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Relevance of the natural HDAC inhibitor sulforaphane as a chemopreventive agent in urologic tumors

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ABSTRACT

Due to an increased understanding of molecular biology and the genomics of cancer, new and potent agents have been approved by the Food and Drug Administration (FDA) to fight this disease. However, all of these drugs cause severe side effects and resistance inevitably develops, re-activating tumor growth and dissemination. For this reason, patients turn to natural compounds as alternative or complementary treatment options, since it has been found that natural plant products may block, inhibit, or reverse cancer development. The present review focusses on the role of the natural compound sulforaphane (SFN) as an anti-tumor agent in urologic cancer. SFN is a natural compound found in cruciferous vegetables from the Brassicaceae family such as broccoli, cauliflower and cabbage. Several epidemiologic and clinical studies have documented chemopreventive properties of SFN, making it an interesting candidate for additive cancer treatment. SFN shows remarkable anti-tumor effects in vitro and in vivo without exerting toxicity. The review summarizes the current understanding of SFN and provides insights into its molecular mode of action with particular emphasis on epigenetic tumor control.

1. Introduction

Treatment of advanced cancer is based on a variety of strategies including hormonal therapy, targeted therapy, radio-, immuno- and/or chemotherapy. Unfortunately, none of these approaches are likely to cure cancer once tumor cells have spread to distant organs and tissues. Rather, drug resistance inevitably develops, resulting in tumor re-activation. Besides limited therapeutic success, conventional cancer treatments are accompanied by severe side effects, further limiting their benefit.

Dissatisfaction with the efficacy of anti-tumor drugs and the strong side effects caused by conventional treatment have driven many cancer patients to seek “alternative” or “complementary” (CAM) care options. Their predominant objectives include boosting the immune system, actively contributing to tumor therapy, and lowering the risk of cancer relapse [1,2].

The prevalence of CAM use among cancer patients ranges between 30% to almost 90%, depending on the country and culture, level of education, tumor type and stage (among others) [3–5]. Patient-oriented questionnaires for oncological patients have revealed that the oral use of natural herbs is one of the most commonly applied CAM methods. However, although epidemiologic and clinical studies have documented chemopreventive properties of particular herbal agents [1,6,7], information on their efficacy to reverse, suppress, or prevent cancer is still limited.

This review focusses on the use of the herbal compound, sulforaphane (SFN), in the treatment of urological cancers. SFN is known to act on the epigenetic regulation of gene expression by suppressing histone deacetylases (HDACs). This feature is highly clinically relevant, since 90% of all cancers can be attributed to epigenetic modifications [8]. Indeed, there is no doubt that the expression of HDACs is considerably increased in hematological and solid malignancies and correlates with a poor prognosis [9]. Eighteen HDACs have been identified in humans and divided into four classes, whereby the kind of alteration of a particular HDAC subtype partially depends on the tumor entity [10]. In general, class I HDACs (HDAC 1–3, HDAC 8) serve as potent trigger factors for cell growth, proliferation, differentiation and apoptosis, while class II HDACs (HDAC 4–7, HDAC 9–10) (class II HDACs may also be subdivided into class IIa (HDAC 4, 5, 7, 9) and class IIb (HDAC 6, 10)) are involved in tumor angiogenesis [11]. Overexpression of class III HDACs (sirtuins 1–7) has been shown to promote migration, growth and metastasis [11]. The role of the class IV HDAC 11 in

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tumorigenesis has not been finally evaluated, however, recent data point to the function as a cell cycle and DNA damage regulator [12]. In addition, HDAC 6 in association with HDAC 11 has also been suggested to modulate the expression of IL-10 as a transcriptional activator [13].

Due to the fundamental significance of HDAC for cellular de-differentiation processes, HDAC-inhibition has been proposed as a strategy to re-balance transcription of those genes dysregulated in cancer. Four US Food and Drug Administration (FDA) approved HDAC inhibitors are currently in clinical use, the pan-HDAC inhibitors Vorinostat, Belinostat, and Panobinostat, along with the class I HDAC inhibitor Romidepsin, for the treatment of T-cell lymphoma or multiple myeloma [14,15]. Yet, all of these agents typically are associated with severe negative side effects and resistance develops, thus limiting their use. It is, therefore, not surprising that the interest in phytochemicals with HDAC-inhibitory properties has been revived in the hope that particular natural agents may substantially improve the current therapeutic protocol with no or minimal toxicity.

When considering natural HDAC inhibitors, it must be emphasized that lifestyle factors are closely associated with a loss of “normal” epigenetic cell control, a switch to pathologically altered gene transcription and neoplastic development. Nearly one third of all cancers can be traced back to inadequate nutrition [16–18]. Vice versa, a diet rich in ingredients derived from fruits, vegetables, spices or cereals may prevent cancer to the same degree [19]. Supporting this notion, a multicenter prospective study (EPIC) carried out in 23 centers in 10 European countries has pointed to a significant association between dietary factors and cancer [20]. Kanherkar et al. postulated that targeting the epigenetic machinery by adequate phytochemicals might be one innovative strategy to revolutionize personalized medicine [21]. Therefore, the possibility that SFN as a natural HDAC inhibitor might prevent, delay, or reverse epigenetic alterations in cancer simply via consumption of a specific diet is unquestionably attractive.

In fact, high consumption of cruciferous vegetables from the Brassicaceae family such as broccoli, cauliflower and cabbage (species: Brassica oleracea) has been associated with beneficial effects in inhibiting cancer development. All of these vegetables are rich in glucosinolates, the precursors of sulforaphane, which are chemically composed of stable *N*-hydroxysulfates with a sulfur-linked β-*d*-glucopyranose moiety and a variable amino acid-derived side chain (Fig. 1) The glucosinolates themselves do not represent the bioactive compounds. Rather, the breakdown products of glucosinolates after enzymatic hydrolysis by myrosinase are the relevant substances exerting chemopreventive properties.

In the intact plant, glucosinolates and myrosinase are spatially separated, whereby myrosinase is located in myrosin cells and glucosinolates are stored in vacuoles [22,23]. Upon physical damage of the plant, e.g. during food preparation or chewing, these two compounds come in contact and myrosinase (serving as a thioglucohydrolase) induces the hydrolysis of the glucosinolates, leading to an unstable aglucon intermediate. Depending on the specific parent glucosinolate and the reaction conditions (pH-value, presence of ferrous ions and activity of the epithiospecifier protein (ESP)), the intermediate is converted into stable isothiocyanates or nitriles. Glucoraphanin, the primary glucosinolate in broccoli [24], is then broken down to SFN (1-isothiocyanato-4-(methylsulfinyl)butane), SFN nitril, (4-(methylsulfonyl) butyl and isothiocyanate-(4 R)-(methylsulfonyl) butane (Fig. 1) [25]. Besides the plant's myrosinase driven metabolization process, human enteric microflora may also convert ingested glucosinolates with their own myrosinase during gastrointestinal passage [26].

2. Epidemiologic and clinical studies

Epidemiologic studies point to chemopreventive effects of SFN induced by the consumption of cruciferous vegetables. A prospective study involving 47,909 men demonstrated an inverse association between total broccoli or cabbage intake and bladder cancer risk [27]. This finding has recently been corroborated in case-control studies [28,29].

Clinical trials have only been performed on prostate cancer patients. In a double-blind, randomized, placebo-controlled multicenter trial including 78 patients with increasing PSA level after radical prostatectomy, daily oral administration of 60 mg of SFN for 6 months followed by 2 months without treatment led to a reduced PSA doubling time, and the PSA increase was significantly lower in the SFN- than in the placebo group [30]. Compliance and tolerance were good, and no dose-limiting toxicity was observed. A phase II clinical trial of prostate cancer patients with biochemical recurrence after radical prostatectomy, who received a daily oral SFN administration of 200 µmol/day, revealed a significant lengthening of the on-treatment PSA doubling time, compared to pre-treatment [31]. Adverse side effects were not detected. Finally, global gene expression analyses of men with high-grade prostate intra-epithelial neoplasia before, during, and after a 12 month broccoli-rich diet (400 g/week) demonstrated complex changes of signaling pathways associated with inflammation and carcinogenesis [32].

The effective SFN concentration necessary to exert chemopreventive

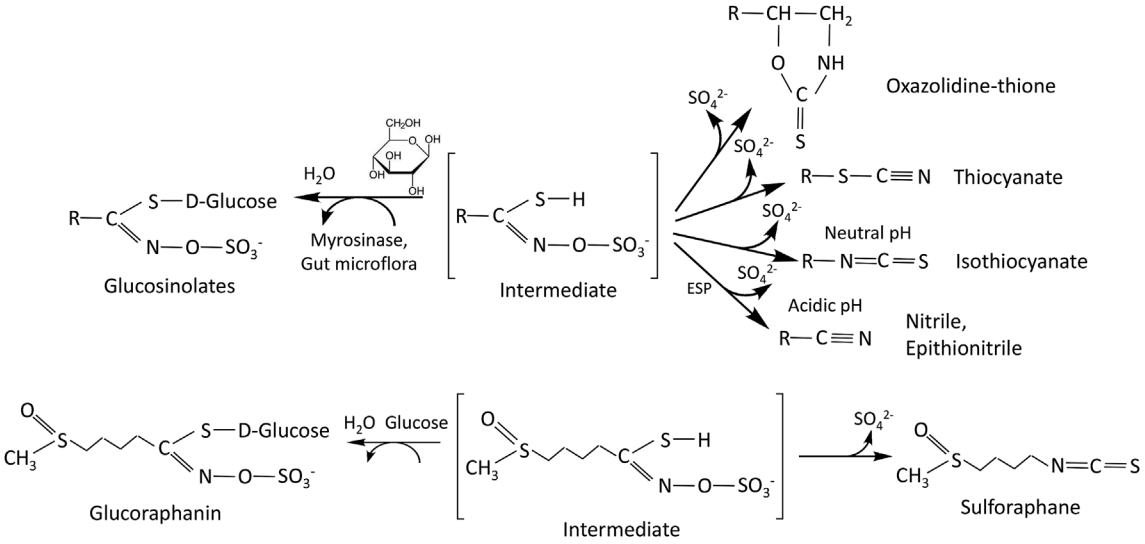

Fig. 1. Top: General structure of sulfur-containing glucosinolates and their breakdown products after hydrolysis by myrosinase or gut microflora. ESP = epithiospecifier protein. Bottom: Conversion of glucoraphanin to sulforaphane.

effects has not yet been determined in clinical trials. However, consumption of at least one serving of raw broccoli per month (average intake 3.9 servings/month) reduced disease-specific and overall mortality of bladder cancer patients [33]. Cipolla et al. in treating prostate cancer patients with SFN, employed an oral dose of 60 mg/day but provided no further details about SFN metabolism or bioavailability [30]. The urine concentration of dithiocarbamates, a group of SFN metabolites, has been evaluated in clinical trials with healthy volunteers to be about 20 μM after consuming 50 g/day [34] or 70 g/day broccoli sprouts [35]. This accords with a concentration shown to be effective in preclinical studies.

3. HDAC interaction

The interaction of SFN with HDAC has been shown both clinically and preclinically. In human subjects, a single dose of 68 g broccoli sprouts significantly inhibited HDAC activity in peripheral blood mononuclear cells 3 and 6 h following consumption. Based on an in vivo prostate cancer xenograft model, the daily consumption of 7.5 μmol SFN per animal for 21 days evoked a significant decrease in HDAC activity in the xenografts, as well as in the prostates and mononuclear blood cells, along with an increase of acetylated histones H3 and H4. Both HDAC suppression and increased histone acetylation correlated well with tumor growth blockage [36].

The same effect was seen in mice bearing colon cancer cells, fed with 10 μmol SFN/animal. The authors concluded that inhibition of HDAC activity was coupled to an increase of acetylated histones H3 and that H4 might contribute to the cancer chemoprotective and therapeutic effects of SFN [37].

Interestingly, SFN may act differently on tumor than on normal cells. Comparative analysis of SFN's action in normal, benign hyperplasia and cancerous prostate epithelial cells revealed a selective cell cycle arrest and apoptosis in the benign hyperplastic and cancer but not the normal cells. Several class I and II HDAC proteins decreased in LNCaP and PC3 prostate cancer cells (HDAC 3, HDAC 4, HDAC 6, HDAC 8), whereas SFN caused only a transient reduction of HDAC activity in the normal cells. In particular, HDAC 6 seemed to play the central role in controlling the mitotic cell cycle arrest [38]. In another prostate cancer in vitro model, employing TRAMP C1 cells, HDAC 1, HDAC 4, HDAC 5, and HDAC 7 were significantly decreased and acetylation of histone H3 was increased by SFN [39]. HDAC6 was not evaluated in this investigation. Therefore, whether HDAC 6 inhibition represents a specific mechanism of SFN in prostate cancer is not yet clear.

Individual HDACs inhibited by SFN in bladder cancer cells included HDAC 1, HDAC 2, HDAC 4, and HDAC 6 (acetylation status of histones H3 or H4 remained unaltered) [40]. In human colon cancer cells HDAC 1, HDAC 2, HDAC 3, and HDAC 8, but not HDAC 6, were potently targeted by SFN [41]. Spurling and coworkers assumed that HDAC 3 might be the central ligand for SFN in this tumor entity [42].

Nonetheless, although the chemopreventive role of SFN is associated with its HDAC inhibitor activity, the effect of SFN on chromatin composition and dynamic folding is poorly understood. Based on computer modeling, it has been speculated that metabolic conversion of SFN to SFN-cysteine via the mercapturic acid pathway might generate the HDAC inhibitor properties, since the cysteine moiety occupied most of the HDAC active site [43]. Recently, an SFN-induced decrease in gene expression in prostate cancer cells has been shown to correlate not only with changes in chromatin structure but also with alterations in chromatin composition. Acetylation of histone H3 lysine 18 was associated with repression of the human telomerase reverse transcriptase (hTERT) promoter region. Additionally, the chromatin compactor, MeCP2, was enriched over regions of the hTERT promoter, with increased nucleosome density [44].

4. Molecular mechanism

The chemopreventive properties of SFN are multifaceted. SFN blocks tumor cell proliferation and induces apoptosis by altering transcription of apoptotic and cell cycle regulating genes in several prostate, renal and bladder cancer cells in vitro and in vivo [45,46].

Experiments on prostate cancer cells revealed an up-regulation of the cyclin-dependent kinase (cdk) inhibitor p21 under SFN [38], presumably triggered by phosphorylation of the checkpoint kinase Chk2 [47]. Besides p21, the anti-mitogenic protein p27 has been identified to be a pivotal target of SFN in bladder cancer [48]. Cell cycle arrest also correlated with reduction in protein levels of cyclin D1, cyclin E, cdk1, cdk4, and cdk6, and down-regulation of the mitosis inducer Cdc25C (evidenced in prostate cancer cells). The SFN-caused effects were closely associated with an increased Ser(10) phosphorylation of histone H3 [47]. Still, entity specific differences should be considered. In fact, studies on renal cancer cells have recently presented evidence that the cdk1-cyclin B and cdk2-cyclin A axis may become up-regulated by SFN [46], and experiments on bladder cancer cells reveal either no changes of cyclin D [48] or diminished expression following SFN treatment [49].

SFN-induced apoptosis has been associated with up-regulation of Bax, down-regulation of Bcl-2 and activation of caspases 3, 9 and 8, along with cleavage of procaspase 3 and poly(ADP-ribose)polymerase (PARP) [50]. Choi et al. has pointed to a marked decrease in the levels of inhibitor of apoptosis (IAP) family proteins (cIAP1, cIAP2 and XIAP), which was accompanied by inhibition of nuclear translocation of p65-nuclear factor kappaB (NFkappaB) [51]. Animal studies point to a reduced appearance of tumors, including karyopyknosis and angiogenesis, induced by SFN via induction of caspase 3 and down-regulation of survivin, a member of the IAP family [52]. Notably, dietary administration to rats of a freeze-dried aqueous extract of broccoli sprouts significantly inhibited bladder cancer development and progression, which has been associated with induction of glutathione S-transferase and NAD(P)H:quinone oxidoreductase 1 in the bladder, enzymes that are important protectants against oxidants and carcinogens [53].

Attention has also been paid to SFN triggered up-regulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), TRAIL-R1/DR4, TRAIL-R2/DR5, with subsequent blockade of PI3K/AKT and MEK/ERK pathways [54]. Of these targets, the Akt downstream target “mechanistic target of rapamycin” (mTOR) considerably contributes to cell growth control observed under SFN in prostate [55], kidney [46,56] and bladder cancer cells [49].

Transcriptome analysis has identified the transcription factors Sp1 and Sp3 as important mediators of the SFN effect on growth and apoptosis in prostate cancer cells. Suppression of Sp1 by SFN, and to a lesser extent of Sp3, decreased prostate cancer cell proliferation and increased the expression of the apoptosis related genes Bid, Smac/Diablo, and ICAD. Sp1/Sp3 is also involved in regulating survivin, p21 and cdk (e.g. cdk4 and cdk6). Cross-communication between Sp1/Sp3 and hTERT and NFkappaB has been documented as well [57]. Deactivating NFkappaB by SFN may also result in the reduced production of immune and inflammatory mediators, as has been demonstrated on murine monocytic and human embryonic kidney cells [58]. However, no further reports are available dealing with this issue. Therefore, whether suppression of the immune response by SFN may inhibit carcinogenesis and/or tumor progression cannot be satisfactorily answered [59]. It cannot be excluded that deactivating cytokines such as IL1β, IL-6, and TNFα may provoke a transition from inflammation to cancerous growth [23].

Advanced cancers are characterized by enhanced cell migration and mobility. It is, therefore, intriguing that SFN interferes with the tumor cell invasion cascade by down-regulating matrix metalloproteinases (MMP), particularly MMP-1, MMP-2, MMP-7, and MMP-9 [54]. Suppression of the focal adhesion kinase (FAK)/ERK and FAK/Akt signaling pathways has been suggested to precede the step initiating MMP

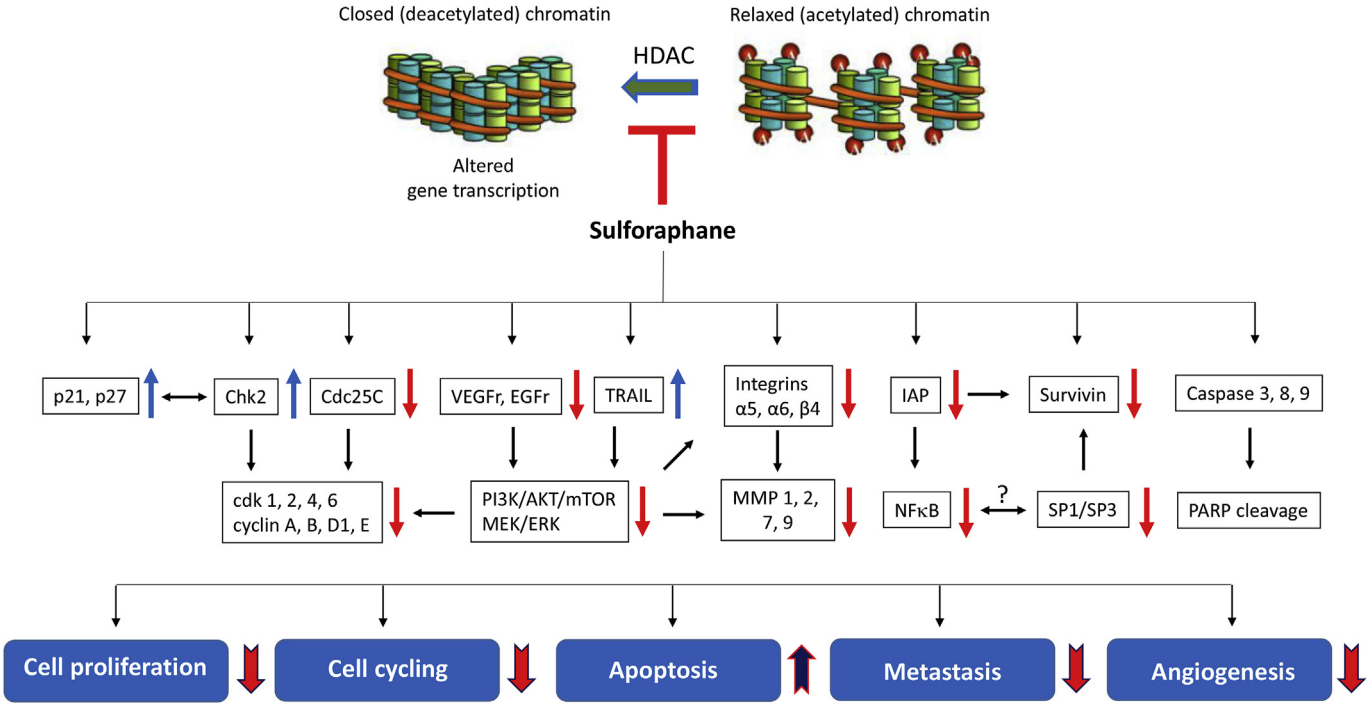

Fig. 2. Simplified depiction of the proposed mechanisms of SFN on tumor development and progression.

deactivation [60]. Aside from being relevant for tumor cell invasion and metastasis, MMPs serve as crucial mediators for angiogenesis. Bertl and colleagues reported that SFN reduced the angiogenic growth of endothelial cells due to the inhibition of MMP-9 in coordination with suppression of the key angiogenesis parameter, vascular endothelial growth factor (VEGF) [61]. Furthermore, other metastasis relevant molecules targeted by SFN have been identified, including the epidermal growth factor receptor (EGFR) [62], the C-X-C motif chemokine receptor 4 (CXCR4 receptor) [63,64], and the receptor CD44v6 [65], all of which are diminished in presence of SFN. Recent experiments on renal cancer cell lines have presented evidence of a close association between SFN-triggered suppression of the integrin subtypes α5, α6, and β4 and loss of chemotactic tumor cell movement [46].

In recent years, it has been acknowledged that microRNAs (miRNAs) are involved in the regulation of gene expression at the epigenetic level, thereby playing an important role in several steps of cancer cell development and progression. Among the epigenetic players, HDACs function as the key regulators of miRNA expression. Correlative studies on RCC patients treated with the HDAC inhibitor Vorinostat have shown that modulation of specific miRNAs is associated with clinical benefit [66]. Concordantly, epigenetic manipulation of miRNA-320a suppressed androgen receptor activity and growth of prostate cancer cells [67]. The influence of SFN on miRNAs in urologic tumor entities has not been evaluated yet. However, kinetic validation of HDAC inhibitors derived from plant material demonstrated a close correlation between post-transcriptional modification, up-regulating of miRNAs involved in tumor suppression and anti-mitotic effects in cervix carcinoma cells [68]. SFN itself down-regulated miRNA-21 in colon cancer cells, leading to hTERT deactivation and apoptosis induction [69]. SFN-induced repression of miRNAs, which function as tumor oncogenes, has also been observed. This includes the pro-apoptotic miRNA-15a-5p/16-1-5p cluster (evaluated in mantle cell lymphoma cells [70]) and miRNA-29 (related to breast ductal carcinoma cells [71]).

5. Bioavailability of SFN

Although SFN per se exerts distinct anti-cancer effects, its application is restricted because of low water solubility and poor oral bioavailability. Delivery systems such as emulsions, nanoemulsions, liposomes, biopolymer nanoparticles, and microgels might help to overcome these problems [72]. Various nano-particulate delivery systems have been used to increase the absorption of natural products, including a rotary-evaporated film-ultrasonication method, nanoemulsification with isopropyl myristate/glycerin, and liposomal incorporation. Natural compounds manufactured with nanoparticulate delivery systems have demonstrated improved bioavailability and optimization of its anti-inflammatory, anti-angiogenic, and anticancer effects [73].

PEGylated gold coated Fe_3O_4 magnetic nanoparticles (PEGylated Fe_3O_4@Au NPs) have recently been used to endorse SFN maintenance as an effective and promising anti-tumor treatment. SFN loaded PEGylated Fe_3O_4@Au NPs not only induced apoptosis and necrosis but also inhibited migration of human breast adenocarcinoma cells to a stronger extent than did free SFN [74]. Encapsulating SFN within monomethoxypoly (ethylene glycol)-poly (ε-caprolactone) (mPEG-PCL) resulted in SFN-loaded mPEG-PCL (SFN/mPEG-PCL) micelles that exhibit a sustained release of SFN from the micelles) and induction of apoptosis in a breast cancer cell model [75]. SFN-encapsulated microspheres for cancer epigenetic therapy have also been developed and exhibit efficacy in delivery with the potential to enhance the therapeutic effect of SFN in melanoma tumor-bearing mice [76].

Shi and coworkers have created tetrazole analogues by replacing the methyl group with heterocyclic moieties, or replacing the sulfoxide group with sulfide or sulfone [77]. All of these analogues were significantly more potent than the SFN mother compound in terms of caspase-3 activation and reduced growth of the ALDH + subpopulation of breast cancer stem cells.

6. Conclusions and open questions

There is no doubt that a diet rich in cruciferous vegetables may exert chemopreventive properties. Based on a variety of models and systems, SFN has been demonstrated to block tumor development and progression by suppressing relevant signaling networks (Fig. 2). Though SFN is meanwhile available as a purified compound, it has not

been approved for treating cancer.

The limited bioavailabilty remains a hurdle, necessitating further investigation. The development of genetically altered plants with significantly higher amounts of glucoraphanin might be a suitable strategy to overcome this problem. Optimization of the plant preparation is a further challenge. Nano-encapsulation and the synthesis of potent SFN analogues may also circumvent the low bioavailability of SFN. Identifying novel biomarkers of SFN might be helpful in allowing an accurate measurement of intake. Nevertheless, we should be aware that bioavailability and pharmacokinetics of SFN also depend on further factors such as myrosinase activity and gastrointestinal microbiota composition [78].

Studies have evaluated differences in the microbiome between high-risk and low-risk populations, or between healthy individuals and patients with latent or metastatic disease [79]. Although SFN targets multiple signal transduction pathways, the question arises whether this compound exerts different activities in healthy and diseased individuals and in patients suffering from early versus late stage cancer. Indeed, not all patients may respond similarly to SFN, particularly when the tumor is considerably advanced. Presumably, SFN's mode of action may (at least partially) depend on the cellular differentiation status. Normal, healthy cells could be protected from undesired epigenetic alterations, whereas pathologically altered cancer cells might be re-directed to their initial physiologic state or driven to apoptosis. Further studies, therefore, are necessary to identify how SFN targets healthy and pathological cells.

The optimum dosage of SFN has not been defined. Although several investigations point to a regimen with maximum tolerated SFN-doses, lower concentrations may work equally well or even be more effective in particular cases.

Presently, patient related data has been restricted to prostate and bladder cancer. Future studies should include further, genetically heterogeneous cancer types to explore whether the antitumor potential of SFN is generalizable or rather depends on the tumor entity. Clinical trials should also consider the potential of SFN as an anti-cancer compound integrated within existing treatment strategies.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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