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Conducting bioinformatics analysis to predict sulforaphane-triggered adverse outcome pathways in healthy human cells

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ABSTRACT

Sulforaphane (SFN) is a naturally occurring molecule present in plants from *Brassica* family. It becomes bioactive after hydrolytic reaction mediated by myrosinase or human gastrointestinal microbiota. Sulforaphane gained scientific popularity due to its antioxidant and anti-cancer properties. However, its toxicity profile and potential to cause adverse effects remain largely unidentified. Thus, this study aimed to generate SFN-triggered adverse outcome pathway (AOP) by looking at the relationship between SFN-chemical structure and its toxicity, as well as SFN-gene interactions. Quantitative structure-activity relationship (QSAR) analysis identified 2 toxophores (Derek Nexus software) that have the potential to cause chromosomal damage and skin sensitization in mammals or mutagenicity in bacteria. Data extracted from Comparative Toxicogenomics Database (CTD) linked SFN with previously proposed outcomes via gene interactions. The total of 11 and 146 genes connected SFN with chromosomal damage and skin diseases, respectively. However, network analysis (NetworkAnalyst tool) revealed that these genes function in wider networks containing 490 and 1986 nodes, respectively. The over-representation analysis (ExpressAnalyst tool) pointed out crucial biological pathways regulated by SFN-interfering genes. These pathways are uploaded to AOP-helpFinder tool which found the 2321 connections between 19 enriched pathways and SFN which were further considered as key events. Two major, interconnected AOPs were generated: first starting from disruption of biological pathways involved in cell cycle and cell proliferation leading to increased apoptosis, and the second one connecting activated immune system signaling pathways to inflammation and apoptosis. In both cases, chromosomal damage and/or skin diseases such as dermatitis or psoriasis appear as adverse outcomes.

1. Introduction

Sulforaphane (SFN) is a naturally occurring molecule, found in plants from *Brassica* family. It is present in the form of glucoraphanin, a precursor that becomes bioactive after hydrolytic reaction mediated by plant myrosinase or human gastro-intestinal microbiota [1]. It gained popularity in the scientific community at the end of the last century when extensive epidemiological cohort and case-control studies revealed an inverse association between cancer risk and consumption of cabbage, broccoli, cauliflower, and brussels sprouts [2]. Moreover, results from clinical studies showed that SFN has potential to improve clinical parameters such as blood glucose level and lipid profile as well as molecular parameters of oxidative stress [3]. Preliminary evidences also indicated that SFN could be used for treatment of mental disorders such as autism spectrum disorder, depression and schizophrenia [4]. As a small, lipophilic molecule, it has an absolute bioavailability higher than other phytochemicals and potential to cross blood-brain barrier [5]. Electrophilic isothiocyanate group of SFN provokes the reaction with nucleophilic cysteine residues in proteins and has been identified as the pharmacophore [6]. SFN is rapidly metabolized by

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glutathione-S-transferase to SFN-glutathione which then enters the pathway of consecutive enzymatic reactions leading to the step-by-step hydrolysis and formation of cysteinyl-glycine, cysteine, and N-acetylcysteine (NAC) conjugates, which are excreted in urine [7].

Sulforaphane is described as an antioxidant compound with the ability to inhibit cell proliferation, cause apoptosis and stop cell cycle. Here, the most studied underlying mechanisms include induction of NF-E2–related factor 2 (Nrf2) transcription factor and suppression of Kelch-like ECH-associated protein 1 (Keap1) [2,8]. For example, Keum et al. suggested that activation of Nrf2 and antioxidant response element (ARE) by SFN is critical for induction of cytoprotective heme oxygenase-1 (HO-1) in human hepatoma HepG2 cells [9]. Moreover, SFN inhibits cytochrome P450 enzymes and activates phase II enzymes, known direct and indirect antioxidants via Nrf2 transcription factor pathway, and consequently increase the tissue glutathione (GSH) levels [10,11]. Interestingly, in TRAMP C1 prostate cancer cells, SFN treatment increased the expression of Nrf2 and NAD(P)H quinone oxidoreductase 1 (NQO1) by inhibiting DNA methyltransferases and histone deacetyltransferases, leading to the cancer cell growth arrest [12]. Anti-cancer properties of SFN were also shown in human colon cancer cells, where it activated cdc2 kinase and induced G2/M arrest with a slight induction of p21 expression. As a result, growth of cancer cells was inhibited, and they entered the apoptotic process [13]. However, the most recent data indicated that SFN might stimulate hepatocarcinogenesis by interfering with the same transcriptional factor. Zheng et al. (2022) reported that, after 3 months of intraperitoneal injection of 25 mg/kg of SFN to male C57BL/6 mice, significant diethylnitrosamine-induced tumor growth and high number of hepatomas were seen. These parameters were in correlation with increased expression of Nrf2 and NQO1 indicating that once tumor is formed, SFN may promote cancer cell growth through activation of Nrf2 pathway [14]. Moreover, we previously demonstrated the importance of the colon cancer patient's genome signature in the light of the risk-to-benefit profile of SFN therapy. Conducted *in silico* investigation showed that SFN has potential to stimulate immune-suppressive processes and promote tumor aggressiveness of colon cancer cells [15]. Another toxicity study showed that injection of high doses of SFN (150–300 mg/kg) in mice produced sedation, hypothermia, impairment of motor coordination, decrease in skeletal muscle strength, as well as leucopenia [16]. However, to the best of our knowledge, its in-depth toxicity profile and potential to cause adverse effects are still unclarified.

Toxicity predictions have significantly advanced in recent years with the constant development and enhancement of *in silico* methods. Software like Derek Nexus, GenRA or HazardExpert are applying machine-learning algorithms to predict the toxicity of a given compound based on its toxic fragments [17]. The (quantitative) relationship between the structure and activity of molecules ((Q)SAR) analysis in toxicology has proven to be useful for predicting genetic toxicity endpoints of pharmaceutical impurities and assessing the mutagenicity of tobacco flavors [18] and non-cancerogenic chemicals from the National Toxicology Program database [19]. Moreover, Kianpour et al. (2021) showed that it was possible to use QSAR model together with molecular descriptors for prediction of oral acute toxicity of organophosphate compounds [20]. Thus, implementation of QSAR methods in toxicology is not only timesaving and cost-effective, but also able to direct further in vivo testing and reduce the use of experimental animals. The advantages and disadvantages of this method are explained in more details elsewhere [21–23]. In addition, due to the constant improvements of the use of computational sciences in toxicology it became possible to predict links between chemicals and adverse outcomes (AO) that can impact our health. Mechanistic studies and systems toxicology approach allow generation of pathways that lead to the predicted outcomes called adverse outcome pathways (AOPs) [24]. The concept of AOPs stands for structured linear connection between molecular initiating event (MIE) to an adverse outcome (AO) via different key events (KEs) through key event relationships (KERs) [25]. As KEs represent biological events that can be measured, causal relationship between them can be extracted from biological networks, making an AOP a subnetwork of the cascade biological events [26]. The development of online resources which collect the formerly detected relationships between chemical exposures and changes in gene expressions that are able to induce variations in biological parameters (e.g. contribute to pathogenesis of various diseases) can be used for data-mining, analysis and discussion of observed links [27]. Further application of these data has been proven useful in identification of mechanisms of toxicity. Moreover, AOP framework can help in guiding researchers toward key toxic events and assays, thus reducing the number of animal experiments [28]. This concept found great application in regulatory toxicity testing of chemicals but also in drug discovery and drug toxicity testing due to improved mechanistic understanding of human disease pathways that often cannot be investigated in animal models [29].

Therefore, this study aimed to generate SFN-triggered AOP by looking at the relationship between SFN-chemical structure and its toxicity, as well as SFN-gene interactions in healthy human cells. Moreover, the study objective was to predict KEs and KERs that can lead to the proposed AOs.

2. Methodology

2.1. Predicting toxic potential of sulforaphane

2.1.1. Structure-related toxicity prediction

Derek Nexus (Deductive Estimation of Risk from Existing Knowledge) is commercial software that can be used to qualitatively predict the toxicity of the test substance based on QSAR. These toxic effects prediction program uses rules based on hypotheses derived from the sources of published toxicological and chemical data. The Derek Nexus software database contains a range of information which link a particular chemical structure and toxic effect and thus covers a wide range of toxicological effects. The main advantage of this database lies in the areas of mutagenicity, carcinogenicity and skin sensitization [30,31].

The program has a graphical interface for input of the previously prepared or created chemical structures that will be analyzed. It is automatically checked whether the structure is chemically correct (e.g. that there is no incorrect valence of the compound). Based on the database, the system recognizes parts of the structure that can interact with biological systems and lead to toxic effects, called the warning structures or toxophores. Each identified toxophore is accompanied by the summary of the data, indicating the hazard that was identified, as well as the list of all the references stating the potential toxicity [19,30].

SMILES formula D, L-sulforaphane - CS (= O) CCCCN = C = S, taken from the PubChem website, was uploaded into the Derek Nexus software for *in silico* toxicity predictions [32].

2.1.2. Chemical read-across toxicity prediction

Next, the generalized read-across (GenRA) approach was used to check the prediction of SFN-induced toxicities/adverse outcomes. The GenRA uses information about how a chemical with known data behaves to make a prediction about the behavior of another chemical that is "similar" but does not have as much data available in the literature [33]. In other words, GenRA assists in identifying source analogues and making predictions of in vivo toxicity effects for a target substance. Moreover, it is implemented in the U.S. Environmental Protection Agency's (EPA) CompTox Chemicals Dashboard to provide public access to a GenRA module structured as a read-across workflow [34]. The similarity can be annotated as c (for chemistry/structural similarity) or b (for bioactivity) depending on the similarity context selected as calculated by the Jaccard index (ranges from 0 to 1; 0 indicating dissimilarity and 1 showing identical chemicals). This software automatically filters analogues by the availability of in vivo toxicity data. Analogues may be identified by using chemical or bioactivity descriptors. There are 3 different types of chemical descriptors included - Morgan fingerprints,

torsion fingerprints and chemotype ToxPrints. The predictions are binary outcomes of the presence or absence of toxicity with quantitative measures of uncertainty (AUC and p-value) [34]. For the purpose of this investigation analogues were chosen by ToxPrints chemotype similarity and filtered by ToxRef data.

2.2. Data-mining for chemical-gene/protein-disease linkage

Comparative Toxicogenomics Database (CTD; http://ctdbase.org/) was used for identification and extraction of the chemical-gene/protein-disease interactions between SFN and proposed toxic (adverse) outcomes. CTD is a publicly available resource with scientifically relevant information that enables integration of data towards the better understanding of the relationships between chemicals, genes/proteins, phenotypes, diseases, organisms, and exposure data [35,36]. The analysis reported here was based on the data downloaded in June 2022.

2.3. Protein-protein interaction network

Protein-protein interaction (PPI) network is a crucial step in toxicogenomic analysis as it helps in generating information about the interconnections between genes/proteins in the investigated set. Moreover, identification of the first-order interacting proteins enables in-depth understanding of protein complexes that function in groups [37]. NetworkAnalyst 3.0 (https://www.networkanalyst.ca/) is a web tool for network mapping and analyses of genes sets extracted from the CTD database. STRING interactome was set as the background data with the confidence cutoff score of 900 [38].

2.4. Over-representation analysis

Extracted gene sets from the generated networks were further analyzed with the REACTOME pathways-based database (https://reactome.org/) to identify as much as possible information about the enriched pathways/biological processes related to the gene/protein complexes linked to SFN. Captured pathways could be proposed as key events related to detected toxic/adverse outcomes.

Enrichment analysis was performed via the ExperssAnalyst tool (https://www.expressanalyst.ca/) that uses *ExpressAnalystR*, the underlying R package synchronized with ExpressAnalyst web server. The over-representation analysis (ORA) statistical method was applied aiming to determine in which biological pathways pre-defined in the REACTOME database, gene subsets from our data are present more than would be expected (over-represented). A significance level of 0.05 (p-value < 0.05) was used to select the most relevant associations. Recognized pathways were further used as potential key events related to SFN and detected adverse outcomes.

2.5. Prioritization of the molecular pathways

The AOP-helpFinder tool was used to rank the linkage between SFN and biological pathways detected in the ORA that can lead to proposed AOs. AOP-helpFinder (http://aop-helpfinder.u-paris-sciences.fr/index.php) is a web text-mining tool that uses graph theory to calculate the scores aiming to prioritize the findings about the stressor – biological event connection [24]. It assists in rapid evaluation of existing knowledge from PubMed database, currently screening more than 30 million available abstracts [39]. The text mining part is used to gather information about co-mentioned words (for example, SFN and a biological event) in an abstract from the scientific literature, while the graph theory allows systematization of the findings via calculated position score. This score determines the position of the co-occurred terms in an abstract [40]. In order to capture the links between SFN and enriched molecular pathways, as fully as possible, the existing information related to SFN synonyms and chemical terms were retrieved using the PubChem database (NIH) (Table S1).

Next, NaviGO web-tool (https://kiharalab.org/web/navigo/views/goset.php) was used to calculate the functional similarity and associations between detected biological pathways.

In this study, we applied relevance semantic similarity score (simRel) for computing functional similarity of a pair of GO terms/biological pathways, c1 and c2:

$$sim_{Rel}(c_1, c_2) = c \in \overset{\max}{S}(c_1, c_2)\left(\frac{2 \cdot \log p(c)}{\log p(c_1) + \log p(c_2)} \cdot (1 - (c))\right)$$

The first term considers the relative depth of the common ancestor c to the depth of the two terms c1 and c2, while the second term takes into account how rare it is to identify the common ancestor c by chance [41].

Finally, to decipher the type of interactions between SFN and detected pathways, CTD Chemical–Phenotype Interaction Query tool was used, which retrieves both increased and decreased linkages between the tested chemical and phenotype of interest.

The workflow of the present investigation is shown in the Fig. 1.

3. Results

3.1. Structure-related toxicity prediction

The chemical structure of D, L-sulforaphane was uploaded into the Derek Nexus software in the SMILES format (CS(=O)CCCCN=C=S) and 3 potential toxic effects were retrieved: chromosomal damage in vitro in mammal, mutagenicity in vitro in bacterium, and skin sensitization in mammal. Two toxophores (structural alerts) were recognized: isocyanate and isothiocyante that might be responsible for predicted toxic effects with plausible evidences, meaning that these findings are likely to be true and valid. Details about SFN chemical characteristics recognized by the software are shown in the supplementary material (Fig S1). To further investigate the structure-related toxicity, GenRA approach was applied, but no statistically significant data were found (Fig S2). Thus, in order to investigate how SFN could contribute to the chromosomal damage, mutagenicity or skin sensitization mechanistical analysis were performed.

These analyses directed further *in silico* investigation which aimed to describe the AOP network linked to SFN using a systems toxicology approach.

3.2. The relationship between SFN and toxic effects

For comprehensive mechanistic studies, available databases were screened aiming to collect as much as possible information about the molecular targets, biological pathways, and potential AO/toxic effects triggered by SFN at different levels of the biological organization. As MIE occurs at the molecular level and initiates the SFN interaction with the organism, CTD could be useful for detection of the first contact between SFN and human organism by exploring SFN-interacting genes.

Moreover, CTD database was used to identify the linkage between SFN and reported toxic effects on the gene/protein level. Batch Query tool extracted all curated and inferred associations between SFN and predicted toxic effects – adverse outcomes. Inferred (predicted) associations are based on the data from published literature which separately describes the connections between chemicals and genes and, on the other hand, genes and diseases. In this case, these interactions are based on the assumption that if SFN interacts with gene A, and gene A is associated with disease B, then SFN has an association with the disease B. The database retrieved 6 chromosome-related pathology processes affected by SFN, namely Chromosome 17 Deletion, Chromosome 1q21.1 Deletion Syndrome, 1.35-Mb, Chromosome 20q11-q12 Deletion Syndrome, Chromosomal Aberrations, Chromosomal Breakage, and Chromosomal Deletion, while no SFN-mutagenicity associations were found. Moreover, the link between SFN and skin diseases was obtained and 44 conditions were identified. Table 1 shows detailed list of SFN – toxic

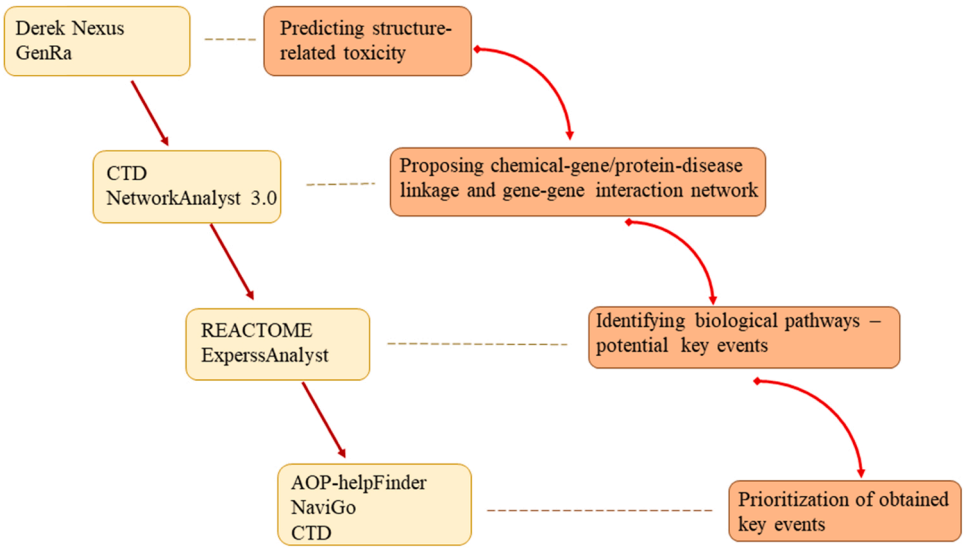

Fig. 1. Study workflow: Generating SFN-induced AOP. Step1: Identification of potential structure-related adverse outcomes; Step2: Data mining for chemical interfering genes/proteins and generation of first-order gene/protein networks; Step3: Identification of biological pathways – potential key events; Step 4: Prioritization of obtained key events and generation of adverse outcome pathway.

effects associations along with interfering genes and interference score.

As expected, the highest interference score was obtained for contact dermatitis (37.65), psoriasis (20.86), and chloracne (15.63), followed by skin neoplasms (15.11) and allergic contact dermatitis (14.85). To decipher how SFN could lead to toxic skin-related effects, reported interfering genes were downloaded and further analyzed. The total of 11 genes/proteins involved in the chromosome-related pathology processes and 146 genes/proteins reported as SFN – skin diseases interfering genes were extracted from CTD and further used in this *in silico* study (Table S2). Binary interactions observed between SFN and collected genes are presented in Table S3, along with the doses which provided these effects. As shown, SFN was able to increase and decrease gene expression in different cell types, including both normal and tumor cells. The effect of SFN on AKT1, C3, CXCL8, CYP1A1, GCLC, GSR, IL1B, KEAP1, NQO1, TNF, TNFAIP3, TXNRD1, and UGT1A1 genes/proteins was hormetic, or in other words, dose and time-dependent or cell type specific. Although some interactions were observed in mouse or rat cells, CTD database collects chemical-gene relations only if they are also relevant for humans. Statistically significant change in gene mRNA expression was usually seen after 24 h of cell exposure to SFN. However, only 6 h were enough to increase GCLC and NFE2L2 expression in human keratinocytes. On the contrary, melanoma cells were exposed to SFN for 48 h and 24 h before decreased AKT1 phosphorylation and TYR expression, respectively, were captured. Moreover, SFN-mediated dysregulation of genes listed in the Table S3 might trigger biological pathways that lead to proposed adverse effects in healthy human cells (chromosomal pathology processes or skin diseases).

3.3. Protein-protein interaction network

Comprehensive gene expression profiling and network analytics were simultaneously performed in NetworkAnalyst for group of genes/proteins linked to chromosomal pathology processes and skin diseases. For 11 and 146 previously detected genes/proteins, first-order interacting genes/proteins or, in other words, all genes/proteins directly connected to the uploaded set of genes, were pinpointed. Identification of the first-order protein partners is an important step for better understanding of complex human protein interactome [42] and further explains the proposed MIE defined as SFN-gene interaction. Thus, 11 unique (seed) genes/proteins connected to chromosomal pathology processes generated a network of 490 proteins mutually connected with 612 edges while 146 unique (seed) skin disease-related genes/proteins retrieved a network with 1986 proteins and 3625 edges (connections) (Figs. 2 and 3). Genes/proteins detected in created networks are then extracted for further investigation.

3.4. Over-representation analysis and pathways prioritization

To propose potential key events that can lead to the predicted adverse outcomes, ORA was performed with previously downloaded sets of genes/proteins. For both groups, 120 molecular pathways from REACTOME database were retrieved (p-value < 0.05). From detected pathways, 72 were common for chromosomal-related and skin diseases, among which „disease“ and „immune-system“ were identified. These 2 pathways were excluded from further analyses due to their non-specificity. NaviGO tool was used to calculate the sematic similarity between the detected pathways. The distance between the nodes (biological pathways) is based on the functional similarity of GO terms in clusters. Fig. 4 shows that shared pathways cluster in 2 groups: immune system [26] and cell cycle regulation [10], while others [34] do not show significant similarity to each other (Fig. 4A). Additionally, in each group (SFN-chromosomal pathology processes and SFN-skin diseases) ORA recognized 48 unique pathways were detected. Similarly, pathways related to chromosomal pathology processes grouped into 3 clusters, one related to DNA damage repair [30] and 2 other related to cell maturation [11] and division [7] (Fig. 4B), while those linked to skin diseases did not show significant functional similarity between each other (Fig. 4C).

To prioritize biological pathways triggered by SFN and related to chromosomal pathological processes and skin diseases AOP-helpFinder was used. The total of 2321 connections between 19 enriched pathways and SFN identified in the available literature are listed in the Table 2. Detected pathways/potential key events were then manually clustered based on the biological processes they are involved in (data retrieved from REACTOME). Moreover, no connection between SFN and unique pathways was found in the mined scientific publications.

According to the CTD Chemical–Phenotype Interaction Query tool, SFN inhibits cell division by stimulating the negative regulation of mitotic phase of the cell cycle and decreasing cell population proliferation. Moreover, SFN stimulates the apoptotic process. Finally, curated interactions from CTD revealed that SFN can stimulate inflammatory response in both *Homo sapiens* and *Mus musculus* and positively regulate signal transduction in human cell lines.

Table 1
Associations between SFN and suggested toxic effects along with the interfering genes and interference score (CTD Batch Query Tool).

Disease name	Disease category	Interfering genes	Interference score
Chromosomal Aberrations	Pathology process	CDK6, GSTM1, GSTP1, GSTT1	2.04
Chromosomal Breakage	Pathology process	CAT, GSTM1, MYC, NFE2L2	2.10
Chromosomal Deletion	Pathology process	CDKN2A	3.88
Chromosome 1q21.1 Deletion Syndrome, 1.35-Mb	Pathology process	GJA5	4.51
Chromosome 17 Deletion	Pathology process	TP53	3.08
Chromosome 20q11-q12 Deletion Syndrome	Pathology process	EPB41L1	4.75
Acanthosis Nigricans	Skin disease	AHR	3.43
Alopecia	Skin disease	AHR, ABCC2, AR, HR, PARP1, TNFRSF10A	4.48
Arthritis, Psoriatic	Skin disease	BMP4, CD68, CXCL8, HLA-C, IL12B, NOS2, TNF	13.1
Atrichia with Papular Lesions	Skin disease	HR	4.81
Blister	Skin disease	ADAM17	3.32
Chloracne	Skin disease	BTG2, CDK6, CYCS, GADD45A, GSTM1, GSTM3, ITGB2, KRT17, TGM1	15.63
Crouzon Syndrome with Acanthosis Nigricans	Skin disease	FGFR3	4.49
Dermatitis	Skin disease	HLA-B, ITGB2, PARP1, VCAM1	2.69
Dermatitis, Irritant	Skin disease	HLA-DPA1, HLA-DPB1	3.56
Dermatitis, Occupational	Skin disease	ALDH2, BDKRB2, HLA-DMA, KNG1	6.42
Dermatitis, Atopic	Skin disease	AHR, CCDC80, CCL11, CCL5, CYP1A1, IFNG, IL1B, IL33, IL6, MAPK8, S100A8	8.48
Dermatitis, Allergic Contact	Skin disease	BCL2, CASP8, CCR2, CYP1A1, ETS2, F13A1, FGL2, HSD11B1, IFI30, IFNG, IL2, IL32, IPCEF1, ITGAM, MEOX1, MXD1, NFE2L2, PLAT, QPCT, SAT1, SLC2A3, SPP1, TNF, UPP1	14.85
Dermatitis, Contact	Skin disease	AHR, AKR1B10, AKR1C2, BCL2, CCN2, CYP1A1, CYP1B1, DDIT3, FABP4, FTH1, G6PD, GCLC, GSR, GSTP1, HLA-DMA, HMOX1, HSP90AA1, KRT4, NQO1, NQO2, NRG1, PIR, S100A8, SAA1, SLC7A11, SOD1, TLR4, TXN, TXNRD1, UGT1A1	37.65
Dermatomyositis	Skin disease	HLA-B, IL1B, TNF	3.89
Drug Eruptions	Skin disease	CYP1A1, CYP2E1, HLA-B, HLA-DPB1, IFNG, IL2, KNG1, RELA, TNF	2.49
Drug Hypersensitivity Syndrome	Skin disease	LRG1, ORM1	4.34
Eczema	Skin disease	CD14	2.98
Exanthema	Skin disease	HLA-B, MVK	4.39
Hair Diseases	Skin disease	EIF2AK4	3.89
Hyperpigmentation	Skin disease	AHR	2.41
Hypotrichosis simplex	Skin disease	APCDD1	4.51
Keratoacanthoma familial	Skin disease	TGFBR1	4.44
Keratosis	Skin disease	AHR, CAT, KEAP1, NFE2L2, TP53	9.32
Lichenoid Eruptions	Skin disease	CXCL8, IL6	4.89
Marie Unna congenital hypotrichosis	Skin disease	HR	4.84
Melanoma, Cutaneous Malignant	Skin disease	BAP1, CDKN2A, TERT	5.62
Melanosis	Skin disease	ALDH2	3.13
Nail Diseases	Skin disease	LAMA3	3.54
Nephrogenic Fibrosing Dermopathy	Skin disease	ACTA2, COL1A1	5.0
Pemphigoid, Benign Mucous Membrane	Skin disease	PTGER3	4.0
Pemphigoid, Bullous	Skin disease	CXCL8	2.49
Pemphigus	Skin disease	C3	3.16
Pruritus	Skin disease	AHR	3.96
Psoriasis	Skin disease	CAT, CSF2, HLA-C, IFIH1, IL12B, IL1B, IL6, NFKBIA, NOS2, PCNA, PPARG, PTTG1, REL, RIGI, SOD2, STAT3, TNF, TNFAIP3, TP53	20.86
Pyogenic arthritis, pyoderma gangrenosum, and acne	Skin disease	IL1B	3.10
Scleroderma	Skin disease	ACTA2, CCN2, CNR2, HDAC5, RHOB, S1PR5, SIRT1, TGFBR1, TNF	8.24
Skin Abnormalities	Skin disease	SOD2	2.41
Skin Fragility-Woolly Hair Syndrome	Skin disease	DSP	4.67
Skin Neoplasms	Skin disease	AKT1, AQP3, CASP8, EPHX1, GSTT1, HIF1A, KRT17, NFE2L2, NOTCH1, NOTCH3, NOTCH4, NPPA, PTGS2, PTK2B, RELA, SOD2, TGFB1, TGFBR1, TP53, TRP53, TYR	15.11
Skin Ulcer	Skin disease	ITGB2, LAMA3, NGF	4.14
Steatocystoma Multiplex	Skin disease	KRT17	4.64
Stevens-Johnson Syndrome	Skin disease	ALB, CFP, EP300, HLA-B, HLA-C, IFNG, LRG1, NOS2, ORM1, PARP1, PTGER3, RB1, VCP	3.70
Urticaria	Skin disease	ALB, GSTM1, HLA-DPB1, ICAM1, IL1B, TGFB1, TNF, VCAM1	2.32
Vitiligo	Skin disease	CASP7, IFIH1, NFE2L2, TNF	5.63

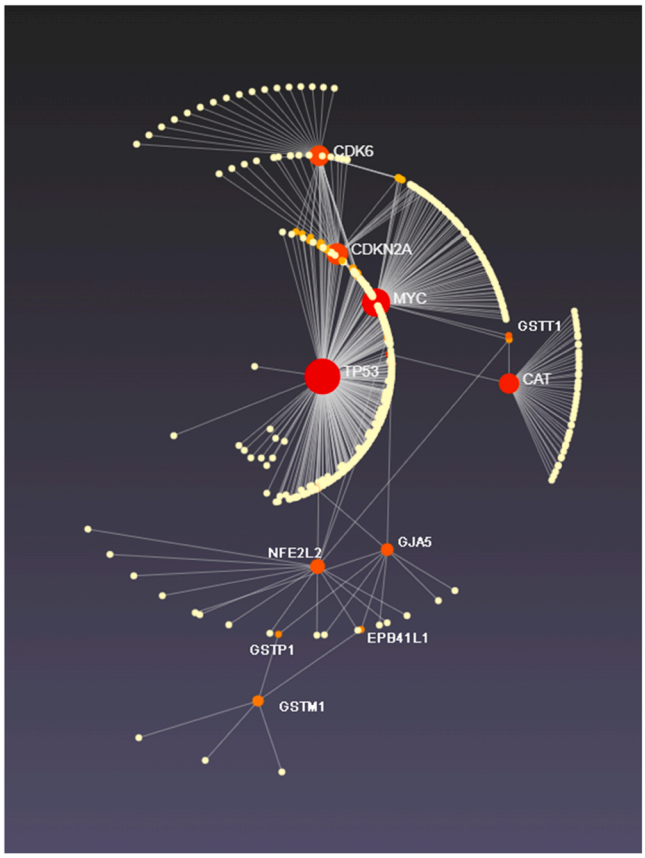

Fig. 2. The first-order gene network for SFN-chromosomal pathology processes related genes where red and orange nodes represent seed genes. Generated with NetworkAnalyst; STRING confidence score 900.

Finally, results obtained in this *in silico* investigation and expert opinion developed based on the data available in the literature and AOP-Wiki database (https://aopwiki.org/) facilitated the generation of SFN-triggered AOP shown in Fig. 5. Increased apoptosis, cell cycle disruption, decreased cell proliferation, and immune system inflammation were defined in AOP-Wiki database as KEs with IDs: KE:1365, KE:1505, KE:1812, and KE:1225, respectively.

4. Discussion

The QSAR analysis with Derek Nexus software, detected 2 toxophores of SFN, isocyanate and isothiocyante that could potentially induce chromosomal damage or skin sensitization. To further decipher and understand the link between SFN and predicted AOs, additional *in silico* tests/mechanistic studies were performed.

4.1. Sulforaphane predicted adverse outcomes

The CTD database found a connection between SFN and 6 chromosome related pathology processes through 11 genes/proteins that function in the PPI network of 490 proteins. In other words, even though SFN interacted with only 11 genes, network analysis showed that it could indirectly affect large number of genes/proteins involved in the regulation of biological processes. According to ORA, these genes/proteins controlled 120 molecular pathways related to cell cycle, immune system and signal transduction. Similarly, toxicogenomics data extracted from the CTD pointed out direct interactions between SFN and 146 genes/proteins leading to 44 skin diseases. This set of genes generated a PPI network of 1986 proteins mutually connected with 3625 edges. Interestingly, conducted ORA resulted in 120 molecular pathways among which 72 were common to pathways related to SFN-chromosomal pathology processes. Finally, by screening previously published data, AOP-helpFinder was able to capture the link between SFN and only 19 out of 120 molecular pathways (Table 2). The highest number of connections was extracted for SFN and apoptosis (1581), followed by the cell cycle (487) and regulation of mitotic cell cycle (110). Each of the detected pathway might represent KE in the network of SFN-induced AOs. However, in order to answer the question about KER between them and better understand the possible SFN-triggered AOPs, data available in the literature and AOP-Wiki database was analyzed by our experts. AOPwiki is a primary repository for all the generated AOPs developed by the Organization for Economic Co-operation and Development (OECD) as one of the tools from the AOP Knowledge Base (AOP-KB) [43].

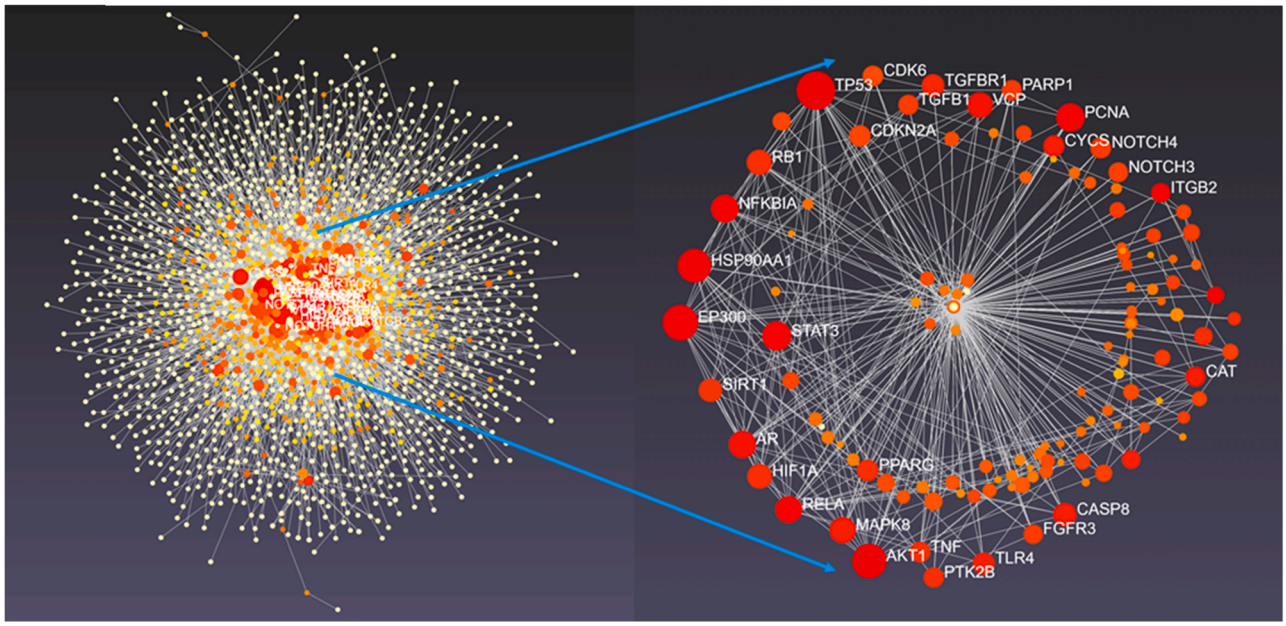

Fig. 3. The first-order gene network for SFN-skin diseases related genes with pin-pointed seed genes. Generated with NetworkAnalyst; STRING confidence score 900.

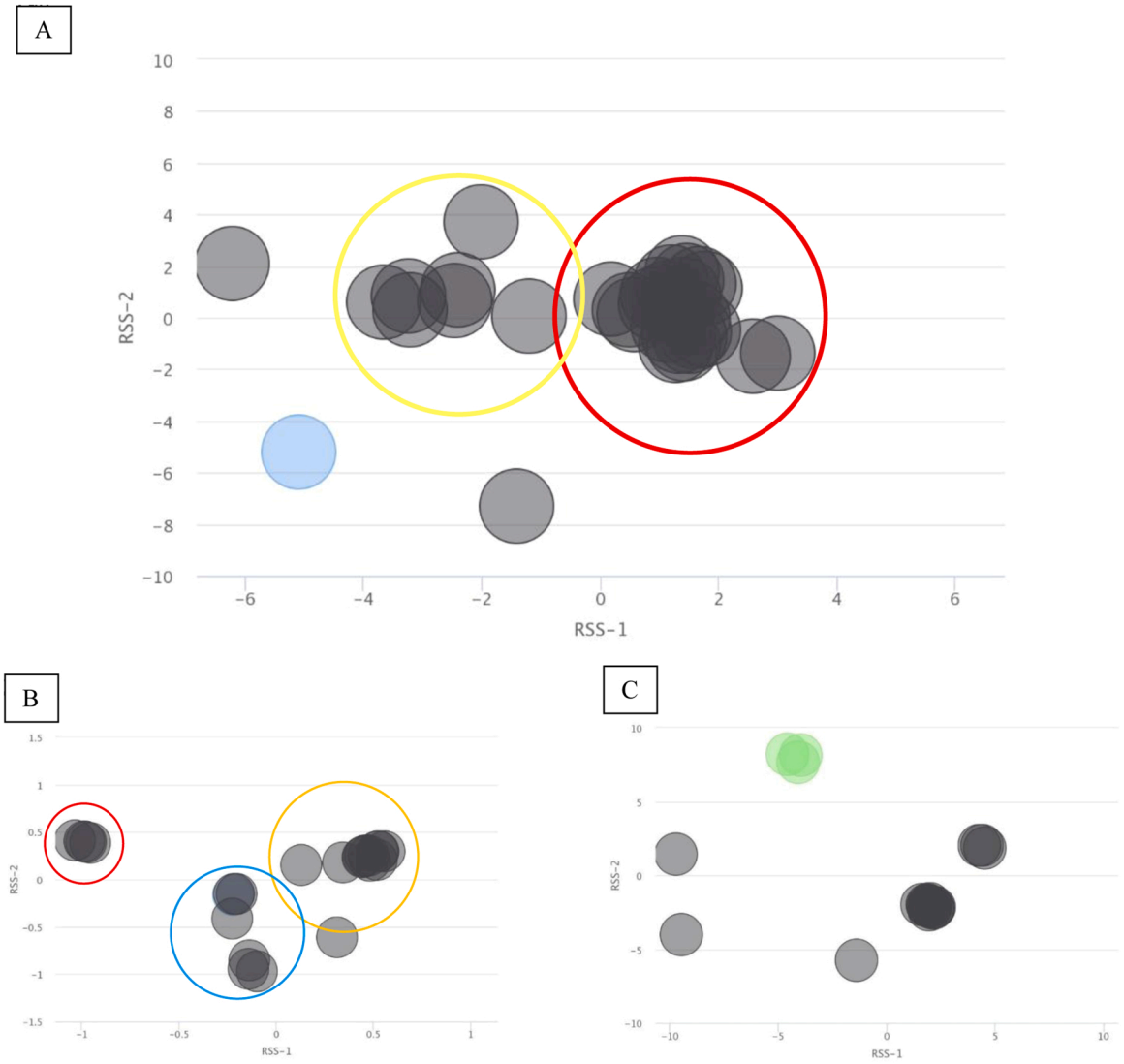

Fig. 4. (A) Clustering biological pathways common to chromosomal pathology processes and skin-diseases based on relevance semantic similarity score (RSS): red circle – biological pathways related to immune system; yellow - biological pathways related to cell cycle. (B) Clustering biological pathways related to chromosomal pathology processes: red circle – biological pathways related to mitotic phase of cell division; blue circle – biological pathways related to cell maturation; orange circle – biological pathways related to DNA damage repair. (C) Clustering biological pathways related to skin-diseases. Two-dimensional graph was made in NaviGo web-tool.

4.2. Sulforaphane – genes interactions

Supplementary Table 3 shows the potential of SFN to induce or inhibit expression of genes linked to chromosomal pathology processes or skin diseases in different cell types, including normal and tumor cells. Although, only 4 interactions (reduced AKT1 phosphorylation, increased GCLC and NFE2L2 expression, and decreased TYR expression) were detected in skin cells, this type of analysis allowed the prediction of more human-relevant chemical-gene interactions based on the available data. For example, it was noted than SFN increases the expression of CD14, which is known marker of atopic dermatitis [44]. Moreover, CD14 expression correlates with the progression of skin diseases such as skin fibrosis in patients with early diffuse cutaneous systemic sclerosis [45]. Similarly, SFN was identified as an inducer of CASP8 gene, a caspase which increased activation in vitiligo and psoriatic skin cells leads to pyroptosis [46], a newly discovered type of a highly inflammatory form of lytic programmed cell death [47]. Aberrant NOTCH signaling has been linked to the development of psoriasis and skin cancers [48]. Oto et al. (2014) explained that NOTCH molecules localized in epidermis of normal skin direct the expression of proliferating basal cells synthesize keratin 14 (K14) and suprabasal cells express keratin 10 (K10), proteins whose irregular expression and synthesis leads to anomalies of epidermis in psoriatic skin [49]. Knowing that SFN modifies NOTCH3 and NOTCH4 signaling might suggest that SFN should be used with caution in patients with psoriasis. Moreover, it was reported that IFN-γ induces regenerative epidermal phenotype of psoriasis through IL-1 activation [50]. As seen in the Table 2, SFN could reduce IFN-γ protein activity inhibiting previously mentioned psoriasis regenerative mechanism. Another example is SFN-HIF1A inhibitory interaction. The loss of HIF1A and HIF2A in mice model causes dry flaky skin, impaired permeability barrier, and enhanced sensitivity to cutaneous allergens via suppression of filaggrin production in primary keratinocytes [51].

4.3. Grouping molecular pathways into clusters

Detected molecular pathways were grouped into 4 clusters: Apoptosis, Cell Cycle, Cell proliferation, and Signal Transduction, while CTD Chemical–Phenotype Interaction Query tool was used for identification of types of interactions between SFN and defined clusters.

Table 2
Prioritized pathways related to SFN, chromosomal pathological processes and skin diseases detected with AOP-helpFinder tool.

Pathway	Connections	Cluster
Apoptosis	1581	Apoptosis
Intrinsic Pathway for Apoptosis	4	Apoptosis
APC/C-mediated degradation of cell cycle proteins	3	Cell Cycle
Cell Cycle	487	Cell Cycle
Cell Cycle Checkpoints	14	Cell Cycle
G1/S DNA Damage Checkpoints	15	Cell Cycle
G1/S Transition	1	Cell Cycle
Regulation of mitotic cell cycle	110	Cell Cycle
S Phase	32	Cell Cycle
Downstream signaling of activated FGFR	3	Cell proliferation
PI3K/AKT activation	12	Cell proliferation
PIP3 activates AKT signaling	21	Cell proliferation
Signaling by NOTCH	18	Cell proliferation
Signaling by EGFR	3	Cell proliferation
Innate Immune System	4	Immune System inflammation
Signaling by Interleukins	1	Immune System inflammation
Constitutive PI3K/AKT Signaling in Cancer	9	Signal Transduction
PI3K/AKT Signaling in Cancer	1	Signal Transduction
Signaling by NGF	2	Signal Transduction

4.3.1. *Sulforaphane – cell cycle – cell proliferation – apoptosis linkage*

Positive apoptotic anti-cancer potential of SFN and its ability to cause cell cycle disruption are documented in pancreatic, prostate, breast, lung, cervical, and colorectal cancers [52]. The underlying mechanisms are well-developed and includes activation of caspase 9 [53] and caspases 3/7 [54]. Caspase 9 is one of the initiator caspases that activates caspase 3, the most important of the executioner caspases in the intrinsic apoptotic pathways [55]. Moreover, in combination with carboplatin, SFN was able to arrest cell cycle of cancer cells in the G0/G1 phase and consecutively upregulated the expression of Bax, cytochrome C, apoptosis-inducing factor, caspase-9 and -3, and cleaved poly ADP ribose polymerase. As a result, SFN-carboplatin mixture promoted apoptosis and inhibited proliferation of lung cancer cells [56]. Moreover, when lung cancer cells were treated with 30 μM of SFN, an increased G2/M phase population, along with a decreased polyploid fraction of cells were seen, which suggested a functional G2/M arrest. However, in this case, the major mode of cell death was necrosis, while the percentage of apoptotic cells were lower [57]. Apoptotic and anti-proliferative effect of SFN was also seen in colorectal cancer cell lines where combinational treatment of SFN and salinomycin inhibited the PI3K/Akt pathway and increased the Bax/Bcl-2 ratio [58]. Another proposed mechanism of SFN-cell proliferation suppression is the inhibition of IL-6/ΔNp63α/Notch axis [59] or PI3K/Akt pathway [60]. Consistently, the CTD revealed that SFN could increase apoptosis in human cell lines. Notably, increased apoptosis is defined in the AOPwiki database as KE:1365, while disrupted cell cycle can be found under KE:1505. Moreover, there is a moderate level of evidence that disrupted cell cycle leads to apoptosis. In addition, decreased cell proliferation is defined as KE:1821 which leads to growth decrease (KE:1521) with moderate level of evidence [61].

Furthermore, even though the impact of SFN on cell growth, proliferation and death was already described, the association between these KE and chromosomal pathology processes and/or skin diseases required further explanations. For example, contact dermatitis represent a polymorphic inflammation of the skin which lead to keratinocyte apoptosis when it becomes chronic condition [62]. Moreover, in case of allergic contact dermatitis, the over-activation of immune system in the dermis results in enhanced secretion of proinflammatory cytokines and chemokines which ultimately activate cytotoxic effector T cells that induce keratinocytes apoptosis [63]. Thus, it might be suggested that SFN has potential to augment the acute reaction of skin barrier to the irritants/allergens and speed up the transition from acute to chronic phase. Another skin disease associated with increased keratinocytes apoptosis is the histologic manifestation of sunburned skin. Proposed mechanisms involve the direct activation of death receptor by cytokines or direct contact with intraepidermal lymphocytes, via TNF and Fas receptors, respectively [64]. In addition, sun lights can induce direct DNA-damage and upregulate the expression of p53 protein, which activates Bax/Bak complex, subsequently leading to apoptosis by stimulating the activation of apoptotic induction of protease-activating factor (Apaf-1), caspase-9, and caspase-3 [65]. In other words, SFN might contribute to photosensitivity by inducing programed skin cells death. However, there are conflicting results in the published literature. For example, Wu et al. (2019) detected therapeutic effect of SFN in a murine model of atopic dermatitis through the activation of the Nrf2/HO-1 axis and suppression of Janus kinase 1/STAT3 signaling pathway [66]. Similarly, when human HaCaT keratinocytes were treated with SFN and phenylethyl isothiocyanate increased expression of Nrf2-dependent genes, γGCS, HO-1, NQO1, was seen 6 h after 5 μM of SFN and 10 μM of phenylethyl isothiocyanate were added to the cell medium. Subsequent activation of antioxidant pathways could protect human skin against UVR-induced skin apoptosis [67]. In the same experimental settings, SFN alone was able to induce Nrf2 transcription factor, phase-2 and antioxidant enzymes [68]. Notably, Arcidiacono et al. demonstrated that in higher doses (5 μg/mL which is equivalent to 28 μM), SFN has the ability to induce apoptosis of human epidermal melanocytes [69], indicating

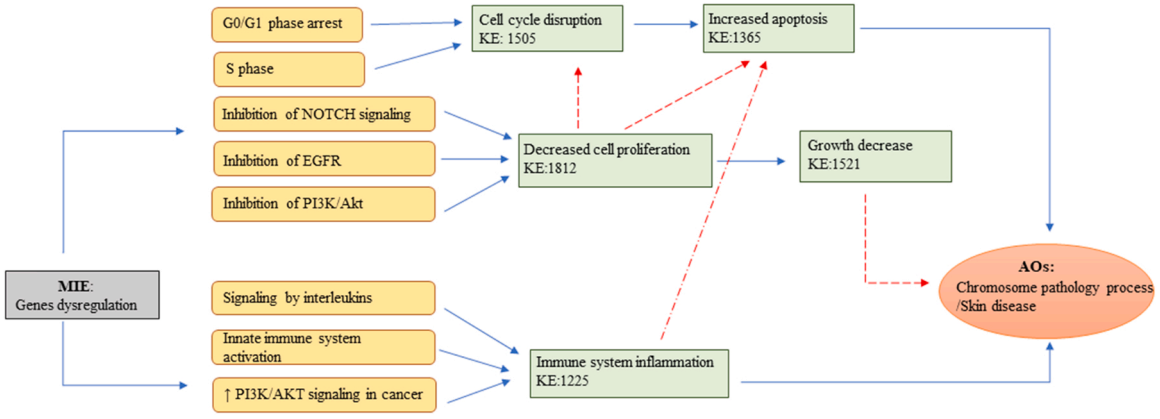

Fig. 5. Proposed SFN-induced adverse outcome pathway leading from genes dysregulation (MIE) to chromosomal pathology process and/or skin disease (AOs) through cell cycle disruption (KE1), decreased cell proliferation (KE2), increased apoptosis (KE3), or immune system inflammation (KE4). Blue lines represent evidence-based KER, red lines are predicted KER.

dose-dependent beneficial effects of SFN on human skin. Moreover, 8 μM of SFN induced CASP8 gene expression in human cancer cells after 24 h of exposure [70], suggesting that apoptotic characteristics of SFN could also be time-dependent. Sulforaphane-mediated activation of Nrf2-pathways has also been proposed as a mechanism which ameliorates skin aging in male mice [71], as well as in normal human keratinocytes when treated with the combination of patented extract Fernblock® XP, obtained from *Polypodium leucotomos* and SFN [72]. Thus, SFN dose and treatment duration should be carefully selected to ensure the best risk-to-benefit ratio.

Acquired chromosomal abnormalities affect a single line of cells with restricted distribution and might be involved in different pathogenesis, including cancer [73]. The basis of both direct and indirect DNA damage is the activation of molecular pathways that lead to growth arrest and apoptosis. If cells fail to activate repair mechanisms, the high expression of chromosomal instability occurs [74]. Therefore, it might be hypothesis that chromosomal pathology processes are in tight connection with SFN-induced skin diseases. The SFN-mediated DNA damage and apoptosis induction could precede the chromosomal instability in keratinocytes and thus, skin diseases.

4.3.2. Sulforaphane – signal transduction – immune system, inflammation linkage

Sulforaphane is recognized as immunomodulator, and thus, it is not surprising that Chemical–Phenotype Interaction Query CTD tool predicted SFN-mediated stimulation of immune system potentially leading to inflammation. Additionally, the immune system inflammation was recognized by AOPwiki as KE:1225.

The effects of SFN on immune cells have been extensively studied. The induction of Nrf2 and inhibition of NFkβ leads to activation of HO-1, glutathione and phase II enzymes secretion, while phase I enzymes and histone deacetylase remain inhibited. Consequently, SFN exerts chemo preventive properties by maintaining redox balance, cellular homeostasis and stimulating immune response [75]. Shen et al. (2021) reported that SFN improves the cytotoxicity of CAR-T cells by modulating the PD-1/PD-L1 pathway and stimulating the secretion of proinflammatory cytokines [76]. Moreover, some data indicate that SFN effectively inhibited the spread of metastatic tumor cells through stimulation of cell-mediated immune response by upregulation of IL-2 and IFN-gamma, and downregulation of proinflammatory cytokines IL-1beta, IL-6, TNF-alpha, and GM-CSF [77]. Interestingly, Lee et al. (2012) showed that SFN-stimulated generation of reactive oxygen species and activation of PI3K/Akt signaling regulate cell survival in human mesothelioma cells [78].

The dysregulation of the crosstalk between immune and skin cells contributes to the pathogenesis of inflammatory skin diseases such as psoriasis. Moreover, it was suggested that keratinocyte necroptosis plays a critical role in triggering skin inflammation [79]. Another well-known immune-mediated skin condition is psoriasis, where Th17/IL-17 axis has been identified as a key factor. The main sources of IL-17 are $CD4^+$ T helper cells, named Th17, which initiate the binding of IL-17 to its receptor in keratinocytes, leading to their proliferation and release of inflammatory mediators and chemokines [80,81]. Similarly, IL-17 and IL-22 are reported to contribute to skin barrier dysfunction and the development of atopic dermatitis, another common inflammatory skin disease [82]. Thus, SFN-immune response interaction could alter the homeostasis between immunity and skin and contribute to the development of inflammatory skin diseases.

5. Conclusion

The safety profile of SFN and its potential to cause AOs has not been extensively studied. Moreover, our recent work suggested that SFN should be carefully applied in cancer patients, in whom expression of TIMP1, CCL20, SPP1, AURKA, CEP55, NEK2, SOX9 and CDK1 was found increased, and expression of CRYAB, PLCE1, MMP28, BMP2 and PLAC8 was found decreased [15]. Thus, this investigation aimed to further elucidate the potential of SFN to cause AOs and untie the underlying mechanisms by generating an AOP via combining bioinformatics analysis and systems toxicology approach. The QSAR analysis revealed that SFN contains 2 toxophores that could induce chromosomal damage and/or skin sensitization. However, available literature contains conflicting data about the impact of SFN on skin cells, suggesting that its beneficial effects are dose and time-dependent. Furthermore, data-mining for SFN-gene interactions and subsequential network analysis showed that SFN might stimulate or inhibit 490 and 1986 genes/proteins involved in pathogenesis of chromosomal damage or skin diseases, respectively. Interactions on the gene level are defined as MIE, which leads to a series of KEs, mutually linked via KERs, ultimately forming AOP. Thus, it might be hypothesized that SFN stimulates apoptosis-related genes such as CASP7 and CASP8 leading to the disruption of biological pathways involved in the cell cycle and increased apoptosis, as well as triggers inflammatory genes (CXCL8, TNF) connecting activated immune system signaling pathways to inflammation and apoptosis. Finally, chromosomal pathology processes and/or skin diseases such as dermatitis or psoriasis appear as potential SFN-induced AOs. Proposed framework could be used for predicting adverse/side effects of any chemical with limited toxicology data and further direct toxicology research.

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CRediT authorship contribution statement

Dragica Bozic: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Software, Writing – original draft, Visualization. **Katarina Živančević:** Methodology, Supervision. **Katarina Baralić:** Methodology, Supervision. Supervision. **Evica Antonijević Miljaković:** Supervision. **Aleksandra Buha Đorđević:Marijana Ćurčić:** Writing – review & editing. **Zorica Bulat:** Writing – review & editing. **Biljana Antonijević:** Writing – review & editing. **Danijela Đukić-Ćosić:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition, Project administration.

Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

No data was used for the research described in the article.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114316.

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