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Glutathione- and thioredoxin-related enzymes are modulated by sulfur-containing chemopreventive agents

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Abstract

We studied the effects of sulfur-containing chemopreventive agents, including allyl sulfides and isothiocyanates, on human redox networks. Isothiocyanates inhibited isolated redox-active enzymes in a time- and dose-dependent manner. As shown for the most active compound, benzyl isothiocyanate (BITC), on thioredoxin reductase, the inhibition has an initial competitive part (K_i=6.1±1.0 μM) followed by a time-dependent irreversible inhibition (k_2=72.8±25.5 M^{-1} s^{-1}). Also, glutathione reductase and glutathione S-transferase were irreversibly modified by BITC. Sulforaphane led to irreversible inhibition of the studied redox enzymes, but with 5–10 times lower k_2 values. In contrast, allyl sulfides had only moderate effects on the tested enzymes. However, diallyl disulfide was found to react directly with reduced glutathione (k_2=100 M^{-2} s^{-1}). This reaction might contribute to enhanced oxidative stress and the induction of the selenoprotein glutathione peroxidase as determined on activity and transcript levels. All chemopreventive agents tested induced transcript levels of genes associated with cell cycle arrest and apoptosis. This upregulation was accompanied by a dose-dependent decrease in cell number. Our data indicate that modulation of cellular redox networks is likely to contribute to the effects of sulfur-containing chemopreventive agents.

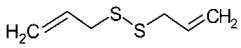
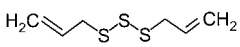
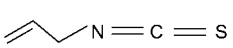
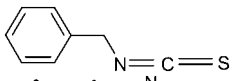
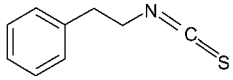
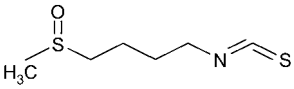
Keywords: A549 cells; allyl sulfides; enzyme inhibition; isothiocyanates; redox networks; transcript levels.

Introduction

Naturally occurring allyl sulfides and isothiocyanates (see Table 1) have been confirmed to act as chemopreventive agents (Wu et al., 2005a) and are abundant in daily-consumed vegetables and traditional herbal medicine (Conaway et al., 2001; Khanum et al., 2004). The proposed mechanisms of chemoprevention – resulting e.g., in cell cycle arrest and apoptosis (Sykiotis and Papavassiliou, 2006) – are complex and not yet fully elucidated. Proteins and pathways involved in mediating the effects of allyl sulfides and isothiocyanates include the mitogen-activated protein kinases, death receptors, caspases, the inhibitor of apoptosis (IAP) family of proteins, heat shock proteins, as well as the B-cell chronic lymphocytic leukemia/lymphoma 2 (BCL2) family (Wu et al., 2005b; Brigelius-Flohé and Banning, 2006; Fisher et al., 2007; Shukla and Kalra, 2007). In addition, reactive oxygen species (ROS) seem to play a pivotal role in starting and promoting anticancer effects of chemopreventive agents (Ahmad et al., 2001; Diamond et al., 2001; Afaq et al., 2002). The effects of chemopreventive agents on phase I and phase II enzymes (Hecht, 1999; Kwak et al., 2001; Steinkellner et al., 2001; Brigelius-Flohé and Banning, 2006) have been demonstrated, including the inhibition of glutathione S-transferase (GST) by benzyl isothiocyanate (BITC) (Ralat and Colman, 2004). Furthermore, glutathione peroxidase (GPx) activation might contribute to the chemopreventive effects of genistein (Suzuki et al., 2002). Systematic and comparative studies investigating the effects of sulfur-containing chemopreventive agents on the major glutathione- and thioredoxin (Trx)-dependent enzymes have, still, not yet been conducted. As thioredoxin reductase (TrxR) is well expressed in A549 cells and considered to contribute to regulating the redox balance in non-small cell lung carcinoma through activation of factors important for transcription and tumor growth, this cell line is suitable to study redox regulatory effects of chemopreventive agents (Soini et al., 2001).

The Trx and the glutathione system interact with a whole range of important cellular pathways (Fernandes and Holmgren, 2004; Gromer et al., 2004). Glutathione reductase (GR) recycles glutathione from its disulfide and keeps it in the functional status. GR and the related selenoprotein TrxR influence cell proliferation, cell cycle regulation, and apoptosis by regulating caspase-3 and BCL2-associated X protein (BAX), blocking tumor necrosis factor α (TNF-α)-induced ROS generation and activating nuclear factor-κB (NF-κB) and the tumor suppressor P53 (Cassidy et al., 2006; Chen et al., 2006; Hansen et al., 2006). Based on their importance for tumor cell growth and multiplication, GR and TrxR as well as GST and Trx represent attractive candidates for cancer therapy (Gromer et al., 2004; Deponte et al., 2005; Ahmadi et al., 2006; Urig et al., 2006). Trx, the physiological substrate of TrxR, provides reducing equivalents to ribonucleotide reductase and Trx peroxidases for DNA synthesis and detoxification of peroxides (Schallreuter and Wood, 2001; Gromer et al., 2004). Trx is also implicated in the control of transcription and translation and acts as a protein-folding catalyst (Gromer et al., 2004). The tripeptide glutathione, a substrate for GSTs and GPx, main-

Table 1 Characteristics of the sulfur-containing chemopreventive compounds used in this study.

	Name	Abbreviation	Molecular formula	Molecular weight	Molecular structure
Allyl sulfides	Diallyl disulfide	DADS	$C_6H_{10}S_2$	146.3	
	Diallyl trisulfide	DATS	$C_6H_{10}S_3$	178.3	
Isothiocyanates	Allyl isothiocyanate	AITC	$CH_2{=}CHCH_2NCS$	99.15	
	Benzyl isothiocyanate	BITC	C_8H_7NS	149.2	
	Phenethyl isothiocyanate	PEITC	C_7H_5NS	135.2	
	Sulforaphane	SF	$C_6H_{11}NOS_2$	177.3	

ly acts as antioxidant but is also conjugated with xenobiotics to enhance their water solubility and excretion. Supporting also the rearrangement of protein disulfide bonds with or without glutathionylation, glutathione is indispensable for the maintenance of the intracellular and extracellular redox balance (Kern and Kehrer, 2005). The ratio of reduced over oxidized glutathione is a sensitive parameter of the redox milieu and is associated with cell survival via redox modulation of molecular factors, including activator protein 1 (AP-1), NF-κB and protein tyrosine phosphatases 1-B (Filomeni et al., 2002). GPx, reducing hydroperoxides enzymatically and efficiently, can be upregulated by P53 and has been reported to modulate cancer cell growth (Gladyshev et al., 1998; Liu et al., 2004). Furthermore, GSTs play an important role in cancer etiology and resistance to chemotherapy (Parl, 2005).

In this report, we studied the influence of six different sulfur-containing chemopreventive agents (Table 1) on enzyme activity and transcript levels of the major cellular glutathione and Trx-dependent enzymes. In addition, we provide cell biological and transcriptome data on cell cycle arrest and apoptosis in A594 cells.

Results

Influence of sulfur-containing chemopreventive agents on the activity of purified enzymes

The inhibitory effects of sulfur-containing compounds on the activity of isolated human TrxR, GR and GST were determined as described in the material and methods section. Results are summarized in Table 2.

For the two allyl sulfides tested, high concentrations in the upper micromolar range were required to observe enzyme inhibition. The greatest inhibitory effect was observed for the reaction of diallyl disulfide (DADS) with hTrxR, which led to 50% enzyme inhibition in the DTNB assay after 30 min preincubation at a concentration of 380±50 μM. These interactions are unlikely to be of biological significance.

The inhibitory potential of the isothiocyanates was, however, much stronger with IC_{50} values in the lower micromolar range and showed a time dependency (Table 2). BITC, the most effective inhibitor, led to 50% immediate TrxR inhibition in the Trx assay at a concentration of 30±10 μM. After 30 min incubation, this value dropped down to 3.8±2 μM. As this combination was identified to have the greatest inhibitory effects, the type of inhibition was studied in more detail. When systematically varying substrate (DTNB) against BITC concentrations and representing the data in a Dixon or Lineweaver-Burk plot, the competitive component of the inhibition became evident. The K_i value for competitive inhibition of hTrxR by BITC was calculated to be 6.1+1.0 μM, according to the equation $K_i = K_m[I]/(K_m' - K_m)$.

This competitive inhibition was paralleled by a time-dependent irreversible inhibition of TrxR. By determining the enzyme inhibition at different inhibitor concentrations and time points, a k_2 value of 72.8±25.5 M^{-1} s^{-1} was determined at 25°C. Also, GR (k_2=26.1±5.5 M^{-1} s^{-1}), and GST (k_2=7.0±2.1 M^{-1} s^{-1}) were irreversibly modified by BITC. Sulforaphane (SF) also led to irreversible inhibition of the studied redox enzymes, but with 5–10 times lower k_2 values. All values are summarized in Table 2.

The time-dependent inhibition of TrxR and GR by BITC was NADPH-dependent and could not be reversed by dialysis or dilution suggesting that covalent interactions contribute to the inhibition observed.

GPx was not significantly affected by any of the sulfur-containing chemopreventive agents chosen (all IC_{50}s >500 μM without incubation, data not shown).

Activities of Trx- and glutathione-dependent enzymes are altered by sulfur-containing chemopreventive agents in A549 cells

A 24 h treatment of A549 cells with different concentrations of DADS and diallyl trisulfide (DATS) led to an increase in TrxR activity, which became significant for DADS (up to 165% of the original activity, Figure 1A). Also, GPx activities were found to be enhanced (up to ca. 200% of the original activity for different DADS and DATS concentrations) (Figure 1C). GR activity decreased slightly at higher concentrations; for GST, no change in activity was observed (Figure 1B,D).

All four isothiocyanates decreased TrxR and GR activities significantly and dose-dependently, which clearly

Table 2 Inhibition of purified human thioredoxin reductase (TrxR), glutathione reductase (GR), and glutathione S-transferase (GST) by sulfur-containing chemopreventive agents.

	TrxR (Trx assay)		TrxR (DTNB assay)		GR			GST	
	No incubation	30 min incubation	No incubation	30 min incubation	k_2 (M^{-1} s^{-1})	No incubation	30 min incubation	k_2 (M^{-1} s^{-1})	No incubation	30 min incubation	k_2 (M^{-1} s^{-1})
DADS[a] (μM)	≥500	≥500	≥500	380±50	nd	≥500	≥500	nd	≥500	≥500	nd
AITC (μM)	100±25	13±5	300±30	20.5±5	nd	≥500	100±20	nd	≥500	≥500	nd
BITC (μM)	30±10	3.8±2	40±9	3.3±2	72.8±25.5	900±90	29±7	26.1±5.5	390±60	40±15	7.0±2.1
PEITC (μM)	500±80	50±9	250±50	75.0±15	nd	≥500	50±10	nd	≥500	≥500	nd
SF (μM)	≥500	30±8	2500±500	40±10	17.3±7.7	5000±1000	45±12	4.7±2.8	3500±500	≥500	0.76±0.12

Given are the inhibitor concentrations required for 50% enzyme activity inhibition in comparison to an untreated control without preincubation and after 30 min preincubation. In addition, for the TrxR DTNB assay as well as for GR and GST, the second order rate constants are given as determined for the time-dependent inhibition with BITC and SF at 25°C. In a typical experiment, the values reported are mean±SD of three independent assays.

[a]As enzyme inhibition by DATS was even less pronounced than by DADS, data on DATS were not included in the Table.

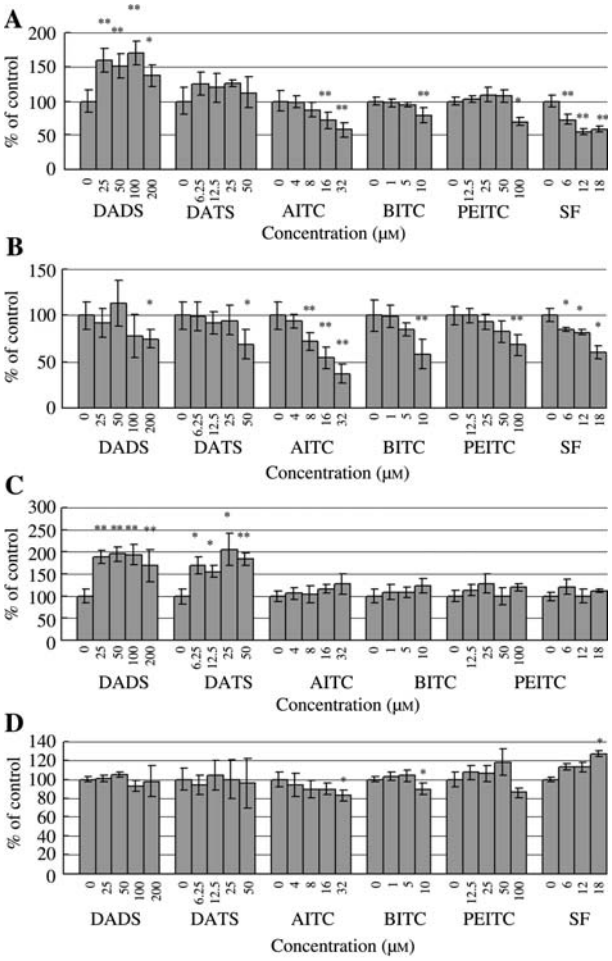


Figure 1 Changes of enzyme activities after 24 h treatment of A549 cells with different sulfur-containing compounds.
(A) TrxR activity was significantly regulated after treatment with DADS, AITC, BITC, PEITC and SF. (B) GR activity decreased dose-dependently after isothiocyanate treatment. (C) GPx activity was significantly induced by treatment with DADS and DATS. (D) GST regulation was not pronounced.
*$p<0.05$, **$p<0.01$.

supports the data on the isolated enzymes. GPx and GST activities were hardly affected (Figure 1C,D).

Interaction of DADS with glutathione

During the kinetic studies on GR and DADS, a particularly strong background reaction became visible which was based on a non-enzymatic reaction between GSH and DADS. This phenomenon was studied in more detail. The reaction between GSH by DADS was measured on the basis of GSSG end-point determinations in the GR assay system. Different concentrations of DADS and GSH were incubated for 0, 15 and 30 min and the amount of GSSG produced was determined. The reaction was found to be time- and concentration-dependent. Assuming that $d[GSSG]/dt = k\,[DADS]\,[GSH^2]$ (Cornish-Bowden, 2001), a third-order rate constant of $k = 100\ M^{-2}\ s^{-1}$ was estimated for the reaction at 28°C. *In vivo* this reaction might lead to a decrease of GSH concentrations and/or increased fluxes of GSSG. This reaction might contribute to enhanced oxidative stress and the induction of GPx

as determined on activity and transcript levels (see above and below).

Transcript levels of redox enzymes are altered by sulfur-containing chemopreventive agents in A549 cells

In parallel with the enzyme activities, we determined the mRNA transcript levels of the enzymes studied after 24 h exposure of A549 cells to the six chemopreventive agents. The upregulation of GPx activity by DADS and DATS was paralleled by an increase in mRNA by >200% (Table 4). In contrast, the isothiocyanates did not have a major influence on GPx activity or transcript levels. An upregulation of GSTpi mRNA was observed only for the higher allyl sulfide concentrations.

The decrease in GR activity induced by the six chemopreventive agents as well as the decreased TrxR activity under isothiocyanate treatment was not reflected by changes in mRNA levels. This might indicate enhanced damage or degradation of the proteins which were not (yet) compensated for. The enhanced TrxR activity induced by allyl sulfides, particularly DADS, was visible on the mRNA level as a trend. Interestingly, Trx was dose-dependently upregulated under most chemopreventive agents tested.

For superoxide dismutase 1 and 2 (SOD1/SOD2), a slight upregulation could be determined on transcript levels for all chemopreventive agents tested. In addition, we determined the mRNA levels of glutathione peroxidase 2 (gastrointestinal GPx, GPx2) and catalase (CAT). However, these transcript levels were changed by less than 30% by any of the six compounds and did not show reproducible trends (data not shown).

Effects of allyl sulfides and isothiocyanates on cell viability and cell cycle distribution

In comparison with a normally growing control, all six sulfur-containing compounds tested led to a dose-dependent decrease in cell numbers of A549 cells (Figure 2A). These observations prompted us to further investigate the effect of the compounds on apoptosis and cell cycle distribution. FACS analysis showed that DADS (Wu, 2005), DATS (Wu, 2005), allyl isothiocyanate (AITC) and BITC dose-dependently and significantly increased apoptosis in A549 cells (Figure 2B). In comparison, phenethyl isothiocyanate (PEITC) and SF did not lead to significant induction of apoptosis under the experimental conditions chosen. The used compounds showed varying effects on cell cycle distribution as the observed cell cycle arrest was dose- and compound-specific. At the concentrations tested, DADS (Wu, 2005), DATS (Wu, 2005), AITC and SF arrested A549 cells predominantly in the G2/M phase in a dose-dependent manner (Figure 2C). In the case of BITC, G2/M arrest occurred at 3 μM, but disappeared at 10 μM. In contrast to the G2/M arrest induced by most of the compounds, PEITC at 25, 50 and 100 μM increased the G0/G1 population in A549 cells by 10, 15 and 20%, respectively.

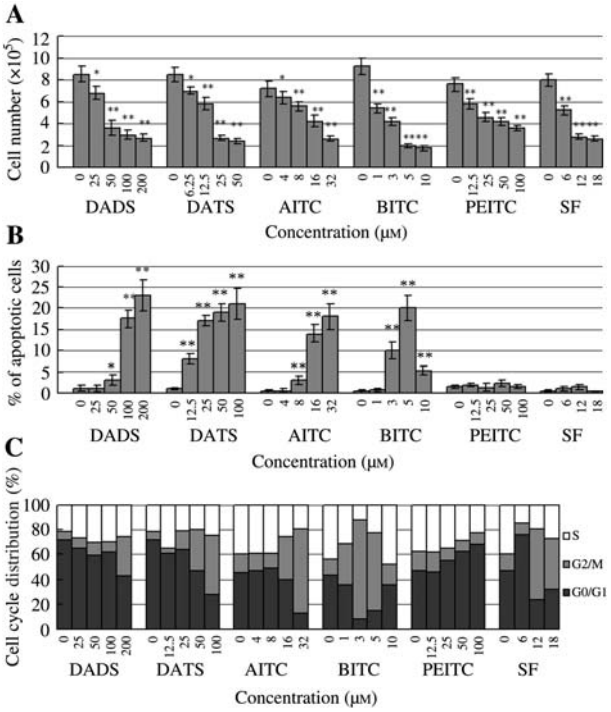


Figure 2 Cell numbers, apoptosis and cell cycle distribution of A549 cells after 24 h treatment with different chemopreventive compounds.
(A) Cell numbers decreased dose-dependently under allyl sulfide and isothiocyanate treatment. (B) Dose-dependent increase of apoptosis was observed for all compounds except for PEITC and SF. (C) Analyzed compounds showed different effects on cell cycle distribution. Chosen agents led to cell cycle arrest at G1/G0 or G2/M in a dose-dependent manner. Data on apoptosis and cell cycle distribution after DADS and DATS treatment are from Wu (2005).
*$p<0.05$, **$p<0.01$.

Allyl sulfides and isothiocyanates modulate transcript levels of genes involved in apoptosis and cell cycle regulation

It is well known that P53, cyclin-dependent kinase inhibitor 1 A (CDKN1A), N-myc downstream regulated gene 1 (NDRG1), growth arrest and DNA-damage-inducible gene α (GADD45A), survivin and the X-linked IAP (XIAP; baculoviral IAP repeat-containing protein 4, BIRC4) are related to cell proliferation, apoptosis and cell differentiation (Giovannini et al., 2004; Stein et al., 2004; Kreishman-Deitrick et al., 2005; Zhan, 2005; Cassidy et al., 2006). We thus assumed that transcription of these genes should be modulated during cell cycle arrest and apoptosis caused by our sulfur-containing compounds. Indeed, CDKN1A, but also NDRG1, and GADD45A transcript levels were found to be significantly upregulated (Table 4). These upregulations were mainly seen at the higher compound concentrations.

Discussion

The mechanism by which SF and other dietary isothiocyanates protect cells was traditionally supposed to be a nuclear factor E2-related factor 2 (Nrf2)-mediated

induction of phase 2 detoxification enzymes. According to the literature, a disturbed redox equilibrium leads to modulation of redox sensitive transcription factors like Nrf2, AP-1 and NF-κB that sense and transduce changes in the cellular redox status and subsequently modulate gene expression responses. Nrf2 binds to antioxidative response elements (AREs) and induces transcription of phase II detoxification (sometimes accompanied by repression of phase I enzymes) and antioxidative enzymes (Brigelius-Flohé and Banning, 2006; Nair et al., 2007). These induced proteins enhance cell defense against oxidative damage and promote the removal of carcinogens. However, there is increasing evidence that multiple mechanisms are activated in response to SF and other isothiocyanates. These comprise mainly the induction of apoptotic pathways and the inhibition of proliferation via regulation of cell cycle progression. Furthermore, the suppression of cytochrome P450 enzymes, inhibition of angiogenesis and anti-inflammatory activity are described (Gamet-Payrastre, 2006; Myzak and Dashwood, 2006a,b; Zhang et al., 2006b; Juge et al., 2007).

The mechanisms responsible for the cancer protective effect of the allyl sulfides are less well studied, but in general seem to comprise similar components. Recently, it has been shown that the allyl sulfides affect phase I and II drug-metabolizing enzymes via activation of CAR and Nrf-2 (Fisher et al., 2007). Furthermore, ROS generation is augmented and processes for ROS scavenging are modulated, as well as cell proliferation, cell cycle progression and apoptosis are affected (Herman-Antosiewicz and Singh, 2004; Shukla and Kalra, 2007). Pinto et al. suggested that β-elimination reactions with cysteine S-conjugates in garlic may target redox-sensitive signal proteins at sulfhydryl sites, thereby regulating cell proliferation and/or apoptotic responses (Pinto et al., 2006).

An alternative mechanism to explain the chemopreventive action of the dietary sulfur-containing compounds, allyl sulfides and isothiocyanates, is reviewed in Myzak and Dashwood (2006a,b). For example, DADS and SR also display histone deacetylase inhibitory activity leading to acetylation of histones. It is important to note that the downstream effectors of histone deacetylase inhibition contain essentially the same effectors identified in many cancer prevention studies, such as p21, BAX and caspases. These again play significant roles in leading to cell cycle arrest and/or apoptosis.

In our study, we intended to study direct effects (on activity of purified enzymes) and indirect effects (activity in cell extract, transcript levels) of sulfur-containing chemopreventive compounds on redox enzymes. Furthermore, transcriptional changes of players connected to cell cycle arrest and apoptosis were monitored.

Disulfide reductases as early targets of isothiocyanates

All six chemopreventive compounds chosen are rather lipophilic, and therefore able to pass cell membranes and to interact with cytosolic enzymes. Thus, a direct interaction between thiol-dependent enzymes and the sulfur-containing compounds might be one of the early events after treatment.

According to our kinetic data on isolated enzymes, GR and TrxR are target enzymes for the direct interaction with isothiocyanates (Table 2). As studied in detail for BITC and hTrxR as an example for the isothiocyanates, this inhibition has an initial competitive component followed by a time-dependent irreversible modification. This is also reflected in the cell culture data (Figure 1) which show a dose-dependent decrease of enzyme activities. For DADS and DATS, no marked inhibition of disulfide reductases – neither on isolated enzymes nor in cell culture – could be observed.

GR and TrxR belong to the same family of FAD-dependent homodimeric oxidoreductases. Both enzymes are reduced by NADPH and have a similar four-domain architecture including a redox-active disulfide-dithiol active site (Williams, 1992). The major difference between the two proteins is the additional cysteine-selenocysteine motif of hTrxR which is located on a flexible C-terminal extension, picks up electrons from the N-terminal redox center and transfers them to a broad range of substrates (Arscott et al., 1997). Thus, TrxR is highly susceptible to modifications by electrophilic compounds (Gromer et al., 2004). Isothiocyanates act with their electrophilic central carbon and can be enzymatically and non-enzymatically transformed into their dithiocarbamates in the presence of GSH (Kolm et al., 1995; Kong et al., 2001). Their terminal N=C=S formation, being rather reactive, might also be able to interact with the thiol-redox centers of disulfide reductases (Heiss and Gerhauser, 2005). Our enzymatic data after several steps of dialysis indeed confirmed an irreversible inhibition of NADPH-reduced TrxR and GR. The NADPH-dependency, i.e., the prereduction of the enzyme as prerequisite for the inhibition, was observed earlier for different TrxR and GR inhibitors and indicates that the active site of the protein must be reduced to be covalently modified (Becker et al., 2001; Deponte et al., 2005; Urig et al., 2006). Covalent modification of intracellular disulfide reductases is likely to decrease their activity irreversibly and to accelerate their degradation. Several studies have reported an upregulation of TrxR upon treatment with SF (Hintze et al., 2003a,b, 2005; Zhang et al., 2003; Heiss and Gerhauser, 2005; Hintze and Theil, 2005; Wang et al., 2005; Brigelius-Flohé and Banning, 2006; Jakubikova et al., 2006; Bacon et al., 2007; Campbell et al., 2007). Our study shows a dose-dependent inhibition of TrxR by the three isothiocyanates and by SF, as demonstrated on the isolated enzyme and in A549 lung adenocarcinoma cells after 24 h treatment. In the cells, this inhibition was paralleled by a trend towards upregulation of TrxR transcript levels (Table 4). As shown by Heiss and Gerhauser (2005), and also in our own studies (Becker et al., 2001), an inhibition of TrxR can lead to a compensatory upregulation of the enzyme at a later time point. This upregulation clearly depends on the cell type as, e.g., the sensitivity of Caco-2 cells to SF was much lower (150%) than of HepG2 cells (Bacon et al., 2007). Thus also in our experiment, the observed enzyme inhibition is likely to induce a long-term compensatory upregulation. In our

study, this compensatory upregulation of transcript levels had just begun after 24 h and was not yet reflected on the protein activity level. Therefore, the differences between our and other studies do not reflect contradictory results. Rather, they are due to the cell type and the exposure times chosen and represent early and late events of the same biological phenomenon.

The realization of enhanced selenoprotein transcripts depends on adequate selenium supply. Because we demonstrated a marked upregulation of the selenoprotein glutathione peroxidase 1 (cytosolic GPx; GPx1), the selenium concentration of approximately 30 nM in the cell medium should be high enough to maintain a functional selenoprotein pool and allow regulation. Upregulation of TrxR activity was previously shown to be the highest at nanomolar selenium levels and lower micomolar SF levels (Hintze et al., 2005). On the other hand, selenium did not affect SF-induced TrxR1 mRNA levels in a different report (Wang et al., 2005). This is also described as selenium-independent induction of TrxR, i.e., induction of TrxR by oxidative stress mediated by a DNA response element in the TrxR promoter region (Hintze et al., 2003b).

Heiss and Gerhauser (2005) described SF as a novel inhibitor of TrxR enzymatic activity *in vitro*. A short-term treatment of macrophages with SF resulted in the inhibition of TrxR activity *in vivo* with a half-maximal inhibitory concentration of 25.0±3.5 μM, whereas after a 24-h treatment with 25 μM SF, TrxR activity was more than 1.5-fold elevated. Our kinetic data on isolated enzyme support these previous reports and demonstrate that after an initial competitive inhibition of TrxR, SF leads to the irreversible modification of hTrxR.

The induction of TrxR production on the mRNA level can be a consequence of long-lasting inhibitory effects in cells. The cellular consequences of TrxR inhibition is realized by different events, such as decrease of TrxR and Trx activity, increased production of ROS and decreased levels of GSH (Nordberg and Arner, 2001). As a consequence of decreased levels of reduced Trx, in part due to decreased levels of TrxR and increased cellular oxidative stress, the expression of Trx and TrxR can be induced (Nordberg and Arner, 2001). For TrxR, this is supported by a constitutively high transcription in combination with oxidant-induced stabilization of TrxR mRNA (Rundlof et al., 2001; Rundlof and Arnér, 2004). If no restoration by overexpression events takes place, cells undergo apoptosis or necrosis. In our study, SF and the other chemopreventive compounds tested led to an increase of Trx mRNA. For all compounds, except AITC, also a trend towards overexpression of TrxR was determined.

The question is, under which conditions an upregulation of TrxR is really desirable, having in mind that activators of TrxR and Trx may ultimately promote tumor growth, effectively outweighing any beneficial antioxidant properties. In an established tumor, TrxR overexpression may contribute to drug resistance. Here, inhibition of TrxR may support a successful single, combinatory or adjuvant cancer therapy (Urig and Becker, 2006).

In contrast to the direct interaction of isothiocyanates with TrxR and also GR, the allyl sulfides tested in this study were much less reactive on the isolated enzymes. Thus, the mechanism of action of the allyl sulfides seems to differ fundamentally from the isothiocyanates. The direct inhibitory effects on isolated GR and TrxR were – even after incubation – weak. In cell culture, GR was only slightly inactivated (<10%) at the highest allyl sulfide concentrations tested. Interestingly, DADS, and to a lower extent DATS, rather increased the activity of TrxR after 24 h in the cell culture. This increase was accompanied by a slight increase on mRNA level. Overexpressed TrxR is expected to support the development of drug resistance in cancer cells, to protect cells from ROS and to induce cellular differentiation (Becker et al., 2000; Nalvarte et al., 2004; Smart et al., 2004).

GST is not a major direct target of sulfur-containing chemopreventive compounds

With maybe the exception of BITC (IC_{50}=390±60 μM), none of the six compounds tested inhibited GST directly at biologically meaningful concentrations (all IC_{50}s >500 μM). For BITC and SF a time-dependent modification could be determined, but with low k_2 values of 7 and 0.76 M^{-1} s^{-1}. A contribution of this inhibition to the biological effects of the compounds can however not be excluded. As shown previously, GST incorporates 2 mol of BITC per mol of enzyme subunit and mass spectrometric analysis after proteolytic digests revealed that Tyr103 and Cys47 are modified equally by the inhibitor (Ralat and Colman, 2004). The inhibition of GST by BITC was still too weak to become evident on cell culture level (Figure 1D). Treatment with isothiocyanates leads to a moderate but significant induction of GST on the mRNA level (Figure 1D). For the higher concentrations of the allyl sulfides, an upregulation of GST transcripts became more pronounced, which might be a result of an enhanced oxidative challenge induced by these compounds. Such an induction is explainable through induction of phase II detoxification enzymes as one intensely studied mechanism for the preventive action of isothiocyanates. This was also shown previously for DADS and DATS (Singh et al., 1998; Wu et al., 2002; Fukao et al., 2004) and is modulated through CAR (constitutive androstane receptor) and Nrf2 (nuclear factor E2-related factor2) activation (Fisher et al., 2007).

Upregulation of GPx is likely to contribute to the chemopreventive effects of allyl sulfides

Both allyl sulfides significantly and dose-dependently increased GPx activity in cell culture and upregulated GPx1 transcript levels at all concentrations tested. Increased levels of ROS are known to occur shortly after DADS treatment and represent an initial effect of garlic oil extracts (Wu et al., 2005b). Thus, the enhanced expression of GPx is likely to represent a compensatory mechanism for protecting cells against oxidative damage (Lei, 2001). Direct inhibitory effects on GPx activity were hardly observed for any of the compounds tested. A total of 200 μM DADS led to a maximal GPx inhibition of 20% after 30 min incubation. Although this is a weak interaction, and we observed a maximal upregulation of transcript levels by a factor of 1.4 at the highest DADS con-

centration, it cannot be excluded that this inhibitory effect on GPx contributes to the long-term induction of the enzyme.

Isothiocyanates did not act markedly on GPx under chosen conditions – neither on the isolated enzymes, nor in cell culture or on GPx transcript levels (maximal elevation 130%). The concerted regulation of GR, TrxR, GPx, GST, glutathione and other redox components define the complexity of the redox regulation that defends an organism against oxidative and electrophilic species. These protective regulations principally work in both healthy and transformed cells; however, malignant cells are likely to be more susceptible to oxidative stress and disturbed redox equilibria (Xiao et al., 2003; Nair et al., 2007). This is likely to contribute to the cytotoxic effects of chemopreventive compounds against tumor cells. However, in the long term, induction of (mild) oxidative stress and/or inhibition of antioxidant enzymes are likely to enhance the antioxidant capacity of cells. This might contribute to the chemopreventive effects of sulfur-containing compounds on healthy cells.

GSH depletion by DADS

The role of ROS in DADS-modulated apoptosis and cell cycle arrest as well as a DADS-dependent increase of ROS production in A549 cells have been reported previously (Wu et al., 2005b). Under conditions of oxidative stress, the elevated ROS levels are often accompanied by decreased levels of GSH after short-term treatment, and thus impairing the redox capacity of the glutathione system and promoting glutathionylation (Ghezzi et al., 2002; Miyamoto et al., 2003). The modulation of cellular sulfhydryl homeostasis has been described to be modulated by allyl sulfides by Pinto et al. (2006). Alkylation and short-term depletion of cellular glutathione was also previously shown for isothiocyanates and results in a delayed but prolonged increase in GSH levels in isothiocyanate-treated cells (Zhang et al., 2006b). The direct interaction between DADS and GSH, as described in this study, may additionally contribute to the prooxidant effects of the compound and to the induction of antioxidant response enzymes, such as GPx and GST.

Cell signaling pathways are modulated by sulfur-containing compounds

The modulation of thiol-dependent enzymes as well as Trx and glutathione metabolism has significant influence on cell cycle regulation, apoptosis and necrosis (Filomeni et al., 2002; Gomez et al., 2004). Therefore, thiol-dependent redox systems may be targeted by chemoprevention and chemotherapy (Nair et al., 2007).

As the induction of cell cycle arrest and/or apoptosis are among the most attractive discussed mechanisms for the preventive action of isothiocyanates and allyl sulfides, we studied changes on the transcriptional level of genes connected to these cellular events (Brigelius-Flohé and Banning, 2006; Gamet-Payrastre, 2006; Myzak and Dashwood, 2006a,b; Zhang et al., 2006b; Juge et al., 2007; Nair et al., 2007; Shukla and Kalra, 2007). Previous reports indicate that sulfur-containing compounds target molecules, such as MAPKs (JNK, ERK1/2 and P38), P53, NF-κB, members of the BCL-2 family and caspases (Wu et al., 2005b). Thus, we were interested in the effects of sulfur-containing chemopreventive compounds on the mRNA levels of NDRG1, GADD45A, XIAP and survivin in addition to P53 and CDKN1A.

Indeed, for CDKN1A, NDRG1 and GADD45A a significant upregulation was found in the cancer cell line A549 under treatment with allyl sulfides and isothiocyanates. CDKN1A (P21) is a cyclin-dependent kinase inhibitor acting in a P53-dependent manner. This cyclin-dependent kinase inhibitor was, e.g., shown previously to be upregulated by SF (Myzak et al., 2004; Parnaud et al., 2004) and BITC (Zhang et al., 2006a). Although the main role of CDKN1A is thought to be exerted during the G1 phase of the cell cycle, accumulating data have shown that it could be required for the G2/M block induced by isothiocyanates and allyl sulfides (Gamet-Payrastre, 2006; Shukla and Kalra, 2007). CDKN1A expression was previously shown to be induced by SF dose-dependently in parallel to increasing histone acetylation. Moreover, SF increased the amount of acetylated histone H4 associated with the CDKN1A promoter (which lacks an ARE) indicating a direct effect of deacetylase inhibition on CDKN1A transcription (Myzak et al., 2004). This theory is in accordance with our data, as CDKN1A transcription was significantly upregulated by all of the used sulfur-containing chemopreventive compounds. However, histone deacetylase inhibition will have to be analyzed for the used isothiocyanates and allyl sulfides in a comparative manner. In response to a variety of cellular stressors, it was shown that CDKN1A leads to apoptosis (Ueno et al., 1999; Smith et al., 2000).

Regulations of NDRG1 are observed under different physiological and pathophysiological conditions, such as hypoxia, cellular differentiation, heavy metal intoxication and neoplasia (Lachat et al., 2002). NDRG1 is repressed in neoplastic disease and its upregulation decreases tumor malignancy (Qu et al., 2002). Furthermore, it has been demonstrated that NDRG1 is necessary but not sufficient for P53-dependent caspase activation and apoptosis (Stein et al., 2004). GADD45a, an 18-kDa protein, is induced by genotoxic compounds and other cellular stressors and appears to be an important component in the cellular defense network to maintain genomic stability (Zhan, 2005). It can be regulated by P53, but is able to work also in a P53 independent way. Therefore, it has been characterized as one of the important players that participate in the intricate cellular responses to genotoxic stress (Zhan, 2005).

The upregulation of the transcript levels of these four proteins indicates the activation of pathways connected to cell cycle arrest and apoptosis of the treated A549 cells. Although the proteins are reported to be P53 downstream genes, changes in P53-mRNA levels did not reach the level of significance in our study. However, other authors have reported that P53 may be regulated by the protein level and/or phosphorylation after treatment with sulfur-containing compounds (Huang et al., 1998; Hong et al., 2000; Kuang and Chen, 2004).

The present results suggest that studied sulfur-containing chemopreventive compounds inhibit the proliferation of A549 cells via cell cycle arrest and apoptosis in

association with the modulation of Trx and glutathione networks. Allyl sulfides seem to primarily induce oxidative stress, which is likely to be supported by the direct reaction of the compounds with GSH (see also Pinto et al., 2006). This oxidative stress might result in the observed upregulation of GPx and GST. As shown by Fisher et al. (2007), phase 2 enzymes can be induced by allyl sulfides, which are modulated through CAR and Nrf2. In addition, isothiocyanates are likely to interact directly with GR and TrxR – and potentially with other physiological thiols – which finally also results in a disturbed redox balance. The disturbed redox balance is then obviously sensed by redox-sensitive transcription factors, such as Nrf2, AP-1 and NF-κB leading to specific transcriptional changes, which consequently end in cell cycle arrest and/or apoptosis. Owing to their high metabolic and proliferation rate, tumor cells are particularly susceptible to a disturbed redox equilibrium. In healthy cells, a continuous slight oxidative stress is rather expected to trigger antioxidative defense systems, and thus protecting from cancer development.

Materials and methods

Chemicals and culture medium

DADS, purity ≥80% (GC), was purchased from Sigma (St. Louis, MO, USA). DATS [purity ≫70% (GC) and (Lan and Lu, 2003)] was from Shanghai Harvest Pharmaceutical Co. Ltd. (Shanghai, China). AITC, BITC and PEITC were obtained from Fluka [Buchs, Switzerland; AITC: purity ≥98% (GC), BITC: purity ≥97.0% (GC), PEITC: purity ≥98.0% (GC)]. Sulforaphane-D,L, purity ≥90% (HPLC), was from Sigma (Munich, Germany). Low glucose Dulbecco's modified Eagle's Medium (DMEM), fetal calf serum (FCS) and antibiotics (penicillin, 100 U/ml; streptomycin 0.1 mg/ml) were from Gibco (Paisley, UK). All reagents were dissolved in filter-sterilized dimethyl sulfoxide (DMSO; Sigma) prior to addition to cultures (final concentration in the medium maximum, 0.5%).

Cell culture

Non-small cell lung cancer A549 cells were provided by the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). A549 cells were cultivated in DMEM with 10% FCS (containing 30 nM selenium as determined by atom absorption spectroscopy) enriched with penicillin and streptomycin at 37°C, 5% CO_2. Cells were sub-cultured and 2.5×10^5 cells were seeded in 25 cm^2-wells. When the attached cells reached 40–45% confluency, cells were treated with 25, 50, 100 or 200 μM DADS; 6.25, 12.5, 25 or 50 μM DATS; 4, 8, 16 or 32 μM AITC; 1, 5 or 10 μM BITC; 12.5, 25, 50 or 100 μM PEITC or with 6, 12 or 18 μM SF, for 24 h. The dosages of the different compounds were chosen on the basis of preliminary inhibitory tests (screenings on 96-well microtiter plates; less than 10% cell death in the culture after 24 h incubation) and data from the literature (Sundaram and Milner, 1996a,b; Sakamoto et al., 1997; Otsubo et al., 2004; Heiss and Gerhauser, 2005; Wu et al., 2005a,b). Control cells were treated with less than 5% DMSO. Also, the exposure time was chosen on the basis of previous reports (Heiss and Gerhauser, 2005; Hintze and Theil 2005; Hintze et al., 2005; Wang et al., 2005; Bacon et al., 2007; others have chosen a 24 h treatment period).

Cell harvesting and evaluation of cell viability

Cells were collected after 24 h treatment with the different agents. Cells were washed three times with cold phosphate buffered saline (PBS) and then trypsinized. Medium containing fetal bovine serum was used to deactivate the trypsin, followed by centrifugation at 400 *g* and 4°C for 4 min. Cells were resuspended in PBS and the numbers of viable and non-viable cells were counted by measuring Trypan Blue exclusion using a hemocytometer.

Measurement of DNA distribution and apoptosis

Cells were harvested and washed twice with ice-cold PBS before overnight fixation with 70% ethanol. After fixation, cells were washed twice with ice-cold PBS and stained with 50 μg/ml propidium iodide for 30 min before being analyzed in a FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA). Cells with sub-diploid DNA content (Sub-G1) were counted as apoptotic cells. At least 10 000 cells per sample were analyzed. The DNA histograms were acquired and analyzed with CellQuest Pro (Becton Dickinson) and Modfit (House, San Jose, CA, USA) to estimate cell cycle distribution by the percentage of sub-diploid DNA.

Quantitative real-time PCR

Total RNA was isolated with the Qiagen RNA miniprep kit (Qiagen, Hilden, Germany) and aliquots of 800 ng of each sample were reversely transcribed to cDNA (Reverse-iT™ RTase Blend, Abgene, Hamburg, Germany) using $p(dT)_{15}$ primers (Roche Diagnostics, Mannheim, Germany). The QuantiTect SYBR Green PCR kit (Qiagen) was used in the real time PCR approach on a Rotor-Gene 3000 real time PCR cycler (Corbett Research, Sydney, Australia). The required primers (Table 3) were designed in our laboratory or obtained from the Real Time PCR Database (RTPrimerDB Real Time PCR Primer and Probe Data, http://medgen.ugent.be/rtprimerdb/). The Rotor-Gene 6.0 software was used to analyze the PCR results and to determine cycle threshold values. All primers used were tested previously in a normal PCR to ensure their target gene specificity. Data collected were based on the threshold cycle and the reaction efficiency for target genes and reference genes in treated cells and untreated control cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 18S rRNA (ribosomal RNA) were used as internal reference genes. In our experiments, each RT-PCR run was carried out in quadruplet and in general, the whole series was reproduced in an independent experiment. Using this approach the obtained data usually differed within a range of ±30%.

Determination of TrxR activity

Human placenta TrxR was purified and analyzed as described previously with slight modifications (Gromer et al., 1998). The K_m value of the enzyme preparation for hTrxC73S was determined to be 13 μM, which is in accordance with previous reports (Urig et al., 2006).

To study TrxR activity, two different assay systems were used at 25°C, either 200 μM NADPH or 3 mM DTNB were used as substrates in TrxR assay buffer (100 mM potassium phosphate, 2 mM EDTA, pH 7.4) and the production of TNB^- anions ($\varepsilon_{412\,nM}$=13.6 $mM^{-1}\,cm^{-1}$) was followed spectrophotometrically. In parallel assays, TrxR activity was determined using its physiological substrate Trx (20 μM of recombinant, completely oxidized TrxC73S; this mutant does not form inactive dimers in the assay) and 100 μM NADPH and the initial change in absorbance was followed at 340 nM (Ahmadi et al., 2006).

Table 3 Overview of transcripts studied by quantitative real-time PCR.

	Gene	GenBank acc. no.	Sense (5′→3′)	Antisense (5′→3′)
Housekeeping gene	GAPDH (glyceraldehyde-3-phosphate dehydrogenase)	NM_002046	GTGGTCTCCTCTGACTTCAACA	CTGTAGCCAAATTCGTTGTCATAC
	18S rRNA (ribosomal RNA)	M10098	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
	CAT (catalase)	NM_001752.2	TTTCCCAGGAAGATCCTGAC	ACCTTGGTGAGATCGAATGG
Redox-related genes	GPx1 (cytosolic GPx, glutathione peroxidase1)	NM_000581	GTTCGAGCCCAACTTCATGCT	ATCGTTGCGACACACCGGAG
	GPx2 (gastrointestinal GPx, glutathione peroxidase2)	AY785560.1	TAAGTGGGCTCAGGCCTCTCT	GGTCATAGAAGGACTTGGCAATG
	GR (glutathione reductase)	X15722	AGACCTATTCAACGAGCTTTACC	CCTGCAGCATTTCATCACACC
	GSTpi (glutathione S-transferase pi)	NM_000852.2	GGAGACCTCACCCTGTACCA	GGGCAGTGCCTTCACATAGT
	SOD1 (superoxide dismutase 1)	AY450286.1	AGGGCATCATCAATTTCGAG	TGCCTCTCTTCATCCTTTGG
	SOD2 (superoxide dismutase 2)	NM_001024465.1	AAGGGAGATGTTACAGCCCAGATA	TCCAGAAAATGCTATGATTGATATGAC
	Trx1 (thioredoxin1)	NM_003329	GGATGACTGTCAGGATGTTGC	ATTCATTAATGGTGGCTTCAAGC
	TrxR1 (thioredoxin reductase 1)	NM_003330	TGCCACTGGTGAAAGACCAC	CAAGAAATCCAGCGCACTCC
	P53 (tumor suppressor P53; TP53)	AY429684.1	TCAACAAGATGTTTTGCCAACTG	ATGTGCTGTGACTGCTTGTAGATG
Cell cycle and apoptosis-related genes	CDKN1A (cyclin-dependent kinase inhibitor 1 A)	NM_078467	GACCTGTCACTGTCTTGTACCCTT	GTAGAAATCTGTCATGCTGGTCTG
	SURVIVIN (baculoviral IAP repeat-containing 5, BIRC5)	NM_001168.2	CGAGGCTGGCTTCATCCACT	ACGGCGCACTTTCTTCGCA
	XIAP (X-linked inhibitor of apoptosis, baculoviral IAP repeat-containing 4, BIRC4)	NM_001167.2	CCGTGCGGTGCTTTAGTTGT	TTCCTCGGGTATATGGTGTCTGAT
Genes related to differentiation	NDRG1 (N-myc downstream regulated gene 1)	NM_006096	TGGAGATTGAGCGACCAATG	CACAGTCCGCCATCTTGAG
	GADD45A (growth arrest and DNA-damage-inducible, α)	NM_001924.2	AGTCAGCGCACGATCACTGT	TGTTGATGTCGTTCTCGCAG

Table 4 Changes of transcript levels of redox, cell cycle and apoptosis-related genes after treatment with allyl sulfides and isothiocyanates.

	Treatment (μM)																						
	DADS				DATS				AITC				BITC			PEITC				SF			
	25	50	100	200	6.25	12.5	25	50	4	8	16	32	1	5	10	12.5	25	50	100	3	6	12	18
TrxR	1.2	1.4	1.0	1.4	1.0	1.3	1.5	1.5	0.9	0.7	0.6	0.8	1.4	1.0	1.3	1.5	1.2	1.0	1.2	1.1	1.0	1.0	1.3
Trx	1.3	1.6	2.0	2.5	1.2	1.6	1.9	2.3	1.2	1.3	1.4	1.7	1.0	1.0	1.4	1.8	1.6	1.4	1.6	1.4	1.6	nd	nd
GR	nd	nd	1.4	1.2	1.0	1.4	0.9	0.8	0.7	0.9	0.7	0.8	1.6	1.3	1.1	1.8	1.7	0.9	0.8	1.0	0.8	0.5	0.7
GPx1	2.5	3.4	3.1	2.7	2.1	2.4	2.1	2.3	1.1	0.9	1.1	1.1	0.9	0.6	1.1	1.3	1.2	1.3	1.3	1.0	0.9	0.9	1.1
GSTpi	1.6	1.9	2.3	2.5	1.2	1.9	2.3	2.2	1.1	1.0	1.7	1.8	1.1	1.5	1.5	1.4	0.9	1.2	1.4	0.9	0.6	1.1	1.7
SOD1	1.0	1.0	1.3	1.3	1.2	1.4	1.3	1.2	nd	1.2	1.3	1.2	0.9	1.1	1.2	1.6	1.4	1.0	1.8	1.0	nd	1.7	1.9
SOD2	1.2	1.2	1.5	1.5	1.7	2.4	1.8	1.7	nd	1.7	1.4	1.6	1.3	1.6	1.6	1.9	1.6	nd	1.6	1.2	nd	1.1	2.4
CDKN1A	1.1	2.3	3.5	1.8	1.8	2.6	2.2	2.2	1.1	2.3	2.0	3.0	1.0	1.6	3.3	2.6	2.3	3.2	5.3	0.8	1.8	4.0	3.6
NDRG1	1.4	1.7	1.6	1.1	1.2	2.2	2.0	3.2	1.7	1.5	1.1	2.9	0.9	0.7	1.1	1.7	1.3	0.9	1.6	1.0	0.9	1.0	2.3
GADD45A	0.9	1.6	3.2	2.3	1.9	3.8	4.3	8.9	1.5	1.5	2.7	2.5	1.1	1.4	5.2	1.9	1.6	1.8	4.1	1.1	1.7	3.6	2.0
P53	1.3	1.4	0.8	0.9	0.8	0.8	0.6	0.7	0.9	0.8	1.0	1.1	1.1	1.1	0.8	1.6	1.7	1.3	1.8	1.1	1.0	1.4	1.4

All values were determined in quadruplet within one experiment. All values represent means of at least two independent experiments. Using this approach the obtained data usually differed within a range of ±30%. This means that from values between 0.7 and 1.3 only trends can be deduced. The transcript levels of GPx2, CAT, survivin and XIAP were not found to be markedly (>30%) regulated and did not show a clear and reproducible trend under drug treatment. All values shown in this paper were normalized for GAPDH. However, for all transcripts the same trends were observed when normalizing for 18S rRNA (data not shown).

To measure TrxR activity in cell extracts, lysates ($\geq$40 µl) were incubated in a total volume of 400 µl with 3 mM DTNB in TrxR assay buffer for at least 60 s to allow for the oxidation of endogenous thiols. Once the initial reaction was completed and a stable background reaction was obtained, the TrxR-specific reaction was started with 200 µM NADPH. The background reaction, which was regularly less than 10% of the TrxR catalyzed reaction, was subtracted in each assay (Ahmadi et al., 2006).

Determination of GR activity

Human recombinant GR (kindly provided by Prof. Heiner Schirmer, Heidelberg, Germany) was produced and assayed according to Nordhoff et al. (1993). Activity (in the cell-free system or cell extracts) was determined in GR assay buffer (20.5 mM KH_2PO_4, 26.5 mM K_2HPO_4, 1 mM EDTA, 200 mM KCl, pH 6.9) in the presence of 100 µM NADPH (ε_{340nM}=6.22 mM^{-1} cm^{-1}) and 1 mM GSSG as substrates. The change in absorbance at 340 nM was monitored spectrophotometrically at 25°C.

Determination of GPx activity

GPx from human erythrocytes was obtained from Sigma (G4013). GPx activity (in the cell-free system or cell extracts) was determined at 340 nM according to Beutler (1984). Assays were carried out in 100 mM Tris, 0.5 mM EDTA, pH 8.0, and in the presence of 1 U/ml human GR, 2 mM GSH and 100 µM NADPH at 25°C. *tert*-Butyl hydroperoxide (ad 70 µM) was used as substrate. Before adding the peroxide substrate, GSH, GR, NADPH and GPx were incubated together for 10 min at 25°C. Observed background reactions were subtracted. Because of various background reactions, reliable data for time-dependent inactivation of GPx could not be obtained.

GST activity

Human placenta pi class GST was obtained from Sigma (G8642). GST activity (in the cell-free system or cell extracts) was measured by the rate of increase in the concentration of S-dinitrophenylglutathione (DNB-S-GSH; $\varepsilon_{340\ nM}$=9.6 mM^{-1} cm^{-1}) generated by the reaction of 0.5 mM chloro-dinitrobenzene (CDNB) with 1 mM GSH in 84 mM KH_2PO_4, 16.6 mM K_2HPO_4, 1 mM EDTA, pH 6.5 (Beutler, 1984). For the time-dependent experiments, GST was incubated with inhibitor in assay buffer, and then CDNB was added followed by the addition of GSH.

Enzyme inhibition

To determine the direct influence of allyl sulfides and isothiocyanates on purified enzymes, the compounds were added to the enzymes (final concentration 2–10 mU/ml) in the respective assay mixtures (containing NADPH for GR and TrxR) and enzyme activity was measured directly (for IC_{50} values) or after 30 min of incubation. The concentrations of inhibitor leading to 50% enzyme inhibition were calculated for each compound and time point.

The type of inhibition was studied in more detail for hTrxR and BITC as this combination was identified to have the lowest IC_{50} value. For determining the inhibition constant (K_i) for the reversible component of the inhibition, approximately 3 nM hTrxR was mixed with 200 µM NADPH and variable inhibitor concentrations (5, 10, 20, 30, 40 µM) in assay buffer. The reactions were started with DTNB (20 µM–0.8 mM which was systematically varied against the inhibitor concentrations) at 25°C. In parallel, the K_m value for DTNB in the absence of inhibitor was determined. When representing the data in a Dixon or Lineweaver-Burk plot, the competitive component of the inhibition became evident. The K_i value for competitive inhibition of hTrxR by BITC was calculated according to the equation $K_i=K_m[I]/(K_m'-K_m)$ (Cornish-Bowden, 2001).

The time-dependent irreversible inhibition of TrxR, GR and GST by the two most efficient inhibitors, BITC and SF was studied at 25°C. For hTrxR, enzyme (76 nM) was incubated with 200 µM NADPH (to guarantee reduction of the active site) and BITC (0, 5, 10, 17, 33, 40 µM) or SF (0, 10, 20, 30, 40, 50, 100 µM). For GR, enzyme (40 nM) was incubated with 100 µM NADPH and BITC (30 and 50 µM) or SF (50 and 100 µM). For GST, enzyme (2.1 µM) was incubated with 0.5 mM CDNB and BITC (40 and 80 µM) or SF (0.5, 1, and 2 mM). For all incubations and assays the above standard assay buffers were chosen. At various time points (0, 2, 5, 10, 15, 20, 30 min) samples were drawn, assayed in the standard assays, and the percentage of inhibition in comparison with an untreated control was determined. The inhibitory reactions followed second order and could be described by the equation $v=k_2\times[I]\times[E]$ (Cornish-Bowden, 2001). Applying this equation to the different experimental series resulted in the k_2 values given in Table 2.

To determine enzyme activities in extracts of cells treated with the chemopreventive compounds, cells were harvested as described above and then sonicated for 3×10 s followed by centrifugation at 136 000 *g* and 4°C for 30 min. The Bradford assay was used to determine the protein concentration of the cell extracts. All enzyme activity assays were conducted at 25°C as described above.

Tests for irreversibility of TrxR and GR inhibition

For analysis of irreversible inhibition, hTrxR or hGR (ad 10 mU/ml) were incubated with 100 µM BITC in the presence or absence of 100 µM NADPH for 30 min and their residual activity was determined in the Trx-reducing assay and the GSSG-reducing assay, respectively, in comparison with a control sample. Then, the samples were dialyzed three times for 1 h at room temperature against 1000-fold volumes of buffer. Subsequently, the samples were tested for protein content and enzyme activities.

Reaction between GSH and DADS

Different concentrations of DADS (100–200 µM) were incubated with 0.4–2 mM GSH in GR assay buffer for 0, 15 and 30 min. Then, 150 µM NADPH was added, the initial absorbance was monitored and the reactions were started by adding GR (1 U/ml). The absolute changes in absorbance were determined at 340 nM and reflect the amount of GSSG generated during the incubation period. Adequate control samples (with only GSH and only DADS in DMSO, as well as NADPH oxidize activity of GR) were accounted for. On the basis of the time- and concentration-dependent reactions, rate constants were calculated.

Statistical analysis

Data are expressed as mean±SD. Statistical analysis was performed using SAS 8.0 software package (SAS, Cary, NC, USA). Parametric and non-parametric tests were applied according to the data collected. *p*-Values <0.05 were considered to be statistically significant.

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