Universidade de Aveiro Departamento de Biologia
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CATARINA FORTUNA DOS REMÉDIOS

EFEITO CITOTÓXICO DO SULFORAFANO EM CÉLULAS DE OSTEOSSARCOMA HUMANO

CYTOTOXIC EFFECT OF SULFORAPHANE IN HUMAN OSTEOSARCOMA CELLS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, Ramo de Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Helena Cristina Correia de Oliveira, Investigadora Pós-Doutoramento do Departamento de Biologia da Universidade de Aveiro, e co-orientação da Professora Doutora Maria da Conceição Lopes Vieira dos Santos, Professora Associada com Agregação do Departamento de Biologia da Universidade de Aveiro, e do Doutor José Miguel Pimenta Ferreira de Oliveira, Investigador Pós-Doutoramento do Departamento de Biologia da Universidade de Aveiro.

Aos meus pais, irmã e F. que todos os dias encurtam os quilómetros que nos separam. À Bia, por todo o amor, por todo o apoio, por toda uma vida, por tudo.

o júri

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"I find hope in the darkest of days, and focus in the brightest. I do not judge the universe."

Dalai Lama

palavras-chave

Osteossarcoma, MG-63, sulforafano, citotoxicidade, terapia do cancro, ciclo celular, apoptose, expressão génica

resumo

O osteossarcoma é o tipo mais comum de tumor maligno do osso. Esta doença apresenta uma elevada incidência em crianças e adolescentes. Adicionalmente, metástases, ocorrendo principalmente nos pulmões, afectam uma percentagem considerável dos pacientes com osteossarcoma. Os tratamentos actuais incluem ressecção cirúrgica, quimioterapia e radioterapia, mas os pacientes com osteossarcoma apresentam uma baixa capacidade de resposta a estas opções de tratamento. Assim, é urgente desenvolver novas abordagens de tratamento. O sulforafano é um fitocomposto que exibe actividade quimiopreventiva e quimioterápica. Numerosos estudos têm demonstrado que o sulforafano modula muitos eventos associados ao cancro, como susceptibilidade a agentes carcinogénicos, ciclo celular, apoptose, angiogénese e metastização. Apesar de bem caracterizados em diversos tumores, os efeitos do sulforafano são ainda insuficientemente conhecidos no osteossarcoma, assim como o mecanismo de acção subjacente. O objectivo desta dissertação foi avaliar os potenciais efeitos citostático e apoptótico do sulforafano e revelar o mecanismo de acção por trás destes efeitos. Para isso, a linha celular de osteossarcoma humano MG-63 foi exposta a sulforafano (no intervalo de 0 a 20 μM) durante 24 e 48 h. Parâmetros como confluência, morfologia celular, viabilidade celular, ciclo celular, clastogenicidade, apoptose, actividade específica de caspases e expressão de genes associados ao ciclo celular foram analisados após o tratamento com sulforafano. As culturas expostas ao sulforafano apresentaram uma diminuição da confluência, acompanhada de alterações na morfologia celular. O sulforafano também induziu uma diminuição da viabilidade celular dependente da dose e do tempo de exposição que pode ser explicada pelo aumento de células apoptóticas e por citostaticidade com bloqueio na fase G_2/M. Este bloqueio do ciclo celular foi mediado por Chk2, pela sub-expressão de *CDC25C* e, provavelmente, pela sub-expressão de *CCNB1*, assim como de *CDK2*. Um efeito clastogénico foi também detectado após 48 h de tratamento com sulforafano. Estes dados sugerem que o sulforafano deve ser considerado um candidato a agente quimioterápico no tratamento de osteossarcoma, embora sejam necessários mais estudos para lançar luz sobre vias de sinalização moduladas pelo sulforafano, assim como sobre a possível citotoxicidade deste fitocomposto em osteoblastos normais.

keywords Osteosarcoma, MG-63, sulforaphane, cytotoxicity, cancer therapy, cell cycle, apoptosis, gene expression

abstract Osteosarcoma is the most common type of malignant bone tumor. This disease shows high incidence in children and adolescents. Additionally, metastases occurring mainly at the lungs affect a considerable percentage of osteosarcoma patients. Current treatments include surgical resection, chemotherapy, and radiotherapy, but osteosarcoma patients show poor responsiveness to these treatment options. Thus, it is urgent to develop novel treatment approaches. Sulforaphane is a phytochemical that displays chemopreventive and chemotherapeutic activity. Many studies have been shown that sulforaphane modulates many cancer-related events, as susceptibility to carcinogenic agents, cell cycle, apoptosis, angiogenesis, and metastasis. Although well characterized in several tumors, the effects of sulforaphane are still poorly understood in osteosarcoma, as well as the underlying mechanism of action. The aim of this dissertation was to evaluate the potential cytostatic and apoptotic effects of sulforaphane and to unveil the mechanism of action behind these effects. In order to do that, the human osteosarcoma cell line MG-63 was exposed to sulforaphane (in the 0 to 20 µM range) for 24 and 48 h. Parameters as confluence, cell morphology, cell viability, cell cycle, clastogenicity, apoptosis, caspase specific activity, and expression of cell cycle-related genes were analyzed after sulforaphane treatment. Cultures exposed to sulforaphane showed a decrease in confluence, accompanied by alterations in cell morphology. Sulforaphane also induced a dose- and time-dependent impairment of cell viability, which can be explained by an increase of apoptotic cells and cytostaticity with G_2/M phase arrest. This cell cycle blockage was mediated by Chk2 and *CDC25C* downregulation, and probably *CCNB1* as well as *CDK2* downregulation. A clastogenic effect was also detected after 48 h of sulforaphane treatment. These data suggest that sulforaphane should be considered as a chemotherapeutic agent candidate in osteosarcoma treatment, although more studies are needed to shed insight on signaling pathways modulated by sulforaphane, as well as the possible cytotoxicity of this phytochemical on normal osteoblasts.

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List of Abbreviations

Akt	RAC-alpha serine/threonine-protein kinase
Apaf-1	Apoptotic protease-activating factor 1
ARE	Antioxidant response element
ATCC	American Type Culture Collection
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ATM- and Rad3-related protein
Bak	B-cell lymphoma protein 2-antagonist/killer 1
Bax	B-cell lymphoma protein 2-associated X protein
Bcl-2	B-cell lymphoma protein 2
Bcl-X_L	B-cell lymphoma protein 2-related protein, long isoform
Bid	BH3-interacting domain death agonist
BLM	Bloom syndrome protein coding gene
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
CCNB1	Cyclin B1 coding gene
Cdc25	Cell division cycle 25
CDC25C	M-phase inducer phosphatase 3 coding gene
Cdc25C	M-phase inducer phosphatase 3
CDK	Cyclin-dependent kinase
CDK1	Cyclin-dependent kinase 1 coding gene
Cdk1	Cyclin-dependent kinase 1
CDK2	Cyclin-dependent kinase 2 coding gene
Cdk2	Cyclin-dependent kinase 2
Cdk4	Cyclin-dependent kinase 4
CDKN2A	Cyclin-dependent kinase inhibitor 2A coding gene
cDNA	Complementary DNA

CDS	Coding DNA sequence
CHEK1	Checkpoint kinase 1 coding gene
CHEK2	Checkpoint kinase 2 coding gene
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CIN	Chromosomal instability
Cip	Cyclin-dependent kinase interacting protein
CKI	Cyclin-dependent kinase inhibitor
c-Myc	Myc proto-oncogene protein
Ct	Cycle threshold
CYP	Cytochrome P450 enzyme
Cyt c	Cytochrome c
DEPC	Diethylpyrocarbonate
DISC	Death-inducing signaling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FADD	Fas-associated death domain
Fas	Tumor necrosis factor receptor superfamily member 6
FasL	Fas ligand
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FPCV	Full peak coefficient of variation
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase coding gene
GLS	Glucosinolate
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2

Hh	Hedgehog
HIF-1	Hypoxia-inducible factor 1
HIF-1α	Hypoxia-inducible factor 1-alpha
ICAD	Inhibitor of caspase-activated DNase
Ink4	Inhibitor of cyclin-dependent kinase 4
ITC	Isothiocyanate
KDR/flk-1	Kinase insert domain receptor/ Fetal liver kinase 1
Keap1	Kelch-like ECH-associated protein 1
Kip	Kinase inhibitor protein
Mdm2	Human homologue of murine double minute 2
α-MEM	α-Minimum Essential Medium
MIRA-1	Mutant p53 reactivation and induction of rapid apoptosis
MMP	Matrix metalloproteinase
MMP-2	Matrix metalloproteinase 2
MMP-9	Matrix metalloproteinase 9
MPF	Maturation-promoting factor
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyl tetrazolium bromide
Myt1	Membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase
***p*NA**	*p*-nitroanilide
Noxa	Phorbol-12-myristate-13-acetate-induced protein 1
Nrf2	Nuclear factor erythroid 2-related factor 2
OS	Osteosarcoma
$p15^{Ink4b}$	Cyclin-dependent kinase inhibitor 2B
$p16^{Ink4a}$	Cyclin-dependent kinase inhibitor 2A
$p18^{Ink4c}$	Cyclin-dependent kinase inhibitor 2C
$p19^{Ink4d}$	Cyclin-dependent kinase inhibitor 2D

$p21^{Cip1}$	Cyclin-dependent kinase inhibitor 1A
$p27^{Kip1}$	Cyclin-dependent kinase inhibitor 1B
p53	Tumor protein p53
$p57^{Kip2}$	Cyclin-dependent kinase inhibitor 1C
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PRIMA-1	p53 reactivation and induction of massive apoptosis
PS	Phosphatidylserine
Puma	B-cell lymphoma protein 2-binding component 3
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
Rb	Retinoblastoma-associated protein
RecQ4	ATP-dependent DNA helicase Q4
RECQL4	ATP-dependent DNA helicase Q4 coding gene
RFU	Raw fluorescence unit
RNA	Ribonucleic acid
RNase	Ribonuclease
ROS	Reactive oxygen species
RUNX2	Runt-related transcription factor 2 coding gene
Runx2	Runt-related transcription factor 2
SD	Standard deviation
SFN	Sulforaphane
Smac/DIABLO	Second mitochondria-derived activator of caspase/Direct IAP-binding protein with low pI
TGF-β	Transforming growth factor β
TNF	Tumor necrosis factor

TP53	p53 coding gene
TRADD	Tumor necrosis factor receptor-associated death domain
TRAF2	Tumor necrosis factor receptor-associated factor 2
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TRAIL-R1	Tumor necrosis factor-related apoptosis-inducing ligand receptor 1
VEGF	Vascular endothelial growth factor
Wee1	Wee1-like protein kinase
Wnt	Wingless-type
XIAP	X-linked inhibitor of apoptosis protein

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I. INTRODUCTION

1.1. Cancer

1.1.1 General considerations about cancer

Cancer is a major burden worldwide. Economically developed countries present overall cancer incidence rates twice as high as the ones found in developing countries (Jemal *et al.*, 2011), mainly due to the Western lifestyle. According to the American Cancer Society, it is estimated that 1,638,910 men and women will be diagnosed with cancer and 577,190 men and women will die of cancer in the United States in 2012, being these rates slightly higher for men than for women and with lung, colorectal, breast, and prostate cancers accounting for the largest numbers of new cases and deaths (Siegel *et al.*, 2012). In Europe, cancer was listed as the second most common cause of death after cardiovascular diseases (OECD, 2012) with approximately 3.2 million estimated new cases and 1.7 million estimated deaths in 2008 (Ferlay *et al.*, 2010). Recent data project 1,283,101 cancer deaths to occur in Europe during the current year, with lung (262,250), colorectal (163,106), breast (88,101), and prostate (69,960) cancers as the main causes (Malvezzi *et al.*, 2012), similarly to the United States.

From a simplistic point of view, cancer can be defined as a group of diseases in which abnormal cells proliferate in an uncontrolled manner and spread to other tissues. However, during the past few decades, an immense body of knowledge evidenced the inherent complexities of cancer, but fortunately also unveiled its characteristics and the underlying molecular mechanisms.

The etiological factors of cancer vary considerably from case to case. Chemical agents, lifestyle (e.g., diet, obesity, tobacco smoke, alcohol consumption), infections, radiation, genetic problems, and hormones are some of the factors already proved to correlate with carcinogenesis (Langeberg *et al.*, 2007; Khan and Shrivastava, 2010; Khan *et al.*, 2010; Vahakangas, 2011). However, the general established conception is that cancer results from cumulative genomic alterations that lead to atypical RNA and protein profiles, which

in the end originate the abnormal cancer phenotype. Such alterations are often found in genes that in the cancer scenario behave as either tumor suppressors or oncogenes (Hahn and Weinberg, 2002). The range of cancer-inducing genomic changes is vast, yielding from intragenic mutations to aneuploidy events. Loss of function of tumor suppressors is usually directly or indirectly related with intragenic mutations (Malkin *et al.*, 1990) and deletions or allelic loss (Wei *et al.*, 1999; Yoshimoto *et al.*, 2007) of the corresponding genes. In the case of oncogenes, mutations (Akagi *et al.*, 2007), chromosomal translocations (MacKenzie *et al.*, 1993), and genomic amplifications (Birkeland *et al.*, 2012) are the three major events explaining gain of function. In recent years, epigenetic alterations, heritable alterations in gene expression without changes in the primary DNA sequence (Sadikovic *et al.*, 2008a), were also found to be main contributors for cancer development. Variations in the DNA methylation profile of genes or gene promoters are common drivers of carcinogenesis (Futscher *et al.*, 2004), but histone modifications (Kondo *et al.*, 2007) and changes in nucleosome positioning (Medina and Sanchez-Cespedes, 2008) are important players in that process, as well.

Based on these and other findings of the past decades and on the assumption that the molecular machinery owned by mammalian cells is equable, Douglas Hanahan and Robert A. Weinberg suggested that most – if not all – human cancers share the same functional capabilities that allow cancer cells to survive, proliferate, and disseminate (Hanahan and Weinberg, 2000). The six hallmarks of cancer proposed by these authors in 2000 include:

1) Self-sufficiency in growth signals. In order to grow and proliferate, normal cells require the binding of growth signals to transmembrane receptors and the subsequent activation of intracellular signal transduction cascades that culminate in a pro-growth cellular response. Tumor cells can reduce the dependency on exogenous signals by different means, such as producing their own growth signals (e.g., platelet-derived growth factor (PDGF) (Furuhashi *et al.*, 2004)) and overexpressing growth signal receptors (e.g., human epidermal growth factor receptor 2 (HER2) (Loi *et al.*, 2011)), thus breaching homeostasis and achieving abnormal growth rates.

2) Insensitivity to antigrowth signals. Besides growth signals, a normal cell is also exposed to antigrowth signals from the environment. As the name suggests, these molecules act as antiproliferative signals, inducing either a quiescent state or cell differentiation. Deregulation of proteins involved in signaling pathways transducing

antigrowth signals is often found in cancers and usually such proteins are intimately related with cell cycle progression. One example is retinoblastoma-associated protein (Rb), a major transducer of antigrowth signals that inhibits transcription factors, preventing cell cycle progression (Jonsson *et al.*, 2012).

3) Evasion of apoptosis. Together with proliferation rates, the life span of a tumor cell is an important determinant of the overall tumor progression. Apoptosis is the cell death pathway commonly activated to maintain tissue homeostasis, but cancer cells seem to be resistant to this mechanism. Alterations in apoptotic mediators are extremely abundant in cancers. Amongst such mediators, p53, a tumor suppressor also known as "guardian of the genome" due to its function when DNA damage occurs, is frequently found to suffer different deregulations (Malkin *et al.*, 1990; Pogribny and James, 2002), preventing activation of the apoptotic cascade.

4) Limitless replicative potential. Within a normal tissue, cells undergo a finite number of replications, after which they become senescent. This event can be explained by the existence of telomeres, thousands of short nucleotide sequence repeats protecting the ends of chromosomes. During each replication cycle, chromosomes lose telomeric DNA, resulting in a progressive telomere shortening throughout cell life that eventually leads to death (Londono-Vallejo and Wellinger, 2012). However, tumor cells appear to undergo unlimited replications, thanks to the expression of functional telomerase, the DNA polymerase that adds DNA sequence repeats to the 3' ends of chromosomal DNA for telomere maintenance, which is almost absent in normal cells (Chen and Chen, 2011).

5) Sustained angiogenesis. A critical step in tumor progression is the maintenance of the expansion rate. As the tumor becomes larger, the inner cells of the tumor mass experience unfavorable conditions and often die due to the lack of blood supply that enables delivery of nutrients and oxygen, as well as removal of waste products and carbon dioxide. In order to surpass short blood supply and to continue to grow, tumors must activate an "angiogenic switch" and induce angiogenesis, the process of blood vessel formation from quiescent blood vessels (Hanahan and Folkman, 1996). Under hypoxic conditions, hypoxia-inducible factor 1 (HIF-1), an heterodimer possessing an oxygen sensor subunit (HIF-1α), is responsible for prompting angiogenesis in several human cancers via transcription factor activity that results in overexpression of vascular endothelial growth factor (VEGF); VEGF is a major growth signal that binds to tyrosine

kinase receptors of endothelial cells, stimulating proliferation and generation of new blood vessels (Semenza, 2000). Both oxygen deprivation (Shweiki *et al.*, 1992) and genetic alterations causing increased activity of HIF-1 (Maxwell *et al.*, 1999; Ravi *et al.*, 2000) seem to be two reasonable mechanisms behind angiogenesis in tumors.

6) Tissue invasion and metastasis. This pivotal step in tumor development comprises the escape of tumor cells from the initial tumor mass, the invasion of other tissues (adjacent or distant), and the settlement of new colonies: metastatic or secondary tumors. The fifth hallmark is intrinsically connected with the metastatic process providing the main route for primary tumor cells evasion and displacement (Zetter, 1998). Cancer cells that acquired this capability often present loss of molecules mediating cell-cell adhesion (e.g., cadherins (Yoshida *et al.*, 2001)). Other feature that enhances the invasive potential of tumor cells is the upregulation of matrix metalloproteinases (MMPs) (Lafleur *et al.*, 2005; Kryczka *et al.*, 2012), zinc-dependent extracellular matrix-degrading proteases.

These functional capabilities are attained through a multistep tumorigenic process driven by the accumulation of genomic alterations, but one particular lesion may give rise to several capabilities at the same time (Hanahan and Weinberg, 2000).

Four years later, Dunn, Old, and Schreiber proposed a seventh hallmark of cancer: avoidance of immunosurveillance (Dunn *et al.*, 2004). In truth, highly immunogenic cancer cells can be detected and destroyed by the immune system, but the remaining ones are spared due to their weak immunogenic phenotype (immunoediting) and ability to dismantle components of both arms of the immune system (Dunn *et al.*, 2004).

In 2008, Guido Kroemer and Jacques Pouyssegur discussed the complex interactions between these seven hallmarks and tumor cell metabolism (Kroemer and Pouyssegur, 2008). Many tumor cells undergo a “metabolic switch” in which their energy production is highly limited to glycolysis instead of mitochondrial oxidative phosphorylation even in normoxic conditions, a concept brought up by Otto Warburg many decades ago (Warburg, 1956). Underlying this metabolic reprogramming is, among others, the upregulation of glucose transporters and enzymes of the glycolytic pathway (Jones and Thompson, 2009).

Last year, Hanahan and Weinberg (2011) revisited their former list of hallmarks, highlighted the importance of tumor microenvironment in the carcinogenic process, included the mechanisms that have been discussed in the meantime, and considered new

hallmarks: genome instability and mutation, and tumor-promoting inflammation. The first refers to the acceleration in the accumulation of mutations by the cancer cell through a variety of mechanisms, such as the collapse of DNA damage sensors or DNA damage repairing machinery (Jackson and Bartek, 2009). The second, explores the ability of tumor cells to induce an inflammatory response in the tumor microenvironment, that promotes several steps of the carcinogenic process; innate immune cells play here an important role by releasing reactive oxygen species (ROS) and growth factors in the vicinity of the tumor (Qian and Pollard, 2010).

Figure 1 illustrates the establishment of cancer hallmarks throughout the years as result of the incessant research on this subject.

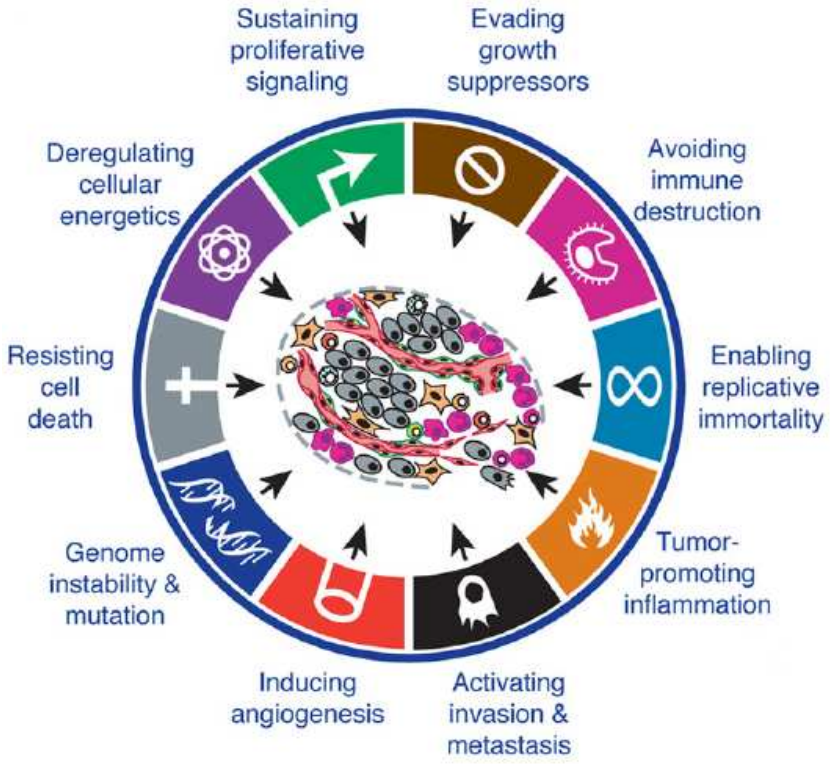

Figure 1. Cancer hallmarks established over the past decade. The 2011 up-to-date Hanahan and Weinberg's scheme enclosing: the six acquired hallmarks of cancer cells (sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis) initially proposed by these authors in 2000; a seventh hallmark (avoiding immune destruction) recommended by Dunn and colleagues in 2004; the deregulated cellular energetics integrated by Kroemer and Pouyssegur in 2008; and two enabling characteristics (genome instability and mutation, and tumor-promoting inflammation) added by Hanahan and Weinberg in 2011. Adapted from Hanahan and Weinberg (2011).

1.1.2. Target mechanisms in cancer therapy

The compelling body of research and knowledge accomplished in the past decades lead to a better understanding of carcinogenesis and the molecular mechanisms behind it, and concomitantly to the progress in identifying novel targets for mechanism-based targeted therapies in human cancers. These therapies target specific key molecules that are involved in aberrant signaling pathways, thus modulating proliferation, differentiation, and death of cancer cells (Cattley and Radinsky, 2004). Such specificity can bring advantages for cancer treatment, as it is expected to show more effective results in cancer cells than current treatment approaches and less harmful effects on normal cells. As is already apparent, the mechanisms underlying cancer hallmarks offer attractive therapeutic targets and, in the next sections, two of these mechanisms – cell cycle and apoptosis – will be described in more detail and some targeted therapies exploiting them will be enumerated.

1.1.2.1. *Cell cycle*

Mammalian cell proliferation consists of repeated cell cycles that are divided in two main stages: mitosis (M), the process of nuclear division, and interphase, the period from the end of one mitosis to the beginning of the next one. Mitosis is subdivided in prophase, metaphase, anaphase, and telophase, whereas interphase comprises G_1 (growth phase), S (DNA synthesis phase), and G_2 (gap phase) phases (Norbury and Nurse, 1992). Non-proliferating cells, which accounts for the majority of cells in the human body, enter a quiescent state at G_1 that prevents further divisions – the G_0 phase. As many other processes, cell cycle is strongly regulated by a wide number of proteins.

Progression through the cell cycle is controlled by active protein kinase complexes, composed of a catalytic cyclin-dependent kinase (CDK, a family of serine/threonine protein kinases) and a regulatory cyclin. Throughout the cell cycle, CDK protein levels remain steady, but cyclin protein levels oscillate due to cyclic synthesis and degradation, leading to a periodically activation of CDKs (Pines, 1995). Consequently, CDKs are sequentially activated by specific cyclins during different phases, inducing cell cycle progression by phosphorylation of downstream proteins (Figure 2).

CDK activity can also be counteracted by CDK inhibitors (CKIs) from two distinct families: Cip/Kip (for CDK interacting protein/kinase inhibitor protein), which includes $p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$; and Ink4 (for inhibitor of cyclin-dependent kinase 4 (Cdk4)), that

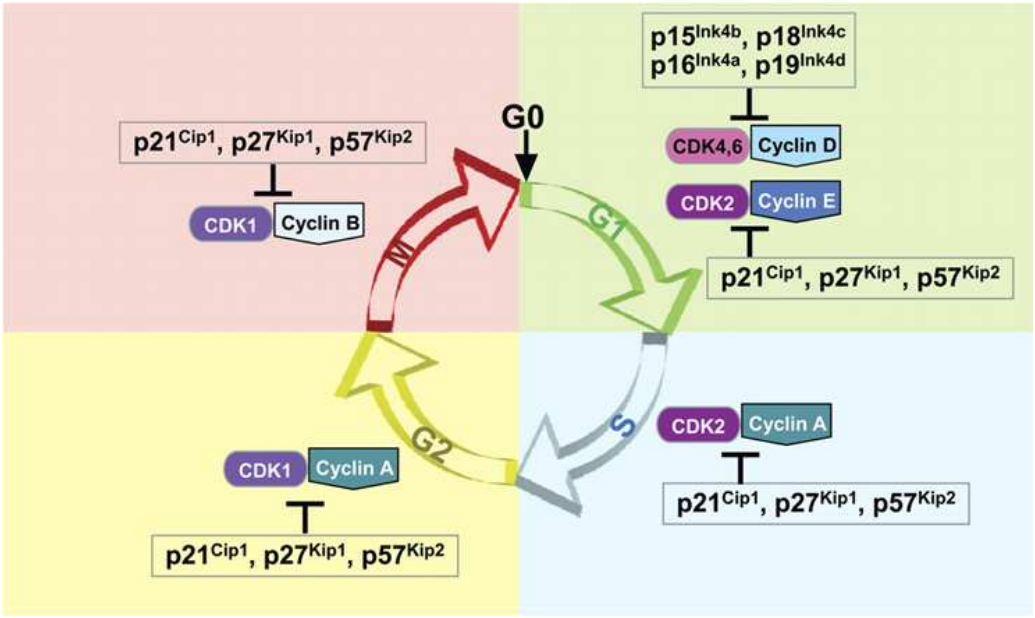

Figure 2. General scheme of the main cell cycle phases of mammalian cells and its key regulators. The specific CDK/cyclin associations required in each cell cycle phase and the CKIs that negatively regulate the main drivers of cell cycle progression. Adapted from Fuster *et al.* (2010).

comprises $p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$, $p19^{Ink4d}$. The Cip/Kip proteins interact with and inhibit various CDK/cyclin complexes (Harper *et al.*, 1995), whereas Ink4 proteins specifically bind to G_1 CDKs, preventing their association with cyclin D (Carnero and Hannon, 1998) (Figure 2). In turn, CKIs are regulated by different internal and external signals. For instance, $p21^{Cip1}$ expression is transcriptionally activated by the tumor suppressor p53 when DNA damage is detected (el-Deiry *et al.*, 1993) and the expression and activation of $p15^{Ink4b}$ and $p27^{Kip1}$, respectively, are influenced by transforming growth factor β (TGF-β) (Reynisdottir *et al.*, 1995).

In addition, CDK activity is regulated by phosphorylation/dephosphorylation on conserved threonine and tyrosine residues. For example, Wee1-like protein kinase (Wee1) and membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase (Myt1) inactivate cyclin-dependent kinase 1 (Cdk1) by tyrosine-15 and/or threonine-14 phosphorylation, and dephosphorylation of these sites is required for re-establishment of Cdk1 activity, which is mediated by phosphatases of the cell division cycle 25 (Cdc25) family (Medema and Macurek, 2012). Besides these molecules, others involved in

different signaling pathways (e.g., PI3K/Akt/mTOR pathway) can interact and modulate the cell cycle machinery.

Investigation of cell cycle deregulations in the cancer context resulted in the development of some agents that target components of these pathways. Alvocidib is a small molecule that inhibits CDKs, inducing cell cycle arrest (Lee and Sicinski, 2006). NVP-BEZ235, an imidazo [4,5-*c*] quinoline derivative, induces G_1 arrest by targeting the ATP-binding cleft of PI3K (for phosphatidylinositol 3-kinase) and mTOR (for mammalian target of rapamycin), whose activity is frequently altered in human cancer cells (Maira *et al.*, 2008).

1.1.2.2. *Apoptosis*

Apoptosis, also known as programmed cell death, is a controlled type of cell death that plays an important role in tissue homeostasis. This process occurs in physiological conditions (e.g., embryonic development, normal cell turnover), but also as a response to pathological conditions (e.g., immune reactions) and cell injuries (e.g., radiation, hypoxia, harmful chemical agents) (Norbury and Hickson, 2001). Apoptosis is generally characterized by distinct morphological and biochemical characteristics. These hallmarks of apoptosis include expression of phosphatidylserine (PS) in the outer layer of the cell membrane, cell shrinkage, retraction of pseudopods, rounding up of the cell, membrane blebbing, ultrastructural modification of cytoplasmic organelles, chromatin condensation, DNA fragmentation, and loss of membrane integrity (Kroemer *et al.*, 2005). At the end of the apoptotic process, the cellular content is enclosed into apoptotic bodies ("budding"), which are subsequently engulfed and degraded by phagocytic cells. Due to the restriction of the cellular constituents in apoptotic bodies, the rapid phagocytosis of apoptotic remains by surrounding cells and the absence of anti-inflammatory cytokines production by the latter cells, apoptosis is in essence not associated with inflammatory reactions (Kurosaka *et al.*, 2003). Another feature of apoptosis is the activation of a group of enzymes belonging to the caspase (cysteine-aspartic acid protease) family that function as initiators and executioners of the apoptotic process (Lavrik *et al.*, 2005). The activation of caspases can occur through two major pathways (Figure 3).

The extrinsic or death receptor pathway is triggered when external signals bind to surface cell death receptors (e.g., tumor necrosis factor (TNF), Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL) bind to TNF receptors, TNF receptor superfamily member 6 (Fas), and TRAIL receptors, respectively) (Hengartner, 2001). Then, the

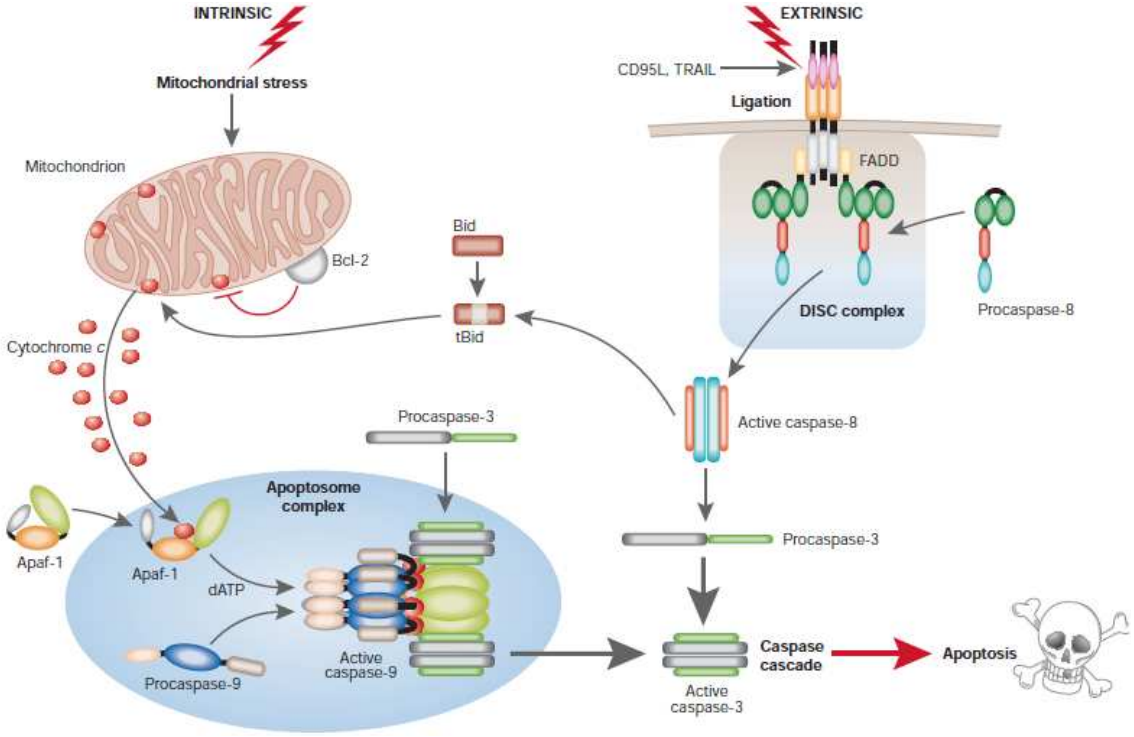

Figure 3. The intrinsic and extrinsic pathways to caspase activation in apoptosis. Activation of caspase 9 by apoptosome complex and caspase 8 by DISC complex as a result of the triggering of mitochondrial and death receptor apoptotic pathways, respectively. The initiator caspases 8 and 9 cleave procaspase 3, leading to the activation of an executioner caspase cascade that ends in apoptosis. Members of the Bcl-2 family modulate apoptosis through the control of mitochondrial Cyt c release. Extracted from MacFarlane and Williams (2004).

intracellular domain of death receptors recruits adapter proteins, such as TNF receptor-associated death domain (TRADD) or Fas-associated death domain (FADD), as well as procaspase 8, forming a ligand-receptor-adapter protein complex, named death-inducing signaling complex (DISC), that activates the initiator caspase 8 (Schneider and Tschopp, 2000).

On the other hand, the intrinsic or mitochondrial pathway integrates various intracellular signals (e.g., irreparable DNA damage, hypoxia, severe oxidative stress) that cause mitochondrial permeability and release of pro-apoptotic molecules, such as cytochrome c (Cyt c) into the cytoplasm. Once released, Cyt c binds to apoptotic protease-activating factor 1 (Apaf-1), which in turn recruits procaspase 9, resulting in the formation of the apoptosome complex and activation of the initiator caspase 9 (Kroemer *et al.*, 2007). The release of Cyt c from the mitochondria is partly regulated by B-cell lymphoma protein 2

(Bcl-2) family members, with anti-apoptotic (e.g., Bcl-2, Bcl-2-related protein, long isoform (Bcl-X_L)) and pro-apoptotic (e.g., Bcl-2-associated X protein (Bax), Bcl-2-antagonist/killer 1 (Bak), BH3-interacting domain death agonist (Bid)) activity (Reed, 1997).

Each pathway culminates in the activation of a proteolytic cascade involving the sequential breakdown of procaspases 3, 6, and 7, and hence their activation, by the corresponding initiator caspases. This execution phase leads to the cleavage of downstream cellular substrates that in part explain the biochemical and morphological changes during apoptosis.

Alternatively, a third less well-known apoptotic pathway can occur, in which it is believed that caspase 12 is activated by the adapter protein TNF receptor-associated factor 2 (TRAF2) as a result of an injured endoplasmic reticulum (O'Brien and Kirby, 2008). In addition, DNA damage can lead to apoptotic death through a p53-dependent pathway. The pro-apoptotic proteins Bax, phorbol-12-myristate-13-acetate-induced protein 1 (Noxa), and Bcl-2-binding component 3 (Puma) were described as transcriptional targets of p53. Alternatively, p53 can also activate death receptors and repress anti-apoptotic proteins (Amaral *et al.*, 2010).

Some of the described constituents of the apoptotic cascades have been pivotal for the design of new therapies. Anti-apoptotic members of these cascades are promising targets for new therapeutic agents, as ABT-737, a small molecule capable of inhibiting the activity of Bcl-X_L through binding to its BH3 groove (Stauffer, 2007), and Oblimersen, an antisense oligonucleotide that inhibits the expression of Bcl-2 by blocking the translation of its mRNA (Moreira *et al.*, 2006). Mapatumumab, a fully human agonistic monoclonal antibody, is another therapeutic alternative that binds and activates TRAIL-R1 (for TRAIL receptor 1) to induce apoptosis (Carlo-Stella *et al.*, 2007). The induction of p53-dependent apoptosis can also be achieved with PRIMA-1 (for p53 reactivation and induction of massive apoptosis) and MIRA-1 (for mutant p53 reactivation and induction of rapid apoptosis), two compounds that reactivate the function of mutant p53 (Selivanova and Wiman, 2007), and with Nutlin-3 that binds to Mdm2 (human homologue of murine double minute 2, a negative regulator of p53) and inhibits the binding and ubiquitination of p53 (Vassilev, 2007).

1.2. Osteosarcoma

1.2.1. Bone physiology

Throughout life, bone tissue undergoes different processes. Longitudinal and radial growth of bones occurs mainly during childhood and adolescence, a rapid growth phase in an individual's life. The process of bone modeling (change of the general bone shape influenced by physiological or mechanical forces) and bone remodeling (bone renewal to maintain mineral homeostasis and bone strength) are also important for safeguarding the health and performance of skeleton (Clarke, 2008). In one way or another, these critical processes are dependent on the action of two cellular types: osteoclasts and osteoblasts.

Osteoclasts are cells capable of resorbing bone, which is essential for the removal of old, microdamaged bone tissue. These cells bind to bone matrix and secrete H^+ ions and acidified vesicles containing MMPs, cathepsin K, and other enzymes (Teitelbaum *et al.*, 1995). The resorption area is limited by osteoclasts through a rearrangement of its cytoskeleton that forms a sealing zone wherein the degradation of bone tissue occurs (Vaananen *et al.*, 2000).

In contrast, osteoblasts are responsible for formation of new bone matrix on appropriate surfaces. Osteoblasts are generated from osteoprogenitor cells that descend from mesenchymal stem cells, and amongst osteoblasts' functions one can count: synthesis of bone matrix components, such as collagen-related proteins; regulation of the mineralization process of bone matrix via release of vesicles that concentrate calcium and phosphate; and destruction of mineralization inhibitors, such as pyrophosphate or proteoglycans (Anderson, 2003). After bone formation, 50-70% of osteoblasts undergo apoptosis (Clarke, 2008).

Bone remodeling is particularly highly dependent on the coupled action of osteoclasts and osteoblasts that sequentially remove old bone and replace it with newly synthesized and mineralized bone matrix. Thus, any imbalance in the osteoblast physiology may cause severe damages in the bone structure.

1.2.2. Osteosarcoma: epidemiology, clinical aspects, diagnostic methods, histologic features, and etiology

Osteosarcoma (OS) or osteogenic sarcoma is a primary malignant bone tumor characterized by the formation of immature bone or osteoid (the unmineralized, organic component of bone matrix) by tumor cells.

This tumor is the most frequent primary bone sarcoma, representing around 20% of all bone tumors, and the most common primary malignant bone tumor in children and adolescents (Marina *et al.*, 2004; Tang *et al.*, 2008). Nevertheless, OS is considered a rare disease, accounting for only 0.2% of all malignant tumors (Greenlee *et al.*, 2001). OS has a bimodal age distribution, with a first and larger peak incidence during the second decade of life (the adolescent growth spurt) and a minor second peak in elderly adults (Marina *et al.*, 2004). Children and adolescents represent approximately 75% of OS patients (Picci, 2007) and boys are more frequently affected than girls (Marina *et al.*, 2004). A slightly higher incidence of OS in African-American children than in Caucasian children is also reported (Marina *et al.*, 2004). In 80-90% of cases, OS occurs in long tubular bones and is usually originated in the metaphysis. Children, adolescents, and young adults affected with this malignant disease normally present high-grade tumors in long bones of the extremities (rapid bone growth areas), including femur, tibia, and humerus (Marina *et al.*, 2004; Tang *et al.*, 2008; Gorlick and Khanna, 2010). In the second OS peak incidence, corresponding to individuals over the age 60, low-grade tumors are typically found in axial sites (Gorlick and Khanna, 2010). These differences may suggest different underlying mechanisms for the development of OS in younger and older patients.

The most common and early symptom of OS (2-4 months before diagnosis) is pain, caused either by the stretching of the periosteum or bone deterioration due to stress fractures, and generally arises after vigorous physical exercise or trauma (Marina *et al.*, 2004; Ta *et al.*, 2009). Some patients complain about swelling, related to soft tissue extension, and rare systemic symptoms as weight loss, pallor, fever, and anorexia can also be reported (Picci, 2007). In 80% of cases, conventional OS is localized in one bone site, presenting metastases in about 20% of patients (Ta *et al.*, 2009). Still, a great percentage of patients with localized OS (80%) develop metastatic disease after surgical resection, which means that almost all OS patients are affected by metastatic disease (Marina *et al.*, 2004). The most frequent metastatic site is the lung, with secondary tumors occurring in this organ in

approximately 90% of patients with metastatic OS (Longhi *et al.*, 2003; Gorlick and Khanna, 2010). Besides lung, bone metastases are also common, generally appearing after pulmonary metastases are already established (Ta *et al.*, 2009). However, the occurrence of multiple bone metastases may actually reflect multifocal primary tumors (Marina *et al.*, 2004). “Skip metastases” (outside the reactive zone, but within the same bone or across the neighboring joint) and lymph node metastases are rare (Ta *et al.*, 2009) and metastatic disease at OS recurrence can affect the central nervous system (Marina *et al.*, 1993), as well. Death in OS patients is usually caused by progressive pulmonary metastasis that leads to respiratory failure (Marina *et al.*, 2004).

The initial imaging diagnostic modality for OS is commonly radiography, showing sclerotic, lytic or mixed lesions in the affected bone with periosteal elevation and often production of soft tissue swelling (Picci, 2007). Other important diagnostic methods include computed tomography and magnetic resonance imaging, which are useful to predict the tumor extension and determine surgical resection (Panicek *et al.*, 1997). Elevated levels of alkaline phosphatase and lactate dehydrogenase have been reported to have prognostic significance (Marina *et al.*, 2004; Picci, 2007). Biopsy of the affected area for confirmation is a mandatory step in diagnosis.

Conventional OS consists of primary intramedullary high-grade sarcoma and presents a wide spectrum of histologic appearances that share common characteristics: proliferation of malignant mesenchymal stem cells and production of bone and/or osteoid (Marina *et al.*, 2004). Histologically, the World Health Organization classifies conventional OS in three major subtypes - osteoblastic, chondroblastic, and fibroblastic - that reflect the predominant type of matrix in the tumor, as well as the mesenchymal origin of the malignant cells and their ability to differentiate into various cell types (Raymond *et al.*, 2002). Osteoblastic OS is found in 70% of conventional OS cases (Tang *et al.*, 2008) and is characterized by the production of osteoid or bone as the main type of matrix and the presence of malignant plasmacytoid to epithelioid osteoblasts (Marina *et al.*, 2004). Chondroblastic and fibroblastic OS tumors account for 10% of conventional OS, each (Tang *et al.*, 2008). The first subtype shows mostly chondroid matrix with malignant cells within the lacunae, while the second subtype is composed of malignant spindle cells with scarce osteoid (Marina *et al.*, 2004). In addition to the conventional OS subtypes, anaplastic, telangiectatic, giant cell-rich, and small cell OS are recognized as rare OS

subtypes (Tang *et al.*, 2008). Despite the variety found between patient samples and even within a single tumor, the clinical behavior of OS doesn't seem to be affected by this divergence.

The current understanding of OS etiology is still limited. Because the major peak incidence coincides with the adolescent growth spurt, it is believed that the occurrence of OS is correlated with rapid bone growth. This is supported by the fact that girls have an OS peak incidence a little earlier than boys, corresponding to the earlier age of growth spurt (Rytting *et al.*, 2000). Exposure to radiation is the only well-established environmental risk factor already associated with OS (Weatherby *et al.*, 1981; Mark *et al.*, 1994), but the long period between radiation exposure and tumor appearance (10 to 20 years) suggests that this is not relevant for the development of most conventional OS tumors. Yet, radiation can be responsible for the appearance of rare OS cases in adults and the development of secondary OS in individuals that received radiation therapy for treatment of certain primary tumors (Tang *et al.*, 2008). Orthopedic implant-related OS has also been reported in a few patients (Keel *et al.*, 2001). Furthermore, several human genetic disorders and familial cancer syndromes are linked with higher incidence of OS: Li-Fraumeni syndrome, an autosomal dominant disorder characterized by a germline mutation of *TP53*, which encodes p53; Rothmund-Thomson, RAPADILINO, and Bloom syndromes, autosomal recessive disorders associated with mutations in *RECQL4* (in the case of the first two syndromes) and *BLM* (in the case of Bloom syndrome), genes encoding RecQ DNA helicases; hereditary retinoblastoma, caused by a mutation in the Rb tumor suppressor gene; among others (Calvert *et al.*, 2012). OS tumors observed in older age individuals can also relate to Paget's disease (Picci, 2007).

1.2.3. Pathogenesis of osteosarcoma

The underlying molecular mechanisms of OS pathogenesis are characterized by a vastly heterogeneous array of genomic abnormalities, which hampers the identification of consensus changes in the development of this malignant tumor.

High levels of genomic instability, particularly chromosomal instability (CIN, elevated rate of gain or loss of entire chromosomes or sections of chromosomes), are often found in OS tumors (Selvarajah *et al.*, 2006). Numerical CIN (caused by mitotic errors, segmental amplifications/deletions) usually leads to gene copy number alterations, whereas structural CIN (resultant from failure in DNA damage response mechanisms or replication errors)

promotes additional genomic rearrangements and chromosomal breakages. Such variety of outcomes in CIN results in complex and diverse aberrations among OS patients (Martin *et al.*, 2012). Among the most commonly observed numerical abnormalities in OS, one can count gain of chromosome 1, loss of chromosomes 9, 10, 13, and/or 17, and partial/complete loss of the long arm of chromosome 6, whilst structural abnormalities include rearrangements of chromosomes 11, 19, and 20 (Marina *et al.*, 2004). CIN-dependent or -independent genetic alterations found in conventional OS consist of amplifications, deletions, mutations, or loss of heterozygosity affecting diverse tumor suppressors (e.g., RecQ4, $p16^{Ink4a}$, $p15^{Ink4b}$) and oncogenes (e.g., Cdk4, Mdm2) (Martin *et al.*, 2012). Variation in the DNA methylation profile of genes encoding these same proteins is described in OS tumors, as well (Sadikovic *et al.*, 2008a).

Nevertheless, within this lack of consistency reported in genetic alterations of different OS tumors, the importance of the tumor suppressors p53 and Rb in OS pathogenesis was underlined by several studies. Alterations of *TP53* by point mutations are present in 30% of OS tumors (Papachristou and Papavassiliou, 2007), while deletion or loss of heterozygosity has a frequency of approximately 40% (Martin *et al.*, 2012). Functional inactivation of p53 at the post-translational level cannot be forgotten, since the oncoprotein Mdm2, a p53 inhibitor that promotes its degradation and downregulation of its transcription, is frequently amplified in OS metastases and at OS recurrences (Miller *et al.*, 1996). *RB1*, the gene encoding Rb, presents mutations and loss of heterozygosity associated with inactivation of Rb in 50% of OS tumors (Wadayama *et al.*, 1994). Together with the amplification of Cdk4 gene found in 10% of OS cases (Martin *et al.*, 2012), Rb inactivation leads to an uncontrolled cell cycle through G_1/S progression. Additionally, inactivation of p53 and Rb causes CIN *in vivo* (van Harn *et al.*, 2010). The association of p53 and Rb with OS is further supported by the high risk of OS in individuals carrying a germline mutation of *TP53* or *RB1* (Calvert *et al.*, 2012).

As for the potential cancer cell responsible for OS development, our understanding is rather restricted. OS arises from mesenchymal cells that have or acquire the capacity to produce osteoid (Gorlick *et al.*, 2003). OS tumors exhibit osteoblast-like features, but the presence of distinct histologic forms of OS and the broad range of differentiation status of OS cells (highly differentiated, poorly differentiated, undifferentiated) suggest that the cell of origin is preosteoblast and retains the potential for pluripotent differentiation (Gorlick *et*

al., 2003; Haydon *et al.*, 2007; Gorlick and Khanna, 2010). Actually, emerging data suggest that OS should be regarded as a differentiation disease caused by genetic and epigenetic alterations that disrupt differentiation of mesenchymal stem cells (Tang *et al.*, 2008). Several signaling pathways, such as wingless-type (Wnt), bone morphogenic protein (BMP), and Hedgehog (Hh), play an important role in regulating osteogenic differentiation (Yuasa *et al.*, 2002; Zhou *et al.*, 2008). Transcription factors are also key regulators of this process. Among them, runt-related transcription factor 2 (Runx2) is a central player in osteogenic lineage commitment and terminal differentiation, and its transcription regulation and activity are complexly affected by numerous signaling pathways (including the ones described above), interaction with Rb, and epigenetic modifiers (Lian *et al.*, 2004; Tang *et al.*, 2008; Martin *et al.*, 2011). The disruption of Runx2 signaling by these interactors may interrupt osteoblast differentiation and *RUNX2* overexpression resulting from gain and amplification of chromosome 6p12-p21, which encompasses the *RUNX2* gene, is also probably involved in this pathogenesis (Won *et al.*, 2009; Martin *et al.*, 2011).

1.2.4. Conventional treatment approaches in osteosarcoma

Surgical resection of localized OS tumors is crucial for the treatment of this disease. Currently, the surgical strategies to deal with OS include amputation, limb salvage (removal of tumor without amputation, with subsequent replacement of bones and joints with allografts or prosthetic devices), and rotationplasty, being limb salvage the choice for 80-90% of patients (Picci, 2007). However, surgical treatment alone frequently fails in the eradication of OS because high-grade OS patients usually present micro-metastases (Marina *et al.*, 2004). When surgery is not practicable, radiotherapy can be used, but presents limited effects (Picci, 2007). During the last three decades, the development of chemotherapeutic agents, as well as the efficient combination of chemotherapy regimens, improved the outcome for patients with OS. In fact, before the use of effective chemotherapy, surgical resection and/or radiotherapy only allowed 2-year overall survival rates of 15-20% (Gorlick *et al.*, 2003; Marina *et al.*, 2004). Nowadays, multimodal treatment programs are frequent, in which chemotherapy is not only applied after surgery, but also as a pre-surgery tool (neoadjuvant chemotherapy) (Gorlick *et al.*, 2003). The most common chemotherapeutic agents used in OS treatment are Doxorubicin, Cisplatin, Ifosfamide, and Methotrexate, generally used in combination, and the mechanisms of

action of these drugs are described in Table 1. Other drugs, such as taxanes, may be used to sensitize cancer cells to conventional chemotherapeutic agents.

When metastatic OS occurring in the lung is diagnosed, the nodules should be resected and pre- and post-operative chemotherapeutic treatment should be the same as that for patients with localized disease (Picci, 2007). In the case of metastatic OS located in other sites, the probability of tumor eradication is less than 5% (Bacci *et al.*, 2003). Unfortunately, treatment failure is rather common in metastatic patients and recurrence of OS located in the lung occurs in 90% of them (Gorlick *et al.*, 2003; Picci, 2007).

Although the integration of chemotherapeutic agents in OS treatment programs has contributed to the achievement of better overall survival rates, recurrence of OS is still a major problem (Chou and Gorlick, 2006). The failure of current treatments suggests acquired resistance to chemotherapy and/or ability of metastatic cells to develop a protective microenvironment in the lung (Gorlick *et al.*, 2003).

Table 1. Mechanisms of action of conventional chemotherapeutic agents used in osteosarcoma treatment. Adapted from Ta *et al.* (2009).

Agent	Mechanism of action
Doxorubicin	Intercalates at point of local uncoiling of the DNA double helix and inhibits the synthesis of DNA and RNA
Cisplatin	Binds directly to tumor DNA and inhibits the synthesis of DNA through the formation of DNA cross-links
Ifosfamide	Causes crosslinking of DNA strands, inhibiting the synthesis of DNA and protein
Methotrexate	Inhibits the synthesis of purine and thymidylic acid by binding dihydrofolate reductase

1.2.5. MG-63: an *in vitro* model for osteosarcoma

The past few decades have been prolific in the development of cell isolation and culturing techniques, conducting to the emergence of multiple cell culture models. The use of cell lines allows the study of general cell biology, biocompatibility testing, drug discovery,

among others. In particular, cancer cell lines have been a useful instrument towards a better comprehension of this malignancy, and the subsequent identification of promising therapeutic agents and novel targets for specific therapies (Masters 2002; Sharma *et al.*, 2010). In general, OS cell lines derived from patient samples retain many markers of the osteoblast phenotype (Pautke *et al.*, 2004), but their altered molecular background and their high proliferative rates explain the selection of these cell lines as *in vitro* OS models.

The human OS MG-63 cell line is derived from a 14 years old Caucasian male and is deposited in the American Type Culture Collection (ATCC) center. In culture, these cells develop as an adherent monolayer, present a fibroblast-like morphology, and require 48 h for one population doubling (Mohseny *et al.*, 2011). MG-63 cells size is approximately 1/6 of the size of normal osteoblasts in culture and, contrarily to normal osteoblasts, MG-63 cells do not vary their cell size dependent on cell density and do not show branching cell processes (Pautke *et al.*, 2004).

A molecular characterization with immunohistochemical and genetic data from MG-63 demonstrated that these cells present disturbances that affect important signaling pathways. A homozygous deletion of *CDKN2A*, the gene coding for cyclin-dependent kinase inhibitor 2A (also known as $p16^{Ink4a}$), was found in these cells (Ottaviano *et al.*, 2010). The complete deletion of *CDKN2A* may have serious effects on carcinogenesis, since $p16^{Ink4a}$ is no longer available to perform G_1 cell cycle control through the inhibition of Cdk4. Moreover, MG-63 cells presented a wild-type *TP53* and a normal chromosomal band 17p13.1 (harboring the *TP53* gene); however, levels of *TP53* transcripts were found to be extremely low and p53 protein was not detectable by immunohistochemical staining (Ottaviano *et al.*, 2010). The downregulation of p53 can be explained by post-transcriptional regulation mechanisms of this protein (Vogelstein *et al.*, 2000), but rearrangements of *TP53* can also lead to the absence of *TP53* transcripts in the *TP53* wild-type cell line MG-63 (Chandar *et al.*, 1992). The lack of p53 may lead to uncontrolled proliferation of these OS cells, due to its crucial role in cell cycle and apoptosis, as well as in other relevant pathways. In addition, the important role of Runx2 is not limited to osteoblast lineage determination and differentiation, but a cell cycle-dependent expression of *RUNX2* is required for the regulation of cell cycle progression at G_1 in osteoblasts (Galindo *et al.*, 2005). In MG-63 cells, Runx2 protein levels are constitutively expressed throughout the cell cycle (Lucero *et al.*, 2012), suggesting the involvement of this protein

in the disrupted proliferative mechanism of this OS model. Furthermore, Sadikovic and colleagues reported that MG-63 present genomic imbalance, different DNA methylation profiles, and changes in gene expression when compared to normal osteoblasts, particularly showing hypomethylation and gain of OS-related genes accompanied by overexpression of these genes (Sadikovic *et al.*, 2008b).

Overall, the genetic scenario commonly found in OS patients and shared by MG-63 cells makes this cell line a representative *in vitro* model of OS.

1.3. Sulforaphane

1.3.1. Cruciferous vegetables, glucosinolates, and isothiocyanates

Research on the action of bioactive constituents of plants has shown, during the last decades, their anti-carcinogenic properties and epidemiologic studies confirm that consumption of fruits and vegetables, particularly cruciferous vegetables, may decrease the frequency of certain cancer types (Beliveau and Gingras, 2007; Hayes *et al.*, 2008).

Cruciferous vegetables belong to the Brassicaceae family (formerly known as Cruciferae), and include economically important plants, as broccoli, cabbage, cauliflower, kale, bokchoy, Brussels sprouts, radish, and mustards. Among the phytochemicals that can be found at high concentrations in cruciferous vegetables is the group of glucosinolates (GLSs). This well-characterized group is responsible for the bitter or hot taste intensively present in mustard, radish, and horseradish (Hayes *et al.*, 2008). GLSs are β-thioglucoside-N-hydroxysulfates that when enzymatically hydrolyzed by the action of myrosinase (β-thioglucoside glucohydrolase) originate different compounds (Fimognari and Hrelia, 2007), as illustrated in Figure 4. One of the possible products of GLSs hydrolysis is a group of bioactive sulphur-containing compounds, named isothiocyanates (ITCs). GLSs and myrosinase are stored in separate cell compartments and their release is triggered by cell damages, such as microbial attack, insect predation, and food processing (e.g., chewing, cutting, boiling) (Fimognari and Hrelia, 2007). However, recent data show that GLSs hydrolysis can also be mediated by the microflora of the mammalian gastrointestinal tract (Lai *et al.*, 2010). This means that besides the GLSs conversion occurring before the digestive process, further formation of ITCs may take place by myrosinase of the gastrointestinal tract microflora.

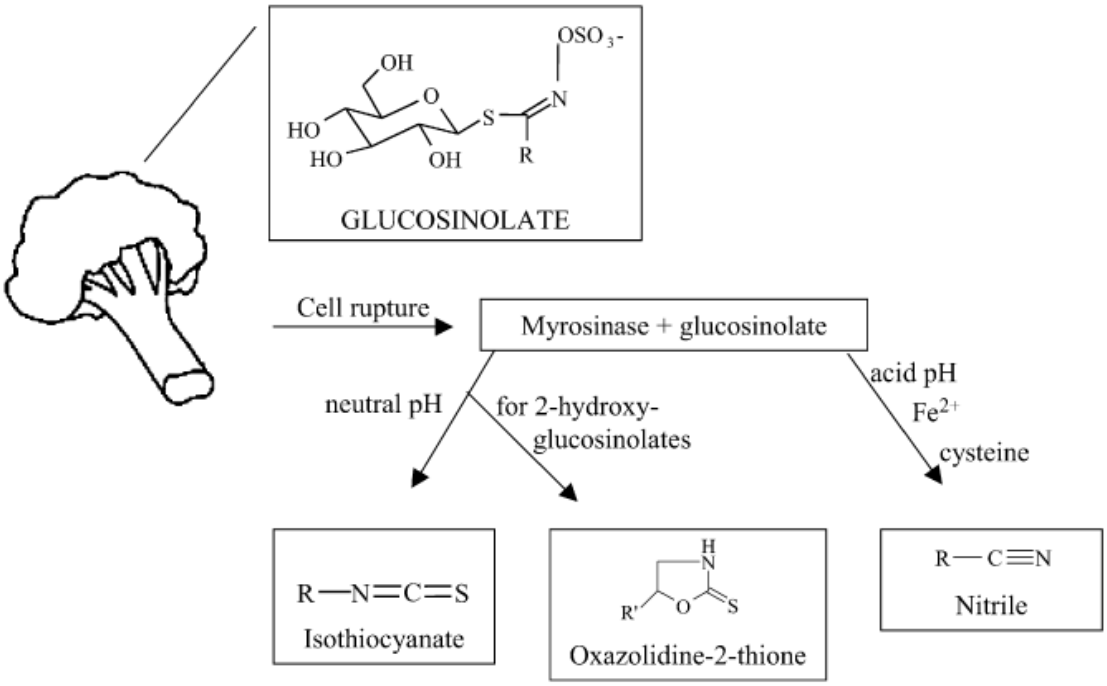

Figure 4. Bioconversion pathway of glucosinolates. The hydrolysis of GLSs catalyzed by myrosinase results in glucose release and formation of an instable intermediate compound (R-C(-SH)=N-O-SO_3^-), which, dependent on the reaction conditions, can originate ITCs, nitriles, or oxazolidine-2-thiones, among others. Extracted from Fimognari and Hrelia (2007).

1.3.2. Sulforaphane and cancer

Considerable research interest has been given in the past years to one particular ITC compound – sulforaphane (SFN, 1-isothiocyanato-4-(methylsulfinyl)-butane) – due to the variety of exerted anti-cancer effects (Clarke *et al.*, 2008). SFN is the hydrolysis product of the GLS precursor glucoraphanin (4-(methylsulfinyl)butyl glucosinolate) (Figure 5) and is found at particular high levels in broccoli and broccoli sprouts (Fahey *et al.*, 1997).

The great interest in SFN is related to its ability to simultaneously modulate multiple cellular targets involved in different stages of cancer development that include: DNA protection by modulation of carcinogen-metabolizing enzymes; inhibition of cell proliferation and induction of apoptosis; and inhibition of tumor angiogenesis and metastases formation. Therefore, SFN is able to prevent cancer initiation, as well as act on cancer cells as a therapeutic agent (Fimognari and Hrelia, 2007).

The basis for anti-cancer SFN effects rely on the highly electrophilic central carbon in the -N=C=S group. This structural feature enables SFN to bind to proteins and modify their

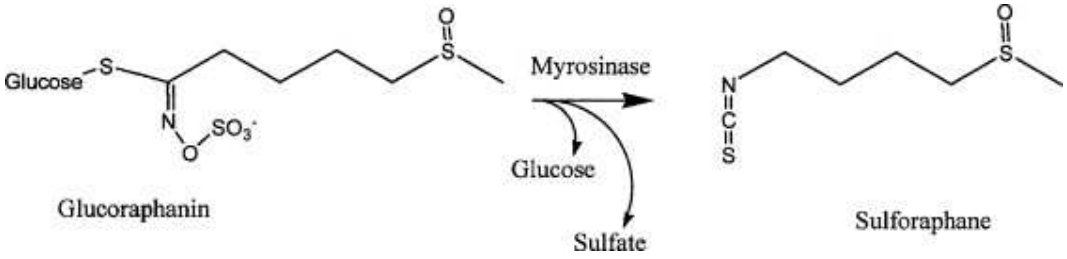

Figure 5. Enzymatic conversion of glucoraphanin to sulforaphane and the respective chemical structures. Extracted from Cwik *et al.* (2010).

function, controlling various physiological processes, including the ones directly involved in the cancer phenotype (Fimognari *et al.*, 2012). Another interesting property of SFN that makes this ITC a promising candidate in cancer therapy is the lack of systemic toxicity. A phase I clinical study evaluated broccoli sprouts preparations of GLSs (25 to 100 µM) and ITCs (25 µM), mainly containing SFN, and no significant cytotoxicity was observed with any of the preparations (Shapiro *et al.*, 2006). Moreover, the *in vitro* and *in vivo* effects of SFN lead to the acceptance of this ITC as a promising anti-cancer agent and several ongoing phase I and phase II clinical trials are evaluating SFN potential in the treatment of breast and prostate cancers (USNIH, 2012).

In the next sections, some of the pathways affected by SFN activity in the chemopreventive and chemotherapeutic perspective will be described.

1.3.3. Chemopreventive and chemotherapeutic pathways targeted by sulforaphane

1.3.3.1. *Modulation of Phase I and Phase II enzymes*

The first step of cancer prevention is the blockage of carcinogens that may cause DNA damage and, consequently, cancer initiation. When these compounds enter the body, they are subjected to metabolic reactions by the cytochrome P450 enzymes (CYPs) or Phase I enzymes, causing gain of hydrophilic properties. At the same time, carcinogens are converted to highly reactive intermediates that can bind and damage macromolecules, such as DNA, RNA, and proteins (Juge *et al.*, 2007). Chemopreventive mechanisms of action of SFN involve the detoxification of carcinogens by inhibition of Phase I enzymes through regulation of the expression and function of different CYPs (Maheo *et al.*, 1997; Langouet *et al.*, 2000).

On the other hand, Phase II enzymes also play an important role in carcinogenics detoxification, contributing to their elimination and hence protecting cells against them (Higdon *et al.*, 2007). The effect of SFN at this level is based on the induction of Phase II enzymes and a large number of proteins involved in this cellular defense mechanism were found to be significantly induced by this ITC: epoxide hydrolase (Thimmulappa *et al.*, 2002), glutathione reductase (Wu and Juurlink, 2001; Hu *et al.*, 2006), glutathione peroxidase (Cornblatt *et al.*, 2007), glutathione S-transferase (Zhang *et al.*, 1992), thioredoxin and thioredoxin reductase (Hu *et al.*, 2006), among others.

The induction of Phase II enzymes by SFN is in part accomplished by the regulation of their expression. SFN reacts with thiol groups on Kelch-like ECH-associated protein 1 (Keap1), which promotes nuclear factor erythroid 2-related factor 2 (Nrf2) dissociation from Keap1, allowing the activation of antioxidant response element (ARE)-driven gene expression of Phase II enzymes (Zhang *et al.*, 1992).

1.3.3.2. *Induction of cell cycle arrest*

The anti-proliferative effect of SFN on different cancer cells is the result of cell cycle arrest at G_0/G_1, G_2/M, or S phase through diverse mechanisms. In this respect, SFN can directly regulate the expression of cyclins and CDKs that control progression through specific cell cycle phases (Singh *et al.*, 2004b; Cho *et al.*, 2005; Kim *et al.*, 2011). In addition to the control of the main drivers of cell cycle progression, SFN can modulate the expression of other proteins that influence the activity of the former. Levels of Cdc25 phosphatases were found to be downregulated in prostate cells upon SFN exposure (Singh *et al.*, 2004b) and members of the Cip/Kip family (e.g., $p21^{Cip1}$, $p27^{Kip1}$) that act as CKI usually suffer SFN-induced upregulation in cancer cells (Parnaud *et al.*, 2004; Shan *et al.*, 2006; Shen *et al.*, 2006). The disruption of microtubules by inhibition of tubulin polymerization was also reported by Jackson and Singletary and associated with SFN-induced cell cycle arrest (Jackson and Singletary, 2004a). Microtubules formation is essential for a correct mitotic process and the inhibition of mitotic spindle formation during this cell cycle phase prevents a normal outcome in cell division.

1.3.3.3. *Induction of apoptosis*

Resistance to apoptosis in cancer cells is a major problem encountered in cancer therapy. SFN is a well-known apoptotic inducer and the mechanisms underlying this effect seem to

be dependent on the cancer cell type. In non-tumorigenic and tumorigenic malignant glioblastoma cell lines (T98G and U-87MG, respectively) SFN induced apoptosis through different ways; both caspase-dependent and caspase-independent pathways are suggested to be involved in the SFN-induced apoptosis in these cells (Karmakar *et al.*, 2006). Different upstream regulators of apoptosis, as p53 (Fimognari *et al.*, 2002; Fimognari *et al.*, 2004), second mitochondria-derived activator of caspase/direct IAP-binding protein with low pI (Smac/DIABLO) (Karmakar *et al.*, 2006), and Bax (Singh *et al.*, 2004a), are proposed to mediate this programmed cell death after SFN exposure.

Interestingly, SFN ability to surpass Doxorubicin resistance to the induction of apoptosis in cells carrying different p53 mutations was suggested by Fimognari and collaborators (2007); the induction of apoptosis in these cells was achieved by mediation of selective activation of certain executioner caspases after exposure to a combination of SFN and Doxorubicin (Fimognari *et al.*, 2007).

In recent years, SFN was identified as a histone deacetylase (HDAC) inhibitor and its role as a modulator of epigenetic processes that lead to apoptosis is described in the literature, as well. SFN was reported to induce apoptosis via promotion of the expression of genes encoding pro-apoptotic proteins, as $p21^{Cip1}$ (Myzak *et al.*, 2006b). In this *in vitro* study, histone-induced $p21^{Cip1}$ gene repression was reverted by the increase of acetylated histones H3 and H4. *In vivo* experiments support this mechanism of apoptosis induction by SFN, as mice treated with SFN were reported to present significant inhibition of HDAC activity in the colonic mucosa with concomitant increase in acetylated histones H3 and H4 (Myzak *et al.*, 2006a).

1.3.3.4. *Inhibition of angiogenesis and metastasis*

An important event that enables tumor progression – angiogenesis – can also be modulated by SFN. Using human umbilical vein endothelial cells (HUVEC cell line) as an *in vitro* model for angiogenesis, Asakage and colleagues demonstrated that SFN inhibited the proliferation of these cells in a dose-dependent manner, as well as tube formation on matrigel; according to the authors the antiproliferative effect was dependent on cell apoptosis (Asakage *et al.*, 2006). Moreover, an *in vitro* study performed with HMEC-1, an immortalized human microvascular endothelial cell line, showed that SFN interferes with angiogenic signaling pathways, inhibiting mRNA expression of two angiogenesis-associated transcription factors (HIF-1α and Myc proto-oncogene protein (c-Myc)) in a

concentration range of 0.8 to 25 µM and transcription of VEGF receptor KDR/flk-1 (for kinase insert domain receptor/fetal liver kinase 1), and affects basement membrane integrity (Bertl *et al.*, 2006).

Other crucial step of carcinogenesis is the formation of metastases. Evidences show that the use of SFN in combination with TRAIL is more effective in inhibiting metastatic markers (Shankar *et al.*, 2008). SFN also reduced the invasive potential of B16F-10 melanoma cells through inhibition of activation of MMP-2 and MMP-9, accompanied by 95.5% inhibition of lung tumor nodule formation (Thejass and Kuttan, 2006).

II. OBJECTIVES

Despite the remarkable advances in OS therapy over the past few decades, OS patients still present high resistance rates to the current treatment approaches (Chou and Gorlick, 2006), demanding urgent action on the discovery/development of new therapeutic agents. The cytotoxic effect of SFN has been described for multiple cancers, as well as other human diseases. The ability of modulate apoptosis and cell cycle progression are desired properties to be found in chemotherapeutic agents and SFN has proven to exert them at different stages of the carcinogenic process (Fimognari and Hrelia, 2007). However, literature lacks information about the effects of SFN in OS and the mechanisms that lead to these effects have not been fully understood yet.

Therefore, the main objectives of the present dissertation were to evaluate the potential of SFN as a cytostatic and pro-apoptotic agent for OS cells and to provide insight about the mechanisms of action of SFN underlying the modulation of cell cycle progression and apoptotic pathways. In this context, the work presented in this dissertation purposes to:

1) assess the viability status of cells;
2) evaluate cell distribution throughout cell cycle;
3) elucidate the type of cell death behind cytotoxicity;
4) measure the activity level of different caspases in order to determine the activated apoptotic pathway(s);
5) and quantify the expression of genes encoding proteins involved in cell cycle regulation, after exposure of an *in vitro* OS model to different concentrations of SFN.

III. MATERIALS AND METHODS

3.1. Materials and reagents

α-Minimum Essential Medium (α-MEM), fetal bovine serum (FBS), fungizone, penicillin-streptomycin, Trypsin-ethylenediaminetetraacetic acid (EDTA), 1X phosphate-buffered saline (PBS) pH 7.2, TRIzol reagent, and the kit DNase I - Amplification grade were purchased from Life Technologies (Carlsbad, CA, USA). D,L-sulforaphane (SFN), dimethyl sulfoxide (DMSO), 3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI), ribonuclease (RNase), bovine serum albumin (BSA), and Bradford reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol and chloroform were obtained from Merck Millipore (Darmstadt, Germany). FITC Annexin V Apoptosis Detection Kit I was purchased from BD Biosciences (San Jose, CA, USA) and APOPCYTO Caspase-3 Colorimetric Assay Kit from MBL International Corporation (Woburn, MA, USA). Caspase-8 Colorimetric Activity Assay Kit and Caspase-9 Colorimetric Activity Assay Kit were purchased from Chemicon-Millipore (Temecula, CA, USA). RNeasy Mini Kit and Omniscript RT Kit were obtained from Qiagen (Hilden, Germany). Kanamycin positive control RNA and iQ SYBR Green Supermix were purchased from Promega (San Luis Obispo, CA, USA) and Bio-Rad (Hercules, CA, USA), respectively.

3.2. Cell culture

The human OS cell line MG-63 (CRL-1427, ATCC, Vanassas, VA, USA) was used as an *in vitro* OS model and was kindly provided by INEB (Instituto Nacional de Engenharia Biomédica, Universidade do Porto, Portugal). MG-63 cells were cultured in α-MEM supplemented with 10% FBS, 2.5 µg/mL fungizone, and penicillin-streptomycin (100 U/mL-100 µg/mL). Cells were cultured in 75 cm^2 culture flasks with 10 mL supplemented medium and maintained at 37 °C in a 5% CO_2 humidified atmosphere. Cell cultures were routinely observed under an inverted microscope to verify their confluence, the morphology and viability of cells, and the absence of microorganism contaminations.

Subculture was performed every 3-4 days, when cultures reached approximately 70% confluence. After removal of the culture medium, cells were washed with 5 mL PBS and incubated with 3 mL Trypsin-EDTA (0.25% trypsin, 1 mM EDTA) for 5 min, to cleave proteins that enable cell adherence to the flask and cell-cell adhesion. After cell detachment from the culture flask, trypsin was inactivated by adding 6 mL supplemented medium to the culture. Cells were harvested, counted using a TC10 Automated Cell Counter (Bio-Rad, Hercules, CA, USA), and seeded ($1x10^5$ cells/mL) in a new culture flask with a final volume of 10 mL of supplemented medium.

3.3. Sulforaphane preparation and cell exposure

D,L-sulforaphane was dissolved in DMSO in order to prepare 10 mM SFN stock solutions, which were stored at -20 ºC. Before every treatment, SFN stock solutions were thawed and diluted in supplemented medium to a concentration of 100 µM SFN. This solution was transferred to a syringe (Terumo, Leuven, Belgium) and forced through a 0.2 µm syringe filter (VWR International, Radnor, PA, USA) with a cellulose acetate membrane to obtain a sterile SFN solution. The desired SFN concentrations (5, 10, and 20 µM) were then prepared by serial dilutions with supplemented medium from the sterile 100 µM SFN solution.

For each experiment, cells were seeded and allowed to adhere for 24 h (37 ºC, 5% CO_2). After this period, the medium was removed and replaced by fresh medium containing SFN at final concentrations of 5, 10, and 20 µM or fresh medium without SFN (0 µM SFN) in the case of control cells. SFN-treated cultures were exposed to <0.5% DMSO. The effect of SFN was measured after exposure periods of 24 and 48 h in the same temperature and controlled atmosphere conditions.

3.4. Characterization of confluence and cell morphology

Cells were visualized under an inverted microscope in order to characterize, in a qualitative manner, general aspects of cells that can reflect their functional status and viability. Confluence and cell morphology (e.g., shape, volume, attachment/detachment status) of SFN-treated cultures were described and compared with control cultures.

3.5. Cell viability

The effect of SFN on cell viability was evaluated by the colorimetric MTT assay. This assay depends on the ability of mitochondrial enzymes to reduce MTT, a soluble, yellow tetrazolium salt, into an insoluble, purple formazan product. The formazan crystals can be dissolved in various organic solvents and measured spectrophotometrically. Because reduction of MTT can only occur in metabolically active cells, the amount of formazan produced is proportional to the number of viable cells.

The MTT assay was performed according to Twentyman and Luscombe (1987) with slight modifications. Cells were seeded in 96-well plates at $1x10^5$ cells/mL and $0.7x10^5$ cells/mL for 24 and 48 h exposure periods, respectively, in a final volume of 100 µL supplemented medium/well. The initial number of cells to use was selected with the view to obtain control samples yielding an absorbance between 0.75 and 1.25. After each exposure period, 50 µL MTT solution (1 mg/mL PBS) were added to each well and cells were incubated for 4 h at 37 ºC, 5% CO_2, in darkness. At this point, the medium and the MTT solution were aspirated from the wells using a syringe with a needle (Terumo, Leuven, Belgium) in order to avoid the disturbance and removal of the produced formazan crystals. Cells were incubated with DMSO (150 µL/well) for 2 h with gentle shaking, at room temperature and protected from light, to dissolve the formazan crystals. The absorbance of each well was measured at λ = 570 nm using a Synergy HT Multi-Mode Microplate Reader controlled via Gen5 Data Analysis Software (BioTek Instruments, Winooski, VT, USA). One hundred and fifty microliters DMSO/well were used as blank and the mean absorbance of blank replicates was subtracted from the total absorbance of each sample. Relative cell viability was calculated using the following equation:

$$\text{Relative viability (\%)} = (A_{570} \text{ of sample} / \text{mean } A_{570} \text{ of control}) \times 100$$

3.6. Cell cycle and clastogenicity analysis

The measurement of the DNA content of single cells is one of the most universal applications of flow cytometry. Analyzing the content of DNA also provides information about the cell cycle, allowing one to determine the percentage of cells within a population that are undergoing G_1/G_0, S, and G_2/M phases. This analysis relies on the use of dyes (e.g., PI) that bind specifically and stoichiometrically to nucleic acids and which

fluorescence is enhanced on binding. The cell cycle status of a population can then be reflected on a histogram plotting the number of cells vs. DNA content.

For this experiment, cells ($1.5x10^5$ cells/mL) were seeded in 6-well plates, each well containing a final volume of 2 mL supplemented medium. At the end of each exposure period, control and treated cells were washed with 1 mL PBS/well, trypsinized using 360 µL Trypsin-EDTA/well and 720 µL supplemented medium/well to inactivate the trypsin, and harvested. Subsequently, cell suspensions were centrifuged at 300 x *g* for 5 min, the supernatant was carefully removed and discarded, and the pellet was resuspended in 1 mL PBS. Another centrifugation was performed in the same conditions and cells were fixed with 1 mL 85% ethanol at 4 ºC, after discarding the supernatant. Samples were stored (-20 ºC) until flow cytometric analysis.

At the time of analysis, fixed cells were centrifuged (300 x *g*, 5 min), resuspended in 800 µL PBS, and filtered through a 35 µm nylon mesh (to separate aggregated cells and remove large debris) into a 5 mL tube. Afterwards, 50 µL RNase (50 µg/mL) and 50 µL PI (50 µg/mL) were added to each sample followed by an incubation period of 20 min at room temperature, in darkness. This step ensures RNA elimination and DNA staining by RNase and PI, respectively. Finally, the relative fluorescence intensity of PI-stained nuclei was measured through a COULTER EPICS XL Flow Cytometer (Coulter Electronics, Hialeah, FL, USA) equipped with an air-cooled argon-ion laser turned at 15 mW and operating at 488 nm. The amplification was adjusted so that the peak corresponding to the diploid peak was positioned at channel 200. At least 5,000 events were analyzed for each sample. Acquisitions were made using SYSTEM II Software version 3.0 (Coulter Electronics, Hialeah, FL, USA). Doublets (clustered cells), nuclei with associated cytoplasm, partial nuclei, and other debris were excluded from the analysis by gating a region that encloses only single cells on the cytogram of the fluorescence light intensity pulse integral vs. the fluorescence light intensity pulse height. The cell cycle analysis was conducted based on the histogram outputs and percentages were estimated as number of nuclei in each cell cycle phase / total number of nuclei x 100. In order to assess the putative clastogenic effect of SFN on MG-63 cells, full peak coefficient of variation (FPCV) of G_1/G_0 nuclei was determined in each histogram as diagnostic for clastogenic damage, as recommended by Misra and Easton (1999).

3.7. Apoptosis assessment

Apoptosis consists of multiple biochemical events that trigger characteristic morphological and functional changes in the cell, culminating in its death. Amongst all, the loss of plasma membrane asymmetry is one of the earliest morphologic alterations occurring during the apoptotic process, in which the membrane phospholipid PS is translocated from the inner to the outer leaflet of the plasma membrane. Annexin V, a 35-36 kDa calcium-dependent phospholipid-binding protein, shows high affinity for PS, binding to cells that present this phospholipid on the surface of the plasma membrane. When conjugating Annexin V with a fluorochrome, such as fluorescein isothiocyanate (FITC), this conjugate can be used as a sensitive probe for flow cytometric analysis of cells undergoing apoptosis. Also, since non-viable cells and cells undergoing late stages of apoptosis lose plasma membrane integrity and are permeable to viability probes (e.g., PI), the simultaneous use of FITC Annexin V and PI allows to distinguish early and late apoptotic cells. Therefore, using FITC Annexin V and PI in conjunction in a flow cytometric analysis will enable the quantification of: viable cells (both FITC Annexin V and PI negative); cells in early apoptosis (FITC Annexin V positive and PI negative); cells in late apoptosis (both FITC Annexin V and PI positive); and necrotic cells (FITC Annexin V negative and PI positive).

Apoptosis assessment was performed using the FITC Annexin V Apoptosis Detection Kit I. In this assay, $1x10^5$ cells/well in 2 mL supplemented medium were seeded in 6-well plates. After SFN exposure, cells were washed with 1 mL PBS/well and trypsinized (360 µL Trypsin-EDTA/well). After trypsin inactivation with 720 µL supplemented medium/well, cells were collected, briefly vortexed, and counted. Cell suspensions were centrifuged for 5 min at 4 ºC and 300 x *g*, the supernatant was discarded, and 1 mL cold PBS was added without resuspension of the pellet. This previous step was repeated entirely, but cells were then resuspended in 1X Annexin V Binding Buffer (1 volume of 10X Annexin V Binding Buffer to 9 volumes of distilled H_2O) at $1x10^6$ cells/mL. One hundred microliters of each cell suspension were transferred to a 5 mL tube and 5 µL FITC Annexin V and 5 µL PI were added to each sample. After incubation (15 min, room temperature, in darkness), 400 µL 1X Annexin V Binding Buffer were added to each tube and samples were analyzed by flow cytometry within 1 h. The following controls were used to set up compensation and quadrants: unstained cells; cells stained with FITC Annexin V (no PI); and cells stained with PI (no FITC Annexin V). At least 10,000 events

were analyzed for each sample and percentages were estimated as number of cells in each quadrant / total number of cells x 100.

3.8. Protein quantification

The Bradford protein assay is a protein determination method based on the binding of Coomassie Brilliant Blue G-250 dye to proteins, which leads to the conversion of the protonated red cationic form in the unprotonated blue form of the dye (Bradford, 1976). The latter shows A_{max} = 595 nm, allowing the spectrophotometric quantification of protein-dye complexes at this wavelength, and hence protein content determination. When needed, total protein in samples was quantified by the Bradford protein assay. Briefly, 5 µL of cell extract of unknown protein concentration were incubated with 250 µL Bradford reagent in a flat-bottom, clear polystyrene 96-well microplate for 10 min, protected from light and shaken. The buffer of each sample was used as blank. Absorbance of samples was read at λ = 595 nm in the previously described microplate reader. A BSA standard curve was generated by measuring the absorbance of samples with known concentrations of BSA (in the 0 to 1.4 mg/mL ultrapure H_2O range) and protein concentration of the cell extracts was determined by comparison. Whenever needed, cell extracts were diluted in the respective buffers to fit in the BSA concentration range.

3.9. Caspase activity assessment

After activation by a variety of signals, caspases recognize and cleave a wide number of different substrates, leading to the activation of different apoptotic pathways. The detection of caspase activity is recognized as a standard method to measure apoptosis. Active caspases recognize 4-5 amino acid sequences in a substrate molecule. For caspases 3, 8, and 9 this sequence is DEVD, IETD, and LEHD, respectively. In the present work, the assessment of the activity of these three caspases relied on the use of a synthetic substrate consisting of a peptide, enclosing the specific sequence recognized by each caspase, labeled with the chromophore *p*-nitroanilide (*p*NA) at the C-terminal side. *p*NA is released from the labeled synthetic substrate on cleavage by active caspase. Because the amount of free *p*NA is proportional to the amount of caspase activity present in a sample, caspase activity can be determined by measuring spectrophotometrically the absorbance of free *p*NA at λ = 405nm.

3.9.1. *Caspase 3 activity*

Caspase 3 activity was measured using the APOPCYTO Caspase-3 Colorimetric Assay Kit according to manufacturer's protocol, with few alterations. For this assay, $2.5x10^6$ cells were seeded in 150 mm cell culture dishes using 20 mL supplemented medium and subjected to control and SFN treatment (10 and 20 µM) conditions for 48 h. Ended this period, cells were washed with 10 mL PBS and trypsinized with 8 mL Trypsin-EDTA, which was inactivated with 16 mL supplemented medium. After determining the cell density, $1.5x10^6$ cells were collected from each sample and centrifuged at 400 x *g* for 5 min, at 4 ºC. The supernatant was discarded and cells were resuspended in 100 µL ice-cold Cell Lysis Buffer and incubated on ice for 10 min. Cell lysates were centrifuged at 10,000 x *g* for 5 min at 4 ºC, to precipitate cellular debris, and the supernatant (cell extract) was transferred to new microtubes and placed on ice. Total protein concentration of the cell extracts was measured by the Bradford protein assay, as described above. Fifty microliters of 2X Reaction Buffer containing 10 mM dithiothreitol (DTT), 50 µL of cell extract, and 5 µL 10 mM Caspase-3 Substrate (Substrate DEVD-*p*NA) were added to each well of a polystyrene 96-well microplate. To measure the blank absorbance, wells with 50 µL Cell Lysis Buffer instead of cell extract were prepared. Also, to verify that the signal detected by the kit was generated by protease activity, 1 µL 1 mM Inhibitor DEVD-FMK was added to wells already containing the cell extract and the reaction buffer, adding the Caspase-3 Substrate after 10 min. The microplate was then covered with a plate sealer and incubated at 37 ºC, protected from light, for 18 h. Absorbance in the wells was measured at λ = 405 nm in a microplate reader. For construction of a *p*NA standard curve, 100 µL *p*NA standards (0 to 500 µM *p*NA prepared by serial dilutions from a 100 mM *p*NA Standard in Cell Lysis Buffer) were added to empty wells and the absorbance was measured at λ = 405 nm.

3.9.2. *Caspase 8 and caspase 9 activity*

The activity of caspase 8 and caspase 9 was measured using the Caspase-8 Colorimetric Activity Assay Kit and Caspase-9 Colorimetric Activity Assay Kit, respectively, following the manufacture's instructions with slight modifications. For both assays, 150 mm cell culture dishes with 20 mL supplemented medium were used and cells were seeded at a concentration of $2.5x10^6$ cells/dish. After 48 h of SFN exposure, treated (10 and 20 µM

SFN) and untreated cells (control) were washed (10 mL PBS), trypsinized (8 mL Trypsin-EDTA and 16 mL supplemented medium for trypsin inactivation), and counted. From each sample, $1.5x10^6$ cells were harvested and centrifuged at 1,500 rpm for 10 min, at 4 ºC. The pellet was resuspended in 100 µL chilled 1X Cell Lysis Buffer (1 volume of 5X Cell Lysis Buffer to 4 volumes of ultrapure H_2O) and incubated on ice for 10 min, followed by another centrifugation at 10,000 x *g* for 5 min, at 4 ºC. The supernatant (cell extract) was transferred to new microtubes (placed on ice) and the total protein concentration of each cell extract was measured by the Bradford protein assay. An assay mixture containing 20 µL 5X Assay Buffer, 50 µL of cell extract, 20 µL ultrapure H_2O, and 10 µL Caspase-8 Substrate (2.5 mg/mL Ac-IETD-*p*NA) or Caspase-9 Substrate (3 mg/mL Ac-LEHD-*p*NA) was prepared in wells from a 96-well microplate for each sample. Additional wells containing the same volumes of 5X Assay Buffer and cell extract, but with 15 µL ultrapure H_2O, were pre-incubated with 5 µL Caspase-8 Inhibitor (100 µM Ac-IETD-CHO) or Caspase-9 Inhibitor (100 µM Ac-LEHD-CHO), for 10 min at room temperature, being the corresponding substrate added subsequently. In the case of blank samples, this mixture consisted of 20 µL 5X Assay Buffer, 70 µL ultrapure H_2O, and 10 µL Caspase-8 Substrate or Caspase-9 Substrate. The microplate was sealed and incubated for 2 h at 37 ºC, in darkness. Wells' absorbance was read at λ = 405 nm using a microplate reader. One hundred microliters *p*NA standards (0 to 500 µM) were added to empty wells, after diluting the provided 10 mM *p*NA Standard in 1X Assay Buffer (1 volume of 5X Assay Buffer to 4 volumes of ultrapure H_2O), and the absorbance was measured at λ = 405 nm. A *p*NA standard curve was constructed for extrapolation of caspase activity in samples.

3.9.3. *Determination of caspase specific activity*

For all caspase activity assays, the specific activity of caspase was calculated as follows: the mean of the replicate blank readings was subtracted from the absorbance values of each sample; the corresponding free *p*NA concentration was determined using the *p*NA standard curve; caspase activity present in each sample was calculated using the equation:

Caspase activity (CA) = (free *p*NA concentration (µM) x 0.1 mL) / incubation hour

Finally, specific activity of caspase in each sample was estimated by the formula:

Specific Activity = CA / (total protein concentration (mg/mL) x 0.1 mL)

3.10. Gene expression

3.10.1. *Primers*

For primer design, the consensus coding DNA sequence (CDS) of the genes of interest was acquired from the National Center for Biotechnology Information "Gene" database (http://www.ncbi.nlm.nih.gov/gene/) (when multiple CDSs were found for one gene, the largest overlap region from the different CDSs was obtained from the multiple sequence alignment tool Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/)). Specific primers were selected using Primer3 version 0.4.0. (http://frodo.wi.mit.edu/primer3/) (Rozen and Skaletsky, 2000). Primer pairs were tested for specificity using the University of California Santa Cruz In-Silico PCR database (http://genome.ucsc.edu/cgi-bin/hgPcr). Table 2 shows the oligonucleotide primer list for quantitative reverse transcription polymerase chain reaction (qRT-PCR) to analyze the expression of *CHEK1*, *CHEK2*, *CDK1*, *CDK2*, *CDC25C*, and *CCNB1*. Amplification of the housekeeping gene *GAPDH* was performed as an internal reference for relative quantification. The following external control primers (for kanamycin positive control) were used as control for *GAPDH* expression stability relative to total RNA amounts: Kan-F: 5'-AGCATTACGCTGACTTGACG-3'; Kan-R: 5'-AGGTGGACCAGTTGGTGATT-3'.

3.10.2. *RNA extraction*

Cells were seeded ($1x10^5$ cells/well) in 6-well plates with 2 mL supplemented medium. After 48 h of SFN exposure (10 µM), medium from treated and untreated cultures was discarded and cells were washed twice with 1 mL PBS/well. Cell lysis was performed adding per well 333 µL TRIzol reagent. Cell lysate of replicates of the same condition were transferred to a 2 mL RNase-free tube and stored frozen (-80 ºC) until analysis. At the time of analysis, 200 µL chloroform were added to each tube, shaken on vortex for 10 s, and incubated at room temperature for 2 min. The TRIzol-chloroform mixtures were poured into previously centrifuged (1 min at maximum rpm) 2 mL Phase Lock Gel tubes (5 Prime, Hamburg, Germany) and went through a centrifugation at 12,000 x *g* for 15 min. The aqueous top phase (containing the RNA) was transferred to a 1.5 mL RNase-free tube (about 600 µL) and an identical volume of RNase-free 70% ethanol (in

Table 2. Specific oligonucleotide primers used in qRT-PCR for gene expression analysis.

Target gene	Product	Forward primer (5'-3')	Reverse primer (5'-3')
CHEK1	Checkpoint kinase 1 (Chk1)	CCCGCACAGGTCTTTCCTT	CGTGTCATTCTTTTGACCAACC
CHEK2	Checkpoint kinase 2 (Chk2)	CACAGCTCTACCCCAGGTTC	CACAACACAGCAGCACACAC
CDK1	Cdk1	GGGTAGACACAAAACTACAGGTCAA	GGAATCCTGCATAAGCACATC
CDK2	Cyclin-dependent kinase 2 (Cdk2)	ACCTCCCCTGGATGAAGATG	AGATGGGGTACTGGCTTGGT
CDC25C	M-phase inducer phosphatase 3 (Cdc25C)	CCTATGCATCATCAGGACCAC	GGCTCATGTCCTTCACCAGA
CCNB1	Cyclin B1	GCTGAAAATAAGGCGAAGATCAA	ACCAATGTCCCCAAGAGCTG
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	ACACCCACTCCTCCACCTTT	TACTCCTTGGAGGCCATGTG

diethylpyrocarbonate (DEPC)-treated H_2O) was added to each tube to promote selective binding of RNA to the RNeasy membrane in the next step. The RNeasy Mini Kit was used from this point on with adaptations to its protocol. From each sample, 600 µL were transferred to an RNeasy Mini Spin Column placed in a 2 mL Collection Tube, centrifuged for 15 s at 10,000 rpm, and the flow-through was discarded. The same column and tube were reused to repeat the previous step with the remaining volume of sample. Seven hundred microliters Buffer RW1 were added to the column, followed by a centrifugation at 10,000 rpm for 15 s and the discard of the flow-through. This step ensures the efficient removal of biomolecules, such as carbohydrates,proteins, fatty acids, etc., that are non-specifically bound to the RNeasy membrane, allowing the binding of large RNA molecules (>200 bases) in the column. Then, 500 µL Buffer RPE were added to the column and, after a centrifugation at 10,000 rpm for 15 s, the flow-through was discarded. This step was performed with the view to remove traces of salts. The previous step was entirely repeated, performing a centrifugation for 2 min to dry the RNeasy membrane, ensuring that no ethanol was carried over. The RNeasy Mini Spin Column was then carefully transferred from the Collection Tube to a 1.5 mL RNase-free tube, 40 µL RNase-Free Water were added directly to the RNeasy membrane, and samples were centrifuged at 10,000 rpm for 1 min to elute the RNA. A second elution was performed using the eluted water and applying it again onto the column, followed by a centrifugation at 10,000 rpm for 2 min. Samples were stored at -80 ºC until further analysis.

3.10.3. *cDNA synthesis*

Samples were thawed and RNA quality and quantity was determined spectrophotometrically using NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). Contaminant genomic DNA was degraded prior to complementary DNA (cDNA) synthesis using the kit DNase I - Amplification grade with few alterations to the provided protocol. Two micrograms of total RNA of each sample were diluted in DEPC-treated H_2O (achieving a final volume of 6 µL) and mixed with 2 ng 1.2 kb kanamycin positive control RNA. To the previous mixture, 1 µL DNase I (1 U/µL) and 1 µL 10X DNase I Reaction Buffer were added. An incubation at 37 ºC for 15 min was performed to allow enzymatic reaction. DNase I was then inactivated with 1 µL 25 mM EDTA/tube during incubation at 65 ºC for 10 min. After this, tubes were spun down to collect condensation and placed on ice. The reverse transcription was accomplished using the Omniscript RT Kit, with

modifications to the supplied protocol. Nine microliters Reverse Transcription mastermix (2 µL 10X Buffer RT, 2 µL dNTP Mix (5 mM each deoxyribonucleotide triphosphate), 4 µL Oligo dT18 (5 mM), and 1 µL Omniscript Reverse Transcriptase (4 U/µL)) were added to each DNase-treated tube and incubated at 37 ºC for 1 h. After enzymatic reaction, cDNA samples were spun down and diluted in 380 µL ultrapure H_2O. cDNA samples were stored at -20 ºC until qRT-PCR reaction.

3.10.4. *qRT-PCR*

Amplification of target cDNA was performed by qRT-PCR using an iCycler iQ5 PCR Thermal Cycler (Bio-Rad, Hercules, CA, USA). In brief, wells of a 96-well PCR plate (Bio-Rad, Hercules, CA, USA) were filled with 7.5 µL 2X iQ SYBR Green Supermix (100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM each dNTP, 50 U/mL iTaq DNA polymerase, 6 mM $MgCl_2$, SYBR Green I, 20 nM fluorescein, stabilizers), 3.75 µL forward and reverse primer mix (1.5 µM final concentration each primer), and 3.75 µL cDNA sample. Additionally, No Template Controls were used, in which instead of cDNA, 3.75 µL ultrapure H_2O were added. The PCR plate was then carefully sealed and qRT-PCR was carried out. For all primer pairs, the conditions for PCR were as follows: initial denaturation at 95 ºC for 6 s, followed by 60 cycles comprising denaturation at 94 ºC for 5 s, annealing at 58 ºC for 15 s, and extension at 72 ºC for 15 s. In addition, the following melting program was performed to confirm product specificity: temperature increase from 68 ºC to 95 ºC, with 0.5 ºC increments for 6 s each. Two independent PCR runs were performed per cDNA sample from each biological sample. Raw fluorescence unit (RFU) data were obtained from the iQ5 Optical System Software (Bio-Rad, Hercules, CA, USA) and were used only when the corresponding PCR reactions were validated by melting curve analysis. Determination of gene expression levels was based on the Pfaffl method (Pfaffl, 2001). RFU data from baseline-normalized curve fits were used to calculate the cycle threshold (Ct) values and average efficiency parameters through Real-Time PCR Miner (http://www.miner.ewindup.info/Version2) (Zhao and Fernald, 2005). All genes were normalized to the housekeeping gene *GAPDH*. *GAPDH* was validated to total RNA by comparing *GAPDH* Ct values with Ct values from the external kanamycin RNA transcript, which was directly proportional to total RNA present in the sample.

3.11. Data and statistical analysis

For all assays, at least three independent experiments with replicates were performed. In the case of gene expression, two independent experiments were analyzed and two technical replicates were performed per PCR run. All data are expressed as mean ± standard deviation (SD). The statistical analysis was performed using SigmaPlot for Windows version 11.0 (Systat Software Inc., San Jose, CA, USA). The assessment of statistical significance between control and SFN-treated groups was performed using one-way or two-way analysis of variance (ANOVA), followed by the Holm-Sidak's test ($p<0.05$).

IV. RESULTS

4.1. Characterization of confluence and cell morphology

The visualization of control and SFN-treated cells under an inverted microscope revealed drastic changes in the overall appearance of cultures exposed to SFN (Figure 6). Adherent control cells showed a typical MG-63 fibroblast-like morphology, reaching a confluence of 70% and 90% after 48 and 72 h, respectively. With the increase of SFN concentration in the medium, the confluence of the cultures gradually decreased and the number of detached cells increased, for both times. In the culture medium of cells exposed to higher doses of SFN (10 and 20 µM), the amount of cell debris was also visibly higher. Morphological changes in attached cells were observed within SFN-exposed cultures, namely cell enlargement and a rounded shape.

4.2. Cell viability

The assessment of SFN-induced cytotoxicity was conducted by the MTT assay and is depicted in Figure 7. After 24 h exposure, cell viability was not significantly reduced by the concentrations of 5 and 10 µM SFN when compared to control. A decrease in cell viability was only observed for the concentration of 20 µM SFN ($p<0.05$). However, after 48 h, cell viability was reduced by SFN in a dose-dependent manner ($p<0.05$). The percentage of relative viability decreased in cells exposed to SFN concentrations of 5, 10, and 20 µM. Moreover, SFN showed a time-dependent effect. When comparing the effect of SFN between both exposure periods, cell viability significantly decreased after a longer exposure time for all SFN concentrations.

4.3. Cell cycle and clastogenicity analysis

After 24 h exposure, SFN increased the percentage of cells at G_2/M phase in a dose-dependent manner (Figure 8A). This trend was statistically significant for cultures exposed to 10 and 20 µM SFN. Simultaneously, after 24 h exposure, all SFN doses decreased the percentage of cells at G_1/G_0 ($p<0.05$). The 48 h exposure's results followed the same trend,

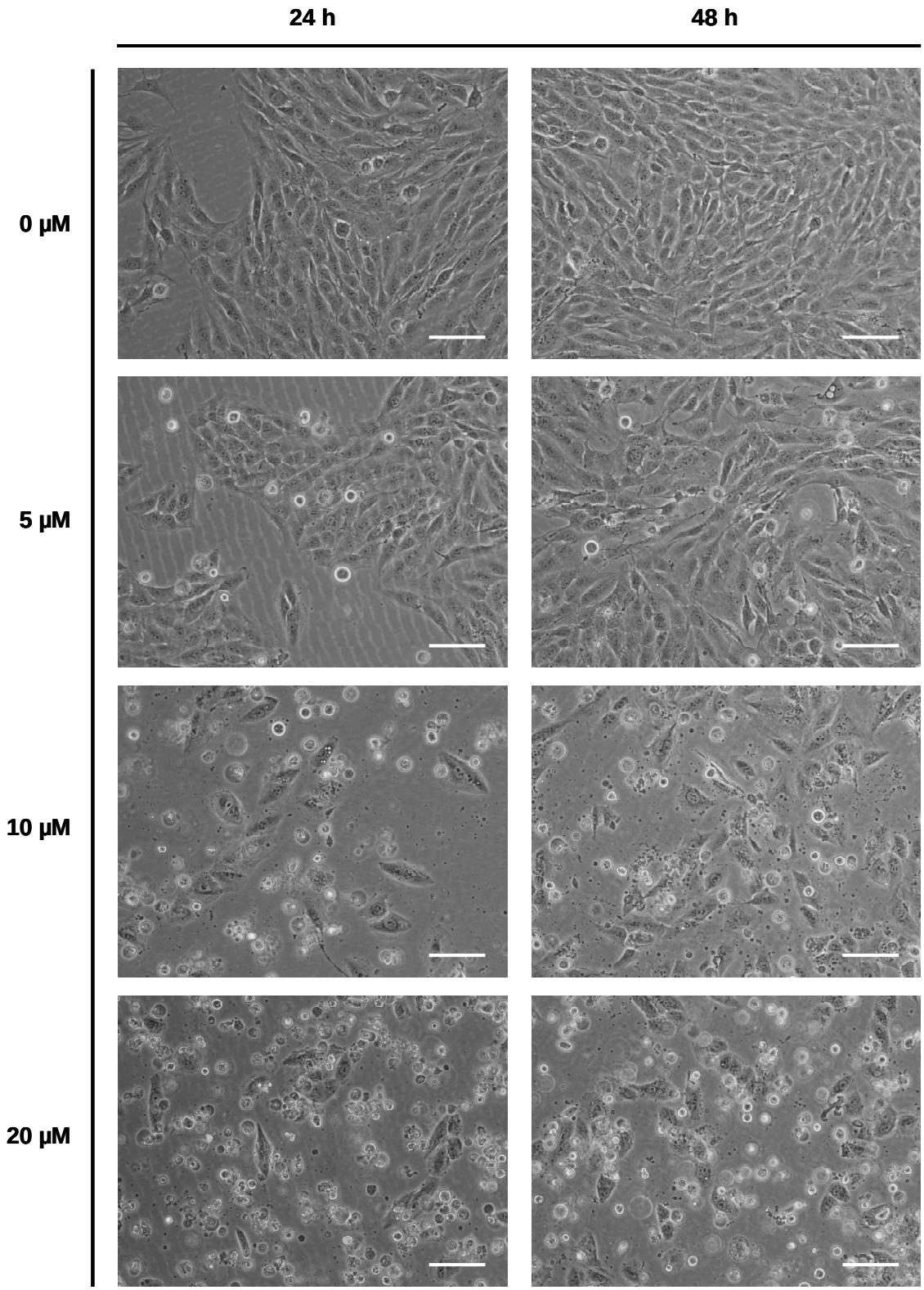

Figure 6. Microscopic visualization of control and sulforaphane-treated MG-63 cells. Confluence and cell morphology of cultures exposed to 0, 5, 10, and 20 µM SFN after 24 and 48 h (100x magnification). Bar = 100 µm.

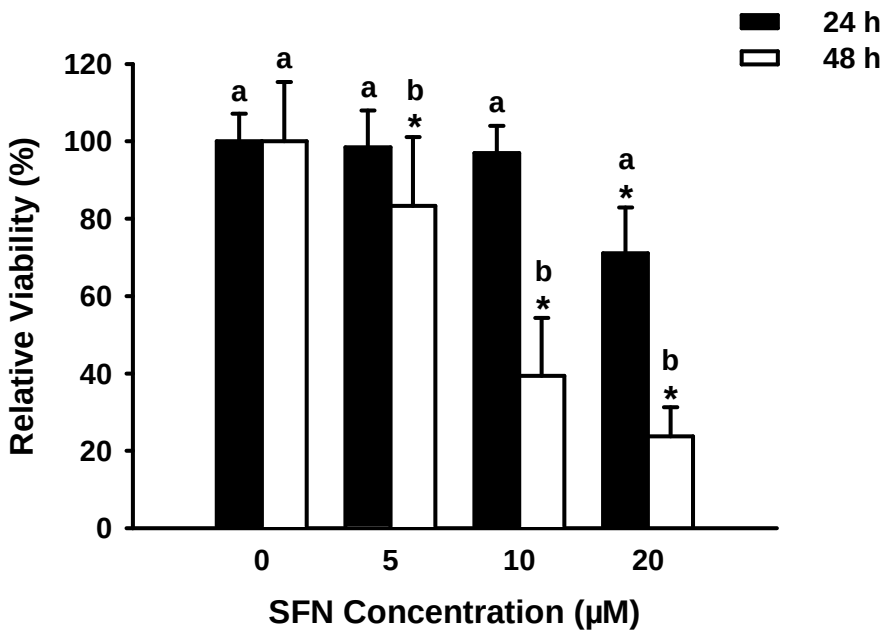

Figure 7. Cytotoxic effect of sulforaphane on MG-63 cells. Viability of cells exposed to 5, 10, and 20 µM SFN for 24 and 48 h relatively to control (100%). * significant differences between control and SFN-treated cells within time ($p<0.05$). a, b, significant differences between times within a concentration ($p<0.05$).

with all tested SFN concentrations exhibiting greater magnitude of response (Figure 8B). After 48 h and compared to control, SFN-treated cultures presented significantly higher percentages of cells at G_2/M for all tested SFN concentrations. Inversely, for the same exposure period, the percentage of cells at G_1/G_0 phase decreased with every SFN dose ($p<0.05$). As for 24 h exposure, after 48 h exposure, the S phase was not affected by SFN ($p<0.05$). Collectively, these data show a dose-dependent effect of SFN on cell cycle distribution.

Clastogenicity evaluation by the FPCV of the G_1/G_0 peak showed that SFN didn't induce significant damage when administered for 24 h, as FPCV values from control and treatments were similar (Figure 9A). Yet, for the longer exposure period of 48 h, FPCV values revealed that SFN exerted a clastogenic effect ($p<0.05$), given by increasing FPCV values in 5, 10, and 20 µM SFN-treated cultures, as shown in Figure 9B.

4.4. Apoptosis assessment

When treated for 24 and 48 h with 5 µM SFN, cells didn't present significant differences in the viability status comparing to control. Untreated and 5 µM SFN-treated cultures induced

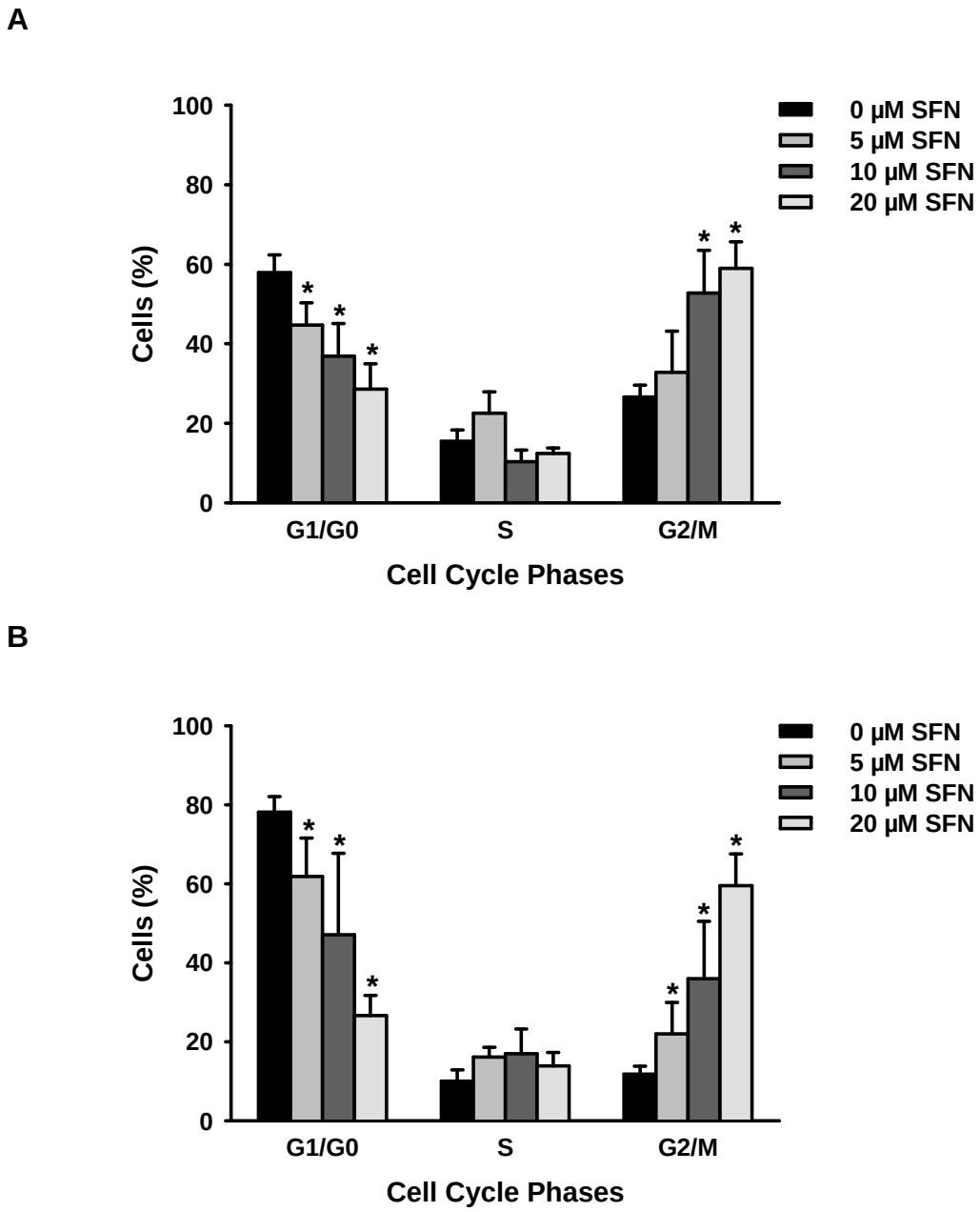

Figure 8. Cytostatic effect of sulforaphane on MG-63 cells. Cell cycle dynamics of MG-63 cells exposed to SFN for (A) 24 and (B) 48 h. * significant differences between control and SFN-treated cells ($p<0.05$).

similar percentages of viable, early apoptotic, late apoptotic, and necrotic cells after 24 h (Figure 10A). In the meantime, results of 5 µM SFN-treated and untreated cultures after 48 h showed a resembling trend with no significant differences between their viability status (Figure 10B).

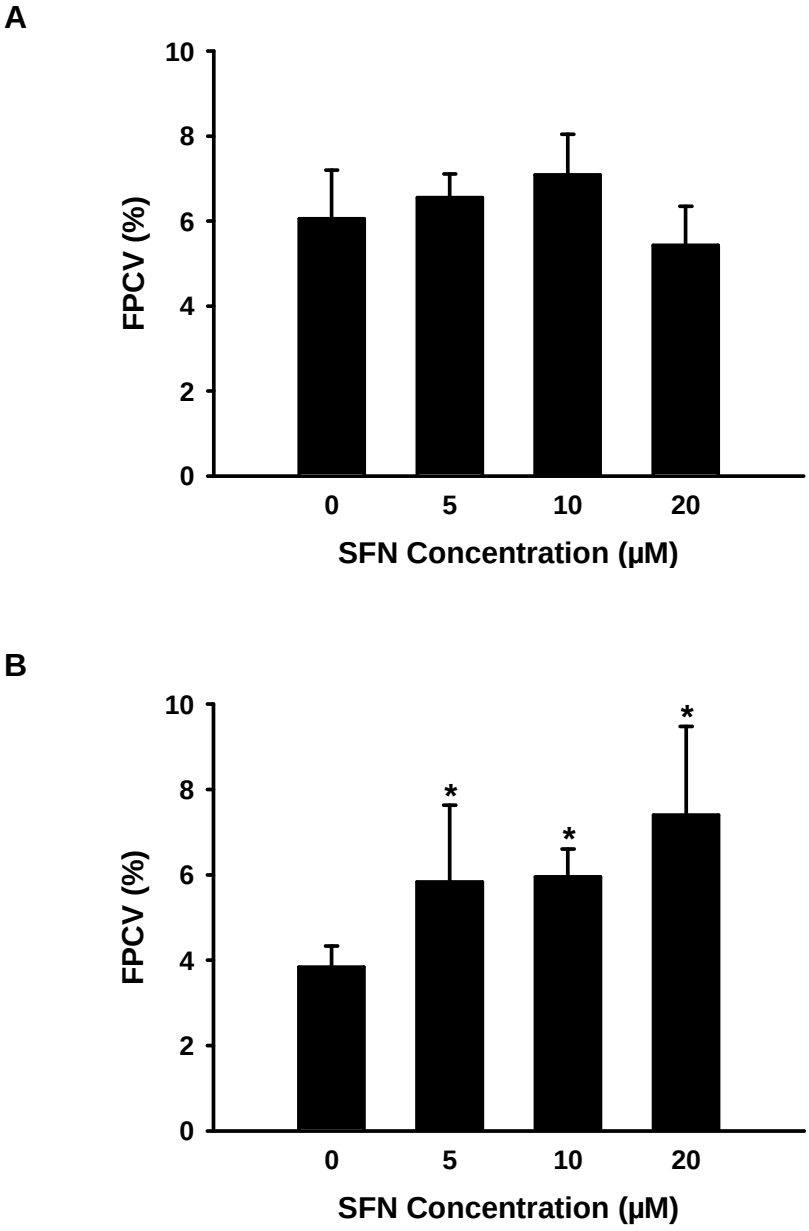

Figure 9. Sulforaphane-induced clastogenicity. G_1/G_0 peak FPCV percentage of MG-63 cells exposed to 0, 5, 10, and 20 µM SFN for (A) 24 and (B) 48h. * significant differences between control and SFN-treated cells ($p<0.05$).

Nevertheless, results show induction of apoptosis by the highest SFN doses. As depicted in Figure 10, for both 24 (Figure 10A) and 48 h (Figure 10B) exposure times, cultures exposed to 10 and 20 µM SFN presented a lower number of viable cells compared to control ($p<0.05$) with a concurrent increase of cells undergoing early and late apoptosis ($p<0.05$).

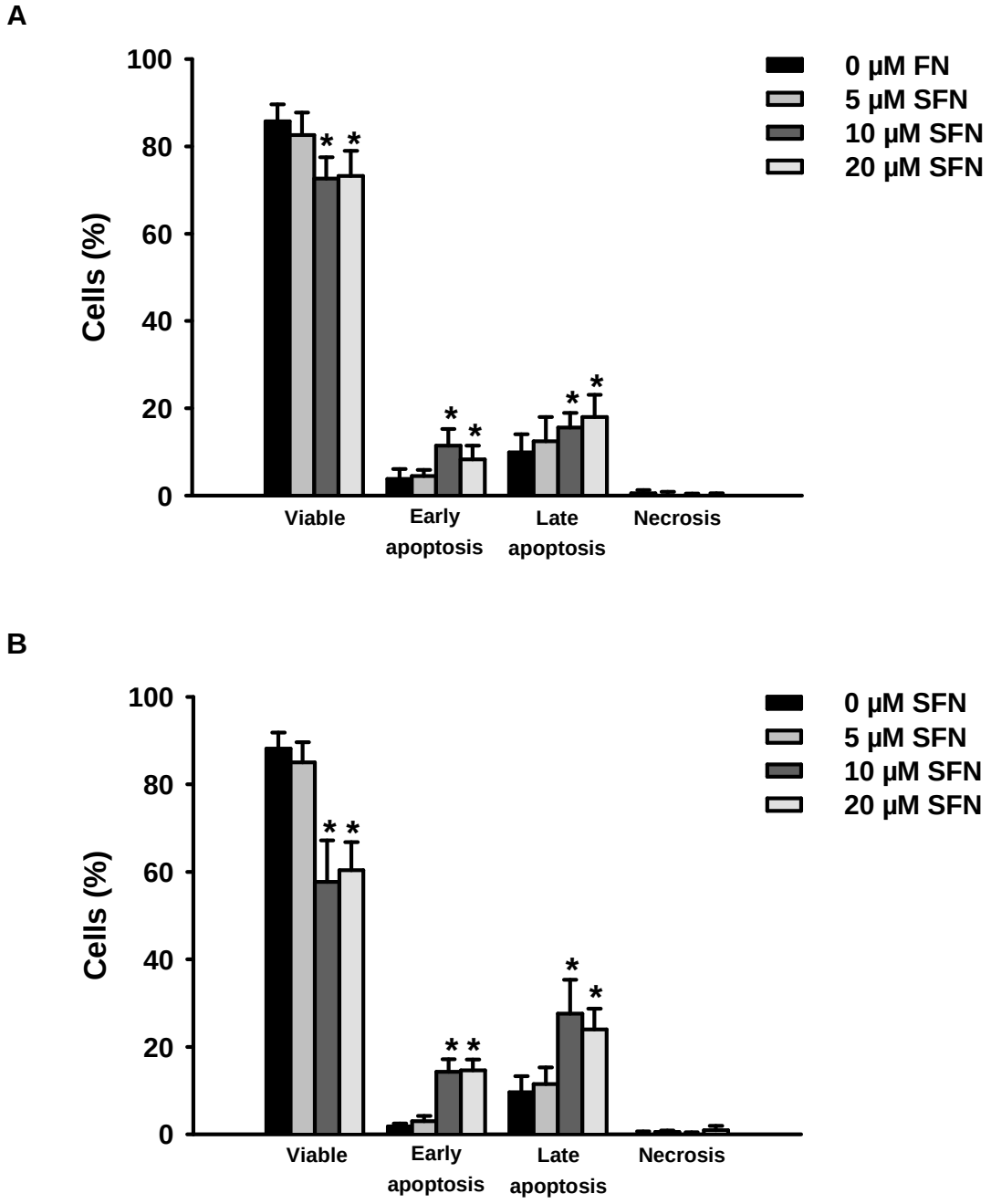

Figure 10. Activation of apoptosis in MG-63 cells exposed to sulforaphane. Percentage of viable, early apoptotic, late apoptotic, and necrotic cells after 0, 5, 10, and 20 µM SFN treatment for (A) 24 and (B) 48 h. * significant differences between control and SFN-treated cells ($p<0.05$).

Furthermore, the cytotoxicity of SFN does not appear to be associated with the induction of necrosis. Necrotic cells were found at similar percentages in every condition for 24 and 48 h experiments.

4.5. Caspase activity assessment

As pointed earlier, 10 and 20 µM SFN doses were able to induce a significant increase of apoptotic cells in treated cultures, and this effect was more pronounced after 48 h. For this reason, the enzyme activity of caspase 3, caspase 8, and caspase 9 was only measured in cultures exposed to 10 and 20 µM SFN for 48 h.

Caspase 3 specific activity (Figure 11A) was enhanced with increasing SFN concentrations. Nevertheless, this trend was not statistically significant. The assessment of caspase 8 showed a slight increase of the specific activity of this enzyme in cultures treated with 10 µM SFN and a slight decrease in cultures exposed to 20 µM SFN, when comparing to control, but none of the differences were significant (Figure 11B). As represented in Figure 11C, caspase 9 specific activity was also enhanced in cultures treated with both SFN concentrations. Again, these values were not statistically different from caspase 9 activity found in control.

4.6. Gene expression

Because of the more pronounced effects obtained in cell cycle distribution after 48 h of SFN treatment, expression of genes involved in cell cycle regulation was only quantified in cultures exposed to SFN for the described period. Also, cultures exposed to 5 and 20 µM SFN were not used in this analysis due to the less intense effects shown in cell cycle modulation and the insufficient total amount of RNA obtained after RNA extraction, respectively. Consequently, gene expression was measured in control cells vs. 10 µM SFN-treated cells (Figure 12).

Expression levels of the analyzed cell cycle genes were in general reduced by SFN, with the exception of two genes. After 48 h, expression of *CDK2* and *CCNB1* in 10 µM SFN-treated cultures was lower than in control, but without statistical significance. In contrast, the same SFN dose significantly downregulated the expression of *CHEK1* and *CDC25C*. On the other hand, 10 µM SFN was found to upregulate the expression of *CHEK2* and *CDK1*, the latter showing a significant increase in the expression level.

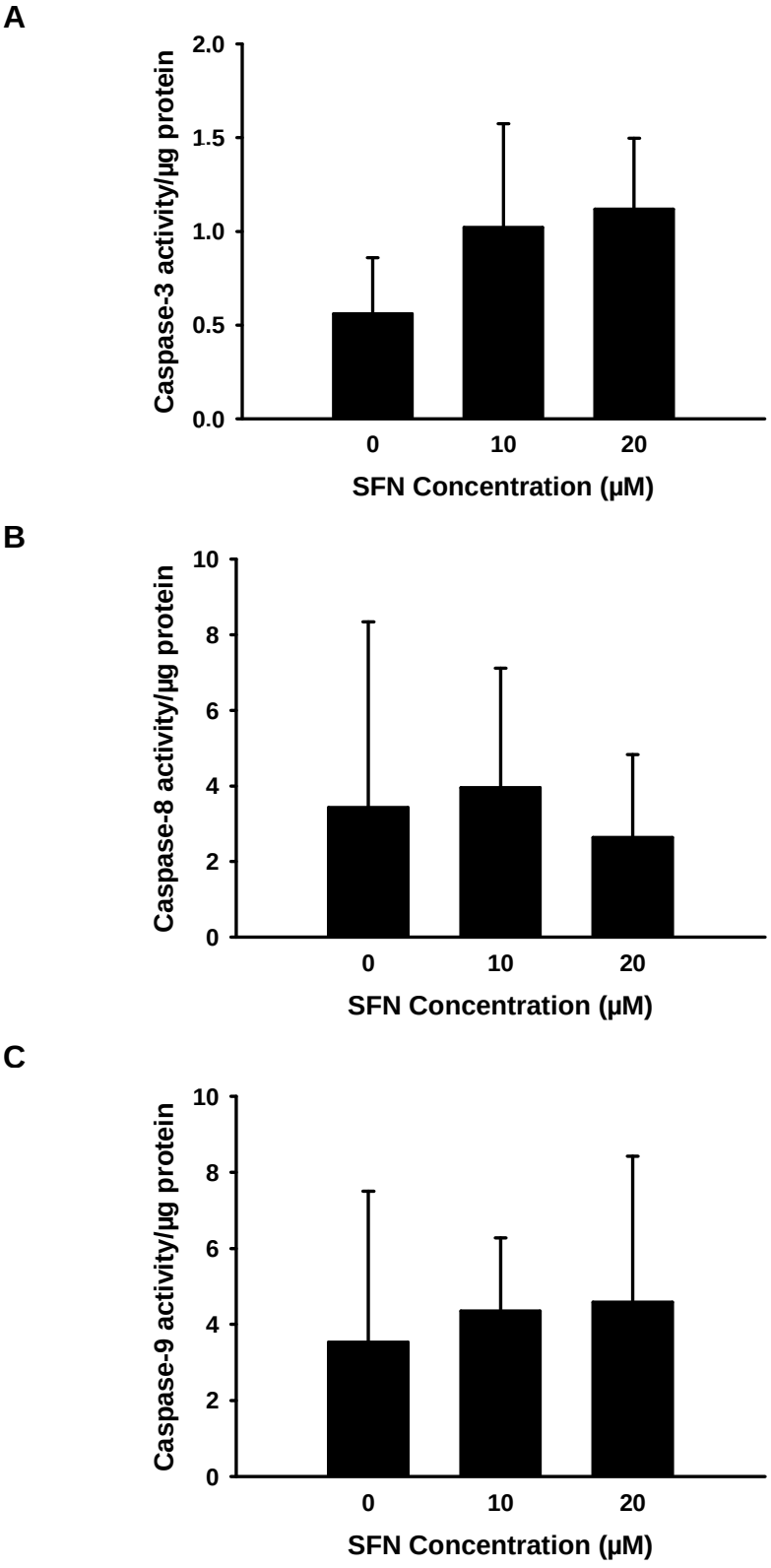

Figure 11. Specific activity of two initiator caspases and one executioner caspase after sulforaphane treatment. MG-63 cells were treated with 0, 10, and 20 µM SFN and the activity of (A) caspase 3, (B) caspase 8, and (C) caspase 9 was measured after 48 h.

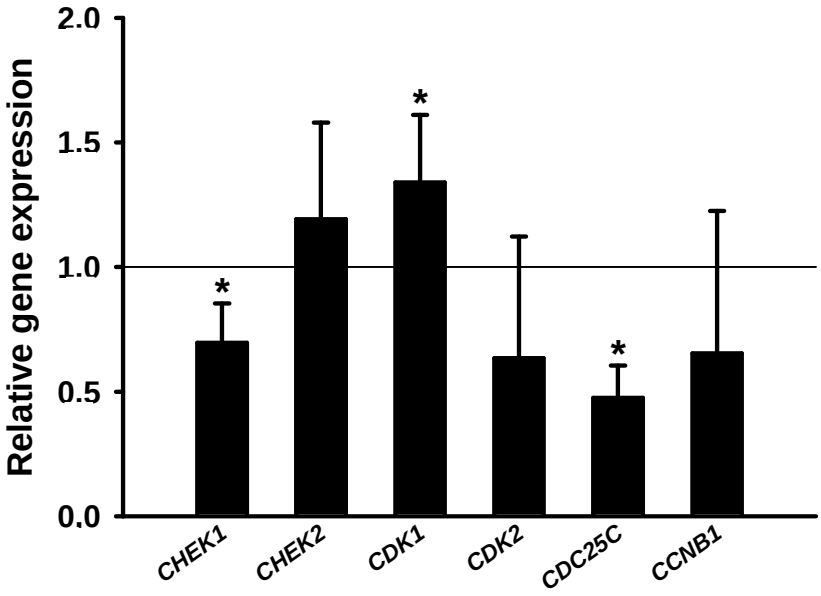

Figure 12. Relative expression of cell cycle-related genes after exposure to 10 µM sulforaphane for 48 h. Expression values of *CHEK1*, *CHEK2*, *CDK1*, *CDK2*, *CDC25C*, and *CCNB1* were normalized to the housekeeping gene *GAPDH*. Data are presented as the expression of each gene in cultures treated with 10 µM SFN relatively to the expression obtained in untreated cultures. * significant differences between control and SFN-treated cells ($p<0.05$).

V. DISCUSSION

Cancer is a complex disease, in part due to the multiplicity of genomic perturbations governing the cellular network that allows the survival of cancer cells. Such perturbations vary widely between different types of cancer, and even individuals presenting the same type of cancer are often found to carry tumors that are driven by their own sets of genomic changes. As a result, the effectiveness of current cancer treatment is frequently undermined.

Bone sarcomas, in which OS is comprised, represent 5-10% of children and young adults' malignancies (Skubitz and D'Adamo, 2007). Nevertheless, these sarcomas are somewhat rare in an overall view of cancer, accounting for merely 0.2% of all cancers (Greenlee *et al.*, 2001). Because of the relative rarity of OS incidence, research on this pathology is sometimes hindered by the low number of available study subjects and the poor understanding of the biology of this tumor. Additionally, current treatment options for OS seem to fail in achieving successful survival rates and induce aggressive toxicity (Bacci *et al.*, 2003; Patel *et al.*, 2004; Duffaud *et al.*, 2012; Ebb *et al.*, 2012).

SFN, a naturally occurring ITC, has evidenced to modulate several key signaling pathways in different types of cancer, both *in vitro* and *in vivo*. Induction of apoptosis and blockage of cell cycle progression in cancer cells are two well-established SFN properties that make this phytochemical a promising agent in cancer therapy (Fimognari and Hrelia, 2007).

Considering the above mentioned, this dissertation aimed to investigate the potential of SFN as a chemotherapeutic agent for OS, evaluating its cytotoxicity in an *in vitro* human OS model and clarifying some of the molecular mechanisms leading to it. MG-63 cells were exposed to 5-20 µM SFN for 24 and 48 h, after which several parameters were assessed. The range of SFN doses was selected based on the literature.

Results show that SFN induced a cytotoxic effect on MG-63 cells, promoting a decrease in cell viability in a dose- and time-dependent manner. Several studies support a magnification of SFN cytotoxicity with higher doses and longer exposures in different

human cancer cells, but this effect appears to be highly dependent on the cell type (Misiewicz *et al.*, 2005; Yeh and Yen, 2005; Chaudhuri *et al.*, 2007; Jin *et al.*, 2007).

The cell cycle dynamics of MG-63 cells after SFN treatment was also inquired through DNA content analysis by flow cytometry. For both exposure periods, the S phase presented non-significant alterations; contrarily, SFN is shown here to diminish the number of cells in G_1/G_0 phase and concurrently raise the G_2/M phase cell population, again in a dose-dependent manner. This accumulation of cells with DNA content = 4N shows that SFN induces a G_2/M phase arrest in this human OS model. As the majority of cancer cell lines affected by SFN, the progression of MG-63 cell cycle was blocked at G_2/M phase and for that some mechanisms have been unveiled.

The G_2/M arrest is commonly a consequence of DNA damage and this arrest typically enables the cell machinery to repair the DNA lesions, thereby maintaining the genome integrity and preventing the propagation of defective DNA to daughter cells. This is generally achieved by the activation of the G_2/M DNA damage checkpoint through the recognition of DNA damage by molecular sensors that activate a cascade of kinases; the most downstream ones, in turn, directly target cell cycle key controllers, blocking the transition to mitosis (Medema and Macurek, 2012). Macip and colleagues reported induction of a reversible G_2/M arrest by sublethal doses of ROS via Chk1 phosphorylation (Macip *et al.*, 2006) and, actually, production of ROS has been correlated with SFN-induced cell cycle arrest at G_2/M. Mitochondrial DNA deficient Rho-0 variants of LNCap and PC-3, two human prostate cancer cell lines, were shown to be more resistant to several SFN effects, including G_2/M cell cycle arrest, than the corresponding wild-type cells, with mitochondria-derived ROS playing a key role in the initiation of SFN cytotoxicity (Xiao *et al.*, 2009); another study supports ROS-mediated blockage of G_2/M transition in prostate cancer cells exposed to SFN (Cho *et al.*, 2005). Interestingly, data generated by our group also showed that the increase in ROS production is correlated with G_2/M cell cycle arrest in MG-63 after SFN treatment (Costa *et al.*, 2012). Remarkably, the generation of ROS induced by SFN appears to be tumor cell specific (Antosiewicz *et al.*, 2008).

Matsui and collaborators found an upregulation of $p21^{Cip1}$ in MG-63 after SFN treatment that contributed to G_2/M blockage (Matsui *et al.*, 2007) and the same was observed in U2-OS, another OS cell line (Kim *et al.*, 2011). These results reinforce the potential of SFN as a chemotherapeutic agent for OS, since they reveal that the expression of $p21^{Cip1}$, a CKI

whose expression is tightly regulated by the transcription factor p53, is modulated by SFN even when p53 is inactivated (a common alteration in OS).

If the DNA damage is reparable, the G_2/M checkpoint is deactivated and cells can progress to mitosis after restoring the DNA integrity. Nevertheless, it has also been proposed that SFN may induce ROS-independent G_2/M arrest by disruption of tubulin polymerization and spindle assembly. A correct segregation of chromosomes during mitosis requires formation of the mitotic spindle, which includes polymerization of α- and β-tubulin heterodimers into microtubules (Walczak and Heald, 2008). Mi and collaborators demonstrated that in A549, a carcinomic human alveolar basal epithelial cell line, SFN directly targets α- and β-tubulin inducing conformational changes (probably due to covalent bonding to cysteine residues), which results in microtubules disruption and tubulin polymerization inhibition (Mi *et al.*, 2008). Perturbations in microtubules dynamics were also presented in other SFN reports (Jackson and Singletary, 2004a; Jackson and Singletary, 2004b; Jackson *et al.*, 2007; Azarenko *et al.*, 2008) and were always associated with G_2/M arrest. This effect was confirmed by recent data from our group showing that SFN triggers microtubules disruption in MG-63 cells (unpublished observations).

The variation in DNA content measured by the FPCV of the G_1/G_0 peak can be used as diagnosis for clastogenic damage within a cell population. The dispersion of nuclear DNA content measured by this flow cytometric parameter reflects chromosomal perturbations (e.g., chromosome breakage and reattachment, loss of chromosome fragments) that result in unequal distribution of DNA in the daughter cells and magnification of the genomic destabilization by further divisions (Misra and Easton, 1999). The significant increase of the FPCV values for all SFN doses, after 48 h of treatment, suggests that SFN leads to clastogenic damage in MG-63 cells exposed for longer periods to this ITC. Different genotoxic effects of SFN have already been demonstrated. DNA single (Sestili *et al.*, 2010) and double (Singh *et al.*, 2004b; Sekine-Suzuki *et al.*, 2008; Sestili *et al.*, 2010) strand breaks were found in different transformed and non-transformed cell lines after treatment with SFN. In fact, DNA breaks were previously detected in SFN-treated MG-63 cells in a dose-dependent manner after 48 h exposure, but the type of breaks (single or double) remains unclear (Pinto, 2011). Moreover, micronuclei and nucleoplasmic bridges may arise during nuclear division from lagging whole chromosomes/acentric chromosome fragments and from dicentric chromosomes caused by misrepair of double strand DNA

breaks/telomere end fusions, respectively (Fenech, 2007). Exposure to SFN increases the frequency of such nuclear alterations in MG-63 cells (Remédios *et al.*, 2012). Yet, and as for other SFN effects, the induction of micronuclei appears to depend on the cell type, dose, and culture conditions, since SFN is also able to prevent micronuclei formation induced by selected mutagens (Fimognari *et al.*, 2005). Taking into account the previously discussed and the results from the flow cytometric analysis, the G_2/M arrest in this OS model was probably activated by SFN-induced DNA damage.

Apoptosis plays a vital role in the maintenance of tissue homeostasis. Depending on the extent of the DNA damage that triggers cell cycle arrest, cells can re-enter cell cycle progression (as stated before) or, in case DNA damage is not reparable, cells can activate the apoptotic machinery and die through programmed cell death (Medema and Macurek, 2012). To examine whether apoptosis could be involved in the loss of cell viability driven by SFN, the FITC Annexin V assay was performed. Data indicate that the lowest SFN dose (5 µM) was not able to induce apoptosis in MG-63, either for 24 or 48 h treatments. Nevertheless, for the same exposure periods, 10 and 20 µM SFN significantly reduced the viability within the cell populations and, at the same time, increased cell death through apoptosis, as shown by the higher number of MG-63 cells with early and late apoptotic characteristics. Kim and colleagues used the same range of SFN doses and found apoptotic cell death in another OS cell line, evidenced by the FITC Annexin V assay and the apoptotic characteristic ladder pattern of DNA fragmentation, which was confirmed by the proteolytic cleavage of the caspase 3 substrates poly (ADP-ribose) polymerase (PARP) and inhibitor of caspase-activated DNase (ICAD) (Kim *et al.*, 2011), supporting the results of this dissertation. Although the molecular basis of apoptosis in MG-63 cells was not identified, several *in vitro* and *in vivo* studies report SFN-induced apoptosis through a multitude of mechanisms: upregulation of gene and/or protein expression of pro-apoptotic factors, as Bax (Gamet-Payrastre *et al.*, 2000; Fimognari *et al.*, 2002; Singh *et al.*, 2004a; Choi and Singh, 2005; Yeh and Yen, 2005; Devi and Thangam, 2012), Bak (Choi and Singh, 2005), and p53 (Fimognari *et al.*, 2002; Park *et al.*, 2007; Devi and Thangam, 2012); downregulation of protein expression of anti-apoptotic factors, as X-linked inhibitor of apoptosis protein (XIAP) (Choi and Singh, 2005; Choi *et al.*, 2007), Bcl-2 (Jackson and Singletary, 2004b; Singh *et al.*, 2004a; Yeh and Yen, 2005; Park *et al.*, 2007; Pledgie-Tracy *et al.*, 2007; Hamsa *et al.*, 2011; Sharma *et al.*, 2011; Devi and Thangam, 2012;

Wang *et al.*, 2012), and Bcl-X_L (Yeh and Yen, 2005; Park *et al.*, 2007); and upregulation of protein expression of intrinsic apoptotic pathway mediators, as Apaf-1 (Choi and Singh, 2005; Choi *et al.*, 2007). Noteworthy, the cytotoxicity exerted by SFN in MG-63 was not associated with necrosis, other type of cell death considered less controlled than apoptosis and characterized by cellular swelling, release of intracellular components into the microenvironment, and induction of inflammatory response (de Bruin and Medema, 2008).

Taken together, these results support the aspects described in the qualitative characterization of MG-63 cells after SFN treatment. Regarding the loss of confluence, control cells proliferated normally, whereas SFN-treated cells suffered a blockage in cell cycle, preventing a regular growth rate. Apoptosis also contributed to this outcome, as well as to the increased amount of detached cells and cell debris in the medium of cultures exposed to 10 and 20 μM SFN, the same doses that succeed in the activation of the apoptotic program in these cells. Microscopic visualization also revealed morphological changes (enlargement and rounded shape) in attached cells of treated cultures. Chauduri and collaborators reported the same cell shape in another monolayer cancer cell line upon SFN treatment (Chaudhuri *et al.*, 2007). Targeting of α- and β-tubulin by SFN can justify the changes in the cell aspect. These proteins are components of the microtubule cytoskeleton and, as referred before, SFN directly binds to them and induces conformational changes that lead to the disruption of microtubules and impairment of tubulin polymerization (Jackson *et al.*, 2007); collapse of the microtubule cytoskeleton was associated with acquisition of a rounded shape in other cells upon SFN exposure (Mi *et al.*, 2008). In addition, a proteomic analysis of A549 cells treated with SFN revealed that actin, other tubulin chains, vimentin, tropomyosin, and desmin (constituents of different cytoskeletal structures) are potential protein targets of this ITC (Mi *et al.*, 2011). Thus, a cytoskeleton rearrangement of MG-63 cells due to SFN-microtubule proteins interaction may be the foundation for the observed morphological alterations of these OS model.

The effect of SFN on apoptosis induction prompted the next series of experiments wherein an attempt to determine the triggered apoptotic pathway(s) was made. Caspase 8 and caspase 9 were chosen as representatives from the extrinsic and intrinsic apoptotic pathways, respectively, as they initiate the corresponding apoptotic pathways, and their activity was measured. The activity of caspase 3, the first executioner of the caspase cascade that leads to apoptosis and substrate of caspases 8 and 9, was also explored. Our

data do not clearly demonstrate the activated apoptotic pathway. Although results are indicative of an increment of caspase 9 activity with 10 and 20 µM SFN and the same effect for caspase 8 activity after 10 µM SFN when compared to control, the lack of statistical significance in the obtained differences restrain any assumptions on the subject. Data from caspase 3 assessment also points to a boost in its activity by the tested SFN doses, but once more not statistically significant However, these negative results must be interpreted cautiously, because a substantial variability in the caspase activity of samples from the same condition was observed in every caspase assessed (as shown by the high SD values), which can cover a difference in the activity of these enzymes between untreated and treated cultures when one actually exists. Moreover, the profile of caspase 3 activity, even though not showing statistical magnitude, is sustained by the results from the FITC Annexin V assay that demonstrate undoubtedly apoptotic cell death. As for other human cancer cell lines, SFN-induced apoptosis seems to occur through different pathways. Activation of caspase 8 is reported in pancreatic cancer cells (Pham *et al.*, 2004) and Barrett's adenocarcinoma (Qazi *et al.*, 2010), while procaspase 9 cleavage or caspase 9 activation occur in different colon cancer cell lines (Pappa *et al.*, 2006) and medulloblastoma (Gingras *et al.*, 2004) upon SFN administration. Also, the apoptotic pathway induced by SFN can vary within a cancer type, as Pledgie-Tracy and team found in different breast cancer cell lines (Pledgie-Tracy *et al.*, 2007). On the other hand, intrinsic and extrinsic pathways are not exclusively activated in apoptosis caused by SFN. Studies suggest that SFN is able to activate both mitochondria- and death receptor-mediated pathways in the same cell line (Singh *et al.*, 2004a; Nishikawa *et al.*, 2009; Balasubramanian *et al.*, 2011). Alternatively, a caspase 12-dependent apoptosis is possible to occur as well, as determined by Karmakar and colleagues in SFN-treated glioblastoma cells (Karmakar *et al.*, 2006). In the case of MG-63 cells, more experiments are required to better understand the cause of the variability found across this work and to clarify whether SFN-induced apoptotic death is driven by caspase 8, caspase 9, both, or even other alternative caspases.

Next, to elucidate if a different expression of cell cycle-related genes could be involved in the cell cycle arrest induced by SFN in the used OS model, qRT-PCR analysis of G_2/M key regulators was performed. The completion of G_2 phase and progression through mitosis requires activation of Cdk1/cyclin B1 complex, also known as maturation-promoting factor

(MPF), in which the kinase activity that triggers the access to mitosis relies on Cdk1 (Lindqvist *et al.*, 2007). Cyclin B1 is the regulatory element of MPF and without it the complex lacks activity. After SFN treatment, *CDK1* (coding for Cdk1) expression levels significantly increased. In contrast, levels of *CCNB1* (coding for cyclin B1) suffered a slight reduction that was not considered statistically significant. Nevertheless, a considerable variability was found in the expression of this gene and the possibility that SFN plays an inhibitory role in it shouldn't be excluded. Considering this, mitotic entry could have been hindered through the direct downregulation of cyclin B1 by SFN, which is supported by other studies (Singh *et al.*, 2004b; Balasubramanian *et al.*, 2011), including in OS cells (Kim *et al.*, 2011).

Furthermore, the activity of Cdk1 is negatively regulated by phosphorylation (e.g., Wee1 and Myt1 kinases), which can be reversed by the phosphatase Cdc25C (Medema and Macurek, 2012). Expression levels of *CDC25C* in SFN-treated cells showed a significant decrease relatively to untreated ones, in this work as in others (Singh *et al.*, 2004b; Herman-Antosiewicz *et al.*, 2007), suggesting an important role for Cdc25C depletion, and hence Cdk1/cyclin B1 inactivation, in SFN-induced G_2/M cell cycle arrest. Therefore, the potential higher expression of Cdk1 at the protein level can be overcome through the downregulation of Cdc25C that leads to an accumulation of phosphorylated (inactive) Cdk1.

The G_2/M checkpoint-mediated cell cycle arrest in response to DNA damage is at least in part established through inhibition of Cdc25C activity by the serine/threonine protein kinases Chk1 and Chk2 (Boutros *et al.*, 2007); these kinases integrate signals from the DNA damage sensors Ataxia telangiectasia mutated (ATM) and ATM- and Rad3-related protein (ATR). For this reason, expression levels of *CHEK1* and *CHEK2* encoding Chk1 and Chk2, respectively, were quantified. SFN had a negative effect on the expression of *CHEK1*. Contrarily, *CHEK2* presented a slightly higher level of mRNA upon SFN treatment, but not significant. These results suggest that inhibition of Cdc25C (already less expressed in SFN-treated MG-63 cells) was prompted mainly by Chk2, as it presented at least a constant level of expression. The role of Chk2 in SFN-induced G_2/M blockage was noticed in human prostate cancer cells and was confirmed by an increase in the resistance to this effect in the same cells transfected with Chk2-specific small interfering RNA duplexes (Singh *et al.*, 2004b).

In addition, the role of p53-independent G_2/M arrest in MG-63 was investigated. The MG-63 cell line carries rearrangement of the p53 gene and low levels of transcripts are produced, which results in virtually no expression of wild-type p53 in this cell line (Chandar *et al.*, 1992; Ottaviano *et al.*, 2010). Since null mutations of *CDK2* have been found associated with defective G_2/M arrest (Chung and Bunz, 2010), the *CDK2* gene expression was also investigated. As for the case of cyclin B1 gene, MG-63 cells exposed to SFN presented lower, although not significantly lower, levels of Cdk2. However, given the high variability in gene expression observed, it is not conclusive whether this gene is downregulated upon exposure to SFN.

Figure 13 shows the alterations in gene expression found across the present work that may be involved in the G_2/M cell cycle arrest obtained after exposure of MG-63 cells to 10 µM SFN. These last results concerning gene expression should be cautiously interpreted due to different reasons: a) data were obtained from two independent experiments; b) some gene profiles show high SD values that may be hiding existing differences between treated and untreated cells; and c) the level of gene expression does not always relate with the level of protein expression or protein activity.

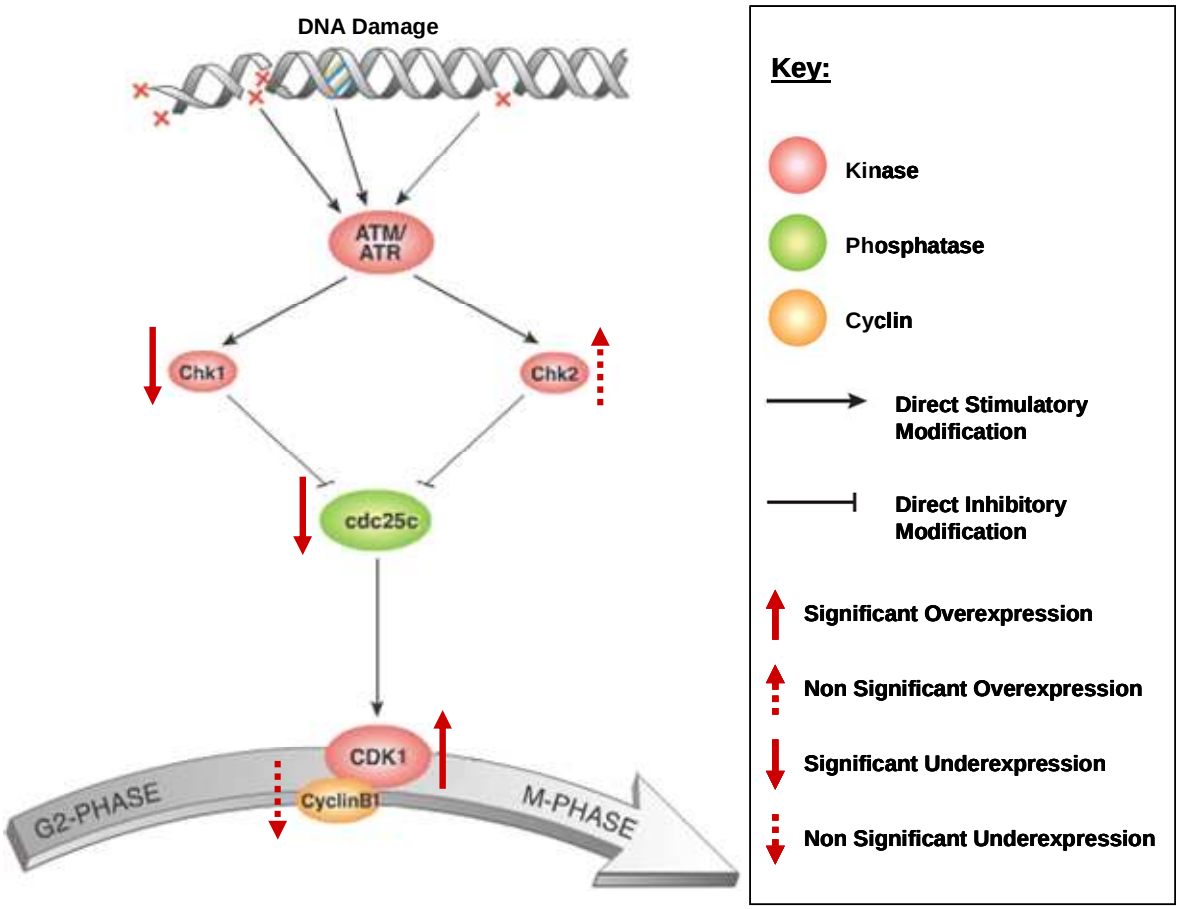

Figure 13. Sulforaphane-induced changes in gene expression of MG-63 that may contribute to activation of G_2/M DNA damage checkpoint. Adapted from Cell Signaling Technology Inc. (2010) (http://www.cellsignal.com/reference/pathway/Cell_Cycle_G2M_DNA.html).

VI. CONCLUSION AND FUTURE PERSPECTIVES

The present study supports the cytotoxic effect of SFN already described for several tumors and provides evidences that SFN should be considered a promising candidate for OS treatment. SFN caused visible cell morphological changes, cell cycle arrest at G_2/M phase, and apoptosis in the human OS model MG-63, the last two events most likely triggered by SFN-induced DNA damage. Beyond that, preliminary results show that cell cycle arrest was mainly mediated by Chk2, *CDC25C* downregulation, and probably *CCNB1* as well as *CDK2* downregulation.

Nevertheless, the effect of SFN on OS should be further investigated in order to expand our knowledge about the molecular mechanisms underlying these effects. The analysis of expression profiles of other genes, such as apoptotic-related genes, would be important to fully characterize cellular events reported in the present dissertation. Also, protein quantification (e.g., Western blot) would be very interesting to perform, with particular attention to the different protein forms (activated/inactivated, phosphorylated/dephosphorylated, etc.), enabling a comparison with the gene expression profile and a better understanding of the signaling pathways that lead to certain events induced by SFN. New chemotherapeutic agents for OS (and other cancers) should display a sufficiently large therapeutic window and at the same time spare normal cells. A comparative study with different human OS cell lines (e.g., Saos-2, U2-OS, HOS, NOS-1, etc.) and a normal osteoblast cell line is advisable to determine whether SFN effects are tumor selective and whether it exerts a similar cytotoxicity in a broad variety of tumor phenotypes. Lastly, a great deal of attention must be given to the fact that SFN is able to induce clastogenicity. Such effect should be carefully considered in an already inherently unstable genomic context as cancer cells.

VII. REFERENCES

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