Nrf2 targeting by sulforaphane: a potential therapy for cancer treatment

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Abstract

In the past decades, extensive studies ~~have~~ reported the potential chemopreventive activity of sulforaphane, an isothiocyanate derived from glucoraphanin, occurring in large amounts in *Brassica* genus plants. Sulforaphane was found to be active against several forms of cancer. A growing body of data shows that sulforaphane acts against cancer at different levels, from development to progression, through pleiotropic effects. In this review, we discuss the available experimental and clinical data on the potential therapeutic role of sulforaphane against cancer. Its effects range from the protection of cells from DNA damage to the modulation of the cell cycle via pro-apoptotic, anti-angiogenesis and anti-metastasis activities. At molecular level, sulforaphane modulates cellular homeostasis *via* the activation of the transcription factor Nrf2. Although data from clinical studies are limited, sulforaphane remains a good candidate in the adjuvant therapy based on natural molecules against several types of cancer.

Keywords: Antioxidant, Nrf2, Sulforaphane, Cancer.

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1. Introduction

Food plants may be considered the oldest drugs in the world, because of the presence of bioactive components, often called nutraceuticals, which show a variety of physiological and pharmacological properties with limited side effects. In the last two decades, a plethora of scientific evidences showed that food plants contain thousands of non-nutritional compounds able to play a crucial role in wellness and health improvement. Vegetables such as those belonging to the *Brassica* genus (Brassicaeae) are a rich source of important micronutrients and phytochemicals including vitamins C, E, carotenoids and secondary plant metabolites, including polyphenols, terpenes, coumarins and, especially, the sulfur containing glucosinolates, responsible for the typical pungent aroma and taste of *Brassica* genus plants (Manchali et al., 2012). Upon cellular disruption, the release of endogenous myrosinase enzymes induces the hydrolysis of glucosinolates leading to the release of bioactive substances such as isothiocyanates, nitriles and thiocyanates (Cabello-Hurtado et al., 2012). Isothiocyanates possesses potential role in health management and treatment of diseases induced by oxidative stress and chronic inflammation. For instance, the intake of sulforaphane, an isothiocyanate derived from glucoraphanin, has been associated with reduced risk of cardiovascular diseases (especially myocardial infarction and atherosclerosis) (Joshipura et al., 1999; Cornelis et al., 2007; Mirmiran et al., 2009), neurodegenerative pathologies (Kraft et al., 2004; Danilov et al., 2009), metabolic syndrome and types 1/2 diabetes (Xue et al., 2008; Zhang et al., 2014), osteoarthritis and rheumatoid arthritis (Facchini et al., 2011; Ko et al., 2013) and different types of cancers, such as breast, lung, prostate, as well as colorectal (Verhoeven et al., 1996; Feskanich

et al., 2000; Voorrips et al., 2000; Neuhouser et al., 2003; Joseph et al., 2004; Conzatti et al., 2014).

Since the early 1990s, epidemiological evidence suggested that a high consumption of plant-derived foods and beverages decreases the incidence of some forms of cancer (Block et al., 1992). Over the last three decades, the evidence supporting this recommendation has been confirmed by a large number of *in vitro* investigations leading to the identification of compounds responsible for these protective activities and, in some cases, their mechanisms of action (Key, 2011). Limiting the attention to isothiocyanates, these compounds have been associated with a decreased risk of cancer through different mechanisms, such as immune system stimulation, prevention of oxidative stress, inhibition of malignant transformation following carcinogenic mutations (Fahey et al., 1997; Myzak et al., 2004; Clarke et al., 2011).

Among isothiocyanates, sulforaphane was known to protect cells from DNA injury through the modulation of Phase II enzymes, and to alter the expression and activity of proteins related to cell cycle regulation, apoptosis and angiogenesis, which play a crucial role in cancer. Moreover, sulforaphane was found to be able to influence nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2), a transcription factor that, in humans, plays a critical role against inflammation in multiple tissues through activation of phase II enzymes and, indirectly, through suppression of the nuclear factor-kappa beta (NF-κB) signaling pathway (Kim and Keum, 2016; Menegon et al., 2016).

Considering the enormous body of literature available on this topic, the purpose of the present review article is to assess the recent works on the influence of sulforaphane on cancer development and progression with particular emphasis on its interaction with the Nrf2

transcription factor. The work also includes a short paragraph on sulforaphane bioavailability, limiting, for lack of space, the information related to its dietary sources, chemistry and extraction.

2. Sulforaphane

Glucosinolates (GSL) are a large family of sulphur- and nitrogen-containing compounds found mostly in cruciferous vegetables, including *Brassica*, mostly broccoli, and white and red cabbages (Tarozzi et al., 2013; Ares et al., 2015). Their chemical structure is a β-thioglucoside *N*-hydroxysulfates, a sulphur-linked β-D-glucopyranose moiety and a variable side chain (Angelino and Jeffery, 2014) (Fig. 1). GSL themselves are biologically inactive, and must be enzymatically hydrolysed to become bioactive by myrosinases, a group of plant enzymes which catalyse the formation of bioactive isothiocyanates, in a series of reactions widely referred to as the "mustard-oil bomb" (Matile, 1980). When plants undergo the tissue damage (through chewing, chopping, etc.), myrosinases interact with their substrates and the products of hydrolysis are formed (Angelino and Jeffery, 2014). These enzymes are heat sensitive; therefore, cooked vegetables no longer support the hydrolysis reaction, unless the cooking process time is very short. Thus, boiling for more than a minute, or steaming for more than 4 or 5 min cause loss of myrosinase activity (Wang et al., 2012). However, exogenous myrosinase, purified from white mustard *Sinapis alba*, is able to hydrolyse broccoli GSL (Angelino and Jeffery, 2014). Isothiocyanates may be also formed through the action of gut bacteria (Atwell et al., 2015). Among the various GSL, the most well-known is glucoraphanin (4-methylsulfinylbutyl

glucosinolate) (Fig. 1A), which is the glucosinolate precursor of the bioactive isothiocyanate sulforaphane (Fig. 1B). The sulfoxide group on the side chain of glucoraphanin can undergo reversible oxidation or reduction to form glucoerysolin or glucoerucin, respectively, each of which can be hydrolyzed by myrosinase to form the bioactive analogues of sulforaphane, erysolin and erucin, respectively (Angelino and Jeffery, 2014).

It has been reported that the glucoraphanin content in broccoli sprouts is about 20 times higher than in the mature plants (Fahey et al., 1997). Therefore, a high glucoraphanin content is crucial for a high level of sulforaphane production. The highest content of sulforaphane, ranging from 273 to 3632 µg/g, was measured in ripe seeds and in 8-11 day old sprouts (López-Cervantes et al., 2013; Li et al., 2014). Sulforaphane (4*R*-1-isothiocyanato-4-(methylsulfinyl)-butane) (Fig. 1B) was first isolated and identified in 1992. Natural sulforaphane is chiral and possesses the *R* configuration; however, identical activity was noticed for either *R*-sulforaphane or the synthetic racemic form (*R*,*S*) (Ganin et al., 2013). On the other hand, it was shown that quinone reductase and glutathione *S*-transferase activities were enhanced by *R*-sulforaphane, while the *S*-sulforaphane had no effect (Razis et al., 2011). As a result, there has been a focus on improving the purification of enantiopure *R*-sulforaphane from natural sources. De Nicola (De Nicola et al., 2014) and Han and Row (Han and Row, 2011) established new method for isolation of glucoraphanin. Several analytical methods have been used to determine sulforaphane content and C_{18} RP-HPLC silica phase resulted the most common and effective method. In fact, being sulforaphane a labile compound, with poor solubility in water, it may precipitate when aqueous mobile phases are employed (Budnowski et al., 2013).

To provide bigger amount of pure compound, an efficient separation method was proposed by Liang et al. Sulforaphane was purified by high-speed counter-current chromatography (HSCCC) technique and the ethyl acetate extract of broccoli seed meal was subjected to two-phase solvent extraction with a system which included *n*-hexane, ethyl acetate, methanol, and water (1:5:1:5; v/v). Before extraction, the seeds were homogenized and mixed with water in order to hydrolyse glucosinolates through endogenous myrosinase (2 h at 25°C). It has been reported that 850 mg of the ethyl acetate extract contained 186 mg of purified sulforaphane in a single run (Liang et al., 2008).

Because of its lipophilicity and molecular size, sulforaphane easily diffuses into the enterocytes. After administration of either sulforaphane, GSL, or even its main source, broccoli, the maximum concentration in plasma occurs in the range of 1 - 3 h (Tarozzi et al., 2013). Sulforaphane absorption is affected by the presence of glucoraphanin. Conversion efficiency to the bioactive sulforaphane is determined by chewing intensity rate, genotype of glutathione-*S*-transferase, as well as gut flora which can hydrolyse the glucophoranin when plant enzymes are inactivated, such as during cooking. Sulforaphane, for example, after rapid distribution into the cells of gut epithelium, is transformed through the mercapturic acid metabolic pathway. It is initially conjugated with glutathione, a reaction catalyzed by glutathione S-transferase (GST) enzymes. Finally, at the end this process, sulforaphane-N-acetylcysteine is generated, an important step for the subsequent elimination from the organism (Tortorella et al., 2015). Atwell et al. (Atwell et al., 2015) compared the sulforaphane absorption from a raw broccoli and its sprout and the extract of myrosinase-treated broccoli sprout. Consumption of sprouts ensures the 3–5 times higher level of total sulforaphane metabolites in plasma and urine compared to extract

consumption. Even though myrosinase-treated broccoli sprout extract provided more sulforaphane, its absorption was lower than absorption from fresh broccoli sprouts. Those differences strongly suggest that conversion from glucoraphanin is not the only factor which influences absorption. It was emphasised that minerals and fibre in fresh sprouts may enhance sulforaphane transport across cell membranes and increase the contact time between the target compound and gut bacteria (Atwell et al., 2015).

Egner et al. studied the bioavailability of sulforaphane from two extracts including lyophilised aqueous broccoli sprout extract containing high level of glucoraphanin and treated with gut microflora to obtain the active compound compared to the same extract treated with myrosinase from *Raphanus sativus* sprouts. The latter preparation had much higher bioavailability, than the glucoraphanin-rich (70 and 5%, respectively) (Egner et al., 2011).

In volunteers who consumed crushed broccoli, raw or cooked, the content of glucoraphanin in cooked broccoli was significantly higher than sulforaphane content in raw broccoli (about 7-fold). However, when sulforaphane-derived metabolites were determined in blood and urine, higher amounts were detected when broccoli were eaten raw (bioavailability equal to 37%), compared to the cooked broccoli (bioavailability 3.4%). Based on this relatively low recovery, the authors formulated different hypotheses: i. no complete conversion of glucoraphanin into sulforaphane occurred; ii. not all sulforaphane was absorbed from the gut; iii. alternative metabolic routes are available for its metabolism (Vermeulen et al., 2008).

3. Sulforaphane and cancer

Cancer is a multi-factorial disease responsible of an increasing morbidity and mortality worldwide. The definition of the eleven hallmarks of cancer, described by Hanahan and Weinberg (Hanahan and Weinberg, 2011), highlights its complexity and heterogeneity supporting the finding of new molecules able to impact on the several targets of cancer. Sulforaphane, similarly to other phytochemicals, is a pleiotropic compound that, acting on several hallmarks of cancer, possesses chemopreventive and chemotherapeutic potentiality (Clarke et al., 2008; Lenzi et al., 2014) (Fig. 2).

In the course of tumor evolution, the hallmarks acquired are at first genomic instability followed by sustained proliferative signaling and evasion of anti-growth signaling, resistance to apoptosis and replicative immortality, deregulated metabolism and tumor-promoting inflammation, the support of microenvironment, immune system evasion, angiogenesis and tissue invasion and metastasis (Hanahan and Weinberg, 2011).

3.1 Genomic instability

In vitro experiments demonstrated that sulforaphane protects cells from DNA damage. This effect is mainly mediated by sulforaphane activity on Phase II enzymes. For example, at the concentration of 10-20 μg/ml, this compound protects human lymphocytes from DNA damage induced by low levels of pesticide mixture (Tope and Rogers, 2009). It is also able to reduce the formation of DNA adducts in HepG2 cell line and human hepatocytes exposed to PhIP (amino-methyl-phenyl-imidazol pyridine), increasing mRNA expression of UDP-glucuronosyltransferase 1A1 and glutathione S-transferase, but without any impact on the enzymes of DNA repair (Bacon et al., 2003).

In primary cultures of human hepatocytes, sulforaphane reduces the formation of DNA adducts caused by the hepatocarcinogen aflatoxin B *via* the transcriptional inhibition of CYP3A4 and CYP1A2 genes (Gross-Steinmeyer et al., 2010). In MCF-10F, mammary epithelial cells, sulforaphane (0.1 - 2.0 μM) reduces the DNA adducts induced by benzo[a]pyrene- and 1,6-dinitropyrene through the increased expression of glutathione-S-transferase and NAD(P)H-quinone reductase protein expression (Singletary and MacDonald, 2000).

In HeLa S3 cells, sulforaphane inhibiting PARP-1, reduces H_2O_2-induced poly (ADP-ribosyl)ation and DNA single-strand break repair; in particular, it has been demonstrated that, not sulforaphane but its cellular metabolites, glutathione conjugates and the erucin, a reduced analog of sulforaphane, are crucial for the reduction of PARP-1 activity (Piberger et al., 2015).

It is interesting the inhibitory activity played by sulforaphane against histone deacetylases (HDACs) (Myzak et al., 2004; Nian et al., 2009), chromatin modifiers that alter significantly chromatin structure and gene expression. HDACs proteins influence DNA damage and repair pathways (Robert et al., 2011). Sulforaphane is able to down-regulate HDAC activity and induce histone hyper-acetylation in tumor cells (Myzak et al., 2004). In particular, in human colon cancer cells, a molecular docking study shows the interaction of sulforaphane with the allosteric site of HDAC3, causing a weakening of the interactions between HDAC3 and its co-repressor SMRT; therefore, sulforaphane enhances the acetylation and degradation of important repair proteins, such as CtIP, and the consequent DNA damage accumulation leads to cell death. Since in colon cancer cells HDAC3 is overexpressed, sulforaphane might preferentially target the DNA damage/repair pathways in cancer cells, with reduced effect on non-cancer cells (Rajendran et al., 2013).

A study focused on sulforaphane effect on NER (nucleotide excision repair), an important cellular repair pathway, shows that this compound significantly reduces the early stage of NER against the (+)-anti-benzo[a]pyrene 7,8-diol-9,10-epoxide induced DNA lesion in colorectal carcinoma cells (Piberger et al., 2014). In this case, the authors illustrate a potential negative effect exerted by sulforaphane, which, targeting the zinc binding domain of the xeroderma pigmentosum A, essential for NER, inhibits genome repair, suggesting to consider carefully the applicative outcomes of this natural compound.

3.2 Sustained proliferative signaling and evasion of anti-growth signaling

Altered expression and activity of proteins involved in the cell cycle regulation and anti-growth signals evasion play an important role in tumorigenesis. Sulforaphane modulates cell cycle acting on key regulators such as Cyclins, Cyclins-dependent kinases (CDKs) and CDKs inhibitors in a manner dependent upon cell type, dose and time of treatment. Sulforaphane induces cell cycle arrest in G1, S and G2/M phases, as reported by several studies (Shan et al., 2006; Shen et al., 2006) (Tang and Zhang, 2004; Yoo et al., 2013) (Singh et al., 2004; Kim et al., 2011; Chang et al., 2013), in various cancer model. Moreover, sulforaphane is also able to interfere with important signaling pathway implicated in growth regulation. It acts against the pro-survival phosphoinositide-3-kinase (PI_3K/AKT) and mitogen-activated protein kinases (MAPKs) pathways. For example, in human bladder cancer cells the anti-cancer effect exerted by sulforaphane is mainly mediated by modulation of p38MAPK activity (Shan et al., 2010); in human gastric cancer cells, inhibition of MAPKs induces apoptosis and inhibits cell migration (Mondal et al., 2016). In four different breast cancer cell lines, where PI_3K/AKT signaling

pathway is hyperactive, sulforaphane decreases phosphorylation of AKT (Pawlik et al., 2013). Sulforaphane, in pancreatic cells, inhibits both PI_3K/AKT and MEK/ERK pathways activating the transcription factor FOXO and inducing, consequently, cell cycle arrest and apoptosis (Roy et al., 2010).

Few data also reported the capacity of sulforaphane to interfere with Wnt signaling; for example, through the Wnt/β-catenin signaling, it down-regulates miR-21 enhancing temozolomide-induced apoptosis in glioblastoma cells (Lan et al., 2015). Similarly, in leukemia stem cells, sulforaphane potentiates imatinib effect through inhibition of the Wnt/β-catenin functions (Lin et al., 2012).

3.3 Resistance to apoptosis and replicative immortality

The evasion from the apoptotic processes may lead to tumor development, progression, and drug resistance. It has been widely demonstrated that sulforaphane, in several cell lines, possesses pro-apoptotic effects (Park et al., 2007; Hsu et al., 2013; Bergantin et al., 2014), inducing caspases activation, elevated expression of pro-apoptotic Bcl-2 proteins, PARP cleavage and nuclear chromatin condensation. In bladder cancer cells, sulforaphane activates the intrinsic apoptotic pathway unbalancing the ratio Bax/Bcl-2, down-regulating IAP family proteins, activating caspase-9 and -3, and inducing the cleavage of PARP. Since the observation that sulforaphane causes an increment in endoplasmic reticulum (ER) stress-associated proteins, it has been postulated that its pro-apoptotic effect is related to the activation of these signaling pathways (Jo et al., 2014).

In HT29, colon cancer cells, sulforaphane acting on Cdc2 kinase, blocks cell cycle in G2/M phase and consequently induces apoptosis; in particular, it has been showed that the sulforaphane requires a functional proteasome-dependent degradation system (Parnaud et al., 2004). The apoptotic induction by sulforaphane could be also mediated by the MAPK signaling pathways, like JNK and P38 in human gastric cancer (Mondal et al., 2016), or ERK and JNK in pancreatic cancer cells (Xu et al., 2006).

Replicative immortality, when acquired by somatic cells with genetic damage, leads to accumulation of aberrations that trigger autonomous growth, invasiveness, and therapeutic resistance. Potential targets against these events are telomerase and the regulatory subunit of telomerase hTERT (Block et al., 2015). Sulforaphane is able to act on telomerase, that play a critical role also in genomic stability; it creates a protective cap that avoids the recognition of the chromosomal termini (Wright and Shay, 2005). In Hep3B cells, sulforaphane reduces telomerase activity and cell viability by inhibition of hTERT expression; in particular, Moon et al. suppose that the increment of ROS, induced by this compound, is essential for the downregulation of transcription and of post-translational modification of hTERT in suppression of telomerase activity (Moon et al., 2010). Moreover, it has been demonstrated that sulforaphane influences telomerase activity through epigenetic regulation. In two prostate cancer cell lines, this molecule modulates the histone post-translational modifications with consequent reduction of the expression and activity of hTERT (Abbas et al., 2016). Finally, in breast cancer cell lines, sulforaphane (2.5 - 10 μM) represses hTERT by impacting epigenetic pathways, in particular through decreased DNA methyltransferases activity (DNMTs) (Meeran et al., 2010).

3.4 Angiogenesis, tissue invasion and metastasis

Sulforaphane is able to inhibit tumor development through regulation of angiogenesis. The ability of this molecule to modulate the activity of critical actors of angiogenesis has been verified in several *in vitro* models. Using an immortalized human microvascular endothelial cell line (HMEC-1), it has been shown how sulforaphane interferes with all the important steps in neo-vascularization. The molecule inhibits, in time- and concentration-dependent manner (0.8 - 25 μM), hypoxia-induced expression of vascular endothelial growth factor (VEGF), HIF-1α and c-Myc, being the latter two important transcription factors involved in angiogenesis. Moreover, it acts on several additional targets of angiogenesis, such as basement membrane integrity and endothelial cell proliferation, migration and tube formation (Bertl et al., 2006).
Sulforaphane, in HCT116 cells, inhibits tumor progression and angiogenesis blocking HIF-1α and VEGF expression (Kim et al., 2015). The molecule, regulating FOXO transcription factor, can inhibit angiogenesis also in human umbilical vein endothelial cells (HUVECs) (Asakage et al., 2006); moreover, the nuclear translocation of FOXO3a is enhanced by the MEK and AKT kinases inhibitors (Davis et al., 2009). In human tongue squamous cell carcinoma and prostate cancer cells, sulforaphane inhibits expression of HIF-1α lowering its synthesis with a consequent reduction of VEGF expression; probably, this effect is mediated by JNK and ERK signaling pathways (Yao et al., 2008). In bovine aortic endothelial (BAE) cells, sulforaphane (15 μM) inhibits angiogenesis suppressing mitotic progression and altering the polymerization of mitotic microtubules. Moreover, the authors demonstrated that daily administration of this molecule (100 nmol/day, i.v.) to female Balb/c mice bearing VEGF-impregnated Matrigel plugs suppresses angiogenesis progression (Jackson et al., 2007).

Metastasis, it is a complex process that includes loss of adhesion, migration, invasion and proliferation of cancer cells. Sulforaphane can impact on metastasis; in fact, an *in vivo* model has been used to examine metastasis in KPL-1 human breast cancer cell xenografts in female athymic BALB/c mice. Treating these mice with sulforaphane (intraperitoneal injection of 25 or 50 mg/kg for 26 days), it has been observed a dose-dependent reduction of proliferation, an increase of apoptosis of the primary tumor cells and in particular a reduction of axillary lymph node metastasis (Kanematsu et al., 2011). The crucial enzymes of tumor metastasis, able to degrades the extracellular matrix, metastasis matrix metalloproteinases (MMPs), are targets of sulforaphane. For example, the inhibition of migration and invasion activities induced by sulforaphane in oral carcinoma cell lines has been associated to the inhibition of MMP-1 and MMP-2 (Jee et al., 2011). Sulforaphane, such as other isothiocyanates, inhibits C6 glioma cell invasion and migration, by reducing FAK/JNK-mediated MMP-9 expression (Lee et al., 2015). In prostate cancer, sulforaphane inhibits invasion by activating ERK1/2, with consequent upregulation of E-cadherin (an invasion inhibitor) and downregulation of CD44v6 and MMP-2 (invasion promoters) (Peng et al., 2015). The combination of sulforaphane and quercetin synergistically reduces the proliferation and migration of melanoma (B16F10) cells by a decrease of MMP-9 protein expression in the mouse tumors (Pradhan et al., 2010). A different target has been evidenced in B16F-10 melanoma-induced metastasis-bearing C57BL/6 mice, where sulforaphane acting on cell-mediated immune response (CMI) inhibits the propagation of metastatic cancer cells. It induces upregulation of IL-2 and IFN-γ and downregulation of IL-1beta, IL-6, TNF-α, and GM-CSF (Thejass and Kuttan, 2007).

3.5 Tumor-promoting inflammation and the tumor microenvironment

Inflammation is linked to several phases of tumorigenesis. The transcription factor NF-κB, one of the main targets of inflammation, is constitutively activated in various human malignancies; it is responsible of the up-regulation of inflammatory cytokines, genes encoding adhesion molecules, anti-apoptotic genes, and growth factors. In MCF-10A cells, 12-O-tetradecanκoylphorbol-13-acetate (TPA) stimulates the NF-κB signaling with a consequent expression of COX-2. In this cellular model, sulforaphane inhibits the phorbol ester induction of NF-κB, inhibiting two pathways, ERK1/2 and NF-κB -activating kinase (NAK). In MCF7 cells, sulforaphane suppressing the NF-κB pathway inhibits TPA-induced MMP-9 expression and cell invasion (Lee et al., 2013).

Often, in tumor cells, drug treatment can generate resistance via NF-κB; and sulforaphane is able to overcome this resistance. For example, pancreatic cancer cells are resistant to apoptosis triggered by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) by activating NF-κB signaling. The combined treatment, sulforaphane plus TRAIL, overcomes the NF-κB dependent resistance (Kallifatidis et al., 2009). Similarly, sorafenib, a multi-kinase inhibitor, activates NF-κB in pancreatic cancer stem cells, with consequent survival and re-growth of spheroids; the combined treatment with sulforaphane completely inhibits sorafenib-induced NF-κB, ameliorating the elimination of cancer stem cells (Rausch et al., 2010).

It is known that cancer cells communicate with the tumor microenvironment allowing tumor progression. For example, in the tumor microenvironment, the hypoxia generates an aggressive tumor phenotype. It has been demonstrated that, in this conditions, sulforaphane can bypass the chemo-resistance of cancer cells. Sulforaphane inhibits cell proliferation of leukemia cell line

(REH) maintained in hypoxic conditions with consequent DNA damage and activation of Bax and p53 gene (Fimognari et al., 2014). In ovarian carcinoma cell line A2780 exposed to hypoxia, sulforaphane modulates several oncogenic pathways; it activates anticancer signaling (p53, ARE, IRF-1, Pax-6 and XRE) and suppresses those related to tumor progression (AP-1 and HIF-1). In such a way, sulforaphane impacts on CA IX, target of HIF-1, leading to reduced pH regulation and migration of cancer cells (Pastorek et al., 2015).

Mature adipocytes, differentiated from adipose mesenchymal stem cells (MSCs), induce cytokine signaling within the tumor microenvironment leading to breast cancer progression. Sulforaphane can promote MSC self-renewal and inhibit adipogenic differentiation, reducing communication between cytokine and breast cancer cells, thereby, reducing cell migration and tumor development (Li et al., 2013).

3.6. Clinical studies

Data reported above consistently show the ability of sulforaphane to interfere with the main hallmarks of cancer acting in a different way, on different targets, depending often on the cell model investigated. Considering the high heterogeneity of cancer, the multi-functionality of the molecule can be seen as a strong point in favor of its anticancer properties. In fact, to address a complex multifactorial disease, such as cancer, a multi-targeted strategy is necessary with the advantage to reduce side effects, compared to conventional therapy. This aspect is critical and is strictly connected to the bioavailability of the molecule. As clearly indicated in the above paragraphs, the large part of studies, if not all, have been conducted *in vitro*. However, the promising effects of sulforaphane might be overestimated. Although, sulforaphane is considered

a decently bioavailable compound, its plasma conjugates concentration remains of about 100 nM after the ingestion of 200 g of broccoli (Vermeulen et al., 2008). Even if this amount is easily detectable respect to other phytochemicals, it is 1-2 order of magnitude far from the concentrations applied on cell lines. However, the lipophilic nature and the low molecular weight of sulforaphane are important physico-chemical attributes to investigate in order to improve its diffusion into cells and a rapid absorption.

These criticisms have been indirectly confirmed by the analysis of clinical studies which remain still limited and largely describe the effects of broccoli sprout extracts. We retrieved about 20 completed or ongoing clinical trial from ClinicalTrials.gov, but only few of them where cancer-related (Table 1). A study design, named "POUDER trial" aims to test in patients with advanced pancreatic ductal adenocarcinoma the feasibility of broccoli sprout extracts as supportive treatment, in fact patients will receive during the chemotherapy 15 capsules of these extract per day (corresponding to 90 mg of active sulforaphane) (Lozanovski et al., 2014). This trial is still in the recruiting phase and the results have not been posted yet. A phase II study reports that in patients with recurrent prostate cancer, administration of 200 μmoles/day of sulforaphane-rich extracts for 20 weeks is safe, but it does not lead to significant reduction in PSA levels in the large part of patients. However, based on the safety of the treatment, the authors suggest that further studies, at with higher doses, may support the role of sulforaphane as a prevention agent (Alumkal et al., 2015). Similarly, two other studies are investigating the efficacy of a dietary supplement made by broccoli soup and a high sulforaphane extract (100 μmol) on prostate cancer patients (Table 1; NCT numbers: NCT02404428 and NCT00946309, respectively), but in both cases, no results have been published. A glucoraphanin supplement providing sulforaphane

has been consumed by 54 women with abnormal mammograms and scheduled for breast biopsy. This study concludes that the treatment is safe, but it is not sufficient to ameliorate the level of breast tissue tumor biomarkers (H3K18ac, H3K9ac, HDAC3, HDAC6, Ki-67, p21) (Atwell et al., 2015). Of particular interest are two pivotal clinical studies (Table 1) aimed to demonstrate the preventive effects of broccoli sprouts to enhance the detoxification of some airborne pollutants which are associated to long-term risks of lung cancer and cardiopulmonary diseases. The subjects, enrolled in a high polluted area of China, were treated with a broccoli sprout-derived beverage providing 600 μmol glucoraphanin and 40 μmol sulforaphane daily. The presence of sulforaphane metabolites constantly detectable in urine over a period of 12 weeks indicated that bioavailability did not decline (Egner et al., 2011). An implementation of this trial (NCT number: NCT02656420 in Table 1) designed with the aim to evaluate a high, medium and low dose of broccoli sprout beverage has been completed, but the result are not known yet. Finally, it is worthwhile to mention the attempt to ameliorate sulforaphane bioavailability and stability using commercially available formulations, such as Sulforadex®, a complex of sulforaphane and alpha-cyclodextrins. Studies on healthy male subjects receiving escalating doses of Sulforadex® have been completed to evaluate drug safety, tolerance, pharmacokinetics and pharmacodynamics, but results are not available.

The results of these few clinical trials only partially confirm the promising anticancer potential of sulforaphane observed in pre-clinical experiments. Probably, this could be a consequence of its instability, or, as discussed in the next paragraphs, the different/opposite effects of sulforaphane in the complex tumor microenvironment respect to *in vitro* studies may account for the ambiguous results observed in the clinical trials. Therefore, it would be interesting, in future

studies, to use nanoparticles or other vehicles to enhance its delivery and use genetic mouse models recapitulating the complexity of tumor development in human body.

4. Sulforaphane in chemoprevention

In a recent report based on large-scale genomics studies combined with cancer epidemiology, the authors clearly confirmed that approximately 80% of adult cancers can be caused by environmental factors (Wu et al., 2016). A contemporary study by Zhang *et al.* (Zhang et al., 2015) in a population of 1000 children with cancer found that extrinsic factors play an equally critical role in pediatric malignancies. Only 8.5% of these little patients were born with a potential oncogenic mutation, a value very close to that predicted by Wu et al. (2016). The authors concluded that "these results are important for strategizing cancer prevention, research and public health". The concept that tumors may be primarily the result of exposure to adverse external factors, rather than only the result of intrinsic causes and genetic predisposition ("bad luck hypothesis") is the basis of the studies on cancer chemoprevention (Russo, 2007; Russo et al., 2010; Zhang et al., 2015). As historically defined, chemoprevention is intended as the use of "pharmacological agents to impede, arrest or reverse carcinogenesis at its earliest stages" (Sporn, 1991; Sporn and Suh, 2002). Cells have evolved to avoid or counteract the toxic impact of carcinogens. Starting from evolutionary speculations, reactive oxygen species (ROS) are the most attractive to demonstrate chemopreventive activity of natural molecule from plant kingdom. In fact, in plant tissues, phytochemicals act principally as anti-oxidants against ultraviolet radiations associated cellular damage. Phytochemicals also counteract plant tissues infections by

virus and parasites. Previous idea was that sulforaphane and other phytochemicals, acting as antioxidants, directly scavenged radical species before they damage DNA, proteins and cellular lipids. However, the explanation of a direct antioxidant effect of phytochemicals inside animal cells is too simplistic and far from experimental reality for two main reasons: their low bioavailability and the complex metabolic conversion in whole organism after their ingestion in food matrix. More realistically, different authors imagine that ROS can modify redox-sensitive amino acids in many proteins, including phosphatases, kinases, ion channels, and transcription factors such as Nrf2.

The chemopreventive role of sulforaphane is mainly explicated by the modulation of Phase I and Phase II enzymes. Phase I metabolism enzymes control the activation of pro-carcinogens, mediated by enzymes such as cytochrome P450 (CYPs) (Rushmore and Kong, 2002), that can be inhibited by sulforaphane. In rat hepatocytes, the molecule inhibits the activities of CYP1A1 and 2B1/2 dose-dependently (Maheo et al., 1997), and, in primary culture of human hepatocytes, it decreases mRNA level of CYP3A4 and consequently its activity (Maheo et al., 1997; Gross-Steinmeyer et al., 2010). The structure of isothiocyanates can modulate their properties; in fact, it has been demonstrated that sulforaphane and similar compounds reduce CYP1A1 and CYP1A2 activity, but the effect induced by sulforaphane is weaker compared to those of alyssin (1-isothiocyanato-5-methanesulfinylpentane) which possesses one additional CH_2-group (Skupinska et al., 2009).

The prominent effect of sulforaphane in chemoprevention is associated to its capacity to activate the transcription factor Nrf2, with the final induction of the phase II detoxification enzymes (Thimmulappa et al., 2002; Houghton et al., 2016). The relevance of this role is linked to the low

concentration of sulforaphane necessary to stimulate the expression of Nrf2 target genes, comparable to its bioavailable amount (Cornblatt et al., 2007). Moreover, compared to other phytochemicals, such as carotenoids, sulforaphane (0.2 μM) possesses the higher inducer activity on Quinone reductase (NQO1), one of the most important Nrf2-activated enzyme (Fahey et al., 2005; Houghton et al., 2016).

A critical analysis of the Nrf2 induction by sulforaphane and the functional consequences of this interaction is discussed in the following paragraphs.

4.1. Nrf2 as the major molecular target liked to sulforaphane cellular effects

Nrf2, originally identified by the laboratory of Yuet Wai Kan (Moi et al., 1994), is a transcription factor relating to the cap'n'collar (CNC) basic-region leucine zipper (bZIP) family. Nrf2 is the master regulator of about 500 transcripts controlling cellular homeostasis through different and interconnecting effects. It is thought that Nrf2 allows adaptation and survival of nucleated cells under conditions of stress by regulating gene expression of different networks. The mostly studied and well-known effect regards the modulation of intracellular redox status. Antioxidant response element (ARE), are DNA sequences found in the 5′-neighboring region of the phase II and antioxidant genes. Nrf2 is an ARE-binding transcription factor playing a crucial role in the ARE-mediated gene expression (Liu et al., 2007). In basal conditions, Nrf2 is mainly controlled by Kelch-like ECH-associated protein 1 (Keap1), a Cullin (Cul)-3/Rbx1 ubiquitin ligase substrate adaptor protein that mediates constant ubiquitination and proteasomal degradation of Nrf2 protein. In particular, Keap1 could be considered a sort of "cellular sensor" for a wide array

of sulfhydryl-reactive small molecules, called “inducers”. These molecules chemically modify the sensor cysteines of Keap1 (Cys-151, Cys-226/Cys-613, Cys-273/Cys-288, and Cys-434) leading to Nrf2 stabilization, and finally to the expressions of downstream transcripts (Dinkova-Kostova et al., 2002; Hayes and Dinkova-Kostova, 2014). Among Nrf2 transcriptional products, best characterized are antioxidant and drug-metabolizing enzymes (for example phase II enzymes: UDP glucuronosyltransferase, UGT, NAD (P) Quinone oxidoreductase, NQO-1 and glutathione S-transferase GST), as well as proteins that participate in glucose, lipid, and nucleotide metabolism. Recent studies show that Nrf2 generally modulates the expression of cytoprotective systems including antioxidant, anti-inflammatory and detoxification enzymes in addiction to proteins associated with the repair or removal of damaged macromolecules (Hayes and Dinkova-Kostova, 2014; Dodson et al., 2015). To extend the intricate puzzle of signaling molecules regulated by Nrf2, different reports show that Keap1 is not the unique regulator of its activity. There are also different protein kinases (for example glycogen synthase kinase, GSK-3), or other interacting proteins, such as p62/sequestosome-1 (p62/SQSTM1) and p21Cip1/WAF1, which interfere with the formation of the ubiquitylation-competent Keap1-Nrf2 complex and influence Nrf2 stability and activity (Hayes and Dinkova-Kostova, 2014).

Sulforaphane is a potent activator of the Nrf2 (Hong et al., 2005; Kensler et al., 2013). We retrieved more than 400 articles on PubMed, searching for “sulforaphane and Nrf2”. To synthesize all these studies and discern between positive and potentially deleterious effects of sulforaphane, we found useful to refer to specific cellular context. In fact, recent studies on tumor cell biology demonstrate that Nrf2 induction is not always a positive effect in cancer cells (for example in liver) if the antioxidant defense act as a molecular shield against excessive ROS

produced by an altered metabolism or an inflammatory microenvironment (Karin and Dhar, 2016).

4.2. Sulforaphane and Nrf2 in cancer cell lines and animals models

Due to their high reactivity, cysteine and methionine amino acids are more susceptible to oxidation than other residues. In particular, cysteine oxidation can affect protein–protein interactions, protein degradation and induce post-translational modifications (Dodson et al., 2015). In the specific case of Nrf2 protein stabilization and activation, Keap-1 co-factor is the critical player. To reinforce the hypothesis that Keap1 is a major repressor of Nrf2 is the observation that disruption of Keap1 in the mouse or knockdown of Keap1 in human cells is sufficient to increase the abundance and activity of the transcription factor (Hong et al., 2005; Hayes and Dinkova-Kostova, 2014). Hong *et al* identified the sensor cysteine modified in Keap1 by sulforaphane using a very sensitive liquid chromatography-tandem mass spectrometry method (Hong et al., 2005). This study, however, indicates that sulforaphane displays a pattern of Keap1 modification in the Kelch domain rather than in the central linker domain. This was an unexpected result compared to classical Nrf2 inducers that modify Keap1 by alkylation. Moreover, the modification of Keap1 *in vivo* by sulforaphane is not followed by Keap1 ubiquitination. For this reason the authors suggests a novel mechanism for Nrf2 stabilization. Nrf2 or Keap-1 phosphorylation by specific kinases could be an interesting and more realistic alternative (Keum, 2011). Until now, it is not demonstrated a direct involvement of sulforaphane in this type of post-transduction modifications. We believe that a critical issue in these studies is

represented by the enormous difference between sulforaphane doses used *in vivo* (5-20 μM) respect to those used *in vitro* (starting from 200 μM until 1 mM).

In a recent study, Liu et al hypothesized that androgen deprivation therapy could reduce intracellular ROS level in prostate cancer (PCa) and sensitize these tumor cells to radiation (Liu et al., 2015). This paper demonstrates that sulforaphane could improve the effects of radiotherapy in prostate cancer acting as an Nrf2 activator (Liu et al., 2015). Using prostate cancer cells (PC3 cell line) as *in vitro* model and transgenic mouse (TRAMP mice) as experimental animal model, they showed that Nrf2 activation by sulforaphane could sensitize this kind of tumor cells to radiation, lowering basal ROS levels. Therefore, sulforaphane could be used as a radio-sensitizing agent in prostate cancer if clinical trials will confirm the pre-clinical results. However, these studies must be interpreted with caution before designing a clinical trial. In fact, the indirect antioxidant effect of sulforaphane through Nrf2 activation in cancer cells (for example liver or pancreas) could be a double edged sword in this context. Additional studies on animal models, need to be done to verify the contradictory effects of Nrf2 activation. In mice, disruption of Nrf2 augments sensitivity to carcinogens; however, paradoxically, in advanced forms of cancer Nrf2 is always upregulated. It is not yet clear if Nrf2 upregulation drives carcinogenesis, or this represents an "adaptive effect". To shed light in this complex matter, in a recent study Knatko *et al.*, demonstrated the *in vivo* effects of sulforaphane in healthy human subjects and in SKH-1 hairless mice, an animal model characterized by a constitutive activation of Nrf2 (Knatko et al., 2015). The authors demonstrated that the incidence, multiplicity and burden of solar-simulated ultraviolet (UV) radiation-mediated cutaneous tumors in mice over-expressing Nrf2 were lower than in wild type counterparts. It is

well known that UV radiation is one of the most common environmental carcinogen and is involved in the etiology of cutaneous squamous cell carcinomas, mainly in immunosuppressed individuals. If mice are treated with the immunosuppressive agent azathioprine, the group receiving an Nrf2 pharmacologic activator showed a comparable protective effect against UV radiation induced cutaneous damage. Finally, the authors demonstrate that in human subjects, topical applications of sulforaphane extracts reduced the degree of solar-skin erythema, used "as a marker for cutaneous damage and skin cancer risk". The final consideration is that Nrf2, in advanced human cancers, is an indicator of "metabolic adaptation" and sulforaphane could have a chemopreventive role if timely and efficaciously delivered in pre-cancerous tissues.

Besides this example, strong evidence suggest the presence of a "dark side" of Nrf2 in cancer which may be intercepted by sulforaphane with clinical consequences which are difficult to predict. In fact, stable upregulation of Nrf-2 sustains survival pathways and may protect cancer cells. Several excellent review articles have been published on this topic in the recent years (Kensler and Wakabayashi, 2010; Muller and Hengstermann, 2012; Xiang et al., 2014; Chartoumpekis et al., 2015; Huang et al., 2015). One of the key point regards the observation that Nrf2 is constitutively over-expressed in many types of cancer cells, as well as in samples deriving from cancer patients. In addition, elevated levels of Nrf2 are associated with poor prognosis in cancer patients (Solis et al., 2010; Sasaki et al., 2013). Genetic ablation of Nrf2 in Nrf2-KO mice where lung tumors were induced by urethane, failed to engraft in nude mice, compared to those from Nrf2 wild-type mice which grew progressively (Satoh et al., 2013). From molecular point of view, over-expression of Nrf2 may be due to several independent mechanisms, such as somatic mutations in Keap1 or Nrf2 which occur frequently in several

forms of cancers. As an example, loss-of-function mutations in Keap1 weaknesses its repressive effect on Nrf2 (Ohta et al., 2008). Furthermore, K-Ras, B-Raf, and Myc oncogenes can mediate the increased transcriptional activity of Nrf2 and ROS reduction, both events that may facilitate tumorigenesis and cancer progression (DeNicola et al., 2011). Similarly, Keap1 can be silenced by epigenetic mechanisms causing CpG hypermethylation in its promoter region (Hanada et al., 2012). In addition, activation of defensive autophagy in cancer cells may cause degradation of Keap1 resulting in a hyper-active Nrf2 transcriptional activity (Lau et al., 2010). An interesting hypothesis to explain the controversial role of the Nrf2-Keap1 axis in cancer cells has been formulated by Kensler and Wakabayashi (Kensler and Wakabayashi, 2010). They postulated a U-shape like dose-response trend to explain the opposite effect of Nrf2-Keap1 pathway in cancer prevention versus cancer progression. Following this model, only when the expression level of Nrf2 falls within a specific pharmacological range, between "the biologically effective dose" and the "maximal-tolerated dose" the preventive effects prevail. Outside this range, when Nrf2 is too low or absent, as in the case of Nrf2-KO mice mentioned above, or when it expression is elevated, e.g., following Keap1 inactivation, the risk of cancer is increased (Kensler and Wakabayashi, 2010). In this scenario, the question which emerges, related to the present review, is how sulforaphane can interfere with this model? In the same article, Kensler and Wakabayashi also suggest that the genetic manipulation of Keap1 (e.g. mutation, deletion) has much greater effects than the transient activation of Nrf2 due to nutraceutical intervention (e.g., treatment with isothiocyanates, organosulfides, phenols or other bioactive phytochemicals). In fact, comparing the expression levels of detoxication and lipid metabolism-associated genes in hepatocyte-specific Keap1-KO mice versus wild-type mice treated with the oleanane triterpenoid CDDO-Im

(1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl]), they observed that both magnitude and duration of pathway activation were much more intense in Keap1-KO, suggesting that the "peak" of Nrf2 activation due to small molecules treatment can modulates signaling "over a rather small dynamic range", compared to the "highly persistent" activation consequent to genetic manipulation of the pathway (Yates et al., 2009; Kensler and Wakabayashi, 2010). To confirm this view, a pharmacokinetic-pharmacodynamic study of sulforaphane in rats demonstrated that the expression of Nrf2-target genes in blood lymphocytes was maximal after 1–2 h and returned to basal level after 24 h suggesting a less harmful effect compared to other Nrf2 chemical inducers, e.g. arsenic, which maintain the pathway chronically active for prolonged time enhancing the "dark side" effects of Nrf2 (Wang et al., 2012; Lau et al., 2013; Huang et al., 2015).

4.3. Nrf2 and sulforaphane effects in healthy cells and in animal models of inflammation

From previous studies emerges that, when activated, Nrf2 detaches from Keap1, moves to the nucleus and dimerizes with other bZIP proteins (Maf proteins) to act as a trans-activation complex in the ARE nucleotidic sequences. To identify novel gene targeted by Nrf2, Chorley et al. designed chromatin immunoprecipitation (ChIP)-sequencing experiments in human lymphoblastoid cells incubated with sulforaphane (Chorley et al., 2012). They validated particular candidate genes using parallel ChIP experiments and Nrf2-knock-out cell lines. The interesting finding of this study is that the majority of DNA sequences with high affinity for Nrf2 were near likely new members of the Nrf2 pathway. Among the candidate genes potential to be Nrf2-dependent, the authors found retinoid X receptor alpha (RXRA). This result opens new

perspective to Nrf2 regulation involving retinoids. In particular, the authors focalize their studies on retinoid and sulforaphane treatment and adipogenesis. In 3T3-L1 mouse cells, an adipocyte differentiation system, sulforaphane affect RXRA expression. Treatment of these cells for 2-8 days with 10 μM sulforaphane inhibited adipogenesis. Moreover, 3T3-L1 cells with stable silencing for Nrf2, showed delayed RXRA expression that impaired adipocytes differentiation. This study suggests a potential therapeutic role of Nrf2 activators, such sulforaphane, in a cellular context liked to metabolic pathways regulated by retinoids.

Various studies demonstrate that Nrf2 plays a critical anti-inflammatory role in various tissues through the activation of phase II enzyme and, indirectly, via inhibition of the NF-κB related pathway. In a recent paper, Sun et al. studied the anti-inflammatory effects of sulforaphane as Nrf2 activator in a mouse model of Duchenne muscular dystrophy (Sun et al., 2015). They treated 4-week-old male mdx mice with sulforaphane by gavage and measured inflammatory markers in the skeletal muscles. In particular, they showed that sulforaphane increased the expression of HO-1, a typical Nrf2 dependent transcript in muscle tissue. Specific effect of sulforaphane on HO-1 expression dependent from Nrf2 has been also demonstrated in a different study where inflammation was subsequent to oxidative stress in retinal ischemia-reperfusion (I/R) injury animal model (Pan et al., 2014). The authors emphasize the importance of HO-1 in this contest for its potent indirect anti-oxidative functions. In fact, HO-1 degrades heme to carbon monoxide (CO), iron and biliverdin. I/R injury is common in various human diseases, including different types of retinopathies (diabetic retinopathy, acute glaucoma, retinal artery occlusions and retinopathy of prematurity). In this study, rats were intraperitoneally injected with sulforaphane or vehicle once a day for 7 days. To demonstrate the direct involvement of

Nrf2/HO-1 dependent anti-oxidative/anti-inflammatory response, animals were also subjected to protoporphyrin IX zinc (II) (ZnPP) treatment at 24 h before I/R injury. Subsequently, they induced I/R by raising the intraocular pressure and measured apoptosis and infiltrating inflammatory cells induced by oxidative stress in retinal tissue. Following this approach, they demonstrated that sulforaphane protected retinal cell through the expression of Nrf2 and HO-1, measured by immunofluorescence analysis and western blotting. This effect was reversed if animals were pre-treated with ZnPP, used as HO-1 inhibitor. They conclude that the neuroprotective effect of sulforaphane could open new horizons in a possible therapy for I/R-induced injury in different human pathologies.

We can summarize that "phyto-complexity" of natural molecules as sulforaphane in animal nucleated cells, could be "simply" explained by their initial interaction with the intricate complexity of cellular pathways regulated by Nrf2. This "phyto-complexity", however, should be carefully evaluated when we would extrapolate positive results from a cellular model system and translate them in animal models and, finally, in well-designed human clinical trial. This complexity especially emerges in the chemopreventive field and chemotherapeutic studies of sulforaphane in cancer research and could explain the initial failure of some clinical trials in humans.

5. Conclusion and future prospective

A huge body of data implies that plant phytochemicals have health benefits against chronic pathologies. Considering that cancer rates are increasing worldwide, there is an urgent need for

new remedies. Plants are a virtually endless source of substances and a suitable candidate for the development of new anti-cancer agents. The evaluation of data from literature has revealed that sulforaphane, which is derived from glucoraphanin and occurs at a high concentration in *Brassica* genus plants, is a pleiotropic compound able to act on several hallmarks of cancer. In summary, the results of the reported investigations have demonstrated that sulforaphane acts on different levels, from the development to the progression of cancer (Fig. 2). Sulforaphane, in fact, protects cells from DNA injury, through the modulation of Phase II enzymes, and modulates the cell cycle via the activation of key regulators (i.e. Cyclins, CDKs and CDK inhibitors), inducing cell cycle arrest. Moreover, sulforaphane is also able to interfere with important signaling pathways involved in growth regulation, acting as a pro-apoptotic agent, and an angiogenesis and metastasis regulator. At the molecular level, several studies have shown that sulforaphane is a significant activator of the transcriptor factor Nrf2 and this explains its pleiotropicity in different cellular and animal models.

Most of the published clinical trials describe the effects of extracts obtained from *Brassica* genus plants (especially broccoli sprout extracts) and they are not focused on the effects of sulforaphane alone. However, although there is negligible clinical trials, pre-clinical data strongly show that sulforaphane is a good potential candidate as functional molecule against cancer.

We cannot ignore to seriously consider the Janus Bifront behavior of Nrf2-Keap1 pathway which appears beneficial when Nrf2 is transiently activated and harmful if persistently induced. This generated the paradox that Nrf2 shows oncogenic properties in cancer cells and mediates resistance against chemotherapy, such as cisplatin, doxorubicin, and etoposide, as well as

radiotherapy (Wang et al., 2008). As discussed above, nutraceutical intervention, including sulforaphane and other phytochemical, may be seen as deleterious in a genetic background where Nrf2 is over-expressed, making a substantial difference between primary prevention, when disease is absent, and adjuvant therapy in cancer patients. In the latter case, extreme caution is necessary and supplementation with sulforaphane should be considered after a precise measurement of Nrf2 activation. However, also in this complex scenario, a lot of work can be done and recent findings open the way to new investigation. As an example, the observation that Nrf2 can be directly or indirectly in regulating the metabolic pathways triggered by cancer cells to survive, opens new perspectives (Chartoumpekis et al., 2015). In fact, malignant cells may benefit of the protective role of Nrf2 occurring in normal cells to sustain their growth, taking advantage of the Nrf2 capacity to increase the expression of genes encoding enzymes involving in NADPH generation and pentose phosphate pathway and other. Over-expressed Nrf2 also induces lipogenesis via PPARγ activation. All these events falls in the so-called induction by Warburg effect which explain the preference of cancer cells to utilize the aerobic glycolysis to produce ATP instead of the oxidative phosphorylation (Warburg, 1956). On the other hand, Nrf2 is also able, directly or indirectly to reduce the expression of lipogenic enzymes, such as ATP citrate lyase, acetyl-CoA carboxylase-1 and fatty acid synthase which block *de novo* lipogenesis and inhibit cancer cell growth (Chartoumpekis et al., 2015). Future studies will be devoted to test the capacity of sulforaphane, in mono-treatment or in association with other compounds, to selectively enhance the beneficial effects of Nrf2 pathway.

These considerations open the question on how to selected the ideal groups of healthy people or at risk group as target for the chemopreventive effects of sulforaphane. Considering the clinical

studies reported in Table 1 and commented above (Kensler et al., 2005; Egner et al., 2011), target populations are those located in regions where the exposure to human carcinogens is elevated. Currently, intervention on healthy subjects not clearly exposed to environmental risk can be useless since the beneficial effects are difficult to demonstrate without improving the knowledge of new, specific biomarkers (Kensler and Wakabayashi, 2010).

In conclusion, considering the activity of sulforaphane on Nrf2, documented by hundreds of research articles, its pleiotropic effect and the promising results of *in vivo* studies, sulforaphane could be included among potential chemopreventive and chemotherapeutic agents. Therefore, future clinical trials are needed to ascertain the possible beneficial effects of sulforaphane for adjuvant therapy against cancer.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Abbreviations

Histone deacetylases HDACs

Phosphoinositide-3-kinase PI_3K/AKT

Mitogen-activated protein kinases MAPKs

Human telomerase reverse transcriptase hTERT

Hypoxia-inducible factor-1alpha HIF-1α

Nuclear factor kappa-B NF-κB

Reactive oxygen species	ROS
Cytochrome P450	CYPs
Nuclear factor (erythroid-derived 2)-like 2	Nrf2
Kelch-like ECH-associated protein 1	Keap1
Heme oxygenase-1	HO-1

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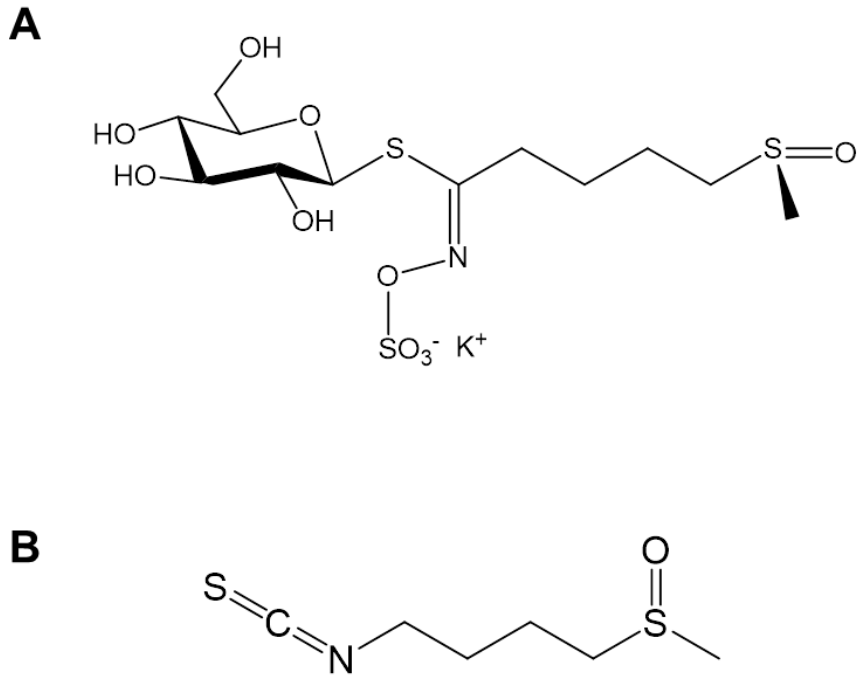

Figure 1.

Figure 1. A. Chemical structure of 4-methylsulfinylbutyl glucosinolate, potassium salt (Glucoraphanin). B. Chemical structure of 4R-1-isothiocyanato-4-(methylsulfinyl)-butane (Sulforaphane).

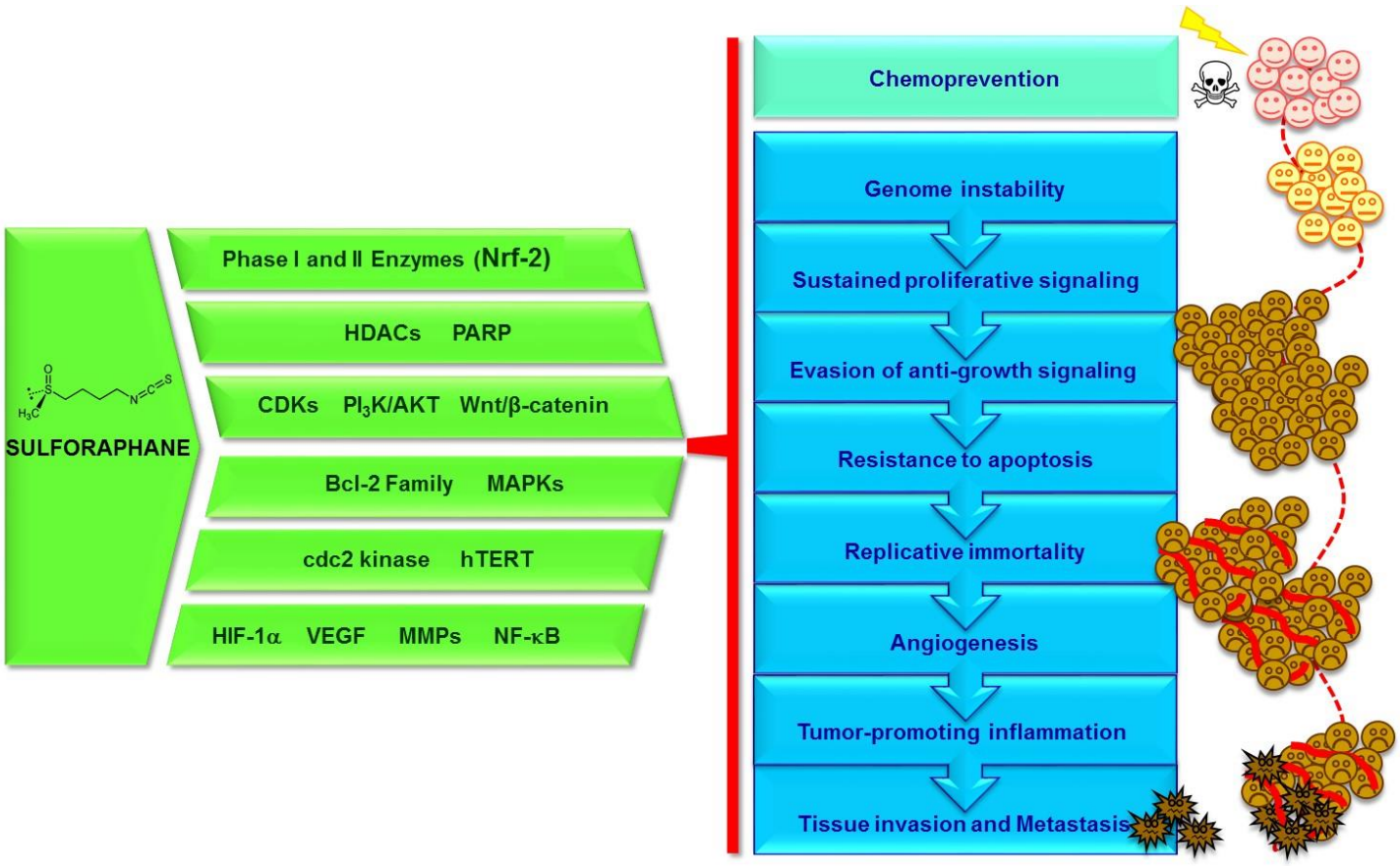

Figure 2.

Figure 2. Sulforaphane against cancer, main targets and effects (see text for details).

Table 1. Clinical trials on sulforaphane and cancer (adapted from https://clinicaltrials.gov/)

NCT Number	Study Title	Assigned interventions	Phase	Status	Condition
NCT01879878	Pilot Study Evaluating Broccoli Sprouts in Advanced Pancreatic Cancer (POUDER Trial) (Lozanovski et al., 2014)	Dietary Supplement: Verum, broccoli sprout grain	ns	Recruiting	Pancreatic Ductal Adenocarcinoma
NCT01228084	The Effects of Sulforaphane in Patients With Biochemical Recurrence of Prostate Cancer (Alumkal et al., 2015)	Sulforaphane-rich broccoli sprout extracts given 200 μmol (total daily) orally	II	Completed with results	Adenocarcinoma of the Prostate; Recurrent Prostate Cancer
NCT02404428	Effect of Sulforaphane on Prostate Cancer PrEvention-imagING Evaluation (Beneforte extra)	Dietary supplement: Beneforte extra broccoli soup one portion (300 g each) per week containing glucoraphanin-enriched broccoli	Ns	Recruitment terminated. Estimated completion date Sep 2016	Prostate Cancer
NCT00946306	In Vivo Effects of Sulforaphane	High Sulforaphane	I	Enrolling by	Prostate Cancer

9	Supplementation on Normal Human Prostate	Extract (Broccoli Sprout Extract) 100 μmol sulforaphane, every other day for 5 weeks	II	invitation	
NCT00843167	Sulforaphane: A Dietary Histone Deacetylase (HDAC) Inhibitor in Ductal Carcinoma in Situ (DCIS) (Atwell et al., 2015)	Dietary Supplement: broccoli sprout extract given orally	II	Completed with results	Breast Cancer; Precancerous Condition
NCT00982319	Evaluating the Effect of Broccoli Sprouts (Sulforaphane) on Cellular Proliferation, an Intermediate Marker of Breast Cancer Risk	Consistent doses of broccoli sprout extract (sulforaphane) dissolved in mango juice	II	Completion date November 2013. No study results posted	Breast Cancer
NCT01437501	Broccoli Sprout Intervention in Qidong, P.R. China (Kensler et al., 2005; Egner et al., 2011)	Broccoli Sprout Extract Beverage: 600 μmol glucoraphanin and 40 μmol sulforaphane dissolved in 100 mL dilute pineapple and	II	Completed with results	Environmental carcinogenesis

		lime juice daily			
NCT02656420	Broccoli Sprout Dose Response	High, medium and low doses of Broccoli Sprout: Beverage (100 ml) containing glucoraphanin-rich (600 – 300 – 120 μmol) and Sulforaphane rich (40 – 20 - 8 μmol) broccoli sprout powder mixed in pineapple juice, lime juice and water.	I II	Completion date March 2016. No study results posted	Environmental carcinogenesis
NCT02055716	A Randomised, Double-blind, Placebo-controlled, Multiple Ascending Dose Study to Evaluate the Safety, Tolerance, Pharmacokinetics and Pharmacodynamics of Sulforadex® in Healthy Male Subjects Following Daily Dosing for 7 Days	Sulforadex®* 100 mg or 300mg size 00 acid resistant HPMC capsules	I	Completion date May 2014. No study results posted	Prostate Cancer
NCT0194836	A Randomised, Double-blind,	Single ascending doses of	I	Completion date	Prostate Cancer

2	Placebo-controlled, Single Ascending Dose Study to Evaluate the Safety, Tolerance, Pharmacokinetics and Pharmacodynamics of Sulforadex® in Healthy Male Subjects	Sulforadex®* administered to healthy male subjects	February 2013. No study results posted

ns = not specified

*Sulforadex® is patent-protected complex of sulforaphane and alpha-cyclodextrin in a stable powder (Evgen Pharma)