Fábio França Vieira e Silva

Investigating the antitumoral activity and mechanism of action of a xanthone derivative

Department of Biomedical Sciences and Medicine

2020

Fábio França Vieira e Silva

Investigating the antitumoral activity and mechanism of action of a xanthone derivative

Master in "*Oncobiologia: Mecanismos moleculares do cancro*"

Work under the supervision of:

Hassan Bousbaa, Ph.D

Affiliation – IUCS, Instituto Universitário de Ciências da Saúde

Patrícia Manuela Areias da Silva, Ph.D

Affiliation – IUCS, Instituto Universitário de Ciências da Saúde

Álvaro Tavares, Ph.D

Affiliation – Departamento de Ciências Biomédicas e Medicina da Universidade do Algarve

Department of Biomedical Sciences and Medicine

2020

Investigating the antitumoral activity and mechanism of action of a xanthone derivative

Authorship Statement

I declare to be the author of this work, which is original and unpublished. Authors and papers consulted are duly cited in the text and are listed in the included references.

“Todas às vitórias ocultam uma abdicação.”

(Simone de Beauvoir 1908 - 1986)

"Que todos os nossos esforços estejam sempre focados no desafio à impossibilidade.

Todas as grandes conquistas humanas vieram daquilo que parecia impossível."

Charles Chaplin (1889 - 1977)

The work presented in this thesis was conducted at:

Drug Discovery, Delivery & Toxicology Group | Mitosis & Cancer Biology

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

and

Natural Products and Medicinal Chemistry Group

CIIMAR-UP - Interdisciplinary Centre of Marine and Environmental Research, University of Porto | ciimar.up.pt

Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos s/n

4450-208 Matosinhos, Portugal

FINANCIAL SUPPORT

This work was supported by CESPU - Cooperativa de Ensino Superior Politécnico e Universitário Crl [grant number ComeTarget_CESPU_2017 and ComeTax-PFT-IINFACTS-2019]. This research was partially supported by FCT/MCTES - Foundation for Science and Technology from the Minister of Science, Technology and Higher Education and European Regional Development Fund (ERDF) under the projects, co-financed by COMPETE 2020, Portugal 2020, PTDC/SAU-PUB/28736/2017 (POCI-01-0145-FEDER-028736) and within the scope of UIDB/04423/2020, UID/QUI/5000612019, and UIDP/04423/2020 (Group of Natural Products and Medicinal Chemistry). ACH thanks FCT for her PhD grant (SFRH/BD/140844/2018). DRPL thanks FCT for her PhD grant (SFRH/BD/140844/2018). JXS thanks for the FCT PhD Programmes, specifically by the BiotechHealth Programme (PD/00016/2012), and for the grants (SFRH/BD/98105/2013 and SFRH/BD/116167/2016). To Departamento de Química da Universidade de Aveiro (Portuguese NMR network) for the NMR analysis.

Acknowledgements

Agradeço a Deus, por sempre reservar muitas bençãos em meu caminho, por ter me mantido forte o suficiente para passar, longe das pessoas que amo e sozinho, por um momento tão delicado que foi esta pandemia. Mesmo diante de tantos percursos, sei que fui abençoado por ter estado saudável e com energia suficiente para que fosse possível concluir todos os planejamentos iniciais que tinha para o meu projeto de mestrado.

Agradeço ao meu orientador, o professor Dr. Hassan Bousbaa, por ter me dado à possibilidade de aprender em seu laboratório, por toda a sua motivação, profissionalismo, paciência e disponibilidade, e principalmente, por ter aceitado o desafio de pensar um projeto que fosse construtivo e que me desse à possibilidade de emergir no mundo da investigação. Terei gratidão eterna pela oportunidade de ter integrado tua equipa e de ter aprendido um pouco com a tua experiência. Cada conversa, ensinamento e incentivo, estarão guardados comigo.

Agradeço à minha coorientadora professora Dra. Patrícia Silva, por todos os inúmeros ensinamentos, por tantas horas do seu trabalho que foram destinadas a mim, e por sempre ter acreditado e confiado no meu trabalho. Este trabalho não seria possível sem todo esforço mútuo que empenhamos para que ele acontecesse. O aluno não é nada se não tiver ao seu lado um grande mestre, alguém que saiba partilhar seu conhecimento sem jamais pedir nada em troca, pelo simples prazer de ensinar. Agradeço imensamente à amizade que construímos gradativamente ao longo deste tempo, e que certamente levarei para vida. Todos os conselhos e risadas que compartilhamos, estarão guardados comigo.

Agradeço também ao meu coorientador o professor Dr. Álvaro Tavares, por ter se mostrado além de um grande mestre, um grande amigo.

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

Agradeço à professora Dra. Raquel Andrade, por sua amizade e apoio para que eu ultrapassasse alguns dos meus maiores desafios durante o decorrer do mestrado.

Agradeço também à professora Dra. Isabel Palmeirim, por sua ajuda em momentos difíceis e por toda sua paciência quando precisei de alguém para conversar.

À minha amiga e companheira do mestrado, Renata Sandres, que compartilhou junto a mim tantas dificuldades, horas de estudos, mas que hoje compartilha também esta vitória. Só nós sabemos o quanto foi difícil todo este processo, e que com certeza nos fez ainda mais fortes e resilientes.

Agradeço também aos meus companheiros de laboratório, Ana Henriques, Joel Pedrosa, Bárbara Fernandes, Ricardo Gonçalves, e aos professores, professora Dra. Leonor Delgado e professor Dr. Luís Monteiro, por todos os ensinamentos diários, por toda paciência e apoio, por toda amizade, risadas, almoços, conselhos, conversas e bons momentos compartilhados. Nenhuma conquista é concretizada sozinho! Vocês fazem parte desta juntamente a mim.

À Isabel, Zélia, Paula e Matilde, que além de cuidarem dos laboratórios, cuidaram de mim como um filho, obrigado pelas guloseimas e por tantas conversas e risadas nos horários vagos.

Agradeço imensamente também aos meus pais Tânia Mara e Sebastião Machado, e minhas irmãs, Sarah e Cíntia, que sempre estiveram ao meu lado em todos os momentos da minha vida, sendo meu verdadeiro alicerce e fazendo com que o impossível se tornasse possível. Serei eternamente grato por terem comprado junto a mim o desafio de ter que me afastar (temporariamente) da medicina dentária e a viver em um novo país para embarcar em uma nova jornada, e por terem me ajudado a entender que para que eu pudesse dar dois passos à frente, talvez fosse preciso antes um para trás. Eu nunca conseguirei agradecer o suficiente por todo amor que recebo de vocês, nem mesmo todas as palavras de agradecimento do mundo seriam suficientes para agradecer e demonstrar o tamanho do amor que sinto por vocês.

Aos amigos, que estiveram ao meu lado, mesmo que virtualmente, sempre me ouvindo e me aconselhando a ser alguém melhor dia após dia. Eu só tenho gratidão por cada vez mais ter a certeza de que eu tenho os melhores amigos do mundo. Obrigado por cada ligação, mensagem ou chamada de vídeo, sejam as que fazíamos

para eu desabafar sobre as dificuldades de estar vivendo sozinho em outro país, sejam as que fazíamos para que continuássemos participando uns da vida dos outros da mesma maneira. Toda compreensão que sempre tiveram pelas minhas ausências que foram necessárias neste percurso, só demonstram que os 7482 km que nos separam físicamente não influenciaram em nada nos nossos sentimentos, pelo contrário, apenas reafirmaram o nosso amor. Não há distância que impeça o amor verdadeiramente incondicional.

Thesis outline

This thesis is organized into seven chapters. All figures are copyright.

Chapter I refers to the introduction, and has the role of providing an initial overview of the issues addressed directly or indirectly in the rest of the study. Initially, an overview of the cell cycle, its regulation and control is given. In the sequence, a greater emphasis is given to mitosis, which is described in detail from its cascade of events to the molecular machinery of the mitotic spindle. Still at this stage, the dynamics that occur between kinetochore-microtubules, correlated errors and their due corrections are described. Next, we provide an overview of the Spindle Assembly Checkpoint (SAC), dissecting the functions triggered by this mechanism, as well as the proteins involved in this important cellular control mechanism. Following, an introduction was given about drugs that use the targeting of mitosis for cancer therapy, namely through microtubules, providing an overview of current approaches, their limitations and future directions. Finally, a correlation was made between xanthones and cancer, demonstrating how this class of compounds (as well as their derivatives) is already used as a starting point in the development of new anticancer drugs.

Chapter II concerns what motivated the project, as well as its specific objectives.

Chapter III refers to the materials and methods used throughout the study, so that it was possible to dissect the mechanism of action of the compound.

Chapter IV will demonstrate the results about the compound's mechanism of action, through: in vitro characterization of the compound's antimitotic activity, identification of the underlying mechanism of action and evaluation of the combined treatment of PX2 with paclitaxel in promoting cell death of tumoral cells.

Chapter V provides a discussion, correlating previous studies and the present study.

Chapter VI provides general conclusions about the mechanism of action of PX2 and the prospects for future research.

Chapter VII contains a list of all references cited in the course of the thesis.

The information presented in the chapters is submitted for publishing in international peer review scientific journals and presented at congresses, according to the following:

Fábio França, Patrícia M. A. Silva, Ana C. Henriques, José X. Soares, Daniela R. P. Loureiro, Carlos M. G. Azevedo, Carlos M. M. Afonso and Hassan Bousbaa. A pyranoxanthone as a potent antimitotic and sensitizer of cancer cells to low doses of paclitaxel in Toxicology Letter. 2020. *(submitted).*

Fábio França, Patrícia M. A. Silva, Ana C. Henriques, José X. Soares, Daniela R. P. Loureiro, Carlos M. G. Azevedo, Carlos M. M. Afonso and Hassan Bousbaa. Investigating the antitumoral activity of the compound pyranoxanthone 2 and its mechanism of action, at oral communication format, in 3ª Reunião Internacional Rede Académica das Ciências da Saúde da Lusofonia (RACS). 2020.

Fábio França, Patrícia M. A. Silva, Ana C. Henriques, José X. Soares, Daniela R. P. Loureiro, Carlos M. G. Azevedo, Carlos M. M. Afonso and Hassan Bousbaa. Investigating the antitumoral activity of the compound pyranoxanthone 2 and its mechanism of action, in e-poster communication format, in 2º Encontro Nacional de Jovens Investigadores em Oncologia.

GENERAL INDEX

List of figures

List of tables

List of abbreviations, acronyms and symbols

A375-C5 – Melanoma cell line

Akt/PKB – Protein kinase B

AMPk – AMP-activated protein kinase

Ang – Angiopoietin

APAF – Apoptotic protease activating factor

APC/C – Anaphase promoting complex/cyclosome

ATP – Adenosine triphosphate

AURK – Aurora kinase

Bcl – B-cell lymphoma

Bub – Budding uninhibited by benzimidazole

BubR1 – Budding uninhibited by benzimidazole-related 1

CAKs – Cdk-activating kinases

cAMP – Cyclic adenosine monophosphate

Cdc – Cell division cycle

Cdks – Cyclin-dependent kinases

Chk – Checkpoint kinase

CIN – Chromosomal instability

CENP – Centromere-associated protein

CKIs – Cdk inhibitors

cm – Centímetros

C-Mad2 – Closed Mad2

CO2 – Carbon dioxide

CYP – Cytochrome P450

Cyt-c – cytochroma C

DAPI – 4’,6-diamidino-2-phenylindole

DF – dilution factor

DMSO – Sulfur dioxide dimethyl

DMXAA – 5,6-dimethylxanthenone-4-acetic acid

DNA – Deoxyribonucleic acid

DIC – Dynein intermediate chain

DiM – Death in mitosis

DTT – Dithiothreitol

ECM – Extracellular matrix

EGF – Heparin-binding

EMAP – Endothelial monocyte-activating polypeptide

EMT – Epithelial mesenchymal transition

EPO – Epothilone

ERK – Extracellular signal-regulated kinases

ET – Endothelins

FBS – Fetal bovine serum

FGF – Fibroblast growth factors

G0 – Gap0

G1 – Gap1

G2 – Gap2

GDP – Guanosine diphosphate

GF – growth factors

GI50 – Cell growth inhibition of 50%

GRB – Growth factor receptor-bound

GTP – Guanosine triphosphate

h – Hours

HCl – Hydrochloric acid

HER – Human epidermal growth factor receptor

HIF – Hypoxia-inducible factors

HT-29 – Human colorectal adenocarcinoma cell line

HepG2 – Hepatocellular carcinoma cell line

IL – Interleukin

IRS – insulin receptor substrate

KP – Kinase protein

KT – Kinetochore

KT-MT – Kinetochore-microtubule

Lis – Platelet Activating Factor Acetylhydrolase

M – Molar

Mad – Mitotic arrest deficient

MAPK – Mitogen activated protein kinase

MAPs – Microtubule-associated proteins

MCAK – Centromere-associated kinesin

MCC – Mitotic checkpoint complex

MCF-7 – Human breast adenocarcinoma cell line

MDR – Multidrug resistance

MG-132 – Carbobenzoxy-Leu-Leu-leucinal

MI – Mitotic index

min – Minutes

mL – Milliliter

mM – Millimolar

M phase – Mitosis

Mps1 – Monopolar spindle 1 kinase

MRP – Multiresistant protein

MTAs – Microtubule-targeting agents

MTOC – Microtubule-organizing center

MT – Microtubule

MYT – Myelin transcription

NF-Kb – Nuclear factor kappa B

NCI-H460 – Non-small cell lung cancer

nM – Nanomolar

O-Mad2 – Open-Mad2

PAI – Plasminogen activator inhibitor

PBS – Phosphate-buffered saline

PDGF – Platelet-derived growth factor

PE – Plating efficiency

P-gp – P-glycoprotein

PHD – Hydroxylase proline

PI – Propidium iodide

PI3-k – Phosphoinositide 3-kinase

PIP 2 – Phosphatidylinositol 4,5-bisphosphate

PIP 3 – Phosphatidylinositol 3,4,5-triphosphate

PBD – P21-Rho-binding domain

PMD – Post-mitotic death

Plk – Polo-like kinase

PMD – Post-mitotic death

PTEN – Phosphatase and tensin homolog gene

PTX – Paclitaxel

PX2 – Pyranoxanthone 2

Q – Quadrants

Rb – Retinoblastoma

RHEB – Ras homolog enriched in brain

RNA – Ribonucleic acid

RNase – Ribonuclease

rpm – Rotations per minute

RPMI – Roswell Park Memorial Institute

RTK – Receptor tyrosine kinases

RZZ – Rod/ZW10/Zwilch

Scc – Double-strand-break repair protein

S-phase – Synthesis-phase

SAC – Spindle assembly checkpoint

SD – Standard deviation

Ska – Spindle and kinetochore associated

SOS – Son of sevenless gene

SRB – Sulforodamine B

T47D – Human breast carcinoma cell line

Thr – Threonine

TGF – tumor growth factor

TNF – Tumor necrosis factor

TSC – Tuberous sclerosis gene

TSP – Thrombo-spondin

Tyr – Tyrosine

TUNEL – Terminal deoxynucleotidyl transferase-mediated nick end labeling

VEGF – Vascular endothelial growth factor

XIAP – X-linked inhibitor of apoptosis protein

WHO – World Health Organization

α – Alpha

β – Beta

+TIPs – Microtubule plus-end-tracking proteins

°C – Degree Celsius

µg – Microgram

µM – Micromolar

Abstract

Microtubule-targeting agents (MTAs) remain a gold standard for the treatment of several cancer types. By interfering with microtubules dynamic, MTAs induce a mitotic arrest followed by cell death. This antimitotic activity of MTAs is dependent on the spindle assembly checkpoint (SAC), which monitors the integrity of the mitotic spindle and proper chromosome attachments to microtubules in order to ensure accurate chromosome segregation and timely anaphase onset. However, the cytotoxic activity of MTAs is restrained by drug resistance and/or toxicities, and had motivated the search for new compounds and/or alternative therapeutic strategies. Here, we have determined the mechanism of action of the xanthone derivative pyranoxanthone 2 identified as a potent inhibitor growth of cancer cells. We found that cancer cells treated with the pyranoxanthone 2 exhibited persistent defects in chromosome congression during mitosis that were not corrected over time, which induced a prolonged SAC-dependent mitotic arrest followed by massive apoptosis. Importantly, pyranoxanthone 2 was able to potentiate apoptosis of cancer cells treated with nanomolar concentrations of paclitaxel. Our data identified the potential of the pyranoxanthone 2 as a new potent antimitotic with promising antitumor potential, either alone or in combination regimens.

Keywords: mitosis, kinetochore-microtubule attachments, pyranoxanthone, paclitaxel, cancer, apoptosis

Resumo

O cancro é uma doença em ascensão, e sua incidência e mortalidade aumentaram consideravelmente nos últimos anos. Como a idade é o principal fator etiológico, é possível relacionar a população mais longeva atualmente como um dos indicadores desse aumento, bem como as mudanças no estilo de vida no mundo e fatores ambientais. As taxas de mortalidade têm sido tão altas que em muitos países ultrapassam as taxas de mortalidade por acidente vascular cerebral e doenças coronárias. Os fatores de risco do cancro podem incluir exposições ocupacionais, agentes infecciosos, determinantes sociais, fatores de estilo de vida e mudanças genéticas e epigenéticas, tornando-se uma doença de alta complexidade. Muitas interações heterotípicas ocorrem no ambiente tumoral e as células estromais normais associadas a tumores são participantes ativos no desenvolvimento tumoral. A compreensão das muitas etapas do processo tumorigênico é necessária para que novas abordagens terapêuticas surjam, como resultado da compreensão da evolução da doença e de seus mecanismos adjacentes.

Os tratamentos mais comumente usados para combater o cancro são quimioterapia, radioterapia e cirurgia, ou uma associação de ambas. Uma importante abordagem quimioterapêutica usa a interrupção da maquinaria mitótica para potencializar a paragem do ciclo celular e/ou morte das células cancerígenas. Os "*microtubule-targeting agents*" (MTAs) são os principais agentes antimitóticos utilizados em clínica atualmente, isto porque os microtúbulos são parte essencial no processo de divisão e inibir sua dinâmica de polimerização acaba por ativar checkpoint mitótico (SAC), que monitora a integridade do fuso mitótico e as ligações cromossômicas adequadas aos microtúbulos, a fim de garantir a segregação cromossômica precisa e o início oportuno da anáfase. Com o SAC ativo, não é possível ocorrer a transição da metáfase para a anáfase e ocorre uma paragem do ciclo celular seguido, na maioria das vezes, de morte celular.

Entretanto, a atividade citotóxica dos MTAs não está limitada apenas às células cancerosas, e por este motivo, as toxicidades frequentemente se manifestam de maneira negativa, causando efeitos adversos, como, por exemplo, neuropatia

periférica. Além disso, em todas as fases do tratamento, pode haver mecanismos de resistência à droga, onde a célula cancerígena tenta emitir rotas de escape no contexto de morte celular.

O interesse agora é encontrar novas moléculas que contornem os mecanismos de resistência e que sejam mais direcionados a determinados alvos. Aqui, determinamos o mecanismo de ação do derivado da xantona pyranoxantona 2 identificado como um potente inibidor do crescimento de células cancerosas. Descobrimos que as células cancerígenas tratadas com a pyranoxantona 2 exibiram defeitos persistentes no processo de congressão cromossômico durante a mitose que não foram corrigidos ao longo do tempo, o que induziu uma paragem em mitose dependente de SAC seguida por apoptose maciça. É importante ressaltar que a pyranoxantona 2 foi capaz de potenciar a apoptose de células cancerosas tratadas com concentrações clinicamente relevantes de paclitaxel. Nossos dados identificaram o potencial da pyranoxantona 2 como um novo antimitótico potente com potencial antitumoral promissor, sozinho ou em regimes de combinação.

Palavras-chave: mitose, ligações cinetocoro-microtúbulos, piranoxantona, paclitaxel, cancro, apoptose

Chapter I

Introduction

1 The eukaryotic cell cycle - General overview

Cell cycle is the set of processes that occur in the cell from its appearance to the cell division process, where a parental cell will gives rise to two daughter cells (Sullivan and Morgan, 2007; Harashima, Dissmeyer and Schnittger, 2013). In eukaryotic cells, this process is divided into two main phases: interphase and mitosis. This process is responsible for the renewal of damaged or aged cells. During this period, a cell goes through several processes, such as cell growth, multiplication of its genetic material and cell division (Harashima, Dissmeyer and Schnittger, 2013).

Interphase is the longest period of cell cycle and occurs between two consecutive cell divisions. The interphase is divided into three stages: Gap1 (G1); synthesis phase (S); and Gap2 (G2) (Nigg, 2001; Sullivan and Morgan, 2007; Harashima, Dissmeyer and Schnittger, 2013) (**Figure 1**). At this stage, a cell is in intense metabolic activity for its growth. The chromosomal filaments remain disconnected within the nucleus, constituting a complex of DNA chromatin and proteins of eukaryotic cells. It is during the interphase that the chromosomal DNA is active and in constant production of RNA molecules. It is also during interphase that cell grows and chromosomal DNA duplicates, preparing the cell for further division. Mitosis is also divided in stages, simply: Prophase, Prometaphase, Metaphase, Anaphase and Telophase (**Figure 1**).

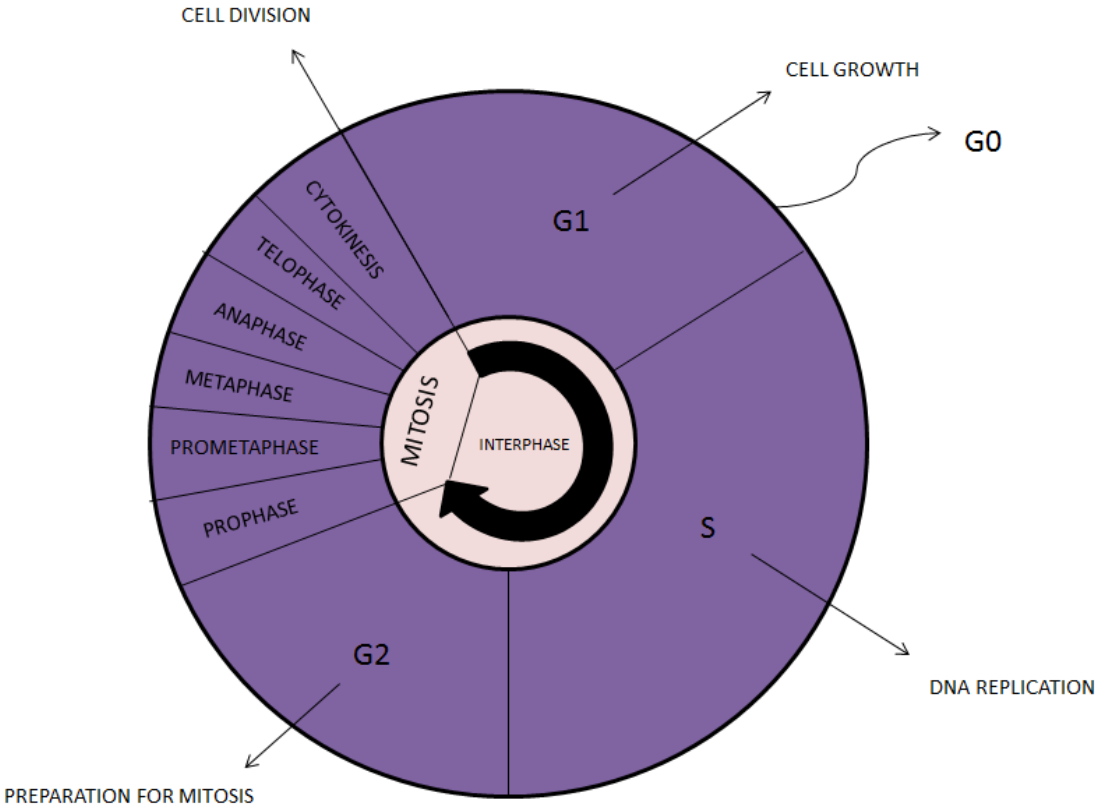

Figure 1 Cell cycle representation. The interphase is divided into G1, S and G2 phases. G0 is a resting phase. Mitosis is divided into Prophase, Prometaphase, Anaphase and Telophase. Cytokinesis ends the process with the cytoplasmic division of the cell.

The G1 phase is usually the longest and most variable phase (depending on the cell type) that precedes the duplication of chromosomal DNA. It is at this stage that the membrane, structural proteins, cytoplasmic organelles and RNAs increase in size, although there is only one copy of chromosomes per cell (Harashima, Dissmeyer and Schnittger, 2013). Because of this characteristic of structural duplication, this stage is recognized by cell growth. There are cells that do not go through this state and remain in it until their death, when the cell receives no stimulus to perform its division and ends up fulfilling only its cellular role with normal metabolism, without duplicating the genetic material or dividing.

It is also in the G1 phase that the restriction point (R) occurs, which is known as a decisive point for the cell to enter in the cell cycle. This restriction point is responsible

for dictating the fate of most cells and is regulated by extracellular growth factors (Vermeulen, Berneman and Van Bockstaele, 2003; Malumbres and Barbacid, 2009).

Through the reception of extracellular growth factors, some cells pass the restriction point and proceed to the S phase, while some cells, in the absence of suitable growth factors, leave the cell cycle and enter a non-proliferative state called G0. In this stage, the cells are inactive and can remain in G0 or reverse this state and return to their activity (**Figure 1**) (Sanchez *et al.*, 1997; Sullivan and Morgan, 2007).

It is in the S-phase that the process of duplication of genetic material occurs. In this stage, the cell is performing its metabolic functions and, in the end, the centrosomes and DNA are completely replicated (Doxsey, 2001; Nigg, 2001; Sullivan and Morgan, 2007; Harashima, Dissmeyer and Schnittger, 2013). In the G2 phase, occurs the duplication of centrioles, production of glycolysis to form the mitotic spindle, the cell completes growth and is prepared for mitosis.

Mitosis, or M phase, is the step that results in nuclear division and cytokinesis, the next step, occurs cytoplasmic division that generates daughter cells (Weinert, 1997; Harashima, Dissmeyer and Schnittger, 2013).

The order of the events in the cell cycle must be controlled, so that the time and direction are accurate. This is because it is a unidirectional process, on which subsequent events depend directly on the success of previous events to be carried out in the correct way (Murray, 2004; Sullivan and Morgan, 2007). There is, therefore, a network of regulatory proteins responsible for this control of the cell cycle that occurs through signal transduction pathways allowing events with a high level of complexity that are separated in time or space to also be connected.

The main regulatory proteins are cyclin-dependent kinases (CDKs), which refers to a family of serine/threonine protein kinases that are activated at the cycle checkpoints to regulate the process of entry and exit between phases (**Figure 2**) (Vermeulen, Van Bockstaele and Berneman, 2003; Malumbres and Barbacid, 2009; Lim and Kaldis, 2013). When activated, CDK is responsible for inducing downstream processes through

the phosphorylation of selected proteins (Morgan, 1995; Malumbres and Barbacid, 2009; Lim and Kaldis, 2013).

CDK activity is regulated by cyclins, but also by phosphorylation in conserved threonine and tyrosine residues (Vermeulen, Van Bockstaele and Berneman, 2003; Lim and Kaldis, 2013) that end up inducing conformational changes that consequently increase the binding of cyclins (Jeffrey *et al.*, 1995; Paulovich and Hartwell, 1995; Murray, 2004).

Cyclins are a family of proteins that control the progression of a cell through the cell cycle. These can be divided into four classes: G1 (D) cyclins, G1/S (E) cyclins, S (A) cyclins and G2/M cyclins (A and B) and are produced and degraded depending on the phase, where cyclin-CDK interactions are considered to be largely responsible for controlling the progression of the cell cycle (**Figure 2**) (Nigg, 2001; Murray, 2004).

The three type D cyclins (cyclin D1, cyclin D2, cyclin D3) bind to the CDK4 and CDK6 and CDK-cyclin D complexes, being essential for entry into G1 (Kato et al., 1994). Another G1 cyclin is cyclin E, which binds to CDK2 and functions as a regulator of G1 to S phase progression (Ohtsubo et al., 1995). G1 cyclins stimulate and control cell cycle entry in response to extracellular factors, and G1/S cyclins trigger cell progression through the restriction point and initiate DNA and centrosome replication (Doxsey, 2001; Nigg, 2001; Murray, 2004).

Cyclin A binds to CDK2 forming an important bond that occurs during the S phase (Girard et al., 1991; Walker and Maller, 1991). Cyclins A are responsible for DNA replication and their expression remains high throughout the S, G2 phase and in early mitosis to promote early mitotic events. At the end of phase G2 and at the beginning of phase M, the cyclin A complex is associated with CDK1 to promote entry into mitosis (Nigg, 2001; Murray, 2004).

Mitosis is regulated by cyclin B in the CDK1 complex (Arellano and Moreno, 1997). Cyclins A and B contain a complex responsible for their destruction, while cyclins D and E contain a PEST sequence (proline-rich segment (P), glutamic acid (E), serine residues (S) and threonine (T)), which are protein sequences that allow efficient proteolysis of

ubiquitin-mediated cyclin at the end of a cell cycle phase (Glotzer, Murray and Kirschner, 1991; Rechsteiner and Rogers, 1996; Dacatur and Portnoy, 2000). Cyclin B increases its expression levels as the cell approaches mitosis while its degradation in return after ubiquitination by the anaphase/cyclosome promoter complex (APC), followed by destruction in the proteasome that leads to the mitotic exit (Nigg, 2001; Murray, 2004).

Unlike the levels of CDK protein that remain stable during the cell cycle, the levels of its activating proteins, cyclins, increase and decrease during the cell cycle, so that they activate the respective CDKs at the precise moment (Evans *et al.*, 1983; Malumbres and Barbacid, 2009; Lim and Kaldis, 2013).

For this complete CDK activation to occur, a CAK (CDK activating kinase) needs to phosphorylate an amino acid in its active site, which in this case is a threonine residue 160 (Thr 160). On the other hand, an additional double phosphorylation regulated by the protein kinase Wee1 ends up inhibiting CDK activity, requiring dephosphorylation that is performed by means of a phosphatase known as Cdc25 used for reactivation (Mailand *et al.*, 2000).

Cell cycle inhibitory proteins, called CDK inhibitors (CKI), can neutralize CDK activity, binding only to CDK or the CDK-cyclin complex (Vermeulen, Van Bockstaele and Berneman, 2003). CKIs perform inhibitory phosphorylation on the tyrosine residue 15 (Tyr 15) and threonine residue 14 (Thr 14) of CDKs and work as brakes to stop the progression of the cell cycle (Sanchez *et al.*, 1997; Lim and Kaldis, 2013).

CKIs are regulated by internal and external signals. The expression of p21 depends on transcriptional control of the p53 tumor suppressor gene. The p53 gene acts transcriptionally under the p21 gene, where the p21 gene promoter contains a p53 binding site that allows this action (El-Deiry *et al.*, 1993; Lim and Kaldis, 2013). The expression and activation of the p15 and p27 proteins leads to an increased response to the transforming growth factor β (TGF-β), which contributes as an important regulatory factor in cell growth (Waga *et al.*, 1994; Reynisdóttir *et al.*, 1995; Dewidar *et al.*, 2019).

Each checkpoint has a primary focus. The checkpoint that occurs in G1 aims to review DNA damage. The checkpoint that occurs during the S phase is intended to verify that adequate DNA duplication has occurred. The checkpoint for the G2 phase consists of an additional check of the DNA content for non-replicated DNA or damage to it. Finally, the checkpoint that occurs during mitosis, called the spindle assembly checkpoint, which prevents the cell from dividing while all the chromosomes are not aligned along the metaphase plate. It is concluded that the cell frequently checks during the cell cycle whether the DNA transmitted to the daughter cells is of good quality and in adequate quantities (**Figure 2**) (Clarke and Giménez-Abián, 2000; Vermeulen, Van Bockstaele and Berneman, 2003; Musacchio and Salmon, 2007).

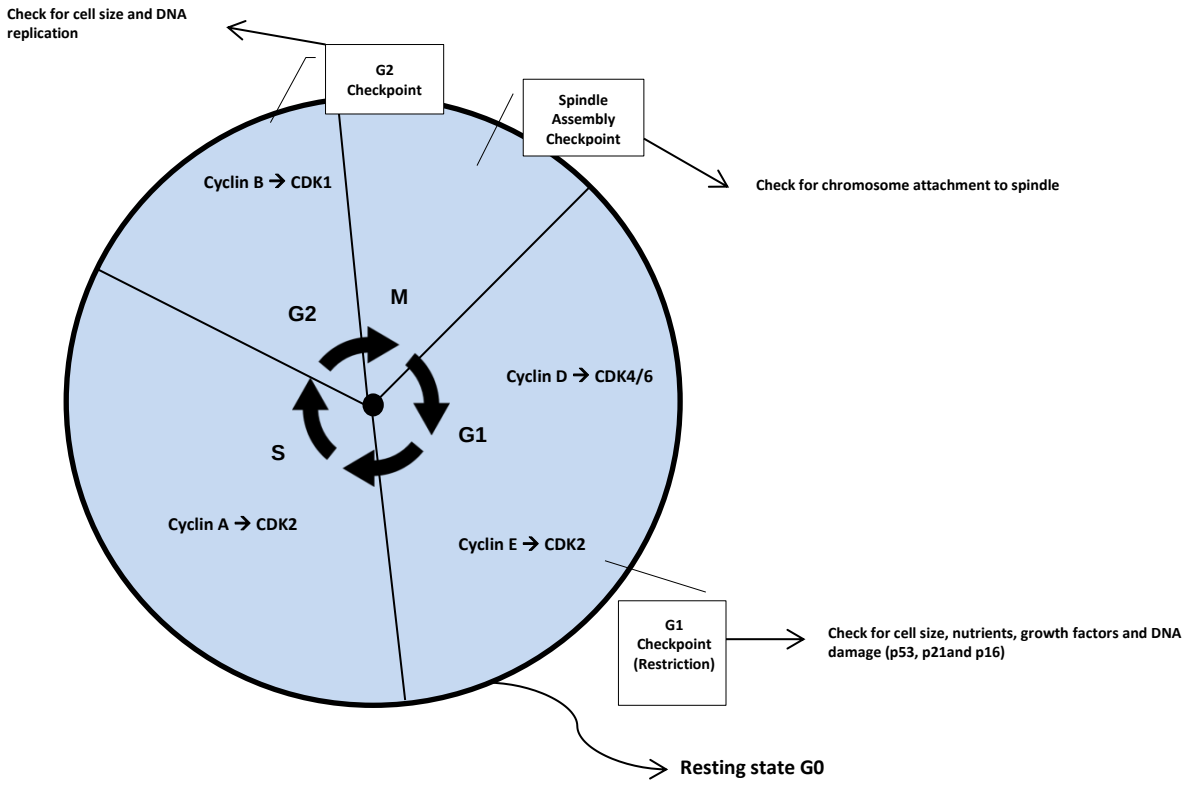

Figure 2 CDK/Cyclin interactions and checkpoints throughout the cell cycle. Cyclin D binds with CDK4/6 during the G1 phase and cyclin E with CDK2. These interactions are decisive to dictate whether or not to enter G0 through the checkpoint that occurs in G1 check for cell size, nutrients, growth factors and DNA damage (p53, p21and p16). Cyclin A binds with CDK2 during phase S and Cyclin B with CDK1 during phase G2 where a new decisive checkpoint occurs to check for cell size, nutrients, growth factors and DNA damage (p53, p21and p16). In mitosis there is the Spindle Assembly Checkpoint that verifies a correct alignment of the chromosomes in the mitotic spindles and allows the advance from metaphase to anaphase.

These control mechanisms in each phase transition that are known as checkpoints have a function of detecting errors and causing a stop until the damage is repaired (Weinert, 1998). In turn, a loss of function causes genomic instability, a typical characteristic in the development of tumorigenesis (Hartwell, 1992; Visconti, Della Monica and Grieco, 2016). Cell cycle checkpoints can be grouped according to their role in DNA damage, during interphase or in mitosis progression. These checkpoints stop or delay the progression of the cell cycle from phase G1, before DNA replication, in phase S, during DNA replication or in phase G2, before mitosis (Weinert, 1998; Zhou and Elledge, 2000; Nigg, 2001).

Checkpoints in response to DNA damage occur when the checkpoints interrupt the cell cycle with the intention of providing time until DNA repair is complete. Sometimes, DNA damage checkpoints occur during the cycle, that is, before the cell enters the S phase (checkpoint G1-S) or after DNA replication (checkpoint G2-M) (Zhou and Elledge, 2000). There also appear to be other checkpoints for DNA damage during the S and M phases (Vermeulen, Van Bockstaele and Berneman, 2003).

The restriction point (R) is known as a point of no return in the G1 phase, which is decisive for the cell to enter the cell cycle (Vermeulen, Van Bockstaele and Berneman, 2003). In case of DNA damage, the checkpoint in phase G1 generates an accumulation of tumor suppressor p53 and cells with damaged DNA are prevented from entering phase S until the error is repaired (Shieh *et al.*, 1997; North and Hainaut, 2000).

The Rb protein plays an essential role in regulating G1 progression and is probably involved as a key component of the molecular network that controls the restriction point. Rb binds and regulates a large number of cellular proteins, including members of

the E2F family of transcription factors, which in turn regulate the expression of many genes that encode proteins involved in cell cycle progression and also in DNA synthesis (**Figure 3**) (DeGregori, Kowalik and Nevins, 1995; Johnson and Walker, 1999; Nevins, 2001; Sherr and McCormick, 2002).

Through activation of the transcription factor E2F, cyclin E is induced during the progression of cells by the G1 phase (Ohtani, Degregori and Nevins, 1995; Geng *et al.*, 1996) and is associated with CDK2 so that the cells make the transition from phase G1 to phase S and, in addition, cyclin E/CDK2 participates in the maintenance of Rb in the hyperphosphorylated state, causing a positive feedback loop for the accumulation of active E2F (**Figure 3**) (Johnson and Walker, 1999). In turn, cyclin A is also partially regulated by E2F, where its accumulation in the G1/S phase transition is observed, a characteristic that persists in phase S. Cyclin A is initially associated with CDK2 and, in sequence, in phase S, is associated with CDK1. The kinase activity associated with cyclin A is critical for entry into phase S, completion of phase S and entry into phase M (Johnson and Walker, 1999; Nevins, 2001; Sherr and McCormick, 2002; Giacinti and Giordano, 2006; Burkhart and Sage, 2008).

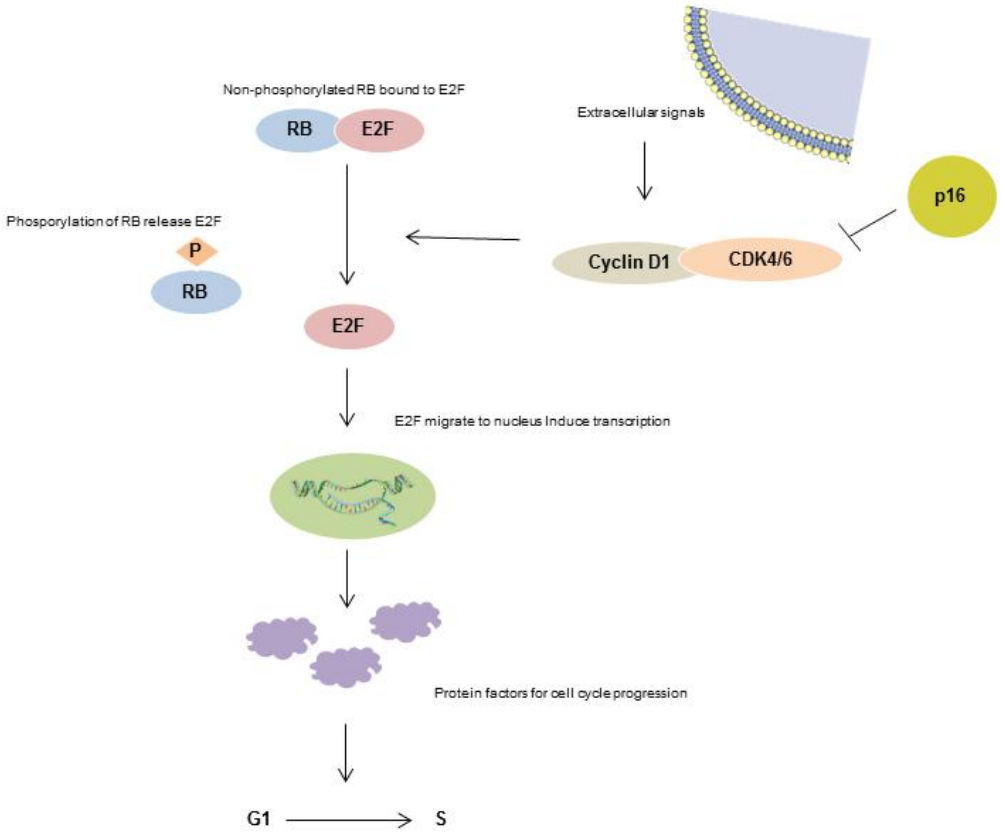

Figure 3The role of RB protein in the progression of the G1 phase. The Rb protein plays an essential role in regulating G1 progression and is involved as a key component of the molecular network that controls the restriction point. Rb binds to the transcription factor E2F which in turn regulates the expression of many genes that encode proteins involved in the progression of the cell cycle and also induces the activation of cyclin E which is associated with cdk2 so that cells make the transition from the G1 phase to the S phase.

Phase G2, as in the other phases, contains a checkpoint that responds to DNA damage and causes a delay in the entry of mitosis, so that DNA repair is possible. Mitosis, in turn, is regulated by CDK1 in association with cyclins A, B1 and B2 (King, Jackson and Kirschner, 1994; Arellano and Moreno, 1997; Zhou and Elledge, 2000). For cells to get out of mitosis cyclins A and B must be degraded and the association of cyclins B/CDK1 kinases is responsible for regulating this process of destruction. After mitosis, cells return to the starting point and return to G1 and, sequentially, at the restriction point, they must decide whether to start a new cell cycle (Johnson and Walker, 1999).

The spindle assembly verification point (SAC), responsible for mitosis, certifies that there was bipolar fixation of the chromosomes with the spindle microtubules and alignment in the metaphase equatorial plate in the transition from metaphase to anaphase (Musacchio and Salmon, 2007).

2 Mitosis

2.1 General description

Schrader in 1944 was already kicking off the complex study of mechanisms involving the cell cycle, in particular mitosis, when he said: "*We came to the conclusion that mitosis is composed of a large complex of mechanisms. As previous investigations tend to be made in the composition of the entire mitotic mechanism, when exposed, it appears to be quite simple. One can explain the fact that almost all hypotheses were built around the idea that a single type of force underlies all mitotic activity and that changes and adjustments in that force and on mobile devices explain the entire execution cycle*".

Mitosis represents a phase of the cell cycle where the genomic material is equally distributed to daughter cells in order for the genome to be successfully transmitted. Mitosis is divided into five stages, which occur continuously and are respectively named: prophase, prometaphase, metaphase, anaphase and telophase (**Figure 4**).

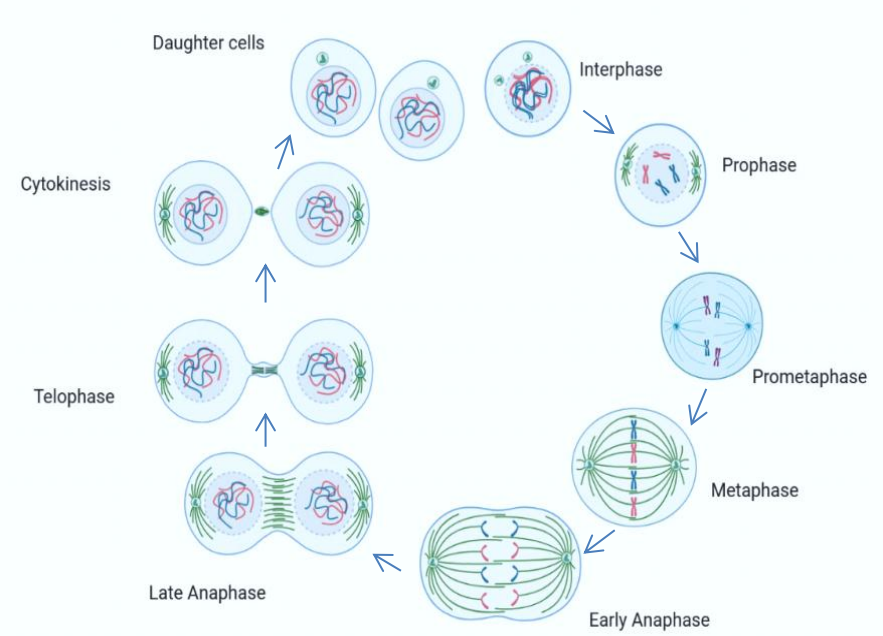

Figure 4 Cell morphology during phases of mitosis. Mitotic division begins with prophase through the condensation of chromatin on chromosomes and the initial formation of the spindle. In prometaphase, microtubules carry out the process of ''search and capture'' of kinetochores in chromosomes in order to align them along the metaphase plate. Metaphase is established by the bipolar orientation of all chromosomes and their alignment midway between the cell poles. In anaphase there is the separation of sister chromatids and their direction to the poles by microtubules. In telophase, the chromatin decondense and reorganizes the nuclear envelope. Cytokinesis ends the division process with the cytoplasm being distributed throughout the daughter cells causing their separation.

In the initial stage of the prophase, the cell begins to break some structures and form others, setting the stage for the division of chromosomes. Chromosomes initiate a condenser with a recognized chromosome end where two sister chromatids hold together by the centromere. Attachment and condensation employ the cohesion and condensin complexes, which associate with replicating DNA and become additionally active in preparation for mitosis (Lavoie, Hogan and Koshland, 2002).

The centrosomes separate and begin to migrate to opposite sides of the cell to initiate the matrix of bipolar microtubules for the assembly of the mitotic spindle. The centrosome is a large organelle consisting of a pair of centrioles that are orthogonally positioned and surrounded by amorphous pericentriolar material and which are referred to as the main microtubule organizing center (MTOC) (Ou and Rattner, 2004). The mitotic spindle, in turn, is a structure made of microtubules, strong fibers that are

part of the cell's "skeleton", whose function is to organize the chromosomes and move them during mitosis. The mitotic spindle grows between the centrosomes as they separate (Conduit, Wainman and Raff, 2015).

Still in prophase, the nucleolus, a part of the nucleus where ribosomes are formed, disappears, and this is a sign that the nucleus is about to break. Cdc2 phosphorylates and leads to depolymerization of the nuclear lamina, followed by the fragmentation of the nuclear membrane into particles, which eventually fuse to form new child nuclei in the telophase. In addition, the endoplasmic reticulum and the Golgi apparatus also fragment into vesicles, and can, at the end of the cell cycle, be distributed to daughter cells in cytokinesis (De Matteis and Luini, 2008).

At the end of the prophase and the beginning of the prometaphase, the mitotic spindle begins to capture and organize the chromosomes. Prophase ends and prometaphase begins (by definition) when the chromosomes start to interact with spindle microtubules. In many organisms this occurs when the nuclear envelope break down. The chromosomes complete the condensation and, therefore, become very compact and the nuclear envelope breaks, causing the release of the chromosomes. The mitotic spindle grows larger and some microtubules begin to "capture" chromosomes. The microtubules bind to chromosomes through the kinetochore, an arrangement of proteins found in the centromere, regions of the DNA of each sister chromatid (Kline-Smith *et al.*, 2004). The kinetochores of the sister chromatids are positioned on the opposite sides of the chromosome, so that they are connected to the microtubules that come out of the opposite poles of the spindle. The chromosomes move back and forth until they line up on the metaphase plate in the center of the spindle (Chng *et al.*, 2008).

Metaphase starts from the moment when the captured chromosomes establish bipolar connections with microtubules of opposite poles of the axis. This bi-orientation process until the chromosomes reach alignment in the equatorial region of the axis is known as a congress (Silva *et al.*, 2011).

The transition from metaphase to anaphase is a delicate and important moment of mitosis. An important checkpoint of the cell cycle monitors the alignment of

chromosomes on the metaphase axis. Once this alignment is verified, the cell begins anaphase. The transition from metaphase to anaphase is the result of ubiquitin-mediated proteolysis of the main regulatory proteins, triggered by the activation of an ubiquitin ligase, which is the so-called anaphase promoter complex (Lub *et al.*, 2014).

In anaphase, the sister chromatids are segregated and pulled towards the opposite poles of the axis. During telophase, the spindle is disassembled so that a single centrosome is associated with a set of chromosomes. At this moment, the nuclear envelope reorganizes and re-involves chromosomes and other nuclear components in two daughter nuclei (Maciejowski and Hatch, 2020).

A contractile groove forms between the nuclei and causes the cell to divide in two through the action of actin and myosin, characterizing the step of cytokinesis, part of the cell division process during which the cytoplasm of a single eukaryotic cell divides into two daughter cells (Green, Paluch and Oegema, 2012).

2.2 Kinetochore-microtubules interactions

Microtubules (MTs) are hollow cylindrical polymers of heterodimeric α-tubulin and β-tubulin subunits, where the tubulin dimers are polarized, with β-tubulin exposed at one end (positive end) and α-tubulin at the other end (negative end) (Allen and Borisy, 1974; Nogales *et al.*, 1999). It is through this polarity that motor proteins are able to transport charges in vivo (Wittmann, Hyman and Desai, 2001). Microtubules have a highly dynamic activity, being able to polymerize and depolymerize over many cycles, this activity being responsible for several cellular functions such as cell division, polarization and migration. The transitional process, which goes from polymerization to depolymerization, is caused by the rapid loss of GTP-tubulin subunits and oligomers from the end of the microtubules, and is called catastrophe, while the reverse transition receives the name of rescue (**Figure 5**) (Walker *et al.*, 1988; Akhmanova and Steinmetz, 2015).

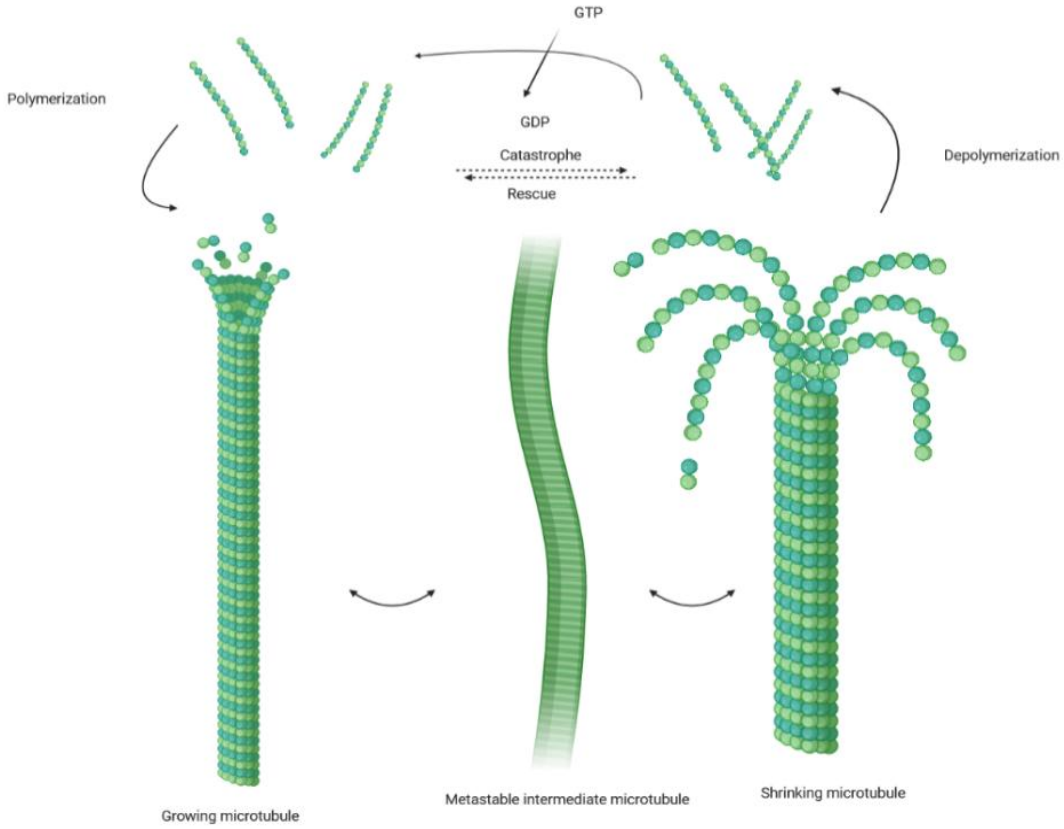

Figure 5 Microtubules dynamics. Microtubules have a highly dynamic activity, being able to polymerize and depolymerize. The process, which goes from polymerization to depolymerization, is caused by the rapid loss of GTP-tubulin subunits and oligomers and is called a catastrophe, while the reverse transition is called a rescue.

The microtubules of the mitotic spindle, during metaphase, are arranged in a symmetrical and fusiform structure with the negative ends oriented towards the poles and the positive ends facing the cellular cortex. It is through the orientation of the positive ends that the spindle microtubules are divided into three classes: astral, interpolar and kinetochore microtubules (McIntosh and Euteneuer, 1984).

The astral microtubules leave the centrosomes and diffuse into the cytoplasm with the positive ends in contact with the cellular cortex. They are assistants in the separation and positioning of the spindle poles in the cell. The interpolar microtubules go from the centrosomes towards the cell center and remain in the middle zone of the spindle, serving as a connection point between the two poles, providing stability to the spindle (Mastronarde *et al.*, 1993; Sharp, Rogers and Scholey, 2000). The kinetochore

microtubules are responsible for connecting the spindle poles to the chromosomes through initial lateral fixation in the kinetochore which is then converted into a mature final fixation for the bi-orientation of the chromosomes (Rieder and Salmon, 1998).

The dynamic instability of microtubules and their interactions with other cell structures are controlled by numerous factors, which are grouped into a large group of proteins associated with microtubules (MAPs) (Akhmanova and Steinmetz, 2008). Some microtubules from neighboring asters interact through engines and MAPs to form interpolar fibers that are directly linked in keeping the shaft poles separate (McIntosh and Pfarr, 1991).

The kinetochore plays a central role in the microtubules attachments to chromosomes and has a trilaminar proteinaceos morphology: the innermost layer, known as internal kinetochore plate, which is composed of a disc of densely compacted material and continuous with centromeric heterochromatin, the kinetochore plate external, which is a structure without DNA, dense in electrons, being the main binding site of the spindle microtubules and the third middle layer, which is responsible for separating and connecting the inner and outer regions of the kinetochore (Rieder, 1982; McEwen *et al.*, 1998).

Mature kinetochores can connect and put pressure on microtubules, their mounting characteristics and original signals that delay or start the operation until they are silenced by the MT axis coupling (Rieder and Salmon, 1998). To perform these functions, countless proteins are needed (Yu *et al.*, 2000).

Located in the fibrous crown of the kinetochore are the proteins involved in the SAC signaling pathway, such as Bub1, BubR1, Bub3, Mad1, Mad2 and Mps1 that target Cdc20 and APC/C. Also located in the fibrous crown of the kinetochore are some components involved in the fixation of kinetochore-microtubules and/or silencing of SAC as RZZ complex (Rod, Zw10, Zwilch), Spindly, dinein, dinactin, Lis1, CENP-E, CENP-F and Ska complex (Ska1, Ska2, Ska3), besides of microtubule tracking proteins plus end (+ TIPs) (Barbosa *et al.*, 2011).

The kinetochore proteins network is responsible for favoring the efficient alignment of chromosomes in the metaphase plate and consequently promoting the proper segregation of chromosomes (Wittmann, Hyman and Desai, 2001).

The “search and capture” model proper the capture of chromosomes, through their kinetochore, by the spindle microtubules is obtained gradually after the nuclear envelope is broken in the prometaphase, where the chromosomes are released in the cytosol and are accessible to the microtubules by the mitotic spindle. The microtubules probe the cytoplasm, through episodes of stretching and shortening its positive extremities, to find and capture the chromosome (Maiato, Sampaio and Sunkel, 2004).

Chromosomes are successfully aligned on the metaphase plate when they become bi-oriented, a condition known as amphitelic attachment, obtained at the moment when the sister kinetochores are connected to opposite poles of the spindle. It is this geometry that manages to guarantee that an accurate segregation of the sister chromatids to the daughter cells will be carried out in anaphase (Maiato and Sunkel, 2004; Cheeseman and Desai, 2008).

However, some errors can occur and cause improper connections that compromise the correct segregation of chromosomes. There are three types of incorrect connections of known kinetochore microtubules: monotelic, syntelic and merotelic (**Figure 6**) (Tanaka *et al.*, 2005; Tanaka, Stark and Tanaka, 2005; Tanaka, 2008). Monotelic attachment of the kinetochore is caused when one sister kinetochore is disconnected and the other remains connected to the microtubules from just one pole. The syntelic attachment is when the two sister kinetochores are connected to microtubules on the same pole as the spindle. Merotelic attachments occur when one sister kinetochore binds to microtubules at both poles (Kelly and Funabiki, 2009).

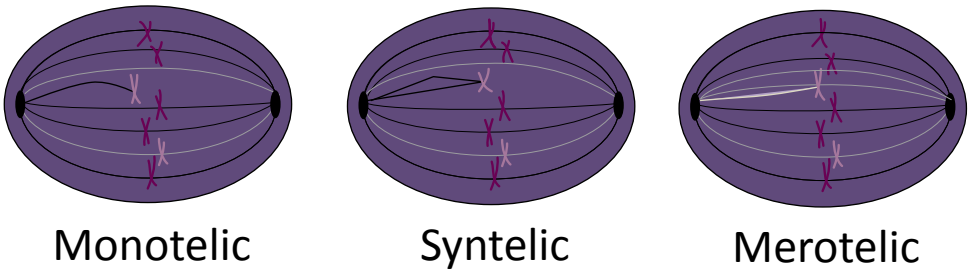

Figure 6 Representation of attachments errors between microtubules and kinetochores of the monotelic, synthetic and merotelic type. The monothelic fixation of the kinetochore represents a disconnected sister kinetochore and the other connected to the microtubules from just one pole. The synthetic bond is when the two sister kinetochore are connected to microtubules in the same pole as the spindle. Merothelic bonds occur when a sister kinetochore binds to microtubules at both poles.

Adequate tension between sister kinetochores is a factor that influences the detection and correction of merotelic and syntelic bonding errors that distinguishes the different bonding states of sister kinetochores. Aurora B kinase acts as a voltage sensor to correct connection errors by destabilizing them (Nicklas, Ward and Gorbsky, 1995; Kelly and Funabiki, 2009), as well as the polo-like kinase 1 (Plk1), located in kinetochores during prometaphase (Ahonen *et al.*, 2005; Liu, Davydenko and Lampson, 2012; Suijkerbuijk *et al.*, 2012).

2.3 The spindle assembly checkpoint

The stable union between sister chromatids is mediated by Cohesin, a multimeric protein ring structure that surrounds replicated sister chromatids (Nasmyth and Haering, 2009). Cohesin is maintained throughout the S, G2 phase and in the initial mitosis, when the chromosomes are aligned in the center of the cell in the microtubule spindle apparatus. In anaphase the Cohesin ring opens and the union between sister chromatids is lost by the proteolytic activity of the enzyme Separase, allowing the spindle forces to pull them to opposite sides of the cell. Separase is under the control of Securin, which when connected prevent the cleavage of Cohesins, thus delaying the onset of anaphase. Securin levels are regulated by the anaphase/cyclosome promoter complex, APC/C, which is a multi-subunit E3 ubiquitin ligase that targets proteins for degradation by the 26S proteasome (Morgan, 1999; Reddy *et al.*, 2007; Stegmeier *et*

al., 2007). The ubiquitination of APC/C leads to Securin being degraded causing the activation of Separase, responsible for the cleavage of one of the Cohesin subunits, Scc1. The sister chromatids are then able to segregate in the mitotic spindle and anaphase is possible to occur (Nasmyth, 2001; Holland and Cleveland, 2009; Przewloka and Glover, 2009). The central role of the spindle assembly checkpoint is to prevent anaphase from entering until all the chromosomes are precisely positioned and attached to the axis (Musacchio and Salmon, 2007; Nezi and Musacchio, 2009).

SAC was discovered and initially described after pioneering work that led to the concept of cell cycle checkpoints (Hartwell and Weinert, 1989), through genetic screening in the yeast Saccharomyces cerevisiae (Hoyt, Totis and Roberts, 1991; Li and Murray, 1991) that managed to identify most of the main components, such as Mad1, Mad2, Mad3, Bub1 and Bub3. Later, other components of SAC such as Mps1 were described (Weiss and Winey, 1996).

This discovery showed that all of them were located in unattached kinetochores, and that the downstream targets were the APC/C, which is an ubiquitin E3 ligase that acts in association with various proteins for degradation proteolytic, including mitotic cyclins (Santaguida and Musacchio, 2009).

Since then, SAC has been described as the main responsible for monitoring the presence of misaligned chromosomes and preventing the onset of anaphase (Musacchio and Salmon, 2007) and involves the performance of different proteins in this process, such as Aurora B located in the internal kinetochore, Mad1 and Mad2 that identifies kinetochores and microtubules disassociated or with coupling errors, in addition to Mad2 also being part of the Mitotic Checkpoint Complex (MCC) signaling, among others (Li *et al.*, 1997; Luo *et al.*, 2002).

MCC is the main APC/C inhibitor generated by SAC and its substrate securin elucidates the securin-separase-cohesin pathway (Nasmyth and Haering, 2009), that provide a structure that describes how SAC regulated the onset of anaphase and the mitotic exit. The understandings arising from this discovery expand to questions that involve how the SAC is assembled in kinetochores, how it generates the MCC and how the MCC inhibits APC/C (**Figure 7**).

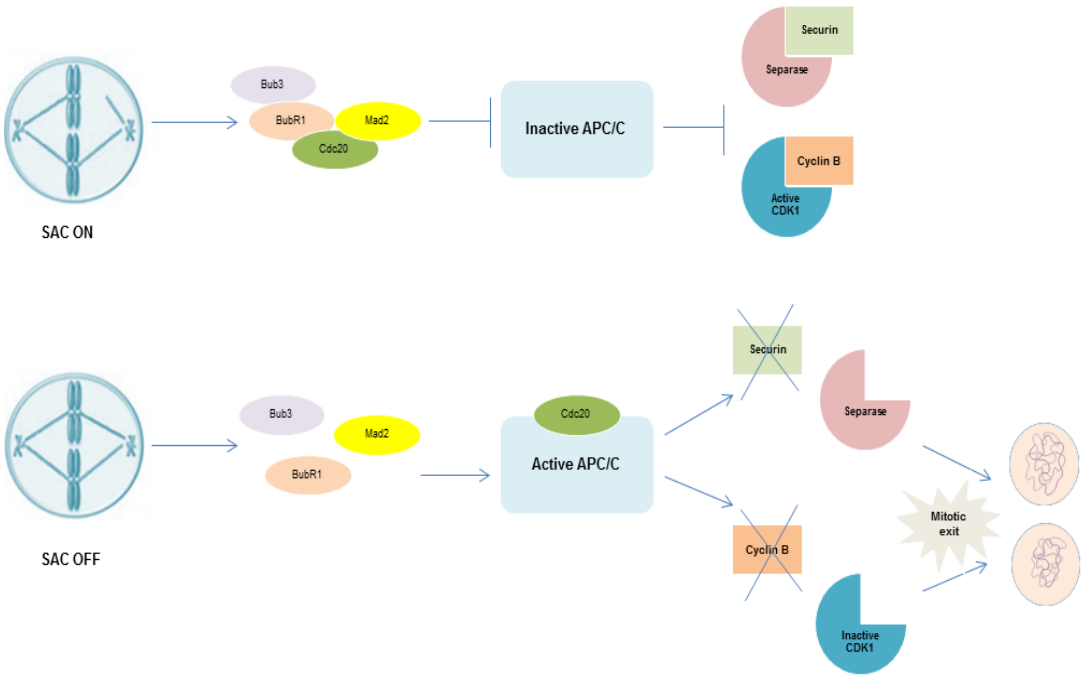

Figure 7 SAC pathway representation. When there are unconnected kinetochores Bub3, Mad2 and BubR1 bind to CDC20, forming the MCC. This prevents the activation of APC/C and inhibition of cell progression to anaphase due to the sequestration of CDC20. With the alignment of all chromosomes, the MCC is disassembled and the CDC20 is free to activate the APC/C, which causes the degradation of Cyclin B and Securin in the proteasome, releasing the Cdk1 that promotes the mitotic exit and the Separase that separates the sister chromatids.

Regarding the assembly of the SAC, the proteins belonging to it are recruited to the kinetochores in a gradual manner, with Bub1 (Hoyt, Totis and Roberts, 1991; Meraldi, Draviam and Sorger, 2004; Perera *et al.*, 2007) connecting first in the initial prophase and recruiting most of the downstream SAC components, such as BubR1, Bub3, Mad1 and Mad2 (Acquaviva *et al.*, 2004; Kim *et al.*, 2012).

Mad2 was the first SAC component identified in vertebrate cells (Li and Benezra, 1996; Luo *et al.*, 2004) and it is important to understand that Mad2 adopts two distinct conformations, one when it is decoupled, and adopts an open conformation which is called O-Mad2, however after binding to Mad1 (or Cdc20), create the closed conformation, which is called C-Mad2 (Sironi *et al.*, 2001; De Antoni *et al.*, 2005).

O-Mad2 is attracted and linked to the Mad1-C-Mad2 complex that captures Cdc20, thus creating a C-Mad2-Cdc20 complex, completing the first step in assembling the MCC (De Antoni *et al.*, 2005). Thus, the activity of the Mad1-C-Mad2 nucleus in the kinetochore is extremely important in the SAC mechanism to demonstrate that a kinetochore is detached.

Mps1 emerged as an important regulator of the Mad2 model mechanism that led to the understanding that in mitosis, concomitant with the kinetochore set, the activity of this protein is responsible for attracting the recruitment of the RZZ complex, which, in turn, is the responsible for recruiting Mad1-C-Mad2 (Hewitt *et al.*, 2010; Maciejowski *et al.*, 2010; Santaguida *et al.*, 2010).

Generating the C-Mad2-Cdc20 complex may be enough to alert the cell to the presence of unbound kinetochores, but it is not enough to block the onset of anaphase. BubR1/Mad3 are responsible for acting downstream of Mad1-C-Mad2 to block the onset of anaphase (Hardwick *et al.*, 2000; Nilsson *et al.*, 2008). In fact, the formation of the C-Mad2-Cdc20 complex is only the beginning of the APC/C activation cascade, that is, the formation of the MCC (Sudakin, Gordon K.T. Chan and Yen, 2001).

The MCC is assembled from two subcomplexes, the Mad2-Cdc20 complex and the Mad3/BubR1-Bub3 complex (Musacchio and Salmon, 2007). And while the formation of Mad2-Cdc20 is catalyzed by unattached kinetochores, BubR1-Bub3 are present throughout the cell cycle (Hardwick *et al.*, 2000; Chen, 2002). It is well established that Mad2 is important for BubR1/Mad3 to be bound to Cdc20 (Hardwick *et al.*, 2000; Fang, 2002; Davenport, Harris and Goorha, 2006; Burton and Solomon, 2007; Nilsson *et al.*, 2008; Sczaniecka *et al.*, 2008).

As for the inhibition of APC/C, kinetochore-activated Mad2 is an important inhibitor of anaphase, through binding to Cdc20 that prevents it from activating APC/C (Li *et al.*, 1997; Fang, Hongtao and Kirschner, 1998). Even with the discovery of MCC (Sudakin, Gordon K.T. Chan and Yen, 2001) and the demonstration that BubR1 potentiates Mad2-mediated inhibition of APC/C (Fang, 2002), the view remains that Mad2 is the ultimate inhibitor of APC/C downstream. However, there is some current evidence to show that BUBR1, and not Mad2, is the main APC/C inhibitor.

The MCC-activated APC/C has the ability to recruit cyclin B1 and lower securin (Herzog *et al.*, 2009), which suggests that MCC, and therefore BubR1, interfere in some way in substrate binding. The Cdc20 and APC/C link is mediated by different subunits of the APC/C, which change depending on whether the SAC is active or disabled (Acquaviva *et al.*, 2004).

It has already been proven that the shape and composition of kinetochores directly affect the fixation of microtubules (Howell *et al.*, 2004). Even though the shape changes are mainly carried out by traction forces, in the upper eukaryotic discharges, a subset of kinetochore components is removed through the stable fixation of the microtubule. The removal of Mad1 and Mad2 from kinetochores is essential for the efficient inactivation of SAC (Maldonado and Kapoor, 2011) and is mediated by the microtubule motor protein called dynein (Howell *et al.*, 2004).

Spindly dynein-mediated removal of connected kinetochore is a key point for the removal of RZZ, Mad1 and Mad2 from connected kinetochore. After the microtubules are attached, the removal of Mad1-C-Mad2 from the kinetochores prevents the formation of new MCC complexes. In addition to preventing the assembly of a new MCC complex, extinguishing the SAC signal requires that several existing inhibitory complexes be disassembled to release Cdc20 to activate APC/C and promote mitotic exit (Henriques *et al.*, 2019).

Taking into account the role of SAC in preventing cells from prematurely segregating their genetic material, its accurate activity is of great importance as a guarantee that mitotic events will occur correctly, preventing the genomic imbalance and abnormal cell proliferation that characterizes the tumor tissue (Bharadwaj and Yu, 2004), which suggests a relationship between the malfunctioning of the SAC and CIN of tumors.

3 Cancer

3.1 Overview

Cancer is a rise disease, and its incidence and mortality have increased considerably in recent years (Bray et al., 2018). As age is the main etiologic factor, it is possible to relate the longest-lived population currently one of the indicators of this increase, as

well as changes in lifestyle in the developing world. Mortality rates have been so high that in many countries it exceeds death rates from stroke and coronary heart disease (Bray *et al.*, 2018). According to the World Health Organization (WHO), there were 18.1 million new cases in 2018 and 9.6 million deaths. Lung cancer represents the highest incidence rates (11.6% of total cases) and also mortality (18.4% of total deaths), followed by breast cancer (11.6%), colorectal cancer (10.2%) and prostate cancer (7.1%) in relation to the incidence, and colorectal cancer (9.2%), stomach cancer (8.2%) and liver cancer (8.2%) in relation to mortality (Bray *et al.*, 2018).

Incidence rates vary, with higher rates of prostate, colorectal, breast and lung cancer considerably higher in the most affected countries compared to the least used countries. On the other hand, liver, stomach and cervical cancer are more common in less developed countries, because these cancers often have etiological factors attributed to the infection (Iarc., 2012; World Health Organization, 2012).

These regional differences directly interfere with patients' prognosis and treatments when looking at the overview. A good example is shown through data related to the numbers of liver cancer, which is very common in underdeveloped countries, and because it displays a high mortality rate, it ends up increasing disproportionately the cancer mortality rates in the countries. As global demographic and epidemiological transitions signal an increase in low and middle income countries in the coming years, they indicate a trend of socioeconomic influence in mortality rates (Siegel, Miller and Jemal, 2019).

Cancer risk factors can include occupational exposures, infectious agents, social determinants, lifestyle factors and genetic and epigenetic changes (Toporcov and Wünsch Filho, 2018). Tobacco, alcohol and diet are some of the etiological factors related to several types of cancer (Vineis and Wild, 2014).

Neoplasm is a type of change in normal cells that can give rise to the tumor or not. The benign tumor grows locally without invading adjacent tissues, whereas the malignant tumor consists of invasion of neighboring tissues and eventually metastasis, which is the growth in distant and distinct locations from the primary tumor (Evan and d'Adda di Fagagna, 2009; Hanahan and Weinberg, 2011). There are tumors arising from

different cell types, namely: carcinoma, sarcoma, neuroectodermal and hematopoietic, which originate in cells of the epithelial tissue, cells of the connective tissue, cells of the central and peripheral nervous system and blood cells, respectively.

The tumor progression process happens due to a sequence of mutations that occur at random and epigenetic changes that in turn will control the proliferation of cells, their survival and other characteristics related to the phenotype. The tumorigenic process is in fact a process that involves several steps, that is, it is a chronic process. The best example to demonstrate this evolution in a multi-step process is the intestinal epithelium (Loupakis *et al.*, 2009).

Involved genes code for proteins with various functions that complement each other in transforming a normal cell into a cancer cell. Tumor progression is divided into four main phases: initiation, carcinoma in situ, invasion and metastasis (**Figure 8**) (Evan and d'Adda di Fagagna, 2009; Hanahan and Weinberg, 2011). At initiation, normal cells mutate and initiate processes of uncontrolled division.

This process occurs over several generations and will give rise to a first tumor. In carcinoma in situ, the tumor has not yet invaded other adjacent tissues and is where additional mutations occur that give cells the ability to evade apoptosis, however at this stage the presence of nutrients is limited. The invasion is a phase marked by angiogenesis, where the tumor cells start to access blood vessels and from there obtain the necessary nutrients for their growth. In metastasis, which is the final stage, the tumor acquires capacities that allow it to spread to other tissues, even if they are far from the place of origin (**Figure 8**) (Evan and d'Adda di Fagagna, 2009).

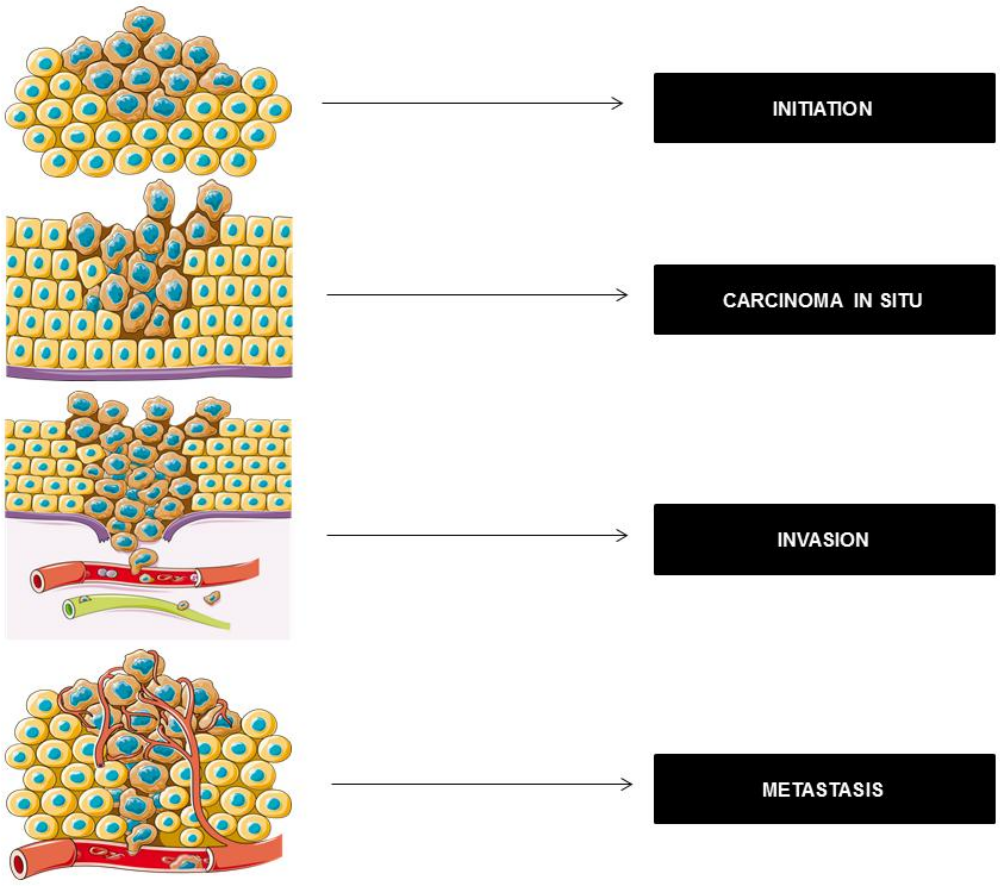

Figure 8 Tumor progression. Four main phases mark tumorogenesis: initiation, carcinoma in situ, invasion and metastasis. In the beginning, normal cells mutate and initiate processes of uncontrolled division and give rise to a first tumor. In carcinoma in situ, the tumor has not yet invaded other adjacent tissues. Invasion is a phase marked by angiogenesis, where tumor cells begin to access blood vessels. In metastasis, the tumor acquires abilities that allow it to spread to other tissues.

The three types of cancerous genes are oncogenes, tumor suppressor genes and DNA repair genes. Mutations are abnormal changes in the DNA of a gene. The building blocks of DNA are called bases and it is this sequence of bases that determines the gene and its function. Mutations are nothing more than changes in the arrangement of the bases that make up a gene (Loeb, Loeb and Anderson, 2003).

A genetic mutation can affect the cell in several ways, either by interrupting the production of proteins, or by altering the protein itself or causing it to no longer function as it should or not work at all (Loupakis *et al.*, 2009). We have 2 copies of most genes, one from each chromosome in a pair. For a gene to stop working completely and potentially cause cancer, both copies must have mutations.

There are 2 main types of genetic mutations: somatic and germline. Somatic mutations are acquired by a somatic cell and these mutations are passed on to daughter cells during cell proliferation, that is, each time a cell divides it needs to replicate its DNA, mistakes happen at random. Germline mutations are present in the germ cells and passed on to the descendants (Salk, Fox and Loeb, 2010).

Somatic mutations, regardless of their structural nature, can be classified according to their consequences that cause cancer. Driver mutations are the ones that give rise to the growth advantage of the cells that carry them. The rest of the mutations are passengers, which means that they do not confer growth advantage, but were already present in an ancestral cancer cell when it acquired one of its drivers. An important driver subclass is a mutation that confers resistance to therapy, which generally gives cancer cells limited growth advantage in the absence of therapy (Stratton, Campbell and Futreal, 2009). The genes involved in the process of tumorigenesis can be: oncogenes, tumor suppressor genes and DNA repair genes (Loeb, Loeb and Anderson, 2003).

3.2 Molecular basis

Given the numbers indicated, it is very clear the need to advance in understanding the biological and molecular aspects of cancer. Six characteristics, described by Hanahan & Weinberg in 2000, provide a logical structure that allows us to understand the various steps of tumor pathogenesis in humans, which demonstrate the evolution that happens from a cell in its normal state to a neoplastic state, progressively. Despite understanding the complexity of cancer, an innate and indisputable resource is a high rate of abnormal cell proliferation, which is considerably higher than normal cells, as well as the ability of these invading cells from other tissues to spread to any other organ (Hanahan and Weinberg, 2000; Evan and d'Adda di Fagagna, 2009).

What increases the complexity of the disease, when one imagines which tissues are treated in several different cell types, promotes heterotypic interactions with each other. In these interactions, normal stromal cells associated with tumors are active participants in tumors, not passive spectators, directly contributing to the development and expression of certain striking features of tumors (Hanahan and

Weinberg, 2011). To manage its migration and proliferation, a cancer cell can avoid patterns of use, in order to contribute to cell proliferation, cell survival and cell communication. These processes are regulated in normal cells by redundant routes, which makes it understood that a cell to become cancerous needs several mutations in different genes.

The characteristics described for Hanahan & Weinberg in 2000 and 2011 are responsible for maintaining proliferative signaling, and are the consequences of genetic mutations in various genes. Cancer cells develop their own proliferation rate by manipulating positive signals to stimulate growth. Cancerous cell communication, whether paracrine or autocrine, increases the display of receptors on the cell surface or allows activation of receptors downstream of the pathway. Through sequestration in the pericellular space and extracellular matrix and the actions of a complex network of proteases, sulfatases and possibly other enzymes that release and activate them, the bioavailability of growth factors is regulated in a highly specific and localized manner (Hanahan and Weinberg, 2011). This proliferative signaling in cancer cells can be sustained in several ways, such as through the production of growth factor ligands, which produce an autocrine proliferative stimulation response through the expression of cognate receptors. Cancer cells can also send signals to stimulate normal cells within the stroma tumor-associated, which reciprocate by providing cancer cells with various growth factors (Bhowmick, Neilson and Moses, 2004; Cohen *et al.*, 2008).

Several oncogenes directly influence cellular behavior through signals in the cytoplasm. The cancer cell acts by mimicking the normal growth signal, ranging from quiescence in normal tissue to motility. Changes in extracellular growth signals, transcellular transducers of these signals and intracellular circuits that convert these signals into action, are molecular strategies that aim to reach the signal. The manufacture of its own growth factors (GF) by the cancer cell makes it independent of GFs from other cells within the tissue. For example, the production of PDGF (platelet-derived growth factor) and TGFα (tumor growth factor α) by glioblastomas and sarcomas, respectively, adequately demonstrate this production (Alimandi *et al.*, 1997). In addition, cancer cells can alter the expression of extracellular matrix receptors (integrins) favoring those that transmit signs of progress (Lukashev and

Werb, 1998; Giancotti and Ruoslahti, 1999). The SOS-Ras-Raf-MAP kinase pathway can be activated through GF receptors activated by ligands and pro-growth integrins coupled to the components of the extracellular matrix (**Figure 9**) (Howe *et al.*, 1998; Giancotti and Ruoslahti, 1999).

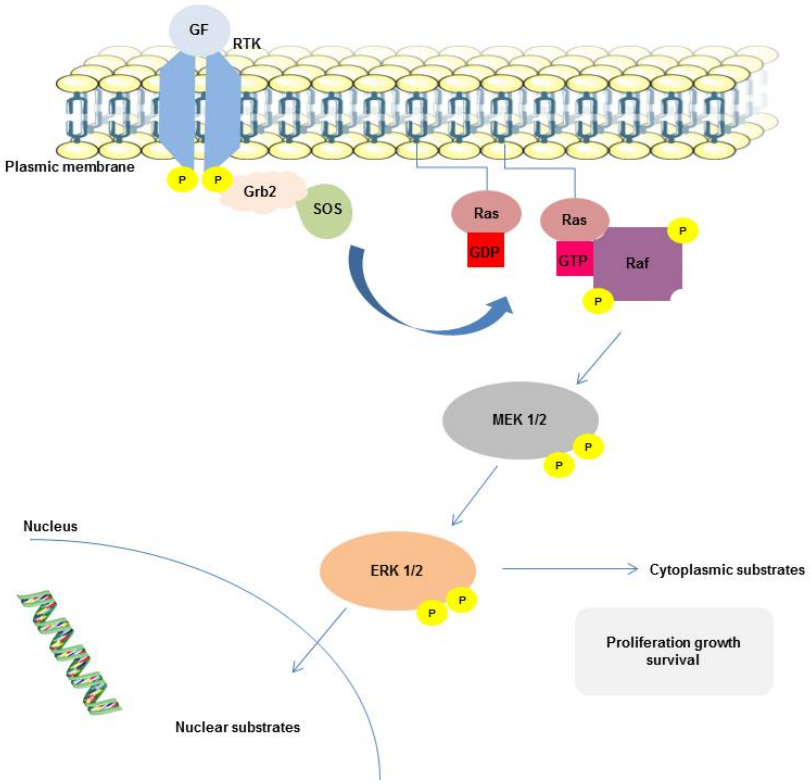

Figure 9 SOS-Ras-Raf-MAP kinase pathway. The binding of growth factors to receptor tyrosine kinases stimulates the autophosphorylation of specific tyrosines at receptors. The phosphorylated receptor binds to GRB2, which is an adapter protein that removes SOS from the plasma membrane. SOS is a factor that changes guanine nucleotides and displaces the GDP of Ras and thus allows the binding of GTP. Ras linked to GTP recruits and activates Raf which initiates a cascade of protein phosphorylation by the first phosphorylation of MEK which in turn phosphorylates ERK. Phosphorylated ERK goes from the cytoplasm to the nucleus, where it phosphorylates a number of transcription factors.

tumorogenesis, according to Medema & Bos, in 1993, "*25% of human tumors, Ras proteins are present in structurally altered forms that enable them to release a flux of mitogenic signals into cells, without ongoing stimulation by their normal upstream regulators*". Going against the above, in 2010, Davies & Samuels confirmed that "*40%*

of human melanomas contain activating mutations affecting the structure of the B-Raf protein, resulting in constitutive signaling through the Raf to mitogen-activated protein (MAP)-kinase pathway".

The role that this signaling pathway plays interferes with several other pathways, through connections that allow extracellular signals to influence multiple biological effects in cells, such as the direct interaction of the Ras protein with PI3-kinase, which sends growth signals into the cell generating its survival (Downward, 1998). Some mutations in the catalytic subunit of the phosphoinositide 3-kinase (PI3-kinase) isoforms have already been found in several types of tumors, and play an important role in the signaling of the cancer cell, by hyperactivating the PI3-kinase signaling circuit, including its main Akt/PKB signal transducer (**Figure 10**) (Medema and Bos, 1993; Jiang and Liu, 2008; Yuan and Cantley, 2008).

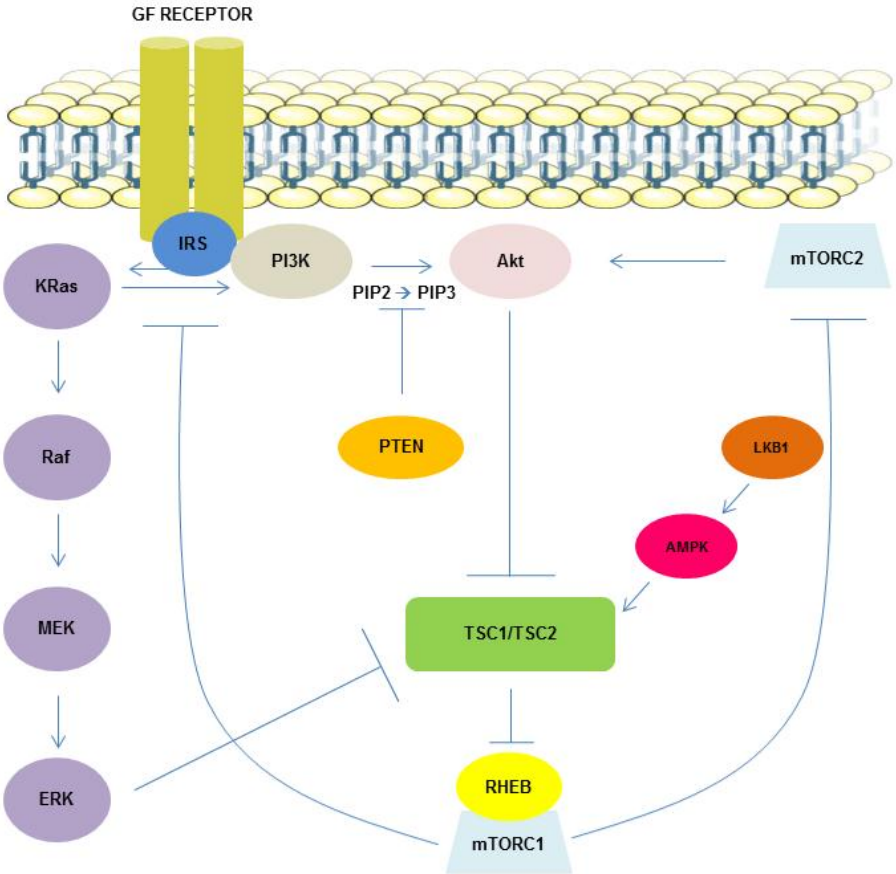

Figure 10 PI3K-AKT-mTOR and RAS pathways association. The PI3K-AKT-mTOR pathway is triggered by the activation of several growth factor receptor tyrosine kinases. PI3K proteins are recruited to the plasma membrane through adapter proteins, such as members of the insulin receptor substrate (IRS) family. This interaction leads to phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) to generate phosphatidylinositol 3,4,5-triphosphate (PIP3) which in turn activates AKT which phosphorylates the protein complex formed by TSC1 and TSC2, dissociating it O. The TSC1-TSC2 complex in turn acts in the negative regulation of the activity of the mTOR1 kinase (which performs a negative feedback loop to prevent over activation of AKT. The PI3K-AKT-mTOR pathway can be increased by activating molecular changes in the subunits PI3K, AKT and mTOR or even some loss of function changes in PI3K, PTEN, TSC1, TSC2 and LKB1 regulatory subunits. The activation of the tyrosine kinase growth factor receptor and also induces KRAS-RAF-MEK-ERK signaling The active ERK can further contribute to the activation of mTORC1 through the dissociation of the TSC1-TSC2 complex, while KRAS can in parallel reinforce the activation of PI3K.

Hanahan & Weinberg in 2011 concluded that defects in these feedback mechanisms can increase proliferative signaling. The consequent excessive proliferative signaling can trigger cellular senescence. This demonstrates the importance of negative feedback loops that often play a fundamental role in controlling different types of signaling to ensure homeostatic regulation of the signal flow that occurs within intracellular circuits (Amit *et al.*, 2007; Cabrita and Christofori, 2008; Mosesson, Mills and Yarden, 2008; Wertz and Dixit, 2010). For example, PTEN phosphatase, which controls the levels of PI3-kinase through the degradation of its product, phosphatidylinositol (3,4,5) triphosphate (PIP3), where the loss-of-function mutations in PTEN, often lost through methylation of the promoter, end up amplifying the PI3K signaling (**Figure 10**) (Jiang and Liu, 2008; Yuan and Cantley, 2008). Excessively high signaling by oncoproteins such as RAS, MYC and RAF can generate responses contrary to cells, induction of cellular senescence and/or apoptosis (Wendel *et al.*, 2004; Evan and d'Adda di Fagagna, 2009; Collado and Serrano, 2010).

The cancer cell also needs to survive, to generate certain insensitivity to the anti-growth signals of the cancer cells. These signals come through soluble growth inhibitors and immobilized inhibitors embedded in the extracellular matrix and on the surfaces of nearby cells. Anti-growth signals manage to block cell proliferation through two distinct mechanisms: forcing a cell out of the active proliferative cycle to the resting state (G0) - which is reversible - and can return to its active state when extracellular signals permit, or inducing the cell to permanently abandon its

proliferative potential when induced to enter post-mitotic states (Hanahan and Weinberg, 2000).

The retinoblastoma protein (pRb) and its two relative's proteins p107 and p130 exhibit a fundamental role in anti-proliferative signals. In its hypophosphorylated state, pRb blocks proliferation by sequestering and altering the function of E2F transcription factors, which are responsible for controlling the expression of essential genes that allow progression from the G1 to the S phase (Geng *et al.*, 1996). The RB protein plays a fundamental role in the cell's decision to continue or not continue in cycle of growth and division, through the integration of signals from several extracellular and intracellular sources (Sherr and McCormick, 2002; Deshpande, Sicinski and Hinds, 2005; Burkhart and Sage, 2008).

TGFβ suppresses the expression of the c-myc gene which regulates the G1 cell cycle machinery (Barnard, Lyons and Moses, 1990). TGFβ synthesizes the proteins $p15^{Ink4b}$ and p21, which are responsible for blocking the cyclin CDK complexes which are responsible for the phosphorylation of pRb (Waga *et al.*, 1994; Datto *et al.*, 1997).

The pRb and p53 protein are considered the two canonical suppressors of proliferation, but in reality they are just operators that are part of a much larger network, connected to functional redundancy (Soussi, 2000; Hanahan and Weinberg, 2011). Cancer cells must avoid negative regulation of cell proliferation, causing deregulation of cell proliferation suppression, and this happens through mutations in tumor suppressor genes, such as p53, pRB and TGF-β (Hanahan and Weinberg, 2000). The main types of somatic mutations that lead to inactivation of tumor suppressor genes are those that lead to changes in the structure of the protein encoded by the gene in question and mutations that lead to a decrease in the protein-methylation expression of promoters, translocations. Several classes of tumor suppressor genes are related, with several levels of regulation.

The main types of somatic mutations lead to the activation of proto-oncogenes are those that cause changes in the structure of the protein encoded by the gene in question and mutations that lead to increased protein expression. For example, RAS,

WNT and Myc involved in the healthy cell cycle and can become oncogenic when they promote tumorigenesis (Hunter, 1991).

The capacity of evading apoptosis is crucial in the malignant development of cancer cells and increases with resistance to therapy (Adams and Cory, 2007; Hanahan and Weinberg, 2011). Apoptotic machinery has as main regulators components upstream and effector components downstream (Adams and Cory, 2007).

These regulators are part of two main circuits: one responsible for receiving and processing extracellular death-inducing signals (the extrinsic apoptotic program, which has components, for example, the Fas ligand/Fas receptor), and the other responsible for detecting and integrate intracellular signals (the intrinsic program). The intrinsic apoptotic program is recognized as a major barrier to cancer pathogenesis (Thornberry and Lazebnik, 1998; Hanahan and Weinberg, 2011). This process ends up activating a normally latent protease (caspases 8 and 9, respectively), and from there begins a proteolytic cascade that involves effector caspases responsible for the apoptosis execution phase, where the cell is disassembled and then consumed by its neighbors and by phagocytic cells (Thornberry and Lazebnik, 1998).

The mitochondria have a fundamental role in the apoptotic process, receiving pro-apoptotic signals and release a potent catalyst for apoptosis, the cytochrome C (**Figure 11**) (Green and Kroemer, 1998; Wallace, 2012). The p53 protein is an important agent in the apoptosis process, but even though it is functional, it is not always able to repair the cell. This gene is classified as an apoptotic regulator, which inhibits pro-apoptotic genes, allowing the proliferation of cells that contain DNA damage. Anti-apoptotic Bcl-2 inhibits Bax, which consequently induces apoptosis and induces the release of cytochroma C. In the case of overexpression of Bcl-2, even though p53 is functional, there will be no apoptosis, resulting in a positive stop in the process and the consequent overexpression of p53 that allows the proliferation of cells that contain DNA damage (**Figure 11**) (Harris, 1996; Downward, 1998; Soussi, 2005).

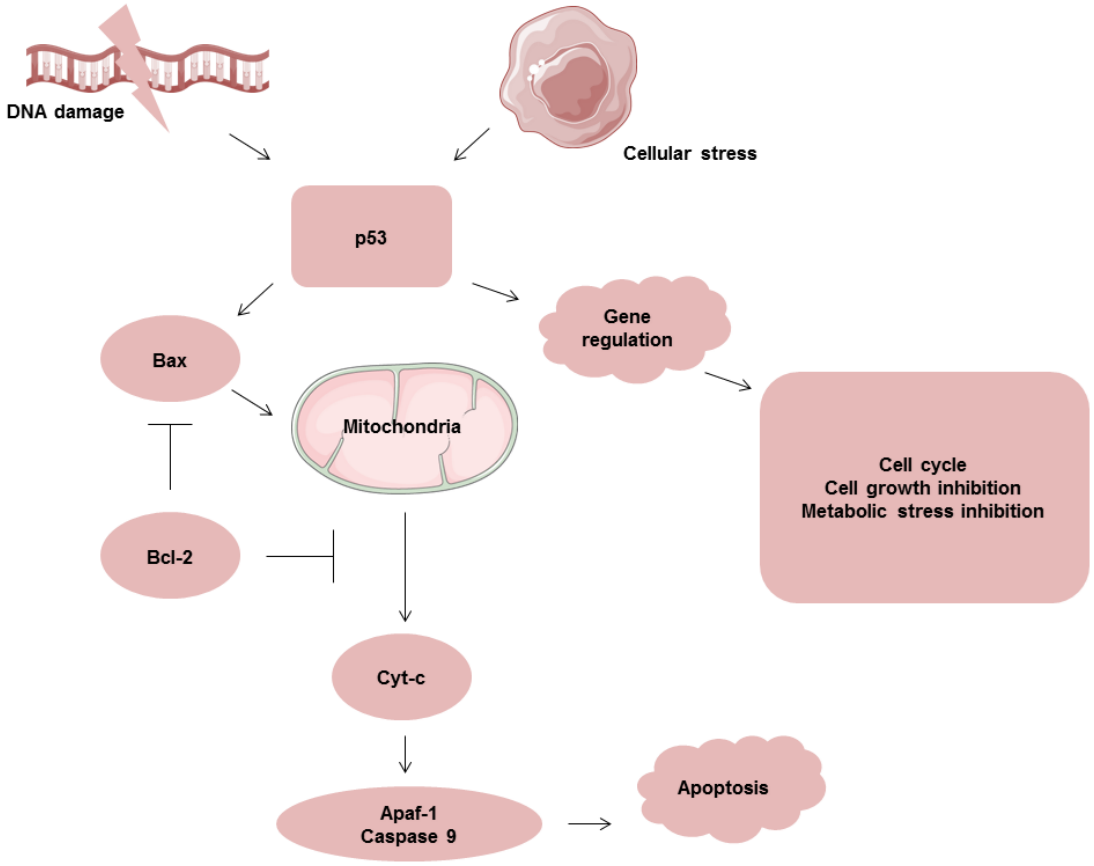

Figure 11 The role of p53 pathway in apoptosis. The scheme shows that in case of DNA damage or cellular stress, the p53 protein plays a central role in the regulation of apoptosis that goes beyond its role in regulating gene transcription. The Bcl-2 demonstrated is an anti-apoptotic gene that acts by inhibiting Bax, which consequently induces apoptosis and induces the release of cytochroma C by the mitochondria.

Resistance to apoptosis can be acquired by cancer cells in several ways, but the most common form is through a mutation involving the tumor suppressor gene p53, considered an important pro-apoptotic regulator. The functional inactivation resulting from its product has been observed in several types of cancer, and for a long time. In 1996, Harris reported that "*more than 50% of human cancers result in the removal of a key component of the DNA damage sensor that can induce the cascade of apoptotic effectors*".

Some anti-apoptotic genes are intended to promote survival, such as those involved in the PI3K/AKT pathway, and these genes depend on extracellular factors such as IGF 1/2 and IL-3, for intracellular signals emanating from Ras (Downward, 1998), or by the

loss of the tumor suppressor PTEN, which is a phospholipid phosphatase that attenuates the signs of AKT survival (**Figure 10**) (Cantley and Neel, 1999).

Autophagy is a cellular physiological response that, like apoptosis, operates at low basal levels in cells, but can be strongly induced in certain states of cellular stress, such as nutrient deficiency. Autophagy machinery is similar to apoptosis, and has regulatory and effector components (Mizushima, 2007; Levine and Kroemer, 2008). In contrast to apoptosis, which is a process by which the cell becomes almost invisible and is soon consumed by neighbors, necrotic cells become swollen and explode (Zong and Thompson, 2006). Necrosis is historically known as organismic death, as a form of exhaustion and collapse throughout the system. However, currently, the conceptual landscape is changing and Galluzzi & Kroemer in 2008 defined necrosis as a cell death clearly under genetic control in some circumstances, and not as a random, undirected process. In addition, necrotic cells can recruit inflammatory cells from the immune system as a consequence of necrosis (Galluzzi and Kroemer, 2008; Grivennikov, Greten and Karin, 2010; Rabinowitz and White, 2010).

In addition to the characteristics already mentioned, mention should also be made of the unlimited replicative potential of cancer cells, where the cell is able to evade senescence or apoptosis by maintaining telomeric DNA. This happens through the expression of Telomerase, an enzyme whose activity extends the telomeres and protects the DNA preventing the triggering of states of senescence or apoptosis (Hanahan and Weinberg, 2011). Telomerase is an enzyme that adds repetitive sequences to the 3 'end of the chromosome using an RNA template for DNA production (it is a type of reverse transcriptase or RNA-dependent DNA polymerase). Multiple evidences are indicative that telomeres, the structures made up of repetitive rows of proteins and non-coding DNA that form the ends of chromosomes, are centrally involved in the capacity for unlimited proliferation (Wright and Shay, 2000; Blasco, 2005). Senescence and apoptosis are therefore considered crucial anti-cancer defenses of our cells, representing two important barriers to proliferation implanted to prevent the growth of clones of pre-neoplastic and neoplastic cells.

Like normal tissues, tumors require sustenance in the form of nutrients and oxygen as well as an ability to evacuate metabolic wastes and carbon dioxide. The tumor-associated neovasculature, generated by the process of angiogenesis, addresses these needs (Hanahan and Folkman, 1996). The well-known prototypes of angiogenesis inducers and inhibitors are vascular endothelial growth factor-A (VEGF-A) and thrombo-spondin-1 (TSP-1), respectively. The well-known prototypes of angiogenesis inducers and inhibitors are vascular endothelial growth factor A (VEGF-A) and thrombo-spondin-1 (TSP-1), respectively (Hanahan and Weinberg, 2011).

The angiogenic process begins to happen when a quiescent vessel receives an angiogenic signal such as VEGF, FGFs or chemokines and from there pericytes separate from the blood vessel wall, and are freed from the basement membrane by proteolytic degradation mediated by matrix metalloproteinases. Endothelial cells will move apart and the nascent vessel expands and VEGF increases the permeability of the endothelial cell layer causing the extravasation of proteins and the establishment of a temporary extracellular matrix. Endothelial cells migrate to this extracellular matrix in response to integrins. Proteases release angiogenic molecules stored in the ECM (VEGF, FGF) and remodel the ECM in a medium that supports angiogenesis to form an infused tube and to prevent a massive displacement for the angiogenic signal, a single endothelial cell is selected to lead the beginning of the nascent vessel (Hanahan and Folkman, 1996; Carmeliet, 2005; Gabhann and Popel, 2008; Ferrara, 2009).

Maturation occurs through a process in which endothelial cells return to a quiescent state and secrete proteins that induce recruitment and lining of new blood vessels with pericytes/vascular smooth muscle cells. Namely, platelet derived growth factor (PDGF), heparin-binding EGF (heparin-binding EGF), Angiopoietin (Ang-1), transforming growth factor β (TGF-β) and ephrin-B2. Metalloprotease inhibitors and plasminogen activator inhibitor (PAI-1) promote deposition of the basement membrane. Junctions between cells are re-established to ensure optimum perfusion and blood vessel waterproofing. Pericytes/vascular smooth muscle cells provide structural stability and the ability to resist the forces exerted by blood pressure. VGF gene expression can be over-regulated by both hypoxia and oncogene signaling (Hanahan and Folkman, 1996; Carmeliet, 2005; Gabhann and Popel, 2008; Ferrara, 2009).

The hypoxic response leads to the production of angiogenic factors by cancer cells, for example, VEGF, angiopoietin and erythropoietin. Hypoxic cancer cells secrete chemoattractive molecules for recruiting macrophages, such as VEGF, Endothelial monocyte-activating polypeptide II (EMAPII) and Endothelins (ET-2, ET-RA, ET-RB). Macrophages recruited to hypoxic areas of the tumor will also express and secrete VEGF, increasing angiogenesis and recruiting more macrophages. Hypoxia induces a dramatic change in macrophage gene expression. TNFα, TGFβ, IL-1 and IL-6, which are cytokines secreted by inflammatory cells associated with the tumor, play an important role in the stabilization of HIFα and induction of hypoxia-regulated EMT. TNFα induces the activation of the transcription factor NF-kb, which stabilizes SNAIL and induces HIFα transcription. IL-6 lowers cell adhesion generating induction of SNAIL and TWIST expression leading to a decrease in E cadherin expression and increased cell mobility that induces vimentin and cadherin N expression. IL-1 induces HIF1-α transcription through NF-κb activation and lowers cell adhesion, inducing SNAIL expression that lowers cadherin E expression. TGF-β increases the stability of HIF-1α protein by inhibiting the expression of PHD2 protein (hydroxylase proline 2) (Hanahan and Folkman, 1996; Carmeliet, 2005; Gabhann and Popel, 2008; Ferrara, 2009).

Cancer cells tend to progress to higher pathological degrees of malignancy, leading to local invasions and metastases, as a consequence of changes in the shape of the cells and impairment of their connection to other cells and to the extracellular matrix promoted by E-cadherin. E-cadherin promotes unregulated growth, because it lowers the expression on the cell surface of proteins that induce growth inhibition by contact. That is, if we lower the expression of E-Cadherin, which is a transmembrane and cytoskeleton protein that connects cells to each other, they continue to grow even close to each other, as the cells become mobile. This protein, when mutated or deregulated, arouses stronger invasive cancer characteristics, while the increased expression of this protein has been identified as an antagonist of invasions and metastases (Hanahan and Weinberg, 2011).

Sooner or later, primary masses of tumors generate pioneer cells that move and invade adjacent tissues and travel to distant sites to establish new colonies (Sporn, 1996). The cancer cells have separated from the primary tumor, invade the circulatory

and lymphatic systems, manage to evade the immune attack, leak into distant capillary beds and invade and proliferate in other distant organs. For the metastatic process to be completed, many steps need to happen, involving an epithelial mesenchymal transition, an accumulation of mutations in stem cells, a macrophage facilitation process and a macrophage origin involving transformation or fusion hybridization with neoplastic cells (Seyfried and Huysentruyt, 2013).

The interaction between cancer cells and neoplastic stromal cells has been identified as essential in the acquired capacity for invasive growth and metastasis (Kalluri and Zeisberg, 2006; Joyce and Pollard, 2009; Egeblad, Nakasone and Werb, 2010; Qian and Pollard, 2010). Associated cancer cells typically developed changes in their shape and in their connection to other cells and to the extracellular matrix (Hanahan and Weinberg, 2011).

As already mentioned, the expression of E-cadherin helps to maintain cell quiescence and its increased expression is considered an invasion and metastasis antagonist, while its low expression potentiates these phenotypes. The low expression and occasional mutation of E-cadherin seen in human carcinomas demonstrate its link as a key suppressor in the ability to invade and metastasize (Cavallaro and Christofori, 2004; Van Roy and Berx, 2008). Macrophages also play an important role in this process, promoting local invasion on the periphery of the tumor and providing matrix degrading enzymes, such as metalloproteinases and cysteine-cathepsin proteases (Mohamed and Sloane, 2006; Palermo and Joyce, 2008; Joyce and Pollard, 2009; Kessenbrock, Plaks and Werb, 2010).

For many years, pathologists have recognized that some tumors are invaded by cells of the innate and adaptive arms of the immune system, which is reflected in inflammatory conditions that arise in non-neoplastic tissues (Flier, Underhill and Dvorak, 1986). Thus, inflammation has an opposite effect to that of defense, contributing to factors that support proliferative signaling, survival factors that limit cell death, pro-angiogenic factors, extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion and metastasis and inductive signals that lead to EMT activation (Karnoub and Weinberg, 2006; DeNardo, Andreu and Coussens, 2010;

Grivennikov, Greten and Karin, 2010; Qian and Pollard, 2010). Most tumors are infiltrated with immune cells. These cells release reactive chemical oxygen species (ROS), which are actively mutagenic, accelerating the tumor process (Qian and Pollard, 2010).

The chronic cell proliferation that represents the essence of the neoplastic disease involves not only the unregulated control of proliferation, but also the corresponding adjustments of the energy metabolism, which perform a reprogramming of the metabolism in order to stimulate cell growth and division. Normal cells break down glucose through a biochemical process called glycolysis, which occurs only under anaerobic conditions. Tumor cells, in turn, prefer to degrade glucose through aerobic glycolysis, that is, what happens even in the presence of oxygen, which even though it is not as efficient in the production of ATP, compensates for the production of intermediates necessary for the biosynthesis of macromolecules.

Glycolytic fuel has been associated with several activated oncogenes, such as the RAS and MYC genes, and also with mutant tumor suppressors, such as p53 (DeBerardinis et al., 2008; Jones & Thompson, 2009). The dependence on glycolysis as an energy generator may be even greater in hypoxic conditions, common in tumors. In this case, the hypoxia response system acts by pleitropy to positively regulate glucose transporters and various enzymes in the glycolytic pathway (DeBerardinis *et al.*, 2008; Jones and Thompson, 2009; Semenza, 2010). Thus, hypoxia increases the levels of the transcription factors HIF1α and HIF2α, which in turn positively regulates glycolysis (Kroemer and Pouyssegur, 2008; Semenza, 2010).

Genomic instability is an important factor that directly influences the process of tumor progression, through the ability of genome maintenance systems to detect and resolve defects in DNA that ensure that spontaneous mutation rates are generally very low during each cell generation. Throughout the mutagenic process in order to accumulate the mutant genes necessary to orchestrate tumorigenesis, cancer cells are able to generate an increase in mutation rates (Negrini, Gorgoulis and Halazonetis, 2010; Salk, Fox and Loeb, 2010). There is a diverse variety of defects that affect various

components of DNA maintenance machinery, often referred to as "caregivers" of the genome (Kinzler and Vogelstein, 1997).

Any understanding of this multi-step tumorigenic process is necessary for new therapeutic approaches to emerge as a result of understanding the evolution of the disease and its adjacent mechanisms (Potenta, Zeisberg and Kalluri, 2008).

4 Targeting mitosis in cancer therapy

4.1. Classical antimitotic drugs (antimicrotubules)

The treatments most commonly used to fight cancer are chemotherapy, radiation therapy and surgery, or an association of both. Immunotherapy has also emerged as an important ally in the fight against cancer, which implies biotherapeutic treatment resulting in an increase in the capacity of cancer cells to recognize cells of the immune system (Vanneman and Dranoff, 2012).

An important chemotherapeutic approach uses the interruption of mitotic machinery to potentiate death or arrest of the cell cycle. Thus, traditional antimitotic agents are those that directly interfere with the dynamics of microtubules, which play a central role in the assembly of the mitotic spindle and the subsequent alignment and segregation of DNA in the daughter cells.

The antimicrotubule agents in use for cancer treatment today are taxanes, vinca alkaloids and epothilones, having already been used in the treatment of several types of cancer as unique agents or in association with other oncological drugs.

Maintaining the symmetrical and always orderly appearance of the spindle microtubules during cell division requires highly accurate dynamics for this critical event during mitosis to occur correctly. As important allies in this organization and execution of the division, there is a huge variety of proteins that cooperate in this alignment and later in the locomotion of chromosomes along the microtubules (Dumontet and Jordan, 2010).

Microtubules are targets of most antimitotic agents, because as they are an essential part in the division process, inhibit their polymerization dynamics end up activating the

spindle assembly checkpoint and preventing the transition from metaphase to anaphase, causing a cell cycle arrest (Masawang *et al.*, 2014; Teixeira *et al.*, 2014). Cells that undergo mitotic arrest and in the face of interruption of spindle formation and chromosomal orientation, the cells remain in a state of prolonged arrest that ends up inducing apoptosis or in a G1 state in cellular senescence (Mitchison, 2012).

Microtubules are structures formed during interphase and are primarily responsible for correct chromosomal segregation and, therefore, mitosis. Due to their rapid dynamics during mitosis compared to interphase, they become an ideal drug target, since cancer cells have increased proliferative activity (Dumontet and Jordan, 2010).

Most MTAs bind to specific sites in microtubules that trigger mechanisms that affect tubulin stability, the main four being called: the laulimalide (stabilizing) site, the taxane/epothilone (stabilizing) site, the colchicine site (destabilizing) or the vinca alkaloid location (destabilizing).

The binding of free β-tubulin to vinca alkaloids and colchicine prevents polymerization in microtubules. Furthermore, if the binding occurs to β-tubulin at the ends of the microtubules, it will trigger the depolymerization of the microtubules and the formation of spirals made of one or two rolled protofilaments (Liu *et al.*, 2014). In contrast, taxanes and epothilones are mainly bound to β-tubulin in microtubules and prevent their depolymerization, leading to uncontrolled formation of microtubules.

In addition, both tubulin polymerization and microtubule depolymerization inhibitors end up significantly lowering the dynamic activity of microtubules in concentrations well below those that would be necessary to affect the total mass of the microtubules and, therefore, become the ideal target, since the effects on mitotic progression are more easily achieved (Liu *et al.*, 2014).

The effect caused by these interruptions in the dynamics of the microtubules and their subsequent cascade leads to a stop in metaphase and apoptosis. Both colchicine and Paclitaxel (the main taxane used in cancer treatment) bind to the side of the tubulin heterodimer facing the lumen of the microtubule, with the location of colchicine being close to the α-β interface and that of Paclitaxel attached to the β monomer. Vinca

alkaloids, on the other hand, bond through a longitudinal contact between the tubulin dimers in or near β-tubulin.

Paclitaxel (Taxol) was discovered in yew bark extracts from Pacifi in the early 1960s, being the first taxane identified, and was approved for use in the treatment of ovarian cancer three decades later, specifically in 1992. Paclitaxel is a drug used for years in anticancer therapy, being approved for use in several types of tumors, such as in the treatment of breast cancer (Bishop *et al.*, 1999), ovarian cancer (Ozols *et al.*, 2003) and lung cancer (Sandler *et al.*, 2006). This drug has the mechanism of action to promote the assembly of tubulin in dysfunctional microtubules, through its connection, which consequently generates a chromosome missegregation in multipolar spindles (Zasadil *et al.*, 2014). Consequently, microtubule dysfunction will inhibit mitosis and cell proliferation, leading to cell death by apoptosis of rapidly proliferating tumor cells.

Despite its effectiveness and proven toxicity, the synergistic action is an important ally to the use of this drug, which is widely used in combination with other chemotherapeutic agents. Clinical protocols can vary from patient to patient, and depend on prior planning of the dosages and periodicity that should be used. Adverse events are different from patient to patient, although frequency and severity are common among types of cancer. The most common and problematic adverse event in clinical practice is peripheral sensory neuropathy (Zasadil *et al.*, 2014).

Docetaxel (Taxotere) is a semi-synthetic derivative of paclitaxel, with the difference that it is more soluble and has started to be used in some types of cancer due to its response to increase prognostic success, such as in metastatic breast cancer (Jones *et al.*, 2005). Both Paclitaxel and Docetaxel have a similar spectrum of clinical activity, including cancer of the ovary, breast, bladder, lung, and among others. Although both have been used clinically for many years as the main therapeutic agents associated with chemotherapy, their usefulness continues to expand to new indications through the verification of effectiveness and new combinations have been proposed with other agents to potentiate associated cell death.

The signaling pathway associated with the HER2 gene influences multiple forms of taxane resistance, such as cell survival, drug efflux and drug metabolism (de Hoon *et al.*, 2012).

Abraxane is paclitaxel with the difference that it is formulated in nanoparticles that are linked to albumin, and that consequently end up, eliminating the need for Cremephor EL in the formulation, which is a vehicle that in itself has demonstrated toxicity and requires pre-medication (Ibrahim *et al.*, 2002). Despite the modest effectiveness of these agents, there are still many associated problems, such as issues related to water solubility, bioavailability, toxicity profile and resistance.

The first vinca alkaloids were extracted from the catharamthus roseus plant, native to Madagascar, and in 1960 its anticancer activities were identified (Liu *et al.*, 2014). Originally they were considered antidiabetic agents, however, it was identified that they had antiproliferative activity. These compounds act by binding to β-tubulin close to the binding sites to guanosine triphosphate (GTP) (vinca domain) that are at the interface of β-α-tubulin heterodimers (Liu *et al.*, 2014). The connection in the vinca domain prevents the straightening of the curved tubulin and consequently ends up interfering in the growth and assembly of the spindle microtubules (Ravelli *et al.*, 2004).

The biochemical effects of vinca alkaloids cause microtubule disruption, elevate oxidized glutathione, alter lipid metabolism and lipid content in the membrane, elevate cAMP and inhibit calmodulin-regulated cAMP phosphodiesterase. However, they have no impact on cellular respiration, glycolysis, nucleic acid or protein synthesis (Bates and Eastman, 2017).

Vinca alkaloids are hydrophobic substances and can be divided into lipid bilayers when they are not loaded, leading to a change in the structure and function performed by cell membranes. The most commonly observed mechanism of action is the ability to interrupt microtubules through high affinity bonds to tubulin. The vinca alkaloids family consists of: vinblastine, vincristine, vinorelbine, vindesine and vinflunine, three of which are all semi-synthetic derivatives of vinblastine (Bates and Eastman, 2017).

Vincristine and Vinblastine are the two oldest microtubule destabilizers in this class of microtubule binding agents and are used in the standard treatment of several types of cancer. Vincristine, for example, is used to treat Non-Hodgkin and Hodgkin's lymphoma and certain types of pediatric cancer, while vinblastine is used to treat Hodgkin's lymphoma, head and neck, lung and breast.

Vinorelbine, a semi-synthetic alkaloid from vinca, has a better preclinical profile than other family members (Moudi *et al.*, 2013) and has been approved for the treatment of lung cancer, in addition to having shown promising activity in breast carcinoma, head and neck, ovary and squamous cells (Burstein *et al.*, 2003; Infante *et al.*, 2009). Adverse effects associated with members of the vinca alkaloids class include mainly neutropenia and peripheral neuropathy, limiting the dose.

Epothilones are a class of 16-membered macrolide compounds targeting microtubules recently identified and isolated for the first time in the 1990s from the myxobacterium Sorangium cellulosum (Bollag et al., 1995). So far, eight epothilone derivatives have been reported, called epothilone A (EPO A), epothilone B (EPO B), epothilone C (EPO C), epothilone D (EPO D), epothilone E (EPO E), epothilone F (EPO F), epothilone G (EPO G) and epothilone H (EPO H) (Cheng, Huang and Huang, 2018).

Epothilones have their mechanisms of action similar to taxanes, promoting the stability of microtubules and, in fact, both share the same binding site. In contrast, some advantages are perceived over taxanes, including greater potency and lower probability of resistance resulting from bombs and drug efflux and mutations in tubulin (Kowalski, Giannakakou and Hamel, 1997; Wartmann and Altmann, 2002), in addition to being formulated in more modern vehicles well tolerated than the cremophore that is used, for example, for Paclitaxel (Watkins *et al.*, 2005; Sessa *et al.*, 2007).

There are differences in toxicities and clinical activity between different types of epothilones and also compared to taxanes. For example, epothilones A and B, cytotoxic metabolites that stabilize microtubules and which are derived from mycobacterium sorangrum cellulosum, demonstrate greater cytotoxicity than taxanes (Jordan and Wilson, 2004). Epothilone B inhibits paclitaxel, because both bind in the same place to tubulin-β, generating competition for this binding. Despite sharing this

point in common, epothilones and taxanes do not show common resistance mechanisms (Rogalska *et al.*, 2013).

Returning to the starting point, we can understand that it is well established that antimitotic compounds compromise the ability of cells to divide causing prolonged mitotic arrest that leads directly to cell death or divide abnormally, with an uneven distribution of DNA (Rieder and Maiato, 2004; Weaver and Cleveland, 2005; Gascoigne and Taylor, 2008), that is, this unsuccessful division can effectively keep cells in cycle, causing the cycle to stop or the cell cycle to die.

The results after treatment with antimitotic agents will depend on the type of cell, tissue and obviously the concentration of the agent used (Gascoigne and Taylor, 2008; Shi, Orth and Mitchison, 2008).

Cell death from apoptosis can occur both during mitosis and in the interphase following mitosis (Gascoigne and Taylor, 2008; Shi, Orth and Mitchison, 2008) due to double strand breaks of DNA after treatment with antimitotic agents (Dalton *et al.*, 2007; Lei and Erikson, 2008).

However, apoptosis is not the only means of cell death in solid tumor treatments (Abend, 2003), and there are other forms of cell cycle arrest that contribute substantially to antitumor efficacy in preclinical models, such as senescence (Roninson, Broude and Chang, 2001). For this reason, caution should be exercised when analyzing apoptosis indices as indicators of sensitivity or resistance to the medication.

As the reactionary effects to antimitotic agents are not limited to cancer cells only, dose-limiting toxicities often manifest in the rapid division of tissue, and are accompanied by adverse effects such as, for example, peripheral neuropathy in the case of anti-microtubule agents.

The multidrug resistance gene 1 (MDR1 or ABCD1) is responsible for the production of P-gp, part of the ABC family, which is responsible for the efflux of many hydrophobic antimitotic drugs, such as taxanes and vinca alkaloids (Nobili *et al.*, 2012). Overexpression of ABCD1 and P-gp are important genes involved in both intrinsic resistance and acquired resistance to the drug (Rebucci and Michiels, 2013).

Multiresistant protein 1 (MRP1) in turn is responsible for a resistance mechanism that transports vinca alkaloids out of the cell. Multiresistant protein 2 (MRP2) is responsible for a resistance mechanism that exports taxanes, while multiresistant protein 7 (MRP7) is responsible for a resistance mechanism that acts on the transport of epothilone B (Dumontet and Jordan, 2010).

Due to the resistance mechanisms, the need arose for the development of drugs that are not sub-extracts of P-gp, such as second and third generation taxanes and epothilones. Through structural modifications, these compounds allow the avoidance of P-gp, being essential to overcome obstacles to cancer resistance (Nobili *et al.*, 2012). Another possible and used strategy is to make use of molecules that reinforce the overexpressed P-gp efflux pumps (Rebucci and Michiels, 2013).

βIII-tubulin is another protein associated with resistance mechanisms, expressed in stressed cells deprived of oxygen and nutrients, which increases the dynamic instability of microtubules and neutralizes the stabilizing action of taxanes and also affects the effectiveness of vinca alkaloids (Kavallaris, 2010; Rebucci and Michiels, 2013). Kinesins associated with mitotic centrosomes (MCAK), stathmin and tau, which are microtubule regulatory proteins, are also frequently associated with resistance to antimitotic drugs (Rebucci and Michiels, 2013).

There are also, resistance mechanisms related to the deregulation of SAC proteins via gene amplification, such as Aurora kinase protein via Aurora-A amplification, which increase cell survival, lower levels of apoptosis induction and improve efflux drugs (Anand, Penrhyn-Lowe and Venkitaraman, 2003; Park *et al.*, 2008).

Overexpression of the HER2 gene is associated with more aggressive tumors and the types of cancer amplified by HER2 are resistant to taxanes, through the regulation of P-gp efflux pumps (de Hoon *et al.*, 2012).

Hypoxia is another important factor of drug resistance to be overcome, as it potentially hinders access and, consequently, the effectiveness of medicines. The state of oxygen deprivation in solid tumors is related to several signaling pathways responsible for controlling the cell cycle and angiogenesis, causing invasion and metastasis to spread.

For example, an increase in resistance due to hypoxia in the treatment with Paclitaxel is overcome by the increase in the levels of cyclin B1, thus, hypoxia reduces the antimitotic activity of Paclitaxel by the negative regulation of cyclin B1 (Teicher, 1994; Dong *et al.*, 2012).

The resistance mechanism varies depending on which medication is being administered. In vinca alkaloids and other drugs that inhibit microtubule assembly, MDR predominates. In contrast, in paclitaxel, epothilone and other drugs that promote the assembly of microtubules, mutations that happen in tubulin occur more frequently (Ganguly, Yang and Cabral, 2011).

Tubulin mutations can confer resistance to antimitotic drugs by distinct mechanisms. One of them, the drop in drug binding as a result of genomic instability, and it is possible that tumors have functionally altered the β-tubulin locus, making it haploid and, therefore, capable of resisting therapy through the acquisition of mutations that have lowered the binding to the medicine. Understanding this mechanism becomes simpler if we consider that microtubules are metastable and highly dynamic structures that can only function within a limited range of stability (Ganguly, Yang and Cabral, 2011).

The mechanisms of anticancer drugs have been predominantly evaluated to determine the influence of these drugs on the real physiology of the human tumor (Kamb, 2005). This can lead to several mechanisms of action to be detected and from this knowledge new compounds can be developed that are able to evade these mechanisms and improve the prognosis of treatment.

4.2 Second generation of antimitotic drugs

With the progress of specialized medicine in oncology, which aims to create drugs that can act on specific targets, several targets for antimitotic drugs have been studied and developed, acting in different phases of the cell cycle (**Figure 12**).

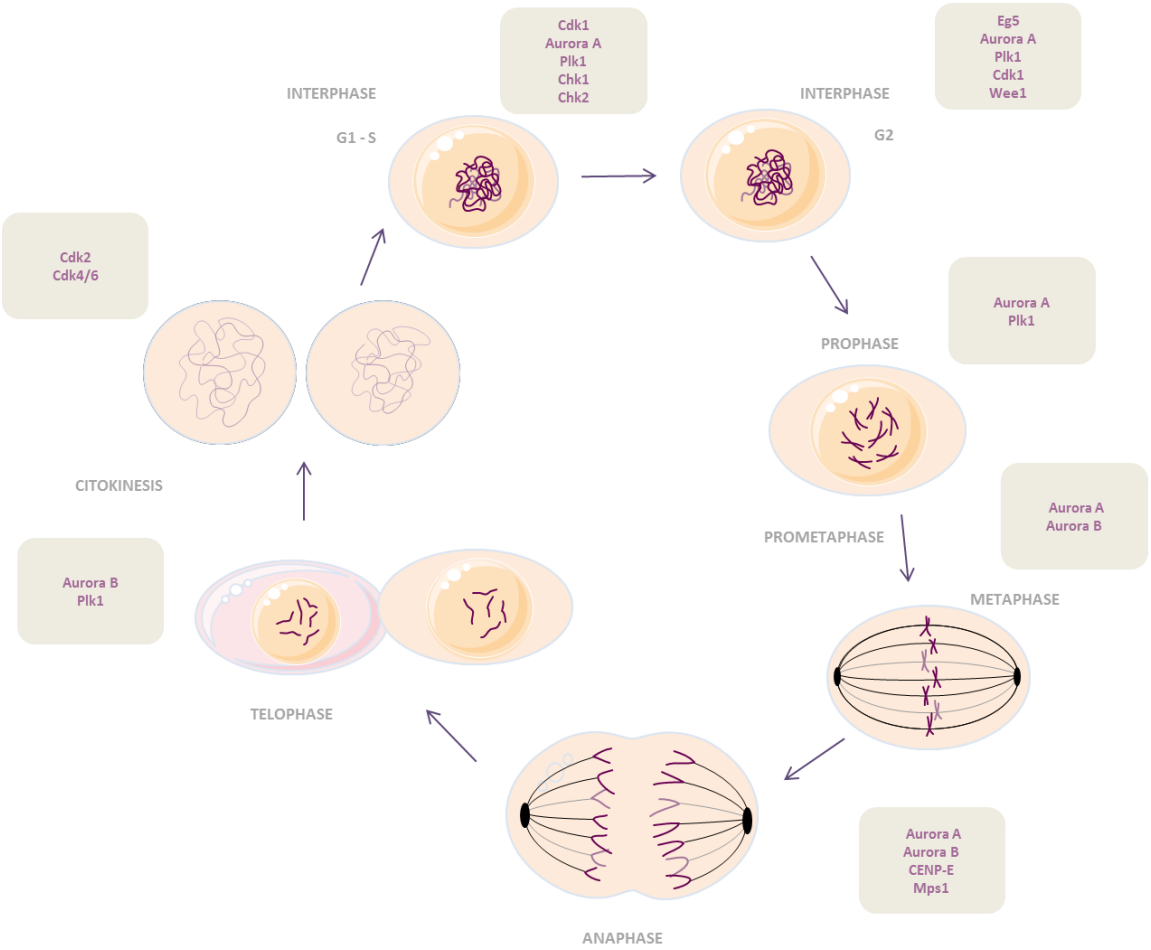

Figura 12 Proteins involved in advancing the cell cycle as targets for antitumor drugs. Many proteins are necessary for the cell cycle to function properly, especially in mitosis, where a multitude of regulatory proteins are responsible for the correct advance of its phases. Some of these proteins that act at different stages of the cell cycle have been approached as important therapeutic targets for new anti-tumor drugs. Inhibitors of these proteins, mentioned in the figure above, are in preclinical or clinical development.

Aurora kinases and Polo type kinases (PLKs) play key roles in normal cell cycle progression through mitosis and represent the two families of kinases that are the best examples of second generation antimitotic targets.

Aurora kinases were identified for the first time in Yeasts (Ipl1), Xenopus (Eg2) and Drosophila (Aurora) and are considered critical regulators of mitosis and are therefore determinants for the normal continuation of the cell cycle (Chan and Botstein, 1993; Glover *et al.*, 1995; Andrésson and Ruderman, 1998; Roghi *et al.*, 1998).

There are three known isoforms of Aurora kinase in humans: Aurora A, Aurora B and Aurora C. Aurora A and Aurora B have their functions associated with mitosis and are structurally interrelated, unlike Aurora C which has its function associated with meiosis. Its catalytic domains are at the C-terminus, being differentiated by only a few amino acids. Greater diversity can be observed in the non-catalytic N-terminal domains and the different sequences presented in this region of Aurora A and Aurora B end up being decisive in directing their interactions with different proteins. This allows them to have different and unique subcellular locations and functions in mitotic cells at different stages.

Some attempts are underway to develop inhibitory drugs for molecules targeting Aurora A, Aurora B or both kinases simultaneously, as they may present different approaches in relation to different types of tumors.

The Aurora A gene (AURKA) is commonly amplified or overexpressed in a variety of tumor types (Bischoff *et al.*, 1998; Crosio *et al.*, 2002; Camacho *et al.*, 2006; Ikezoe *et al.*, 2007; Chng *et al.*, 2008), and this has been an important feature related to the etiology of cancer and a worsened prognosis (Miyoshi *et al.*, 2001; Sakakura *et al.*, 2001; Crosio *et al.*, 2002; Fraizer *et al.*, 2004; Jeng *et al.*, 2004; Guan *et al.*, 2007; Landen *et al.*, 2007). The therapeutic potential in developing drugs that are related to this protein goes against the important oncogenic role of Aurora A in tumor development. The same occurs with Aurora B overexpression, which has also been observed in several types of tumors (Ikezoe *et al.*, 2007) and correlated with a worse prognosis.

This overexpression of Aurora A and B proteins may be related to the increase in protein levels per mitotic cell or also due to an increase in the mitotic index characteristic of tumor cells. Aurora A is expressed several times during the normal cell cycle, the first time being in the G2 stage where it is located in the centrosomes and acts on the maturation and separation of the centrosomes, showing a controversial role in the entry of cells in mitosis, because although Aurora Kinase results in a late mitotic entry (Marumoto *et al.*, 2002), sometimes cells enter mitosis even though this kinase is inactive.

In mitosis Aurora A is located in the centrosomes and in the proximal portion of the incipient mitotic spindles where several proteins interact and phosphorylate that collectively serve to perform different functions in mitosis and cytokinesis (Bischoff and Plowman, 1999; Carmena and Earnshaw, 2003; Giet, Petretti and Prigent, 2005; Barr and Gergely, 2007).

Aurora A inhibition initially leads to the formation of abnormal mitotic, monopolar, bipolar or tripolar spindles with misaligned chromosomes, commonly associated with defects in the separation of centrosomes, which lead to a mitotic stop determined by the activation of the checkpoint of the axis set. Some cells may undergo prolonged mitotic arrest and be taken to apoptosis, while others may come out of mitosis without undergoing cytokinesis, resulting in G1 tetraploidy or may divide at high frequency even with severe chromosomal segregation defects. Both in the case of tetraploidy and in the case of accelerated mitotic divisions, they result in deleterious aneuploidy and subsequent death or arrest of the cell cycle (Gascoigne and Taylor, 2008).

In turn, Aurora B is located in the centromeres in the prophase cells where it plays an important role in defining the spindle bipolarity and in establishing and maintaining the spindle assembly checkpoint (Adams *et al.*, 2001; Murata-Hori and Wang, 2002; Ditchfield *et al.*, 2003). Already in anaphase and telophase, it is located in the central zone and middle body spindle, respectively, and acts in cytokinesis (Giet and Glover, 2001; Yokoyama *et al.*, 2005).

The inhibition of Aurora B through the use of genetic mutations, RNA interference or competitive inhibitors of ATP molecules can lead to several defects in the connection of the spindle microtubules to kinetochores, chromosomal segregation and cleavage groove formation (Adams *et al.*, 2001; Giet and Glover, 2001; Murata-Hori and Wang, 2002; Ditchfield *et al.*, 2003; Honda, Körner and Nigg, 2003; Yokoyama *et al.*, 2005), in addition to preventing the proper formation of the spindle assembly checkpoint, causing the cells come out of mitosis before completing cytokinesis, without a mitotic arrest (Ditchfield *et al.*, 2003). This would result in the formation of G1 tetraploid cells, similar to the inhibition of Aurora A, and would eventually generate death, stop or

undergo additional rounds of DNA replication (duplicate replication), generating a DNA ploidy>4N.

Taking this into account, many Aurora kinase molecule inhibitors have been developed in clinical trials with cancer patients, including selective Aurora A inhibitors, selective Aurora B inhibitors or inhibitors that work together to inhibit both kinases. Understanding that Aurora kinases have a mandatory function in all dividing cells, they can be used in a wide variety of tissues, acting in different treatments of solid and hematological cancers, both in a single agent and in combination.

On the other side of the second generation antimitotics that concern the proteins Polo-kinases Quantum (PLK) initially identified in Drosophila melanogaster (polo) with orthologists also found in yeasts (cdc5 and plo1) and Xenopus (Plx) (Sunkel and Glover, 1988; Llamazares *et al.*, 1991; Kumagai and Dunphy, 1996), it is known that it represents an important regulator of mitosis and that it is structurally and functionally related to the member of the PLK1 mammal family.

The family is still composed of three additional members, PLK2, PLK3 and PLK4. PLK4 has a similar function to PLK1 and acts during mitosis while PLK2 and PLK3 have roles not associated with mitosis (Winkles and Alberts, 2005).

Of all the members of the family of this kinase, PLK1 is the one with the most reported mechanism and, therefore, there are inhibitors of molecules developed against this isoform in preclinical and clinical contexts.

PLKs are highly conserved serine/threonine kinases and differentiated by non-catalytic C-terminal domains that have 60 to 70 amino acids called polo-box domain (PBD) that serve as a binding module through phosphorylation in other proteins mediating interactions protein-protein (Lee *et al.*, 1998; Elia *et al.*, 2003).

The kinase domain and the PBD act mutually to inhibit themselves through intermolecular interaction that occurs in the stages of phases G1 and S, rendering the kinase inactive. During the period in G2 there is phosphorylation of the kinase domain that relieves the interaction with the PBD and makes the temporal control of PLK1.

Cdk1 cyclin can perform the function of phosphorylating proteins by creating anchorage sites for PLK1 PBD (Santamaria *et al.*, 2007; Fu *et al.*, 2008).

The spatial regulation of PLK1 depends on the recruitment of PBD to different regions of the mitotic cell. This allows PLK1 to perform the function of phosphorylating different substrates that consequently trigger divergent mitotic functions. In the G2 phase, PLK1 is located in the centrosomes and in the metaphase in the centromeres and kinetochores, while in anaphase it is present in the middle zone of the spindle and in the middle of the body at the end of the whole process, in cytokinesis.

PLK1 plays several roles, such as regulating the maturation of centrosomes, entering mitosis, anaphase-promoting complex activity, forming and maintaining a bipolar mitotic spindle, cytokinesis and mitotic output (Lane and Nigg, 1996; Toyoshima-Morimoto *et al.*, 2001; Sumara *et al.*, 2004; Eckerdt and Strebhardt, 2006; Petronczki, Lénárt and Peters, 2008).

The inhibition of PLK1 causes activation of the SAC, due to the impediment of being located in the centrosomes and kinetochores, which manifests itself as a mitotic delay of prometaphase that is characterized by a phenotype composed of monopolar or bipolar misaligned mitotic spindles that do not bind stable way to kinetochore (Gilmartin *et al.*, 2009).

In addition to the roles developed within the cell cycle, PLK1 is also involved in regulating the stabilization of telomeres, regulating DNA topoisomerase II and repairing DNA (Li, Wang and Liu, 2008; Svendsen *et al.*, 2009).

Molecule inhibitors that target the catalytic active site of PLK1 are being evaluated in clinical trials for solid and hematological malignancies. The clinical benefit has been observed for some types of tumors both as a single agent and in combination (Schöffski, 2009).

Science has created many promising approaches to cancer treatment in recent decades. Studies of the biochemical processes that occur in the cell cycle exclusive to cancer cells have favored the development of many drugs. Even with the discovery of these new selective agents, cytotoxic drugs will continue to be the basis of

chemotherapy for cancer in the coming years, and of these, compounds with antimitotic activity are among the main cytotoxic agents under development due to the success of taxanes and the widespread use of vinca alkaloids in clinical oncology.

The interest now is in finding new molecules that make resistance mechanisms softer and that are more targeted at certain targets. The renewed interest in antimicrotubules agents was generated by the hope that non-MDR substrates will be discovered that interact with tubulin in close, overlapping or different locations from taxanes or vinca alkaloids that are already widely used in clinical practice.

5 Xanthones and cancer

Xanthones are secondary tricyclic metabolites with a symmetrical structure, derived from dibenzo-γ-pyrone (El-Seedi *et al.*, 2009). Its name comes from the Greek “xanthosin”, which means blonde, due to its yellow color. Xanthones are called "promiscuous binders" because they have the ability to bind to different receptors and exercise a variety of pharmacological activities, acting on a variety of diseases (Masters and Bräse, 2012).

Xanthones were initially identified in only six groups of four families: Gentiana L. and Swertia L. from Gentianaceae Juss.; Garcinia L. and Platonia Mart., from Clusiaceae Lindl.; Calophyllum L. from Calophyllaceae J. Agardh; and Mangifera L. from Anacardiaceae R. Br. (Roberts, 1961).

However, it is now known that xanthones are widely present in higher plants and in endophytic species of fungi (El-Seedi *et al.*, 2010). In 2005 Matsumoto *et al*. described xanthones as biologically active phenols that are found naturally in a restricted group of plants. In 2007, Wätjen *et al*. they said that approximately 200 xanthones were found in nature. However, today, about 1225 xanthones were isolated between 2012 and 2019 from 23 plant families (Matsumoto *et al.*, 2005; Kampkötter *et al.*, 2007; Klein-Júnior *et al.*, 2020).

The xanthones found in nature have their nucleus originating from the acetate and shiquimate pathways, however it is liable to undergo additional metabolic steps that end up leading to a wide range of chemical entities, which can be classified according

to their chemical adjectives such as: bis-xanthones, glycosylated xanthones, prenylated xanthones, simple xanthones, xanthonolignoids and various xanthones (El-Seedi *et al.*, 2010).

Xanthones are compounds formed by a six-carbon conjugated ring structure with multiple carbon double bonds (**Figure 13**). The prenyl group found in these phenols plays a central role in internalization in the cell, which consequently leads to interaction with signal transduction molecules and proteins involved in the permeability transition of mitochondria (Ozols *et al.*, 2003; Kampkötter *et al.*, 2007).

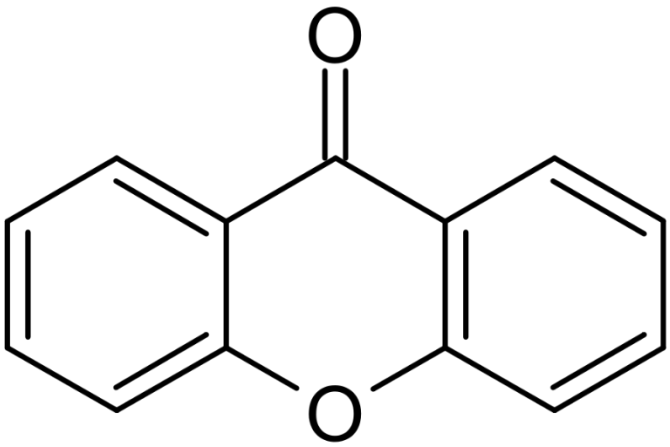

Figure 13 Chemical skeleton of Xanthone. Chemically, Xanthone (9H-xanten-9-one) is formed by two benzene rings and a central γ-pyrone, which gives symmetry to the compound.

Several primitive medicinal plants and some dietary factors have served as a starting point to find new substances that have an antitumor activity so that they can be used as leading compounds in the development of drugs that increase the effectiveness of treatments. A huge number of natural products have already been evaluated as chemopreventive or therapeutic agents, and it was from there that drugs that are used today in clinical practice such as paclitaxel and vincristine have emerged.

Some evidence-based bio-factors for cancer prevention are needed to come to practical use. Polyphenols, for example, were idealized as chemopreventive agents because they already have proven antioxidant activity and possible anti-cancer activity (Sun, 2002) due to inducing cell apoptosis when reaching mitochondria and consequent activation of signal transduction intrinsic apoptotic (Nakagawa *et al.*, 2007; Akao *et al.*, 2008).

Most xanthones were found in natural resources of plant origin, however, some derivatives were isolated from species of marine fungi. For example, Monodictyxanthone analogues isolated from marine algicolous fungus Monodictys putredinis that have shown effective inhibition of the cytochrome P450 (CYP) 1A iso enzyme that converts xenobiotics into carcinogens (Krick *et al.*, 2007). Another example concerns α-mangostin which is found in the pericarps of mangosteen, Garcinia mangostana, and which demonstrated a potent inhibition of cell growth in human cancer through induction of apoptosis (Matsumoto *et al.*, 2005; Nakagawa *et al.*, 2007).

Lee et al. in 2016 demonstrated that α-mangostine treatment lowered the viability of YD-15 tongue mucoepidermoid carcinoma cells and induced cell apoptosis by inhibiting the ERK1/2 and p38-MAPK signaling pathway. Kritsanawong et al. in 2016 they demonstrated very similar results where treatment with α-mangostine in human breast carcinoma (T47D) cells inhibited cell proliferation and induced apoptosis associated with the HER2/PI3K/Akt and MAPK signaling pathways (Franceschelli *et al.*, 2016; Kritsanawong *et al.*, 2016; Pérez-Rojas *et al.*, 2016).

Some studies have also shown that xanthone γ-mangostine also has antiproliferative capacity, and Chang, Wu and Yang in 2013 described that this compound inhibited the proliferation of human colorectal adenocarcinoma (HT-29) cells by apoptosis and has elimination activities free radicals, antiproliferative and apoptotic in hepatocellular carcinoma (HepG2) cells (Chang, Wu and Yang, 2013).

Mangiferin is another xanthone that has shown promising biological activity for the treatment of cancer, where the main mechanism of action is through the inhibition of the NF-κB pathway, which regulates inflammation and manages to block the progress

of the disease (Núñez Selles, Daglia and Rastrelli, 2016). This compound can induce apoptosis by suppressing the activation of NF-κB and Bcl-xL and XAIP expressions and increasing caspase-3 activity (Shoji *et al.*, 2011), in addition to being able to induce cell cycle arrest in the G2/M phase through negative regulation of the CDC2-CyclinB1 pathway in human breast carcinoma cells (MCF-7) (Li *et al.*, 2013).

Psorospermin is a xanthone extracted from the African plant Psorospermum febrifugum (Schwaebe, Moran and Whitten, 2005) and has demonstrated anti-cancer activities through the intercalation of the xanthone group with base pairs of DNA and alkylation of the epoxide by N7-guanine in the presence of topoisomerase II (Hansen *et al.*, 1996; Kwok and Hurley, 1998).

Phomoxanthones are structurally unique xanthone dimers identified in endophytic fungus Phomopsis species and have demonstrated excellent cytotoxic activity against tumor cell lines (Isaka *et al.*, 2001).

Another type of xanthones found in plants and found in abundance in nature is prenylated xanthones. Some specimens of this type have been isolated from Garcinia cantleyana in the Malaysian Peninsula. These analogs are polyprenylated derivatives, and the main examples are 7-hydroxy-forbesione, cantleyanone B and cantleyanone C were considered to be potent cytotoxic agents (Shadid *et al.*, 2007).

Still on this pre-alkylated group of xanthones, gambogic acid is found, which is isolated from the resin of the Garcinia hurbury tree and which has been proven as a potent anticancer agent through the induction of apoptosis (Zhang *et al.*, 2004) and also as an anti-angiogenic that inhibits angiogenesis by suppressing tyrosine phosphorylation induced by vascular endothelial growth factor (VEGF) of VEGF-A receptor 2 (KDR/Flk-1) (Lu *et al.*, 2007).

Several in vivo and in vitro studies have already been carried out to demonstrate the potent anticancer activity of gambogic acid, which acts on several targets, including inducing apoptosis, stopping cell proliferation and autophagy and inhibiting telomerase, having effects anti-metastatic, anti-metastatic, anti-angiogenesis and anti-inflammatory drugs (Kashyap *et al.*, 2016). One of the main mechanisms of action

associated is due to an increase in p53 expression and the consequent suppression of the MDM2 protein (Gu *et al.*, 2009).

Gambogic acid has been studied as an ally to resistance to docetaxel, since this xanthone acts by carrying out negative regulation of survivin, which is an inhibitor of apoptosis protein. The inhibition of topoisomerase II catalytic activity has also been shown to important suppression performed by this xanthone by binding to the ATP domain in the enzyme (Qi *et al.*, 2008).

Although naturally isolated xanthones have been identified as potential new candidates for anticancer drugs, their low amounts found in nature in the face of demand and also their structural limitations have brought the need to develop efficient synthetic methods to modify them and calibrate their pharmacological structures in order to solving the problems. In contrast to this identified variety, xanthones are difficult to obtain and are present in low concentrations in plants.

The biological activity of xanthones depends on the position and types of substituents on the ring nucleus. Structural changes occur through the introduction of various substituents on the aromatic ring portions in the nucleus to provide a greater spectrum of biological activity.

This is how oxygenated xanthones, with a flat structure and known for using DNA intercalation, show their anticancer activity through non-covalent interaction with DNA. Starting from this principle of activity, some compounds conjugated to two xanthone rings by means of appropriate ligands were synthesized.

Bis-xanthones are one example, tied by a 6-carbon unit that effectively inhibits the cell cycle of cancer cells and that the inclusion of a lipophilic ligand in its structure has been improved to increase the penetration of the blood-brain barrier and thus increase its effectiveness (Genovese *et al.*, 2016). Some xanthones such as bisfuranoxanthones are comparable to psorospermin in the cytotoxicity test, but have not shown the same efficiency as DNA alkylators mediated by topoisomerase II.

Oxime and methyloxime coupled xanthones that have been prepared and tested for cytotoxic activity and that have been shown to be potent inhibitors of cancer cell

growth (Liu *et al.*, 2016; Klein-Júnior *et al.*, 2020). Some synthetic oxygenated xanthones have demonstrated anticancer potential through the inhibition of α-glucosidase (Liu *et al.*, 2006).

Protein kinase C (PKC) is also a pharmacological target for some xanthone compounds, namely 3,4-dihydroxy- and 1-formyl-4-hydroxy-3-methoxyxanthones (Saraiva *et al.*, 2003). Synthetic xanthones modified with imidazole substituents showed aromatase inhibitory activity in upper breast cancer compared to fadrozole drug which is used with the same functionality (Recanatini *et al.*, 2001).

5,6-dimethylxanthenone-4-acetic acid (DMXAA) is a xanthone that has held high scientific interest since its discovery due to its excellent pharmacological profile of known potential anticancer drugs (Ching *et al.*, 1991). DMXAA is a vascular rupture agent that can influence the blood supply to the tumor tissue, lowering it and resulting in tumor regression (Gaya and Rustin, 2005).

Xanthones with an epoxy group effectively inhibited the growth of cancer cells (Liou et al., 1993). Epoxide appears to play an important role in the cytotoxicity of this series of xanthones (Lin *et al.*, 1993, 1996).

The structural diversity and biological aspects described identified in xanthones make these secondary metabolites important allies in the search for new and potent anticancer drugs (Matsumoto *et al.*, 2005). Its anticancer and anti-inflammatory activity has shown exciting results due to its effectiveness and low toxicity in normal cells (Gutierrez-Orozco and Failla, 2013; Pérez-Rojas *et al.*, 2016).

The various anticancer and cytotoxic properties of these compounds have been evidenced in several studies in vitro and in vivo, elucidating that xanthones can act in all stages of carcinogenesis: initiation, promotion and progression (Ibrahim *et al.*, 2016; Liu *et al.*, 2017).

In view of everything described, it can be said that xanthones, are among the oldest structures found in the chemical world, are seen as an important ally in the development of anticancer drugs due to their structural diversity and biological

aspects. The biological activities of synthetic xanthone derivatives are dependent on the various substituents and their position.

With all that described, we came to the conclusion that xanthones can be considered potential anticancer therapeutic agents, acting on several mechanisms of action in carcinogenesis (Klein-Júnior *et al.*, 2020) (**Figure 14**). Xanthones have shown hope in anticancer therapy, as they present cytotoxic effects through different mechanisms, such as those mentioned above, for example, inhibiting angiography, through the low expression of vascular endothelial growth factor (VEGF). The anti-metastatic potential is also of great interest, and is achieved by xanthones by inhibiting the expression of matrix metalloproteins. The anti-inflammatory activity also has a multifunctional anti-cancer effect, through a cascade of events that influences tumorigenesis.

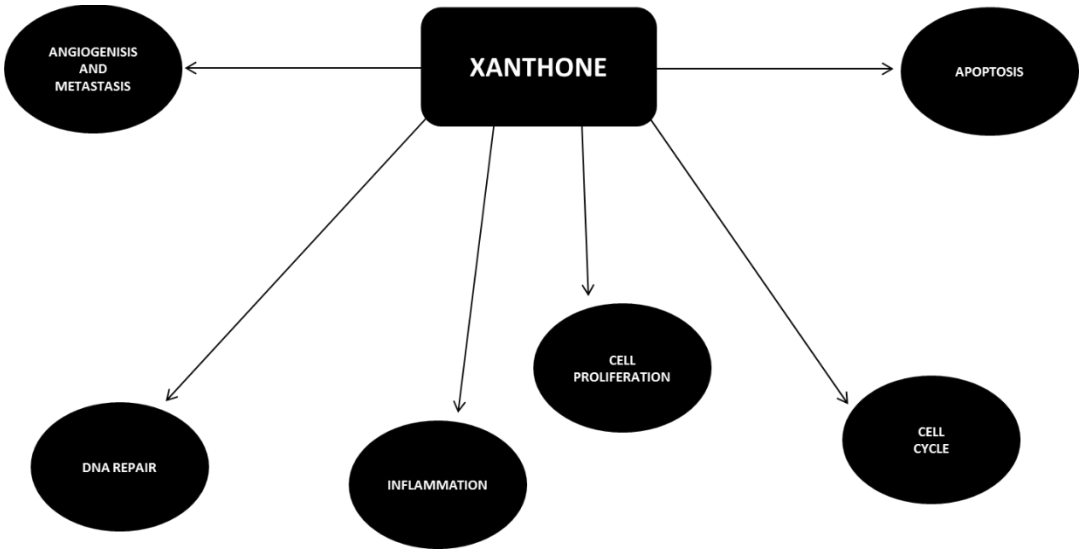

Figure 14 Mechanisms affected by xanthones. Xanthones operate their anticancer activity by interfering with various mechanisms of the tumor cell: Blocking the cell cycle, preventing the proliferation of the tumor cell, directing apoptosis, triggering DNA repair, functioning as an anti-inflammatory agent and preventing the recruitment of cells necessary in angiogenesis and consequently metastasis.

Further studies like ours, which involve biological targets and demonstrate toxicity effects of xanthones will be responsible for the identification and development of these compounds. The elucidation of the exact biological target of xanthone compounds and their mechanisms of action will provide the development of more potent anticancer drugs. As well as the modification of natural xanthone derivatives to target specific targets through the elaborate design of new xanthone analogs with the help of modern medicinal techniques, including molecular modeling, will be responsible for expanding the biological spectrum of these compounds.

Chapter II

Aims

The pyranoxanthone 1 (**Figure 15**) was identified which exhibited a potent growth inhibitory activity against the human tumor cell lines MCF-7 (breast adenocarcinoma) and A375-C5 (melanoma) (Azevedo et al., 2013a). Even though its growth inhibitory mechanism is still unknow, the angular fused ring orientation without substituents appears to be important for the activity. Retaining these features, the pyranoxanthone 2 was designed to assess the significance of the two alkyl groups in the chromene moiety for the antitumor activity (**Figure 15**).

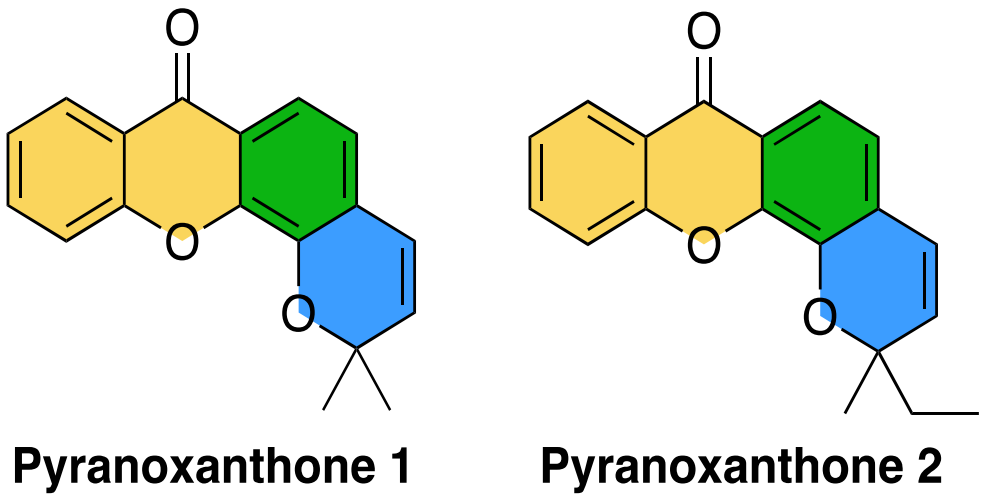

Figure 15 Structure of pyranoxanthone 1 and 2. The xanthone scaffold is highlighted in yellow and green and the benzopyran scaffold is highlighted in green and blue.

Prior to this study, in a series of xanthones, including pyranoxanthone 2, was performed a Sulforhodamine B (SRB) assay with the aim of determined the GI50 of the compound, corresponding to the concentration that was able to cause 50% cell growth inhibition.

The compound pyranoxanthone 2 showed potent growth inhibitory activity against the three human cancer cell lines tested: melanoma (A375-C5), breast (MCF-7) and lung (NCI-H460) with a GI50 value of 5.29 ± 1.19, 6.61 ± 0.78 and 6.67 ± 0.20 µM,

respectively (**Table 1**). This result supports the potential of pyranoxanthone 2 as promising anticancer agent which leads us to explore their cytotoxic mechanism.

Cell line	A375-C5	MCF-7	NCI-H460
GI50 of PX2 (µM)	5.29 µM ± 1.19	6.61 µM ± 0.78	6,67 µM ± 0.20

Table 1 GI50 of compound PX2. Concentration necessary to inhibit the growth of 50% of the cells, initially seeded, after treatment with the compound in each cell line; 5.29 µM on A375-C5, 6.61 µM on MCF-7 and 6.67 µM on NCI-H460.

Hence, in the present study, we report the mechanism elucidation of the lead compound pyranoxanthone 2, identified as a lead compound with potent cytotoxic activity against cancer cells, and determined its potential to increase the sensitivity of cancer cells to low doses of paclitaxel. Our data highlight the potential of the pyranoxanthone 2 as a new and promising antimitotic with effective antitumor activity in vitro.

Chapter III

Materials and methods

1 Compound

Pyranoxanthone 2 (from Faculty of Pharmacy, U. Porto) was reconstituted in sterile dimethyl sulfoxide (DMSO, Sigma-Aldrich) 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, pyranoxanthone 2 was diluted in fresh culture medium at desired concentrations.

2 Cell culture

2.1 Cell lines

Melanoma A375-C5, breast adenocarcinoma MCF-7 and non-small cell lung cancer NCI-H460 (European Collection of Cell Culture, UK) cell lines were grown in RPMI-1640 medium (Roswell Park Memorial Institute, Biochrom), supplemented with 5% heat inactivated FBS (fetal bovine serum, Biochrom). All cell lines were maintained at 37˚C in a humidified incubator (Hera Cell, Heraeus) with 5% CO2.

2.2 Subculture and cellular conditions

Cell lines were grown in 25 cm^3 (VWR) flasks with complete growth culture medium composed of RPMI-1640 (Roswell Park Memorial Institute, Lonza), supplemented with 5% inactivated FBS (fetal bovine serum, Biochrom), and maintained at 37˚C in a humidified incubator (Hera Cell, Heraeus) with 5% CO2. All experiments were carried out in aseptic environments in a level II biosafety cabinet (Telstar, Bio-II-A/P) and the culture solutions used were preheated in a 37˚C water bath (Precisterm) to be at temperature ideal for use.

In order for exponential growth to be maintained, avoiding metabolic stress due to space and nutrient restrictions, the cells were subcultured at most within 3 to 4 days. In this process, the RPMI medium was aspirated and the adherent cells were washed with approximately 2 mL of PBS 1x isosmotic saline obtained through diluted sterile PBS 10x. Then, 500 µL of trypsin 1x (GIBCO, Invitrogen) was added and left for approximately 3 minutes incubated at 37°C to promote cell detachment. After this time, the complete detachment of the cells was verified through an inverted

microscope (Zeiss Primo Vert) and the trypsin neutralized by the addition of fresh RPMI. The resulting cell suspension was transferred to a 15 ml conical tube from which 30 µL of the cell suspension was removed and mixed with 30 µL of 0.4% (v/v) Trypan Blue dye (Sigma-Aldrich) to determine cell density and viability by counting cells per quadrant using a Neubauer chamber.

Cell density (expressed in cells/mL) was determined as the number of viable cells over the number of quadrants considered and multiplied by the dilution factor (DF) which was $0.15x10^{6}$ and the constant of 10,000, as described in the following formula: (number of living cells/quadrants counted) × DF × 10^{4}.

Cell viability (%), in turn, was determined by the percentage of viable cells over the total number of viable cells added to dead cells, and was checked frequently to ensure that the cells were healthy, as they had a viability index above 90%. All three cell lines initially used were subcultured at least twice a week, using a set of dilutions, ranging from 1:5 to 1:20, according to the specific doubling time for each cell line.

2.3 Freezing and thawing the cell line

In order for changes in the characteristics of the genotype and phenotype to be controlled, all cell lines needed to have a low number of passages, which was possible through regular freezes in high concentration, in exponential growth and with the lowest possible number of passages.

The cell suspension was centrifuged at 1000 rpm for 5 minutes (Heraeus Biofuge primo R) from where the sediment containing the cells was removed and resuspended in RPMI culture medium preheated with 10% (v/v) sulfur dioxide dimethyl (DMSO, Sigma-Aldrich) and finally collected in a cryotube (Thermo Scientific) that was placed in a freezing container (Nalgene® Mr FrostyTM Cryo 1°C) with isopropanol, at -80°C. This procedure prevents abrupt and aggressive freezing of the cells, gradually lowering the temperature by 1 °C per minute. 24 hours after this gradual freezing, the cryotube was switched to liquid nitrogen, where it maintained its temperature.

Defrosting, in turn, was carried out by heating to 37°C in a gentle water bath then suspended by centrifugation at 1000 rpm for 5 minutes to remove the DMSO

cryopreservation agent. The supernatant was discarded and the pellet resuspended in 5 mL of fresh RPMI growth medium and finally placed in a 25 cm^3 culture flask and kept at 37°C and 5% CO2, where it was kept until use.

3 Poly-L-Lysine coated coverslips

Poly-L-lysine is responsible for increasing the adhesion of cells to the coverslips minimizing cell loss. The slides were treated with HCl 1 M submerged in a water bath at a temperature of 56˚C for 16 hours. Then, they were cooled to room temperature and washed 5 times with immersion in distilled water and 5 times with immersion in double distilled water. They were washed with 100% ethanol, dried and treated with 500µg/mL of poly-L-lysine (Sigma-Aldrich), under gentle rotational agitation (Digisystems Laboratory Instruments Inc.) for approximately 1 hour. Finally, another wash by immersion in distilled water and in 100% ethanol was performed. At the beginning of each experiment, the slides were again submerged in 70% ethanol and 100% ethanol sequentially, so that they were in aseptic conditions of use.

4 Evaluation of pyranoxanthone 2 activity

4.1 Determination of antimitotic activity

A total of $0.15x10^6$ NCI-H460 cells were seeded in a six-well plate allowing to attach for 24h to be adhered to the surface. Then, cells were treated either with 1.7 µM, 3.4 µM and 5.1µM of the compound pyranoxanthone 2 and 1 µM of Nocodazole (Sigma-Aldrich) as a positive control. The negative control was performed with untreated cells and cells treated with 5.1µM DMSO served as an additional control for the compound's solvent. The compound was left to act for 16 hours and then a cell morphology analysis was performed using phase contrast microscopy and the mitotic index (IM) was determined by counting the cells that showed morphology supposed to be in mitosis and cells that had supposed morphology of being in interphase. The count was established by scoring mitotic figures from ten random microscopic fields or from 2000 mitotic cells. The MI was calculated individually in each well indicative of each condition described above, using the following formula: MI (%) = (number of mitotic cells/Total number of cells) × 100.

4.2 Indirect immunofluorescence

NCI-H460 cells growing on poly-L-lysine-coated coverslips were treated with 1.7µM of pyranoxanthone 2 and controls with untreated cells and 1.7µM of DMSO for additional control of the compound's solvent for 16 hours. Then, cells were fixed with fresh 2% (w/v) paraformaldehyde (Sigma-Aldrich) in PBS for 12 minutes, washed 3 times with PBS for 5 minutes and permeabilized to 0.5% (v/v) Triton X-100 diluted in PBS for 7 minutes. Then the cells were then washed 3 times with PBS for 5 minutes each wash, and after wash, cells were blocked with blocking solution of 10% FBS in PBST (0.05% Tween-20 in PBS) for 30 min at room temperature, followed by 1 hour incubation with primary antibodies diluted in 5% FBS in PBST. The following primary antibodies were used: human anti-CREST (1:4000, gift from E. Bronze-da-Rocha, University of Porto, Portugal); mouse anti-α-tubulin (1:2500, Sigma-Aldrich) mouse anti-BubR1 (1:200, Milipore Chemicon), mouse anti-Mad2L1 (1:200, Sigma-Aldrich). After washing in PBST to three times, cells were incubated for 1 hour with Alexa Fluor 488 and 568 conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA), diluted at 1:1500. Two more washes were performed with PBST and one last wash with PBS. DNA was stained with 2µg/ml 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) diluted in Vectashield mounting medium (Vector, H-1000, Burlingame, CA, USA) that was placed on the blades before mounting with the coverslips. After drying, the coverslips were sealed with varnish and stored at 4˚C.

4.3 Functional assays for kinetochore-microtubule attachments

4.3.1 Cold treatment assay

To assess whether kinetochore-microtubule attachments in pyranoxanthone 2-treated cells were cold-stable, cells in culture medium were subjected at 4°C for 5 minutes, as well as the respective controls. These cells were immediately processed for immunofluorescence using the follows antibodies: human anti-CREST (1:4000, gift from E. Bronze-da-Rocha, University of Porto, Portugal); mouse anti-α-tubulin (1:2500, Sigma-Aldrich). At the end of the immunofluorescence, with the acquisition of the images, the number of kinetochores linked to microtubules and the number of free

kinetochores of 5 cells chosen at random in different fields of each experimental condition were counted, generating a referring average in view of the results obtained.

4.3.2 MG-132 proteasome inhibitor assay

To assess the ability of chromosomes to congress at equatorial zone, cells were divided into two groups under the same experimental conditions, where in one group the addition of 10 µM of the proteasome inhibitor MG-132 (Sigma- Aldrich) for 1 hour in order to stop the cells at the metaphase-anaphase limit, and in the other group they were maintained without the addition of MG-132. Then, immunofluorescence was performed with anti-α-tubulin mouse antibody in the dilution of (1:2500, Sigma-Aldrich) and with the result of the immunofluorescence, a count was performed in 10 random fields of each experimental condition of the number of metaphase cells with the aligned chromosomes and the number of metaphase cells with the misaligned chromosomes.

4.4. Live-cell imaging

For live-cell imaging experiments, $0.12x10^6$ NCI-H460 cells were seeded onto LabTek II chambered cover glass (Nunc, Penfield, NY, USA) containing RPMI, allowed to attach for 24 hours at 37 °C with 5% CO2. Then, cells were treated with 1.7 µM of pyranoxanthone 2 and images were captured at 10 min intervals up to 48 hours under differential interference contrast (DIC) optics, with a 63x 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 1.44, Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA). The number of cells arrested at mitosis or in cell death was scored, based on cellular morphology. Dead cells were classified into death in mitosis (DiM) or post-mitotic death (PMD) when death occurred during or following cell division, respectively.

4.5 Cell death

4.5.1 Terminal staining of dUTP with deoxynucleotidyltransferase (TUNEL) mediation

A total of $0.15x10^6$ of NCI-H460 cells were treated with 1.7 μM of compound pyranoxanthone 2 for 24 hours. After treatment, cells were immediately fixed in 4% paraformaldehyde (w/v) in PBS for 10 minutes, followed by PBS wash and permeabilization with 0.2% (v/v) Triton X-100 in PBS for 5 minutes. The TUNEL labeling was performed using DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA), according to the manufacturer's instructions. DNA was stained with 2 μg/ml DAPI in Vectashield mounting medium. The level of apoptosis was established by counting TUNEL-positive cells in a total of approximately 400 cells in 5 random fields under fluorescence microscope. The apoptotic index was calculated as the percentage of positively TUNEL-stained cells over the cells.

4.5.2 Flow cytometry - Annexin-V-FITC

Cells were treated as described for mitotic index determination. For cell cycle analysis, after 16 and 24 hours, cells were harvested, washed in PBS twice and fixed in cold 70% ethanol at 4˚C for at least 30 minutes. Then, cells were treated with 5 μg/ml of propidium iodide and 100 μg/ml of RNase in PBS for 30 minutes and analyzed in the flow cytometer. For apoptosis detection, cells were harvested and processed with the "Annexin V-FITC Apoptosis Detection Kit" (eBioscience, Vienna, Austria) according to manufacturer's instructions. A positive control, for cell death, with 1 μM DTT was included. Data was analyzed with BD Accuri TM C6 Plus software, version 1.0.27.1 (www.bdbiosciences.com). All flow cytometry analysis was carried out using a BD Accuri™ C6 Plus Flow cytometer (BD Biosciences, Qume Drive, San Jose, CA) and at least 20.000 events per sample were collected.

4.5.3 Colony formation assay

A total of 500 NCI-H460 cells were seeded in six-well plates, allowed to attach for 24h, and treated with 0.85 μM of pyranoxanthone 2, 2 nM of paclitaxel or with a combination of pyranoxanthone 2 and paclitaxel. Untreated and DMSO-treated cells were also included. Forty-eight hours later, cells were washed twice with PBS and

incubated in a drug-free RPMI medium for 8 days. The grown colonies were fixed for 5 minutes with 3.7% paraformaldehyde (w/v) in PBS and stained for 20 minutes with violet crystal (Merck) 0.05% (w/v) in distilled water. The number of colonies for each condition was counted in duplicate dishes from three independent experiments. The plating efficiency (PE) was calculated as the percentage of the number of grown colonies over the number of cells seeded in control. For each condition, the survival fraction was determined as the number of colonies over the number of cells seeded × 1/PE.

5 Statistical analysis

The data are presented as the mean ± standard deviation (SD) of two or three independent experiments and 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 (GraphPad software Inc., CA, USA). The level of statistical significance was established considering the probabilities of * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

6 Image acquisition and processing

For the initial phase with phase contrast microscopy, a Zeiss Primo Vert microscope and a Nikon TE 2000-U microscope with a 10x objective were used. The Nikon microscope used a DXM1200F digital camera, with Nikon ACT-1 software (Melville, NY). For experiments where image acquisition was performed using fluorescence, an Axio Observer Z.1 SD microscope (Carl Zeiss, Germany) was used, coupled to an AxioCam MR3, and with the Plan Apochromatic 63x/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 1.44 (http://rsb.info.nih.gov/ij/).

Chapter IV

Results

1 The pyranoxanthone 2 induces mitotic arrest of cancer cells

Antimitotic agents are capable of causing cell arrest in mitosis. To test the ability of compound pyranoxanthone 2 to interfere in mitosis, cells A375-C5, MCF-7 and NCI-H460 were treated with the value corresponding to once the value of GI50, twice the value of GI50 and three times the value of GI50 in each well, for 16 hours.

As a positive control, 1 μM of cells treated with nocodazole in a well was used. Nocodazole is an antimitotic agent already used in clinical practice that interferes with

As a positive control, 1 μM of cells treated with nocodazole in a well was used. Nocodazole is an antimitotic agent already used in clinical practice that interferes with the dynamics of microtubules, binding to β-tubulin and preventing the formation of one of the two inter-chain disulfide bonds, culminating in stopping the cell cycle in mitosis. As a negative control, a well of untreated cells was used, and for solvent control the value referring to three times the GI50 of each respective DMSO cell line in a well was added.

The analysis was performed under phase contrast microscopy, through the observation of cell morphology, where “rounded” cells are indicative of cells in mitosis. The mitotic arrest was noticeable at both concentrations in the cell lines A355-C5 (**Figure 16A**), MCF-7 (**Figure 16B**) and NCI-H460 (**Figure 16C**). It is markedly larger when compared to untreated control cells and looks similar to the positive control for cells treated with nocodazole.

The mitotic index (MI) was determined by counting the cells that showed morphology indicative of cells in mitosis and cells that by the morphology were supposed to be in interphase. The count was established by scoring mitotic figures from ten random microscopic fields or from 2000 mitotic cells. The IM was calculated individually in each well indicative of each condition described above, in cell lines A355-C5 (**Figure 16A'**), MCF-7 (**Figure 16B'**) and NCI-H460 (**Figure 16C'**) using the following formula: MI (%) = (number of mitotic cells / total number of cells) × 100. In A375-C5 the mitotic index was 37.63% ± 4.07 when treated with 1.3 μM of pyranoxanthone 2, of 41.83 % ± 0.47 when treated with 2.6 μM and 50.09% ± 13.40 when treated with 3.9 μM, being

considerably higher when compared to the negative control with untreated cells where the mitotic index was 17.48% ± 0.77 and with the solvent control using DMSO which was 17.62% ± 0.96, and less than the positive control with nocadozole which was 71.49% ± 5.49. In MCF-7 the mitotic index was 47.09% ± 0.69 when treated with 1.7 µM of pyranoxanthone 2, 53.03% ± 9.11 when treated with 3.4 µM and 43.83% ± 2.05 when treated with 5.1 µM, being considerably higher when compared to the negative control with untreated cells where the mitotic index was 5.22% ± 0.01 and with the solvent control using DMSO which was 6.19% ± 1, 38, and less than the positive control with nocadozole, which was 84.02% ± 1.48. In NCI-H460 the mitotic index was 52.13% ± 3.84 when treated with 1.7 µM of pyranoxanthone 2, 55.71% ± 5.95 when treated with 3.4 µM and 68.51% ± 11.27 when treated with 5.1 µM, being considerably higher when compared to the negative control with untreated cells where the mitotic index was 14.47% ± 2.64 and with the solvent control using DMSO which was 14.69% ± 3,72, and less than the positive control with nocadozole, which was 89.93% ± 0.75. Normally, the number of interphase cells is always greater than that of mitotic cells, this is because the duration of the interphase is abruptly greater than that of mitosis. After treatment with pyranoxanthone 2 the number of cells with morphology suggestive of mitosis was consistently similar to the number of interphase cells, suggesting the idea that pyranoxanthone 2 has antimitotic activity due to stopping in mitosis.

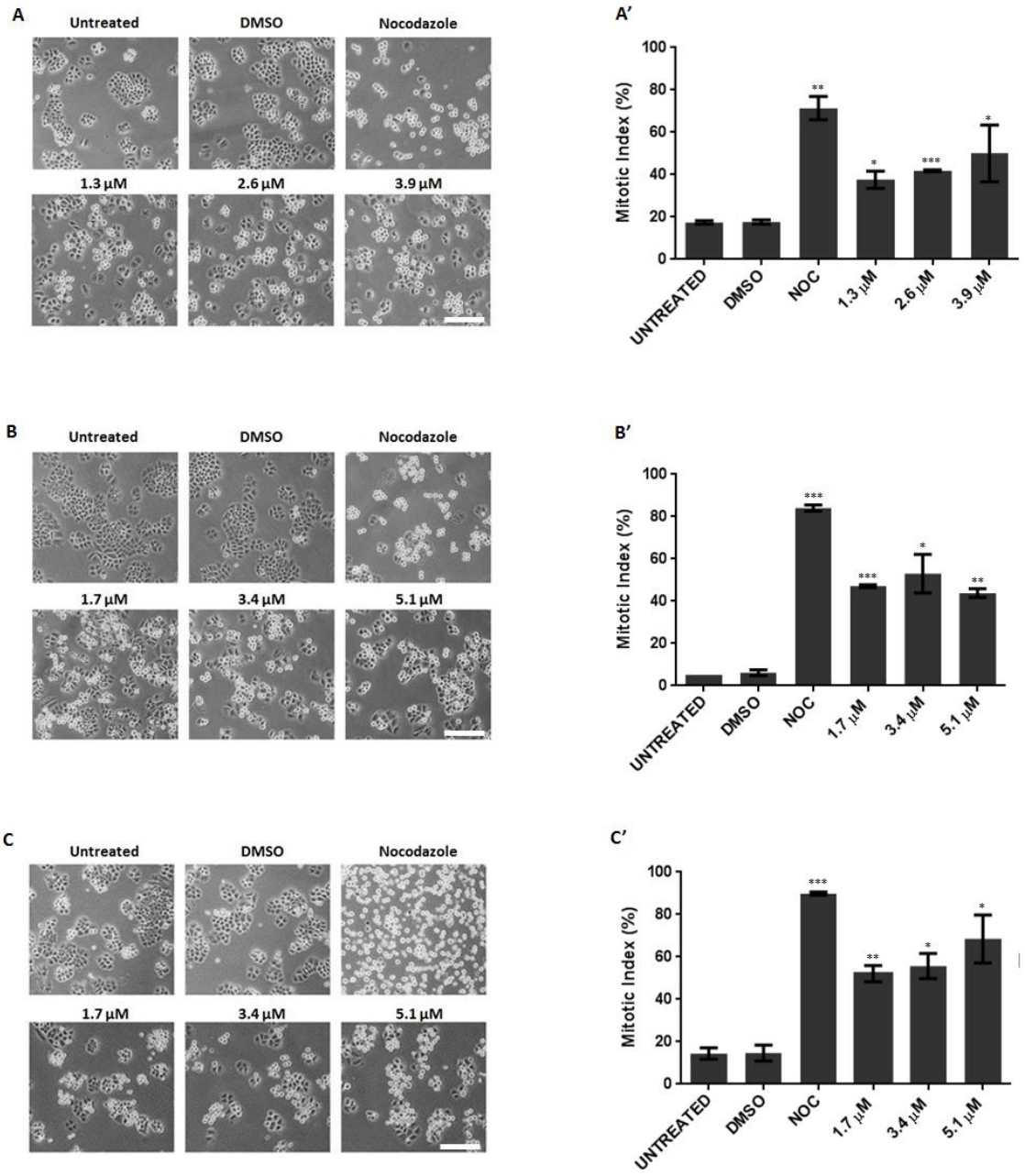

Figure 16 Cancer cells arrested in mitosis, in response to PX2 treatment. Left - Representative phase contrast microscopy images, after 16 hours treatment with PX2, at indicated concentrations, displaying accumulation of mitotic cells (rounded) in **(A)** A375-C5, **(B)** MCF-7 and **(C)** NCI-H460 cancer cell lines. Nocodazole was used as a positive control. DMSO was used as a compound solvent control. Bar, 10 µm. Right – Mitotic index graphs showing accumulation of mitotic cells in **(A')** A375-C5, **(B')** MCF-7 and **(C')** NCI-H460 cancer cells with statistical relevance of * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by unpaired t-test.

2 Treatment with pyranoxanthone 2 leads to chromosome congression defects

Once it was understood that the treatment with the compound pyranoxanthone 2 generated mitotic arrest that was sustained by the constitutive activation of the SAC, we performed a new immunofluorescence in order to understand the mechanism that led to its constitutive activation. The NCI-H460 cells were treated with 1.7 µM of pyranoxanthone 2, for 16 hours, and proceed to immunofluorescence, using an anti-α-tubulin antibody, for staining the spindle microtubules and DAPI staining to visualize the DNA. Due controls were also carried out, negative control with untreated cells and solvent control with 1.7 µM DMSO. In fluorescence microscopy, we observed that the untreated control cells exhibited a bipolar spindle with normal microtubule fibers (**Figure 17A**). In contrast, the cells treated with pyranoxanthone 2 showed an abnormal morphology of the mitotic spindle, with a misalignment of the chromosomes that goes against the previous finding that the morphology of the cells that were stopped in mitosis demonstrated a state similar to that of prometaphase. The number of cells that presented morphology suggestive of metaphase and the number of cells that presented morphology suggestive of prometaphase was then counted so that these data could be compared in order to analyze whether treatment with pyranoxanthone 2 leads to persistent chromosome misalignment phenotype (**Figure 17B**). From this count, a considerable number of cells demonstrated phenotype indicative of prometaphase with misalignment of chromosomes, representing 98.18% ± 7.80, compared to 67.46% ± 7.80 in untreated cells and 69.81% ± 7.99 in solvent control.

To confirm that that the misalignment phenotype persists, a new assay was carried out using MG-132 (**Figure 17C**), which is an enzyme that works as a proteasome inhibitor and that consequently prevents the dissociation and cleavage of cohesins, not allowing the advance of metaphase for anaphase. Two groups were separated under the same experimental conditions, where in one group the addition of 10 µM of the proteasome inhibitor MG-132 for 1 hour in order to stop the cells at the metaphase-anaphase limit, and in the other group they were maintained without the addition of MG-132. Then, immunofluorescence was performed with anti-α-tubulin mouse antibody and with the result of the immunofluorescence, a count was performed in 10 random fields of each experimental condition of the number of metaphase cells with the aligned

chromosomes and the number of metaphase cells with the misaligned chromosomes, and an average was made regarding the results obtained in two independent experiments. The results of this accounting showed that the characteristic persistent phenotype of cells with the chromosomes misaligned with 100% ± 0 of metaphase cells with the chromosomes misaligned both in the presence of MG-132 and in the absence. The negative control in relation to this count showed 33% ± 0.04 when in the presence of MG-132 compared to 72% ± 0.05 when in absence. The solvent control was faithful to the results obtained, demonstrating 30% ± 0.03 when in the presence of MG-132 compared to 77% ± 0.03 when in absence. These results meet the cell's inability to form or maintain a bipolar axis when treated with compound pyranoxanthone 2. The cell stopping in mitosis happens due to defects in the chromosome connections to the microtubules, which in this case are the result of a disturbance in the spindle arrangement of the microtubules. It is then concluded that the treatment with pyranoxanthone 2 in NCI-H460 could affect the morphology of the mitotic spindle by activating the SAC as a consequence and causing the cell to stop in mitosis.

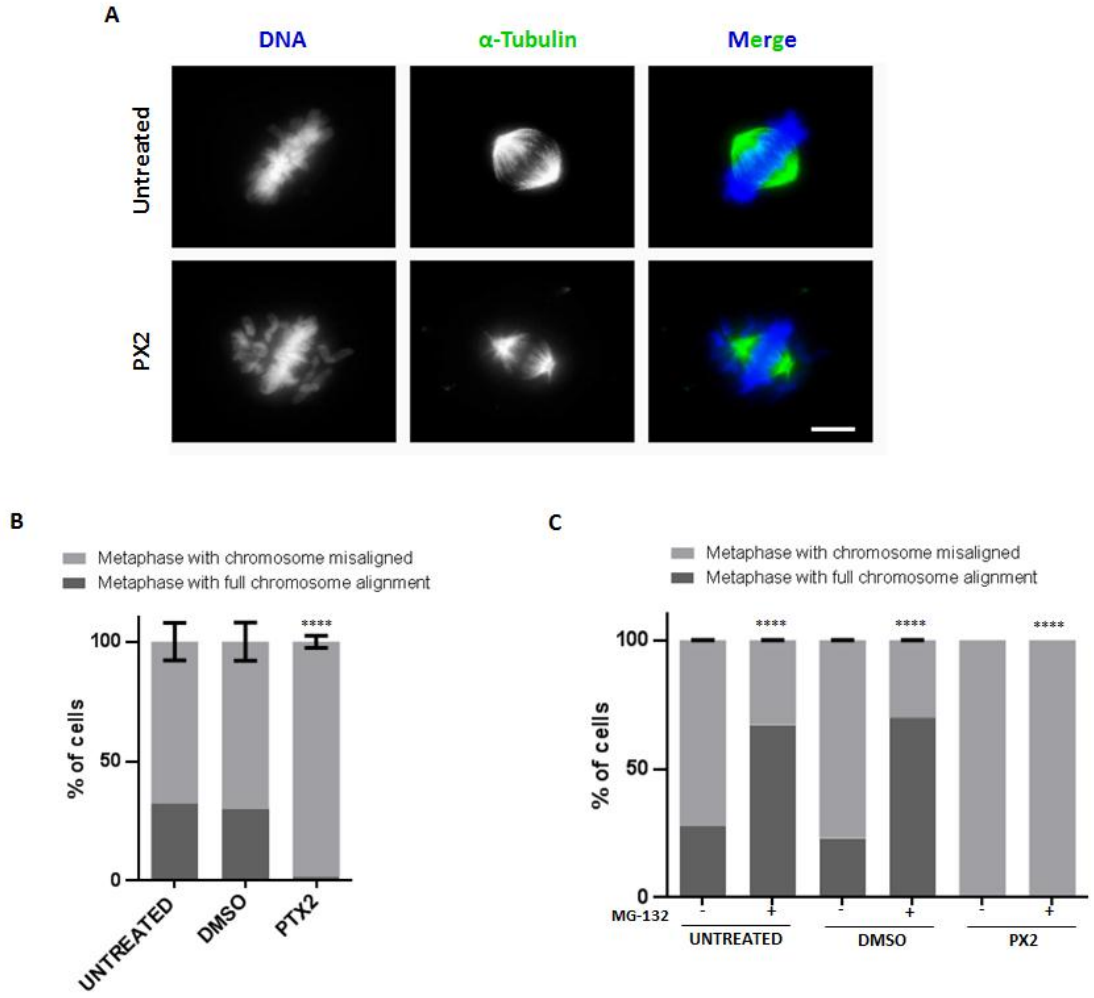

Figure 17 PX2 treatment results in chromosome misalignment phenotype. (A) Immunofluorescence images of a untreated cell (Top), in metaphase, with all chromosomes aligned at equatorial zone, and PX2-treated cell (Bottom), arrested in prometaphase-like state, showing many misaligned chromosomes. Microtubules (green) were stained with anti-α-tubulin antibody and DNA (blue) with DAPI. Bar, 5 µm. **(B)** Graphical representation of data shown in percentage cells of metaphase state or metaphase with misaligned/prometaphase with statistical relevance of (****) p <0.0001 by unpaired t-test. **(C)** Graphical representation of data shown in (A), in absence (-) or presence (+) of proteasome inhibitor MG-132 with statistical relevance of (****) p <0.0001 by two-way ANOVA with Tukey's multiple comparisons test.

3 Treatment with pyranoxanthone 2 interferes with kinetochore-microtubules attachments stability

For the congress of chromosomes to occur correctly and to be able to align themselves in the metaphase plate, it is necessary that stable and functional attachments occur between kinetochores and microtubules, for this reason it was investigated whether these attachments were stable through a cold test. treatment where cells in culture

medium in the wells treated with 1.7 µM of compound pyranoxanthone 2 or negative control with untreated cells respectively were incubated at 4 ° C for 5 minutes and immediately processed for immunofluorescence with anti-CREST anti-α-tubulin antibodies so that they were the robustness of the kinetochore-microtubule attachments and their ability to generate tension and conduct chromosome movement and alignment were tested. The temperature of 4°C induces a disassembly of kinetochore fibers that are unstable, due to this thermal shock (**Figure 18A**).At the end of the immunofluorescence, with the acquisition of the images, the number of kinetochores linked to microtubules and the number of free kinetochores of 5 cells chosen at random in different fields of each experimental condition were counted (**Figure 18B**).

The percentage of kinetochores attached with microtubules in cells treated with pyranoxanthone 2 was 19.52% ± 2.70 while in untreated cells it was 90.08% ± 3.25 (**Figure 18C**), suggesting that the connections between kinetochores and microtubules were unstable and was an important condition for the phenotype of chromosome misalignment presented by the cells and consequent stop in mitosis.

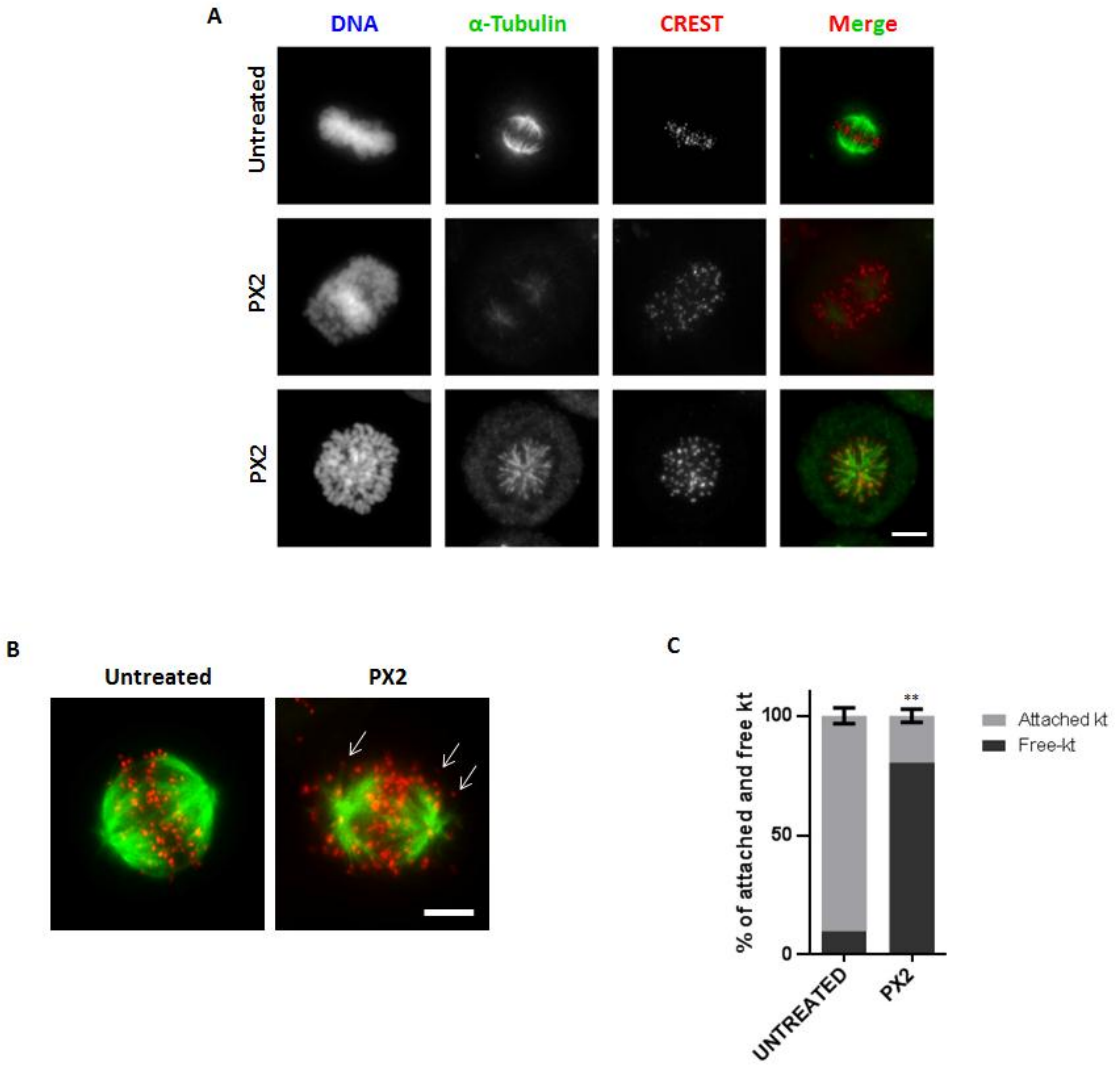

Figure 18 PX2 treatment generates loss of stability of kinetochore-microtubule attachments. (A) Representative immunofluorescence images after cold treatment assay, showing several unattached kinetochores (free CREST red spots) in cells with pyranoxanthone 2 treatment, whereas most kinetochores were attached (red spots with attached green fibers) in untreated cells. Microtubules (green) were stained with anti-α-tubulin antibody, kinetochores (red) with anti-CREST antibody, and DNA (blue) with DAPI. **(B)** Example of untreated cells (right) and treated with 1.7 µM of compound PX2 used for counting kinetochore-microtubules unattached (free CREST red spots pointed by the white arrows) Bar, 5 µm. **(C)** Quantification of cold-stable microtubules (as percentage of attached kinetochores per cell) after treatment with PX2 with statistical relevance of * p <0.05 by unpaired t-test.

4 Treatment with pyranoxanthone 2 elicits spindle assembly checkpoint activation

The increase in the number of mitotic cells when compared to the control shows an eventual blockage of the mitosis output, suggesting permanent activation of the spindle assembly checkpoint (SAC). The SAC is a checkpoint responsible for detecting incorrect connections of microtubules and kinetochores and can prevent progression

to anaphase whenever these errors are detected, keeping the cells in metaphase until all the chromosomes are properly aligned on the metaphase plate. Only from a signal that the chromosomes are aligned do SAC silencing occur with the shutdown of the proteins that form the Mitotic Checkpoint Complex (MCC), releasing Cdc20 to activate the anaphase promoter complex/cyclosome (APC/C) and enable the advance to anaphase. Mad2 proteins are an important part of MCC and are often used to mark microtubule connections to chromosomes. BubR1, in turn, is often used to mark the tension exerted between the sister kinetochores. When these proteins are co-located in the kinetochore, it means that the SAC is active. Therefore, to see if SAC was active in cells treated with the pyranoxanthone 2 compound, we performed an indirect immunofluorescence assay, using anti-Mad2 and anti-BubR1 antibodies, during 16 hours of treatment with 1.7 µM of pyranoxanthone 2, as well as in control negative with untreated cells. The analysis carried out by fluorescence microscopy revealed the presence of the Mad2 protein (**Figure 19A**) and the BubR1 protein (**Figure 19B**) in the kinetochores of the uncoupled chromosomes of the cells that were stopped in mitosis, which suggests activation of the SAC. In contrast, cells not treated with the compound had a kinetochore label on cells that were in prometaphase with chromosomes that were also not coupled, while in cells that metaphase alignment was completed, the staining by the antibodies of the proteins Mad2 and Bubr1 was not more visible in the kinetochore, demonstrating that the proteins had been removed from the kinetochore, generating the silencing of the SAC by interrupting the signaling that activated the SAC. The results described demonstrate that the SAC remained active in these cells that were stopped in mitosis after treatment with pyranoxanthone 2.

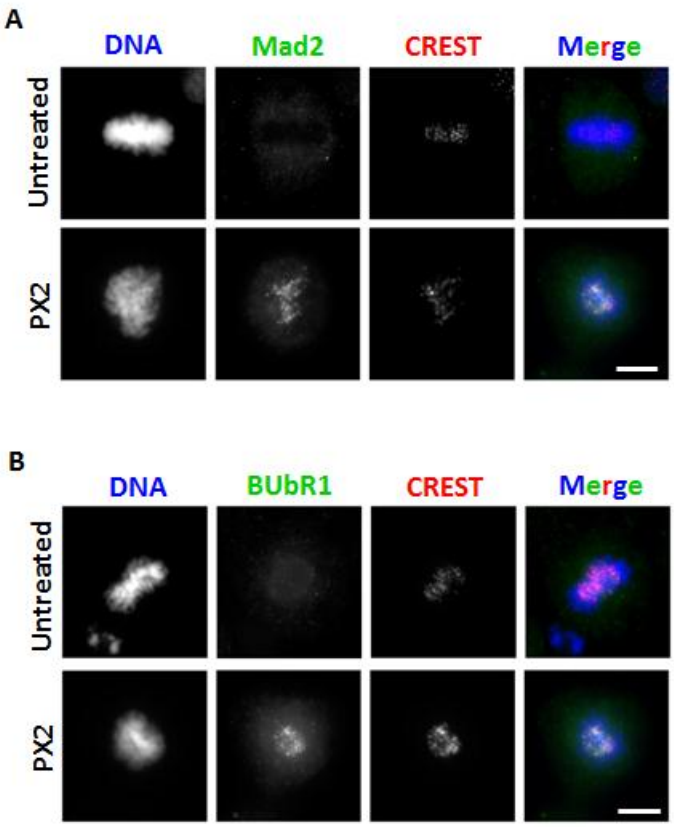

Figure 19 Treatment with PX2 triggers the spindle assembly checkpoint. Immunofluorescence staining using antibodies against **(A)** Mad2 (green), **(B)** BubR1 (green), CREST (red) and DAPI (blue) in untreated and PX2-treated cells, as indicated. In untreated cells, Mad2 and BubR1 is not located in kinetochores, consistent with their normal localization pattern. In PX2-treated cells (bottom panel), these proteins are present in all mitotic cells, indicating mitotic checkpoint activation. Bar, 5 µm.

5 Cell fates of cancer cells arrested in mitosis by the pyranoxanthone 2

In order to further understand the mechanistic underlying the cytotoxic activity of the pyranoxanthone 2, we analyzed the cell fate of mitosis-arrested cells by live-cell imaging using time lapse differential interference contrast (DIC) microscopy, over 48h time course. First, we found that pyranoxanthone 2-treated cells (n= 40) lasted in mitosis (from nuclear envelope breakdown to anaphase onset) 457.8 ± 264.2 min on average, more than 16-fold when compared to the duration of mitosis in untreated cells (28 ± 6.64 min) (**Figure 20** and **supplementary video S1** online at https://youtu.be/Lfu6tMx8fks and **supplementary video S2** online at https://youtu.be/P9DYSUjxiIM). Then, survival fate analysis of each mitotic cell

revealed that 92.5% of pyranoxanthone 2-treated cells died in mitosis, and 7.5% of cells underwent post-mitotic death (**Figure 20 and videos S1 and S2**).

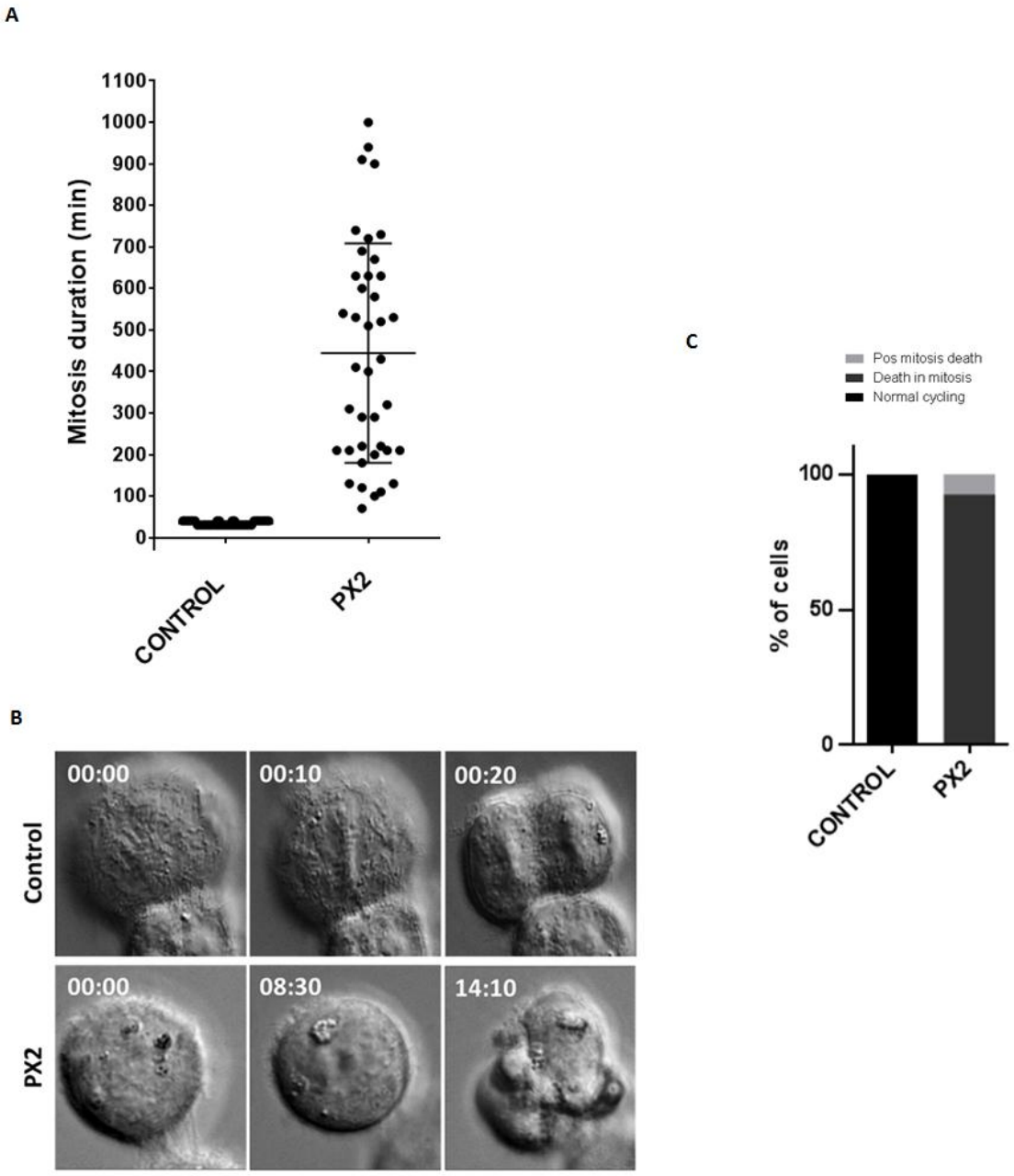

Figure 20 PX2 treatment promotes death in mitosis, after prolonged arrest. (A) Mitosis duration as determined by time-lapse microscopy, in untreated (control) and PX2-treated cells. Each spot represents one cell. **(B)** Representative time-lapse sequences of untreated and PX2-treated cells. Untreated cell undertakes mitosis for about 30 min (top) while PX2-treated cell (bottom) arrests in mitosis during several hours (457.8 ± 264.2 min) followed by death. Numbers indicate times in 00 h:00 min. Movies are available as Supplementary materials. **(C)** Quantification of cell fate after PX2 treatment. 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.

6 Pyranoxanthone 2 induces cell death after prolonged treatment

During the course of characterization of pyranoxanthone 2, it was found that prolonged exposure led to abnormal nuclear morphology, with micronucleation, a characteristic that suggests that the cells were dead, either as a result of prolonged mitotic arrest or due to failure in cell division. To validate that this frequent occurrence of cells showing this morphology was even indicative of dead cells, NCI-H460 cells were exposed for an extended period of 24 hours to treatment with pyranoxanthone 2, and then stained with DAPI, for DNA evaluation. Through this assay an increase in cells with abnormal nuclear morphology was verified, mainly composed of micronuclei (**Figure 21**).

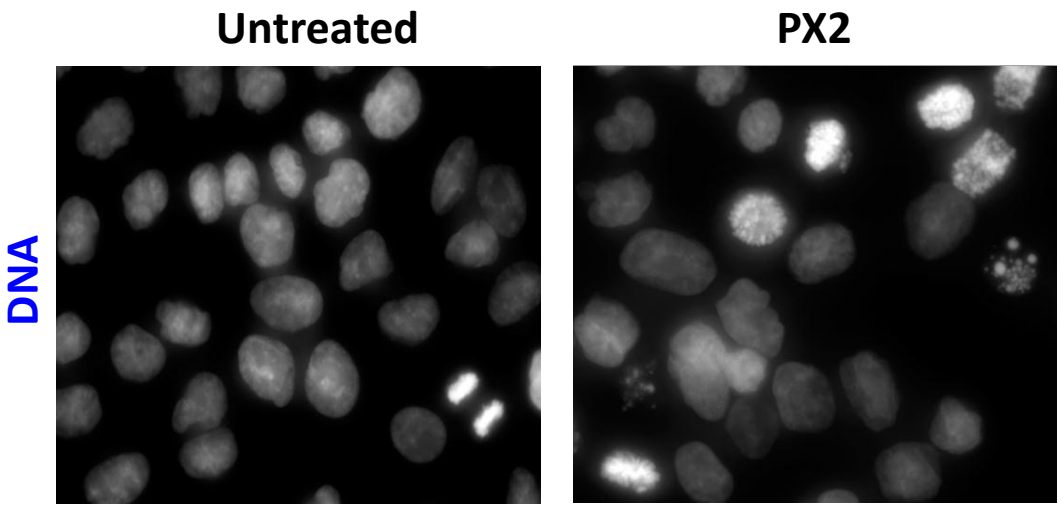

Figure 21 Representative death cells before treatment with PX2. On the right there is a representative field of control, with cells in their normal nuclear morphology, mostly interphasic. On the left the cells were treated with 1.7 µM of the compound pyranoxanthone 2 and many cells with a phenotype suggestive of chromosome misalignment can be observed, and some cells with abnormal nuclear morphology, with micronucleation, characteristic of dead cells. The DNA was stained using DAPI.

From then on the need for answers about the mechanism responsible for the cell death emerged, and to answer this question, NCI-H460 cells were treated with pyranoxanthone 2 for 24 hours and then analyzed using a TUNEL assay, so that it was possible to detect cell death by apoptosis. While the untreated cells used as a negative control exhibited normal, largely non-apoptotic morphology, without positive TUNEL

staining, cells treated with the pyranoxanthone 2 compound showed positive TUNEL micronucleus marking (**Figure 22A**). The level of apoptosis was established by counting TUNEL-positive cells on a total of approximately 400 cells in 5 random fields for each slide corresponding to an experimental point, from which the apoptotic index was calculated, which was the percentage of positively stained cells about total number of cells. The number of apoptotic cells among cells treated with pyranoxanthone 2 was 25.53% ± 1.97, while in untreated cells it was 0.60% ± 0.50 and in solvent control 0.27% ± 0, 12 (**Figure 22B**), suggesting that prolonged exposure to pyranoxanthone 2 leads to cell death, due to apoptosis. To confirm this finding, flow cytometry was performed using the "Annexin V-FITC apoptosis detection kit". Cells were treated with 1.7µM of compound pyranoxanthone 2 in 16 hours and 24 hours of treatment. In addition, the positive control with 1µM DTT and the negative control with untreated cells were included, as well as a well with 1.7 µM DMSO for additional control of the compound's solvent. Apoptosis was detected through analysis using a BD Accuri ™ C6 flow cytometer with the analysis of 20,000 events per sample and the results were determined using use of BD Accuri TM C6 Plus software, version 1.0.27.1. The percentage of apoptotic cells was 13.76% ± 2.24 in the negative control with untreated cells, 11.89% ± 3.49 in the solvent control, 28.49 ± 6.71 in the positive control using DTT, in the treatment for 16 hours with 1.7 µM of compound pyranoxanthone 2 was 13.98% ± 2.61 and in the treatment for 24 hours with 1.7 µM of compound pyranoxanthone 2 was 28.67% ± 3.52 (**Figure 22C**). The quantification of cells was deferred using Q quadrants, which were defined by Q1 = live (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) (**Figure 22D**).

These results support the theory that cell death due to apoptosis occurs when treated with pyranoxanthone 2, and demonstrates through a considerably higher percentage of apoptotic cells after 24 hours of treatment compared to the number of apoptotic cells after 16 hours of treatment, that the time is a crucial factor for apoptosis cell death.

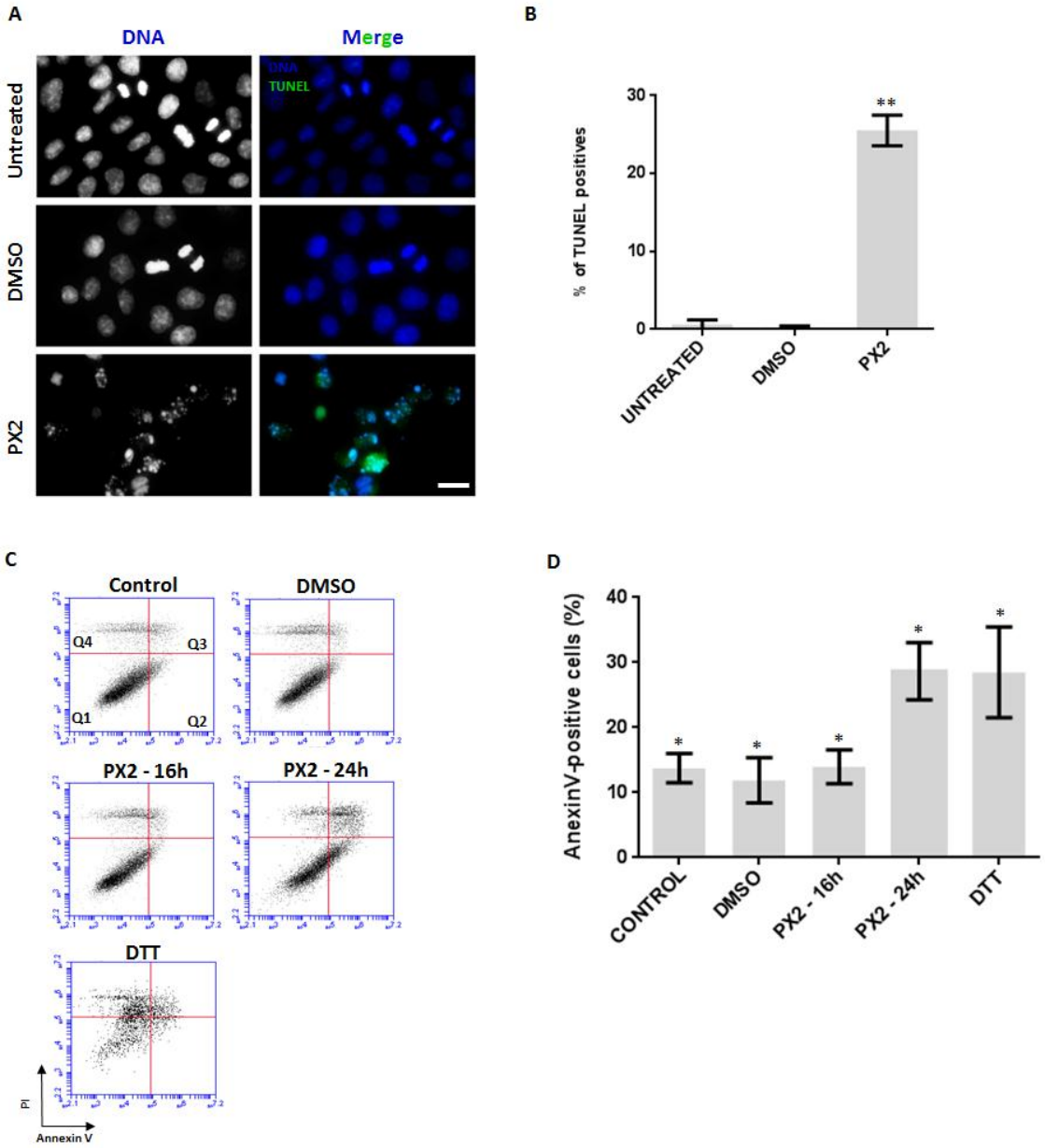

Figure 22 Prolonged exposure to PX2 leads to apoptotic cell death. (A) TUNEL staining showing accumulation of apoptotic cells (green) in cells treated with the compound for 48 h. DNA was counterstained with DAPI (blue). Bar, 5 µm. **(B)** Apoptotic index in control cells and upon 48 h compound treatment, expressed as a percentage of total cells with statistical relevance of ** $p < 0.01$ by unpaired t-test. **(C e D)** Flow cytometry analysis of apoptosis by Annexin V/PI co-staining, 16 and 24 hours after PX2 treatment. **(C)** Representative cytogram and (D) quantification of Annexin V-positive cells are shown with statistical relevance of * $p < 0.05$ by unpaired t-test. The quadrants Q were defined as Q1 = live (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).

7 Treatment with pyranoxanthone 2 enhances paclitaxel cytotoxicity

Taken into account the potent cytotoxic activity of pyranoxanthone 2 and given the limitations of current paclitaxel-based chemotherapy, we explored the potential of pyranoxanthone 2 to increase the sensitivity of cancer cells to clinically relevant concentrations of paclitaxel. To address this question, we performed a long-term proliferation assay. Cells were treated with 0.85 μM of pyranoxanthone 2 (almost 8-fold less GI_{50}), alone or in combination with 2 nM of paclitaxel, for 48h. Eight-days later, treatment with 0.85 μM of pyranoxanthone 2 had no effect on the ability of cancer cells to proliferate and form colonies, behaving as untreated cells (**Figure 23**). Interestingly, when pyranoxanthone 2 was added prior paclitaxel treatment, the inhibitory effect on cancer cell proliferation was significantly higher than that observed in cells treated with pyranoxanthone 2 or paclitaxel alone (**Figure 23**). This suggests that pyranoxanthone 2 treatment sensitizes cancer cells to cytotoxic effect of paclitaxel. Of note, the concentrations of both agents in combination regimens could be significantly lowered.

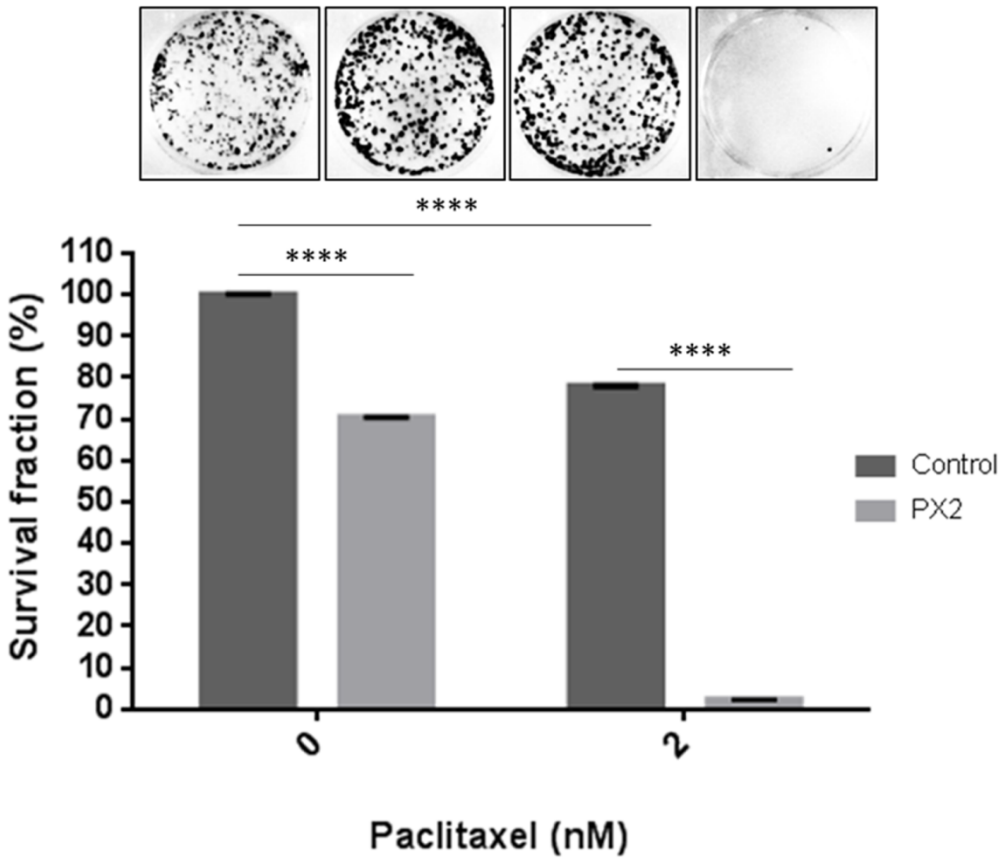

Figure 23 PX2 treatment enhances paclitaxel-mediated cytotoxicity. NCIH460 cells were treated with 0.85 µM of PX2 and/or paclitaxel (PTX) at 2 nM, for 48h. A 8-days colony-formation assay was performed. After washout, the surviving colonies were stained with crystal violet and a representative figure is shown for each condition. Quantification of data shown was performed and the results are the mean from two independent experiments, expressed as % of survival fraction. Statistical relevance of (****) $p < 0.0001$ by two-way ANOVA with Tukey's multiple comparisons test was used.

Chapter V

Discussion

Despite the widespread clinical use in oncology of anti-mitotic drugs that function as anti-microtubule agents, the associated toxicities, whether hematopoietic or neurological, are one of the major problems to be overcome, as well as intrinsic or acquired resistance (Rebucci and Michiels, 2013). This fact limits the efficacy of the medication and, consequently, causes a worse prognosis due to a limited or unspecified response to the medication used. Based on these flaws in the drugs used in current clinical practice, the need arose to explore new compounds that can avoid the mechanisms of resistance and nonspecific toxicity already mentioned.

In this field, several natural products have been evaluated as potential therapeutic agents. Increasing attention has been paid to xanthone derivatives, from natural sources and synthetic routes, to discover new drug candidates with, among others, anticancer properties (Klein-Júnior *et al.*, 2020). Xanthones are natural compounds found remotely in nature when compared to the high demand that would be required for use in clinical practice (Matsumoto *et al.*, 2005; Suksamrarn *et al.*, 2006). For this reason, and to overcome other limitations, they started to be synthesized from natural derivatives of modified xanthones to reach specific targets through the elaborate design of new xanthone analogues, with the help of modern medicinal techniques, including molecular modeling, by expanding the biological spectrum of these compounds (Genovese *et al.*, 2016).

In the present study, we unveiled the mechanism of the cytotoxic activity of the xanthone derivative pyranoxanthone 2. We demonstrated that pyranoxanthone 2 disturbs the mitotic spindle, which lead to unstable kinetochore-microtubules attachments and chromosome misalignment that activate SAC, thereby leading to prolonged mitotic arrest and, subsequently, to cell death.

We demonstrated in this present data the biological activity of this xanthone that emerges as a potent antimitotic agent, which acts through microtubules, interfering with microtubule-kinetochores attachments and preventing the alignment of chromosomes in the equatorial plane of the mitotic spindle in the metaphase plate, thus providing an effective anti-microtubule approach for therapeutic intervention in cancer. Anti-microtubule agents directly interfere with the dynamics of microtubules,

which are structures with a central role in the assembly of the mitotic spindle and in the subsequent alignment of chromosomes and DNA segregation in daughter cells (Masawang *et al.*, 2014; Teixeira *et al.*, 2014). Maintaining symmetrical and orderly appearance of the spindle microtubules during cell division requires highly accurate dynamics for this critical event during mitosis to occur correctly (Dumontet and Jordan, 2010).

Antimitotic agents in general are important pillars in anticancer therapy, because cancer cells have an increased potential for cell proliferation making the events and structures involved in this process important therapeutic targets (Gascoigne and Taylor, 2008; Shi, Orth and Mitchison, 2008). In the present study, antimitotic activity was perceived by calculating the mitotic index, which was determined by counting the cells that showed morphology indicative of cells in mitosis and cells that by the morphology were supposed to be in interphase. In A375-C5 cell line (melanoma cancer) the mitotic index was 37.63% ± 4.07 when treated with 1.3 µM of pyranoxanthone 2, of 41.83% ± 0.47 when treated with 2.6 µM and 50.09% ± 13.40 when treated with 3.9 µM, being considerably higher when compared to the negative control with untreated cells where the mitotic index was 17.48% ± 0.77 and with the solvent control using DMSO which was 17.62% ± 0.96, and less than the positive control with nocadozole which was 71.49% ± 5.49. In MCF-7 cell line (breast cancer) the mitotic index was 47.09% ± 0.69 when treated with 1.7 µM of pyranoxanthone 2, 53.03% ± 9.11 when treated with 3.4 µM and 43.83% ± 2.5 when treated with 5.1 µM, being considerably higher when compared to the negative control with untreated cells where the mitotic index was 5.22% ± 0.01 and with the solvent control using DMSO which was 6.19% ± 1, 38, and less than the positive control with nocadozole, which was 84.02% ± 1.48. In NCI-H460 cell line (lung cancer) the mitotic index was 52.13% ± 3.84 when treated with 1.7 µM of pyranoxanthone 2, 55.71% ± 5.95 when treated with 3.4 µM and 68.51% ± 11.27 when treated with 5.1 µM, being considerably higher when compared to the negative control with untreated cells where the mitotic index was 14.47% ± 2.64 and with the solvent control using DMSO which was 14.69% ± 3.72, and less than the positive control with nocadozole, which was 89.93% ± 0.75. The number of interphase cells, which should be considerably higher than that of mitotic cells due

to the interphase duration time compared to mitosis, remained similar to the number of mitotic cells, indicating the arrest and accumulation of cells in mitosis. This phase microscopy assay served as a principle that pyranoxanthone 2 has antimitotic activity through the analysis of suggestive morphology of cells in mitosis and cells in interphase, however the confirmation came through flow cytometry in order to certify the cell fate and a immunofluorescence so that it was possible to analyze through DNA immunofluorescence microscopy.

Through a new immunofluorescence using anti-α-tubulin and DNA dialing DAPI, we see that the treatment with the pyranoxanthone 2 cell line of lung cancer NCI-H460 created dominant phenotype misaligned chromosomes and consequently stop mitosis due to its interaction with the microtubules of the mitotic spindle, consistent with other antimitotic agents already used in clinical practice. Paclitaxel is an example of antimitotic acting as anti-microtubule (Jones *et al.*, 2005). This phenotype reinforces an increased antimitotic activity with considerable number of cells demonstrating phenotype indicative of prometaphase with misalignment of chromosomes, representing 98.18% ± 7.80, compared to 67.46% ± 7.80 in untreated cells as a result of the metaphase stop resulting from this misalignment of the chromosomes on the plate metaphase and that prevents progress to anaphase. This impediment of metaphase advance in anaphase is the result of a series of signals that are sent by the cell for non-alignment account of chromosomes that these failures are remedied or eliciting a cell death mechanism configured (Musacchio and Salmon, 2007).

The mitotic arrest caused by pyranoxanthone 2 also demonstrates a critical role for SAC as demonstrated in the results. The proteins that make up the MCC are co-located in the coupled kinetochore when the SAC is active, and this characteristic was maintained after treatment with pyranoxanthone 2. This finding was demonstrated by marking the proteins BUBR1 and MAD2, which are a key component of the MCC and are located in the kinetochore when SAC is active.

SAC is constitutively active in the presence of this chromosome misalignment phenotype and ends up keeping APC/C inactive and preventing progress to anaphase. The molecular pathway of the SAC is composed of a set of proteins that have a variety

of functions ranging from the detection of errors in the attachment between microtubules and kinetochores to the generation of the signal that inhibits the progression of mitosis until the errors and the SAC is silenced. This whole set of proteins is co-located in unbound kinetochores, and that the downstream target is APC/C, an ubiquitin E3 ligase that acts in association with several proteolytic degradation proteins, including mitotic cyclins that will allow the progress towards anaphase (Santaguida and Musacchio, 2009).

As already mentioned, with the SAC active, the APC/C is inactive, this triggers a non-degradation of the securin, which is associated with the separase and prevents the cleavage of cohesins that allow the advance to anaphase, as well as triggering a non-degradation of the cyclin B1 which prevents cells from leaving mitosis. This set of events, mitotic block and sustained activation of SAC create conditions that trigger an accumulation of apoptotic signal that ends up sensitizing tumor cells to cell death due to apoptosis. Therefore, cells exposed to antimitotic agents have their ability to activate apoptosis, varying according to the time of stop (Barbosa *et al.*, 2011).

Therefore, different mechanisms keep SAC active due to the non-alignment of chromosomes in the metaphase plate, and thus prevent its silencing. The use of pyranoxanthone 2 xanthone ends up allowing apoptotic signals to accumulate and irreversibly mark the cell for death.

Once it was concluded that this stop in mitosis was a consequence of a change in the cell phenotype, we decided to further analyze the dynamics of microtubules and kinetochore-microtubule connections, and we came to the conclusion that after treatment with pyranoxanthone 2 these connections became less stable, which justifies the non-coupling in certain points of the cells. The percentage of kinetochores attached with microtubules in cells treated with pyranoxanthone 2 was 19.52% ± 2.70 while in untreated cells it was 90.08% ± 3.25, suggesting that the connections between kinetochores and microtubules were unstable and was an important condition for the phenotype of chromosome misalignment presented by the cells and consequent stop in mitosis.

This goes against, for example, treatment with Paclitaxel, which affects the dynamics of microtubules and kinetochore-microtubule bonds, inducing non-silencing of the SAC, and preventing the cell from leaving mitosis (Jones *et al.*, 2005).

Observing our experimental findings that demonstrated cells with morphology indicative of cell death, our study was directed to quantify and qualify this cell death. From there, the results demonstrated that this would in fact lead to a considerable increase in cells killed by apoptosis when treated with pyranoxanthone 2. Faced with the stop in mitosis of the cells and without being able to subvert this phenotype, the cells ended up going into apoptosis.

This massive death as a result of stopping mitosis is the main focus of anticancer therapy using antimitotics (Dalton *et al.*, 2007; Lei and Erikson, 2008). For our study we performed an assay with TUNEL staining where the number of apoptotic cells among cells treated with pyranoxanthone 2 was 25.53% ± 1.97, while in untreated cells it was 0.60% ± 0.50, which demonstrated a very significant increase in apoptotic cells when cells were treated with compost.

A flow cytometry performed using the "Annexin V-FITC apoptosis detection kit" was also performed with the intention of demonstrating the number of apoptotic cells, as well as the time it would take until these cells entered apoptosis. The results demonstrated the percentage of apoptotic cells was 13.76% ± 2.24 in untreated cells, 13.98% ± 2.61 in the treatment for 16 hours with 1.7 µM of compound pyranoxanthone 2 and 28.67% ± 3.52 in the treatment for 24 hours with 1.7 µM of compound pyranoxanthone 2, demonstrating that the time of action of the drug would also be decisive to increase the number of apoptotic cells.

Interestingly, when we tested nanomolar concentrations of pyranoxanthone 2 in co-treatment with clinically relevant concentrations of paclitaxel, in a long-term colony formation assay, we found an enhancement of the anti-proliferative activity. This finding is probably due: (i) to the mitotic arrest induced by pyranoxanthone 2 treatment which delays cell cycle progression giving time to paclitaxel in order to exert their its cytotoxic activity; and/or (ii) to a potentiation of chromosome segregation errors resulting from the additive effect of pyranoxanthone 2 and paclitaxel. Indeed,

both pyranoxanthone 2 and paclitaxel at nanomolar concentrations (<10 nM) promote missegregated chromosomes and aneuploidy (Zasadil *et al.*, 2014).

Importantly, enhancing chromosome missegregation and aneuploidy was previously reported as a possible strategy to sensitize cancer cells to paclitaxel and docetaxel (Janssen, Kops and Medema, 2011; Reck *et al.*, 2014; Maia *et al.*, 2015).

Moreover, lowering the concentrations of both paclitaxel and pyranoxanthone 2 is expected to minimize cytotoxicity and side effects. Xanthone derivatives have also exhibited therapeutic potential in combinatorial therapeutic modalities. For instance, 5,6-dimethylxanthone-4-acetic acid (DMXAA) exhibited either alone or in combination in vitro and in vivo anticancer activity in non-small cell lung cancer by the regulation of proteins involved in signaling pathways, such as cell cycle progression and apoptosis (Liu *et al.*, 2017; Ribeiro *et al.*, 2019). Although DMXAA alone did not show a striking anti-tumor activity in patients, pre-clinical results showed that a co-administration of DMXAA with other drugs has an increase in anti-tumor activity, through activation of immune system by TNF-α induction (Zhou, Yao and Joshi, 2002). In fact, co-treatment regimens constitute an appealing strategy to kill cancer cells more effectively than individual treatments, namely with chemotherapeutics (Henriques *et al.*, 2019).

Chapter VI

Conclusion

Pyranoxanthone 2 synthesized from prior knowledge that xanthone derivatives work as a potent antitumor agent and appears as a potent antimitotic agent that can serve as a starting point for new candidates for anticancer drugs that act through microtubules causing stop in mitosis and consequently cell death. The mitotic arrest caused by pyraxanthone 2 demonstrates a critical role for SAC, as demonstrated in the results of the markings with the BUBR1 and MAD2 proteins. The proteins that make up the MCC are co-located in the coupled kinetochore when the SAC is active, and this characteristic was maintained constitutively after treatment with pyranoxanthone 2, leaving the SAC constantly active (**Figure 24**). It was also concluded that the stop in mitosis is a consequence of a change in the cellular phenotype related to the dynamics of the microtubules and the kinetochore-microtubule attachments that became unstable after the treatment, causing the non-attachment in certain points of the cells. The results showed a considerable increase in dead cells when treated with pyranoxanthone 2, because in the face of arrest in mitosis, and without being able to subvert this phenotype, the cells ended up going into apoptosis (**Figure 24**). Tests carried out to analyze the combinatorial action with low doses of paclitaxel demonstrated that the synergistic action between the drugs is positive, leading to an increase in cell death. In summary, the present data elucidate the mechanics underlying the xanthone derivative pyranoxanthone 2, identifying it as a potent antimitotic agent with promising potential as an anticancer drug, alone or in combinatorial regimens. Future studies are needed to further explore the anticancer potential of pyranoxanthone 2, elucidating, for example, the behavior in non-tumor cells. In vivo trials will also be able to deepen and bring new perspectives on how the drug behaves in a tumor environment and its adjacent mechanisms, in addition to supervising its toxicity in healthy tissues, adverse effects and resistance mechanisms.

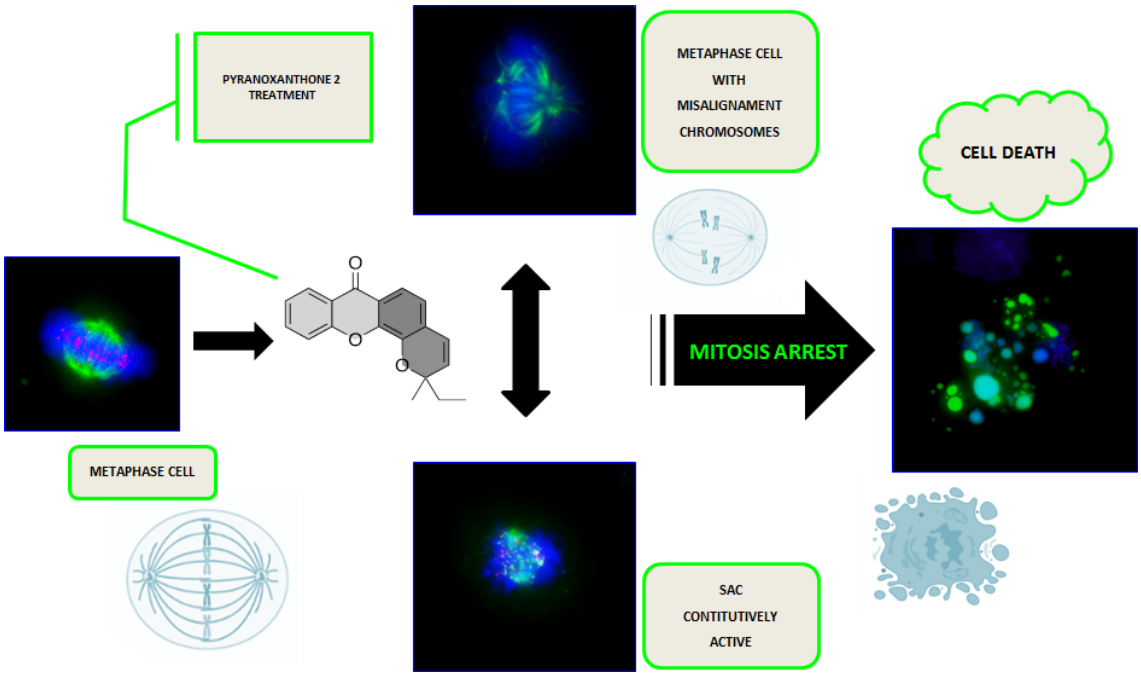

Figure 24 Mechanism of action of PX2. Treatment with PX2 causes the cell arrest in mitosis due to a phenotype of misalignment of chromosomes in the mitotic spindle, which ends up leading to a constitutive activation of the SAC and preventing the advance of metaphase to anaphase. This prolonged stoppage of cells in mitosis ends up causing massive cell death due to apoptosis.

Chapter VII

References

Abend, M. (2003) 'Reasons to reconsider the significance of apoptosis for cancer therapy', *International Journal of Radiation Biology*. doi: 10.1080/09553000310001632958.

Acquaviva, C. *et al.* (2004) 'The anaphase promoting complex/cyclosome is recruited to centromeres by the spindle assembly checkpoint', *Nature Cell Biology*. doi: 10.1038/ncb1167.

Adams, J. M. and Cory, S. (2007) 'The Bcl-2 apoptotic switch in cancer development and therapy', *Oncogene*. doi: 10.1038/sj.onc.1210220.

Adams, R. R. *et al.* (2001) 'Essential roles of Drosophila inner centromere protein (INCENP) and aurora B in histone H3 phosphorylation, metaphase chromosome alignment, kinetochore disjunction, and chromosome segregation', *Journal of Cell Biology*. doi: 10.1083/jcb.153.4.865.

Ahonen, L. J. *et al.* (2005) 'Polo-like kinase 1 creates the tension-sensing 3F3/2 phosphoepitope and modulates the association of spindle-checkpoint proteins at kinetochores', *Current Biology*. doi: 10.1016/j.cub.2005.05.026.

Akao, Y. *et al.* (2008) 'Anti-cancer effects of xanthones from pericarps of mangosteen', *International Journal of Molecular Sciences*. doi: 10.3390/ijms9030355.

Akhmanova, A. and Steinmetz, M. O. (2008) 'Tracking the ends: A dynamic protein network controls the fate of microtubule tips', *Nature Reviews Molecular Cell Biology*. doi: 10.1038/nrm2369.

Akhmanova, A. and Steinmetz, M. O. (2015) 'Control of microtubule organization and dynamics: Two ends in the limelight', *Nature Reviews Molecular Cell Biology*. doi: 10.1038/nrm4084.

Alimandi, M. *et al.* (1997) 'Epidermal growth factor and betacellulin mediate signal transduction through co-expressed ErbB2 and ErbB3 receptors', *EMBO Journal*. doi: 10.1093/emboj/16.18.5608.

Allen, C. and Borisy, G. G. (1974) 'Structural polarity and directional growth of

microtubules of Chlamydomonas flagella', *Journal of Molecular Biology*. doi: 10.1016/0022-2836(74)90381-7.

Amit, I. *et al.* (2007) 'A module of negative feedback regulators defines growth factor signaling', *Nature Genetics*. doi: 10.1038/ng1987.

Anand, S., Penrhyn-Lowe, S. and Venkitaraman, A. R. (2003) 'AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol', *Cancer Cell*. doi: 10.1016/S1535-6108(02)00235-0.

Andrésson, T. and Ruderman, J. V. (1998) 'The kinase Eg2 is a component of the Xenopus oocyte progesterone-activated signaling pathway', *EMBO Journal*. doi: 10.1093/emboj/17.19.5627.

De Antoni, A. *et al.* (2005) 'The Mad1/Mad2 complex as a template for Mad2 activation in the spindle assembly checkpoint', *Current Biology*. doi: 10.1016/j.cub.2005.01.038.

Arellano, M. and Moreno, S. (1997) 'Regulation of CDK/cyclin complexes during the cell cycle', *International Journal of Biochemistry and Cell Biology*. doi: 10.1016/S1357-2725(96)00178-1.

Barbosa, J. *et al.* (2011) 'The spindle assembly checkpoint: Perspectives in tumorigenesis and cancer therapy', *Frontiers in Biology*. doi: 10.1007/s11515-011-1122-x.

Barnard, J. A., Lyons, R. M. and Moses, H. L. (1990) 'The cell biology of transforming growth factor β', *BBA - Reviews on Cancer*. doi: 10.1016/0304-419X(90)90013-Q.

Barr, A. R. and Gergely, F. (2007) 'Aurora-A: The maker and breaker of spindle poles', *Journal of Cell Science*. doi: 10.1242/jcs.013136.

Bates, D. and Eastman, A. (2017) 'Microtubule destabilising agents: far more than just antimitotic anticancer drugs', *British Journal of Clinical Pharmacology*. doi: 10.1111/bcp.13126.

Bharadwaj, R. and Yu, H. (2004) 'The spindle checkpoint, aneuploidy, and cancer',

Oncogene. doi: 10.1038/sj.onc.1207374.

Bhowmick, N. A., Neilson, E. G. and Moses, H. L. (2004) 'Stromal fibroblasts in cancer initiation and progression', *Nature*. doi: 10.1038/nature03096.

Bischoff, J. R. *et al.* (1998) 'A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers', *EMBO Journal*. doi: 10.1093/emboj/17.11.3052.

Bischoff, J. R. and Plowman, G. D. (1999) 'The Aurora/Ipl1p kinase family: Regulators of chromosome segregation and cytokinesis', *Trends in Cell Biology*. doi: 10.1016/S0962-8924(99)01658-X.

Bishop, J. F. *et al.* (1999) 'Initial paclitaxel improves outcome compared with CMFP combination chemotherapy as front-line therapy in untreated metastatic breast cancer', *Journal of Clinical Oncology*. doi: 10.1200/JCO.1999.17.8.2355.

Blasco, M. A. (2005) 'Mice with bad ends: Mouse models for the study of telomeres and telomerase in cancer and aging', *EMBO Journal*. doi: 10.1038/sj.emboj.7600598.

Bray, F. *et al.* (2018) 'Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries', *CA: A Cancer Journal for Clinicians*. doi: 10.3322/caac.21492.

Burkhart, D. L. and Sage, J. (2008) 'Cellular mechanisms of tumour suppression by the retinoblastoma gene', *Nature Reviews Cancer*. doi: 10.1038/nrc2399.

Burstein, H. J. *et al.* (2003) 'Trastuzumab and vinorelbine as first-line therapy for HER2-overexpressing metastatic breast cancer: Multicenter phase II trial with clinical outcomes, analysis of serum tumor markers as predictive factors, and cardiac surveillance algorithm', *Journal of Clinical Oncology*. doi: 10.1200/JCO.2003.02.018.

Burton, J. L. and Solomon, M. J. (2007) 'Mad3p, a pseudosubstrate inhibitor of APCCdc20 in the spindle assembly checkpoint', *Genes and Development*. doi: 10.1101/gad.1511107.

Cabrita, M. A. and Christofori, G. (2008) 'Sprouty proteins, masterminds of receptor

tyrosine kinase signaling', *Angiogenesis*. doi: 10.1007/s10456-008-9089-1.

Camacho, E. *et al.* (2006) 'Analysis of Aurora-A and hMPS1 mitotic kinases in mantle cell lymphoma', *International Journal of Cancer*. doi: 10.1002/ijc.21370.

Cantley, L. C. and Neel, B. G. (1999) 'New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.96.8.4240.

Carmeliet, P. (2005) 'Angiogenesis in life, disease and medicine', *Nature*. doi: 10.1038/nature04478.

Carmena, M. and Earnshaw, W. C. (2003) 'The cellular geography of Aurora kinases', *Nature Reviews Molecular Cell Biology*. doi: 10.1038/nrm1245.

Cavallaro, U. and Christofori, G. (2004) 'Cell adhesion and signalling by cadherins and Ig-CAMs in cancer', *Nature Reviews Cancer*. doi: 10.1038/nrc1276.

Chan, C. S. M. and Botstein, D. (1993) 'Isolation and characterization of chromosome-gain and increase-in-ploidy mutants in yeast', *Genetics*.

Chang, H. F., Wu, C. H. and Yang, L. L. (2013) 'Antitumour and free radical scavenging effects of γ-mangostin isolated from Garcinia mangostana pericarps against hepatocellular carcinoma cell', *Journal of Pharmacy and Pharmacology*. doi: 10.1111/jphp.12111.

Cheeseman, I. M. and Desai, A. (2008) 'Molecular architecture of the kinetochore-microtubule interface', *Nature Reviews Molecular Cell Biology*. doi: 10.1038/nrm2310.

Chen, R. H. (2002) 'BubR1 is essential for kinetochore localization of other spindle checkpoint proteins and its phosphorylation requires Mad1', *Journal of Cell Biology*. doi: 10.1083/jcb.200204048.

Cheng, H., Huang, H. and Huang, G. (2018) 'Synthesis and antitumor activity of epothilone B', *European Journal of Medicinal Chemistry*. doi: 10.1016/j.ejmech.2018.08.055.

Ching, L. M. *et al.* (1991) 'Induction of natural killer activity by Xanthenone analogues of flavone acetic acid: Relation with antitumour activity', *European Journal of Cancer and Clinical Oncology*. doi: 10.1016/0277-5379(91)90067-N.

Chng, W. J. *et al.* (2008) 'The centrosome index is a powerful prognostic marker in myeloma and identifies a cohort of patients that might benefit from aurora kinase inhibition', *Blood*. doi: 10.1182/blood-2007-06-097774.

Clarke, D. J. and Giménez-Abián, J. F. (2000) 'Checkpoints controlling mitosis', *BioEssays*. doi: 10.1002/(SICI)1521-1878(200004)22:4<351::AID-BIES5>3.0.CO;2-W.

Cohen, S. J. *et al.* (2008) 'Fibroblast activation protein and its relationship to clinical outcome in pancreatic adenocarcinoma', *Pancreas*. doi: 10.1097/MPA.0b013e31816618ce.

Collado, M. and Serrano, M. (2010) 'Senescence in tumours: Evidence from mice and humans', *Nature Reviews Cancer*. doi: 10.1038/nrc2772.

Conduit, P. T., Wainman, A. and Raff, J. W. (2015) 'Centrosome function and assembly in animal cells', *Nature Reviews Molecular Cell Biology*. doi: 10.1038/nrm4062.

Crosio, C. *et al.* (2002) 'Mitotic Phosphorylation of Histone H3: Spatio-Temporal Regulation by Mammalian Aurora Kinases', *Molecular and Cellular Biology*. doi: 10.1128/mcb.22.3.874-885.2002.

Dacatur, A. L. and Portnoy, D. A. (2000) 'A PEST-like sequence in listeriolysin O essential for Listeria monocytogenes pathogenicity', *Science*. doi: 10.1126/science.290.5493.992.

Dalton, W. B. *et al.* (2007) 'Human cancer cells commonly acquire DNA damage during mitotic arrest', *Cancer Research*. doi: 10.1158/0008-5472.CAN-07-5162.

Datto, M. B. *et al.* (1997) 'The viral oncoprotein E1A blocks transforming growth factor beta-mediated induction of p21/WAF1/Cip1 and p15/INK4B.', *Molecular and Cellular Biology*. doi: 10.1128/mcb.17.4.2030.

Davenport, J., Harris, L. D. and Goorha, R. (2006) 'Spindle checkpoint function requires

Mad2-dependent Cdc20 binding to the Mad3 homology domain of BubR1', *Experimental Cell Research*. doi: 10.1016/j.yexcr.2006.02.018.

DeBerardinis, R. J. *et al.* (2008) 'Brick by brick: metabolism and tumor cell growth', *Current Opinion in Genetics and Development*. doi: 10.1016/j.gde.2008.02.003.

DeGregori, J., Kowalik, T. and Nevins, J. R. (1995) 'Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes.', *Molecular and Cellular Biology*. doi: 10.1128/mcb.15.8.4215.

DeNardo, D. G., Andreu, P. and Coussens, L. M. (2010) 'Interactions between lymphocytes and myeloid cells regulate pro-versus anti-tumor immunity', *Cancer and Metastasis Reviews*. doi: 10.1007/s10555-010-9223-6.

Deshpande, A., Sicinski, P. and Hinds, P. W. (2005) 'Cyclins and cdks in development and cancer: A perspective', *Oncogene*. doi: 10.1038/sj.onc.1208618.

Dewidar, B. *et al.* (2019) 'TGF-β in Hepatic Stellate Cell Activation and Liver Fibrogenesis-Updated 2019', *Cells*. doi: 10.3390/cells8111419.

Ditchfield, C. *et al.* (2003) 'Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores', *Journal of Cell Biology*. doi: 10.1083/jcb.200208091.

Dong, X. L. *et al.* (2012) 'Hypoxia decreased chemosensitivity of breast cancer cell line MCF-7 to paclitaxel through cyclin B1', *Biomedicine and Pharmacotherapy*. doi: 10.1016/j.biopha.2011.11.016.

Downward, J. (1998) 'Mechanisms and consequences of activation of protein kinase B/Akt', *Current Opinion in Cell Biology*. doi: 10.1016/S0955-0674(98)80149-X.

Doxsey, S. (2001) 'Re-evaluating centrosome function', *Nature Reviews Molecular Cell Biology*. doi: 10.1038/35089575.

Dumontet, C. and Jordan, M. A. (2010) 'Microtubule-binding agents: A dynamic field of cancer therapeutics', *Nature Reviews Drug Discovery*. doi: 10.1038/nrd3253.

Eckerdt, F. and Strebhardt, K. (2006) 'Polo-like kinase 1: Target and regulator of

anaphase-promoting complex/cyclosome-dependent proteolysis', *Cancer Research*. doi: 10.1158/0008-5472.CAN-06-0358.

Egeblad, M., Nakasone, E. S. and Werb, Z. (2010) 'Tumors as organs: Complex tissues that interface with the entire organism', *Developmental Cell*. doi: 10.1016/j.devcel.2010.05.012.

El-Deiry, W. S. *et al.* (1993) 'WAF1, a potential mediator of p53 tumor suppression', *Cell*. doi: 10.1016/0092-8674(93)90500-P.

El-Seedi, H. *et al.* (2009) 'Naturally Occurring Xanthones; Latest Investigations: Isolation, Structure Elucidation and Chemosystematic Significance', *Current Medicinal Chemistry*. doi: 10.2174/092986709788682056.

El-Seedi, H. *et al.* (2010) 'Recent Insights into the Biosynthesis and Biological Activities of Natural Xanthones', *Current Medicinal Chemistry*. doi: 10.2174/092986710790712147.

Elia, A. E. H. *et al.* (2003) 'The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain', *Cell*. doi: 10.1016/S0092-8674(03)00725-6.

Evan, G. I. and d'Adda di Fagagna, F. (2009) 'Cellular senescence: hot or what?', *Current Opinion in Genetics and Development*. doi: 10.1016/j.gde.2008.11.009.

Evans, T. *et al.* (1983) 'Cyclin: A protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division', *Cell*. doi: 10.1016/0092-8674(83)90420-8.

Fang, G. (2002) 'Checkpoint protein BubR1 acts synergistically with Mad2 to inhibit anaphase-promoting complex', *Molecular Biology of the Cell*. doi: 10.1091/mbc.01-09-0437.

Fang, G., Hongtao, Y. and Kirschner, M. W. (1998) 'Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1', *Molecular Cell*. doi: 10.1016/S1097-2765(00)80126-4.

Ferrara, N. (2009) 'Vascular endothelial growth factor', *Arteriosclerosis, Thrombosis,*

and Vascular Biology. doi: 10.1161/ATVBAHA.108.179663.

Flier, J. S., Underhill, L. H. and Dvorak, H. F. (1986) 'Tumors: Wounds That Do Not Heal', *New England Journal of Medicine*. doi: 10.1056/NEJM198612253152606.

Fraizer, G. C. *et al.* (2004) 'Aurora-A/STK15/BTAK enhances chromosomal instability in bladder cancer cells.', *International journal of oncology*. doi: 10.3892/ijo.25.6.1631.

Franceschelli, S. *et al.* (2016) 'A Novel Biological Role of α-Mangostin in Modulating Inflammatory Response Through the Activation of SIRT-1 Signaling Pathway', *Journal of Cellular Physiology*. doi: 10.1002/jcp.25348.

Fu, Z. *et al.* (2008) 'Plk1-dependent phosphorylation of FoxM1 regulates a transcriptional programme required for mitotic progression', *Nature Cell Biology*. doi: 10.1038/ncb1767.

Gabhann, F. Mac and Popel, A. S. (2008) 'Systems biology of vascular endothelial growth factors', *Microcirculation*. doi: 10.1080/10739680802095964.

Galluzzi, L. and Kroemer, G. (2008) 'Necroptosis: A Specialized Pathway of Programmed Necrosis', *Cell*. doi: 10.1016/j.cell.2008.12.004.

Ganguly, A., Yang, H. and Cabral, F. (2011) 'Overexpression of mitotic centromere-associated kinesin stimulates microtubule detachment and confers resistance to paclitaxel', *Molecular Cancer Therapeutics*. doi: 10.1158/1535-7163.MCT-10-1109.

Gascoigne, K. E. and Taylor, S. S. (2008) 'Cancer Cells Display Profound Intra- and Interline Variation following Prolonged Exposure to Antimitotic Drugs', *Cancer Cell*. doi: 10.1016/j.ccr.2008.07.002.

Gaya, A. M. and Rustin, J. S. (2005) 'Vascular disrupting agents: A new class of drug in cancer therapy', *Clinical Oncology*. doi: 10.1016/j.clon.2004.11.011.

Geng, Y. *et al.* (1996) 'Regulation of cyclin E transcription by E2Fs and retinoblastoma protein', *Oncogene*.

Genovese, S. *et al.* (2016) 'Recent developments in the pharmacology of prenylated xanthones', *Drug Discovery Today*. doi: 10.1016/j.drudis.2016.06.033.

Giacinti, C. and Giordano, A. (2006) 'RB and cell cycle progression', *Oncogene*. doi: 10.1038/sj.onc.1209615.

Giancotti, F. G. and Ruoslahti, E. (1999) 'Integrin signaling', *Science*. doi: 10.1126/science.285.5430.1028.

Giet, R. and Glover, D. M. (2001) 'Drosophila aurora B kinase is required for histone H3 phosphorylation and condensin recruitment during chromosome condensation and to organize the central spindle during cytokinesis', *Journal of Cell Biology*. doi: 10.1083/jcb.152.4.669.

Giet, R., Petretti, C. and Prigent, C. (2005) 'Aurora kinases, aneuploidy and cancer, a coincidence or a real link?', *Trends in Cell Biology*. doi: 10.1016/j.tcb.2005.03.004.

Gilmartin, A. G. *et al.* (2009) 'Distinct concentration-dependent effects of the polo-like kinase 1-specific inhibitor GSK461364A, including differential effect on apoptosis', *Cancer Research*. doi: 10.1158/0008-5472.CAN-09-0945.

Girard, F. *et al.* (1991) 'Cyclin a is required for the onset of DNA replication in mammalian fibroblasts', *Cell*. doi: 10.1016/0092-8674(91)90293-8.

Glotzer, M., Murray, A. W. and Kirschner, M. W. (1991) 'Cyclin is degraded by the ubiquitin pathway', *Nature*. doi: 10.1038/349132a0.

Glover, D. M. *et al.* (1995) 'Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles', *Cell*. doi: 10.1016/0092-8674(95)90374-7.

Green, D. and Kroemer, G. (1998) 'The central executioners of apoptosis: Caspases or mitochondria?', *Trends in Cell Biology*. doi: 10.1016/S0962-8924(98)01273-2.

Green, R. A., Paluch, E. and Oegema, K. (2012) 'Cytokinesis in animal cells', *Annual Review of Cell and Developmental Biology*. doi: 10.1146/annurev-cellbio-101011-155718.

Grivennikov, S. I., Greten, F. R. and Karin, M. (2010) 'Immunity, Inflammation, and Cancer', *Cell*. doi: 10.1016/j.cell.2010.01.025.

Gu, H. *et al.* (2009) 'Gambogic acid reduced bcl-2 expression via p53 in human breast

MCF-7 cancer cells.', *Journal of cancer research and clinical oncology*. doi: 10.1007/s00432-009-0624-2.

Guan, Z. *et al.* (2007) 'Aurora-A, a negative prognostic marker, increases migration and decreases radiosensitivity in cancer cells', *Cancer Research*. doi: 10.1158/0008-5472.CAN-07-1379.

Gutierrez-Orozco, F. and Failla, M. L. (2013) 'Biological activities and bioavailability of mangosteen xanthones: A critical review of the current evidence', *Nutrients*. doi: 10.3390/nu5083163.

Hanahan, D. and Folkman, J. (1996) 'Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis', *Cell*. doi: 10.1016/S0092-8674(00)80108-7.

Hanahan, D. and Weinberg, R. A. (2000) 'The hallmarks of cancer', *Cell*. doi: 10.1016/S0092-8674(00)81683-9.

Hanahan, D. and Weinberg, R. A. (2011) 'Hallmarks of cancer: The next generation', *Cell*. doi: 10.1016/j.cell.2011.02.013.

Hansen, M. *et al.* (1996) 'Molecular details of the structure of a psorospermin - DNA covalent/intercalation complex and associated DNA sequence selectivity', *Journal of the American Chemical Society*. doi: 10.1021/ja960319c.

Harashima, H., Dissmeyer, N. and Schnittger, A. (2013) 'Cell cycle control across the eukaryotic kingdom', *Trends in Cell Biology*. doi: 10.1016/j.tcb.2013.03.002.

Hardwick, K. G. *et al.* (2000) 'MAD3 encodes a novel component of the spindle checkpoint which interacts with Bub3p, Cdc20p, and Mad2p', *Journal of Cell Biology*. doi: 10.1083/jcb.148.5.871.

Harris, C. C. (1996) 'Structure and function of the p53 tumor suppressor gene: Clues for rational cancer therapeutic strategies', *Journal of the National Cancer Institute*. doi: 10.1093/jnci/88.20.1442.

Hartwell, L. (1992) 'Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells', *Cell*. doi: 10.1016/0092-8674(92)90586-2.

Hartwell, L. H. and Weinert, T. A. (1989) 'Checkpoints: Controls that ensure the order of cell cycle events', *Science*. doi: 10.1126/science.2683079.

Henriques, A. C. *et al.* (2019) 'Mitosis inhibitors in anticancer therapy: When blocking the exit becomes a solution', *Cancer Letters*. doi: 10.1016/j.canlet.2018.10.005.

Herzog, F. *et al.* (2009) 'Structure of the anaphase-promoting complex/cyclosome interacting with a mitotic checkpoint complex', *Science*. doi: 10.1126/science.1163300.

Hewitt, L. *et al.* (2010) 'Sustained Mps1 activity is required in mitosis to recruit O-Mad2 to the Mad1-C-Mad2 core complex', *Journal of Cell Biology*. doi: 10.1083/jcb.201002133.

Holland, A. J. and Cleveland, D. W. (2009) 'Boveri revisited: Chromosomal instability, aneuploidy and tumorigenesis', *Nature Reviews Molecular Cell Biology*. doi: 10.1038/nrm2718.

Honda, R., Körner, R. and Nigg, E. A. (2003) 'Exploring the functional interactions between Aurora B, INCENP, and survivin in mitosis', *Molecular Biology of the Cell*. doi: 10.1091/mbc.E02-11-0769.

de Hoon, J. P. J. *et al.* (2012) 'Taxane resistance in breast cancer: A closed HER2 circuit?', *Biochimica et Biophysica Acta - Reviews on Cancer*. doi: 10.1016/j.bbcan.2012.01.001.

Howe, A. *et al.* (1998) 'Integrin signaling and cell growth control', *Current Opinion in Cell Biology*. doi: 10.1016/S0955-0674(98)80144-0.

Howell, B. J. *et al.* (2004) 'Spindle checkpoint protein dynamics at kinetochores in living cells', *Current Biology*. doi: 10.1016/j.cub.2004.05.053.

Hoyt, M. A., Totis, L. and Roberts, B. T. (1991) 'S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function', *Cell*. doi: 10.1016/0092-8674(81)90014-3.

Hunter, T. (1991) 'Cooperation between oncogenes', *Cell*. doi: 10.1016/0092-8674(91)90637-E.

Iarc., I. A. for R. on C. W. H. O. (2012) 'GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide in 2012.', *Globocan*. doi: 10.1002/ijc.27711.

Ibrahim, M. Y. *et al.* (2016) 'α-Mangostin from Garcinia mangostana Linn: An updated review of its pharmacological properties', *Arabian Journal of Chemistry*. doi: 10.1016/j.arabjc.2014.02.011.

Ibrahim, N. K. *et al.* (2002) 'Phase I and pharmacokinetic study of ABI-007, a Cremophor-free, protein-stabilized, nanoparticle formulation of paclitaxel', *Clinical Cancer Research*.

Ikezoe, T. *et al.* (2007) 'A novel treatment strategy targeting Aurora kinases in acute myelogenous leukemia', *Molecular Cancer Therapeutics*. doi: 10.1158/1535-7163.MCT-07-0067.

Infante, J. *et al.* (2009) 'Phase II trial of weekly docetaxel, vinorelbine, and trastuzumab in the first-line treatment of patients with her2-positive metastatic breast cancer', *Clinical Breast Cancer*. doi: 10.3816/CBC.2009.n.004.

Isaka, M. *et al.* (2001) 'Phomoxanthones A and B, novel xanthone dimers from the endophytic fungus Phomopsis species', *Journal of Natural Products*. doi: 10.1021/np010006h.

Janssen, A., Kops, G. J. and Medema, R. H. (2011) 'Targeting the Mitotic Checkpoint to Kill Tumor Cells', *Hormones and Cancer*. doi: 10.1007/s12672-010-0059-x.

Jeffrey, P. D. *et al.* (1995) 'Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex', *Nature*. doi: 10.1038/376313a0.

Jeng, Y. M. *et al.* (2004) 'Overexpression and Amplification of Aurora-A in Hepatocellular Carcinoma', *Clinical Cancer Research*. doi: 10.1158/1078-0432.CCR-1057-03.

Jiang, B. H. and Liu, L. Z. (2008) 'PI3K/PTEN signaling in tumorigenesis and angiogenesis', *Biochimica et Biophysica Acta - Proteins and Proteomics*. doi: 10.1016/j.bbapap.2007.09.008.

Johnson, D. G. and Walker, C. L. (1999) 'CYCLINS AND CELL CYCLE CHECKPOINTS', *Annual Review of Pharmacology and Toxicology*. doi: 10.1146/annurev.pharmtox.39.1.295.

Jones, R. G. and Thompson, C. B. (2009) 'Tumor suppressors and cell metabolism: A recipe for cancer growth', *Genes and Development*. doi: 10.1101/gad.1756509.

Jones, S. E. *et al.* (2005) 'Randomized phase III study of docetaxel compared with paclitaxel in metastatic breast cancer', *Journal of Clinical Oncology*. doi: 10.1200/JCO.2005.02.027.

Jordan, M. A. and Wilson, L. (2004) 'Microtubules as a target for anticancer drugs', *Nature Reviews Cancer*. doi: 10.1038/nrc1317.

Joyce, J. A. and Pollard, J. W. (2009) 'Microenvironmental regulation of metastasis', *Nature Reviews Cancer*. doi: 10.1038/nrc2618.

Kalluri, R. and Zeisberg, M. (2006) 'Fibroblasts in cancer', *Nature Reviews Cancer*. doi: 10.1038/nrc1877.

Kamb, A. (2005) 'What's wrong with our cancer models?', *Nature Reviews Drug Discovery*. doi: 10.1038/nrd1635.

Kampkötter, A. *et al.* (2007) 'Investigations of protective effects of the flavonoids quercetin and rutin on stress resistance in the model organism Caenorhabditis elegans', *Toxicology*. doi: 10.1016/j.tox.2007.02.006.

Karess, R. (2005) 'Rod-Zw10-Zwilch: A key player in the spindle checkpoint', *Trends in Cell Biology*. doi: 10.1016/j.tcb.2005.05.003.

Karnoub, A. E. and Weinberg, R. A. (2006) 'Chemokine networks and breast cancer metastasis', *Breast Disease*. doi: 10.3233/bd-2007-26107.

Kashyap, D. *et al.* (2016) 'Molecular targets of gambogic acid in cancer: recent trends and advancements', *Tumor Biology*. doi: 10.1007/s13277-016-5194-8.

Kato, J. ya *et al.* (1994) 'Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27Kip1) of cyclin-dependent kinase 4 activation', *Cell*. doi: 10.1016/0092-

8674(94)90257-7.

Kavallaris, M. (2010) 'Microtubules and resistance to tubulin-binding agents', *Nature Reviews Cancer*. doi: 10.1038/nrc2803.

Kelly, A. E. and Funabiki, H. (2009) 'Correcting aberrant kinetochore microtubule attachments: an Aurora B-centric view', *Current Opinion in Cell Biology*. doi: 10.1016/j.ceb.2009.01.004.

Kessenbrock, K., Plaks, V. and Werb, Z. (2010) 'Matrix Metalloproteinases: Regulators of the Tumor Microenvironment', *Cell*. doi: 10.1016/j.cell.2010.03.015.

Kim, S. *et al.* (2012) 'Structure of human Mad1 C-terminal domain reveals its involvement in kinetochore targeting', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.1118210109.

King, R. W., Jackson, P. K. and Kirschner, M. W. (1994) 'Mitosis in transition', *Cell*. doi: 10.1016/0092-8674(94)90542-8.

Kinzler, K. W. and Vogelstein, B. (1997) 'Cancer-susceptibility genes. Gatekeepers and caretakers', *Nature*.

Klein-Júnior, L. C. *et al.* (2020) 'Xanthones and Cancer: from Natural Sources to Mechanisms of Action', *Chemistry and Biodiversity*. doi: 10.1002/cbdv.201900499.

Kline-Smith, S. L. *et al.* (2004) 'Depletion of Centromeric MCAK Leads to Chromosome Congression and Segregation Defects Due to Improper Kinetochore Attachments', *Molecular Biology of the Cell*. doi: 10.1091/mbc.E03-08-0581.

Kowalski, R. J., Giannakakou, P. and Hamel, E. (1997) 'Activities of the microtubule-stabilizing agents epothilones A and B with purified tubulin and in cells resistant to paclitaxel (Taxol®)', *Journal of Biological Chemistry*. doi: 10.1074/jbc.272.4.2534.

Krick, A. *et al.* (2007) 'Potential cancer chemopreventive in vitro activities of monomeric xanthone derivatives from the marine algicolous fungus Monodictys putredinis', *Journal of Natural Products*. doi: 10.1021/np060505o.

Kritsanawong, S. *et al.* (2016) 'Antiproliferative and apoptosis induction of α-mangostin

in T47D breast cancer cells', *International Journal of Oncology*. doi: 10.3892/ijo.2016.3399.

Kroemer, G. and Pouyssegur, J. (2008) 'Tumor Cell Metabolism: Cancer's Achilles' Heel', *Cancer Cell*. doi: 10.1016/j.ccr.2008.05.005.

Kumagai, A. and Dunphy, W. G. (1996) 'Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from Xenopus egg extracts', *Science*. doi: 10.1126/science.273.5280.1377.

Kwok, Y. and Hurley, L. H. (1998) 'Topoisomerase II site-directed alkylation of DNA by psorospermin and its effect on topoisomerase II-mediated DNA cleavage', *Journal of Biological Chemistry*. doi: 10.1074/jbc.273.49.33020.

Landen, C. N. *et al.* (2007) 'Overexpression of the centrosomal protein Aurora-A kinase is associated with poor prognosis in epithelial ovarian cancer patients', *Clinical Cancer Research*. doi: 10.1158/1078-0432.CCR-07-0431.

Lane, H. A. and Nigg, E. A. (1996) 'Antibody microinjection reveals an essential role for human polo-like kinase 1 (Plk1) in the functional maturation of mitotic centrosomes', *Journal of Cell Biology*. doi: 10.1083/jcb.135.6.1701.

Lavoie, B. D., Hogan, E. and Koshland, D. (2002) 'In vivo dissection of the chromosome condensation machinery: Reversibility of condensation distinguishes contributions of condensin and cohesin', *Journal of Cell Biology*. doi: 10.1083/jcb.200109056.

Lee, K. S. *et al.* (1998) 'Mutation of the polo-box disrupts localization and mitotic functions of the mammalian polo kinase Plk', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.95.16.9301.

Lei, M. and Erikson, R. L. (2008) 'Plk1 depletion in nontransformed diploid cells activates the DNA-damage checkpoint', *Oncogene*. doi: 10.1038/onc.2008.36.

Levine, B. and Kroemer, G. (2008) 'Autophagy in the Pathogenesis of Disease', *Cell*. doi: 10.1016/j.cell.2007.12.018.

Li, H., Wang, Y. and Liu, X. (2008) 'Plk1-dependent phosphorylation regulates functions

of DNA topoisomerase IIα in cell cycle progression', *Journal of Biological Chemistry*. doi: 10.1074/jbc.M709007200.

Li, Hongzhong *et al.* (2013) 'Mangiferin exerts antitumor activity in breast cancer cells by regulating matrix metalloproteinases, epithelial to mesenchymal transition, and β-catenin signaling pathway', *Toxicology and Applied Pharmacology*. doi: 10.1016/j.taap.2013.05.011.

Li, R. and Murray, A. W. (1991) 'Feedback control of mitosis in budding yeast', *Cell*. doi: 10.1016/0092-8674(81)90015-5.

Li, Y. *et al.* (1997) 'MAD2 associates with the cyclosome/anaphase-promoting complex and inhibits its activity', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.94.23.12431.

Li, Y. and Benezra, R. (1996) 'Identification of a human mitotic checkpoint gene: hsMAD2', *Science*. doi: 10.1126/science.274.5285.246.

Lim, S. and Kaldis, P. (2013) 'Cdks, cyclins and CKIs: Roles beyond cell cycle regulation', *Development (Cambridge)*. doi: 10.1242/dev.091744.

Lin, C. -N *et al.* (1993) 'γ-pyrone compounds. IV: Synthesis and antiplatelet effects of mono- and dioxygenated xanthones and xanthonoxypropanolamine', *Journal of Pharmaceutical Sciences*. doi: 10.1002/jps.2600820103.

Lin, C. N. *et al.* (1996) 'Synthesis and antithrombotic effect of xanthone derivatives', *Journal of Pharmacy and Pharmacology*. doi: 10.1111/j.2042-7158.1996.tb05994.x.

Liu, D., Davydenko, O. and Lampson, M. A. (2012) 'Polo-like kinase-1 regulates kinetochore-microtubule dynamics and spindle checkpoint silencing', *Journal of Cell Biology*. doi: 10.1083/jcb.201205090.

Liu, J. *et al.* (2017) 'Synthesis of xanthone derivatives and studies on the inhibition against cancer cells growth and synergistic combinations of them', *European Journal of Medicinal Chemistry*. doi: 10.1016/j.ejmech.2017.03.068.

Liu, Q. *et al.* (2016) 'Nitric oxide inhibitory xanthones from the pericarps of Garcinia

mangostana', *Phytochemistry*. doi: 10.1016/j.phytochem.2016.08.007.

Liu, Y. *et al.* (2006) 'Synthesis and pharmacological activities of xanthone derivatives as α-glucosidase inhibitors', *Bioorganic and Medicinal Chemistry*. doi: 10.1016/j.bmc.2006.04.014.

Liu, Y. M. *et al.* (2014) 'Tubulin inhibitors: A patent review', *Expert Opinion on Therapeutic Patents*. doi: 10.1517/13543776.2014.859247.

Llamazares, S. *et al.* (1991) 'Polo encodes a protein kinase homolog required for mitosis in Drosophila', *Genes and Development*. doi: 10.1101/gad.5.12a.2153.

Loeb, L. A., Loeb, K. R. and Anderson, J. P. (2003) 'Multiple mutations and cancer', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.0334858100.

Loupakis, F. *et al.* (2009) 'PTEN expression and KRAS mutations on primary tumors and metastases in the prediction of benefit from cetuximab plus irinotecan for patients with metastatic colorectal cancer', *Journal of Clinical Oncology*. doi: 10.1200/JCO.2008.20.2796.

Lu, N. *et al.* (2007) 'Gambogic acid inhibits angiogenesis through suppressing vascular endothelial growth factor-induced tyrosine phosphorylation of KDR/Flk-1', *Cancer Letters*. doi: 10.1016/j.canlet.2007.08.015.

Lub, S. *et al.* (2014) 'Targeting the Anaphase Promoting Complex/Cyclosome (APC/C) in Multiple Myeloma', *Blood*. doi: 10.1182/blood.v124.21.2097.2097.

Lukashev, M. E. and Werb, Z. (1998) 'ECM signalling: Orchestrating cell behaviour and misbehaviour', *Trends in Cell Biology*. doi: 10.1016/S0962-8924(98)01362-2.

Luo, X. *et al.* (2002) 'The Mad2 spindle checkpoint protein undergoes similar major conformational changes upon binding to either Mad1 or Cdc20', *Molecular Cell*. doi: 10.1016/S1097-2765(01)00435-X.

Luo, X. *et al.* (2004) 'The Mad2 spindle checkpoint protein has two distinct natively folded states', *Nature Structural and Molecular Biology*. doi: 10.1038/nsmb748.

Maciejowski, J. *et al.* (2010) 'Mps1 directs the assembly of Cdc20 inhibitory complexes during interphase and mitosis to control M phase timing and spindle checkpoint signaling', *Journal of Cell Biology*. doi: 10.1083/jcb.201001050.

Maciejowski, J. and Hatch, E. M. (2020) 'Nuclear Membrane Rupture and Its Consequences', *Annual Review of Cell and Developmental Biology*. doi: 10.1146/annurev-cellbio-020520-120627.

Maia, A. R. R. *et al.* (2015) 'Inhibition of the spindle assembly checkpoint kinase TTK enhances the efficacy of docetaxel in a triple-negative breast cancer model', *Annals of Oncology*. doi: 10.1093/annonc/mdv293.

Maiato, H., Sampaio, P. and Sunkel, C. E. (2004) 'Microtubule-associated proteins and their essential roles during mitosis', *International Review of Cytology*. doi: 10.1016/S0074-7696(04)41002-X.

Maiato, H. and Sunkel, C. E. (2004) 'Kinetochore-microtubule interactions during cell division', *Chromosome Research*. doi: 10.1023/B:CHRO.0000036587.26566.81.

Mailand, N. *et al.* (2000) 'Rapid destruction of human Cdc25A in response to DNA damage', *Science*. doi: 10.1126/science.288.5470.1425.

Maldonado, M. and Kapoor, T. M. (2011) 'Constitutive Mad1 targeting to kinetochores uncouples checkpoint signalling from chromosome biorientation', *Nature Cell Biology*. doi: 10.1038/ncb2223.

Malumbres, M. and Barbacid, M. (2009) 'Cell cycle, CDKs and cancer: A changing paradigm', *Nature Reviews Cancer*. doi: 10.1038/nrc2602.

Marumoto, T. *et al.* (2002) 'Roles of aurora-A kinase in mitotic entry and G2 checkpoint in mammalian cells', *Genes to Cells*. doi: 10.1046/j.1365-2443.2002.00592.x.

Masawang, K. *et al.* (2014) 'Evaluation of 2',4'-dihydroxy-3,4,5-trimethoxychalcone as antimitotic agent that induces mitotic catastrophe in MCF-7 breast cancer cells', *Toxicology Letters*. doi: 10.1016/j.toxlet.2014.06.016.

Masters, K. S. and Bräse, S. (2012) 'Xanthones from fungi, lichens, and bacteria: The

natural products and their synthesis', *Chemical Reviews*. doi: 10.1021/cr100446h.

Mastronarde, D. N. *et al.* (1993) 'Interpolar spindle microtubules in PTK cells', *Journal of Cell Biology*. doi: 10.1083/jcb.123.6.1475.

Matsumoto, K. *et al.* (2005) 'Xanthones induce cell-cycle arrest and apoptosis in human colon cancer DLD-1 cells', *Bioorganic and Medicinal Chemistry*. doi: 10.1016/j.bmc.2005.06.065.

De Matteis, M. A. and Luini, A. (2008) 'Exiting the Golgi complex', *Nature Reviews Molecular Cell Biology*. doi: 10.1038/nrm2378.

McEwen, B. F. *et al.* (1998) 'A new look at kinetochore structure in vertebrate somatic cells using high-pressure freezing and freeze substitution', in *Chromosoma*. doi: 10.1007/s004120050320.

McIntosh, J. R. and Euteneuer, U. (1984) 'Tubulin hooks as probes for microtubule polarity: An analysis of the method and an evaluation of data on microtubule polarity in the mitotic spindle', *Journal of Cell Biology*. doi: 10.1083/jcb.98.2.525.

McIntosh, J. R. and Pfarr, C. M. (1991) 'Mitotic motors', *Journal of Cell Biology*. doi: 10.1083/jcb.115.3.577.

Medema, R. H. and Bos, J. L. (1993) 'The role of p21ras in receptor tyrosine kinase signaling', *Critical Reviews in Oncogenesis*. doi: 10.1007/978-3-0348-9057-1_1.

Meraldi, P., Draviam, V. M. and Sorger, P. K. (2004) 'Timing and checkpoints in the regulation of mitotic progression', *Developmental Cell*. doi: 10.1016/j.devcel.2004.06.006.

Mitchison, T. J. (2012) 'The proliferation rate paradox in antimitotic chemotherapy', *Molecular Biology of the Cell*. doi: 10.1091/mbc.E10-04-0335.

Miyoshi, Y. *et al.* (2001) 'Association of centrosomal kinase STK15/BTAK mRNA expression with chromosomal instability in human breast cancers', *International Journal of Cancer*. doi: 10.1002/ijc.1200.

Mizushima, N. (2007) 'Autophagy: Process and function', *Genes and Development*. doi:

10.1101/gad.1599207.

Mohamed, M. M. and Sloane, B. F. (2006) 'Cysteine cathepsins: Multifunctional enzymes in cancer', in *Nature Reviews Cancer*. doi: 10.1038/nrc1949.

Morgan, D. O. (1995) 'Principles of CDK regulation', *Nature*. doi: 10.1038/374131a0.

Morgan, D. O. (1999) 'Regulation of the APC and the exit from mitosis', *Nature Cell Biology*. doi: 10.1038/10039.

Mosesson, Y., Mills, G. B. and Yarden, Y. (2008) 'Derailed endocytosis: An emerging feature of cancer', *Nature Reviews Cancer*. doi: 10.1038/nrc2521.

Moudi, M. *et al.* (2013) 'Vinca alkaloids', *International Journal of Preventive Medicine*. doi: 10.2165/00128415-200711380-00080.

Murata-Hori, M. and Wang, Y. L. (2002) 'Both midzone and astral microtubules are involved in the delivery of cytokinesis signals: Insights from the mobility of aurora B', *Journal of Cell Biology*. doi: 10.1083/jcb.200207014.

Murray, A. W. (2004) 'Recycling the Cell Cycle: Cyclins Revisited', *Cell*. doi: 10.1016/S0092-8674(03)01080-8.

Musacchio, A. and Salmon, E. D. (2007) 'The spindle-assembly checkpoint in space and time', *Nature Reviews Molecular Cell Biology*. doi: 10.1038/nrm2163.

Nakagawa, Y. *et al.* (2007) 'Characterized mechanism of α-mangostin-induced cell death: Caspase-independent apoptosis with release of endonuclease-G from mitochondria and increased miR-143 expression in human colorectal cancer DLD-1 cells', *Bioorganic and Medicinal Chemistry*. doi: 10.1016/j.bmc.2007.04.071.

Nasmyth, K. (2001) 'Disseminating the Genome: Joining, Resolving, and Separating Sister Chromatids During Mitosis and Meiosis', *Annual Review of Genetics*. doi: 10.1146/annurev.genet.35.102401.091334.

Nasmyth, K. and Haering, C. H. (2009) 'Cohesin: Its Roles and Mechanisms', *Annual Review of Genetics*. doi: 10.1146/annurev-genet-102108-134233.

Negrini, S., Gorgoulis, V. G. and Halazonetis, T. D. (2010) 'Genomic instability an evolving hallmark of cancer', *Nature Reviews Molecular Cell Biology*. doi: 10.1038/nrm2858.

Nevins, J. R. (2001) 'The Rb/E2F pathway and cancer', *Human Molecular Genetics*. doi: 10.1093/hmg/10.7.699.

Nezi, L. and Musacchio, A. (2009) 'Sister chromatid tension and the spindle assembly checkpoint', *Current Opinion in Cell Biology*. doi: 10.1016/j.ceb.2009.09.007.

Nicklas, R. B., Ward, S. C. and Gorbsky, G. J. (1995) 'Kinetochore chemistry is sensitive to tension and may link mitotic forces to a cell cycle checkpoint', *Journal of Cell Biology*. doi: 10.1083/jcb.130.4.929.

Nigg, E. A. (2001) 'Mitotic kinases as regulators of cell division and its checkpoints', *Nature Reviews Molecular Cell Biology*. doi: 10.1038/35048096.

Nilsson, J. *et al.* (2008) 'The APC/C maintains the spindle assembly checkpoint by targeting Cdc20 for destruction', *Nature Cell Biology*. doi: 10.1038/ncb1799.

Nishihira, T. *et al.* (1993) 'Molecular and cellular features of esophageal cancer cells', *Journal of Cancer Research and Clinical Oncology*. doi: 10.1007/BF01215923.

Nobili, S. *et al.* (2012) 'Overcoming tumor multidrug resistance using drugs able to evade P-glycoprotein or to exploit its expression', *Medicinal Research Reviews*. doi: 10.1002/med.20239.

Nogales, E. *et al.* (1999) 'High-resolution model of the microtubule', *Cell*. doi: 10.1016/S0092-8674(00)80961-7.

North, S. and Hainaut, P. (2000) 'p53 and cell-cycle control: A finger in every pie', *Pathologie Biologie*.

Núñez Selles, A. J., Daglia, M. and Rastrelli, L. (2016) 'The potential role of mangiferin in cancer treatment through its immunomodulatory, anti-angiogenic, apoptopic, and gene regulatory effects', *BioFactors*. doi: 10.1002/biof.1299.

Ohtani, K., Degregori, J. and Nevins, J. R. (1995) 'Regulation of the cyclin E gene by

transcription factor E2F1', *Proceedings of the National Academy of Sciences of the United States of America*. doi: 10.1073/pnas.92.26.12146.

Ohtsubo, M. *et al.* (1995) 'Human cyclin E, a nuclear protein essential for the G1-to-S phase transition.', *Molecular and Cellular Biology*. doi: 10.1128/mcb.15.5.2612.

Ou, Y. and Rattner, J. B. (2004) 'The centrosome in higher organisms: Structure, composition, and duplication', *International Review of Cytology*. doi: 10.1016/S0074-7696(04)38003-4.

Ozols, R. F. *et al.* (2003) 'Phase III trial of carboplatin and paclitaxel compared with cisplatin and paclitaxel in patients with optimally resected stage III ovarian cancer: A Gynecologic Oncology Group study', *Journal of Clinical Oncology*. doi: 10.1200/JCO.2003.02.153.

Palermo, C. and Joyce, J. A. (2008) 'Cysteine cathepsin proteases as pharmacological targets in cancer', *Trends in Pharmacological Sciences*. doi: 10.1016/j.tips.2007.10.011.

Park, J. H. *et al.* (2008) 'Inhibitors of histone deacetylases induce tumor-selective cytotoxicity through modulating Aurora-A kinase', *Journal of Molecular Medicine*. doi: 10.1007/s00109-007-0260-8.

Paulovich, A. G. and Hartwell, L. H. (1995) 'A checkpoint regulates the rate of progression through S phase in S. cerevisiae in Response to DNA damage', *Cell*. doi: 10.1016/0092-8674(95)90481-6.

Perera, D. *et al.* (2007) 'Bub1 Maintains Centromeric Cohesion by Activation of the Spindle Checkpoint', *Developmental Cell*. doi: 10.1016/j.devcel.2007.08.008.

Pérez-Rojas, J. M. *et al.* (2016) 'Synergic effect of α -mangostin on the cytotoxicity of cisplatin in a cervical cancer model', *Oxidative Medicine and Cellular Longevity*. doi: 10.1155/2016/7981397.

Petronczki, M., Lénárt, P. and Peters, J. M. (2008) 'Polo on the Rise-from Mitotic Entry to Cytokinesis with Plk1', *Developmental Cell*. doi: 10.1016/j.devcel.2008.04.014.

Potenta, S., Zeisberg, E. and Kalluri, R. (2008) 'The role of endothelial-to-mesenchymal

transition in cancer progression', *British Journal of Cancer*. doi: 10.1038/sj.bjc.6604662.

Przewloka, M. R. and Glover, D. M. (2009) 'The Kinetochore and the Centromere: A Working Long Distance Relationship', *Annual Review of Genetics*. doi: 10.1146/annurev-genet-102108-134310.

Qi, Q. *et al.* (2008) 'Anti-invasive effect of gambogic acid in MDA-MB-231 human breast carcinoma cells', *Biochemistry and Cell Biology*. doi: 10.1139/O08-104.

Qian, B. Z. and Pollard, J. W. (2010) 'Macrophage Diversity Enhances Tumor Progression and Metastasis', *Cell*. doi: 10.1016/j.cell.2010.03.014.

Rabinowitz, J. D. and White, E. (2010) 'Autophagy and metabolism', *Science*. doi: 10.1126/science.1193497.

Ravelli, R. B. G. *et al.* (2004) 'Insight into tubulin regulation from a complex with colchicine and a stathmin-like domain', *Nature*. doi: 10.1038/nature02393.

Rebucci, M. and Michiels, C. (2013) 'Molecular aspects of cancer cell resistance to chemotherapy', *Biochemical Pharmacology*. doi: 10.1016/j.bcp.2013.02.017.

Recanatini, M. *et al.* (2001) 'A new class of nonsteroidal aromatase inhibitors: Design and synthesis of chromone and xanthone derivatives and inhibition of the P450 enzymes aromatase and 17α-hydroxylase/C17,20-lyase', *Journal of Medicinal Chemistry*. doi: 10.1021/jm000955s.

Rechsteiner, M. and Rogers, S. W. (1996) 'PEST sequences and regulation by proteolysis', *Trends in Biochemical Sciences*. doi: 10.1016/S0968-0004(96)10031-1.

Reck, M. *et al.* (2014) 'Docetaxel plus nintedanib versus docetaxel plus placebo in patients with previously treated non-small-cell lung cancer (LUME-Lung 1): A phase 3, double-blind, randomised controlled trial', *The Lancet Oncology*. doi: 10.1016/S1470-2045(13)70586-2.

Reddy, S. K. *et al.* (2007) 'Ubiquitination by the anaphase-promoting complex drives spindle checkpoint inactivation', *Nature*. doi: 10.1038/nature05734.

Reynisdóttir, I. *et al.* (1995) 'Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-β', *Genes and Development*. doi: 10.1101/gad.9.15.1831.

Ribeiro, J. *et al.* (2019) 'Carboxyxanthones: Bioactive agents and molecular scaffold for synthesis of analogues and derivatives', *Molecules*. doi: 10.3390/molecules24010180.

Rieder, C. L. (1982) 'The Formation, Structure, and Composition of the Mammalian Kinetochore and Kinetochore Fiber', *International Review of Cytology*. doi: 10.1016/S0074-7696(08)61672-1.

Rieder, C. L. and Maiato, H. (2004) 'Stuck in division or passing through: What happens when cells cannot satisfy the spindle assembly checkpoint', *Developmental Cell*. doi: 10.1016/j.devcel.2004.09.002.

Rieder, C. L. and Salmon, E. D. (1998) 'The vertebrate cell kinetochore and its roles during mitosis', *Trends in Cell Biology*. doi: 10.1016/S0962-8924(98)01299-9.

Roberts, J. C. (1961) 'Naturally occurring xanthones', *Chemical Reviews*. doi: 10.1021/cr60214a003.

Rogalska, A. *et al.* (2013) 'Activation of apoptotic pathway in normal, cancer ovarian cells by epothilone B', *Environmental Toxicology and Pharmacology*. doi: 10.1016/j.etap.2013.06.003.

Roghi, C. *et al.* (1998) 'The Xenopus protein kinase pEg2 associates with the centrosome in a cell cycle-dependent manner, binds to the spindle microtubules and is involved in bipolar mitotic spindle assembly', *Journal of Cell Science*.

Roninson, I. B., Broude, E. V. and Chang, B. D. (2001) 'If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells', *Drug Resistance Updates*. doi: 10.1054/drup.2001.0213.

Van Roy, F. and Berx, G. (2008) 'The cell-cell adhesion molecule E-cadherin', *Cellular and Molecular Life Sciences*. doi: 10.1007/s00018-008-8281-1.

Sakakura, C. *et al.* (2001) 'Tumour-amplified kinase BTAK is amplified and

overexpressed in gastric cancers with possible involvement in aneuploid formation', *British Journal of Cancer*. doi: 10.1054/bjoc.2000.1684.

Salk, J. J., Fox, E. J. and Loeb, L. A. (2010) 'Mutational Heterogeneity in Human Cancers: Origin and Consequences', *Annual Review of Pathology: Mechanisms of Disease*. doi: 10.1146/annurev-pathol-121808-102113.

Sanchez, Y. *et al.* (1997) 'Conservation of the Chk1 checkpoint pathway in mammals: Linkage of DNA damage to Cdk regulation through Cdc25', *Science*. doi: 10.1126/science.277.5331.1497.

Sandler, A. *et al.* (2006) 'Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer', *New England Journal of Medicine*. doi: 10.1056/NEJMoa061884.

Santaguida, S. *et al.* (2010) 'Dissecting the role of MPS1 in chromosome biorientation and the spindle checkpoint through the small molecule inhibitor reversine', *Journal of Cell Biology*. doi: 10.1083/jcb.201001036.

Santaguida, S. and Musacchio, A. (2009) 'The life and miracles of kinetochores', *EMBO Journal*. doi: 10.1038/emboj.2009.173.

Santamaria, A. *et al.* (2007) 'Use of the novel Plk1 inhibitor ZK-thiazolidinone to elucidate functions of Plk1 in early and late stages of mitosis', *Molecular Biology of the Cell*. doi: 10.1091/mbc.E07-05-0517.

Saraiva, L. *et al.* (2003) 'Inhibition of protein kinase C by synthetic xanthone derivatives', *Bioorganic and Medicinal Chemistry*. doi: 10.1016/S0968-0896(02)00641-7.

Schöffski, P. (2009) 'Polo-Like Kinase (PLK) Inhibitors in Preclinical and Early Clinical Development in Oncology', *The Oncologist*. doi: 10.1634/theoncologist.2009-0010.

Schwaebe, M. K., Moran, T. J. and Whitten, J. P. (2005) 'Total synthesis of psorospermin', *Tetrahedron Letters*. doi: 10.1016/j.tetlet.2004.12.006.

Sczaniecka, M. *et al.* (2008) 'The spindle checkpoint functions of Mad3 and Mad2

depend on a Mad3 KEN box-mediated interaction with Cdc20-anaphase-promoting complex (APC/C)', *Journal of Biological Chemistry*. doi: 10.1074/jbc.M803594200.

Semenza, G. L. (2010) 'Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics', *Oncogene*. doi: 10.1038/onc.2009.441.

Sessa, C. *et al.* (2007) 'Phase I clinical study of the novel epothilone B analogue BMS-310705 given on a weekly schedule', *Annals of Oncology*. doi: 10.1093/annonc/mdm198.

Seyfried, T. N. and Huysentruyt, L. C. (2013) 'On the origin of cancer metastasis', *Critical Reviews in Oncogenesis*. doi: 10.1615/CritRevOncog.v18.i1-2.40.

Shadid, K. A. *et al.* (2007) 'Cytotoxic caged-polyprenylated xanthonoids and a xanthone from Garcinia cantleyana', *Phytochemistry*. doi: 10.1016/j.phytochem.2007.05.024.

Sharp, D. J., Rogers, G. C. and Scholey, J. M. (2000) 'Microtubule motors in mitosis', *Nature*. doi: 10.1038/35024000.

Sherr, C. J. and McCormick, F. (2002) 'The RB and p53 pathways in cancer', *Cancer Cell*. doi: 10.1016/S1535-6108(02)00102-2.

Shi, J., Orth, J. D. and Mitchison, T. (2008) 'Cell type variation in responses to antimitotic drugs that target microtubules and kinesin-5', *Cancer Research*. doi: 10.1158/0008-5472.CAN-07-6699.

Shieh, S. Y. *et al.* (1997) 'DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2', *Cell*. doi: 10.1016/S0092-8674(00)80416-X.

Shoji, K. *et al.* (2011) 'Mangiferin induces apoptosis by suppressing Bcl-xL and XIAP expressions and nuclear entry of NF-κB in HL-60 cells', *Archives of Pharmacal Research*. doi: 10.1007/s12272-011-0316-8.

Siegel, R. L., Miller, K. D. and Jemal, A. (2019) 'Cancer statistics, 2019', *CA: A Cancer Journal for Clinicians*. doi: 10.3322/caac.21551.

Silva, P. *et al.* (2011) 'Monitoring the fidelity of mitotic chromosome segregation by the spindle assembly checkpoint', *Cell Proliferation*. doi: 10.1111/j.1365-

2184.2011.00767.x.

Sironi, L. *et al.* (2001) 'Mad2 binding to Mad1 and Cdc20, rather than oligomerization, is required for the spindle checkpoint', *EMBO Journal*. doi: 10.1093/emboj/20.22.6371.

Soussi, T. (2000) 'p53 Antibodies in the sera of patients with various types of cancer: A review', *Cancer Research*.

Soussi, T. (2005) 'Analysis of p53 gene alterations in cancer: A critical view', in *25 Years of p53 Research*. doi: 10.1007/978-1-4020-2922-6_12.

Sporn, M. B. (1996) 'The war on cancer', *Lancet*. doi: 10.1016/S0140-6736(96)91015-6.

Stegmeier, F. *et al.* (2007) 'Anaphase initiation is regulated by antagonistic ubiquitination and deubiquitination activities', *Nature*. doi: 10.1038/nature05694.

Stratton, M. R., Campbell, P. J. and Futreal, P. A. (2009) 'The cancer genome', *Nature*. doi: 10.1038/nature07943.

Sudakin, V., Chan, Gordon K.T. and 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*. doi: 10.1083/jcb.200102093.

Sudakin, V., Chan, Gordon K T and Yen, T. J. (2001) 'Checkpoint inhibition of the APC / C in HeLa cells', *Cell*. doi: 10.1083/jcb.200102093.

Suijkerbuijk, S. J. E. *et al.* (2012) 'Integration of Kinase and Phosphatase Activities by BUBR1 Ensures Formation of Stable Kinetochore-Microtubule Attachments', *Developmental Cell*. doi: 10.1016/j.devcel.2012.09.005.

Suksamrarn, S. *et al.* (2006) 'Cytotoxic prenylated xanthones from the young fruit of Garcinia mangostana', *Chemical and Pharmaceutical Bulletin*. doi: 10.1248/cpb.54.301.

Sullivan, M. and Morgan, D. O. (2007) 'Finishing mitosis, one step at a time', *Nature Reviews Molecular Cell Biology*. doi: 10.1038/nrm2276.

Sumara, I. *et al.* (2004) 'Roles of polo-like kinase 1 in the assembly of functional mitotic spindles', *Current Biology*. doi: 10.1016/j.cub.2004.09.049.

Sun, C.-L. (2002) 'Urinary tea polyphenols in relation to gastric and esophageal cancers: a prospective study of men in Shanghai, China', *Carcinogenesis*. doi: 10.1093/carcin/23.9.1497.

Sunkel, C. E. and Glover, D. M. (1988) 'polo, a mitotic mutant of Drosophila displaying abnormal spindle poles.', *Journal of cell science*.

Svendsen, J. M. *et al.* (2009) 'Mammalian BTBD12/SLX4 Assembles A Holliday Junction Resolvase and Is Required for DNA Repair', *Cell*. doi: 10.1016/j.cell.2009.06.030.

Tanaka, K. *et al.* (2005) 'Molecular mechanisms of kinetochore capture by spindle microtubules', *Nature*. doi: 10.1038/nature03483.

Tanaka, T. U. (2008) 'Bi-orienting chromosomes: Acrobatics on the mitotic spindle', *Chromosoma*. doi: 10.1007/s00412-008-0173-5.

Tanaka, T. U., Stark, M. J. R. and Tanaka, K. (2005) 'Kinetochore capture and bi-orientation on the mitotic spindle', *Nature Reviews Molecular Cell Biology*. doi: 10.1038/nrm1764.

Teicher, B. A. (1994) 'Hypoxia and drug resistance', *Cancer and Metastasis Reviews*. doi: 10.1007/BF00689633.

Teixeira, J. H. *et al.* (2014) 'An overview of the spindle assembly checkpoint status in oral cancer', *BioMed Research International*. doi: 10.1155/2014/145289.

Thornberry, N. A. and Lazebnik, Y. (1998) 'Caspases: Enemies within', *Science*. doi: 10.1126/science.281.5381.1312.

Toporcov, T. N. and Wünsch Filho, V. (2018) 'Epidemiological science and cancer control', *Clinics*. doi: 10.6061/clinics/2018/e627s.

Toyoshima-Morimoto, F. *et al.* (2001) 'Polo-like kinase 1 phosphorylates cyclin B1 and targets it to the nucleus during prophase', *Nature*. doi: 10.1038/35065617.

Vanneman, M. and Dranoff, G. (2012) 'Combining immunotherapy and targeted therapies in cancer treatment', *Nature Reviews Cancer*. doi: 10.1038/nrc3237.

Vermeulen, K., Berneman, Z. N. and Van Bockstaele, D. R. (2003) 'Cell cycle and apoptosis', *Cell Proliferation*. doi: 10.1046/j.1365-2184.2003.00267.x.

Vermeulen, K., Van Bockstaele, D. R. and Berneman, Z. N. (2003) 'The cell cycle: A review of regulation, deregulation and therapeutic targets in cancer', *Cell Proliferation*. doi: 10.1046/j.1365-2184.2003.00266.x.

Vineis, P. and Wild, C. P. (2014) 'Global cancer patterns: Causes and prevention', *The Lancet*. doi: 10.1016/S0140-6736(13)62224-2.

Visconti, R., Della Monica, R. and Grieco, D. (2016) 'Cell cycle checkpoint in cancer: A therapeutically targetable double-edged sword', *Journal of Experimental and Clinical Cancer Research*. doi: 10.1186/s13046-016-0433-9.

Waga, S. *et al.* (1994) 'The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA', *Nature*. doi: 10.1038/369574a0.

Walker, D. H. and Maller, J. L. (1991) 'Role for cyclin A in the dependence of mitosis on completion of DMA replication', *Nature*. doi: 10.1038/354314a0.

Walker, R. A. *et al.* (1988) 'Dynamic Instability of Individual Microtubules', *The Journal of cell biology*.

Wallace, D. C. (2012) 'Mitochondria and cancer', *Nature Reviews Cancer*. doi: 10.1038/nrc3365.

Wartmann, M. and Altmann, K. H. (2002) 'The biology and medicinal chemistry of epothilones', *Current Medicinal Chemistry - Anti-Cancer Agents*. doi: 10.2174/1568011023354489.

Watkins, E. *et al.* (2005) 'The Epothilones and Related Analogues-A Review of Their Syntheses and Anti-Cancer Activities', *Current Pharmaceutical Design*. doi: 10.2174/1381612053764742.

Weaver, B. A. A. and Cleveland, D. W. (2005) 'Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death', *Cancer Cell*. doi: 10.1016/j.ccr.2005.06.011.

Weinert, T. (1997) ‘A DNA damage checkpoint meets the cell cycle engine’, *Science*. doi: 10.1126/science.277.5331.1450.

Weinert, T. (1998) ‘DNA damage checkpoints update: Getting molecular’, *Current Opinion in Genetics and Development*. doi: 10.1016/S0959-437X(98)80140-8.

Weiss, E. and Winey, M. (1996) ‘The Saccharomyces cerevisiae spindle pole body duplication gene MPS1 is part of a mitotic checkpoint’, *Journal of Cell Biology*. doi: 10.1083/jcb.132.1.111.

Wendel, H. G. *et al.* (2004) ‘Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy’, *Nature*. doi: 10.1038/nature02369.

Wertz, I. E. and Dixit, V. M. (2010) ‘Regulation of death receptor signaling by the ubiquitin system’, *Cell Death and Differentiation*. doi: 10.1038/cdd.2009.168.

Winkles, J. A. and Alberts, G. F. (2005) ‘Differential regulation of polo-like kinase 1, 2, 3, and 4 gene expression in mammalian cells and tissues’, *Oncogene*. doi: 10.1038/sj.onc.1208219.

Wittmann, T., Hyman, A. and Desai, A. (2001) ‘The spindle: A dynamic assembly of microtubules and motors’, *Nature Cell Biology*. doi: 10.1038/35050669.

World Health Organization (2012) ‘Cancer Fact Sheets’, *Globocan 2012 (Iarc)*.

Wright, W. E. and Shay, J. W. (2000) ‘Telomere dynamics in cancer progression and prevention: Fundamental differences in human and mouse telomere biology’, *Nature Medicine*. doi: 10.1038/78592.

Yokoyama, T. *et al.* (2005) ‘Aurora-B and Rho-kinase/ROCK, the two cleavage furrow kinases, independently regulate the progression of cytokinesis: Possible existence of a novel cleavage furrow kinase phosphorylates ezrin/radizin/moesin (ERM)’, *Genes to Cells*. doi: 10.1111/j.1365-2443.2005.00824.x.

Yu, H. G. *et al.* (2000) ‘The plant kinetochore’, *Trends in Plant Science*. doi: 10.1016/S1360-1385(00)01789-1.

Yuan, T. L. and Cantley, L. C. (2008) ‘PI3K pathway alterations in cancer: Variations on a

theme', *Oncogene*. doi: 10.1038/onc.2008.245.

Zasadil, L. M. *et al.* (2014) 'Cytotoxicity of paclitaxel in breast cancer is due to chromosome missegregation on multipolar spindles', *Science Translational Medicine*. doi: 10.1126/scitranslmed.3007965.

Zhang, H. Z. *et al.* (2004) 'Discovery, characterization and SAR of gambogic acid as a potent apoptosis inducer by a HTS assay', *Bioorganic and Medicinal Chemistry*. doi: 10.1016/j.bmc.2003.11.013.

Zhou, B. B. S. and Elledge, S. J. (2000) 'The DNA damage response: Putting checkpoints in perspective', *Nature*. doi: 10.1038/35044005.

Zhou, J., Yao, J. and Joshi, H. C. (2002) 'Attachment and tension in the spindle assembly checkpoint', *Journal of Cell Science*. doi: 10.1242/jcs.00029.

Zong, W. X. and Thompson, C. B. (2006) 'Necrotic death as a cell fate', *Genes and Development*. doi: 10.1101/gad.1376506.