

## **Chemical aspects of biological activity of isothiocyanates and indoles, the products of glucosinolate decomposition**

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### ABSTRACT

There is growing evidence that cancer chemoprevention employing natural, bioactive compounds may halt or at least slow down the different stages of carcinogenesis. A particularly advantageous effect is attributed to derivatives of sulfur-organic phytochemicals, such as glucosinolates (GLs) synthesized mainly in *Brassicaceae* plant family. GLs are hydrolysed enzymatically to bioactive isothiocyanates (ITC) and indoles, which exhibit strong anti-inflammatory and anti-carcinogenic activity. Highly bioavailable electrophilic ITC are of particular interest, as they can react with nucleophilic groups of important biomolecules to form dithiocarbamates, thiocarbamates and thioureas. These modifications seem responsible for chemopreventive activity, but also for genotoxicity and mutagenicity. It was documented that ITC can permanently bind to important biomolecules such as glutathione, cytoskeleton proteins, transcription factors NF- $\kappa$ B and Nrf2, thiol-disulfide oxidoreductases, proteasome proteins or heat shock proteins. Furthermore, ITC may also affect epigenetic regulation of gene expression, e.g. by inhibition of histone deacetylases. Some other derivatives of glucosinolates, especially indoles, are able to form covalent bonds with nucleobases in DNA, which may result in

genotoxicity and mutagenicity. This article summarizes the current state of knowledge about glucosinolates and their degradation products in terms of possible interactions with reactive groups of cellular molecules.

**Keywords:** glucosinolates, isothiocyanates, indoles, chemoprevention

## 1. Introduction

According to World Health Organization (WHO), cancer and cardiovascular diseases are major reasons of premature deaths in most developed countries, by far outpacing classical lethal diseases, such as infections. The GLOBOCAN statistics [1], for instance, shows that in 2012 alone, 14 million of incidences of different types of cancer were recorded worldwide, of which 8.2 million were deadly; up from 7.8 million of deaths in the year 2008. The WHO predicts that the number of incidences of cancer will be growing globally, reaching the level of 22 million cases by 2022. In 2016, over 1.5 million of new cases were diagnosed in the United States alone; in other words about 4,500 such diagnoses per day. This growing health problem, especially in ageing societies, calls for innovative, efficient strategies of treatment and also, even more so, prophylaxis. Indeed, prevention has numerous advantages, not only for patients, their health and quality-of-life, but also for the society as a whole, ranging from social and economical to cultural and even environmental benefits (*e.g.* less contamination of water with drugs and drug metabolites). One of such strategies of prophylaxis, which is still under-appreciated and consequently underdeveloped, is chemoprevention. This approach takes advantage of chemical compounds, either natural or synthetic, which prevent or inhibit carcinogenic processes in humans [2, 3, 4]. Research available to date suggests that chemopreventive approach easily accessible to anybody involves the frequent consumption of fruits and vegetables in conjunction with physical activity. It has been estimated that such a persistent conduct could diminish the risk of cancer by up to 20% [5, 6]. In particular, the consumption of vegetables belonging to the *Brassicaceae* family, which includes cabbage, cauliflower, broccoli, Brussels sprouts, wasabi, etc., has been suggested by epidemiological studies to decrease significantly the risk of most common types of cancer, such as lung [7,8], stomach [8], breast [9], and prostate cancer [10]. These



anti-carcinogenic properties of Brassica vegetables are ascribed to the characteristic for this genus secondary metabolites – glucosinolates (GLs) [11, 12].

## 2. Biosynthesis of GLs

GLs are  $\beta$ -D-thioglucosides in which glucose is linked *via* sulfur atom with the carbon of sulfonated oxime. These compounds occur in different amounts in roots, leaves, sprouts, as well as in seeds of Brassica plants. The content and the type of GLs depend mainly on the plant genotype, however they are also influenced by the cultivation and climate conditions [13]. In short, the GL biosynthesis (Fig 1) can be divided into three stages: elongation of the side chain, aglycone synthesis and modification of the side chain [14]. Biosynthesis of aglycone is initialized by the conversion of amino acids to aldoxy derivatives. Alanine, methionine, leucine or isoleucine are precursors of aliphatic, phenylalanine and tyrosine – of aromatic and tryptophan – indolic GLs [15-17]. Additionally, a number of non-protein amino acids with complex side chains, for example (mono/di/tri)homomethionine, may also serve as precursors of aliphatic GLs [17].

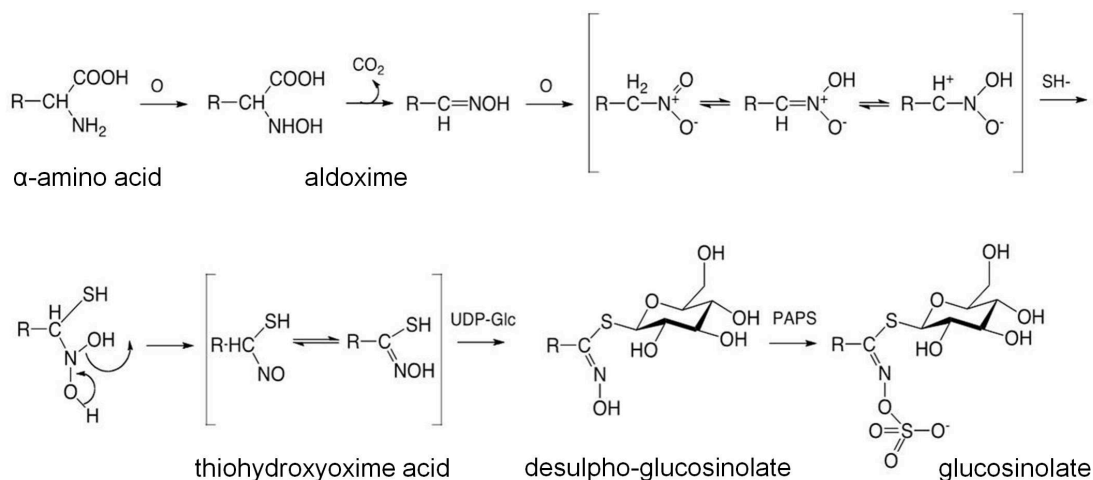


Fig. 1. General scheme of glucosinolate biosynthesis. Glucosinolates similarly to other hydrophilic secondary metabolites are mostly synthesized in cytosol.

The subsequent stages of GL biosynthesis involve rearrangement to thiohydroxy acid, introduction of sulfur from cysteine, transfer of glycosyl moiety from glucose uridino-diphosphate (UDP-Glc) catalysed by UDPglucose: thiohydroximate glucosyltransferase [17] and finally glucoside sulfation by the universal high energy sulfate donor – 3'-phosphoadenosine-5'-phosphosulfate (PAPS)

[13, 16]. This latter stage is catalysed by PAPS: desulfoglucosinolate sulfotransferase [18]. Modification of the side chain can proceed via different routes; mostly by oxidation, desaturation, or hydroxylation [13].

### 3. Degradation of GLs to bioactive derivatives

It is largely believed that GLs do not exhibit biological activity *per se*. The emergence of such activity requires hydrolysis of these compounds by the enzyme myrosinase (EC 3.2.3.1) belonging to the family of thioglucosidases [19]. Myrosinase catalyses the hydrolysis of thioglucosidic bond leading to the formation of unstable intermediate – thiohydroxim O-sulfone, whose further conversions depend on the GL structure, reaction conditions, and additional protein factors (Fig 2). To the latter belong: epithiospecifier protein (ESP) [20], nitrile specifier protein (NSP) [21], epithiospecifier modifying protein linked with myrosinase (ESM) [20] and thiocyanate-forming protein (TFP) [22].

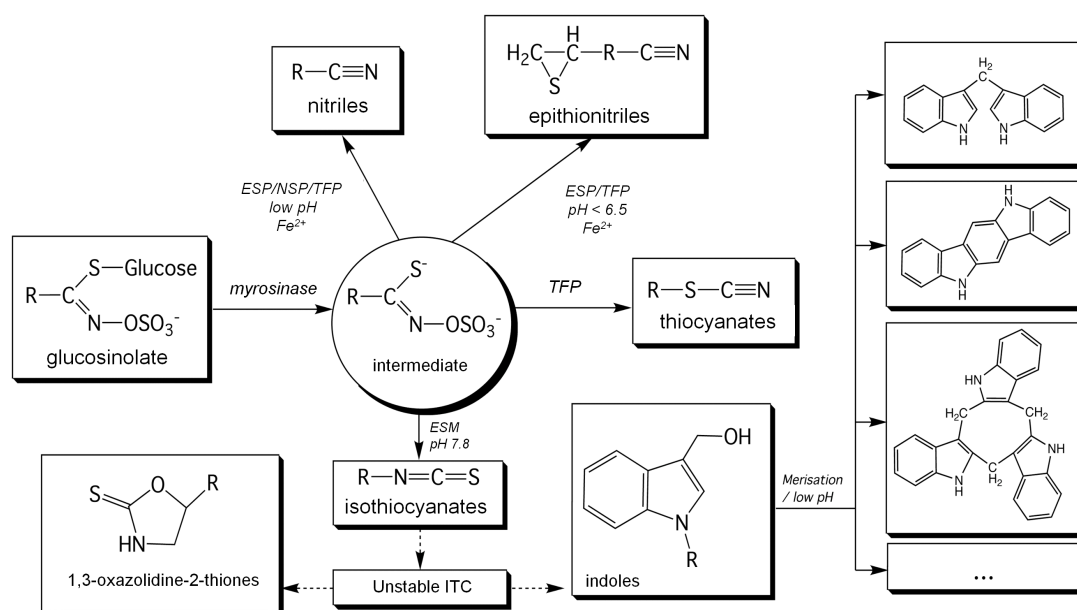


Fig 2. Schematic overview of the hydrolysis of glucosinolates whose direction depends critically on the presence of certain specifier proteins (ESP - epithiospecifier protein, NSP - nitrile specifier protein, TFP - thiocyanate-forming protein, ESM - epithiospecifier modifying protein linked with myrosinase); unstable isothiocyanates include those with indolic side chains or with a  $\beta$ -hydroxy group. These reactions occur after plant tissue disruption when GLs are released from vacuoles and meet myrosinase sequestered in so called myrosine cells.

Table 1. The content of glucosinolates and their degradation products in Brassica plants.

	GLs [ $\mu\text{mol/g dw}$ ]	ITC [ $\mu\text{mol/g dw}$ ]	Indoles [ $\mu\text{mol/g dw}$ ]	Ref
<b>Sprouts</b>				
Brussel sprouts sprouts	16.03 $\pm$ 1.76	0.37 $\pm$ 0.02	0.151 $\pm$ 0.007	[28]
Savoy cabbage sprouts	17.09 $\pm$ 1.39	0.79 $\pm$ 0.02	0.138 $\pm$ 0.006	[28]
White cabbage sprouts	41.30 $\pm$ 7.97	0.25 $\pm$ 0.03	0.073 $\pm$ 0.005	[28]
Mustard sprouts	72.87 $\pm$ 8.49	0.09 $\pm$ 0.04	0.03 $\pm$ 0.01	[34]
Broccoli sprouts	72.95 $\pm$ 8.34	0.39 $\pm$ 0.04	0.31 $\pm$ 0.03	[34]
Tuscan black kale sprouts	90.7 $\pm$ 4.4	62.1 $\pm$ 1.1	nd	[35]
Daikon sprouts	112.7 $\pm$ 4.4	108.8 $\pm$ 5.0	nd	[35]
Sango sprouts	146.6 $\pm$ 3.1	131.9 $\pm$ 2.5	nd	[35]
Radish sprouts	21.42 $\pm$ 3.02	9.67 $\pm$ 0.03	0.009 $\pm$ 0.001	[28]
<b>Edible parts</b>				
Brussel sprouts heads	5.83 $\pm$ 0.47	3.36 $\pm$ 0.23	0.089 $\pm$ 0.005	[28]
Savoy cabbage leaves	3.68 $\pm$ 0.13	1.17 $\pm$ 0.11	0.079 $\pm$ 0.005	[28]
Radish bulb	3.12 $\pm$ 0.29	0.16 $\pm$ 0.02	0.016 $\pm$ 0.001	[28]
White cauliflower	2.63 $\pm$ 0.02	0.16 $\pm$ 0.01	0.062 $\pm$ 0.001	[36]
Purple cauliflower	5.20 $\pm$ 0.59	0.54 $\pm$ 0.02	0.046 $\pm$ 0.004	[36]
White cabbage leaves	7.54 $\pm$ 0.72	4.88 $\pm$ 0.20	0.610 $\pm$ 0.065	[28]
Red cabbage leaves	3.09 $\pm$ 0.10	0.07 $\pm$ 0.01	0.021 $\pm$ 0.001	[36]
<b>Non-edible parts</b>				
White cabbage roots	22.08 $\pm$ 1.97	5.51 $\pm$ 0.11	0.172 $\pm$ 0.003	[28]
White cabbage stump	13.23 $\pm$ 0.68	2.94 $\pm$ 0.14	0.027 $\pm$ 0.001	[28]
White cabbage seeds	21.03 $\pm$ 3.52	4.14 $\pm$ 0.14	0.006 $\pm$ 0.001	[28]
Brussel sprouts stalk	15.69 $\pm$ 0.17	3.07 $\pm$ 0.11	0.094 $\pm$ 0.003	[28]
Brussel sprouts roots	7.98 $\pm$ 0.02	4.49 $\pm$ 0.03	0.163 $\pm$ 0.006	[28]
Brussel sprouts seeds	27.48 $\pm$ 4.89	4.79 $\pm$ 0.04	0.004 $\pm$ 0.001	[28]
Radish stalk	3.24 $\pm$ 0.05	2.19 $\pm$ 0.07	0.077 $\pm$ 0.003	[28]
Radish leaves	4.71 $\pm$ 0.47	4.60 $\pm$ 0.15	0.253 $\pm$ 0.004	[28]
Radish seeds	41.09 $\pm$ 6.7	2.74 $\pm$ 0.07	0.014 $\pm$ 0.001	[28]
Savoy cabbage roots	11.39 $\pm$ 0.18	6.72 $\pm$ 0.02	0.172 $\pm$ 0.005	[28]
Savoy cabbage seeds	33.38 $\pm$ 4.08	23.74 $\pm$ 0.59	0.034 $\pm$ 0.001	[28]

nd – no data

The most promising GL derivatives in cancer chemoprevention are isothiocyanates (ITC) and indoles [23-27], while competing reactions to thiocyanates or nitriles result in the loss of anti-carcinogenic properties [28]. Moreover, there are also reports suggesting undesirable activity of some nitriles, for example nephro- [29] or neurotoxicity [30]. Indolic ITC, derived from GLs with indolic side chain, are very unstable and as a result of further spontaneous rearrangements form corresponding alcohols [27, 31], among which indolo-3-carbinol (I3C, indole-3-methanol) has been most studied [32]. I3C can undergo isomerization to 3,3'-diindolylmethane (DIM) or more complex oligomeric structures; it can also undergo condensation with ascorbic acid to ascorbigen [27]. In the scientific literature, the information on the content and composition of GLs can be found for a large number of *Brassicaceae* plants, native or exposed to different preharvest and postharvest treatments. In this review, we

collected (Table 1) the very limited number of available data from those articles where both the content of GLs and their degradation products belonging to ITC and indole classes are given [28, 34-36]. The data (Table 1) have shown that the GLs content hardly ever matches the content of these most bioactive derivatives.

#### 4. Metabolism of ITC and indoles

ITC are absorbed from the alimentary tract, from which they penetrate *via* both the epithelium and the endothelium capillaries through passive diffusion. Once in the bloodstream, they bind rapidly and reversibly with sulfide groups of the plasma proteins. In this form, ITC migrate to liver via portal vein where in the cells, they readily form conjugates with glutathione (GSH) owing to the susceptibility of isothiocyanate group to nucleophilic attack on its carbon atom characterised by electron deficit. This reaction occurs spontaneously or is catalysed by glutathione S-transferases (GST). It has been shown that ITC rapidly accumulate in cells of various types as glutathione conjugates [37]. They may be then exported by membrane pumps, which belong to the multidrug resistance protein (MRP) family [38]. The dithiocarbamates are further metabolised in the mercapturic acid pathway (Fig 3) [33]. The enzymatic processes taking place in the extracellular matrix consist of a sequential liberation of the two flanking residues, glutamine and glycine, by  $\gamma$ -glutamyltransferase and dipeptidase:cysteinyl-glycine, respectively. The final cysteine conjugates are transported to liver, where they are acetylated by N-acetyltransferase to N $_{\alpha}$ -acetyl derivatives or mercapturic acid. These metabolites are transported to kidneys and are released to urine, then excreted from the organism [39, 40].



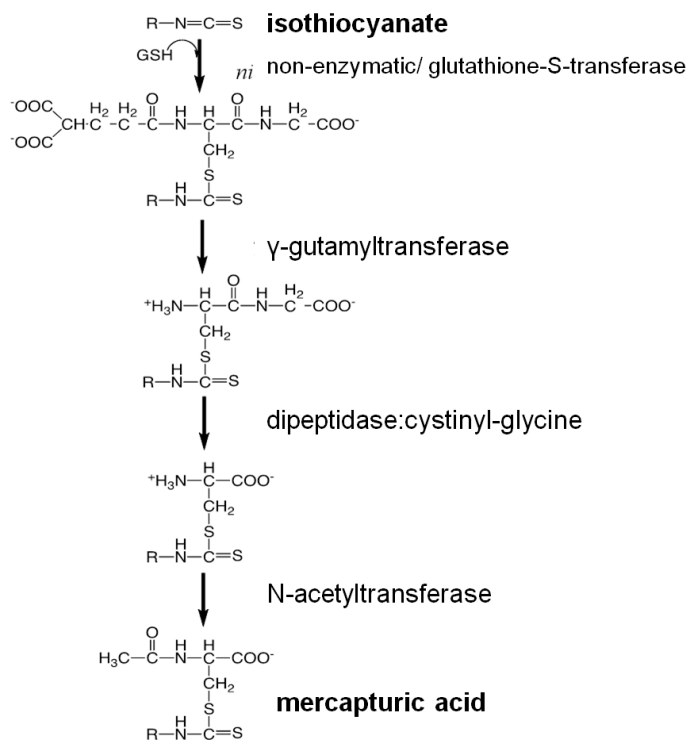


Fig 3. Isothiocyanate metabolism in mammal organisms

The degradation of indolic GL - glucobrassicin - produces I3C or its substituted derivatives depending on substituents in the parent GL structure. The acidic conditions of the stomach were shown to induce the chemical modification of I3C (Fig 4) consisting in the dehydration and formation of di- and trimers [41].

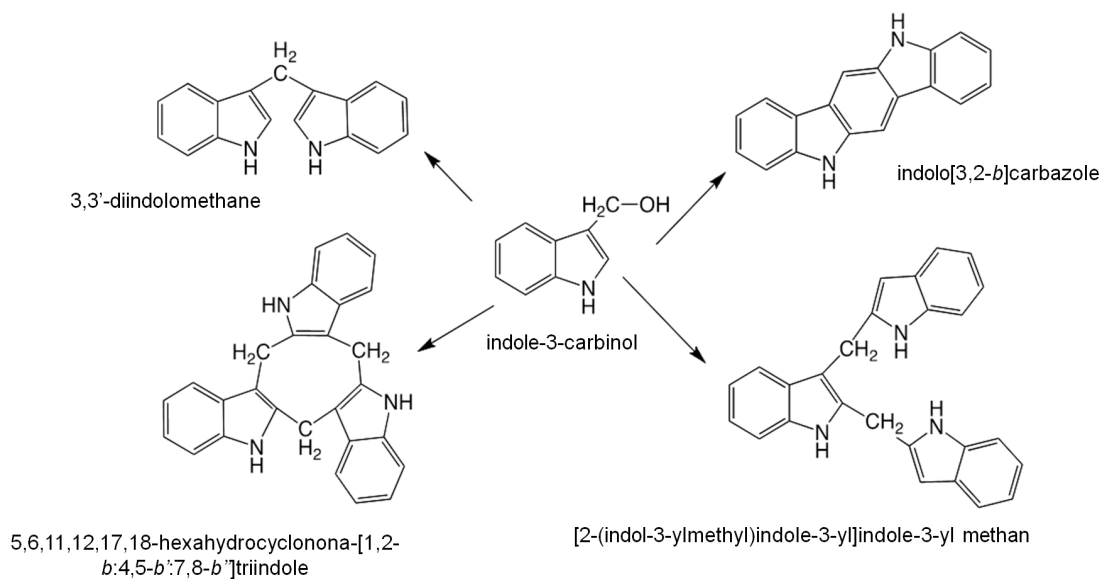


Fig 4. The rearrangements of indolo-3-carbinol molecule under acidic conditions in the stomach [based on 41]

The main products of chemical reactions in the stomach include 3,3'-diindolylmethane (DIM), tri-indolemethane and indole[3,2-b]carbazole [42]. The products of degradation of glucobrassicin and other indolic GLs, may also non-enzymatically react with GSH [43]. Moreover, similarly to other aromatic alcohols, heterocyclic aromatic amines or polycyclic aromatic hydrocarbons, they can be metabolically activated via conjugation with sulfuric acid catalysed by cytosolic enzymes of phase II detoxification – sulfotransferases (SULT) (Fig 5), particularly active in the liver [44]. Such a metabolic pathway of transformation of xenobiotics is not entirely benign, it is known to result in the formation of reactive metabolites with mutagenic properties [45]. This possibility was confirmed *in vitro* for N-methoxy-indole-3-carbinol (NI3C), the derivative of neoglucobrassicin. In the case of an engineered *S. typhimurium* strain with induced SULT expression, the mutagenicity of the above-mentioned derivative(s) in the Ames test was significantly higher when compared to a control strain not expressing this enzyme [46].

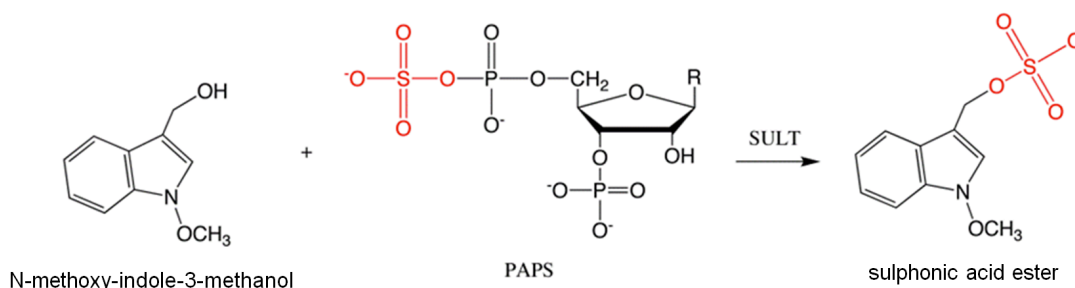


Fig 5. The conjugation of N-methoxy-indole-3-methanol with 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to sulfuric acid ester catalysed by sulfotransferases (SULT)

Also, in genotoxicity studies performed with the recombinant Chinese hamster lung fibroblasts (cell line V79), the expression of gene coding SULT enzyme resulted in the marked increase of sister chromatid exchanges in cells exposed to mentioned indoles [46].

## 5. Chemical reactivity vs. biological properties of ITC and indoles

The biological activity of ITC is ascribed to their two main properties: the relatively easy permeability across biological membranes and the electrophilic character of the isothiocyanate moiety. The first property ensures high bioavailability



of ITC [47] and accumulation sufficient to induce ITC conjugation with GSH and further conversions of the conjugates on the mercapturic acid pathway [33, 38]. The second property is accountable for the ability of ITC to react not only with GSH, but also with other nucleophilic centers, including sulfide, amino and hydroxyl groups, that is moieties whose modification often decides about function of not only individual molecules, but also whole signalling pathways, where even small concentration of regulatory molecule triggers a number of mechanisms in parallel. In addition, ITC may be expected to belong to reactive sulfur species (RSS) modulating biological processes *via* cellular thiolstat, that is thiol/disulfide balance, which assists in sensing, as well as, in maintaining the redox homeostasis within the cell [48].

Reactivity of nucleophilic moieties towards electrophiles can be ordered in a following sequence:  $-\text{SH} > -\text{NH}_2 > -\text{OH}$ , therefore cellular biomolecules containing sulfide groups will be a preferred binding side of ITC. As a result of such a reaction, stable dithiocarbamates are formed (Fig 6). However, the reactivity towards amine or hydroxyl groups may have not less important biological implications.

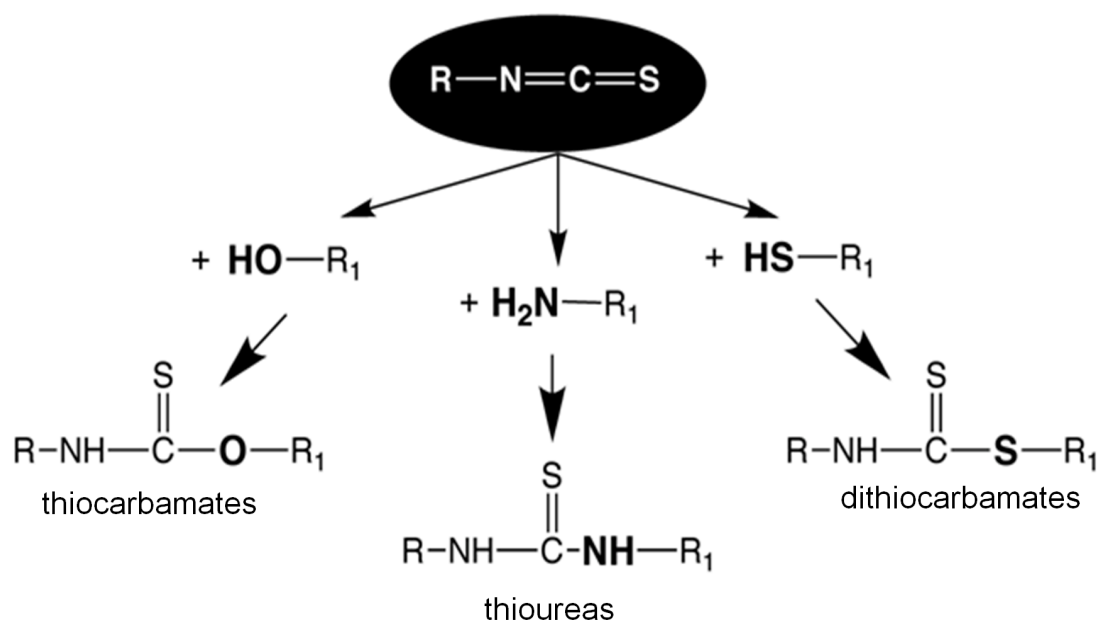


Fig 6. Scheme of reactions of isothiocyanates with main cellular nucleophiles

#### *Reactivity towards sulfide groups*

Due to its high, often millimolar abundance [49], GSH is likely to be the prime target of ITCs in most mammalian cells. This reaction is catalysed by



glutathione *S*-transferases and constitutes the first step on the way to excretion of ITC compounds from the organism [38]. As a consequence, the thiol/disulfide-based “thioredox homeostasis” of the cell is disrupted. Such a temporary decrease of GSH, a major endogenous antioxidant, renders the cell more conducive to the attack of reactive oxygen species (ROS) and activates in the organism the signalling pathway dependent on the transcription factor (Nrf2, nuclear factor (erythroid-derived 2)-like 2) [50]. Nrf2 controls expression of genes whose regulatory region contains the specific sequence ARE (antioxidant responsive element) or EpRE (electrophile responsive element). Such genes are called cytoprotective, because they code, among others, the enzymes responsible for the detoxification of detrimental substances [51, 52]. There are two mechanisms of Nrf2 factor release for translocation to nucleus, which results in the induction of expression of mentioned genes. In both cases, the key stage is the decay of its interaction with another inhibitory protein – Keap 1 (Kelch-like ECH-associated protein 1) [53]. It can occur either due to the change of conformation caused by oxidation of two cysteinyl groups to cystynyl group in protein Keap 1 (the effect of increased concentration of oxidants in the absence of GSH) or due to direct reaction of electrophilic compound with this protein (Fig 7).



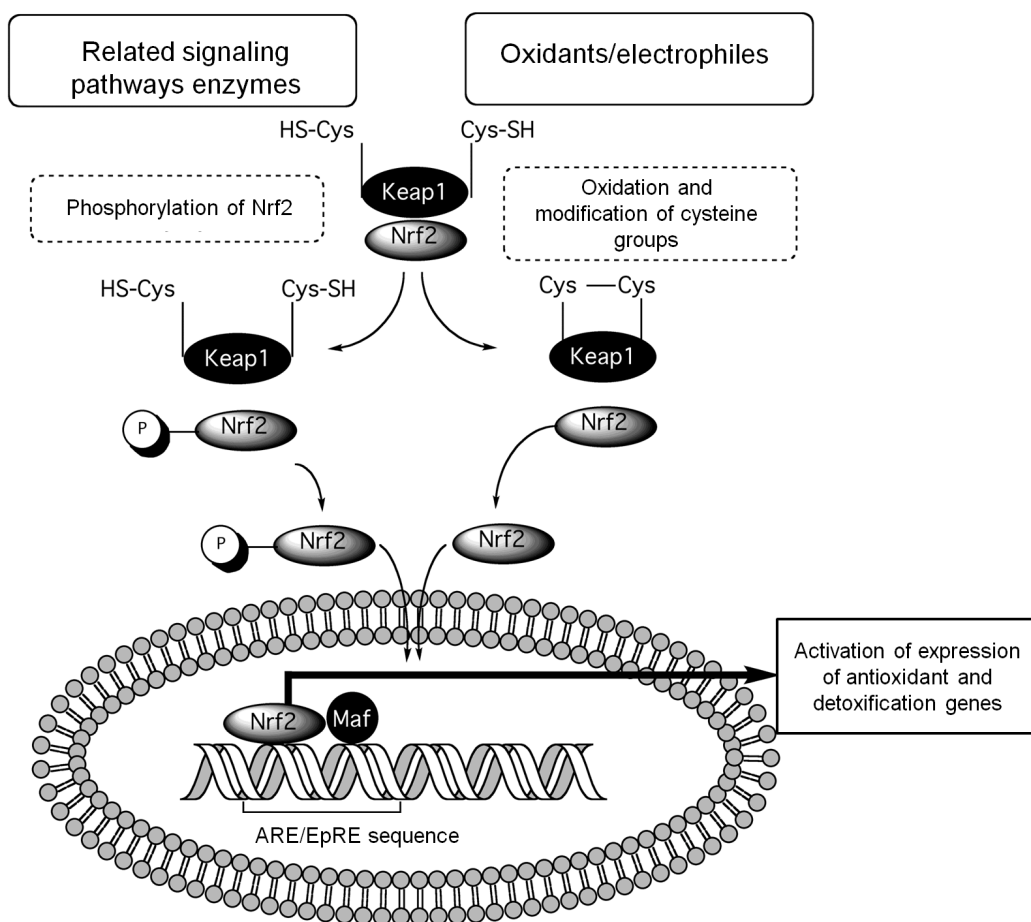


Fig 7. The signalling pathways dependent on Nrf2 transcription factor responsible for the triggering of expression of genes controlled by ARE or EpRE. Prooxidant and/or electrophilic substances may modify Keap1 protein and initiate the translocation of Nrf2 into nucleus. As a result, the expression of antioxidant enzymes and II phase enzymes is stimulated.

ITC can trigger the expression of Nrf2 dependent genes on both these ways, which has been confirmed experimentally [54]. Whilst it is believed that this mechanism is central to the anti-carcinogenic properties of ITC, excessive activation of Nrf2 factor is associated with the development of tumours and increased resistance to chemotherapeutics [55].



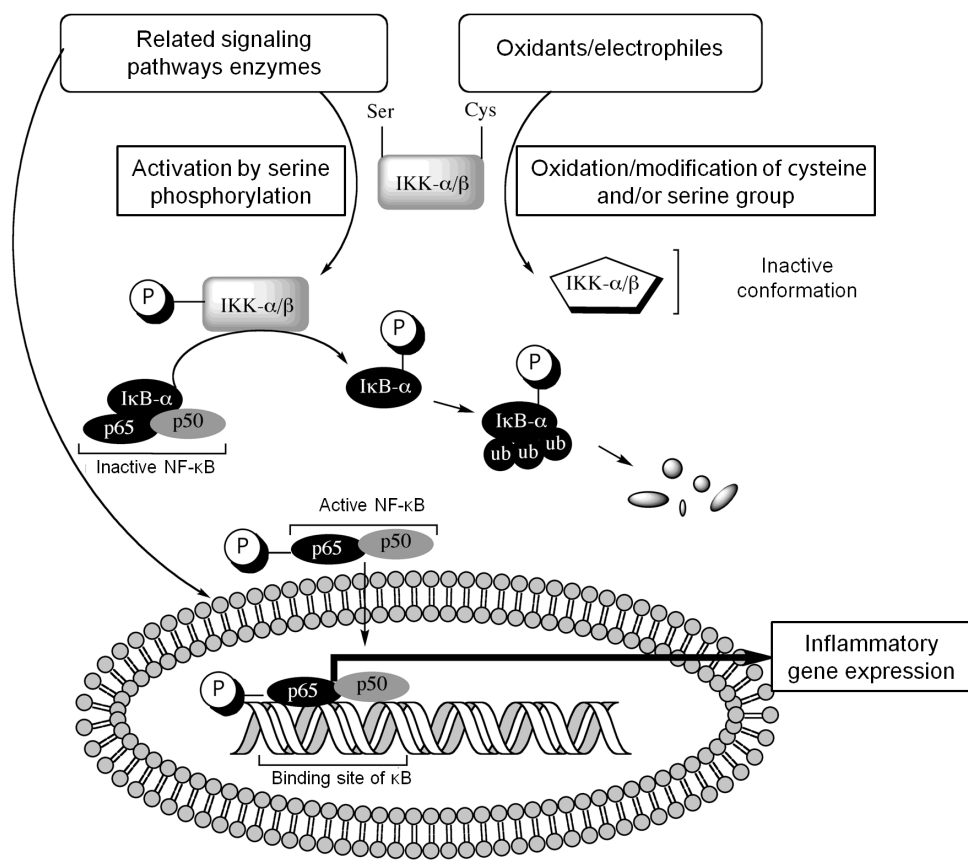


Fig 8. The signalling pathways dependent on NF-κB (dimer p65-p50) transcription factor responsible for the triggering of expression of genes implicated in inflammatory reactions. The modification of Ser or Cys residues of IKK-α/β enzyme by prooxidant and/or electrophilic substances inhibits the catalysed by this enzyme reaction of phosphorylation of IKK-α preventing its release and translocation into nucleus. The inflammatory reaction is brought to the halt

The ability to react with -SH groups lays also at the basis of anti-inflammatory properties of ITC. Inflammation is activated by NF-κB proteins that comprise a family of structurally-related eukaryotic transcription factors involved in the control of a large number of normal cellular and organismal processes, such as immune and inflammatory responses. Multiple signalling pathways are involved in the inflammation. One of the most important is NF-κB, IKKβ-dependent signalling. Several distinct NF-κB proteins can form a variety of homo- and hetero dimers, not all of which are active. NF-κB commonly refers to a p50-p65 heterodimer. This

transcription factor controls the expression of genes that code proteins taking part in the inflammatory reaction, e.g. enzyme cyclooxygenase COX-2 [53]. NF- $\kappa$ B is released from IKK $\alpha/\beta$  protein in a process that can be prevented by chemical modification of -SH group of cysteine or/and probably -OH group of serine in certain positions of this protein amino acid sequence (Fig 8). It follows, that the reaction that stimulates expression of genes protecting the organism against neoplastic transformation in the case of Nrf2 factor, at the same time has inhibitory effect on the development of inflammation dependent on factor NF- $\kappa$ B. The underlying inhibition of these two pivotal cysteine proteins of the “cellular thiolstat” involved in cellular signalling and control, *i.e.* Keap-1 and IKK- $\alpha/\beta$ , explains why ITCs are useful, not only in the prevention of cancer, but also in the fight against neurodegenerative diseases, such as Alzheimer’s, Parkinson’s or Huntington’s disease [56].

Apart from the mentioned mechanisms being the basis of biological activity of ITC, there have been postulated other mechanisms, also stemming from ITC reactivity towards sulfide groups, however less well recognized. In recent years, studies with  $^{14}$ C-labeled ITCs, such as labelled sulforaphane (SFN) and phenylethyl isothiocyanate (PEITC), have aimed to determine the most likely cellular target sites for ITCs [57]. Since then, more than 30 molecular targets have been established, among them  $\alpha$ - and  $\beta$ -tubulins rich in cysteines. These are globular proteins forming protofilaments of cytoskeleton microtubules, long recognized as a promising target of anticancer drugs. Perturbations in their polarization and depolarization, and also more general distortions in the microtubule network may result in an arrest of the cell cycle in phase G2/M and subsequently in the induction of apoptosis [58]. Apart from tubulins, actins may also form a prominent target of electrophilic attack. The exact positions of the bonds between ITCs and actin are still not known; it is presumed that these involve cysteines conducive to oxidation in positions 285 and 374 [59].

Another important example of modification of protein cysteinyl groups by ITC are protein disulfidoreductases, namely glutaredoxin-1 and thioredoxin-1. These small proteins (about 12 kD) are regarded as parts of antioxidant systems regulating sulfide-disulfide homeostasis in cells. They facilitate reversible reduction of disulfide bridge and its reformation by oxidation [60]. Covalent modification of cysteinyl residues by ITC impairs the function of these antioxidant proteins [60] and may lead to the disruption of intracellular thioredox homeostasis and apoptosis in



consequence. As mentioned before, also indolic GL degradation products may react with GSH [43] and thus they may affect cellular thioredox balance as well.

Besides interfering with some of the obvious redox systems in the cell, ITCs are also able to react and hence interact with a range of less apparent cellular regulatory networks, such as the cellular proteasomes, which maintain the cellular protein homeostasis by degradation of spare cellular proteins and therefore are of utmost importance for most processes. The degraded proteins include those responsible for signal transduction, metabolism regulation, cell cycle and apoptosis. It has been demonstrated that ITC directly bind to the catalytic subunit 20S and regulatory subunit 19S bringing about the inhibition of proteasome activity, which in turn blocks proliferation and induces apoptosis, especially in cancer cells [61]. This finding seems significant, because the strategy directed to proteasome inhibition in oncological therapy is currently gaining interest [62]. Unfortunately, the exact site of proteasome modification by ITC is not known. The better recognized situation is in the case of valosine-containing protein. This is a specific ATPase constituting an element of ubiquitin-proteasome system, that is a system of protein degradation associated with endoplasmic reticulum. This protein takes part in the transport of degraded protein substrates to cytosol [63]. ITC compounds modify the unprotected sulfide-cysteine group in position 522, which results in the inhibition of this ATPase. The ability to degrade proteins by proteasome is impaired and as a consequence their accumulation and normal function of cells is endangered. This fact can be exploited in cancer chemoprevention, because overexpression of valosine-containing protein was observed in several types of cancers, e.g. pancreatic cancer [64].

Heat-shock proteins, which protect other protein structures by preventing their aggregation and denaturation and thereby cell apoptosis, are another important group of proteins rich in cysteines. For example, in protein Hsp70, the reactive cysteinyl group is located in the site of nucleotide binding. Modification of this group inhibits ATP-binding, which results in the inactivation of this chaperone protein [65].

Products of GLs degradation also can influence gene expression at the epigenetic level. The suggested impact points to the subtle biochemical changes of DNA or histone proteins, because for at least some ITC and indoles, it has been demonstrated that they are inhibitors of histone deacetylases and/or DNA methyltransferases. This can be beneficial, because cancer cells often exhibit



overexpression of genes coding enzymes responsible for epigenome shaping. These compounds seem also to impact the regulation of gene expression dependent on miRNA [66, 67].

#### *Reactivity towards amine groups*

Other nucleophilic moieties that are present in important cellular biomolecules are amine groups with which ITC can react to form thioureas (Fig 6), stable derivatives with potential significance in carcinogenesis. Although the preferential sites of ITC binding are sulfide groups, it is suggested, that the resultant dithiocarbamates may be unstable under physiological conditions [68]. In contrast, ITC can form very stable compounds with amino groups under alkaline conditions in vitro or under physiological circumstances [69]. Theoretically, ITC released from dithiocarbamates could become linked to amino groups to form stable thioureas. The experiments indicated the diversified reactivity of ITC towards amino groups in amino acids;  $\alpha$ -NH<sub>2</sub> group is more conducive to this reaction than  $\epsilon$ -NH<sub>2</sub>, but thioureas formed as a result of binding with  $\epsilon$ -NH<sub>2</sub> group are more stable [70, 71].

The ability of ITC to react with amino groups has been exploited as a new biomarker of bioavailability of this group of compounds in human organism [72]. As mentioned above, ITCs are excreted from the organism with the urine in the form of acetyl-cysteine derivatives (Fig 5). These thiocarbamates have earlier served as such a biomarker. The problem was however the low stability of these ITC derivatives [73, 71], which can either regenerate free ITC or undergo reactions with other nucleophiles. In the new method, the ability to form covalent stable binding with amino group of lysine present in blood albumin or haemoglobin is used as a biomarker of ITC exposure [72]. Some researchers point out that biological significance of such modifications is not clear, because adducts of xenobiotics with proteins traditionally have been used as a toxicity marker [74]. Whether such adducts indicate toxic or beneficial action of GL derivatives is not known at the moment. ITC can bind also to the N-terminal proline of macrophage migration inhibitory factor – MIF. This factor is an immunological regulator of inflammation and auto-immunological diseases. Its inhibition may have significant therapeutic effects; among others in the treatment of chronic inflammatory states [75].



Apart from ITCs, the ability to bind amino groups of proteins exhibit also indolic alcohols formed from indolic GLs due to their reactivity towards tertiary amino groups in imidazole ring of histidine [76]. In mice treated with neoglucobrassicin, a high level of protein adducts in cecum and colon, liver, lung and blood was detected. In the case of administration of the degradation product N-methoxyindole-3-carbinol, the highest level of protein adducts was detected in the liver, that is, in the tissue where it probably undergoes metabolic activation by SULT enzymes.

Reaction of amino groups of protein side chains to thiourea in an obvious way can distort protein structure, but such a chemical modification of amino groups in nucleobases will also alter DNA structure. Covalent modification of nucleic acids would suggest definitely the detrimental activity of ITC and indoles. The results of in vitro and in vivo studies carried out in recent years have indicated the genotoxic and mutagenic properties of some GL degradation products (Table 2) [77]. For these genotoxic effects, mainly the derivatives of indolic GLs: glucobrassicin,

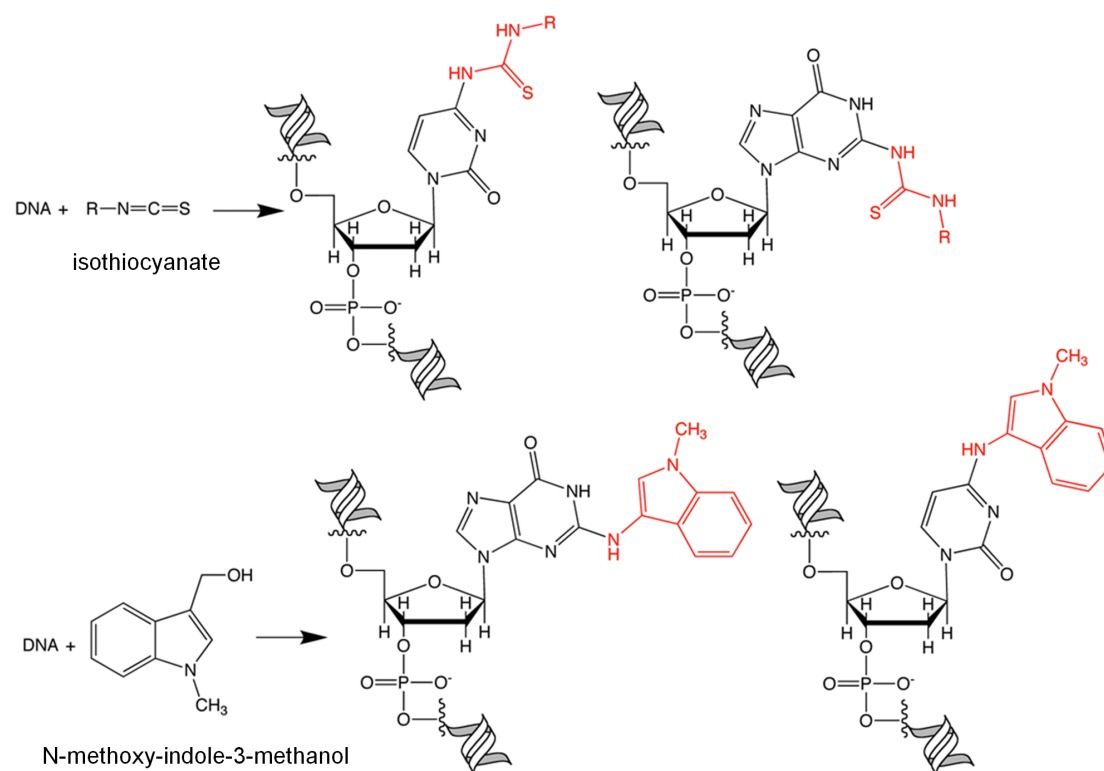


Fig 9. The putative structures of DNA adducts resulting from covalent modification of guanine and cytosine by isothiocyanates or N-methoxy indole-3-carbinol



neoglucobrassicin, 4-methoxyglucobrassicin and 4-hydroxyglucobrassicin, are responsible, because they form DNA adducts at the level 100-times higher than degradation products of aliphatic or aromatic GLs [46, 78, 79]. It was shown that neoglucobrassicin derivative - N-methoxy indole-3-carbinol, which forms DNA adducts with purine nucleobases via nucleophilic substitution; to much lesser extent with cytosine and none with thymine [80, 81]. These adducts result from modification of primary amino groups in nucleobase molecules.

**Table 2. A literature survey concerning the genotoxic effects induced by broccoli extracts or purified glucosinolates**

Method	Results	Extraction method/ individual molecule	Ref
Ames test: <i>S. typhimurium</i> TA 98 and TA 100	<ul style="list-style-type: none"> <li>for both strains dose dependent mutagenic effect</li> <li>addition of S9 fraction and acidifications decreased mutagenic effect</li> <li>heating (T100) – increased mutagenicity</li> </ul>	Fresh broccoli juice	[83]
	<ul style="list-style-type: none"> <li>significant mutagenic effect, no change in the presence of S9 fraction</li> </ul>	Broccoli homogenate in H <sub>2</sub> O/H <sub>2</sub> O:EtOH 1:1	[84]
Differential DNA repair test: <i>E. coli</i>	<ul style="list-style-type: none"> <li>genotoxic effect</li> <li>addition of S9 fraction decreased mutagenic effect</li> </ul>	Fresh broccoli homogente	[83]
Chromosomal abberations and sister chromatid exchange test (SCE) in mammalian cells	<ul style="list-style-type: none"> <li>CHO cells and SV40-transformed Indian muntjac cell line: moderate, dose dependent genotoxicity</li> <li>DNA adduct formation</li> </ul>	Fresh broccoli homogente	[83]

<sup>32</sup> P-postlabeling assay in cell-free system for DNA adduct detection	only in the presence of myrosinase	Plant homogenate	[78,79]
Ames test: <i>S. typhimurium</i> TA 100 with human sulfotransferase expression SULT1A1	<ul style="list-style-type: none"> <li>substantial mutagenicity due to high content of neoglucobrassicin</li> <li>the addition of plant hormone methyl jasmonate which enhances plant stress response increased mutagenicity</li> </ul>	Pak-Choi sprouts	[85]
<sup>32</sup> P-postlabeling assay: mice and rats fed broccoli (respectively 6 and 45 g per animal)	<ul style="list-style-type: none"> <li>detection of one major site of adduction and one less abundant for both broccoli preparations tested (probably resulting from neoglucobrassicin binding)</li> </ul>	Raw or steamed (15 min) broccoli with myrosinase activity retained	[86]
Comet assay: pigs fed diet supplemented with 600 g of broccoli	<ul style="list-style-type: none"> <li>increased DNA fragmentation in colon</li> </ul>	Raw broccoli	[87]
Test „wing-spot” in <i>D. melanogaster</i> fed with broccoli	<ul style="list-style-type: none"> <li>increased genotoxicity both with and without activation</li> </ul>	Raw, lyophilized broccoli from supermarket	[88]
Test on antimutagenicity: feeding experiment in <i>D. melanogaster</i> . Broccoli administered along with alkylating agent methyl methanelsulfonate (MMS)	<ul style="list-style-type: none"> <li>synergistic mutagenic effect</li> </ul>	Organically grown, blanched and lyophilized broccoli	[89]

Ames test: <i>S. typhimurium</i> TA 100 and TA 104	<ul style="list-style-type: none"> <li>• neoglucobrassicin + myrosinase -highly mutagenic</li> <li>• low mutagenicity of N-methoxy-indolo-3-carbinol in TA100 strain</li> <li>• no mutagenicity of nitryle 1-MIM</li> <li>• addition of myrosinase increased genotoxicity of glucosinolates towards one or both strains</li> <li>• neoglucobrassicin induced the greatest number of revertants</li> </ul>	Neoglucobrassicin, N-methoxy-indolo-3-carbinol and nitryle [86,46]
Ames test: <i>S. typhimurium</i> TA 100 with human sulfotransferase expression SULT1A1	<ul style="list-style-type: none"> <li>• neoglucobrassicin and its metabolites exerted strong, dose dependent increase of mutagenicity</li> </ul>	Neoglucobrassicin and its metabolites [79]
Sister chromatid exchange test (SCE) in CHO cell line V79 and transformed V79-hSULT1A1	<ul style="list-style-type: none"> <li>• positive results in the presence of myrosinase for V79-hSULT1A1 cells; this cell line gave stronger response</li> <li>• incubation of 1-MIM with V79 hSULT1A1 cells resulted in a stronger mutagenic effect in HPRT gene mutation test</li> </ul>	Neoglucobrassicin, 1-MIM alcohol [86]
<sup>32</sup> P-postlabeling assay for DNA adduct detection	<ul style="list-style-type: none"> <li>• high level of adducts in DNA isolated from herring sperm</li> </ul>	12 Glucosinolates: aromatic, indolic, aliphatic [78]
UPLC-ESI-MS/MS for DNA adduct determination in vivo	<ul style="list-style-type: none"> <li>• formation of DNA adducts <i>in vitro</i> and <i>in vivo</i> with dG, dC i dA</li> </ul>	N-methoxy-indolo-3-carbinol [80]
UPLC-ESI-MS/MS for DNA adduct	<ul style="list-style-type: none"> <li>• formation of DNA adducts dG, dA i dC in proportion 3:3:1,</li> <li>• neoglucobrassicin formed</li> </ul>	Neoglucobrassicin, N-metoxy-indolo-3- [81]

determination in mice administered 0.6 mmol/kg of glucosinolate or its derivative	<ul style="list-style-type: none"> <li>• adducts mainly in colon</li> <li>• N-methoxy-indolo-3-carbinol generated preferentially DNA adducts in colon, but also in stomach and liver.</li> </ul>	carbinol
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Although covalent DNA modification was best documented for indolic derivatives, the degradation products of aliphatic and aromatic GLs, that is mostly ITC, can also give rise to DNA adducts (Fig 9), though occurring at the lower level [79]. Chemopreventive properties of GL derivatives seem to override their genotoxic properties, however the mentioned observations suggest the necessity of toxicological control of these compounds in order to establish the doses safe for consumption. Such investigations are particularly important in the context of dietary supplements based on *Brassica* plants.

#### *Reactivity towards hydroxyl groups*

Aromatic and especially aliphatic hydroxyl groups are generally considerably less nucleophilic and hence also less reactive when compared to their thiol and amine counterparts. Thus, it is not surprising, that the reactivity of ITCs towards hydroxyl groups is also the lowest in comparison. Still, there are a few examples of proteins whose hydroxyl groups may be modified by ITCs. Most important biologically modifications would concern protein kinases involved in signal transduction, whose function depends on the availability of free hydroxyl groups, which could be blocked by ITCs. The activity of these enzymes, especially when they constitute an element of signalling pathways, is frequently regulated by phosphorylation/dephosphorylation of threonines, serines or tyrosines. The reaction of ITC with –OH groups in these enzymes would lead to the formation of stable thiocarbamates (Fig 6). It can be expected that when such a modification occurs in the case of –OH group that under normal conditions undergoes phosphorylation, this will have impact on the activity of a given kinase and proteins being its substrate. It is likely that such modifications explain certain scenarios reported in the literature, where ITCs inhibit proliferation or stimulate apoptosis via pathways depending on the modulation of activity of MAPK kinases (mitogen-activated protein kinases), being also important in cancer development [82]. Still, there is no documented data



explicitly demonstrating such a - chemically feasible - modification of protein hydroxyl groups by ITCs.

## 6. Conclusions

Relatively high bioavailability of ITC, as well as their reactivity towards moieties present in important biomolecules is behind exceptionally strong and diversified biological activities of these compounds. They can modify proteins whose natural function is monitoring of oxidative and chemical stress, as well as cell protection against negative consequences of ROS and detrimental substances. ITC are a sort of “trial alarm” for the cell, which triggers protective mechanisms thereby preparing the cell to the future threats. This is true until the concentration of ITC does not overcome cellular capabilities of their neutralization. It seems that for the concentrations reachable as a result of consumption of Brassica vegetables the risk of e.g. DNA lesions resulting from covalent modification of DNA by ITC and indoles is negligible, because the reaction with sulfide group of GSH will occur preferentially. However, dietary supplements, containing sometimes very large amounts of these compounds may constitute some genotoxic risk. The impairment of metabolic processes following the administration of ITC in vivo was observed. For example, the toxicity of methyl-ITC caused the withdrawal from agricultural use of this synthetic pesticide. It must be remembered that chemopreventive properties of ITC and indoles involve the same mechanisms, which are exploited by plants producing GLs for protection against other organisms such as bacteria or fungi.

Disclosure of interest

The authors report no conflict of interest.

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