23 A), during 48h. We firstly found that BP-M345-treated cells remain in mitosis for 218.7 ± 335.6 min (Figure 23 B), an average 7-fold higher than untreated cells (31.8 ± 5.6 min) (Figure 23 B and Movie 7). Fate analysis of mitotic cells showed that 35.9 % of BP-M345-treated cells died in mitosis (DiM) (Figure 23 C and Movie 8),

and 20.5% underwent post mitotic death (PMD) (Figure 23 C and Movie 9) , while 43.6% were able to continue normal cycling.

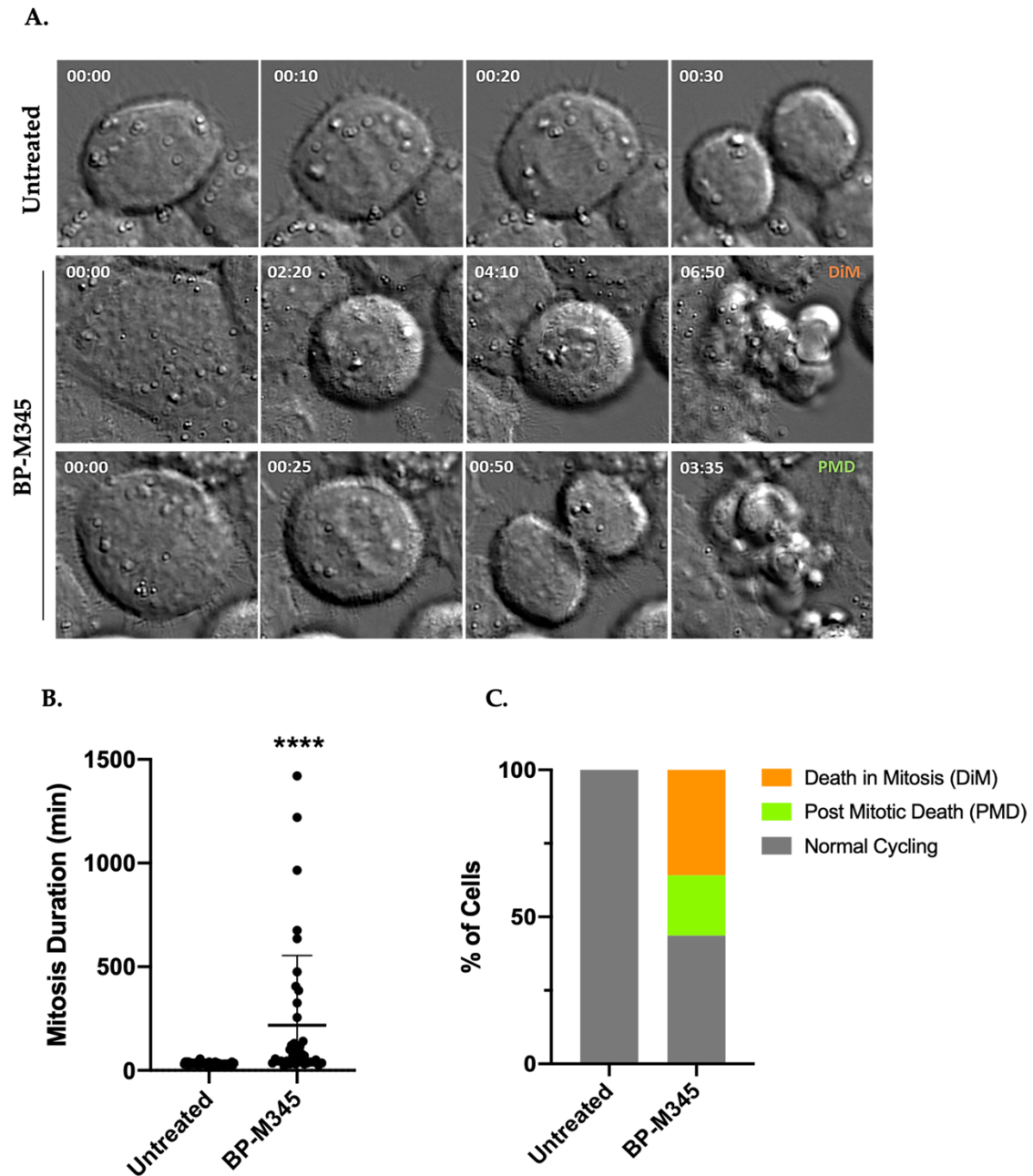


Fig. 23- BP-M345 promotes tumor cell death, mostly in mitosis **(A)** Representative time-lapse sequences of untreated and BP-M345-treated cells. Untreated cells undertake mitosis in 31.8 ± 5.6 min (top) while BP-M345-treated cells arrest in mitosis for 218.7 ± 335.6 , bottom followed by death in mitosis or post mitosis. Numbers indicate times in 00 h:00 min **(B)** Quantification of mitosis duration as determined by time-lapse microscopy in untreated and BP-M345-treated cells showing an increase of time spent in mitosis of tumor cells with statistical relevance of **** $p < 0.0001$ by unpaired t-test. Each spot represents one cell **(C)** Quantification of cell fate after treatment with BP-M345. The percentage of cells undergoing post mitotic death (PMD) and death in mitosis (DiM), and cells with normal cycling, over the total number of cells are represented.

We also evaluated the tumor cells after 24h treatment with BP-M345 by phase contrast microscopy and after DAPI staining (Figure 24 A). By phase contrast microscopy analysis, we found the presence of floating cells indicative of death cells after treatment with BP-M345, in agreement with time-lapse analysis. By DAPI staining we observed that several cells displayed micronucleation and morphology like reminiscent of apoptosis. Thus, to confirm this observation we performed an Annexin V/PI staining in cells treated for 24h with BP-M345 and analyzed in flow cytometer (Figure 24 B). In untreated cells the percentage of apoptosis was residual ($2.7 \pm 1.8 \%$), while in BP-M345-treated cells, it was observed a significant increase in apoptotic cells ($16.8 \pm 6.2 \%$) (Figure 24 C). These results indicated that after a mitotic arrest, imposed by BP-M345 treatment, the cells died by apoptosis.

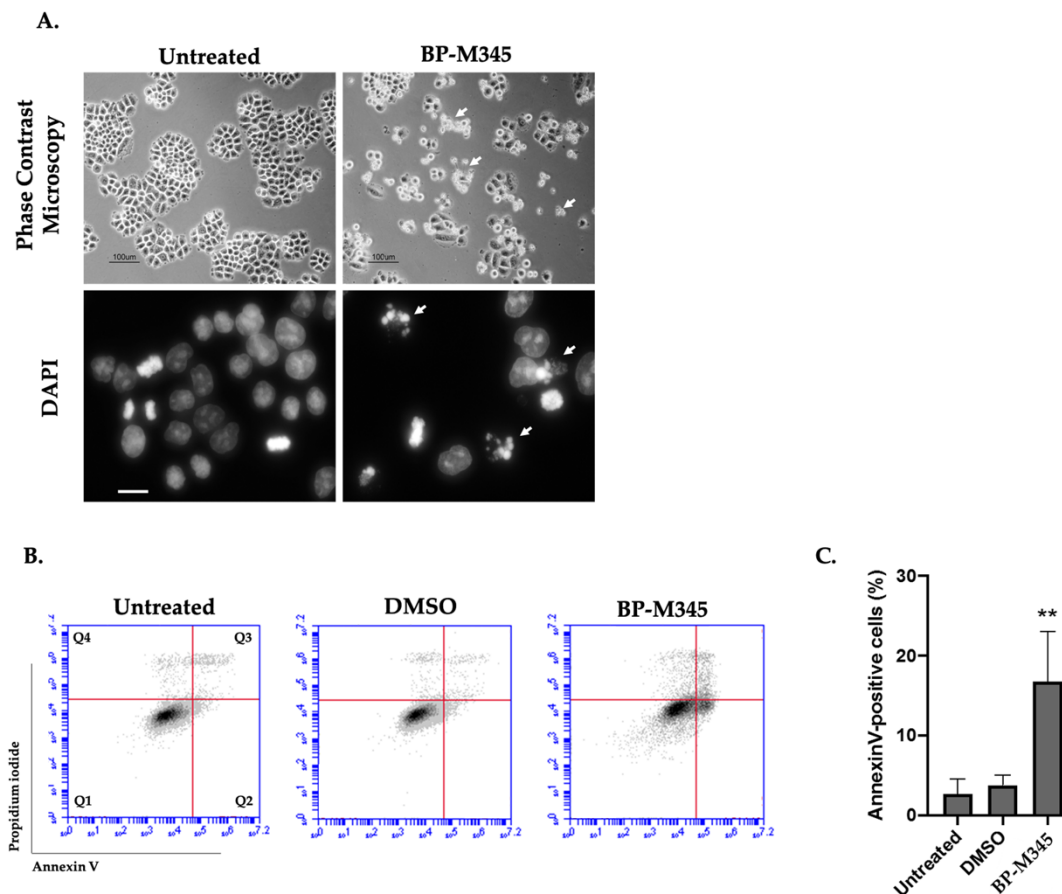


Fig. 24- Tumor cells treated with BP-M345 undergo apoptotic cell death. **(A)**. Representative phase contrast microscopy (Top) and DAPI staining (Bottom) images of untreated and BP-M345-treated cells, after 24 hours exposure, showing accumulation of floating (arrows) and condensing cells (arrows), respectively, suggesting cell death. Bar, 5 μ m **(B)**. Representative flow cytometry histogram of Propidium iodide (PI) versus Annexin V (FITC-A) intensity in untreated and BP-M345-treated cells, at 24 hours. DMSO was used as negative control. The quadrants (Q) were defined as Q1 = live cells (Annexin V- negative/PI-negative), Q2 = early stage of apoptosis (Annexin V-positive/PI-negative), Q3 = late stage of apoptosis (Annexin V-positive/PI-positive) and Q4 = necrosis (Annexin V-negative/PI-positive). **(C)**. Quantification of AnnexinV-positive cells of the data shown in B with statistical relevance of ** $p < 0.01$ by unpaired t-test.

3.6 BP-M345 impairs long-term tumor cell proliferation

To evaluate the long-term effect of BP-M345 on tumor cell proliferation, we performed a colony formation assay. Cells were treated with 0.185 μM of BP-M345 (2-fold less than the GI50) for 48h. Cells were then washed and incubated in a drug-free culture medium. After 6 days, the number of colonies formed was scored. As expected, untreated and DMSO-treated cells extensively proliferated, while cells treated with BP-M345 were unable to form colonies (Figure 25). Therefore, this result suggested that, although the time-lapse assay indicated that 43.6% of BP-M345-treated cells survived 48h post-treatment, the cells would not be able to further proliferate beyond this treatment time, indicating that BP-M345 exerts a long-term inhibitory effect on cell proliferation.

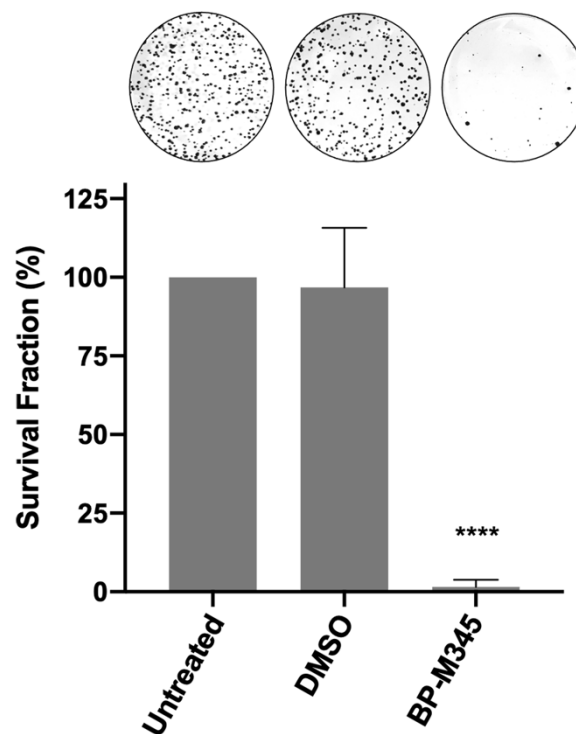


Fig. 25- BP-M345 inhibits long-term tumor cell proliferation. NCI-H460 cells were treated with 0.185 μM of BP-M345 for 48h. Colonies were stained with crystal violet and a representative figure is shown for each condition. Results are the mean of three independent experiments, expressed as % of survival fraction with statistical relevance of **** $p < 0.0001$ by unpaired t-test.

4. Discussion

Antimitotic drugs, such as taxanes and vinca alkaloids, have been widely used in cancer therapy. However, there are some issues that need to be overcome, namely their severe side effects and the resistance mechanisms developed by cancer cells, motivating the need to discover new antimitotics. Here, we uncover the cytotoxic mechanism of the diarylpentanoid BP-M345. We demonstrate that BP-M345 interferes with the stability of KT-MT attachments that leads to chromosome congression defects in NCI-H460 tumor cells. Although most of BP-M345-treated cells exhibited bipolar spindles, they were less robust than in untreated cells. BP-M345-treated cells chronically activate the SAC resulting in mitotic arrest. A significant fraction of cells died by apoptosis in mitosis after a prolonged arrest. These results are consistent with the observation that during a prolonged mitotic arrest the cells accumulate apoptotic signals leading to cell death (Gascoigne & Taylor, 2008). However, other cells were able to exit mitosis. As BP-M345 was inducing misaligned chromosomes, it is possible that these cells exited mitosis with missegregation chromosomes, therefore, generating aneuploid cells. Different cell fates are expected after missegregation errors. The cells could die in the following interphase if the errors are massive, as we observed in the time-lapse assay in which a percentage of cells suffer post mitotic death. Other cells could even become senescent or continue to proliferate (Gascoigne & Taylor, 2008). In the time-lapse assay we observed that a percentage of cells exit mitosis and survived during the period recorded (48h). However, in the colony formation assay when we treated the cells with BP-M345 no colonies were observed. This indicates that the cells that survive during the 48h exposure to BP-M345, were not able to proliferate, possibly due to an accumulation of chromosome missegregation errors. In sum, here we demonstrate that the diarylpentanoid BP-M345 harbors a potent anti-tumor activity *in vitro*, acting as an antimitotic agent. Further *in vivo* studies are required to explore the potential use of BP-M345 in cancer therapy.

Chapter V

General Discussion and Future Perspectives

In this work, we achieved the main goals that we have proposed in the beginning, namely the improvement of the efficacy of the second-generation antimitotic BI2536, and the identification of new antimitotic agents.

Focusing on the first objective, we proposed that the combination of Navitoclax with the mitotic blocker BI2536 could be an interesting approach to improve the efficacy of the Plk1 inhibitor. We first demonstrated that Plk1 was overexpressed in the NCSLC cell lines A549 and NCI-H460. Importantly, overexpression of Plk1 has been associated with poor prognosis in several cancers emphasizing the relevance of targeting Plk1 for the treatment of cancer (Z. Liu et al., 2016). The ability of tumor cells to slip mitosis without dividing represents one of the main reasons for the therapeutical failure of mitotic blockers including Plk1 inhibitors. We showed that the apoptosis potentiator Navitoclax accelerated and enhanced cell death in mitosis, by apoptosis of cells arrested in mitosis by BI2536. As a consequence, mitotic slippage was significantly reduced in the combinational treatment compared to BI236 alone. Hence, Navitoclax by inhibiting the anti-apoptotic proteins BCL-2 and BCL-XL accelerated the accumulation of death signals that reached more quickly the threshold to trigger apoptosis and, consequently, prevented the cell from reaching the threshold of cyclin B degradation that triggers mitotic exit. Moreover, BI2536 and Navitoclax demonstrated a synergistic effect on inducing cell death by apoptosis. Thus, this study demonstrated that combining Navitoclax with BI2536 is a valuable strategy that decreases the risk of mitotic slippage, thereby improving the efficacy of Plk1 inhibitors. Despite mitotic slippage represents one of the main reasons for the therapeutical failure of mitotic blockers in clinical trials, there are other issues that must be addressed to improve the efficacy of the SGA. Patients with the same tumor type respond differently to the same agents, probably due to different genetic and/or epigenetic modifications that alter the sensitivity to a specific drug (Lv et al., 2016; Mountzios et al., 2010). Thus, patient stratification using predictive biomarkers should pave the way to their effective clinical use. For instance, inhibition of Plk1 has been suggested to be more effective in tumors with high levels of Plk1 and mutated p53, thus selection of patients with these tumor characteristics may improve the clinical outcomes of Plk1 inhibitors (Guan et al., 2005; King et al., 2012). Another important aspect is that in clinical trials the most common adverse events in patients treated with SGA, including Plk1 inhibitors, were related to hematologic dysfunction. The development of drug-delivery systems could be a valuable approach to overcome this issue, facilitating better targeting towards cancer cells and in- tumor drug retention, while increasing the therapeutic window (Sgorla et al., 2016).

In the second objective of these thesis, we focused on discovery of new molecules with antimitotic properties. We first analyzed three tetracyclic thioxanthene derivatives of which TX2.1a emerged as hit compound with potent inhibitory growth activity in the tested tumor cell lines. According to the second aim that we have proposed, we further explored whether TX2.1a harbored antimitotic activity. The compound TX2.1a did not block cells in mitosis, although we found that it exhibited fluorescent activity. We also showed that TX2.1a accumulates in rounded-shaped subcellular structures. Further studies are needed to unveil the subcellular localization of TX2.1a, and its relevance for potential application to cancer therapy. Although TX2.1a does not act as an antimitotic agent, it does exhibit antitumor and fluorescent activity which makes it a potential theranostic drug.

Lastly, we showed that the diarylpentanoid BP-M345 exhibited a potent antitumor activity and unveiled their cytotoxic mechanism in the non-small cell lung cancer NCI-H460 cell line. We demonstrated that treatment with BP-M345 compromised the stability of KT-MT attachments which lead to chromosome congression defects and chronic SAC activation resulting in mitotic arrest. We further showed that the cells arrested in mitosis, after treatment with BP-M345, died by apoptosis, either during mitosis or in the following interphase. Moreover, BP-M345 exerted a long-term inhibitory effect on cell proliferation. More studies are required to determine the specific target of BP-M345. Possible targets are the tubulin subunits or proteins that regulates microtubule dynamics since we observed that the mitotic spindle after treatment with BP-M345, exhibited abnormal morphology, including spindles with decreased microtubule density, and some monopolar spindles. For instance, *in vitro* microtubule polymerization assay could permit to access whether BP-M345 is directly targeting microtubules. It will be also interesting in the future to evaluate the therapeutic benefit of combining BP-M345 with currently chemotherapeutical drugs, namely with antimitotics like paclitaxel, which could permit to reduce its dose, in a goal to minimize the side effects. In sum, this study reported a new molecule with promising antitumor activity that acts as an antimitotic agent.

Importantly, all studies were carried out in one cell line, and these results must be validated in other tumor cells. Furthermore, *in vivo* studies are also required to confirm the efficacy of the BI2536 and Navitoclax combination and whether the combination of both compounds at lower doses could minimize their hematologic toxicity. As well as for BP-M345 and TX2.1a, further *in vivo* research will allow explore their anticancer potential and access their side effects, of note that both molecules demonstrated selectivity to tumor cells *in vitro*. Additionally, since mitotic drivers and mitotic blockers differ in their

mechanism of action in a SAC response point of view, it will be also interesting to investigate the combination of Navitoclax with SGAs mitotic drivers, like Mps1 inhibitors and compare its effect to the combination of SGA mitotic blockers and Navitoclax.

Globally, the work presented in this thesis contributed to the development of new strategies to target mitosis for cancer therapy by improving the efficacy of the SGA BI2536 combining it with the apoptosis potentiator Navitoclax, and by providing a new antimitotic agent, the BP-M345, with promising antitumor activity. Additionally, it was also found a new molecule, the TX2.1a as potential theranostic drug against cancer.

Chapter VI

References

- Akhmanova, A., & Steinmetz, M. O. (2015). Control of microtubule organization and dynamics: Two ends in the limelight. In *Nature Reviews Molecular Cell Biology* (Vol. 16, Issue 12, pp. 711–726). Nat Rev Mol Cell Biol. <https://doi.org/10.1038/nrm4084>
- Allan, L. A., Camacho Reis, M., Ciossani, G., Huis in 't Veld, P. J., Wohlgemuth, S., Kops, G. J., Musacchio, A., & Saurin, A. T. (2020). Cyclin B1 scaffolds MAD 1 at the kinetochore corona to activate the mitotic checkpoint. *The EMBO Journal*, 39(12). <https://doi.org/10.15252/embj.2019103180>
- Amaral, J. D., Xavier, J. M., Steer, C. J., & Rodrigues, C. M. (2010). The role of p53 in apoptosis. In *Discovery medicine* (Vol. 9, Issue 45, pp. 145–152). Discov Med. https://doi.org/10.1007/978-1-4615-5287-1_2
- Ataci, N., Kazancioglu, E. O., Kalındemirtas, F. D., Kuruca, S. E., & Arsu, N. (2020). The interaction of light-activatable 2-thioxanthone thioacetic acid with ct-DNA and its cytotoxic activity: Novel theranostic agent. *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*, 239, 118491. <https://doi.org/10.1016/j.saa.2020.118491>
- Ataci, N., Ozcelik, E., & Arsu, N. (2018). Spectrophotometric study on binding of 2-thioxanthone acetic acid with ct-DNA. *Spectrochimica Acta - Part A: Molecular and Biomolecular Spectroscopy*, 204, 281–286. <https://doi.org/10.1016/j.saa.2018.06.001>
- Auckland, P., & McAinsh, A. D. (2015). Building an integrated model of chromosome congression. *J Cell Sci*, 128(18), 3363–3374. <https://doi.org/10.1242/jcs.169367>
- Barbosa, J., Lima, R. T., Sousa, D., Gomes, A. S., Palmeira, A., Seca, H., Choosang, K., Pakkong, P., Bousbaa, H., Pinto, M. M., Sousa, E., Vasconcelos, M. H., & Pedro, M. (2016). Screening a small library of xanthenes for antitumor activity and identification of a hit compound which induces apoptosis. *Molecules*, 21(1). <https://doi.org/10.3390/molecules21010081>
- Barisic, M., Aguiar, P., Geley, S., & Maiato, H. (2014). Kinetochore motors drive congression of peripheral polar chromosomes by overcoming random arm-ejection forces. *Nat Cell Biol*, 16(12), 1249–1256. <https://doi.org/10.1038/ncb3060>
- Barisic, M., e Sousa, R., Tripathy, S. K., Magiera, M. M., Zaytsev, A. V., Pereira, A. L., Janke, C., Grishchuk, E. L., & Maiato, H. (2015). Microtubule detyrosination guides chromosomes during mitosis. *Science*, 348(6236), 799–803. <https://doi.org/10.1126/science.aaa5175>
- Baudino, T. (2015). Targeted Cancer Therapy: The Next Generation of Cancer Treatment. *Current Drug Discovery Technologies*, 12(1), 3–20. <https://doi.org/10.2174/1570163812666150602144310>
- Bernardes de Jesus, B., & Blasco, M. A. (2013). Telomerase at the intersection of cancer and aging. In *Trends in Genetics* (Vol. 29, Issue 9, pp. 513–520). Europe PMC Funders. <https://doi.org/10.1016/j.tig.2013.06.007>
- Blagosklonny, M. V., & Pardee, A. B. (2002). The restriction point of the cell cycle. *Cell Cycle (Georgetown, Tex.)*, 1(2), 103–110.
- Blangy, A., Lane, H. A., D'Hérin, P., Harper, M., Kress, M., & Nigg, E. A. (1995). Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation in vivo. *Cell*, 83(7), 1159–1169. [https://doi.org/10.1016/0092-8674\(95\)90142-6](https://doi.org/10.1016/0092-8674(95)90142-6)

- Bolanos-Garcia, V. M. (2005). Aurora kinases. *Int J Biochem Cell Biol*, 37(8), 1572–1577. <https://doi.org/10.1016/j.biocel.2005.02.021>
- Brito, D. A., & Rieder, C. L. (2006). Mitotic Checkpoint Slippage in Humans Occurs via Cyclin B Destruction in the Presence of an Active Checkpoint. *Current Biology*, 16(12), 1194–1200. <https://doi.org/10.1016/j.cub.2006.04.043>
- Bruinsma, W., Raaijmakers, J. A., & Medema, R. H. (2012). Switching Polo-like kinase-1 on and off in time and space. *Trends Biochem Sci*, 37(12), 534–542. <https://doi.org/10.1016/j.tibs.2012.09.005>
- Carneiro, B. A., & El-Deiry, W. S. (2020a). Targeting apoptosis in cancer therapy. *Nature Reviews. Clinical Oncology*, 17(7), 395. <https://doi.org/10.1038/S41571-020-0341-Y>
- Carneiro, B. A., & El-Deiry, W. S. (2020b). Targeting apoptosis in cancer therapy. In *Nature Reviews Clinical Oncology* (Vol. 17, Issue 7, pp. 395–417). NIH Public Access. <https://doi.org/10.1038/s41571-020-0341-y>
- Cheeseman, I. M., Chappie, J. S., Wilson-Kubalek, E. M., & Desai, A. (2006). The Conserved KMN Network Constitutes the Core Microtubule-Binding Site of the Kinetochore. *Cell*, 127(5), 983–997. <https://doi.org/10.1016/j.cell.2006.09.039>
- Chen, H. C., Kanai, M., Inoue-Yamauchi, A., Tu, H. C., Huang, Y., Ren, D., Kim, H., Takeda, S., Reyna, D. E., Chan, P. M., Ganesan, Y. T., Liao, C. P., Gavathiotis, E., Hsieh, J. J., & Cheng, E. H. (2015). An interconnected hierarchical model of cell death regulation by the BCL-2 family. *Nature Cell Biology*, 17(10), 1270–1281. <https://doi.org/10.1038/ncb3236>
- Cheng, E. H.-Y. ., Wei, M. C., Weiler, S., Flavell, R. A., Mak, T. W., Lindsten, T., & Korsmeyer, S. J. (2001). BCL-2, BCL-XL sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Molecular Cell*, 8(3), 705–711. [https://doi.org/10.1016/S1097-2765\(01\)00320-3](https://doi.org/10.1016/S1097-2765(01)00320-3)
- Chung, V., Heath, E. I., Schelman, W. R., Johnson, B. M., Kirby, L. C., Lynch, K. M., Botbyl, J. D., Lampkin, T. A., & Holen, K. D. (2012). First-time-in-human study of GSK923295, a novel antimitotic inhibitor of centromere-associated protein e (CENP-E), in patients with refractory cancer. *Cancer Chemother Pharmacol*, 69(3), 733–741. <https://doi.org/10.1007/s00280-011-1756-z>
- Claudian, S., & Apperley, J. F. (2018). The argument for using imatinib in CML. *Hematology (United States)*, 2018(1), 161–167. <https://doi.org/10.1182/asheducation-2018.1.161>
- Colicino, E. G., & Hehnly, H. (2018). Regulating a key mitotic regulator, polo-like kinase 1 (PLK1). *Cytoskeleton (Hoboken)*, 75(11), 481–494. <https://doi.org/10.1002/cm.21504>
- Combes, G., Alharbi, I., Braga, L. G., & Elowe, S. (2017). Playing polo during mitosis: PLK1 takes the lead. *Oncogene*, 36(34), 4819–4827. <https://doi.org/10.1038/onc.2017.113>
- Czabotar, P. E., Lessene, G., Strasser, A., & Adams, J. M. (2014). Control of apoptosis by the BCL-2 protein family: Implications for physiology and therapy. In *Nature Reviews Molecular Cell Biology* (Vol. 15, Issue 1, pp. 49–63). Nat Rev Mol Cell Biol. <https://doi.org/10.1038/nrm3722>

- De Martino, D., Yilmaz, E., Orlacchio, A., Ranieri, M., Zhao, K., & Di Cristofano, A. (2018). PI3K blockage synergizes with PLK1 inhibition preventing endoreduplication and enhancing apoptosis in anaplastic thyroid cancer. *Cancer Letters*, 439, 56–65. <https://doi.org/10.1016/j.canlet.2018.09.024>
- Dickens, L. S., Powley, I. R., Hughes, M. A., & MacFarlane, M. (2012). The ‘complexities’ of life and death: Death receptor signalling platforms. *Experimental Cell Research*, 318(11), 1269–1277. <https://doi.org/10.1016/J.YEXCR.2012.04.005>
- Ding, L., Cao, J., Lin, W., Chen, H., Xiong, X., Ao, H., Yu, M., Lin, J., & Cui, Q. (2020). The Roles of Cyclin-Dependent Kinases in Cell-Cycle Progression and Therapeutic Strategies in Human Breast Cancer. *International Journal of Molecular Sciences*, 21(6). <https://doi.org/10.3390/IJMS21061960>
- Ding, S., Xing, N., Lu, J., Zhang, H., Nishizawa, K., Liu, S., Yuan, X., Qin, Y., Liu, Y., Ogawa, O., & Nishiyama, H. (2011). Overexpression of Eg5 predicts unfavorable prognosis in non-muscle invasive bladder urothelial carcinoma. *Int J Urol*, 18(6), 432–438. <https://doi.org/10.1111/j.1442-2042.2011.02751.x>
- Du, H., Wolf, J., Schafer, B., Moldoveanu, T., Chipuk, J. E., & Kuwana, T. (2011). BH3 domains other than bim and bid can directly activate bax/bak. *Journal of Biological Chemistry*, 286(1), 491–501. <https://doi.org/10.1074/jbc.M110.167148>
- Dumontet, C., & Jordan, M. A. (2010). Microtubule-binding agents: a dynamic field of cancer therapeutics. *Nat Rev Drug Discov*, 9(10), 790–803. <https://doi.org/10.1038/nrd3253>
- Ellis, P. M., Chu, Q. S., Leighl, N., Laurie, S. A., Fritsch, H., Gaschler-Markefski, B., Gyorffy, S., & Munzert, G. (2013). A phase I open-label dose-escalation study of intravenous BI 2536 together with pemetrexed in previously treated patients with non-small-cell lung cancer. *Clinical Lung Cancer*, 14(1), 19–27. <https://doi.org/10.1016/j.clcc.2012.04.003>
- Espert, A., Uluocak, P., Bastos, R. N., Mangat, D., Graab, P., & Gruneberg, U. (2014). PP2A-B56 opposes Mps1 phosphorylation of Knl1 and thereby promotes spindle assembly checkpoint silencing. *Journal of Cell Biology*, 206(7), 833–842. <https://doi.org/10.1083/jcb.201406109>
- Famulski, J. K., Vos, L. J., Rattner, J. B., & Chan, G. K. (2011). Dynein/dynactin-mediated transport of kinetochore components off kinetochores and onto spindle poles induced by Nordihydroguaiaretic acid. *PLoS ONE*, 6(1), e16494. <https://doi.org/10.1371/journal.pone.0016494>
- Fan, T. J., Han, L. H., Cong, R. S., & Liang, J. (2005). Caspase family proteases and apoptosis. *Acta Biochimica et Biophysica Sinica*, 37(11), 719–727. <https://doi.org/10.1111/j.1745-7270.2005.00108.x>
- Fanale, D., Maragliano, R., Bazan, V., & Russo, A. (2017). Caretakers and Gatekeepers. In *eLS* (pp. 1–10). American Cancer Society. <https://doi.org/10.1002/9780470015902.a0006048.pub2>
- Fededa, J. P., & Gerlich, D. W. (2012). Molecular control of animal cell cytokinesis. In *Nature Cell Biology* (Vol. 14, Issue 5, pp. 440–447). Nat Cell Biol. <https://doi.org/10.1038/ncb2482>
- Fuchs, Y., & Steller, H. (2011). Programmed cell death in animal development and disease. In *Cell* (Vol. 147, Issue 4, pp. 742–758). Cell.

<https://doi.org/10.1016/j.cell.2011.10.033>

- Galluzzi, L., López-Soto, A., Kumar, S., & Kroemer, G. (2016). Caspases Connect Cell-Death Signaling to Organismal Homeostasis. In *Immunity* (Vol. 44, Issue 2, pp. 221–231). Immunity. <https://doi.org/10.1016/j.immuni.2016.01.020>
- Gascoigne, K. E., & Taylor, S. S. (2008). Cancer Cells Display Profound Intra- and Interline Variation following Prolonged Exposure to Antimitotic Drugs. *Cancer Cell*, 14(2), 111–122. <https://doi.org/10.1016/j.ccr.2008.07.002>
- Gascoigne, K. E., & Taylor, S. S. (2009). How do anti-mitotic drugs kill cancer cells? In *Journal of Cell Science* (Vol. 122, Issue 15, pp. 2579–2585). The Company of Biologists. <https://doi.org/10.1242/jcs.039719>
- Girdler, F., Gascoigne, K. E., Evers, P. A., Hartmuth, S., Crafter, C., Foote, K. M., Keen, N. J., & Taylor, S. S. (2006). Validating Aurora B as an anti-cancer drug target. *J Cell Sci*, 119(17), 3664–3675. <https://doi.org/10.1242/jcs.03145>
- Glaser, K. B., Li, J., Marcotte, P. A., Magoc, T. J., Guo, J., Reuter, D. R., Tapang, P., Wei, R.-Q., Pease, L. J., Bui, M. H., Chen, Z., Frey, R. R., Johnson, E. F., Osterling, D. J., Olson, A. M., Bouska, J. J., Luo, Y., Curtin, M. L., Donawho, C. K., ... Albert, D. H. (2012). Preclinical characterization of ABT-348, a kinase inhibitor targeting the aurora, vascular endothelial growth factor receptor/platelet-derived growth factor receptor, and Src kinase families. *J Pharmacol Exp Ther*, 343(3), 617–627. <https://doi.org/10.1124/jpet.112.197087>
- Global Cancer Observatory. (2020). <https://gco.iarc.fr/>
- Guan, R., Tapang, P., Levenson, J. D., Albert, D., Giranda, V. L., & Luo, Y. (2005). Small Interfering RNA-Mediated Polo-Like Kinase 1 Depletion Preferentially Reduces the Survival of p53-Defective, Oncogenic Transformed Cells and Inhibits Tumor Growth in Animals. *Cancer Res*, 65(7), 2698–2704. <https://doi.org/10.1158/0008-5472.CAN-04-2131>
- Gutteridge, R. E. A., Ndiaye, M. A., Liu, X., & Ahmad, N. (2016). Plk1 inhibitors in cancer therapy: From laboratory to clinics. *Mol Cancer Ther*, 15(7), 1427–1435. <https://doi.org/10.1158/1535-7163.MCT-15-0897>
- Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of Cancer: The Next Generation. *Cell*, 144(5), 646–674. <https://doi.org/10.1016/j.cell.2011.02.013>
- Hauf, S., Cole, R. W., LaTerra, S., Zimmer, C., Schnapp, G., Walter, R., Heckel, A., van Meel, J., Rieder, C. L., & Peters, J.-M. (2003). The small molecule Hesperadin reveals a role for Aurora B in correcting kinetochore-microtubule attachment and in maintaining the spindle assembly checkpoint. *J Cell Biol*, 161(2), 281–294. <https://doi.org/10.1083/jcb.200208092>
- Hayward, D., Alfonso-Pérez, T., Cundell, M. J., Hopkins, M., Holder, J., Bancroft, J., Hutter, L. H., Novak, B., Barr, F. A., & Gruneberg, U. (2019). CDK1-CCNB1 creates a spindle checkpoint-permissive state by enabling MPS1 kinetochore localization. *Journal of Cell Biology*, 218(4), 1182–1199. <https://doi.org/10.1083/jcb.201808014>
- Hedidi, M., Maillard, J., Erb, W., Lassagne, F., Halauko, Y. S., Ivashkevich, O. A., Matulis, V. E., Roisnel, T., Dorcet, V., Hamzé, M., Fajloun, Z., Baratte, B., Ruchaud, S., Bach, S., Bentabed-Ababsa, G., & Mongin, F. (2017). Fused Systems Based on 2-Aminopyrimidines: Synthesis Combining Deprotolithiation-in situ Zincation with N-Arylation Reactions and Biological Properties. *European Journal of Organic*

Chemistry, 2017(39), 5903–5915. <https://doi.org/10.1002/ejoc.201701004>

- Henriques, A. C., Ribeiro, D., Pedrosa, J., Sarmento, B., Silva, P. M. A., & Bousbaa, H. (2019). Mitosis inhibitors in anticancer therapy: When blocking the exit becomes a solution. *Cancer Lett*, 440–441, 64–81. <https://doi.org/10.1016/j.canlet.2018.10.005>
- Hewitt, L., Tighe, A., Santaguida, S., White, A. M., Jones, C. D., Musacchio, A., Green, S., & Taylor, S. S. (2010). Sustained Mps1 activity is required in mitosis to recruit O-Mad2 to the Mad1-C-Mad2 core complex. *Journal of Cell Biology*, 190(1), 25–34. <https://doi.org/10.1083/jcb.201002133>
- Irmeler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J. L., Schröter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L. E., & Tschopp, J. (1997). Inhibition of death receptor signals by cellular FLIP. *Nature*, 388(6638), 190–195. <https://doi.org/10.1038/40657>
- Janssen, A., Kops, G. J. P. L., & Medema, R. H. (2009). Elevating the frequency of chromosome mis-segregation as a strategy to kill tumor cells. *Proc Natl Acad Sci U S A*, 106(45), 19108–19113. <https://doi.org/10.1073/pnas.0904343106>
- Jemaà, M., Galluzzi, L., Kepp, O., Senovilla, L., Brands, M., Boemer, U., Koppitz, M., Lienau, P., Prechtel, S., Schulze, V., Siemeister, G., Wengner, A. M., Mumberg, D., Ziegelbauer, K., Abrieu, A., Castedo, M., Vitale, I., & Kroemer, G. (2013). Characterization of novel MPS1 inhibitors with preclinical anticancer activity. *Cell Death Differ*, 20(11), 1532–1545. <https://doi.org/10.1038/cdd.2013.105>
- Ji, Z., Gao, H., Jia, L., Li, B., & Yu, H. (2017). A sequential multi-target Mps1 phosphorylation cascade promotes spindle checkpoint signaling. *ELife*, 6. <https://doi.org/10.7554/eLife.22513>
- Johnson, D. G., & Walker, C. L. (1999). Cyclins and cell cycle checkpoints. In *Annual Review of Pharmacology and Toxicology* (Vol. 39, pp. 295–312). Annu Rev Pharmacol Toxicol. <https://doi.org/10.1146/annurev.pharmtox.39.1.295>
- Jordan, M. A., & Wilson, L. (2004). Microtubules as a target for anticancer drugs. *Nat Rev Cancer*, 4(4), 253–265. <https://doi.org/10.1038/nrc1317>
- Jorgensen, I., Rayamajhi, M., & Miao, E. A. (2017). Programmed cell death as a defence against infection. In *Nature Reviews Immunology* (Vol. 17, Issue 3, pp. 151–164). Nature Publishing Group. <https://doi.org/10.1038/nri.2016.147>
- Julien, O., & Wells, J. A. (2017). Caspases and their substrates. In *Cell Death and Differentiation* (Vol. 24, Issue 8, pp. 1380–1389). Nature Publishing Group. <https://doi.org/10.1038/cdd.2017.44>
- Kaestner, P., Stolz, A., & Bastians, H. (2009). Determinants for the efficiency of anticancer drugs targeting either Aurora-A or Aurora-B kinases in human colon carcinoma cells. *Mol Cancer Ther*, 8(7), 2046–2056. <https://doi.org/10.1158/1535-7163.MCT-09-0323>
- Kapoor, T. M., Lampson, M. A., Hergert, P., Cameron, L., Cimini, D., Salmon, E. D., McEwen, B. F., & Khodjakov, A. (2006). Chromosomes can congress to the metaphase plate before biorientation. *Science*, 311(5759), 388–391. <https://doi.org/10.1126/science.1122142>
- Kim, H., Rafiuddin-Shah, M., Tu, H. C., Jeffers, J. R., Zambetti, G. P., Hsieh, J. J. D., & Cheng, E. H. Y. (2006). Hierarchical regulation of mitochondrion-dependent

- apoptosis by BCL-2 subfamilies. *Nature Cell Biology*, 8(12), 1348–1358. <https://doi.org/10.1038/ncb1499>
- King, S. I., Purdie, C. A., Bray, S. E., Quinlan, P. R., Jordan, L. B., Thompson, A. M., & Meek, D. W. (2012). Immunohistochemical detection of Polo-like kinase-1 (PLK1) in primary breast cancer is associated with TP53 mutation and poor clinical outcome. *Breast Cancer Res*, 14(2), R40. <https://doi.org/10.1186/bcr3136>
- Kirschner, M., & Mitchison, T. (1986). Beyond self-assembly: From microtubules to morphogenesis. In *Cell* (Vol. 45, Issue 3, pp. 329–342). Cell. [https://doi.org/10.1016/0092-8674\(86\)90318-1](https://doi.org/10.1016/0092-8674(86)90318-1)
- Kung, P.-P., Martinez, R., Zhu, Z., Zager, M., Blasina, A., Rymer, I., Hallin, J., Xu, M., Carroll, C., Chionis, J., Wells, P., Kozminski, K., Fan, J., Guicherit, O., Huang, B., Cui, M., Liu, C., Huang, Z., Sistla, A., ... Murray, B. W. (2014). Chemogenetic evaluation of the mitotic kinesin CENP-E reveals a critical role in triple-negative breast cancer. *Mol Cancer Ther*, 13(8), 2104–2115. <https://doi.org/10.1158/1535-7163.MCT-14-0083-T>
- Kwiatkowski, N., Jelluma, N., Filippakopoulos, P., Soundararajan, M., Manak, M. S., Kwon, M., Choi, H. G., Sim, T., Deveraux, Q. L., Rottmann, S., Pellman, D., Shah, J. V., Kops, G. J. P. L., Knapp, S., & Gray, N. S. (2010). Small-molecule kinase inhibitors provide insight into Mps1 cell cycle function. *Nat Chem Biol*, 6(5), 359–368. <https://doi.org/10.1038/nchembio.345>
- Lauzé, E., Stoelcker, B., Luca, F. C., Weiss, E., Schutz, A. R., & Winey, M. (1995). Yeast spindle pole body duplication gene MPS1 encodes an essential dual specificity protein kinase. *EMBO J*, 14(8), 1655–1663.
- Lee, E. Y. H. P., & Muller, W. J. (2010). Oncogenes and tumor suppressor genes. In *Cold Spring Harbor perspectives in biology* (Vol. 2, Issue 10). Cold Spring Harbor Laboratory Press. <https://doi.org/10.1101/cshperspect.a003236>
- Lénárt, P., Petronczki, M., Steegmaier, M., Di Fiore, B., Lipp, J. J., Hoffmann, M., Rettig, W. J., Kraut, N., & Peters, J.-M. (2007). The Small-Molecule Inhibitor BI 2536 Reveals Novel Insights into Mitotic Roles of Polo-like Kinase 1. *Current Biology*, 17(4), 304–315. <https://doi.org/10.1016/j.cub.2006.12.046>
- Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., & Wang, X. (1997). Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*, 91(4), 479–489. [https://doi.org/10.1016/S0092-8674\(00\)80434-1](https://doi.org/10.1016/S0092-8674(00)80434-1)
- Lima, R. T., Sousa, D., Paiva, A. M., Palmeira, A., Barbosa, J., Pedro, M., Pinto, M. M., Sousa, E., & Vasconcelos, M. H. (2016). Modulation of autophagy by a thioxanthone decreases the viability of melanoma cells. *Molecules*, 21(10). <https://doi.org/10.3390/molecules21101343>
- Liu, C., Zhou, N., Li, J., Kong, J., Guan, X., & Wang, X. (2017). Eg5 Overexpression Is Predictive of Poor Prognosis in Hepatocellular Carcinoma Patients. *Dis Markers*, 2017. <https://doi.org/10.1155/2017/2176460>
- Liu, D., & Lampson, M. A. (2009). Regulation of kinetochore-microtubule attachments by Aurora B kinase. *Biochemical Society Transactions*, 37(5), 976–980. <https://doi.org/10.1042/BST0370976>
- Liu, L., Liu, X., Mare, M., Dumont, A. S., Zhang, H., Yan, D., & Xiong, Z. (2016).

- Overexpression of Eg5 correlates with high grade astrocytic neoplasm. *J Neurooncol*, 126(1), 77–80. <https://doi.org/10.1007/s11060-015-1954-3>
- Liu, M., Wang, X., Yang, Y., Li, D., Ren, H., Zhu, Q., Chen, Q., Han, S., Hao, J., & Zhou, J. (2010). Ectopic expression of the microtubule-dependent motor protein Eg5 promotes pancreatic tumorigenesis. *J Pathol*, 221(2), 221–228. <https://doi.org/10.1002/path.2706>
- Liu, M., Yu, H., Huo, L., Liu, J., Li, M., & Zhou, J. (2008). Validating the mitotic kinesin Eg5 as a therapeutic target in pancreatic cancer cells and tumor xenografts using a specific inhibitor. *Biochemical Pharmacology*, 76(2), 169–178. <https://doi.org/10.1016/j.bcp.2008.04.018>
- Liu, X., & Winey, M. (2012a). The MPS1 family of protein kinases. *Annu Rev Biochem*, 81, 561–585. <https://doi.org/10.1146/annurev-biochem-061611-090435>
- Liu, X., & Winey, M. (2012b). The MPS1 family of protein kinases. *Annual Review of Biochemistry*, 81, 561–585. <https://doi.org/10.1146/annurev-biochem-061611-090435>
- Liu, Z., Sun, Q., & Wang, X. (2016). PLK1, A Potential Target for Cancer Therapy. *Transl Oncol*, 10(1), 22–32. <https://doi.org/10.1016/j.tranon.2016.10.003>
- Logarinho, E., & Bousbaa, H. (2008). Kinetochore-microtubule interactions “in check” by Bub1, Bub3 and BubR1: The dual task of attaching and signalling. *Cell Cycle*, 7(12), 1763–1768. <https://doi.org/10.4161/cc.7.12.6180>
- Logarinho, E., Resende, T., Torres, C., & Bousbaa, H. (2008). The human spindle assembly checkpoint protein Bub3 is required for the establishment of efficient kinetochore-microtubule attachments. *Molecular Biology of the Cell*, 19(4), 1798–1813. <https://doi.org/10.1091/mbc.E07-07-0633>
- Lorusso, P., Chawla, S. P., Bendell, J., Shields, A. F., Shapiro, G., Rajagopalan, P., Cyrus, C., Bruns, I., Mei, J., Souza, F., Rasco, D., Eder, J. P., & Tolcher, A. W. (2018). First-in-human study of the monopolar spindle 1 (Mps1) kinase inhibitor BAY 1161909 in combination with paclitaxel in subjects with advanced malignancies. *Annals of Oncology*, 29, viii138. <https://doi.org/10.1093/annonc/mdy279.410>
- Lv, J.-F., Hu, L., Zhuo, W., Zhang, C.-M., Zhou, H.-H., & Fan, L. (2016). Epigenetic alternations and cancer chemotherapy response. *Cancer Chemother Pharmacol*, 77(4), 673–684. <https://doi.org/10.1007/s00280-015-2951-0>
- Maciejowski, J., Drechsler, H., Grundner-Culemann, K., Ballister, E. R., Rodriguez-Rodriguez, J.-A., Rodriguez-Bravo, V., Jones, M. J. K., Foley, E., Lampson, M. A., Daub, H., McAinsh, A. D., & Jallepalli, P. V. (2017). Mps1 Regulates Kinetochore-Microtubule Attachment Stability via the Ska Complex to Ensure Error-Free Chromosome Segregation. *Developmental Cell*, 41(2), 143–156.e6. <https://doi.org/10.1016/j.devcel.2017.03.025>
- Maiato, H., Gomes, A. M., Sousa, F., & Barisic, M. (2017). Mechanisms of Chromosome Congression during Mitosis. *Biology (Basel)*, 6(1). <https://doi.org/10.3390/biology6010013>
- Mao, Y., Desai, A., & Cleveland, D. W. (2005). Microtubule capture by CENP-E silences BubR1-dependent mitotic checkpoint signaling. *J Cell Biol*, 170(6), 873–880. <https://doi.org/10.1083/jcb.200505040>

- Matson, D. R., & Stukenberg, P. T. (2011). Spindle Poisons and Cell Fate: A Tale of Two Pathways. *Molecular Interventions*, 11(2), 141. <https://doi.org/10.1124/mi.11.2.12>
- McIntosh, J. R., Molodtsov, M. I., & Ataullakhanov, F. I. (2012). Biophysics of mitosis. In *Quarterly Reviews of Biophysics* (Vol. 45, Issue 2, pp. 147–207). NIH Public Access. <https://doi.org/10.1017/S0033583512000017>
- Mohamad Anuar, N. N., Nor Hisam, N. S., Liew, S. L., & Ugusman, A. (2020). Clinical Review: Navitoclax as a Pro-Apoptotic and Anti-Fibrotic Agent. In *Frontiers in Pharmacology* (Vol. 11). Frontiers Media SA. <https://doi.org/10.3389/fphar.2020.564108>
- Mokhtari Brikci-Nigassa, N., Nauton, L., Moreau, P., Mongin, O., Duval, R. E., Picot, L., Thiéry, V., Souab, M., Baratte, B., Ruchaud, S., Bach, S., Le Guevel, R., Bentabed-Ababsa, G., Erb, W., Roisnel, T., Dorcet, V., & Mongin, F. (2020). Functionalization of 9-thioxanthone at the 1-position: From arylamino derivatives to [1]benzo(thio)pyrano[4,3,2-de]benzothieno[2,3-b]quinolines of biological interest. *Bioorganic Chemistry*, 94, 103347. <https://doi.org/10.1016/j.bioorg.2019.103347>
- Moreira, J., Almeida, J., Loureiro, J. B., Ramos, H., Palmeira, A., Pinto, M. M., Saraiva, L., & Cidade, H. (2021). A Diarylpentanoid with Potential Activation of the p53 Pathway: Combination of in silico Screening Studies, Synthesis, and Biological Activity Evaluation. *ChemMedChem*. <https://doi.org/10.1002/cmdc.202100337>
- Mountzios, G., Dimopoulos, M.-A., Soria, J.-C., Sanoudou, D., & Papadimitriou, C. A. (2010). Histopathologic and genetic alterations as predictors of response to treatment and survival in lung cancer: A review of published data. *Critical Reviews in Oncology/Hematology*, 75(2), 94–109. <https://doi.org/10.1016/j.critrevonc.2009.10.002>
- Moyle, M. W., Kim, T., Hattersley, N., Espeut, J., Cheerambathur, D. K., Oegema, K., & Desai, A. (2014). A Bub1-Mad1 interaction targets the Mad1-Mad2 complex to unattached kinetochores to initiate the spindle checkpoint. *Journal of Cell Biology*, 204(5), 647–657. <https://doi.org/10.1083/jcb.201311015>
- Mross, K., Dittrich, C., Aulitzky, W. E., Strumberg, D., Schutte, J., Schmid, R. M., Hollerbach, S., Merger, M., Munzert, G., Fleischer, F., & Scheulen, M. E. (2012). A randomised phase II trial of the Polo-like kinase inhibitor BI 2536 in chemo-naïve patients with unresectable exocrine adenocarcinoma of the pancreas—a study within the Central European Society Anticancer Drug Research (CESAR) collaborative network. *Br J Cancer*, 107(2), 280–286. <https://doi.org/10.1038/bjc.2012.257>
- Müller-Tidow, C., Bug, G., Lübbert, M., Krämer, A., Krauter, J., Valent, P., Nachbaur, D., Berdel, W. E., Ottmann, O. G., Fritsch, H., Munzert, G., Garin-Chesa, P., Fleischer, F., Taube, T., & Döhner, H. (2013). A randomized, open-label, phase I/II trial to investigate the maximum tolerated dose of the Polo-like kinase inhibitor BI 2536 in elderly patients with refractory/relapsed acute myeloid leukaemia. *Br J Haematol*, 163(2), 214–222. <https://doi.org/10.1111/bjh.12518>
- Nakayama, Y., & Inoue, T. (2016). Antiproliferative fate of the tetraploid formed after mitotic slippage and its promotion; a novel target for cancer therapy based on microtubule poisons. *Molecules*, 21(5). <https://doi.org/10.3390/molecules21050663>
- Nijenhuis, W., Vallardi, G., Teixeira, A., Kops, G. J. P. L., & Saurin, A. T. (2014). Negative feedback at kinetochores underlies a responsive spindle checkpoint signal. *Nature Cell Biology*, 16(12), 1257–1264. <https://doi.org/10.1038/ncb3065>

- Nijenhuis, W., Von Castelmur, E., Littler, D., De Marco, V., Tromer, E., Vleugel, M., Van Osch, M. H. J., Snel, B., Perrakis, A., & Kops, G. J. P. L. (2013). A TPR domain-containing N-terminal module of MPS1 is required for its kinetochore localization by Aurora B. *Journal of Cell Biology*, 201(2), 217–231. <https://doi.org/10.1083/jcb.201210033>
- Novais, P., Silva, P. M. A., Amorim, I., & Bousbaa, H. (2021). Second-Generation Antimitotics in Cancer Clinical Trials. *Pharmaceutics* 2021, Vol. 13, Page 1011, 13(7), 1011. <https://doi.org/10.3390/PHARMACEUTICS13071011>
- Núñez, G., Benedict, M. A., Hu, Y., & Inohara, N. (1998). Caspases: The proteases of the apoptotic pathway. In *Oncogene* (Vol. 17, Issue 25, pp. 3237–3245). Oncogene. <https://doi.org/10.1038/sj.onc.1202581>
- Ohashi, A., Otori, M., & Iwai, K. (2016). Motor activity of centromere-associated protein-E contributes to its localization at the center of the midbody to regulate cytokinetic abscission. *Oncotarget*, 7(48), 79964–79980. <https://doi.org/10.18632/oncotarget.13206>
- Ohashi, A., Otori, M., Iwai, K., Nambu, T., Miyamoto, M., Kawamoto, T., & Okaniwa, M. (2015). A novel time-dependent CENP-E inhibitor with potent antitumor activity. *PLoS One*, 10(12). <https://doi.org/10.1371/journal.pone.0144675>
- Ouyang, L., Shi, Z., Zhao, S., Wang, F. T., Zhou, T. T., Liu, B., & Bao, J. K. (2012). Programmed cell death pathways in cancer: A review of apoptosis, autophagy and programmed necrosis. *Cell Proliferation*, 45(6), 487–498. <https://doi.org/10.1111/j.1365-2184.2012.00845.x>
- Pachis, S. T., & Kops, G. J. P. L. (2018). Leader of the SAC: Molecular mechanisms of Mps1/TTK regulation in mitosis. In *Open Biology* (Vol. 8, Issue 8). Open Biol. <https://doi.org/10.1098/rsob.180109>
- Pajtl, K. W., Sadowski, N., Ackermann, S., Althoff, K., Schönbeck, K., Batzke, K., Sch, S., Odersky, A., Heukamp, L., Astrahantseff, K., Künkele, A., Deubzer, H. E., Schramm, A., Spr, A., Thor, T., Lindner, S., Eggert, A., Fischer, M., & Schulte, J. H. (2016). The GSK461364 PLK1 inhibitor exhibits strong antitumoral activity in preclinical neuroblastoma models. *Oncotarget*, 8(4), 6730–6741. <https://doi.org/10.18632/oncotarget.14268>
- Paulraj, F., Abas, F., Lajis, N. H., Othman, I., & Naidu, R. (2019). Molecular pathways modulated by curcumin analogue, diarylpentanoids in cancer. *Biomolecules*, 9(7). <https://doi.org/10.3390/biom9070270>
- Penet, M.-F., Chen, Z., Kakkad, S., Pomper, M. G., & Bhujwala, Z. M. (2012). Theranostic imaging of cancer. *European Journal of Radiology*, 81(0 1), S124. [https://doi.org/10.1016/S0720-048X\(12\)70051-7](https://doi.org/10.1016/S0720-048X(12)70051-7)
- Perez, E. A. (2009). Microtubule inhibitors: Differentiating tubulin-inhibiting agents based on mechanisms of action, clinical activity, and resistance. In *Molecular Cancer Therapeutics* (Vol. 8, Issue 8, pp. 2086–2095). American Association for Cancer Research. <https://doi.org/10.1158/1535-7163.MCT-09-0366>
- Pimenta-Marques, A., & Bettencourt-Dias, M. (2020). Pericentriolar material. *Curr Biol*, 30(12), R687–R689. <https://doi.org/10.1016/j.cub.2020.04.064>
- Pistritto, G., Trisciuglio, D., Ceci, C., Alessia Garufi, & D'Orazi, G. (2016). Apoptosis as anticancer mechanism: Function and dysfunction of its modulators and targeted

- therapeutic strategies. In *Aging* (Vol. 8, Issue 4, pp. 603–619). Impact Journals, LLC. <https://doi.org/10.18632/aging.100934>
- Primorac, I., Weir, J. R., Chirolì, E., Gross, F., Hoffmann, I., van Gerwen, S., Ciliberto, A., & Musacchio, A. (2013). Bub3 reads phosphorylated MELT repeats to promote spindle assembly checkpoint signaling. *ELife*, 2013(2). <https://doi.org/10.7554/eLife.01030>
- Qian, J., García-Gimeno, M. A., Beullens, M., Manzione, M. G., Van der Hoeven, G., Igual, J. C., Heredia, M., Sanz, P., Gelens, L., & Bollen, M. (2017). An Attachment-Independent Biochemical Timer of the Spindle Assembly Checkpoint. *Molecular Cell*, 68(4), 715–730.e5. <https://doi.org/10.1016/j.molcel.2017.10.011>
- Raab, M., Krämer, A., Hehlhans, S., Sanhaji, M., Kurunci-Csacsco, E., Dötsch, C., Bug, G., Ottmann, O., Becker, S., Pachi, F., Kuster, B., & Strebhardt, K. (2015). Mitotic arrest and slippage induced by pharmacological inhibition of Polo-like kinase 1. *Molecular Oncology*, 9(1), 140–154. <https://doi.org/10.1016/j.molonc.2014.07.020>
- Rieder, C L, & Alexander, S. P. (1990). Kinetochore are transported poleward along a single astral microtubule during chromosome attachment to the spindle in newt lung cells. *The Journal of Cell Biology*, 110(1), 81–95. <https://doi.org/10.1083/jcb.110.1.81>
- Rieder, Conly L. (1981). The structure of the cold-stable kinetochore fiber in metaphase PtK1 cells. *Chromosoma*, 84(1), 145–158. <https://doi.org/10.1007/BF00293368>
- Rieder, Conly L, Cole, R. W., Khodjakov, A., & Sluder, G. (1995). The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. *Journal of Cell Biology*, 130(4), 941–948. <https://doi.org/10.1083/jcb.130.4.941>
- Rieder, Conly L, Schultz, A., Cole, R., & Sluder, G. (1994). Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle. *Journal of Cell Biology*, 127(5), 1301–1310. <https://doi.org/10.1083/jcb.127.5.1301>
- Roos, W. P., & Kaina, B. (2013). DNA damage-induced cell death: From specific DNA lesions to the DNA damage response and apoptosis. In *Cancer Letters* (Vol. 332, Issue 2, pp. 237–248). Elsevier. <https://doi.org/10.1016/j.canlet.2012.01.007>
- Roskoski, R. (2019). Cyclin-dependent protein serine/threonine kinase inhibitors as anticancer drugs. *Pharmacological Research*, 139, 471–488. <https://doi.org/10.1016/J.PHRS.2018.11.035>
- Sawin, K. E., LeGuellec, K., Philippe, M., & Mitchison, T. J. (1992). Mitotic spindle organization by a plus-end-directed microtubule motor. *Nature*, 359(6395), 540–543. <https://doi.org/10.1038/359540a0>
- Schaal, C., Pillai, S., & Chellappan, S. P. (2014). The Rb-E2F transcriptional regulatory pathway in tumor angiogenesis and metastasis. In *Advances in Cancer Research* (Vol. 121, pp. 147–182). Adv Cancer Res. <https://doi.org/10.1016/B978-0-12-800249-0.00004-4>
- Schafer, K. A. (1998). The cell cycle: a review. *Veterinary Pathology*, 35(6), 461–478. <https://doi.org/10.1177/030098589803500601>
- Sebastian, M., Reck, M., Waller, C. F., Kortsik, C., Frickhofen, N., Schuler, M., Fritsch,

- H., Gaschler-Markefski, B., Hanft, G., Munzert, G., & von Pawel, J. (2010). The efficacy and safety of BI 2536, a novel Plk-1 inhibitor, in patients with stage IIIB/IV non-small cell lung cancer who had relapsed after, or failed, chemotherapy: Results from an open-label, randomized phase ii clinical trial. *Journal of Thoracic Oncology*, 5(7), 1060–1067. <https://doi.org/10.1097/JTO.0b013e3181d95dd4>
- Sgorla, D., Bunhak, É. J., Cavalcanti, O. A., Fonte, P., & Sarmiento, B. (2016). Exploitation of lipid-polymeric matrices at nanoscale for drug delivery applications. *Expert Opin Drug Deliv*, 13(9), 1301–1309. <https://doi.org/10.1080/17425247.2016.1182492>
- Sharp, D. J., Rogers, G. C., & Scholey, J. M. (2000). Microtubule motors in mitosis. In *Nature* (Vol. 407, Issue 6800, pp. 41–47). Nature. <https://doi.org/10.1038/35024000>
- Shi, J., Zhou, Y., Huang, H. C., & Mitchison, T. J. (2011). Navitoclax (ABT-263) accelerates apoptosis during drug-induced mitotic arrest by antagonizing Bcl-xL. *Cancer Research*, 71(13), 4518–4526. <https://doi.org/10.1158/0008-5472.CAN-10-4336>
- Shi, X., Dou, Y., Zhou, K., Huo, J., Yang, T., Qin, T., Liu, W., Wang, S., Yang, D., Chang, L., & Wang, C. (2017). Targeting the Bcl-2 family and P-glycoprotein reverses paclitaxel resistance in human esophageal carcinoma cell line. *Biomedicine and Pharmacotherapy*, 90, 897–905. <https://doi.org/10.1016/j.biopha.2017.04.043>
- Silva, P. M. A., Reis, R. M., Bolanos-Garcia, V. M., Florindo, C., Tavares, Á. A., & Bousbaa, H. (2014). Dynein-dependent transport of spindle assembly checkpoint proteins off kinetochores toward spindle poles. *FEBS Letters*, 588(17), 3265–3273. <https://doi.org/10.1016/j.febslet.2014.07.011>
- Singh, A., Trivedi, P., & Jain, N. K. (2018). Advances in siRNA delivery in cancer therapy. In *Artificial Cells, Nanomedicine and Biotechnology* (Vol. 46, Issue 2, pp. 274–283). Artif Cells Nanomed Biotechnol. <https://doi.org/10.1080/21691401.2017.1307210>
- Sinha, D., Duijf, P. H. G., & Khanna, K. K. (2019). Mitotic slippage: an old tale with a new twist. In *Cell Cycle* (Vol. 18, Issue 1, pp. 7–15). Cell Cycle. <https://doi.org/10.1080/15384101.2018.1559557>
- Smith, H. L., Southgate, H., Tweddle, D. A., & Curtin, N. J. (2020). DNA damage checkpoint kinases in cancer. In *Expert Reviews in Molecular Medicine* (Vol. 22). Expert Rev Mol Med. <https://doi.org/10.1017/erm.2020.3>
- Stilgenbauer, S., Eichhorst, B., Schetelig, J., Hillmen, P., Seymour, J. F., Coutre, S., Jurczak, W., Mulligan, S. P., Schuh, A., Assouline, S., Wendtner, C. M., Roberts, A. W., Davids, M. S., Bloehdorn, J., Munir, T., Böttcher, S., Zhou, L., Salem, A. H., Desai, M., ... Wierda, W. G. (2018). Venetoclax for patients with chronic lymphocytic leukemia with 17p deletion: Results from the full population of a phase ii pivotal trial. *Journal of Clinical Oncology*, 36(19), 1973–1980. <https://doi.org/10.1200/JCO.2017.76.6840>
- Stucke, V. M., Silljé, H. H. W., Arnaud, L., & Nigg, E. A. (2002). Human Mps1 kinase is required for the spindle assembly checkpoint but not for centrosome duplication. *EMBO Journal*, 21(7), 1723–1732. <https://doi.org/10.1093/emboj/21.7.1723>
- Sudakin, V., Chan, G. K. T., & Yen, T. J. (2001). Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. *Journal of Cell Biology*, 154(5), 925–936. <https://doi.org/10.1083/jcb.200102093>

- Szymiczek, A., Carbone, M., Pastorino, S., Napolitano, A., Tanji, M., Minaai, M., Pagano, I., Mason, J. M., Pass, H. I., Bray, M. R., Mak, T. W., & Yang, H. (2017). Inhibition of the spindle assembly checkpoint kinase Mps-1 as a novel therapeutic strategy in malignant mesothelioma. *Oncogene*, 36(46), 6501–6507. <https://doi.org/10.1038/onc.2017.266>
- Tait, S. W. G., & Green, D. R. (2010). Mitochondria and cell death: Outer membrane permeabilization and beyond. In *Nature Reviews Molecular Cell Biology* (Vol. 11, Issue 9, pp. 621–632). Nat Rev Mol Cell Biol. <https://doi.org/10.1038/nrm2952>
- Tan, N., Malek, M., Zha, J., Yue, P., Kassees, R., Berry, L., Fairbrother, W. J., Sampath, D., & Belmont, L. D. (2011). Navitoclax enhances the efficacy of taxanes in non-small cell lung cancer models. *Clinical Cancer Research*, 17(6), 1394–1404. <https://doi.org/10.1158/1078-0432.CCR-10-2353>
- Tan, T. T., Degenhardt, K., Nelson, D. A., Beaudoin, B., Nieves-Neira, W., Bouillet, P., Villunger, A., Adams, J. M., & White, E. (2005). Key roles of BIM-driven apoptosis in epithelial tumors and rational chemotherapy. *Cancer Cell*, 7(3), 227–238. <https://doi.org/10.1016/j.ccr.2005.02.008>
- Tanaka, K., Mukae, N., Dewar, H., Van Breugel, M., James, E. K., Prescott, A. R., Antony, C., & Tanaka, T. U. (2005). Molecular mechanisms of kinetochore capture by spindle microtubules. *Nature*, 434(7036), 987–994. <https://doi.org/10.1038/nature03483>
- Tanaka, T. U. (2010). Kinetochore-microtubule interactions: Steps towards bi-orientation. *EMBO Journal*, 29(24), 4070–4082. <https://doi.org/10.1038/emboj.2010.294>
- Tanudji, M., Shoemaker, J., L'Italien, L., Russell, L., Chin, G., & Schebye, X. M. (2004). Gene silencing of CENP-E by small interfering RNA in HeLa cells leads to missegregation of chromosomes after a mitotic delay. *Mol Biol Cell*, 15(8), 3771–3781. <https://doi.org/10.1091/mbc.E03-07-0482>
- Tse, C., Shoemaker, A. R., Adickes, J., Anderson, M. G., Chen, J., Jin, S., Johnson, E. F., Marsh, K. C., Mitten, M. J., Nimmer, P., Roberts, L., Tahir, S. K., Xiao, Y., Yang, X., Zhang, H., Fesik, S., Rosenberg, S. H., & Elmore, S. W. (2008). ABT-263: A potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Research*, 68(9), 3421–3428. <https://doi.org/10.1158/0008-5472.CAN-07-5836>
- Tummers, B., & Green, D. R. (2017). Caspase-8: regulating life and death. In *Immunological Reviews* (Vol. 277, Issue 1, pp. 76–89). NIH Public Access. <https://doi.org/10.1111/imr.12541>
- U.S National Library of Medicine. (2021). *ClinicalTrials.gov*.
- Ueda, A., Oikawa, K., Fujita, K., Ishikawa, A., Sato, E., Ishikawa, T., Kuroda, M., & Kanekura, K. (2019). Therapeutic potential of PLK1 inhibition in triple-negative breast cancer. *Lab Invest*, 99(9), 1275–1286. <https://doi.org/10.1038/s41374-019-0247-4>
- Vaupel, P., Schmidberger, H., & Mayer, A. (2019). The Warburg effect: essential part of metabolic reprogramming and central contributor to cancer progression. In *International Journal of Radiation Biology* (Vol. 95, Issue 7, pp. 912–919). Int J Radiat Biol. <https://doi.org/10.1080/09553002.2019.1589653>
- Vleugel, M., Tromer, E., Omerzu, M., Groenewold, V., Nijenhuis, W., Snel, B., & Kops,

- G. J. P. L. (2013). Arrayed BUB recruitment modules in the kinetochore scaffold KNL1 promote accurate chromosome segregation. *Journal of Cell Biology*, 203(6), 943–955. <https://doi.org/10.1083/jcb.201307016>
- Vose, J. M., Friedberg, J. W., Waller, E. K., Cheson, B. D., Juvvigunta, V., Fritsch, H., Petit, C., Munzert, G., & Younes, A. (2013). The Plk1 inhibitor BI 2536 in patients with refractory or relapsed non-Hodgkin lymphoma: A phase I, open-label, single dose-escalation study. *Leuk Lymphoma*, 54(4), 708–713. <https://doi.org/10.3109/10428194.2012.729833>
- Vukušić, K., Buđa, R., & Tolić, I. M. (2019). Force-generating mechanisms of anaphase in human cells. In *Journal of Cell Science* (Vol. 132, Issue 18). The Company of Biologists. <https://doi.org/10.1242/jcs.231985>
- Wang, H., Tao, Z., Feng, M., Li, X., Deng, Z., Zhao, G., Yin, H., Pan, T., Chen, G., Feng, Z., Li, Y., & Zhou, Y. (2020). Dual PLK1 and STAT3 inhibition promotes glioblastoma cells apoptosis through MYC. *Biochem Biophys Res Commun*, 533(3), 368–375. <https://doi.org/10.1016/j.bbrc.2020.09.008>
- Warner, S. L., Munoz, R. M., Stafford, P., Koller, E., Hurley, L. H., Hoff, D. D. Von, & Han, H. (2006). Comparing Aurora A and Aurora B as molecular targets for growth inhibition of pancreatic cancer cells. *Mol Cancer Ther*, 5(10), 2450–2458. <https://doi.org/10.1158/1535-7163.MCT-06-0202>
- Weil, D., Garçon, L., Harper, M., Duménil, D., Dautry, F., & Kress, M. (2002). Targeting the kinesin Eg5 to monitor siRNA transfection in mammalian cells. *BioTechniques*, 33(6), 1244–1248. <https://doi.org/10.2144/02336st01>
- Welburn, J. P. I., Vleugel, M., Liu, D., Yates, J. R., Lampson, M. A., Fukagawa, T., & Cheeseman, I. M. (2010). Aurora B Phosphorylates Spatially Distinct Targets to Differentially Regulate the Kinetochore-Microtubule Interface. *Molecular Cell*, 38(3), 383–392. <https://doi.org/10.1016/j.molcel.2010.02.034>
- Willems, E., Dedobbeleer, M., Digregorio, M., Lombard, A., Lumapat, P. N., & Rogister, B. (2018). The functional diversity of Aurora kinases: a comprehensive review. *Cell Div*, 13, 7. <https://doi.org/10.1186/s13008-018-0040-6>
- Wolf, G., Elez, R., Doermer, A., Holtrich, U., Ackermann, H., Stutte, H. J., Altmannsberger, H. M., Rübsamen-Waigmann, H., & Strebhardt, K. (1997). Prognostic significance of polo-like kinase (PLK) expression in non-small cell lung cancer. *Oncogene*, 14(5), 543–549. <https://doi.org/10.1038/sj.onc.1200862>
- Xie, Y., Wang, A., Lin, J., Wu, L., Zhang, H., Yang, X., Wan, X., Miao, R., Sang, X., & Zhao, H. (2017). Mps1/TTK: a novel target and biomarker for cancer. *Journal of Drug Targeting*, 25(2), 112–118. <https://doi.org/10.1080/1061186X.2016.1258568>
- Yang, H., Zhang, F., Huang, C.-J., Liao, J., Han, Y., Hao, P., Chu, Y., Lu, X., Li, W., Yu, H., & Kang, J. (2019). Mps1 regulates spindle morphology through MCRS1 to promote chromosome alignment. *Molecular Biology of the Cell*, 30(9), 1060–1068. <https://doi.org/10.1091/mbc.E18-09-0546>
- Yang, M., Li, B., Tomchick, D. R., Machius, M., Rizo, J., Yu, H., & Luo, X. (2007). p31comet Blocks Mad2 Activation through Structural Mimicry. *Cell*, 131(4), 744–755. <https://doi.org/10.1016/j.cell.2007.08.048>
- Ye, Q., Rosenberg, S. C., Moeller, A., Speir, J. A., Su, T. Y., & Corbett, K. D. (2015). TRIP13 is a protein-remodeling AAA+ ATPase that catalyzes MAD2 conformation

- switching. *ELife*, 2015(4), 1–44. <https://doi.org/10.7554/eLife.07367>
- Ye, X. S., Fan, L., Van Horn, R. D., Nakai, R., Ohta, Y., Akinaga, S., Murakata, C., Yamashita, Y., Yin, T., Credille, K. M., Donoho, G. P., Merzoug, F. F., Li, H., Aggarwal, A., Blanchard, K., & Westin, E. H. (2015). A novel Eg5 inhibitor (LY2523355) causes mitotic arrest and apoptosis in cancer cells and shows potent antitumor activity in xenograft tumor models. *Mol Cancer Ther*, 14(11), 2463–2472. <https://doi.org/10.1158/1535-7163.MCT-15-0241>
- Yu, K.-W., Zhong, N., Xiao, Y., & She, Z.-Y. (2019). Mechanisms of kinesin-7 CENP-E in kinetochore-microtubule capture and chromosome alignment during cell division. *Biol Cell*, 111(6), 143–160. <https://doi.org/10.1111/boc.201800082>
- Zeng, Y., Li, N., Liu, W., Zeng, M., Cheng, J., & Huang, J. (2020). Analyses of expressions and prognostic values of Polo-like kinases in non-small cell lung cancer. *Journal of Cancer Research and Clinical Oncology*, 146(10), 2447–2460. <https://doi.org/10.1007/s00432-020-03288-6>
- Zhang, G., Kruse, T., López-Méndez, B., Sylvestersen, K. B., Garvanska, D. H., Schopper, S., Nielsen, M. L., & Nilsson, J. (2017). Bub1 positions Mad1 close to KNL1 MELT repeats to promote checkpoint signalling. *Nature Communications*, 8. <https://doi.org/10.1038/ncomms15822>
- Zhang, G., Lischetti, T., & Nilsson, J. (2014). A minimal number of MELT repeats supports all the functions of KNL1 in chromosome segregation. *Journal of Cell Science*, 127(4), 871–884. <https://doi.org/10.1242/jcs.139725>
- Zhu, T., Dou, Z., Qin, B., Jin, C., Wang, X., Xu, L., Wang, Z., Zhu, L., Liu, F., Gao, X., Ke, Y., Wang, Z., Aikhionbare, F., Fu, C., Ding, X., & Yao, X. (2013). Phosphorylation of microtubule-binding protein hec1 by mitotic kinase aurora b specifies spindle checkpoint kinase mps1 signaling at the kinetochore. *Journal of Biological Chemistry*, 288(50), 36149–36159. <https://doi.org/10.1074/jbc.M113.507970>

Appendix

Movie legends

Chapter III

Targeting the anti-apoptotic pathway sensitizes lung cancer cells to Plk1 inhibition

Movie 1- Time-lapse imaging (DIC microscopy) of a normal mitosis in untreated A549 cell. Time is shown in hours:minutes.

Movie 2- Time-lapse imaging (DIC microscopy) of a A549 cell treated with Navitoclax undergoing a normal mitosis.

Movie 3- Time-lapse imaging (DIC microscopy) of a A549 cell treated with BI2536 undergoing death in mitosis after spent more than 13h in mitosis. Time is shown in hours:minutes.

Movie 4- Time-lapse imaging (DIC microscopy) of a A549 cell treated with BI2536 undergoing slippage and remaining alive. Time is shown in hours:minutes.

Movie 5- Time-lapse imaging (DIC microscopy) of a A549 cell treated with BI2536 undergoing slippage followed by death. Time is shown in hours:minutes.

Movie 6- Time-lapse imaging (DIC microscopy) of a A549 cell treated with BI2536 and Navitoclax undergoing cell death in mitosis after arresting in mitosis for only approximately 3h. Time is shown in hours:minutes.

Chapter III

Identifying new antimitotics

Group II

The diarylpentanoid BP-M345 is a potent antitumor agent with antimitotic activity

Movie 7- Time-lapse imaging (DIC microscopy) of a normal mitosis in untreated NCI-H460 cell. Time is shown in hours:minutes.

Movie 8- Time-lapse imaging (DIC microscopy) of NCI-H460 cell undergoing death in mitosis. Time is shown in hours:minutes.

Movie 9- Time-lapse imaging (DIC microscopy) of NCI-H460 cell undergoing post mitotic death. Time is shown in hours:minutes.