Universidade de Aveiro Departamento de Biologia
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Maria Eugénia Marques da Costa

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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 em Microbiologia Clínica e Ambiental, realizada sob a orientação científica da Dra. Helena Cristina Correia de Oliveira, Pós-Doutoranda do Departamento de Biologia da Universidade de Aveiro, do Dr. José Miguel Pimenta Ferreira de Oliveira, Pós-Doutorando do Departamento de Biologia da Universidade de Aveiro e sob co-orientação científica da Professora Doutora Conceição Santos, Professora associada com agregação do Departamento de Biologia da Universidade de Aveiro.

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o júri

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palavras-chave

Sulforafano, Osteosarcoma, Stress Oxidativo, linha celular MG-63, enzimas antioxidantes.

resumo

O sulforafano (SFN) é um composto que pertence à família dos isotiocianatos, sendo o mais estudado deste grupo, principalmente pelas suas potenciais propriedades anti-tumorais. Existe em grande quantidade nas crucíferas, sobretudo nos brócolos. Vários estudos têm demonstrado a capacidade do SFN em causar paragem do ciclo celular, indução da apoptose, aumento de espécies reactivas de oxigénio (ROS), no entanto, os seus efeitos no osteosarcoma, estão ainda pouco compreendidos, principalmente ao nível do stress oxidativo.

O objectivo deste estudo foi investigar a capacidade do SFN induzir, *in vitro*, stress oxidativo numa linha celular de osteossarcoma humano, a MG-63, e compreender as vias de stress oxidativo envolvidas neste processo.

Para tal, as células MG-63 foram expostas a diferentes concentrações de SFN (0, 5, 10 e 20μM) em diferentes períodos (24 e 48h). O crescimento e a morfologia celular foram observados através do microscópio invertido e a viabilidade celular determinada usando o ensaio de *Trypan Blue*. A avaliação do stress oxidativo foi realizada através da análise da actividade das enzimas envolvidas na destoxificação de espécies reativas de oxigénio (ROS), nomeadamente glutationa peroxidase, glutationa reductase e superóxido dismutase. A actividade antioxidante total, a glutationa reduzida e a peroxidação lipídica foram também analisadas, bem como a expressão de genes relacionados com o stress oxidativo (*SOD1, SOD2, GSR, GPX1, GSTM1 e GSTM4* e *CAT)*. Os ensaios enzimáticos, bem como a actividade antioxidante total, foram avaliadas por diferentes ensaios colorimétricos no leitor de microplacas, já a glutationa reduzida e a peroxidação lipídica foram avaliadas por fluorimetria.

Observou-se para ambos os períodos de exposição que a maior concentração de SFN reduziu a viabilidade celular, relativamente ao controlo, bem como a actividade de todas as enzimas analisadas. A actividade antioxidante total aumenta com o tempo e diminui com a concentração de SFN. A actividade das enzimas decresce com o aumento da concentração de SFN (e com o tempo para concentrações de SFN elevadas). Verificou-se também um aumento da glutationa reduzida para concentrações de SFN baixas, embora tenha decrescido para valores de SFN mais elevadas.

A expressão dos genes é concordante em geral com a actividade das respectivas enzimas, ou seja diminui com o aumento da concentração. No entanto é observada uma excepção para a SOD em que a actividade diminui com a concentração enquanto a expressão aumenta. Pode-se observar que a peroxidação lipídica diminui com a concentração.

Como conclusão, estes resultados indicam que o SFN induz stress oxidativo em células de osteossarcoma humano. Propõe-se um modelo de acção para o stress oxidativo induzido por SFN em osteossarcoma.

keywords Sulforaphane, Osteosarcoma, Oxidative Stress, MG-63 Cell line, antioxidant enzymes

abstract

Sulforaphane (SFN) is a compound that belongs to the isothiocyanates family and is the most studied compound of this group, mostly due to its putative anti-tumoral properties. It is found in large quantities in cruciferous vegetables, mainly broccoli.

Several studies have shown the ability of SFN to cause cell cycle arrest, apoptosis induction, reactive oxygen species (ROS) formation however, its effects in osteosarcoma, is still poorly understood, mostly at the stress oxidative level.

The aim of this study was to investigate the ability of SFN in inducing, *in vitro*, oxidative stress in the human osteosarcoma cell line MG-63. It was also an aim to understand the pathways of oxidative stress involved in this process.

For these propose, MG-63 cells were exposed to different concentrations of SFN (0, 5, 10 and 20μM) at different times (24 and 48h). The growth and cell morphology were observed through an inverted microscope, and cell viability determined using Trypan Blue assay. The oxidative stress evaluation was performed by the activity analysis of antioxidant enzymes, namely, glutathione peroxidase, superoxide dismutase and glutathione reductase. The total antioxidant activity (TAA), reduced glutathione and lipid peroxidation were also analysed, as well as the expression of genes related to oxidative stress (*SOD1, SOD2, GSR, GPX1, GSTM1* and *GSTM4 and CAT)*. Enzyme assays and total antioxidant activity were evaluated by different colorimetric assays on a microplate reader, while the reduced glutathione and lipid peroxidation were evaluated by fluorimetry.

It was observed (for both exposure periods) that the highest concentration of SFN reduced cell viability relatively to control, and reduced the activity of all tested enzymes. The total antioxidant activity increased with time and decreased with the concentration of SFN. The activity of enzymes decreased with the increasing concentration of SFN (and with time for the highest SFN concentrations). There was also an increase of the reduced glutathione for low SFN concentrations, while it decreased for higher concentrations of SFN.

Overall, the gene expression is consistent with the corresponding enzymes activities, i.e., the expression decreased with increasing concentrations of SFN. However, SOD was an exception (SOD activity decreased with SFN concentration whereas its expression increased).

It can be observed that lipid peroxidation declined with SFN concentration increase.

Finally, these results indicate that SFN induces oxidative stress in human osteosarcoma cells. It is proposed a model for the pathways involved in this oxidative stress–induced by SFN in osteosarcoma.

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List of Acronymes and Abbreviations

SFN – Sulforaphane

OS – Osteosarcoma

Glucosinolates – S-thioglucoside N-hydroxysulfates

CDK – Cyclin-dependent kinase

CYP – cytochrome P450

$^{\bullet}OH$ – hydroxyl radical

$^{\bullet}O_2^-$ – superoxide anion

$^{\bullet}NO$ – nitric oxide

H_2O_2 – hydrogen peroxide

SOD – Superoxido dismutase

GR – Glutathione reductase

GPx – Glutathione peroxidase

GST – Glutathione-S-t ransferase

CAT – Catalase

GSH – Reduced glutathione

GSSH – Oxidized glutathione

Prxs – Peroxiredoxins

ROS – Reactive oxygen species

RNS – Reactive nitrogen species

MDA – Malondialdehyde

TAA – Total antioxidant Activity

TBARS – Thiobarbituric acid reactive spicies

NrF2 – Nuclear factor E2-related factor 2

ARE – Antioxidative response elements

α-MEM – α-Miniimum Essencial Medium

FBS – fetal bovine sérum

DMSO – dimethyl sulfoxide

BSA – bovine sérum albumin

PBS – Phosphate Buffer Saline without Ca^{2+}/Mg^{2+}

XO – xanthine oxidase

min – minute

s – seconds

h – Hour

nm – nanometers

cm – centimeters

% v/v – percentage volume/volume

mM – milimolar

µL – microlitros

µg/mL – micrograms/milliliter

U/mL – units/milliliter

ºC – Celsius degree

Introduction

1. Osteosarcoma

1.1. General considerations about cancer development and cell physiology

In Europe, it is estimated that around 3.2 million new cases of cancer are diagnosed every year, with predominance of breast, colorectal, prostate and lung cancer occurrences (http://ec.europa.eu/healtheu/health_problems/cancer/index_pt.htm). Moreover, cancer is the second leading cause of death in the United States where it was estimated that, in 2007, over 1.4 million people were diagnosed with cancer.

Cancer is a highly heterogeneous and complex genetic disease since, according to the established model of oncogenesis, the occurrence of consequential mutations in coding regions of oncogenes and tumor suppressor genes is necessary, (un)regulating fundamental cellular processes like cell proliferation, differentiation and apoptosis for cancer development.

The conversion of normal into cancer cells implies a progressive transformation into malignancy by multiple and sequential processes. These imply several alterations in cellular physiology such as high rates of proliferation and angiogenesis (Scholzová et al, 2007; Cuezva et al, 2009).

Diverse studies support that cancer results from disruptions of cell growth and death regulation pathways, which are maintained by the coordinated function of protein-coding genes (e.g. Mannoor et al, 2012). Genetic changes, such as DNA sequence alterations and/or chromosomal changes (e.g. structural or numerical changes) may lead to production of functionally aberrant protein, which may be at the basis of cell transformation into a cancer cell type. Besides DNA sequence alterations, the cell's microenvironment and other epigenetic events may also affect gene expression and thus quantitatively influence the level of 'physiological' protein(s) important in the regulation of cancer (Scholzová et al, 2007; Cuezva et al, 2009). Up to moment, in all these scenarios (m)RNA has been regarded as a 'passive' intermediate but, in the last decade, some evidences suggest that different types of RNA molecules play important roles in gene regulation not only at the level of mRNA processing and regulation of mRNA stability but also at the level of gene transcription (e.g. Scholzová et al, 2007).

In this regard, many studies that were only focused on the analysis of genetic alterations are being questioned due to their limited applicability for the development of effective therapies. On the contrary, it is being now stressed the necessity to develop effective therapies against cancer based on targeting of the biological pathways commonly altered in the cancer cell (Cuezva et al, 2009). Thus, it is fundamental to understand the cell pathways related with tumorigenesis. This knowledge will allow the development of cell molecular markers, which may contribute not only to early tumor detection methods (Mannoor et al, 2012) but also to improve the efficacy of existing therapies such as chemotherapy, etc.

1.2. Osteosarcoma main characteristics

Sarcomas are "*a very heterogeneous group of malignant neoplasms of connective tissues, including bone and soft-tissues*" (vide Demetri, 2012). This type of cancer is rare in comparison with carcinomas and have highest incidence during childhood or infancy (e.g. Kim et al, 2011).

Most primary tumors of the skeleton depend on three varieties of spindle cells: those derived from the fibrous matrix designated by fibrosarcomas, the chondrosarcomas from cartilage and osteosarcomas (OS) that arise from bone (Majó et al, 2010). OS are the most frequent tumor of bone and are highly malignant. OS fundamental characteristic is that they develop from the stem cells that originate normal bone (Majó et al, 2010).

OS are more frequent in younger-aged individuals (Trieb and Kotz, 2001). Its incidence is more notorious in individuals aged 11-20 years, and although it occurs with lower frequency in people aged 60-80, this incidence in younger-aged individuals coincides with the acceleration of bone growth (Kim et al, 2011).

Considering the critical ages and also the lack of effective therapy it is necessary to improve the current research and therapies of this cancer type. Treatment usually includes both chemotherapy and surgery, since chemotherapy alone is incapable of eradicating the primary tumor (Majó et al, 2010).

1.3 . Osteoblast/osteosarcoma cell lines used in *in vitro* studies: emphasis on MG-63

There are several OS cell lines available for *in vitro* studies (e.g. Table 1). The most commonly used are MG-63 and Saos-2 (Mohseny et al, 2011). The MG-63 cell line (used in this work) has typical fibroblasts-like morphology and grows as an adherent monolayer. These cells exhibit a number of characteristics typical of the osteoblast undifferentiated phenotype, including the expression of collagen type I and III, and a low basal expression of alkaline phosphatase (Pautke et al, 2004). Main advantages of the MG-63 cell line rely on their ease of culturing, so providing high amounts of cell material to perform a large number of biochemical and molecular studies related with factors that regulate metabolism of osteoblasts (Bilbe et al, 1996).

Table I: Osteosarcoma cell lines. Osteosarcoma subtype of the original tumor from which cells were derived, N/A- subtype information is not available for cell line. Cells were provided by the different partner institutes of the EuroBoNet network 21–25 or derived from ATCC (from Mohseny et al, 2011). For references marked with * see Mohseny et al, 2011.

Cell line	Subtype	Reference
HOS	N/A	ATCC—CRL-1543
HOS-143B	N/A	ATCC—CRL-8303
HOS-MNNG	N/A	ATCC—CRL-1547
OSA	Fibroblastic	ATCC—CRL-2098
MG-63	Fibroblastic	ATCC—CRL-1427
Saos-2	N/A	ATCC—HTB-85
U2OS	N/A	ATCC—HTB-96
IOR/MOS	Osteoblastic	Benini *et al*[21] *
IOR/OS9	Osteoblastic	Benini *et al*[21] *
IOR/OS10	Fibroblastic	Benini *et al*[21] *
IOR/OS14	Osteoblastic	Benini *et al*[21] *
IOR/OS15	Osteoblastic	Benini *et al*[21] *
IOR/OS18	Osteoblastic	Benini *et al*[21] *
SARG	N/A	Benini *et al*[21] *
KPD	Osteoblastic	Bruland *et al*[22] *
OHS	Osteoblastic	Fodstad *et al*[23] *
HAL	N/A	Høifødt, Oslo[e] *
ZK-58	Osteoblastic	Schulz *et al*[24] *
MHM	Fibroblastic	Kjonniksen *et al*[25] *

2. Isothiocyanates

2.1. Metabolism and bioactivity

Isothiocyanates are compounds already described in literature for some tumor types as possessing potential antitumoral activity (e.g. Clarke et al, 2008; Traka and Mithen, 2009; Brown and Hampton, 2011; Yao et al, 2011). Isothiocyanates are a family of biologically active phytochemicals, which are derived from glucosinolate precursors (Dinkova-Kostova, 2012).

Glucosinolates (S- thioglucoside N-hydroxysulfates) are mainly found in cruciferous plants. There are almost 350 genera and 3000 species of Cruciferae or Brassicaceae family. The best-known species of Brassicaceae are the (commonly known) broccoli, cauliflower, cabbage, kale, brussels sprouts, radish and various mustards (Vasanthi et al, 2009). These species are also known for their richness in bioactive products such as vitamins E, B1 and C, minerals like selenium, flavonoids like quercetin and carotenoids, and, the glucosinolates (Vasanthi et al, 2009). These last are among the most studied bioactive phytocompounds for antitumor properties.

Glucosinolates are anionic, hydrophilic and sulfur-containing secondary metabolites (Zhang et al, 2011; Dinkova-Kostova, 2012). In all glucosinolate-containing plants, the β-thioglucosidase enzymes known as myrosinases are also found, but located in different cell compartments: glucosinolates are found in vacuoles (Zhang et al, 2011) and myrosinases are found in, e.g. idioblasts. When tissues and cells are damaged, glucosinolates are hydrolysed by myrosinase released and form different products, such as isothiocyanates (ITCs), thiocyanates and nitriles, depending on the reaction conditions (Zhang et al, 2011; Dinkova-Kostova, 2012).

The hydrolysis reaction consists on cleavage of thioglycoside by myrosinase enzyme, which releases glucose and forms an unstable non sugar compound named aglycone (R-C(-SH)=N-O-SO3-) (Figure 1). After the formation of this intermediate compound the fragments of aglycone eliminate the sulfate group (SO_4^{2-}) giving rise to isothiocyanates group (R-N=C=S (Figure 1). Besides this product, other compounds are formed after the glucosinolates breakdown, such as thiocyanates and nitriles, etc. The formation of these compounds depends on the reaction conditions (e.g. pH, presence of Fe^{2+}, or temperature) (Zhang et al, 2011; Guerrero-Beltrán et al, 2012). These compounds have bactericidal,

antioxidant, anticarcinogenic effects among others, while intact glucosinolates are believed to be inactive (Zhang et al, 2011).

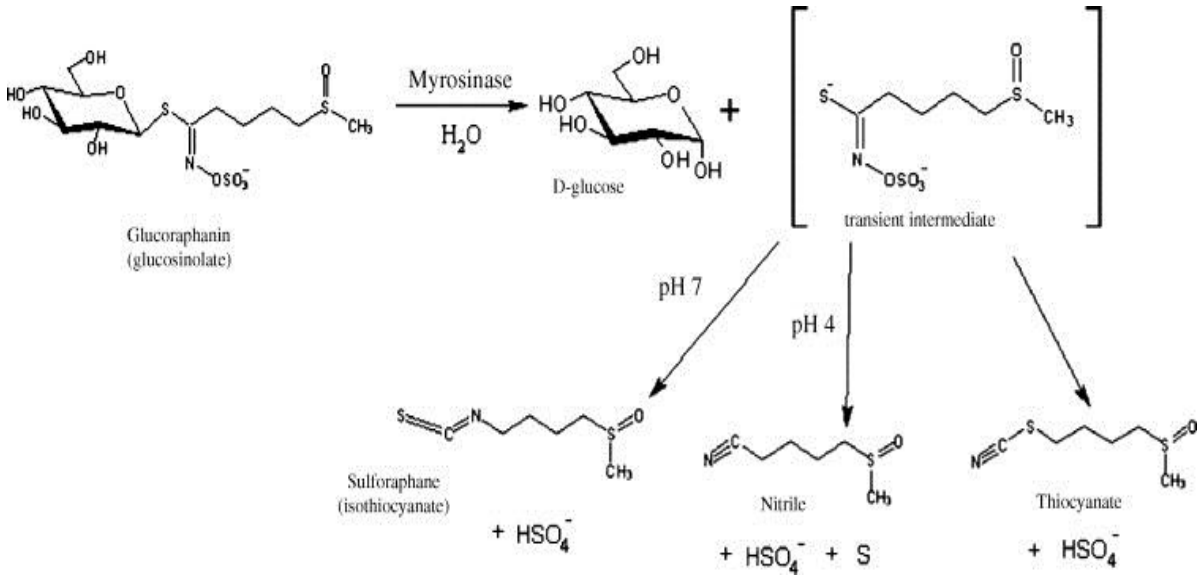

Figure 1: Glucosinolates hydrolysis (from Guerrero-Beltrán et al, 2012).

After the hydrolysis reaction during digestion, isothiocyanates and other products of glucosinolate's hydrolysis (e.g. thiocyanates and nitriles) are released into the intestinal lumen. Some of these products are not adsorbed and may be digested by the intestinal flora. On other hand, a substantial proportion of free isothiocyanates passively diffuses to the intestinal epithelial cells. These isothiocyanates are then conjugated with thiols, such as the reduced form of glutathione (GSH) (Dinkova-Kostova, 2012). After being conjugated with GSH, a reaction catalyzed by glutathione S-transferase (GST), the isothiocyanates become ready to interact with target tissues, which represents the first step in the metabolism of isothiocyanates in biological systems (Dinkova-Kostova, 2012).

As reported above, the isothiocyanates are characterized by high chemical reactivity in contrast to their relatively inert precursors. The central carbon atom of the isothiocyanate group (–N=C=S) is highly electrophilic and reacts avidly with sulfur-, nitrogen-, and oxygen-centered nucleophiles. As such nucleophiles are integral components of aminoacids it is not surprising that proteins and peptides are major cellular targets of isothiocyanates (Dinkova-Kostova, 2012). Furthermore, isothiocyanates have the capacity of react with sulfhydryls groups, which is useful for modification of proteins. Isothiocyanates ability to target the cystein residues existent in the proteins that control key

regulatory pathways leading to cancer has been suggested as being a possible mechanism by which these compounds show anticarcinogenic properties (Zhang and Hannink, 2003).
The isothiocyanates can be divided into different phytochemicals based on their biological activities such as allyl isothiocyanate, benzyl isothiocyanate, phenethyl isothiocyanate, iberin and sulforaphane (SFN) (Yao et al, 2011) (Figure 2).

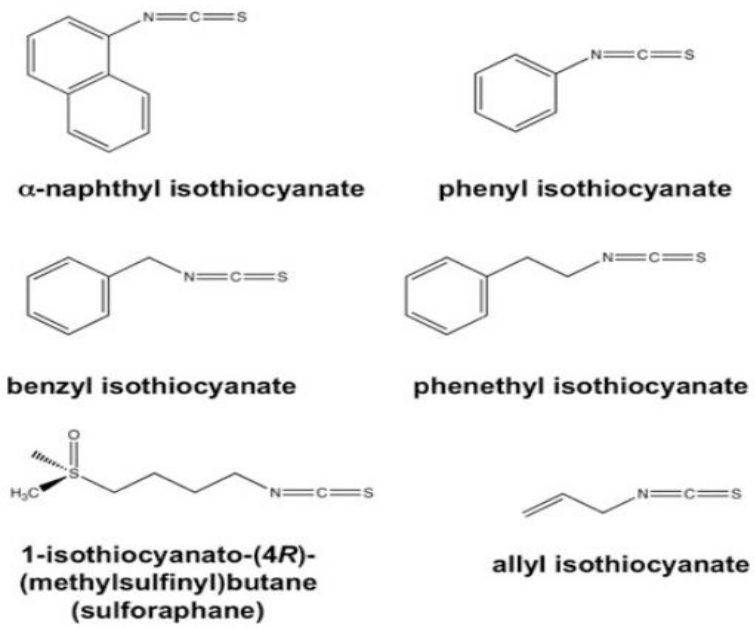

Figure 2: Isothiocyanates chemical structures which have been shown to protect against tumor development (from Dinkova-Kostova, 2012).

In 1992, SFN was first identified as an excellent chemopreventive agent and several studies, regarding the mechanisms of action of this and of other isothiocyanates, were performed. Results show that these compounds induce different responses depending on the stage of carcinogenesis (Clarke et al, 2008). SFN has also the noteworthy capacity of acting in the three phases of the carcinogenic process: tumor initiation, promotion and progression and even in the last steps (angiogenesis and metastasis) (Traka and Mithen, 2009).
Other biological functions of isothiocyanates include their ability to: selectively degrade tubulin (a stable protein that is abundant in the cytoskeleton and is required for cell cycle

progression); induce apoptosis by having a pro-apoptotic activity (Brown and Hampton, 2011; Yao et al, 2011).

As result of the increasing knowledge about the bioactive properties of SFN and isothiocyanates in general, various authors highlight the importance of integrating isothiocyanate-rich crops on the daily diet (e.g. Bonnesen et al, 2001; Brigeuus-Flohe and Banning, 2006). Below we´ll explore some bioactive properties of the particular isothiocyanate, SFN.

2.2. Sulforaphane (SFN) and bioactivity: focus on oxidative stress

Sulforaphane (SFN), or 1-isothiocyanato-4 (methylsulfinyl) butane, is a compound belonging to the isothiocyanates family (mustard oils), as mentioned above (Naumann et al, 2011). This compound derives from glucoraphanin [methyl 4 - sulfinyl - butyl glucosinolate] and is one of the most widely studied isothiocyanates (Shan et al, 2006) (Figure 3). Glucoraphanin is the glucosinolate that by myrosinase (present in plant or in our intestinal flora) activity originates SFN (Clarke et al, 2008; Naumann et al, 2011). It is abundant in broccoli, cauliflower, cabbage, and kale with the highest concentration found in broccoli (Shan et al, 2006; Clarke et al, 2008).

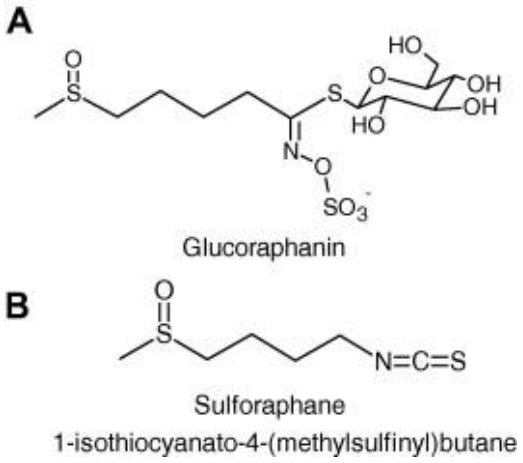

Figure 3: Structures of (A) glucoraphanin and (B) SFN (isothiocyanate hydrolysis product) (from Clarke et al, 2008).

As reported above for isothiocyanates in general, SFN is reported as having high potential as a cancer protective agent, since it reduces the generation of carcinogenic metabolites from consumed xenobiotics (Clarke et al, 2008; Naumann et al, 2011). In line with the previously described, several epidemiological studies have associated the consumption of cruciferous vegetables with a reduced risk of cancer (e.g. Levi et al, 2001; Naumann et al, 2011).

SFN was reported to have anticancer properties being most of the supporting studies performed with *in vivo* animal models (e.g. mice), and some clinical trials for e.g. breast and prostate cancer are ongoing (e.g. http://clinicaltrials.gov/). Recently, some *in vitro* studies testing SFN anti-tumor properties were published (e.g. Fimognari C et al, 2002; Jiang H et al, 2010; Sharma C et al, 2011). However, the *in vitro* experiments of SFN using OS cell lines remain residual (e.g. Kim M et al, 2011). Nevertheless, the use of *in vitro* studies may present several advantages to *in vivo* studies during the screening phases of specific compounds.

Studies in animals indicated that SFN has chemopreventive activity against many carcinogens (Shan et al, 2006; Clarke et al, 2008). Other experiments *in vivo* demonstrated anticancer activity of SFN, e.g. when this compound is orally supplied, it leads to inhibition/retardation of experimental multistage carcinogenesis models including diverse type of cancers such breast, colon, stomach and lung (Priya et al, 2011; Chen et al, 2012). SFN has been described as inhibitor of histone deacetylase *in vivo* and suppressor of tumorigenesis in *Apc*^{min} mice (Myzak et al, 2006). *In vivo* studies were also performed to evaluate if a specific combination therapy would reduce tumor size in a synergistic manner, and the results supported that SFN may be suitable to increase targeting of pancreatic cancer stem cells by Sorafenib (Rausch et al, 2010). A recent *in vivo* study suggests that mice treated with SFN have an inhibition of tumor. SFN extract also reduces the appearance of tumors, the expression of survivin and induce caspase 3 and cytochrome c. Moreover, SFN extract retards the growth of UM-UC-3 xenografts *in vivo*, confirming its future potential in bladder cancer therapy (Wang and Shan, 2012).

The interest in this agent has grown in the last years based on its beneficial pharmacological potential, which includes antitumor activity as mentioned before, antioxidant and anti-inflammatory properties. For example, recently some data support that SFN induced oxidative stress on cancer cells by direct or indirect mechanisms (e.g.

Benedict et al 2012). However, its effect on oxidative status of the cell still remains unclear as the effects depend on the dose and cell type. Moreover, SFN also seems to protect other cells (normal cells) from oxidative stress induced from pro-oxidants (Zachichelli et al, 2012).

It has been proposed that SFN chemopreventive activity results in increased detoxification of xenobiotics and carcinogens (Topè et al, 2009; Qazi et al, 2010; Chen et al, 2012).

A metabolism similar to the classical xenobiotic metabolism is reported to play a key role in tumor initiation process. Conventionally it is developed by two consecutive processes, controlled by phase I and phase II enzymes. Phase I enzymes convert pro-carcinogens into proximate or ultimate carcinogens, while phase II enzymes (e.g. GST) detoxify carcinogens and therefore facilitate their excretion from the body (Clark et al, 2008). This activity has been well studied and reveals diverse responses depending on the stage and the target tissue of carcinogenesis (Clarke et al, 2008). It has been proposed for some cancers that SFN modulates the carcinogenic process through: (a) blocking initiation via inhibiting Phase I enzymes, (b) inducing Phase II enzymes (Clarke et al, 2008), (c) activating antioxidant functions through an increase in GSH levels, (d) inducing apoptosis and inducing cell cycle arrest, (f) initiating anti-inflammatory properties and (g) inhibiting angiogenesis (Juge et al, 2007; Qazi et al, 2010).

The properties mentioned before show that SFN is not only an important chemopreventive agent capable of inhibiting various stages of the carcinogenic process, but also has therapeutic potentially (Zhang, 2002) (Figure 4).

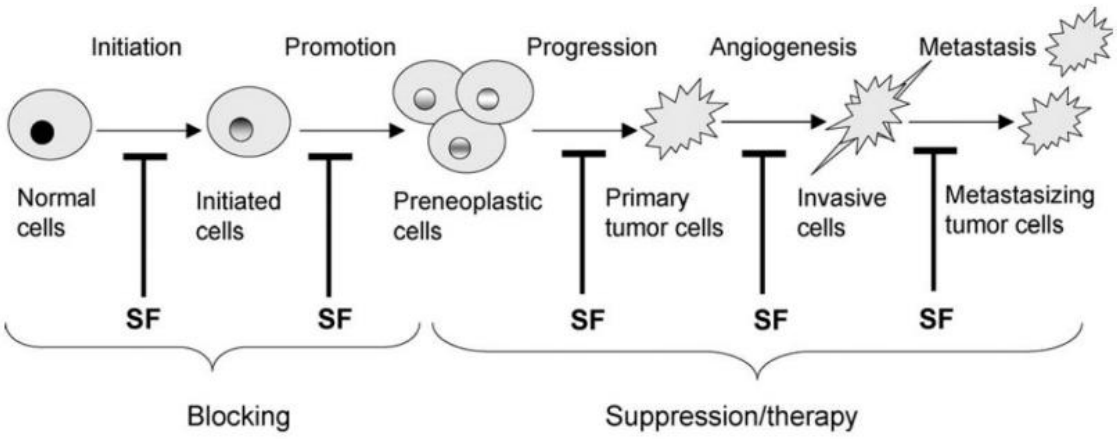

Figure 4: Mechanism of action of SFN in the carcinogenic process, affecting the three phases of tumor initiation and the final stages of carcinogenesis (from Juge et al, 2007).

Consequently, SFN may regulate the metabolism of phase I enzymes by direct interaction with cytochrome P450 (CYP) or indirectly, by regulating the transcriptional levels of this enzymes in the cell. A clear example of phase I enzyme activity regulated by SFN is the reported inhibition of enzymes CYP1A1 and CYP2A2 activities, in breast tumor cells (Skupinskz et al, 2009). SFN was also described as the most potent natural inducer of phase II enzymes in animals and in humans (Talalay, 2000).

SFN is not an antioxidant with direct action but there is substantial evidence that it acts indirectly to increase the antioxidant capacity of animal cells and their capacity to deal with oxidative stress (Fahey and Talalay, 1999) (Figure 5). However, other studies showed that SFN has the ability to increase intracellular ROS levels (Lee et al, 2012). Therefore, the effects of SFN in the oxidative status remain contradictory, depending on complex action mechanisms in cells and most probably depend on the cell type, dose, and the status of the cell.

SFN was also demonstrated to promote apoptosis in some tumor cells. Recently, the induction of apoptosis in human monocytic U937 cells by SNF was demonstrated to be correlated with the generation of intracellular ROS as well as with the loss of mitochondrial membrane potential. This suggests that this pro-oxidant function can play a crucial role in SFN-induced apoptosis (Lee et al, 2012).

Apoptosis plays an important role in the development and maintenance of homeostasis and also in the elimination of cells that are damaged or that are no longer needed to the body. The inappropriate regulation of this mechanism has been implicated in a wide variety of diseases, including cancer. Moreover, the induction of apoptosis may represent a safety mechanism by which several phytocompounds, including SFN, may inhibit such cellular disorders (Elbarbry and Elrody, 2011). The apoptotic process can be executed by the caspases cascade, cysteine proteases that are responsible for the initiation and execution of apoptosis (De Flora et al, 2001) (Figure 5). In this context it has been proposed that SFN is an inducer of apoptosis in several tumor lines such as bladder (Shan et al, 2006), ovary (Bryant et al, 2010), colon (Gamet-Payrastrem et al, 2000) and brain (Van Meir et al, 2010). SFN also induce apoptosis in a dose-dependent manner in osteosarcoma tumor lines such as in murine LM8 cell line (Matsui et al, 2007) and in human osteosarcoma U2-OS (Kim et al, 2011).

There is diverse evidence that SFN may stop the cell cycle at several stages of its progression, leading to inhibition of cancer cells proliferation (Elbarbry and Elrody, 2011). The progression of the cell cycle is regulated by cyclin-dependent kinase (CDK) and cyclins (Juge et al, 2007) (Figure 5). Cell cycle arrest in the G2/M phase induced by SFN was observed in Jurkat T tumor cells (Fimognari et al, 2002) and in breast tumor cells (Jackson and Singletary, 2004), while in bladder carcinoma cells a cell cycle arrest in the G0/G1 phase was observed (Shan et al, 2006).

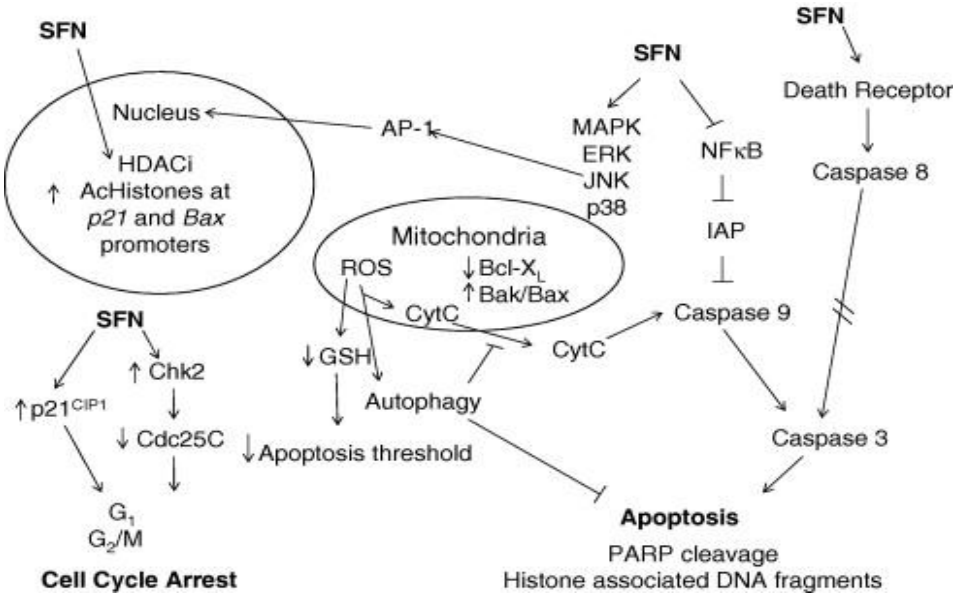

Figure 5: Action mechanisms of chemoprevention by SFN leading to alteration in cell cycle arrest, apoptosis and/or growth inhibition (from Clarke et al, 2008).

SFN interferes with diverse tumors such as human hepatoma HepG2 cells, which cause apoptosis at higher concentrations. However, at low concentrations SFN increases antioxidant defenses, via Nrf2-mediated gene expression (Lee et al, 2012). In prostate cancer PC-3 human cells, SFN inhibits cell growth, which is suggested to be linked with the activation of Erk and JNK signaling (Lee et al, 2012).

Other mechanisms have been reported associated with SFN, such as effects on NFκB transcription factors, inhibition of cyclooxygenase 2 (COX-2), effects on protein kinases, and inhibition of histone deacetylase and of tubulin polymerization. So, SFN can act in several cell pathways (Shan et al, 2006; Chen et al, 2012).

Considering the effects of SFN in inducing apoptosis and cell cycle blockage in osteosarcoma cells, few data are available, except for the *in vitro* studies of Kim et al

(2011), and those of our group where we have recently demonstrated *in vitro* that SFN also induces apoptosis and blocks cell cycle (in G2/M phase transition) in MG-63 cells (Costa et al 2012).

3. Oxidative Stress and Free Radicals

Some emerging data on SFN effects on tumor cells report its interference with the oxidative status of the cell (e.g. Lee et al 2012).

Oxidative stress has been defined as a disturbance in the balance between the production of oxidant species (free radicals) and antioxidant defenses (cellular oxidants and antioxidant enzymes) which may lead to tissue injury (Nathan et al, 2011). This disturbance may be originated from excessive exposure to environmental pollutants, ultraviolet light, or ionizing radiation (Keck and Finley, 2004). The conditions mentioned before can lead to an overload of the cells's antioxidant system, resulting in oxidative damage to proteins and nucleic acids; so oxidative stress is an important contributor to cell/tissue damage. In humans, this may lead to initiation of cancer as well as other degenerative diseases (Keck and Finley, 2004; Nathan et al, 2011).

Free radicals are any chemical species with independent existence that contain unpaired electrons (Halliwell and Gutterige, 1999). These electrons increase the chemical reactivity of an atom or molecule. The most common examples of free radicals are the hydroxyl radical ($^{\bullet}OH$), superoxide anion ($^{\bullet}O_2^-$), transition metals such as iron and copper and nitric oxide ($^{\bullet}NO$) (Halliwell and Gutterige, 1999).

Free radicals are obtained from many biochemical processes in large amounts as an unavoidable by-product of these biochemical processes (Nathan et al, 2011). In addition, these species may be formed in the body in response to diverse causes such as electromagnetic and ionizing radiation (e.g. X-rays and gamma rays) from the environment or acquired directly as oxidizing pollutants such as ozone and nitrogen dioxide. At the physiological level they can be produced by metal-catalyzed reactions, by neutrophils and macrophages activation during inflammation and by mitochondria (Halliwell and Gutterige, 1999).

Free radicals can be also produced by reduction of molecular oxygen during aerobic respiration yielding superoxide and hydroxyl radicals in the mitochondrial electron

transport chain; as by-products of metabolic processes such as oxidation of catecholamines and activation of the arachidonic acid cascade product electrons, which can reduce molecular oxygen to superoxide; in cytochrome P450 enzymes metabolism; in, e.g. vascular endothelium that produces nitric oxide; or in inflammatory cell activation. In addition, free radicals can be produced physiologically in response to environmental stress agents (Valko et al, 2006; Jomova and Valko , 2011).

The presence of unpaired electrons in free radicals confers them a high reactivity, although this varies from radical to radical. As most molecules are not free radicals, the majority of reactions will involve non-radical species. Reactions of a radical with a non-radical (all biological macromolecules are possible targets) produce free radical chain reactions with the formation of new radicals, which in turn can react with further macromolecules. Important examples are lipid peroxidation and protein damage. Lipid peroxidation products [e.g. malondialdehyde (MDA), 4-hydroxynonenal (HNE)] are potent mutagens and have been reported as modulators of signal pathways related to proliferation and apoptosis, processes implicated in carcinogenesis (Matés et al, 2008). From interaction with free radicals, DNA may also suffer damages (see 3.2.1) (Valko et al, 2006; Jomova and Valko, 2011).

In the presence of these species, if antioxidant defenses are deficient, oxidative stress may cause damages in a variety of tissues and the accumulation of these species in DNA, proteins and lipids play a key role in the development of several diseases, such as cancer, atherosclerosis, neurodegenerative diseases and diabetes (Halliwell and Whiteman, 2004; Nathan et al, 2011). As result of free radical generalized attack of cell components, the body has developed a complex antioxidant defense mechanism in order to protect body tissues (Nathan et al, 2011).

3.1. Reactive oxygen species (ROS)

ROS are species with highly reactive capacity, produced during normal metabolic processes: through enzymatic reactions (e.g. respiratory chain) or via non-enzymatic reactions. Examples of ROS are the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^-) (Clarke et al, 2008; Harvilchuck et al, 2009; Priya et al, 2011). The hydroxyl radical, which is considered the most potent oxidant known, has an extremely short half-life and the ability to attack most biological molecules, resulting in the

propagation of free radical chain reactions. This radical can be generated through a variety of mechanisms, such as those mentioned earlier in section 3 (Liochev et al, 2002; Valko et al, 2006).

Superoxide anions are formed when oxygen accepts an electron. They can act as a reducing agent of iron complexes (e.g. cytochrome c) by the Waber-Weiss reaction and it is likely to be an important source of hydroxyl radicals and hydrogen peroxide (Liochev et al, 2002; Valko et al, 2006).

At physiological levels, ROS may be considered as beneficial in chemotherapy and cancer apoptosis, but their role in carcinogenesis has also been proposed. They are produced at a low level by normal aerobic metabolism and play an important role in signaling pathways regulating cell proliferation, differentiation, and activation of transcription factors (Harvilchuck et al, 2009; Nathan et al, 2011; Priya et al, 2011). However these species, present in high levels may cause cellular damage that induce cell death and pathological conditions (Clarke et al, 2008; Harvilchuck et al, 2009). In the last case, when higher levels of ROS are present, this excess causes damage by oxidizing DNA, lipids, proteins, and cellular macromolecules as mentioned before (Smith-Pearson et al, 2008; Harvilchuck et al, 2009) (Figure 6).

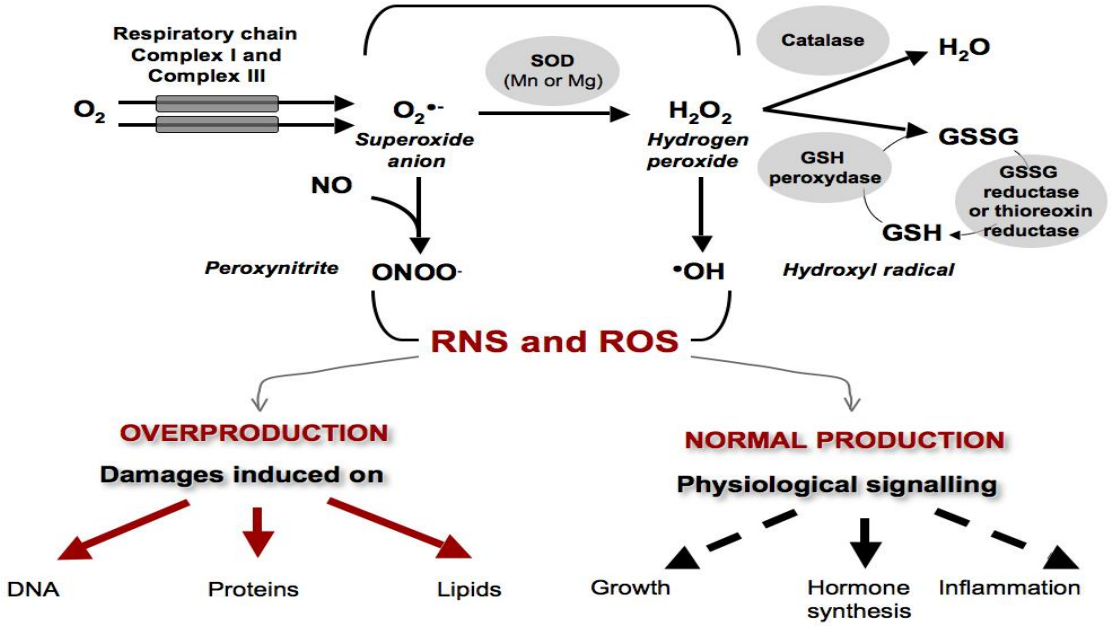

Figure 6: The mitochondria is implicated in the generation of reactive oxygen species (ROS). In most cells, the mitochondrial respiratory chain is recognized as the major site of ROS production in the form of superoxide, hydrogen peroxide and the hydroxyl free radical. Excessive ROS are deleterious for the cell, contributing to a variety of pathological processes (from Nadege et al, 2009).

Oxidative stress, as mentioned before, may result from the increase in ROS, however it may also arise through changes in superoxide dismutase or in other defense mechanisms against ROS (Harvilchuck et al, 2009). The antioxidant defense system is responsible for the balance of ROS as a beneficial substance and has the responsibility of reducing cellular ROS (Smith-Pearson et al, 2008). This system is composed by endogenous antioxidants, more precisely it is formed by enzymatic (superoxide dismutase - SOD; glutathione peroxidase - GPx; glutathione reductase - GRx; catalase - CAT and Peroxiredoxins - Prxs) and non-enzymatic defenses (glutathione - GSH and coenzyme Q10, CoQ10) (Smith-Pearson et al, 2008; Priya et al, 2011).

3.1.1. ROS and Cancer

The complex multi-sequence process of cell conversion from a healthy stage to pre-cancerous and later cancer stages is also known as "initiation–promotion–progression". This model is based on the equilibrium between cell proliferation and cell death (Waisberg et al, 2003). The first step, cell proliferation, is upregulated by protein p53, which plays a primordial role in this mechanism. This protein checks the DNA integrity during cell cycle (Oren, 2003), and triggers mechanisms of DNA repair, for instance to eliminate the oxidized DNA bases that cause mutations. However when the cell damage is too large, p53 may activate cell death by apoptosis at the same time.

The three stages of this model are characterized by multiple events occurring in cascade in the cell (Dreher and Junod, 1996). ROS can act in all these stages of carcinogenesis through modifications on transduction, cell signaling pathways, and/or on other cellular events. These species can interact with DNA in the initiation process, causing non-lethal mutation that can pass cell cycle checkpoints. However excess of oxidative stress can be cytotoxic and promote cell proliferation inducing tumor growth. Many tumor inducers have an inhibiting effect on cellular antioxidant defenses (Dreher and Junod, 1996). ROS can also promote abnormalities in growth factor receptors, changing Ca^{2+} homeostasis, leading to deregulation of many signaling pathways that control cell proliferation or apoptosis (Dreher and Junod, 1996).

Furthermore, ROS also act in the last carcinogenic step, more precisely in tumor angiogenesis and may regulate tumor invasiveness by modulating the gene expression. The stage of tumor progression is characterized by accumulation of additional genetic damage,

leading to the transition of the cell from benign to malignant, involves cellular and molecular changes (Collins, 2000; Nelson and Melendez, 2004).

3.2. Oxidative damage

3.2.1. DNA Damage

Reactive oxygen species can react with almost every molecule in a cell. The hydroxyl radical, for instance, is known to react directly with all components of the DNA molecule causing damage to purine and pyrimidine bases and also the deoxyribose backbone (Dizdaroglu et al, 2002).

Several studies related with cancer tissues refer free radical-mediated DNA damage. ROS-induced DNA damage can involve single- or double stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. DNA damage can result either in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all eventually associated to carcinogenesis (Cooke et al, 2003). Permanent modification of genetic material represents the first step involved in mutagenesis, carcinogenesis and ageing.

Mutations and altered expression in mitochondrial genes encoding for respiratory complexes and in the hypervariable regions of $_{mt}$DNA were identified as good indicators of DNA damage in several cancers (Penta et al, 2001; Vives-Bauza et al, 2006). Several studies have also reported 8-hydroxyguanine (8-OH-G or 8-oxo-G) as a good biomarker for DNA oxidative damage as well a potential biomarker of carcinogenesis (Kasai et al, 2001). Other methods for measuring oxidative DNA damage may involve for example, enzymatic digestion of DNA, the acidic hydrolysis of DNA and others (Collins, 2000).

3.2.2. Lipid peroxidation

All cellular membranes are sensitive to oxidative attack namely those membranes that have polyunsaturated fatty acids (PUFA) in its composition. The process of lipid peroxidation is divided in three stages: initiation, propagation and termination (Marnett, 1999).

The peroxyl radicals (ROO•), after formation, can be arranged via a cyclization reaction to endoperoxides with the final product of the peroxidation process being malondialdehyde (MDA) (Marnett, 1999). This product is mutagenic in bacterial and mammalian cells and

carcinogenic in rats (Mao et al, 1999). Other major aldehyde product resulting from lipid peroxidation is 4-hydroxy-2-nonenal (HNE) which, unlike the weakly mutagenic MDA, is the major toxic product of lipid peroxidation and it also has a powerful effect on signal transduction pathways (Mao et al, 1999). MDA may interact with DNA, creating adducts that can lead to DNA–DNA inter-strand crosslinks or DNA–protein crosslinks (Mao et al, 1999). All of these modifications can be deleterious to cells either being genotoxic and/or carcinogenic (Valko M et al, 2006).

3.2.3. Protein Oxidation

Proteins can also be a target for ROS oxidation. Protein oxidation *in vivo* occurs in the presence of transition metals capable of entering in Fenton reactions (see equation 1, where M^{n+} is a metal). Examples of such transition metals include M^{n+} are Fe, Cu or Cr. Although other metals can catalyze this reaction, these are the most prevalent catalyzers.

$$Eq.1: M^{n+} + H_2O_2 \rightarrow M^{(n+1)} + \ ^{\cdot}OH + OH^-$$

Metal-catalyzed damages of proteins involve oxidative scission, loss of histidine residues, bityrosine crosslinks and introduction of carbonyl groups (Valko et al, 2007).

The hydroxyl radical is one of the ROS that have the capacity to interfere with proteins, since they have the ability to remove from the protein polypeptide backbone the hydrogen atom forming a carbon centered radical, which can readily react with molecular oxygen to form peroxyl radicals (Stadtman, 1990). Protein damage is likely to be repairable and is a known non-lethal event for a cell.

The action of ROS/RNS and ionizing radiation can affect the side chains of all amino acid residues of proteins. However, proline, histidine, arginine, lysine and cysteine residues are sensitive to oxidation by redox metals and may be preferable protein sites of metal binding. This protein-iron complex can react with H_2O_2, forming hydroxyl radical on site which can rapidly promote oxidation process (Stadtman, 2004).

Proteins can also be attacked by nitrosylation reactions. The reaction of nitric oxide with superoxide radical results in the formation of the highly toxic peroxinitrite anion which can nitrosate cystein, nitrate tyrosine and tryptophan and oxidize methionine to methionine

sulphoxide. However, physiological concentration of carbon dioxide, strongly inhibit the process of modification of proteins by peroxynitrite. Even though the nitration of tyrosine is an irreversible process and can interact in phosphorylation and adenylylation process which are important in regulatory pathways (Valko et al, 2006).

The accumulation of oxidized proteins in living systems can be induced by several factors acting alone or in combination. As consequence of these protein-ROS interactions, inter- and intra-protein cross-linkages occur. Therefore, protein carbonyl groups are generated. The concentration of these groups is considered a good biomarker of ROS mediated protein oxidation (Valko et al, 2006).

3.3. Antioxidant systems

Antioxidant is any substance that when present at low concentrations (compared with those of the oxidizable substrate) considerably delays or inhibits its oxidation (Betteridge, 2000).

The antioxidant system used by the cells can be categorized as enzymatic, non-enzymatic and extracellular mechanisms. The enzymatic defense is mediated by a group of enzymes which are capable of metabolize ROS and ROS precursors (e.g. H_2O_2). Non-enzymatic defenses include several molecules that can donate electrons to radical species or by processes that diminish ROS production (Betteridge, 2000; Kim et al, 2010).

3.3.1. Enzymatic Defenses

Antioxidant enzymes are an important component of the antioxidant defense mechanisms (Priya et al, 2011). Cellular antioxidant defenses enzymes include the superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and glutathione reductase (Betteridge, 2000). These enzymes act sequentially to achieve the scavenging of free radicals. Antioxidant defense is also formed by non-enzymatic defenses such as glutathione (Betteridge, 2000) (Figure 7).

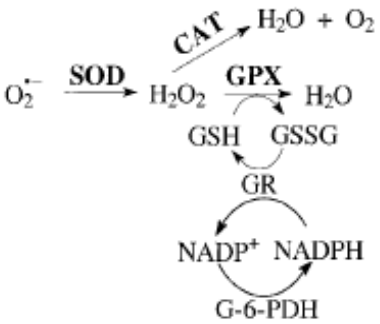

Figure 7: Antioxidant enzyme system. The SODs convert superoxide radical into hydrogen peroxide. The CATs and GPxs convert hydrogen peroxide into water. In this way superoxide radical and hydrogen peroxide, are converted into the harmless product water. GPx requires several secondary enzymes (GR and G-6-PDH) and cofactors (GSH, NADPH, and glucose 6-phosphate) to function (from Li et al, 2000).

Superoxide dismutase (SOD) has the capacity to delay or prevent damage from ROS. There are two SOD isoforms that are differently distributed among intra and extra cellular spaces. The Manganese-containing SOD (MnSOD) located in the mitochondria and Copper/Zinc-containing SOD (Cu/ZnSOD) found in the cytosol. These isoenzymes are capable of catalyzing the dismutation of superoxide anion to hydrogen peroxide and molecular oxygen (equation 2) (Betteridge, 2000; Zelko et al, 2002; Vurusaner et al, 2012).

$$Eq.2: 2O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} 2H_2O_2 + O_2$$

H_2O_2 is a weaker oxidant and can act as an intracellular and extracellular signaling molecule (Betteridge, 2000; Clarke et al, 2008; Harvilchuck et al, 2009). This molecule can react with transition metals (Cu^+, Fe^{2+}) via Fenton reaction when they are in low levels, leading to the formation of deleterious hydroxyl radical, another reactive specie (Kim et al, 2010).

Hydrogen peroxide generated by the reaction controlled by SOD can be reduced by two enzyme systems, glutathione peroxidase (GPx) and catalase (CAT). The first, depending on the protein isoforms, is present in cytosol and in mitochondria (Betteridge, 2000). There are multiple GPx proteins, and at least two different types are present in most human tissues. One GPx form contains selenium in its active center (selenium-dependent: Se-GPx) and the other is a cationic isoenzyme of glutathione S-transferases which is selenium-independent (t-GPx) (Saydam et al, 1997).

Hydrogen peroxide reduction by GPx requires reduced glutathione (GSH) generating H_2O and oxidized glutathione (GSSG) (Peskin and Winterbourn, 2000) as illustrated by equation 3.

$$Eq.3: 2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

CAT can also breakdown H_2O_2 in many tissues decomposing it to water and oxygen. It is localized in peroxisomes and is the primary component of the antioxidant system (Betteridge, 2000). CAT is mostly responsible for the removal of H_2O_2 when it is present in higher concentration in tissue (equation 4) (Aebi, 1984).

$$Eq.4: H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

Glutathione reductase (GRx) is a homodimeric flavoprotein that catalyzes the reduction of glutathione disulfide (GSSG - oxidized glutathione) produced by the reaction of GPx, to form sulfhydryl GSH (reduced glutathione, important cellular antioxidant) in the presence of NADPH as a reducing cofactor. This enzyme is necessary for the functioning of GPx (Saydam et al, 1997; Kim et al, 2010).
When these enzymatic systems work together, they efficiently maintain the oxidant species balance in cells and tissues.

3.3.2. Non-enzymatic defenses

The antioxidant system is also formed by non-enzymatic defenses. These non-enzymatic molecules act as antioxidants and can control oxidative stress. Some examples of these defenses are molecules such as vitamin E, coenzyme Q, β-carotene and other carotenes present within cell membranes, other examples are vitamin C (L-ascorbic acid) and glutathione (-L-glutamyl-L-cysteinylglycine) mostly present in cytosol (Ascenso et al, 2011). Glutathione is the major metabolite involved in determining cellular redox state and functions in the cellular thiol/disulfide system (Yang et al, 2006; Vurusaner et al, 2012). Glutathione is a small tripeptide which is the principal cellular antioxidant present primarily in the reduced form (GSH) (Yang et al, 2006; Kim et al, 2010). This molecule is

involved in the conjugation and detoxification of numerous types of compounds that cause toxicity and carcinogenesis (Kim et al, 2010). Under stable state conditions, the ratio GSH/GSSG is maintained. When excessive oxidation occurs in the cell, the GSH/GSSG ratio decreases, affecting other cellular events such as cell signaling and apoptosis, cellular growth and differentiation. This decrease has been reported to putatively promote tumor development through a mechanism that involves cytotoxicity (Yang et al, 2006).

Besides the molecules mentioned above, tissues are also protected against free radicals by several binding proteins (transferrin, lactoferrin, ceruloplasmin, haptoglobins, hemopexin and albumin) which can sequester metals and keep them in a nonreactive state (Valko et al, 2006). Indeed the mechanism of metal-induced free radicals formation is tightly influenced by the action of cellular antioxidants. These proteins are highly inducible by metals and oxidants and thus, are critical in protection against exogenous oxidants (Qiang, 2010). Moreover, many low-molecular weight antioxidants such as ascorbic acid, alpha-tocopherol, glutathione (GSH), carotenoids, flavonoids, and other antioxidants, are capable of chelating metal ions reducing thus their catalytic activity to form ROS (Jomova and Valko, 2011).

3.3.3. Others Defenses

Besides the above reported antioxidant systems (enzymatic and non-enzymatic systems), cells also have complementary mechanisms in order to regulate ROS production, namely the metabolic uncoupling of mitochondria. The short-cut of the mitochondrial electron chain by uncoupling proteins (UCP) decrease ROS formation where the reduction power of oxidative metabolism is transferred to health production and ROS-formation control (Qiang, 2010).

4. SFN and Oxidative Stress

Several studies have demonstrated that SFN may interfere with oxidative stress. Depending on the cell line or tissue, SFN may induce varying effects on antioxidant enzymes levels and activities, e.g. in some cases an increase is observed in some antioxidant enzymes and in other cases a decrease is observed (Jiang et al, 2003).

Mitochondrial fractions from pulmonary and hepatic tumors of mice are also affected by SFN, since they show a significant increase in the activities of SOD, CAT, GPx and the redox cycle enzymes. In these tumors, a significant increase in CAT and SOD activities is observed, associated with an increase in GSH levels (Priya et al, 2011). The increase of these antioxidant enzymes might have a potential role in the detoxification and elimination of potential carcinogens present in the body, supporting the effects of SFN on this type of tumors (Priya et al, 2011).

In another study, H-SY5Y cells (neuroblastoma) were treated with 2.5 and 5 µmol/L of SFN and did not show any significant changes in SOD and CAT activities. However, the treatment of these cells with 5µmol/L SFN for 24 h induced significant increases of GR but not in GPx activities (Tarozzi et al, 2009).

In PC-3 cells treated with 40µM of SFN for 6 h, the protein levels of Cu,Zn-SOD and Mn-SOD were higher, especially for the latter, an increase in SOD activity was also observed. On the other hand, SFN treatment did not affect CAT activity neither protein levels in those cells (Singh et al, 2005).

Yang and co-workers showed that mouse liver, different brain regions and other tissues express different antioxidant enzyme activities, which may result in different reactions to oxidative stress, depending on the tissue (Yang et al, 2006). An example is the GR activity which is inhibited consistently by SFN in diverse types of cell lines, such as Hepa1c1c7, HepG2, MCF7, MDA-MB-231, LNCaP, and HT-29 cells, while in other cell lines such as HeLa cells GR activity is induced by SFN (Jiang et al, 2003). GR is associated with several cellular mechanisms while maintaining a high ratio GSH/GSSG in the cell, such as protein and DNA biosynthesis and a defensive response against free radicals and ROS. If in some cells inhibition of GR occurs, this leads to an accumulation of GSSG, which generates a state of thiol oxidative stress (Kim et al, 2010). SFN has been described as a stabilizer of glutathione-metabolizing system under oxidative stress (Priya et al, 2011).

In CaCo-2 cells treated with SFN it was observed an increase in GPx, which suggests that SFN induces GPx via Nrf2/Keap1 and leads to a decreased risk of developing gastrointestinal cancers (Banning et al, 2005; Brigeuus-Flohe and Banning, 2006). Figure 8 shows the currently proposed mechanism of action of SFN via Nrf2/Keap1 (Boddupalli et al, 2012).

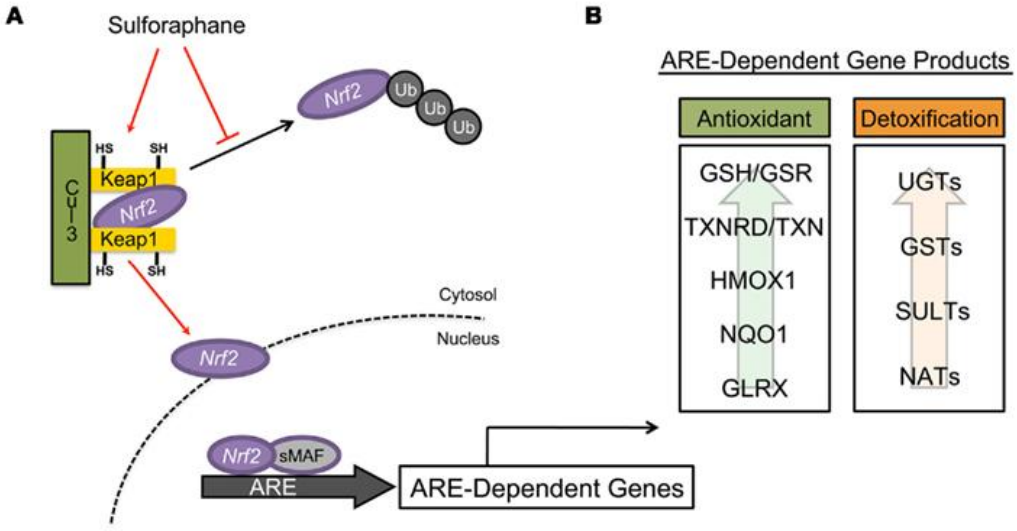

Figure 8: Mechanism of antioxidant response elements (ARE) mediated detoxifying and antioxidant enzyme induction by SFN. (A) Under basal conditions, the transcription factor NrF2 is sequestered within the cytosol by the repressor proteins Keap1 and cullin 3 (CUL3). SFN, through modification of the highly redox-sensitive cysteine residues of KEAP1, facilitates the dissociation of the Keap1/CUL3/NrF2 complex, releasing NrF2, which translocates into the nucleus. In the nucleus, NrF2 heterodimerically pairs with small Maf transcription factors binding to ARE contained within the promoter regions of many enzymes, initiating their transcription. (B) ARE-mediated gene products (antioxidant enzymes) (from Boddupalli et al, 2012).

GSH is extremely important in the cell, a reduction in GSH levels (or increase in oxidized state) in the tissue has been associated with a number of human diseases including Alzheimer, Parkinson, liver disease, heart attack, diabetes, infection caused by HIV and cancer (Yang et al, 2009). Furthermore, a decrease in intracellular GSH levels is likely to occur due to exposure to toxicants, tissue or DNA damage (Yang et al, 2009).

In SH-SY5Y cells, SFN enhances total GSH levels, when the cells are treated with 5 µmol/L of SFN for 24h. In addition, the augmentation in cellular GSH levels induced for the second concentration, a treatment time-dependent relationship (Tarozzi et al, 2009). SFN also leads to the increase of cytosolic GSH levels possibly leading to a simultaneous elevation of the antioxidant enzymes in the mitochondria, avoiding the thiol oxidation of the complex I, an event responsible for mitochondrial dysfunction (Tarozzi et al, 2009).

However, SFN had opposite effects in other cell lines: this compound is an electrophilic molecule capable of reacting with cellular nucleophiles such as GSH, therefore, SFN treatment might cause GSH depletion intensifying oxidative stress (Singh et al, 2005). An

example of this is in PC 3 cells where SFN provoked a rapid and significant depletion of GSH levels. This indicates that SFN induced ROS generation and this is probably mediated by a non-mitochondrial mechanism involving GSH depletion and a mitochondrial component (Singh et al, 2005). Also in this case, SFN action depends on the tissue and concentration.

Objectives

The main aim of the present dissertation was to evaluate if SFN induces oxidative stress in osteosarcoma cells. If so, other aims were proposed: a) what are the main enzymes/pathways affected by SFN (see Figure 7), and b) is this influence in the enzymes related with transcriptional or post transcriptional events.

For the SFN *in vitro* studies the largely used osteosarcoma cell line (MG-63) was used. The SFN effects at the oxidative stress were evaluated on these cells for SOD, GR and GPx activities, GSH levels, lipid peroxidation, and total antioxidant activity. Finally to evaluate putative transcriptional/post-transcriptional effects gene expression for genes related with oxidative stress was also measured. These parameters were evaluated for 24 and 48h of exposure to SFN (0, 5, 10 and 20μM).

The study of antioxidant enzymes and related mechanisms will allow the elucidation of the response of deregulation mechanisms to oxidative stress. This information will contribute to better understand how SFN affects osteosarcoma cell metabolism, and may provide further information for the eventual use of this compound in osteosarcoma therapy.

Material and Methods

1. Cell Culture

Human osteosarcoma MG-63 cell line, supplied by courtesy by the INEB (Instituto de Engenharia Biomédica-Porto, Portugal), was cultured in 75-cm^2 standard culture flasks with α-Minimum Essential Medium (α-MEM) (Life Technologies, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Life Technologies, Carlsbad,

CA), 2.5 µg/mL fungizone (Life Technologies, Carlsbad, CA) and 10000 U/mL penicillin-streptomycin (Life Technologies, Carlsbad, CA). Cells were observed with an inverted microscope (Olympus, Japan). When cultures reached approximately 80% confluence, cells were washed twice with Phosphate Buffer Saline without Ca^{2+}/Mg^{2+} (PBS) (Life Technologies, Carlsbad, CA) and then incubated with trypsinized with Trypsin-EDTA solution (0.25% Trypsin, 1 mM EDTA) (Life Technologies, Carlsbad, CA) (3 mL/75 cm^2) until the disaggregation of cells (2-5 min at 37 °C). When cells were completely detached, complete growth medium was added for trypsin deactivation. Subsequently, cells were resuspended and counted by using a hemocytometer. Afterwards, cells were seeded at 10^5 cell/mL in 10 mL of supplemented medium. After this procedure cells were incubated at 37 °C in a 5% CO_2 humidified atmosphere.

1.1. SFN exposure

D,L-sulforaphane (Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) at a concentration of 10 mM and stored at -20 ºC. For all assays, the exposure procedure was the following: Cells were seeded and allowed to adhere for 24 h, when cells reached 50-60% of confluence, the medium was removed and replaced by fresh medium containing 0, 5, 10, and 20 µM of SFN.

The cultures were routinely visualized for confluence and cell morphology, in order to evaluate the possible effects of SFN. Cells were grown for 24 and 48 h (exposure time) and then harvested for each specific measurement. Depending on the particular assay, cells underwent slightly different seeding and harvesting methods.

2. Cell viability

Cell viability was measured by the Trypan Blue assay. In order to observe the interaction of SFN on cell viability, cells were seeded in a 24-well plate at the concentration of 10^5 cells/mL and left for 24 h to adhere. Subsequently, cells were exposed to 0, 5, 10 and 20 µM of SFN in growth medium during 24 and 48 h. After this period (24 or 48 h), the medium was removed, cells were washed with 200µL of PBS and then 76µL of trypsin were added. When cells were completely detached, growth medium was added for trypsin deactivation (231µL). Afterwards, 20µL of cell suspension were transferred to a microtube

and 20µL of trypan blue dye (TC10^{TM}, Bio-Rad, Hercules, CA) were added. The mixture was incubated for 3 min at room temperature and 10µL were used to determine viability after cell counting in an automated counter (TC10^{TM}, Bio-Rad, Hercules, CA).

3. Protein quantification

Protein quantification was performed through Bradford assay, which is a colorimetric method based in the dye Coomassie Brilliant Blue G-250 (Bradford Reagent) (Sigma-Aldrich, St. Louis, MO) binding to protein. This leads to an absorption peak shift from 465 nm to 595 nm, in which the amount of absorption is proportional to the quantity of sample protein (Bradford MM, 1976).

For this procedure, 5 µL of each sample extract, corresponding to different concentrations and times of exposure, were incubated with 250 µL of Bradford Reagent in the dark for 5 min. The same procedure was applied for the standard curve, using known concentrations of bovine serum albumin (BSA) (Sigma-Aldrich St. Louis, US-MO) instead of sample. The absorbance was read at 595 nm in a microplate reader (Synergy HT Multi-Mode, BioTek®, VT, USA). The concentrations of unknown samples were calculated from standard curve.

4. Enzymatic parameters

For all enzyme assays, cells were seeded in 100-mm culture dishes (Corning®) and exposed to SFN as described above in 1.1. In order to obtain the homogenates for analyses, the method described by Quick (Quick, 2000) was followed with some modifications. Briefly, the culture medium was removed and cells were washed in 1 mL of PBS, after this addition cells was scraped. After this, cells were centrifuged at 1000g for 10 min at 4ºC and the supernatant was discarded. For most enzyme assays, the cell pellet was resuspended in 750µL of cold phosphate buffer (5 mM, pH 7.4) with 1 mM of EDTA. For the GPx enzyme assay, however, after centrifugation, the cell pellet was resuspended in 350µL of cold phosphate buffer and then sonicated once for 30s over ice.

Finally, to clear cellular debris, the homogenates were centrifuged at 12000g for 15 min at 4 °C, and the supernatants were aliquoted to separate vials, one for protein quantification and the remaining vials for each enzyme assay. All samples were stored at -80 °C until

assayed. All enzyme reactions were performed in a 96-well plate, and the absorbance variation was read in a microplate reader (Synergy HT Multi-Mode, BioTek®, VT, USA).

4.1. Superoxide Dismutase (SOD)

SOD is the first line of defense against oxidative stress, namely by acting as a superoxide anion radical scavenger (Zelko et al, 2002). At present, three distinct isoforms of SOD were identified in mammals, CuZn-SOD, Mn-SOD and EC-SOD which differ in their structure and location. SOD catalyzes the dismutation of superoxide anion $O_2^{\bullet-}$ to molecular oxygen and oxygen peroxide (H_2O_2).

Total SOD activity was measured by using Sigma-Aldrich® SOD-assay kit (Sigma-Aldrich, St. Louis, US-MO) and the reaction was performed in accordance to kit instructions. In this system, xanthine oxidase (XO) will form superoxide anion and from this WST-1 will regenerate O_2 forming a colored compound. SOD present in the sample will convert superoxide anion in H_2O_2 leading to colored product decrease.

In this assay, 20 μL of sample were added to 200 μL of a buffer containing WST-1 (WST-Working solution). After this, 20 μL of XO solution were added to initiate the reaction. Absorbance variation was read in a microplate reader in kinetic mode at 440 nm for 5 min. The percentage of inhibition was calculated by the following equation:

$$Eq.1: \quad XO\ \%\ inhibition\ = \frac{(S1 - S3) - (SS - S2)}{(S1 - S3)} \times 100$$

The reaction was always performed with a "blank well" to calculate XO maximum rate (S1), this was formed by miliQ water, WST-Working Solution and Enzyme solution. The other "blank wells" were: a well without XO and sample (S3), a well with only with miliQ water and dilution buffer and other without XO (S2) formed by dilution buffer, WST-working solution and sample. All results were normalized by sample protein concentration and one unit of SOD activity was considered as the enzyme amount which yields a 50% inhibition in WST-1 reduction.

4.2. Glutathione Peroxidase (GPx)

GPx proteins are present as cytosolic, mitochondrial and extracellular enzymes and play a major role in the elimination of hydrogen peroxide (H_2O_2) generated by superoxide dismutase and other organic hydroperoxides (ROOH).

In mammals, the GPx family includes at least five enzymes with different subcellular localizations. In humans, one of these isoforms, GPx1, contains selenium in its active center which is implicated in its catalysis. The other isoform is a cationic isoenzyme of glutathione S-transferases which is selenium-independent (t-GPx) (Halliwell, 1994) GPx uses as a reducing equivalent glutathione (GSH) which is recycled through GR reaction from its dimer form (GSSG) to the reduced one (GSH).

The determination of GPx activity was performed as described by Smith and Levander, (2002) with some alterations.

$$Eq.2: \ 2GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O$$

Briefly, after the procedure described in 4, 50µL of the sample were incubated for 10 min on a 96-well plate, in phosphate buffer (50 mM, pH 7.4 and 5 mM of EDTA) with 2 mM GSH, 1 mM sodium azide (Sigma-Aldrich, St. Louis, MO), NADPH 0.4 mM (Sigma-Aldrich, St. Louis, MO) and 2mM GR, final concentrations The reaction was initiated by the addition of 10µL tert-butyl hydroperoxide solution (Sigma-Aldrich, St. Louis, MO). GPx activity was measured directly by the decay in absorbance of NADPH at 340 nm in a microplate reader. Sample activity was extrapolated using the equation below:

$$Eq.3: U = \frac{(\Delta A/\min_s - \Delta A/\min_{bg}) \times \mathrm{TV}}{\varepsilon \mathrm{d}}$$

Where $\Delta A/min_s$ is the change in absorbance per minute for the sample; $\Delta A/min_{bg}$ is the change in absorbance per minute for the blank; TV is the total volume of the assay (mL); ε is the extinction coefficient and d is the path length value calculated thought the equation:

$$Eq.4\text{: Path length} = \frac{Volume\ (mL)}{\pi r(cm)^2}$$

One enzyme unit of GPx activity is defined as 1 μmol of NADPH oxidized per minute at 37°C. Finally, the GPx specific activity was expressed as a ratio of its activity and the protein content of the samples.

4.3. Glutathione Reductase (GR)

GR plays an important role in cell antioxidant state by producing antioxidant species, this enzyme catalyzes the reduction of glutathione disulfide (GSSG) produced by GPx activity, to the glutathione reduced form (GSH) using NADPH as its source of reducing equivalents. The reaction mentioned in the previous step (GPx activity) produces GSSG which is then recycled to GSH by GR as shown by the equation described below:

$$Eq.5: GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

GR activity was measured directly by the decay in absorbance of NADPH at 340 nm in a microplate reader accordingly to Dringen (Dringen and Gutterer, 2002) with some modifications. The reaction was carried in 100 mM phosphate buffer containing 1 mM EDTA, pH 7.4, at 25°C in a 96-well microplate. The reaction was started by the addition of GSSG solution in 1:1 to the mixture of NADPH and sample. The final concentrations were 1 mM GSSG and 0.2 mM NADPH in a final volume of 320 μL. In parallel with sample measurements, a calibration curve was performed with standard GR to extrapolate sample activity. Finally, GR specific activity was calculated as enzyme activity per minute per protein content of the samples.

4.4. Reduced Glutathione (GSH) quantification

Glutathione is the key antioxidant in animal tissues. It is present in the cells mainly in the GSH reduced form. Intracellular GSH status serves as an indicator of the cell capacity to resist toxic challenge and also appears be a sensitive indicator of the overall health of a

cell**.** For GSH quantification, the Glutathione Assay Kit, Fluorimetric (Sigma-Aldrich, St. Louis, MO) was used. In this system, a thiol probe (monochlorobimane) which can freely pass through the plasma membrane is used and when it binds to reduced glutathione in a reaction catalyzed by glutathione S-transferase (GST), it forms a strongly fluorescent adduct that can be measured in a microplate reader. Briefly, cells were seeded in a fluorometric 96-well plate, in a density of 10^5 cells/mL. After exposure periods the cells were washed with PBS, the kit reagents were added and finally read on microplate reader at 360nm excitation and 485nm emission. In parallel with sample measurement, a calibration curve was performed with GSH standard to extrapolate the sample activity.

In order to normalize GSH levels, the protein amount was determined for each sample. After fluorometric reading, cells were washed with PBS, incubated with the Glutathione Assay Kit's lysis buffer during 30min in shaker. After this, 5µL of homogenate were taken to a new well and 250µL of Bradford Reagent (Sigma-Aldrich, St. Louis, MO) were added. The plate remained in agitation in darkness for 10min and protein amounts were determined after this period.

5. Total antioxidant activity (TAA)

TAA was measured using the Antioxidant Assay KIT Sigma-Aldrich, St. Louis, MO). This assay is based on the capacity of antioxidant molecules in the samples to reduce ABTS (2,2´-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) to produce a radical cation, $ABTS^{\bullet+}$, a green soluble chromogen that can be determined spectrophotometrically at 405 nm in microplate reader.

Cell homogenates were prepared in the same manner as in section 4 using the method described by Quick et al, 2000 with modifications. Cells were washed in 2 mL cold PBS, scraped on ice, and then centrifuged at 1000 g for 10 min at 4 ºC. After this, the pellet was resuspended in assay buffer and sonicated for 30s. The homogenates were centrifuged at 12000 g for 15 min at 4 °C, and the supernatants were stored at -80 ºC until further analysis. ABTS reaction and spectrophotometric measurements were according to the manufacturer's instructions. Values obtained for each sample were compared with the concentration-response curve of standard Trolox solutions. Absorbance values were

normalized to protein amount and expressed as micromoles of Trolox equivalent per milligram of protein.

6. Thiobarbituric acid reactive species (TBARS) assay

Lipid peroxidation analyses were performed by the TBARS assay Kit (Cayman, Michigan, USA) with small modifications to manufacturer instructions. Briefly, cells were seeded in 150-mm culture dishes (Corning®) and exposed to SFN as described above in 1.1. The culture medium was removed and cells were washed first with 2 mL of PBS, then with 3-mL cold PBS and after washing cells were scraped. Subsequently, cells were centrifuged at 1000g for 10 min at 4ºC and the pellet was resuspended in 1mL of cold PBS. Next the suspension was sonicated for 30s over ice and 40µL of homogenate was removed for subsequent protein analysis. To the remaining suspension and standards, TBA SDS solution was added and after this color reagent. Finally 3,5-di-tert-butyl-4-hydroxytoluene (BHT) (Sigma-Aldrich, St. Louis, MO) also was added, in order to decrease oxidation caused by the procedure. The homogenates were placed in a water-bath at 95ºC for 1h and immediately placed on ice for10min. Finally, the homogenates were centrifuged at 5000g for 10 min at 4 °C and the clear supernatant was stored for fluorometric analysis (530nm excitation, 550nm emission). Values obtained for each sample were compared with the concentration-response curve of standard solutions prepared with TBA Malondialdehyde standard. Fluorometric values were normalized with protein.

7. Enzyme gene expression

7.1. RNA extraction and quantification

As preparation for RNase-free work, all bench surfaces, pipettes and material were sprayed with 0.1 mM NaOH/ 1mM EDTA solution, followed by DEPC-Water spray. All consumables used were sterilized in order to denaturate/inactivate RNases.

For this assay 10^4 cells per well were seeded in 12-well plates. Cells were cultured during 24 h for adherence, and then exposed to SFN (control, 5 and 10µM) during 24 and 48 h. After that, cells were washed with PBS, and finally, collected with TRIzol reagent (Life Technologies, Carlsbad, CA). After this, 200 µL of chloroform were added (Merck,

Darmstadt, Germany) to each sample, the solution was shaken and centrifuged at 12000 g for 15 min at 4ºC for phase separation. The aqueous phase (top) containing RNA was collected. One volume of 70% ethanol (RNase-free) was added to the collected aqueous phase. From this point, a QIAGEN RNeasy Mini kit (Qiagen, Dusseldorf, Germany) was used for RNA extraction. All previous solutions were transferred to an RNeasy spin column and centrifuged for 15s at 8000g. Subsequently, the solution was washed and centrifuged successively with: 700 µL of RW1 buffer and twice with 500 µL of RPE buffer for 2 min. In all this steps the flow-through was discarded. The RW1 buffer is used as a stringent washing buffer that efficiently removes biomolecules such as carbohydrates, proteins, fatty acids, etc and RPE buffer is a mild washing buffer that is used to remove traces of salts, which are still on the column due to buffers used earlier in the protocol. Each spin column was then placed in a new RNase-free 1.5mL tube and 40 µL RNAse-free water were added directly to the spin column membrane, proceeding thereafter to centrifugation for 1 min at 8000g to elute the RNA (eluted water was applied again onto the column). Finally, the RNA quality and quantity was determined using NanoDrop (A260/280 ≥ 2-Pure RNA; A260/280 ~ 1.8-DNA contamination or ethanol; A260/230 << 2-contamination with proteins, chaotropic salts like guanidinium isothiocyanate or phenol). RNAs should be: >300 ng/uL; A260/280 ~2 or slightly higher; A260/A230 ~2.

7.2. cDNA synthesis for qPCR

For DNase I incubation, 2 ng of external transcript was added to 2 µg of total RNA in DEPC-treated H_2O (8.0 µL final volume). Then, each diluted RNA sample was incubated at 37°C for 15 min with DNase I (DNase I - Amplification grade, Life Technologies, Carlsbad, CA) to a final 10 µL volume. DNase I was inactivated according to the manufacturer's instructions.

For Reverse Transcription, the QIAGEN Omniscript kit (Qiagen, Duesseldorf, Germany) was used. The final RT mix with DNase-treated RNA contained 0.5 mM each deoxyribonucleotide phosphate (dNTPs), 4 µM Oligo dT18 and Omniscript RT Enzyme. The RT reaction was carried out at 37°C for 1 h. The synthesized cDNA was diluted 20x in milliQ H_2O and stored at -20°C, until qPCR was performed.

7.3. qPCR

For these analyses the iQ5 Bio-Rad thermal cycler (Bio-Rad, Hercules, CA) was used. First, the correct PCR program (cycles, temperatures etc), plate default, fluorochromes (e.g. SYBR green) was chosen. All reagents as well as 96-well PCR plate were kept on ice. The number of wells needed for the analysis was calculated based on the formula indicated below:

$Eq. 6:$

$$[(\text{No.biological samples} \times \text{No.replicates}) + 1] \times \text{No.genes including reference genes}$$

The following qPCR primers were used: SOD1-F: GGTGTGGCCGATGTGTCTAT; SOD1-R: TTCCAGCGTTTCCTGTCTTT; SOD2-F: CCCTGGAACCTCACATCAAC; SOD2-R: CTGAAGAGCTATCTGGGCTGTAA; CAT-F: TGAACTGTCCCTACCGTGCT; CAT-R: TATTGGATGCTGTGCTCCAG; GPX1-F: CGGGACTACACCCAGATGAA; GPX1-R: TCTCTTCGTTCTTGGCGTTC; GSR-F: GATCCCAAGCCCACAATAGA; GSR-R: TCGCTGGTTATTCCTAAGCTG; GSTM_1_F: CAGAGCAACGCCATCTTGT; GSTM_1_R: GCCAGCTGCATATGGTTGT; GSTM_4_F: AGAGCAACGCCATCCTGT; GSTM_4_R: GATTGGAGACGTCCATAGCC. The final individual qPCR reactions contained SYBR green mixture (iQ 2x SYBR Green Supermix) (BioRad, Hercules, CA), 1.5 uM each primer and cDNA. No Template Controls (NTC) were also performed in which milliQ water was added instead of cDNA.

Each cDNA was pipetted into a specific reaction tube (per sample per gene) and then was added a specific volume (depends on number of reaction tubes), to which a pre-mix containing SYBR green and primers was added. After mixing, technical duplicates (15 µL each) were taken to each well in the PCR plate. Moreover, 11.2 µL of reaction mixture (SYBR green and primer mix) and 3.8 µL milliQ water were transferred to individual wells as No Template Control. The plate was carefully sealed and placed on the iQ5 Bio-Rad thermal cycler (Bio-Rad, Hercules, CA) for start the run.

8. Data and Statistical Analysis

For most assays, at least three independent experiments were performed, each with three technical replicates. The statistical analysis was performed using the software SigmaPlot version 11. All analyses of variance were performed with Two-way ANOVA, followed by Holm-Sidak test to evaluate differences between groups and the control exposure. The differences were considered statistically significant when $p < 0.05$. All data are expressed as mean ± standard deviation (SD). For qPCR analysis, two independent experiments were performed with four technical replicates per independent experiment. Cycle threshold (Ct) and average PCR efficiency were estimated from the raw fluorescence data, using the program Real-Time PCR miner (Zhao and Fernald, 2005). These parameters were used to calculate relative gene expression according to the Pfaffl method (Pfaffl, 2001), using GAPDH as a reference gene for data normalization. Data are expressed as mean relative gene expression ± standard error of the mean (SEM).

Results

1. Viability analyses

1.1. Sulforaphane effects in MG-63 cells proliferation and culture confluence

Control MG-63 cells presented typical proliferation and fibroblastic morphology under the growth conditions tested, with total confluence being achieved after approximately 3 days (Figure 9). Cells treated with SFN showed morphological changes such as cell enlargement and loss of adherence, which were more pronounced with increasing SFN concentrations. Cell confluence decreased with increasing SFN concentrations, compared to control for both exposure times (24 h and 48 h).

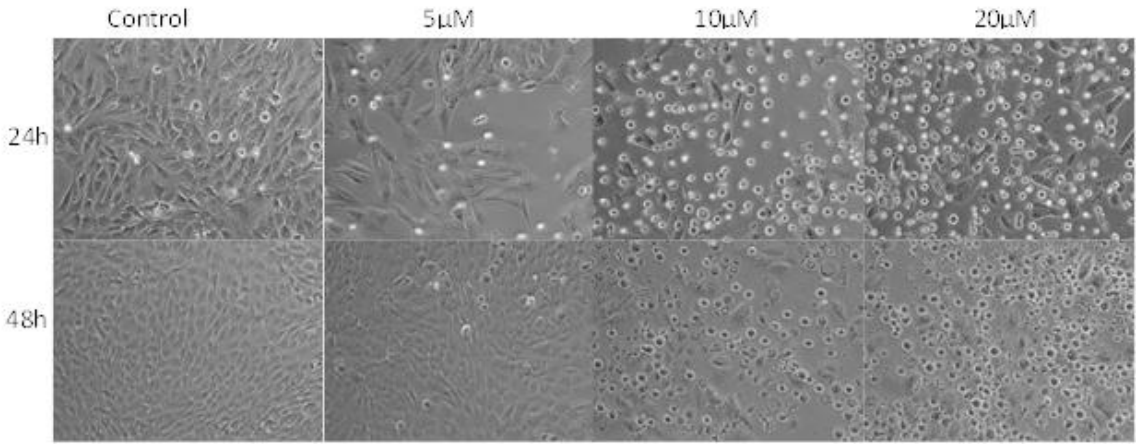

Figure 9: Effect of different concentration and time exposure to SFN on cell viability and morphology in MG-63 cells by inverted microscopy (x100).

1.2. Viability assay

The trypan blue assay was used to asses if SFN could affect cell viability of MG-63 cell line. SFN induced a dose-dependent inhibition of cell viability of MG-63 cell line at both times (Figure 10). For 10 and 20µM SFN concentrations, cell viability was significantly decreased for the 48h exposure period, compared to the control. Moreover, for these concentrations there was significant decrease in cell viability from 24 to 48h.

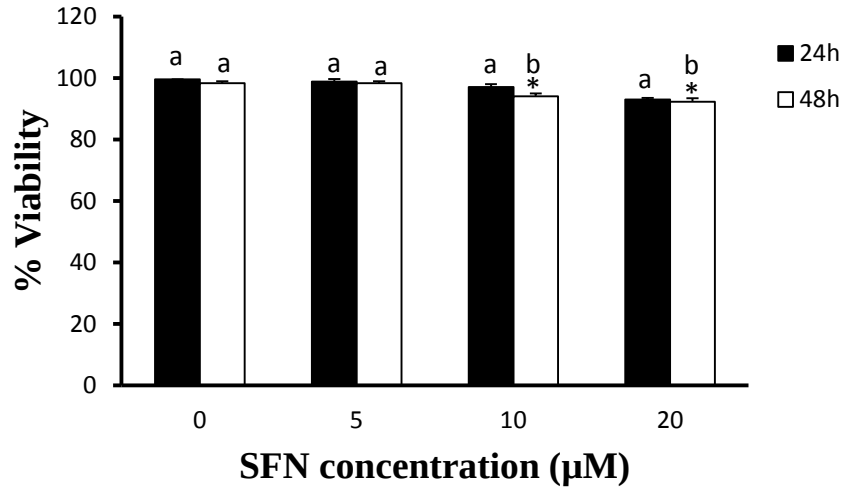

Figure 10: Cell viability by trypan blue assay at 24 and 48 h. Data expressed as percentage viable cells. Values from 4 independent experiments. * indicates significant differences between treatments and control and different letters (a and b) show significant differences for different times ($p<0.05$).

2. Enzyme assays

2.1. Superoxide dismutase activity

A trend for a decrease of SOD activity with increasing SFN concentrations for both times of exposure was found. At 24 h those differences were only significant for the highest SFN concentration tested (20 µM). However, at 48 h of exposure this effect was already significantly at 10 µM SFN. Data also show that for 10 and 20 µM SFN, SOD activity decreased significantly between the 24 and 48 h exposure times (Figure 11).

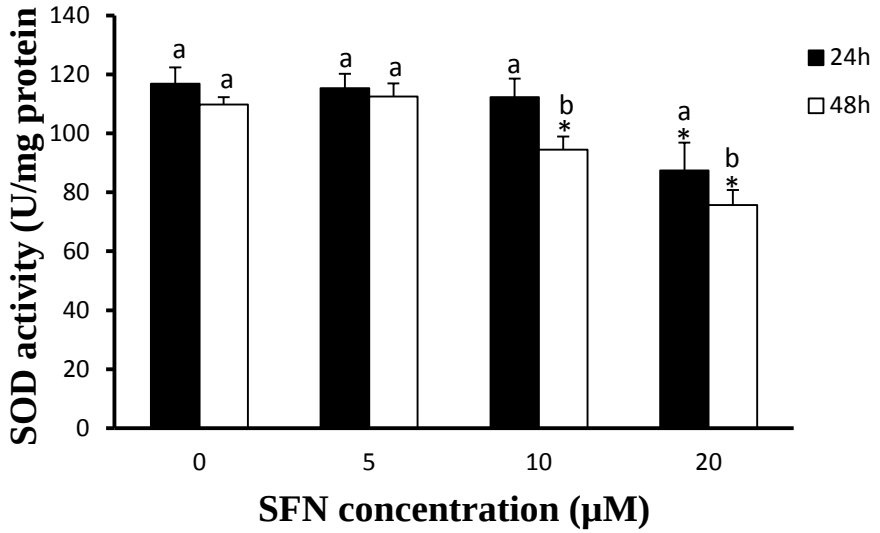

Figure 11: SOD specific activity. Values from 6 independent experiments ± SD. * indicates significant differences between treatments and control and different letters (a and b) show significant differences for different times ($p<0.05$).

2.2. Glutathione peroxidase activity

GPx activity decreased with increasing SFN concentrations (Figure 12). For the control, GPx activity increased significantly from 24 h to 48 h. However, with increasing SFN concentrations, GPx activity did not increase between 24 h and 48 h exposure. Compared to the control, GPx activity was significantly reduced for 10 and 20 µM SFN for both exposure times. Moreover, for the longer exposure period (48 h), exposure to 5µM SFN also resulted in a significant decrease in GPx activity.

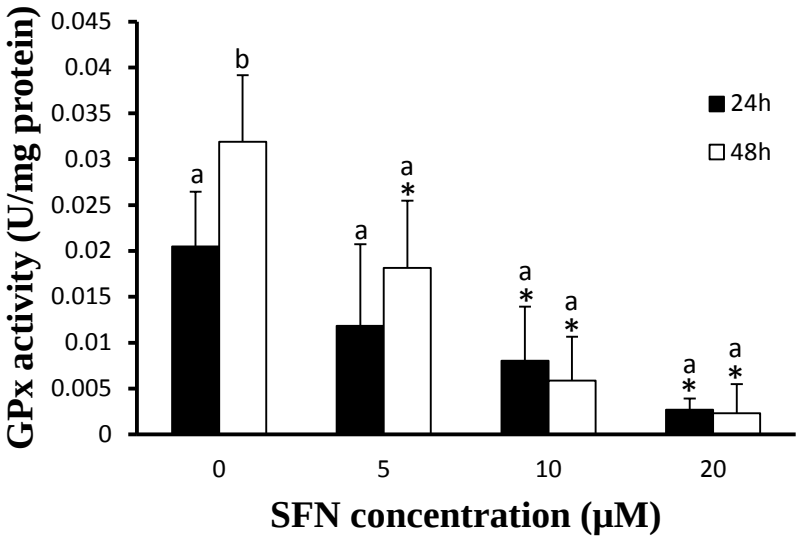

Figure 12: GPx specific activity. Values from 3 independent experiments ± SD. * indicates significant differences between treatments and control and different letters (a and b) show significant differences for different times ($p<0.05$).

2.3. Glutathione Reductase activity

GR activity decreased significantly after 48 h for 10 and 20 µM SFN comparatively to the control (Figure 13). GR activity was also affected between different exposure times for the control and lowest concentration of SFN tested, showing a significant increase from 24 h to 48 h of exposure period. Enzyme activity was extremely low for the longest exposure period tested of 48 h at the highest SFN concentration (20 µM).

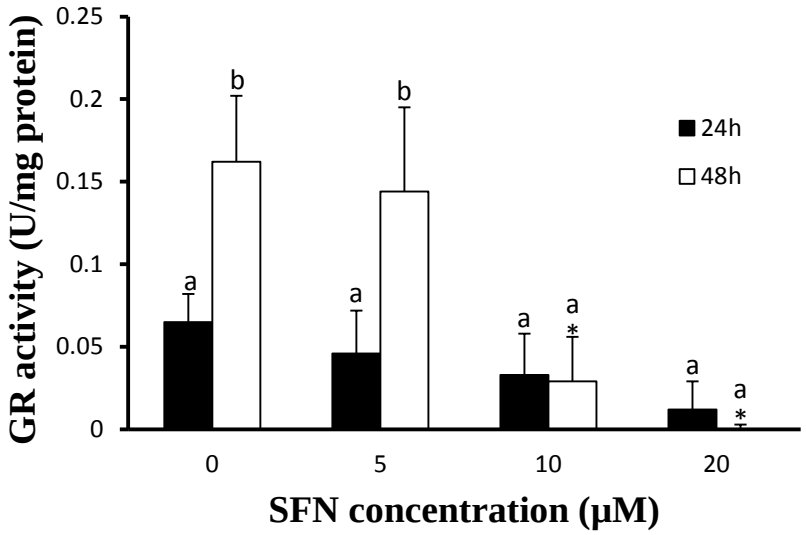

Figure 13: GR specific activity. Values from 4 independent experiments ± SD. * indicate significant differences between treatments and control and different letters (a and b) show significant differences for different times ($p<0.05$).

2.4. Reduced glutathione (GSH)

SFN exposure significantly increased total glutathione content for the shortest exposure period and lowest concentration tested i.e. 24 h exposure to 5 µM SFN. The general trend for GSH levels was an increase after exposure to 5 µM SFN, followed by decreased GSH levels at increasing SFN concentrations (Figure 14).

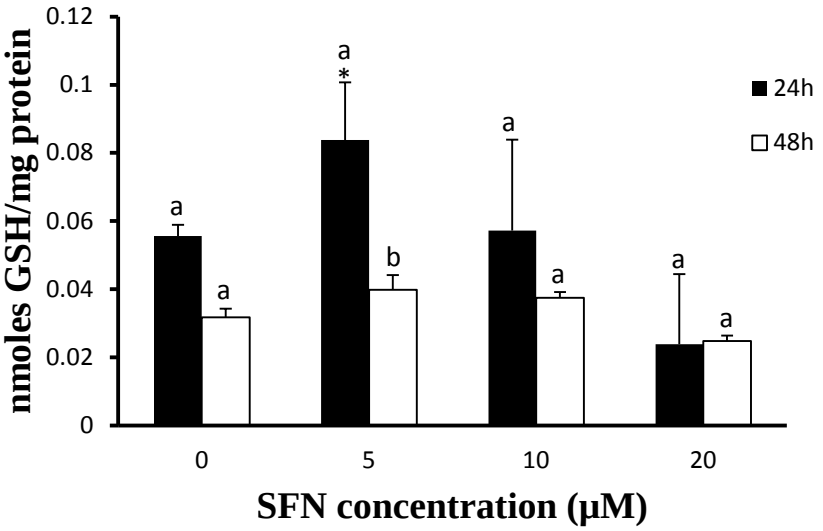

Figure 14: Normalized reduced glutathione levels. Values from 4 independent experiments ± SD. * indicates significant differences between treatments and control and different letters (a and b) show significant differences for different times ($p<0.05$).

3. Total antioxidant activity (TAA)

TAA in MG-63 homogenates was significantly decreased for the dose of 20 µM SFN. This effect was significant for both exposure periods. The length of exposure time also affected TAA, as the longer 48 h period resulted in significantly higher TAA values, relative to 24 h (Figure 15).

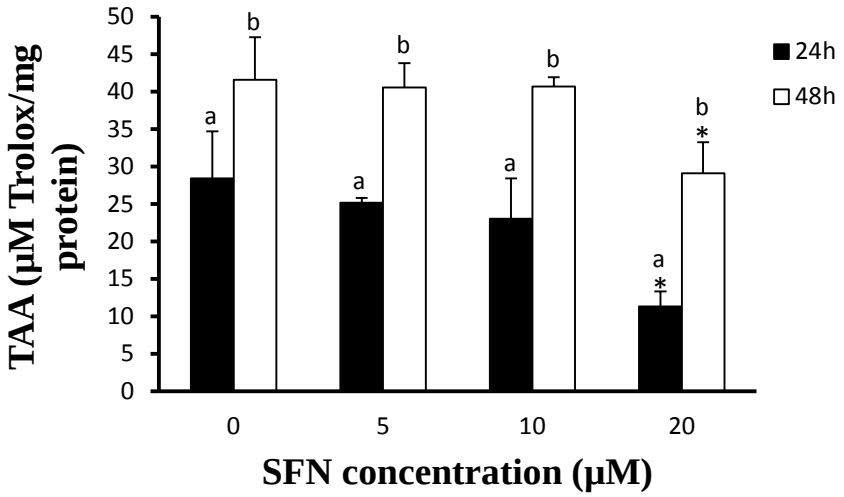

Figure 15: Total Antioxidant activity. Results are expressed as mean ± SD. Values from 3 independent experiments. * indicates significant differences between treatments and control and different letters (a and b) show significant differences for different times ($p < 0.05$).

4. Lipid peroxidation (TBARS)

In this assay, a global decrease in TBARS levels was observed with increasing SFN concentrations (Figure 16). A significant decrease in TBARS levels was observed after 48 h exposure to SFN concentrations between 5 and 20 µM. For the 24 h exposure period, only the largest SFN concentration tested (20 µM) decreased significantly TBARS levels.

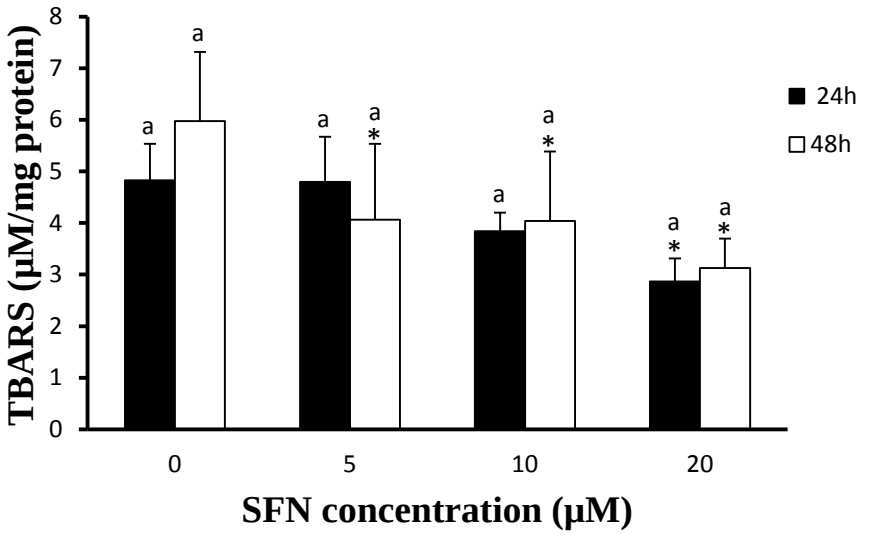

Figure 16: Lipid peroxidation. Results are expressed as mean ± SD. Values from 5 independent experiments ± SD. * indicates significant differences between treatments and control and different letters (a and b) show significant differences for different times ($p < 0.05$).

4.1. Protein quantification in lipid peroxidation assay

In this assay, a global decrease in protein levels was observed with increasing SFN concentrations (Figure 17). A significant decrease in protein levels was observed after 24 and 48 h exposure to SFN concentrations of 10 and 20 µM. The length of exposure time also affected protein levels, as the longer 48 h period resulted in significantly higher protein values, relative to 24 h.

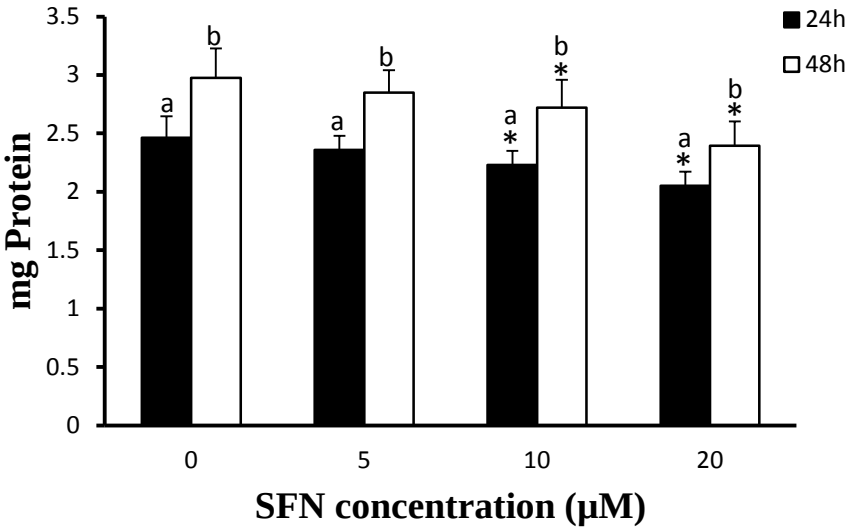

Figure 17: Protein values used in lipid peroxidation assays. Results are expressed as mean ± SD. Values from 5 independent experiments ± SD. * indicates significant differences between treatments and control and different letters (a and b) show significant differences for different times ($p < 0.05$).

5. Enzyme gene expression

Gene expression analysis of enzymes known to be involved in oxidative stress response was performed for control condition and for cells exposed to 10 µM SFN for 48 h. The experimental condition was chosen based on the significant effects produced on cell viability and enzyme activities for SOD, GPx and GR. For a 2-fold cutoff of expression, SOD1 gene, encoding Cu-Zn SOD, was overexpressed in SFN-exposed cells relative to control, whereas GPX and GSTM4 encoding GPx and Glutathione-S-Transferase isoform M4 were underexpressed. GSR gene, enconding GR, and GSTM1 gene, encoding Glutathione-S-Transferase isoform M1 showed expression levels similar to the control, for a 1.5-fold cutoff (Figure 18).

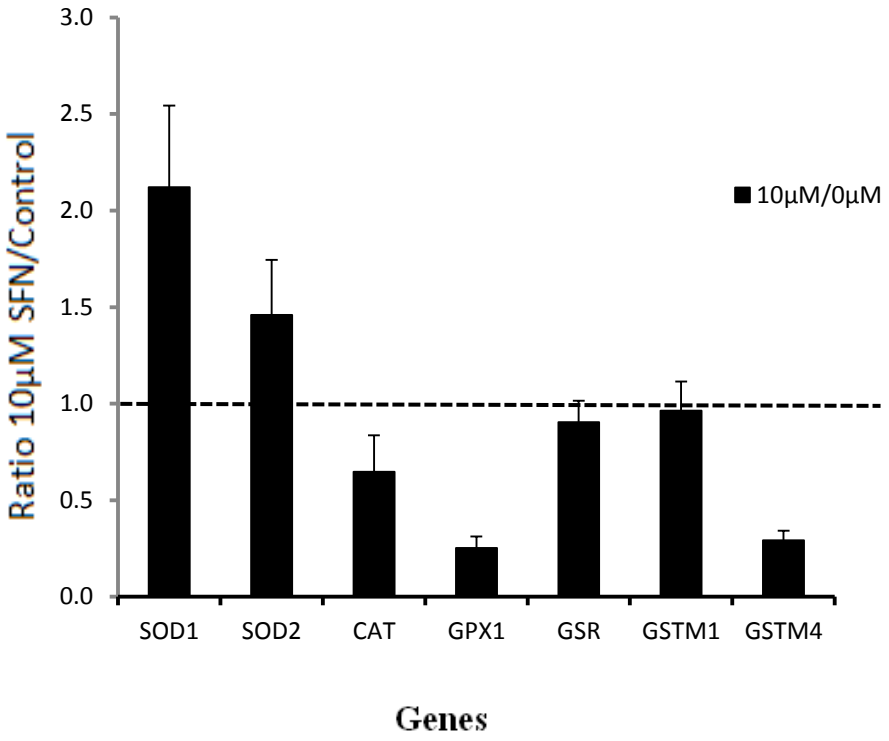

Figure 18: Relative expression (normalized ratios), SFN exposure (10µM) versus control. Gene expression related with the enzymes that interferes with oxidative stress for 48h. Results are expressed as mean ± SD. Method used was the Pfaffl with Ct and average efficiency parameters. Values from 2 independent experiments with 4 replicates each. ---- (reference value)

Discussion

SFN is a plant-derived ITC, which has been shown to display preventive activity against different tumor types. The nature and intensity of SFN effects have been described to depend on SFN concentration, time of exposure, the cell line, or tissue treated (Fimognari et al, 2005; Song et al, 2009). Various ITCs including SFN prevent oxidative damage in the cell, and Hu et al (2007) proposed that this preventive action can be achieved by nuclear factor E2-related factor 2 (Nrf2)-mediated induction of phase II detoxification enzymes. However, other studies reported that this preventive effect may be dose dependent and that SFN may have negative effects, mostly at high doses (e.g. Zanichelli et al, 2012). Also the influence of SFN on the major antioxidant enzymes (besides Phase II) in tumor cells (CAT, SOD, GPX, GR) remain unclear, and this clarification in osteosarcoma was the main aim of this work.

Therefore the present study was undertaken to investigate putative chemotherapeutic effects of SFN on MG-63 osteosarcoma cell line *in vitro*. For this, MG-63 cells were exposed to SFN concentrations previously described as physiologically attainable in the human plasma, e.g. up to 10 µM SFN (Gasper et al, 2005), and also to the higher dose of 20 µM SFN for 24 and 48h.

Data of cell viability showed that SFN exposure caused cell death in a concentration and time dependent manner, so a decrease in cell viability and proliferation occurred, as expected. These gradual concentration and time dependent effects quantitatively supported the visual analyses in which cultures with SFN showed a decrease in confluence and a general increase in the percentage of cells detached from the plate. One of the first lines of antioxidant defenses are the enzymatic antioxidant defenses. Small disturbances in this system can immediately lead to oxidative stress resulting in various damages to cells (Peixoto et al, 2004; Ascenso et al, 2011). The mechanisms by which SFN disrupts oxidative defenses are not fully understood yet. As stated above for other tissues, SFN may interfere with the Nrf2-mediated induction of phase II detoxification enzymes. According to few studies, disturbed redox equilibrium leads to modulation of redox sensitive transcription factors such as Nrf2, AP-1 and NF-kB that transduce alterations in the cellular redox status. Such alterations in cellular redox status can result in gene expression responses. Nrf2 binds to antioxidative response elements (AREs) and induces transcription of phase II detoxification and antioxidative enzymes. These enzymes improve cell defense against oxidative damage and promote the removal of carcinogens (Zhang et al, 2006; Hu et al, 2007; Juge et al, 2007).

The gene expression of the transcription factor Nrf2, was shown to be up-regulated in osteoblasts under exposed to oxidative conditions (Arai et al 2007), but it remains unclear if and how other signaling pathways may also be involved in these cells (e.g. Xu et al 2010). These cells are prone to redox signaling, so being particularly sensitive to alterations on oxidative state (Almeida M, 2011; Zhou H et al, 2011). Moreover, the action of SFN on osteoblasts oxidative stress regulation remains unknown.

As reported above, cellular antioxidant defenses include the SOD, GPx, CAT and GR enzymes that act sequentially to scavenge free radicals (Betteridge, 2000). In our experiment, SOD activity showed a decrease with increasing SFN concentrations for both times of exposure. This decrease in SOD activity is probably due to a perturbation of

cellular redox balance, caused by the excess ROS that can overwhelm the capacity of the antioxidant system. Oxidative stress is a condition of imbalance between ROS formation and cellular antioxidant capacity due to enhanced ROS generation and/or dysfunction of the antioxidant system (Jung and Kwak, 2010). Data also show that for 10 and 20 μM SFN, SOD activity is significantly decreased between the 24 and 48 h exposure times, once again demonstrating the time effect in treated cells.

Activities of the antioxidant enzymes in mouse liver and different brain regions were studied in order to demonstrate that different tissues may also express different enzyme activities, which may result in different reaction to oxidative stress (Yang et al, 2006). An example is the GR activity that was consistently reported as inhibited by SFN in diverse types of cell lines, such as Hepa1c1c7, HepG2, MCF7, MDA-MB-231, LNCaP, and HT-29 cells, while in other cell lines, such as HeLa cells, GR is induced by SFN (Jiang et al, 2003). In this work, the GR and GPx activities suffered a significant decrease after the 48 h of exposure to 10 and 20 μM SFN, comparatively to the control, and an increase for the control and lowest concentration of SFN tested, for both periods. GR enzyme activity was extremely low for the longest exposure period tested of 48 h at the highest SFN concentration (20 μM). These results occur probably because of GSH depletion caused by SFN (data shown in this work), since both enzymes interfere with glutathione cycle. GR recycles glutathione disulfide (GSSG) and keeps it in the functional status (form sulfhydryl GSH) and GPx uses hydrogen peroxide and reduced glutathione (GSH) generating H2O and oxidized glutathione (GSSG) (Saydam et al, 1997; Peskin and Winterbourn, 2000; Hu et al, 2007; Kim et al, 2010). GPx uses the reductive power of reduced glutathione (GSH), so it is linked to the GSH/GSSG cellular content. So, depending (possibly) on the GSH content, alterations in GPx and GR activity suffer also changes, as observed in this work.

GSH is a low molecular thiol that besides being a GPx substrate, also may act, alone, as an antioxidant agent. It is reported that GSH is responsible for almost 80% of total radical scavenging in cell (Singhal et al, 1987).

Studies demonstrating the SFN preventive potential show that it can alleviate cells from chemically-induced oxidative stress (Song et al, 2009; Gan et al, 2010), despite the fact that over time and at high concentrations, SFN may react with and deplete the intracellular GSH pool. SFN conjugates with GSH in rats and humans (Zhang, 2002). Therefore, the conjugating potential of SFN with GSH in cells appears to be critically important for the

induction of phase 2 enzymes (Kim et al, 2003). In this thesis, higher concentration of SFN lead to a decrease of GSH levels, which explains the decrease in GR and GPx activities. These effects cause an increase in cellular ROS levels (data not show). The increase in ROS also corroborates the results obtained in this thesis, namely antioxidant defenses decrease, increase of ROS levels leading to oxidative stress.

Gene expression analysis of enzymes known to be involved in oxidative stress response was performed for control condition and for cells exposed to 10 µM SFN. This concentration was chosen based on the absence of significant differences between control and 5µM and the extensive cell damage found in 20µM SFN exposed samples. The time used for analysis was 48h, since this was the exposure time which showed significant effects on cell viability and enzyme activities for SOD, GPx and GR.

The results mentioned before are in agreement with the results obtained for gene expression, where a decrease in gene expression is observed for GPx and GR. Contrarily to the most of genes analyzed in our work which were either underexpressed (GPx, GSTM4) or unchanged in expression (GSR, GSTM1) for a 1.5-fold cutoff, the SOD genes were overexpressed in SFN-exposed cells relative to control, contrarily to SOD activity data (decrease activity). SFN may decrease SOD activity at a post-transcriptional level. Alternatively, SOD gene expression might change throughout time. So, it is necessary to perform a blotting (protein analysis) to understand the SFN action. In general, our data show that SFN has different effects on the transcription of the studied enzymes: it stimulates the transcription of SOD, while represses the transcription of GPx and of GSTM4).

It is also possible to explain the small increase of GSH since for 10µM of SFN at 48h, a slight decrease was observed in GSTM4 expression. SFN has the capacity to induce the expression of phase II enzymes that may lack the ARE element, such as the different GST variants, so if the GSTs catalyze the conjugation of GSH to a wide variety of endogenous and exogenous electrophilic compounds, it is possible understand the connection of these two molecules (Kim et al, 2003; Townsend et al, 2003). It is feasible to assume that if less GST is produced, free unconjugated GSH levels remain high, leading to an increase in GPx and GR activities for low concentrations at 48h. So, it is possible that SFN at low concentrations leads to an increase in GSH levels. Several studies demonstrate that ITCs, such as SFN may be considered hormetic molecules, which means that these compounds

are able to induce biologically opposite effects at different doses; usually, there is a stimulatory or beneficial effect at low doses and an inhibitory or toxic effect at high doses (Zanichelli et al, 2012). Toxic effects of ITCs may be associated with their pro-oxidant activities. High concentrations of ITCs rapidly accumulate into cells and can react and inactivate –SH groups, as the case of glutathione, which is a potent reactive oxygen scavenger (Zanichelli et al, 2012). Low doses of these compounds have a beneficial effect, leading to an increase of GSH levels (Singhal et al, 1987).

In order to understand the antioxidant process, it is also necessary to analyze catalase. Relatively to this enzyme, the preliminary results indicate that both CAT activity and expression levels decrease upon exposure to SFN (unpublished data). However, more studies are necessary to confirm these preliminary results.

A large number of antioxidants (macro and micro molecules, and enzymes) exist in living organisms, which represent the total antioxidant activity of the system, playing an essential role in preventing oxidative stress. For that reason, quantitative measurement of the total antioxidant capacity may provide important biological information (Ronald et al, 1999). SFN plays significant roles in the prevention of cancer development because this compound acts indirectly by several independent mechanisms, in order to increase the antioxidant capacity of animal cells and their abilities to handle oxidative stress (Priya et al, 2011). In this study an increase was observed in TAA with time for low concentrations, probably due to the induction of some enzymes such as GR and GPx. TAA in MG-63 was significantly decreased for the 20 µM SFN exposure, an effect most significant for the 24 h exposure period. The length of exposure also affected TAA, as the longer 48 h period resulted in significantly higher TAA values, than those obtained for 24 h. Nevertheless, as SFN concentration increased the TAA decreased, and this is consistent with the results obtained, i.e. inhibition of the antioxidant enzymes. Importantly, the cellular TAA decreases only for the higher and most cytotoxic SFN concentration tested (20 µM), which suggests that SFN triggers an antioxidant defense (e.g. by modulating Phase II enzymes) as reported for other tumor cells (Bonnesen et al, 2001).

Lipid peroxidation occurs when ROS are generated close to, or within membranes and attack the fatty acid side chains of membrane phospholipids (Farias et al, 2011). Our results showed a global decrease in TBARS levels with increasing SFN concentration. A

significant decrease in TBARS levels was observed after 48 h exposure to SFN concentrations between 5 and 20 µM. For the 24 h exposure period, only the largest SFN concentration tested (20 µM) was associated with significantly decreased TBARS levels. The results obtained in this assay showing TBARS decrease did not match the other data obtained in this work, e.g. antioxidant enzymes decrease. Since TBARS absolute levels were normalized to total protein amount, the variation in TBARS levels cannot be explained by changes in protein content. More studies in this field are needed, e.g. other lipid peroxidation assays or studies on gene expression of related genes in order to confirm these results or to understand if there is protection of membrane lipids. Finaly it should not be excluded the hypothesis of a putative interference of the SFN *per se* on the TBARS method, so hampering the use of this method.

Overall, these data show that SFN affects antioxidant enzymes (at transcriptional or pos transcriptional levels). Data also show that this regulation is a concerted one of GR, GPx, GST, glutathione and other redox components, which define the complexity of the redox regulation used in cell defenses against oxidative and electrophilic species. This protective regulation is likely to be more susceptible to oxidative stress and disturbed redox balance in malignant cells, and this susceptibility in malignant cells probably contributes to the cytotoxic effects of chemopreventive compounds against tumor cells.

Conclusions and Future Perspectives

In conclusion, these results suggest that SFN can induce physiological alterations on MG-63 cell line by the disruption of cellular redox state. In particular SFN leads to a decrease on SOD, GR, GPx and possibly CAT activity as well. In high concentrations (higher than 10 µM) SFN causes the depletion of antioxidative defenses, namely, SOD, GR, GPx, and GSH content, leadings to oxidative stress inducing damages in the cells leading to cell death (figure 19).

More studies are necessary to support other markers/pathways of the oxidative stress: e.g. meanwhile found CAT activity/expression (data not shown). Also the effects of SFN on mitochondrial parameters related to ROS formation (and eventually with the apoptosis) may provide further evidence to the mechanisms of SFN targeting the tumor/osteosarcoma

cells, and therefore induce oxidative stress on it. So, this may provide a complementary mechanism/pathway of studying SFN as chemotherapeutic drug for this tumor.

Below we propose the current model of action of the SFN on osteosarcoma, based on the data obtained in vitro with MG-63 cells.

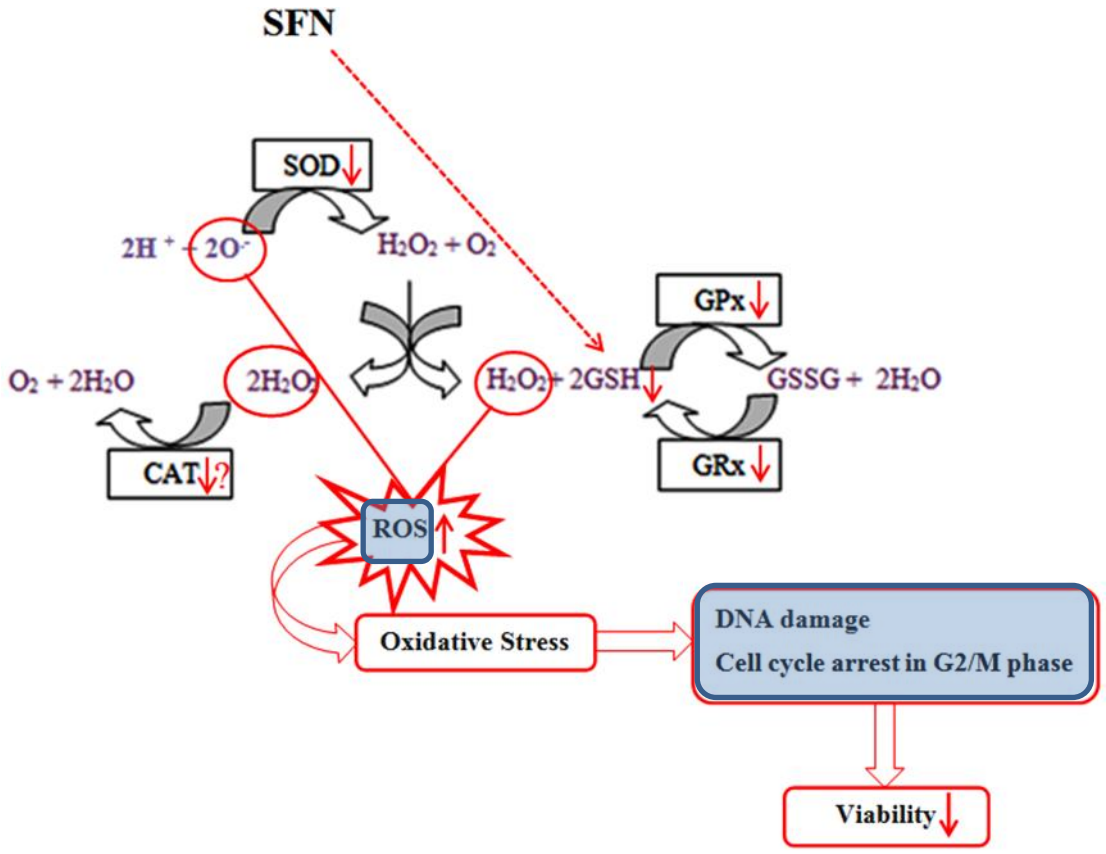

Figure 19: Proposed model of the SFN effects on antioxidant defenses of osteosarcoma cells. This model is based on the data of this work, and on other literature – in blue (e.g. Costa et al, 2012; Pinto, 2011).

References

•Aebi H. Catalase in vitro. Methods in Enzymology. 1984;105:121-6.

•Almeida M. Unraveling the role of FoxOs in bone-Insights from mouse models. Bone. 2011;49(3):319-27.

•Ascenso A, Margarida Ribeiro H, Cabral Marques H, Simoes S. Topical Delivery of Antioxidants. Current Drug Delivery. 2011;8(6):640-60.

• Arai M, Shibata Y, Pugdee K, Abiko Y, Ogata Y. Effects of reactive oxygen species (ROS) on antioxidant system and osteoblastic differentiation in MC3T3-E1 cells. IUBMB Life. 2007;59(1):27-33.

•Banning A, Deubel S, Kluth D, Zhou Z, Brigelius-Flohe R.The GI-GPx Gene Is a Target for Nrf2. Molecular and Cellular Biology. 2005;12:4914-4923.

•Benedict AL, Knatko EV, Dinkova-Kostova AT. The indirect antioxidant sulforaphane protects against thiopurine-mediated photooxidative stress. Carcinogenesis 2012; 33(12):2457-66.

•Betteridge DJ. What is oxidative stress? Metabolism. 2000;49:3-8.

•Bilbe G, Roberts E, Birch M, Evans DB. PCR Phenotyping of Cytokines, Growth Factors and Their Receptors and Bone Matrix Proteins in Human Osteoblast-like Cell Lines. Elsevier Science Inc. 1996;19:437-445.

•Boddupalli S, Mein JR, Lakkanna S, James DR. Induction of phase 2 antioxidant enzymes by broccoli sulforaphane: perspectives in maintaining the antioxidant activity of vitamins A, C, and E. Frontiers in Genetics. 2012;3(7).

•Bonnesen C, Eggleston IM, Hayes JD. Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines. Cancer Res. 2001;61:6120-6130.

•Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry. 1976;72(1-2):248-54.

•Brigeuus-Flohe R and Banning A. Part of the Series: From dietary antioxidants to regulators in cellular signaling and gene regulation - Sulforaphane and selenium, partners in adaptive response and prevention of cancer-Review. Free Radical Research. 2006;40:775-787.

•Brown KK and Hampton MB. Biological targets of isothiocyanates. Elsevier B.V. 2011; 888–894.

•Bryant CS, Kumar S, Chamala S, Shah J, Pal J, Haider M. Sulforaphane induces cell cycle arrest by protecting RB-E2F-1 complex in epithelial ovarian cancer cells. Molecular Cancer. 2010;9:47.

•Butler JM. Chapter 4 – PCR Amplification: Capabilities and Cautions. Advanced Topics in Forensic DNA Typing. 2012;69–97.

•Chen MJ, Tang WY, Hsu CW, Tsai YT, Wu JF, Lin CW, Cheng YM, Hsu YC. Apoptosis Induction in Primary Human Colorectal Cancer Cell Lines and Retarded Tumor Growth in SCID Mice by Sulforaphane. Evidence-Based Complementary and Alternative Medicine. 2012;1-13.

•Clarke JD, Dashwood RH, Ho E. Multi-targeted prevention of cancer by sulforaphane-Mini-review. Cancer Letters.2008; 269:291–304.

•Collins A. Comparison of different methods of measuring 8-oxoguanine as a marker of oxidative DNA damage. Free Radic Res. 2000;32(4):333-41.

•Cooke MS, Evans MD, Dizdaroglu M, Lunec J. Oxidative DNA damage: Mechanisms, mutation, and disease. FASEB Journal. 2003;17(10):1195-214.

•Costa M, Remédios C, Pinto P, Pinho F, Pedrosa T, Oliveira H, Oliveira M, Santos C. Cytotoxic effects induced by Sulforaphane in human osteosarcoma cell line. LINZ 2012 – 17th European Congress on Alternatives to Animal Testing and EUSAAT 2012 – 14th Annual Congress of EUSAAT, 5th – 8th September 2012, University of Linz, Austria.

•Cuezva JM, Ortega AD, Willers I, Sánchez-Cenizo L, Aldea M, Sánchez-Aragó M. The tumor suppressor function of mitochondria: Translation into the clinics- Review. Biochimica et Biophysica Acta 1792. 2009; 1145–1158.

•De Flora S, Izzotti A, D'Agostini F, Balansky RM, Noonan D, Albini A. Multiple points of intervention in the prevention of cancer and other mutation-related diseases. Fundamental and Molecular Mechanisms of Mutagenesis. 2001;9-22.

•Dinkova-Kostova AT. Chemoprotection Against Cancer by Isothiocyanates: A Focus on the Animal Models and the Protective Mechanisms. Top. Curr. Chem.-Springer. 2012;10:128-337.

•Dizdaroglu M, Jaruga P, Birincioglu M, Rodriguez H. Free radical-induced damage to DNA: Mechanisms and measurement. Free Radical Biology and Medicine. 2002;32(11):1102-15.

•Dmetri GD. 209-Sarcomas of soft tissue and bone, and other neoplasms of connective tissues. Goldmans Cecil medicine (24th edition), 2012; 1:1327-1321.

•Dreher D and Junod AF. Role of Oxygen Free Radicals in Cancer Development. European Journal of Cancer. 1996;32(1):30-8.

•Dringen R and Gutterer JM. Glutathione reductase from bovine brain. In: Helmut S, Lester P, editors. Methods in Enzymology: Academic Press; 2002. p. 281-8.

•Elbarbry F, Elrody N. Potential health benefits of sulforaphane: A review of the experimental, clinical and epidemiological evidences and underlying mechanisms. Journal of Medicinal Plants Research. 2011;5:473-484.

•Fahey JW and Talalay P. Antioxidant Functions of Sulforaphane: a Potent Inducer of Phase II Detoxication Enzymes. Food and Chemical Toxicology. 1999;37:973-979.

•Farias ILG, Farias JG, Rossato L, Araujo MCS, Chiesa J, Morsh V, Schetinger MRC. Correlation between TBARS levels and glycolytic enzymes: The importance to the initial evaluation of clinical outcome of colorectal cancer patients. Biomedicine & Pharmacotherapy. 2011;65:395–400.

•Fimognari C, Nusse M, Cesari R, Iori R, Cantelli-Forti G, Hrelia P. Growth inibhition, cell cycle arrest and apoptosis in human T-Cell Leukemia by the isothiocyanates sulforaphane. Carcinogenesis. 2002;23:581-586.

•Gamet-Payrastre L, Pengfei L, Lumeau S. Sulforaphane, a Naturally Occurring Isothiocyanate, Induces Cell Cycle Arrest and Apoptosis in HT29 Human Colon Cancer Cells. Cancer Research. 2000;60:1426–1433.

•Gan NQ, Mi LX, Sun XY, Dai GF, Chung FL, Song LR. Sulforaphane protects Microcystin-LR-induced toxicity through activation of the Nrf2-mediated defensive response. Toxicol. Appl. Pharm. 2010;247: 129-137.

•Gasper AV, Al-janobi A, Smith JA, Bacon JR, Fortun P, Atherton C, Taylor MA, Hawkey C J, Barrett DA, Mithen RF. Glutathione S-transferase M1 polymorphism and metabolism

of sulforaphane from standard and high-glucosinolate broccoli. Am. J. Clin. Nutr. 2005;82:1283-1291.

•Guerrero-Beltrán CE, Calderón-Oliver M, Pedraza-Chaverri J, Chirino YI. Protective effect of Sulforaphane against oxidative stress: Recent advances- review. Experimental and Toxicologic Pathology. 2012; 64(5):503-508.

•Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? The Lancet. 1994;344(8924):721-4.

•Halliwell B and Gutterige JMC. Free Radicals in Biology and Medicine. 1999.

•Halliwell B and Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean?-Review. British Journal of Pharmacology. 2004; 142:231-255.

•Harvilchuck JA, Pu X, Klaunig JE, Carlson GP. Indicators of oxidative stress and apoptosis in mouse whole lung and Clara cells following exposure to styrene and its metabolites. Toxicology. 2009;264:171-178.

•Hu Y, Urig S, Koncarevic S, Wu X, Fischer M, Rahlfs S, Mersch-Sundermann V, Becker K. Glutathione- and thioredoxin-related enzymes are modulated by sulfur-containing chemopreventive agents. Journal of Biological Chemistry. 2007;388:1069-1081.

•Jackson SJ and Singletary KW. Sulforaphane Inhibits Human MCF-7 Mammary Cancer Cell Mitotic Progression and Tubulin Polymerization. Nutrition and Cancer. 2004;134:2229–2236.

•Jiang H, Shang X, Wu H, Huang G, Wang Y, Al-Holou S, Gautam SC, Chopp M. Treatment with Resveratrol and Sulforaphane Induces Apoptosis in Human U251 Glioma Cells. Neurochem Res. 2010;35(1):152-61

•Jiang ZQ, Chen C, Yang B, Hebbar V, Kong ANT. Differential responses from seven mammalian cell lines to the treatments of detoxifying enzyme inducers. Life Sciences. 2003;72:2243-2253.

•Jomova K and Valko M. Advances in metal-induced oxidative stress and human disease. Toxicology. 2011;283(2-3):65-87.

•Juge N, Mithen R.F, Traka M. Molecular basis for chemoprevention by sulforaphane: a comprehensive review. Cellular and Molecular Life Sciences. 2007;64: 1105-1127.

•Jung KA and Kwak MK. The Nrf2 System as a Potential Target for the Development of Indirect Antioxidants. Molecules. 2010;15:7266-7291.

•Kasai H, Iwamoto-Tanaka N, Miyamoto T, Kawanami K, Kawanami S, Kido R, Kido R, Ikeda M. Life style and urinary 8-hydroxydeoxyguanosine, a marker of oxidative DNA damage: Effects of exercise, working conditions, meat intake, body mass index, and smoking. Japanese Journal of Cancer Research. 2001;92(1):9-15.

•Keck AS and Finley JW. Cruciferous vegetables: cancer protective mechanisms of glucosinolate hydrolysis products and selenium. Integr Cancer Ther. 2004;3:5-12.

•Kim BR, Hu R, Keum YS, Hebbar V, Shen G, Nair SS, Kong ANT. Effects of Glutathione on Antioxidant Response Element-Mediated Gene Expression and Apoptosis Elicited by Sulforaphane. Cancer Resreasch.2003;63:7520–7525.

•Kim MR, Zhou L, Park BH, Kim JR. Induction of G2/M arrest and apoptosis by sulforaphane in human osteosarcoma U2-OS cells. Molecular Medicine Reports. 2011;1-6.

•Kim SJ, Jung HJ, Hyun DH, Park EH, Kim YM, Lim CJ. Glutathione reductase plays an anti-apoptotic role against oxidative stress in human hepatoma cells. Biochimie. 2010;92:927-932.

•Lee YJ, Jeong HY, Kim YB, Won SY, Shim JH, Cho MK, Namd HS, Lee SH. Reactive oxygen species and PI3K/Akt signaling play key roles in the induction of Nrf2-driven heme oxygenase-1 expression in sulforaphane-treated human mesothelioma MSTO-211H cells. Food and Chemical Toxicology. 2012;50:116-123.

•Levi MS, Borne RF, Williamson JS. A review of cancer chemopreventive agents. Curr Med Chem. 2001;8:1349-62.

•Li S, Yan T, Yang JQ, Oberley TD, Oberley LW. The Role of Cellular Glutathione Peroxidase Redox Regulation in the Suppression of Tumor Cell Growth by Manganese Superoxide Dismutase1. Cancer Research. 2000;60:3927–3939.

•Liochev SI, Fridovich I. The Haber-Weiss cycle - 70 years later: an alternative view. Redox Report. 2002;7(1):55-7.

•Majó J, Cubedo R, N. Pardo. Treatment of Osteosarcoma–Review. Rev. esp. cir. Ortop. traumatol. 2010;54(5):329-336.

•Mannoor K, Liao J, Jiang F. Small nuclear RNAs in cancer - Review. Biochimica et Biophysica Acta 1826. 2012; 121–128.

•Mao H, Schnetz-Boutaud NC, Weisenseel JP, Marnett LJ, Stone MP. Duplex DNA catalyzes the chemical rearrangement of a malondialdehyde deoxyguanosine adduct. Proceedings of the National Academy of Sciences of the United States of America. 1999;96(12):6615-20.

•Marnett LJ. Lipid peroxidation - DNA damage by malondialdehyde. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis. 1999;424(1-2):83-95.

•Matés J, Segura J, Alonso F, Márquez J. Intracellular redox status and oxidative stress: implications for cell proliferation, apoptosis, and carcinogenesis. Arch Toxicol. 2008;82(5):273-99.

•Matsui TA, Murata H, Sakabe T, Sowa Y, Horie N, Nakanishi R. Sulforaphane induces cell cycle arrest and apoptosis in murine osteosarcoma cells in vitro and inhibits tumor growth in vivo. Oncology Reports. 2007;18:1263-1268.

•Morroni F, Merlicco A, Hrelia S, Angeloni C, Cantelli-Forti G, Hrelia P. Sulforaphane as an inducer of glutathione prevents oxidative stress- induced cell death in a dopaminergic-like neuroblastoma cell line. Journal of Neurochemistry. 2009;111:1161-117.

•Mohseny AB, Machado I, Cai Y, Schaefer KL, Serra M, Hogendoorn PCW, Llombart-Bosch A, Cleton-Jansen AM. Functional characterization of osteosarcoma cell lines provides representative models to study the human disease. Laboratory Investigation. 2011;91:1195–1205.

•Myzak MC, Dashwood WM, Orner GA, Ho E, Dashwood RH. Sulforaphane inhibits histone deacetylase in vivo and suppresses tumorigenesis in Apcmin mice. The FASEB Journal. 2006.

•Nadege B, Patrick L, Rodrigue R. Mitochondria: from bioenergetics to the metabolic regulation of carcinogenesis. Frontiers in Bioscience. 2009;14:4015-4034.

•Nathan FM, Singh VA, Dhanoa A, Palanisamy UD. Oxidative stress and antioxidant status in primary bone and soft tissue sarcoma. BMC Cancer. 2011;11:382.

•Naumann P, Fortunato F, Zentgraf H, Büchler MW, Herr I, Werner, J. Autophagy and cell death signaling following dietary sulforaphane act independently of each other and require oxidative stress in pancreatic cancer. International Journal of Oncology. 2011;1:101-109.

•Nelson KK and Melendez JA. Mitochondrial redox control of matrix metalloproteinases. Free Radical Biology and Medicine. 2004;37(6):768-84.

•Oren M. Decision making by p53: Life, death and cancer. Cell Death and Differentiation. 2003;10(4):431-42.

•Pautke C, Schieker M, Tischer T, Kolk A, Neth P, Mutschler W Milz S. Characterization of Osteosarcoma Cell Lines MG-63, Saos-2 and U-2 OS in Comparison to Human Osteoblasts. Anticancer Research.2004;24:3743-3748.

•Peixoto F, Vicente J, Madeira VM. A comparative study of plant and animal mitochondria exposed to paraquat reveals that hydrogen peroxide is not related to the observed toxicity. Toxicol In Vitro. 2004;18(6):733-9.

•Penta JS, Johnson FM, Wachsman JT, Copeland WC. Mitochondrial DNA in human malignancy. Mutation Research - Reviews in Mutation Research. 2001;488(2):119-33.

•Peskin AV and Winterbourn CC. A microtiter plate assay for superoxide dismutase using a water-soluble tetrazolium salt (WST-1). Clin Chim Acta. 2000;293(1-2):157-66.

•Pinto P. Citoxicidade e Genotoxicidade do Sulforafano emOsteosarcoma Humano. Dissertação de mestrado-Universidade de Aveiro. 2011.

•Priya DK, Gayathri R, Gunassekaran G, Murugan S, Sakthisekaran D. Protective role of sulforaphane against oxidative stress mediated mitochondrial dysfunction induced by benzo(a)pyrene in female Swiss albino mice. Pulmonary Pharmacology & Therapeutics. 2011;24:110-117.

•Qazi A, Pal J, Maitah M, Fulciniti M, Pelluru D, Nanjappa P. Anticancer activity of a broccoli derivative, sulforaphane, in barrett adenocarcinoma: potential use in chemoprevention and as adjuvant in chemotherapy. Transl Oncol. 2010;3:389-99.

•Qiang M. Transcriptional responses to oxidative stress: Pathological and toxicological implications. Pharmacology & Therapeutics. 2010;125(3):376-93.

•Quick KL, Hardt JI, Dugan LL. Rapid microplate assay for superoxide scavenging efficiency. J Neurosci Methods. 2000;97(2):139-44.

•Rausch V, Liu L, Kallifatidis G, Baumann B, Mattern J, Gladkich J, Wirth T, Schemmer P, Büchler MW, Zöller M, Salnikov AV, Herr I. Synergistic Activity of Sorafenib and Sulforaphane Abolishes Pancreatic Cancer Stem Cell Characteristics. Cancer Research - Therapeutics, Targets, and Chemical Biology. 2010;70(12):1-11.

•Reinehr R, Sommerfeld A, Keitel V, Grether-Beck S, Häussinger D. Amplification of CD95 Activation by Caspase 8-induced Endosomal Acidification in Rat Hepatocytes. J Biol Chem. 2008;283(4):2211-22.

•Saydam N, Kirb A, Demir O , Hazan E, Oto O, Saydam O, Guner G. Determination of glutathione, glutathione reductase, glutathione peroxidase and glutathione S-transferase levels in human lung cancer tissues. Cancer Letters. 1997;119:13-19.

•Scholzová E, Malík R, Sevcík J, Kleibl Z. RNA regulation and cancer development-Mini-review. Cancer Letters. 2007; 246:12–23.

• Sharma C, Sadrieh L, Priyani A, Ahmed M, Hassan AH, Hussain A. Anti-carcinogenic effects of sulforaphane in association with its apoptosis-inducing and anti-inflammatory properties in human cervical cancer cells. Cancer Epidemiol. 2011;35(3):272-8.

•Shan Y, Sun C, Zhao X, Wu K, Cassidy A, Bao Y. Effect of sulforaphane on cell growth, G0/G1 phase cell progression and apoptosis in human bladder cancer T24 cells. International Journal Of Oncology.2006;29:883-888.

•Singhal R, Anderson M, Meister A. Glutathione, a first line of defense against cadmium toxicity. FASEB Journal. 1987;1(3):220-3.

•Skupinska MK, Stypulkowski R, Lubelska K, Kasprzycka-Guttman T. Sulforaphane and Its Analogues Inhibit CYP1A1 and CYP1A2 Activity Induced by Benzo[a]pyrene. Biochem Molecular Toxicology. 2009;23:1-11.

•Smith AD and Levander OA. High-Throughput 96-Well Microplate Assays for Determining Specific Activities of Glutathione Peroxidase and Thioredoxin Reductase. Methods in Enzymology. 2002;347;113-121.

•Smith-Pearson PS, Kooshki M, Spitz DR, Poole LB, Zhao W, Robbins ME. Decreasing peroxiredoxin II expression decreases glutathione, alters cell cycle distribution, and sensitizes glioma cells to ionizing radiation and H2O2. Free Radical Biology & Medicine. 2008;45:1178-89.

•Song MY, Kim EK, Moon WS, Park JW, Kim HJ, So HS, Park R, Kwon KB, Park BH. Sulforaphane protects against cytokine- and streptozotocin-induced beta-cell damage by suppressing the NF-kappa B pathway. Toxicol. Appl. Pharm. 2009;235: 57-67.

•Stadtman ER. Metal ion-catalyzed oxidation of proteins: Biochemical mechanism and biological consequences. Free Radical Biology and Medicine. 1990;9(4):315-25.

•Stadtman ER. Role of oxidant species in aging. Curr Med Chem. 2004;11(9):1105-12.

•Talalay P. Chemoprotection against cancer by induction of Phase 2 enzymes. BioFactors. 2000;5-11.

•Topè A, Rogers P. Evaluation of protective effects of sulforaphane on DNA damage caused by exposure to low levels of pesticide mixture using comet assay. Journal of environmental science and health. Part. B, Pesticides, food contaminants, and agricultural wastes. 2009;62-657.

•Townsend DM, Tew KD, Tapiero H. The importance of glutathione in human disease. Biomedicine & Pharmacotherapy. 2003;57:145–155.

•Traka M and Mithen R. Glucosinolates, isothiocyanates and human health. The Phytochemical Society of Europe. 2009;8:269–282.

•Trieb K and Kotz R. Proteins expressed in osteosarcoma and serum levels as prognostic factors-Review. The International Journal of Biochemistry & Cell Biology. 2001;33:11–17.

•Valko M, Leibfritz D, Moncol J, Cronin MTD, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. The International Journal of Biochemistry & Cell Biology. 2007;39(1):44-84.

•Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chemico-Biological Interactions. 2006;160(1):1-40.

•Van Meir E, Hadjipanayis C, Norden A, Shu HK, Wen P, Olson J. The Avenue to a Cure for Malignant Glioma. CA Cancer J Clin. 2010;60:166-193.

•Vasanthi HR, Mukherjee S, Das DK. Potential Health Benefits of Broccoli- A Chemico-Biological Overview. Mini Reviews in Medicinal Chemistry. 2009; 9 (6):749.

•Vives-Bauza C, Gonzalo R, Manfredi G, Garcia-Arumi E, Andreu AL. Enhanced ROS production and antioxidant defenses in cybrids harbouring mutations in mtDNA. Neurosci Lett. 2006;391(3):136-41.

•Vurusaner B, Poli G, Basaga H. Tumor suppressor genes and ROS: complex networks of interactions-Review Article. Free Radical Biology & Medicine. 2012;52:7-18.

•Waisberg M, Joseph P, Hale B, Beyersmann D. Molecular and cellular mechanisms of cadmium carcinogenesis. Toxicology. 2003;192(2-3):95-117.

•Wang F and Shan Y. Sulforaphane retards the growth of UM-UC-3 xenographs, induces apoptosis, and reduces survivin in athymic mice. Nutrition Research. 2012.

•Yang MS, Chan HW, Yu LC. Glutathione peroxidase and glutathione reductase activities are partially responsible for determining the susceptibility of cells to oxidative stress. Toxicology. 2006;226:126-130.

•Yao H, Xu W, Shi X, Zhang Z. Dietary Flavonoids as Cancer Prevention Agents. Journal of Environmental Science and Health, Part C. 2011;29(1):1-31.

•.Xu H, Zhou YL, Zhang XY, Lu P, Li GS. Activation of PERK signaling through fluoride-mediated endoplasmic reticulum stress in OS732 cells. Toxicology. 2010; 9: 277(1-3):1-5.

• Zanichelli F, Capasso S, Cipollaro M, Pagnotta E, Cartenì M, Casale F, Iori R, Galderisi U. Dose-dependent effects of R-sulforaphane isothiocyanate on the biology of human mesenchymal stem cells, at dietary amounts, it promotes cell proliferation and reduces senescence and apoptosis, while at anti-cancer drug doses, it has a cytotoxic effect. Age Dordr. 2012; 34(2):281-93.

•Zanichelli F, Capasso S, Bernardo GD, Cipollaro M, Pagnotta E, Cartení M, Casale F, Iori R, Giordano A, Galderisi U. Low concentrations of isothiocyanates protect mesenchymal stem cells from oxidative injuries, while high concentrations exacerbate DNA damage. Apoptosis. 2012;17:964-974.

•Zelko IN, Mariani TJ, Folz RJ. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radical Biology and Medicine. 2002;33(3):337-49.

•Zhang B, Wang X, Yang Y, Zhang X. Extraction and Identification of Isothiocyanates from Broccolini seeds. NPC-Natural Product Communications. 2011;6:1-2.

•Zhang CC. High cellular accumulation of sulphoraphane, a dietary anticarcinogen, is followed by rapid transporter-mediated export as a glutathione conjugate. Biochemical Society. 2002;364:301±307.

•Zhang DD and Hannink M. Distinct Cysteine Residues in Keap1 Are Required for Keap1-Dependent Ubiquitination of Nrf2 and for Stabilization of Nrf2 by Chemopreventive Agents and Oxidative Stress. Molecular and Cellular Biology. 2003;23(22):8137–8151.

•Zhou H, Newnum AB, Martin JR, Li P, Nelson MT, Moh A, Fu XY, Yokota H, Li J. Osteoblast/osteocyte-specific inactivation of Stat3 decreases load-driven bone formation and accumulates reactive oxygen species. Bone. 2011;49(3):404-11.