RESEARCH ARTICLE

# Effect of sulforaphane on glutathione-adduct formation and on glutathione_*S*_transferase-dependent detoxification of acrylamide in Caco-2 cells

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The toxicity of dietary acrylamide (AA) depends on its biotransformation pathways, in which phase I cytochrome P-450 enzymes transform AA into glycidamide. The phase II enzyme glutathione_*S*_transferase (GST) catalyses the conjugation of AA with glutathione (GSH). GST induction by phytochemicals like sulforaphane (SFN) plays a role in chemoprevention. Here, the effect of SFN on the detoxification of AA through GSH conjugation was studied in Caco-2 cells. GSH adducts with AA and SFN were synthesized, identified by NMR and quantified by LC-MS/MS. Caco-2 cells were treated with either 2.5 mM AA, 10 μM SFN or the combination of both for 24 h. Concentrations of GSH conjugates (GSH-AA, GSH-SFN, SFN-GSH-AA), AA and SFN were analysed by LC-MS/MS. GSH contents and GST activity were determined photometrically. GST activity was increased after treatment of the cells with SFN ($38 \pm 6\%$, $p \leq 0.05$) or AA ($25 \pm 4\%$, $p \leq 0.05$). GSH concentrations decreased after all treatments. Quantitative data of GSH adduct formation showed that the reaction between GSH and SFN is favoured over that between GSH and AA. The data suggest that SFN might impair the GSH-dependent detoxification of AA by SFN-GSH adduct formation and, thus, lower the GSH concentrations available for its reaction with AA.

**Keywords:**
Acrylamide / Caco-2 cells / Glutathione-*S*-transferase / GSH adducts / Sulforaphane

## 1 Introduction

In April 2002, Tareke *et al.* described the formation of acrylamide (AA) in heated, protein- and carbohydrate-rich foods [1]. AA mainly derives from heat-induced reactions between the amino group of asparagine and the carbonyl group of reducing sugars such as glucose. High dietary AA intake has been demonstrated to cause neurotoxic effects [2, 3], initiate several types of cancers [4] and exhibit a genotoxic potential [5–7]. Therefore, the mitigation of AA in thermally processed foods is still sparking a growing body of interest worldwide.

Dietary AA is absorbed in the intestines, where it is transformed into the epoxide glycidamide (GA) by cytochrome P-450 enzymes localized in the enterocytes. GA is converted further downstream into 2,3-dihydroxypropionamide *via* the activity of epoxide hydrolases [6]. Both compounds, AA and GA, are conjugated to glutathione (GSH) by means of the chemopreventive phase II enzyme glutathione_*S*_transferase (GST) and, finally, excreted as mercapturic acids in the urine [8]. AA can likely bind covalently to GSH *via* Michael-type addition of the sulfydryl group in the cysteine moiety of GSH to the reactive terminal double bond of AA [9]. This reaction is favoured at alkaline pH values [10].

Epidemiological data have shown that a diet rich in cruciferous vegetables (*e.g.* broccoli, cabbage and brussels

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**Abbreviations: AA,** acrylamide; **GA,** glycidamide; **GSH,** glutathione; **GST,** glutathione_*S*_transferase; **PE,** papilloma; **SFN,** sulforaphane; **SRM,** selected-reaction monitoring

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sprouts) can reduce the risk of a number of cancers [11, 12]. The cancer chemopreventive activity of these vegetables is partially due to their content in glucosinolates [13]. Sulforaphane (SFN), the isothiocyanate (ITC) obtained from hydrolysis of the glucosinolate glucoraphanin, which occurs in many cruciferous vegetables, *e.g.* broccoli [13, 14], is a potent naturally occurring inducer of phase II enzymes involved in carcinogen detoxification.

Therefore, the general question arises whether cancer protection from AA-induced DNA damage could be accomplished by an induction of GST activity by chemopreventive food ingredients such as ITCs. This hypothesis is further supported by results reported by Lee *et al.* [15] who demonstrated that co-administration of 0.02% AA and 0.5% phenylethyl ITC to rats for 4 wk significantly reduced testicular toxicity compared with rats treated with 0.02% AA solely.

Considering the SFN chemopreventive activity and the important role of GSH in AA detoxification, the main objective of the present investigation was to evaluate whether SFN affects the non-enzymatic and enzymatic formation of AA and GSH adducts in Caco-2 cells. For this purpose, GST activity in Caco-2 intestinal cells was measured to evaluate the enzyme-dependent cellular detoxification of AA that results in GSH-AA adduct formation in the presence of SFN. Formation of GSH-AA, GSH-SFN and also SFN-GSH-AA was studied in Caco-2 cells, as well as in chemical *in situ* experiments with AA, SFN and GSH as reaction precursors. In all experiments, AA, SFN and GSH adducts were identified and quantified by means of LC-MS/MS.

## 2 Materials and methods

### 2.1 Chemicals

ACN (HPLC grade), AA, formic acid, boric acid, diethyl ether, 1,4-dithiothreitol, ethylenediaminetetraacetic acid, GSH, glycerol, sodium hydrogen carbonate, sodium hydroxide, methanol (LiChrosolv grade), hydrochloric acid (32%), trichloroacetic acid, dimethyl sulfoxide and 1-chloro-2,4-dinitrobenzene were purchased from Merck (Darmstadt, Germany). DL-SFN, trypan blue, HEPES and metaphosphoric acid were from Sigma-Aldrich (Deisenhofen, Germany). Deuterium oxide was obtained from Eurisotop (Saint-Aubin, France) while DMEM, streptomycin, penicillin, L-glutamine and FCS were from PAA Laboratories (Linz, Austria).

### 2.2 Chemical synthesis of GSH-AA, GSH-SFN and SFN-GSH-AA adducts

GSH-AA adduct was synthesized by dissolving 50 mg AA ($7 \times 10^{-4}$ mol) and 216.4 mg GSH ($7 \times 10^{-4}$ mol) in sodium hydrogen carbonate buffer (pH 10, 0.5 M) and stirring at room temperature for 1 h. The reaction was stopped by adjusting the pH to 5 using 32% hydrochloric acid. Synthesis of the GSH-SFN adduct was performed by applying the same procedure to 2.5 mg DL-SFN ($1.4 \times 10^{-5}$ mol) and 8.7 mg GSH ($2.8 \times 10^{-5}$ mol). To synthesize the SFN-GSH-AA adduct, 1.7 mg AA ($2.4 \times 10^{-5}$ mol) and 3.4 mg GSH ($1.1 \times 10^{-5}$ mol) were dissolved in boric acid buffer (pH 9.4, 0.2 M) and stirred at room temperature for 1 h. Subsequently, 1.5 mg DL-SFN ($8.5 \times 10^{-6}$ mol) was added and the solution stirred for another term of 1.5 h. Hydrochloric acid (32%) was used to adjust the pH to 5. The adduct structures are shown in Fig. 1. Furthermore, the effects of precursor concentrations, reaction time and pH on adduct formation were tested in experiments comprising precursor concentrations of 2.5 mM AA, 10 μM SFN and 30 μM GSH or equimolar concentrations (100 μM), reaction times of 2 and 24 h and pH values of 7 and 10 (in sodium hydrogen carbonate buffer).

### 2.3 Purification of GSH-AA, GSH-SFN and SFN-GSH-AA adducts

A semi-preparative RP-HPLC system (Bio-TEK Instruments, Eching, Germany) equipped with two pumps (type 522), a gradient mixer (M 800), a Rheodyne injector (250 μL loop) and an UV-detector (type 535) programmed at a wavelength of 210 nm was used for the purification of the GSH-AA, GSH-SFN and SFN-GSH-AA adduct. An analytical Aqua $C_{18}$ HPLC column (5 μm, 12.5 nm, 250 × 10 mm) equipped with a guard column ($C_{18}$, 4.0 × 2.0 mm, both columns purchased from Phenomenex, Aschaffenburg, Germany) as stationary phase as well as water/formic acid (0.1% v/v) and ACN, as A and B mobile phases, respectively, were used. For the isolation of GSH-AA, a flow rate of 2.5 mL/min and a gradient starting with 0% B, arriving at 4% B within 15 min and to 100% B within 20 min, were programmed. The gradient for the purification of GSH-SFN, at a flow rate of 2.5 mL/min, started with 0% B and arrived at 70% B within 20 min and at 100% B within 25 min. To isolate the adduct SFN-GSH-AA from the co-products GSH-AA and GSH-SFN, a flow rate of 3 mL/min was set. The gradient applied started at 0% B, arrived at 30% B within 10 min, at 70% B within 40 min and ended at 100% B within 45 min. The effluent of each pure adduct was collected, pooled and freeze-dried.

### 2.4 Chemical characterization of GSH-AA, GSH-SFN and SFN-GSH-AA adducts

The molecular masses of the compounds were confirmed by MS analysis and chemical structures were identified by NMR analysis.

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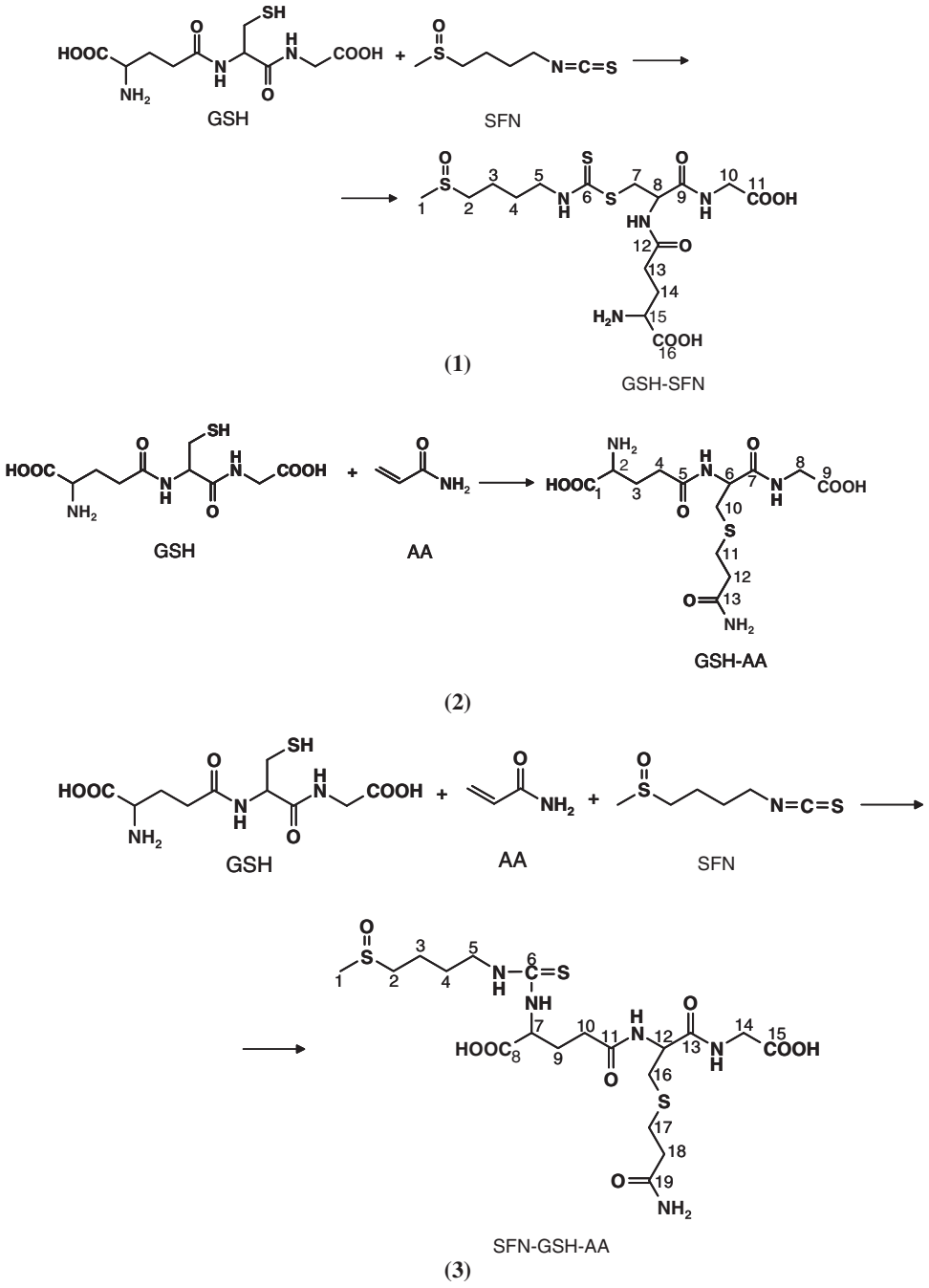


**Figure 1.** Formation pathways and structures of adducts chemically synthesized from GSH, SFN and AA: GSH-SFN (1), GSH-AA (2) and SFN-GSH-AA (3). Carbon atoms of the adducts are numbered arbitrarily for the interpretation of NMR data in the Section 2.

### 2.4.1 MS analyses

For the MS analyses, the samples were dissolved in 0.1% formic acid and were directly injected into the ion source of an ion trap mass spectrometer (LCQ Classic, Thermo Electron, Waltham, USA). An ESI with positive polarity at a flow rate of 5 μL/min, a sheath gas flow rate of 60 arbitrary units, an auxiliary gas flow rate of 5 arbitrary units, a spray voltage of 5 kV, a capillary temperature of 200°C and a capillary voltage of 13 V, respectively, was set.

Compound identification was performed considering the following *m/z* signals as characteristic for each compound: *m/z* 379 for GSH-AA, *m/z* 485 for GSH-SFN and *m/z* 556 for SFN-GSH-AA (Fig. 2). Selected-reaction monitoring (SRM) mass chromatograms of GSH-AA (*m/z* 379→250), GSH-SFN (*m/z* 485→136) and GSH-SFN-AA (*m/z* 556→104) using positive ion LC-ESI-MS/MS were obtained from chemically synthesized standards (Fig. 2, 1), blank cytosol from non-treated cells (Fig. 2, 2) and cytosol of Caco-2 cells treated with AA and SFN (Fig. 2, 3).

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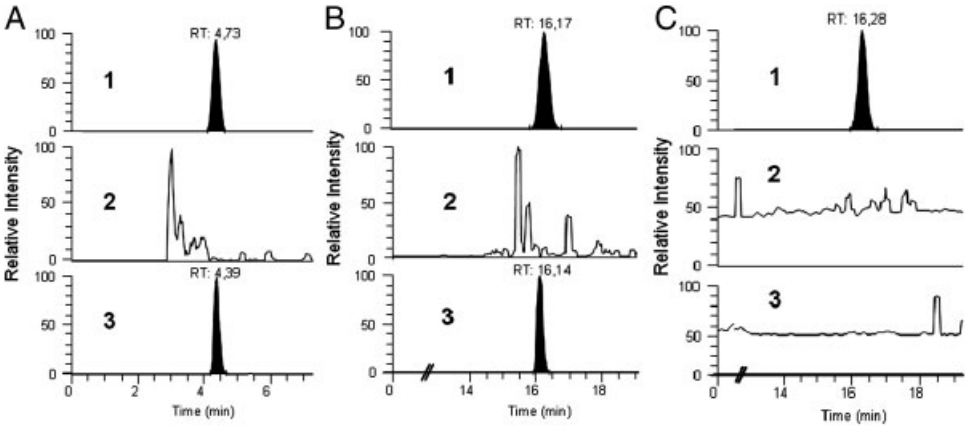


**Figure 2.** SRM mass chromatograms of (A) GSH-AA (*m/z* 379→250), (B) GSH-SFN (*m/z* 485→136) and (C) GSH-SFN-AA (*m/z* 556→104) using positive ion LC-ESI-MS/MS. Chromatogram 1: chemically synthesized standards, 2: blank cytosol (control), 3: cytosol of Caco-2 cells incubated with AA and SFN.

### 2.4.2 NMR analyses

$^{1}H$, $^{13}C$, DQF-COSY, HMQC and HMBC spectroscopies were performed using an AMX 400 spectrometer (Bruker BioSpin, Rheinstetten, Germany) after dissolving the synthesized adducts in deuterium oxide. Chemical shifts are reported relative to residual $H_2O$ (δ 4.65 ppm). Chemical structures of the adducts were identified considering the parameters reported below for each compound.

*GSH-AA*: $^{1}H$ NMR and DQF-COSY (400 MHz, $D_2O$; arbitrary numbering of the carbon atoms refers to structure 2 in Fig. 1) δ 2.23 ppm [m, 2 H, CH, C(3)], δ 2.58 ppm [m, 4 H, 2 × CH, C(4) and C(12)], δ 2.82 ppm [t, 2 H, CH, C(11)], δ 2.89 ppm and 3.06 ppm [2 × dd, 2 H, CH, C(10)], δ 4.01 ppm [s, 2 H, CH, C(8)], δ 4.08 ppm [t, 1 H, CH, C(2)], δ 4.58 ppm [t, 1 H, CH, C(6)]; $^{13}C$ NMR (400 MHz, $D_2O$, HMQC, HMBC; arbitrary numbering of the carbon atoms refers to structure 2 in Fig. 1) δ 26.10 ppm [CH, C(3)], δ 28.00 ppm [CH, C (11)], δ 31.00 ppm [CH, C(4)], δ 33.00 ppm [CH, C(10)], δ 35.10 ppm [CH, C(12)], δ 41.30 ppm [CH,C(8)], δ 52.00 ppm [CH, C(2)], δ 53.00 ppm [CH, C(6)], δ 172.46 ppm [C, C(1)], δ 173.00 ppm [C, C(7)], δ 173.70 ppm [C, C(9)], δ 174.90 ppm [C, C(5)], δ 174.58 ppm [C, C(13)].

*GSH-SFN*: $^{1}H$ NMR and DQF-COSY (400 MHz, $D_2O$; arbitrary numbering of the carbon atoms refers to structure 1 in Fig. 1) δ 1.62 ppm [m, 4 H, CH, C(3) and C(4)], δ 2.24 ppm [m, 2 H, CH, C(14)], δ 2.59 ppm [t, 2 H, CH, C(13)], δ 2.78 ppm [s, 3 H, CH, C(1)], δ 3.01 ppm [t, 2 H, CH, C(2)], δ 3.67 and 3.97 ppm [dd, 2 H, CH, C(7)], δ 3.87 ppm [m, 2 H and 1 H, CH, C(15) and C(5)], δ 4.04 ppm [s, 2 H, CH, C(10)], δ 4.88 ppm [t, 1 H, CH, C(8)]; $^{13}C$ NMR (400 MHz, $D_2O$, HMQC, HMBC; arbitrary numbering of the carbon atoms refers to structure 1 in Fig. 1) δ 20.06 ppm [CH, C(3)], δ 26.51 ppm [CH, C(4)], δ 26,51 ppm [CH, C(14], δ 31.81 ppm [CH, C(13)], δ 35.20 ppm [CH, C(1)], δ 36.07 ppm [CH, C(7)], δ 42.10 ppm [CH, C(10)], δ 47.00 ppm [CH, C(5)], δ 52.80 ppm [CH, C(2)], δ 53.60 ppm [CH, C(8)], δ 54.40 ppm [CH, C(15)], δ 172.40 ppm [C, C(9)], δ 174.05 ppm [C, C(16)], δ 174.20 ppm [C, C(11)], δ 175.12 ppm [C, C(12)], δ 197.30 ppm [C, C(6)].

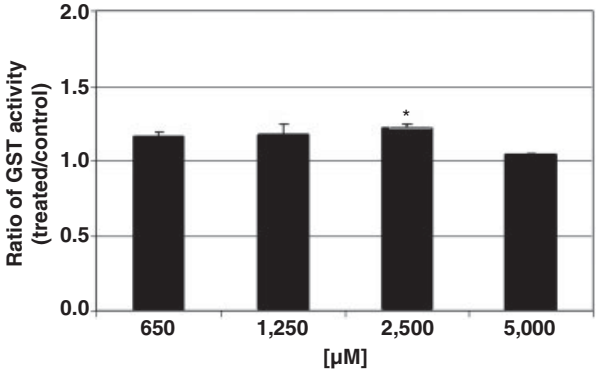


**Figure 3.** Relative activity of GST in Caco-2 cells treated with different concentrations of AA for 24 h (non-treated control cells = 1). Data are presented as mean values ± SD from ≥ three biological replicates (*$p < 0.05$ *versus* control cells).

*SFN-GSH-AA*: $^{1}H$ NMR and DQF-COSY (400 MHz, $D_2O$; arbitrary numbering of the carbon atoms refers to structure 3 in Fig. 1) δ 1.71 ppm [m, 4 H, CH, C(3) and C(4)], δ 2.15 ppm [m, 2 H, CH, C(9)], δ 2.38 ppm [m, 2 H, CH, C(10)], δ 2.52 ppm [t, 2 H, CH, C(18)], δ 2.62 ppm [s, 3 H, CH, C(1)], δ 2.78 [t, 2 H, CH, C(17)], δ 2.86 ppm [m, 2 H and 1 H, CH, C(2) and C(16)], δ 3.03 ppm [dd, 1 H, CH, C(16)], δ 3.80 ppm [m, 4 H, CH, C(14) and C(5)], δ 4.35 ppm [t, 1 H, CH, C(7)], δ 4.50 ppm [m, 1 H, CH, C(12)]; $^{13}C$ NMR (400 MHz, $D_2O$, HMQC, HMBC; arbitrary numbering of the carbon atoms refers to structure 3 in Fig. 1) δ 19.49 ppm [CH, C(3)], δ 26.90 ppm [CH, C(4)], δ 27.70 ppm [CH, C(17)], δ 31.77 ppm [CH, C(10)], δ 33.30 ppm [CH, C(16)], δ 35.30 ppm [CH, C(18)], δ 36.10 ppm [CH, C(1)], δ 42.80 ppm [CH, C(14)], δ 43.00 ppm [CH, C(5)], δ 51.90 ppm [CH, C(12)], δ 52.00 ppm [CH, C(7)], δ 52.10 ppm [CH, C(9)], 52.50 ppm [CH, C(2)], δ 172.00 ppm [C, C(13)], δ 175.30 ppm [C, C(15)], δ 175.70 ppm [C, C(6)], δ 176.10 ppm [C, C(11)], δ 177.50 ppm [C, C(8)], δ 177.7 ppm [C, C(19)].

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### 2.5 LC-MS/MS

HPLC-MS/MS analyses were carried out on a Finnigan Surveyor Plus HPLC-system (Thermo Electron) equipped with a triple-quadrupole mass spectrometer (TSQ Quantum Discovery; Thermo Electron). ESI in positive mode and SRM with a spray voltage of 3.5 kV, a sheath gas pressure of 4.7 Pa, an auxiliary gas pressure of 2.0 Pa, a capillary temperature of 320°C and a capillary offset of 35 V were selected. An Aqua $C_{18}$ column (5 μm, 12.5 nm, 150 × 2 mm, 0.2 mL/min) with a guard column ($C_{18}$, 4.0 × 2.0 mm; both from Phenomenex) was used to separate GSH-AA, GSH-SFN and SFN-GSH-AA. UV detector wavelength was set at 210 nm. A Discovery HS F5 column (5 μm, 12.0 nm, 150 × 2 mm, 0.2 mL/min; Sigma-Aldrich) equipped with a guard column ($C_{18}$, 4.0 × 2.0 mm, Phenomenex) was used to obtain the separation of AA and GA. The detector wavelength was set at 214 nm. The ES technique, in positive mode and SRM with a spray voltage of 4.3 kV, a sheath gas pressure of 4.7 Pa, an auxiliary gas pressure of 2.0 Pa, a capillary temperature of 300 °C and a capillary offset of 35 V were selected for these analyses. All reaction batches were diluted 20-fold prior to LC-MS/MS analysis.

### 2.6 Cell culture

Caco-2 cells (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were cultured in plastic flasks in DMEM supplemented with 2% streptomycin/penicillin, 2% L-glutamine, and 10% FCS, and maintained in a 5% $CO_2$ atmosphere at 37°C. Cells were seeded at a density of $1 \times 10^6$ cells *per* flask (75 $cm^2$) and allowed to grow until confluence. Afterwards, the medium was removed and replaced with DMEM supplemented with 2% streptomycin/penicillin and 2% L-glutamine for 24 h.

For time course experiments with AA, cells were incubated with 2.5 mM AA for 24, 48, 72 and 96 h. Dose response experiments were performed using concentrations of 0.65, 1.25, 2.5 and 5 mM AA at a treatment time of 24 h. AA concentrations were chosen according to experimental conditions reported by Odland *et al.* [16]. SFN experiments comprised four different concentrations (150, 50, 10 and 5 μM SFN) and an exposure time of 24 h. Furthermore, effects of the combined exposure of SFN and AA were tested using AA and SFN in concentrations of 2.5 mM and 10 μM, respectively, for a treatment time of 24 h.

After harvesting, one aliquot of the cells was used to analyse their viability by mixing 180 μL of the cell suspension with 20 μL trypan blue and subsequent counting the number of cells stained dead using a light microscope. In all of the experiments, except for treatment of the cells with 50 μM SFN, cell viability was ≥80% and treated cells did not show any different viability from non-treated control cells (*t*-test >0.05, data not shown). The remaining cells harvested were suspended in HEDG buffer (25 mM HEPES, 1.5 mM EDTA, 1 mM DTT, 10% glycerol, pH 7.4) homogenized and ultracentrifuged at 105 000 × *g* at 4°C for 1 h. The supernatant, containing the cytosol, was stored at −80°C until the quantification of adducts, protein content and the analyses of GST activity.

Samples for GSH quantification were prepared by dissolving the harvested cells in 0.2% aqueous meta-phosphoric acid and centrifugation at 15 700 × *g* for 2.5 min. Supernatants were stored at −80°C until analysis.

### 2.7 Analysis of GST activity and GSH concentrations

GST activity was measured according to Habig *et al.* [17] using 1-chloro-2,4-dinitrobenzene as substrate. Results are expressed as mmol 1-chloro-2,4-dinitrobenzene/mg protein. The protein content was measured according to the procedure of Lowry *et al.* [18] and GSH concentrations were analysed following the procedure reported by Richie *et al.* [19].

### 2.8 Quantitation of adducts formed from GSH with AA or/and SFN in Caco-2 cells

For the extraction of GSH-SFN, SFN-GSH-AA and SFN from the cells, the cytosol was defatted with diethyl ether and proteins were precipitated using trichloroacetic acid. After centrifugation at 4000 × *g* at 4°C for 10 min, the supernatant was loaded onto $C_{18}$ cartridges (Waters, 500 mg, 55–105 μm; Milford, MA, USA), which had been preconditioned with methanol and equilibrated with HEDG buffer. After washing with methanol/water (5/95; v/v), the adducts were eluted with ACN (90%). The eluates were evaporated to dryness and dissolved in formic acid (0.1% in water). The solutions were membrane filtered (45 μm) and diluted (1/10; v/v) with 0.1% formic acid before analysis. For the extraction of GSH-AA, AA and GA, cytosol samples were defatted with diethyl ether and the proteins precipitated with ACN. The supernatant obtained by centrifugation was used for HPLC analysis.

Ten microlitres of each sample were injected into the HPLC-MS/MS system. For GSH-AA, GSH-SFN and SFN-GSH-AA, a mobile phase gradient starting from 100% aqueous formic acid (0.1% formic acid in water) for 5 min and decreasing up to flux 100% of the 0.1% formic acid in ACN phase within 15 min was used. Under these conditions, GSH-AA eluted after 4.7 min, GSH-SFN eluted after 16.2 min and SFN-GSH-AA after 16.3 min (Fig. 2). The gradient for AA and GA started with 100% aqueous formic acid (0.1% formic acid in water) for 5 min, the 0.1% formic acid in ACN was raised to 100% within 2 min and kept at 100% for 5 min. Under those conditions, AA eluted after 3.3 min and GA after 2.8 min.

Analyses were performed with the scan parameters presented in Table 1. Quantitation of the metabolites was

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**Table 1.** Scan parameters for the HPLC-MS/MS analyses of AA, GA and GSH adducts in Caco-2 cells

| Analyte | Precursor ion (*m/z*) | Product ions (*m/z*) | Collision energy (V) |
|---|---|---|---|
| GSH-AA | 379.0 | 250.0 | 14 |
| | | 104.0 | 36 |
| GSH-SFN | 485.0 | 179.0 | 25 |
| | | 136.0 | 23 |
| SFN-GSH-AA | 556.0 | 104.0 | 40 |
| | | 435.0 | 21 |
| AA | 72.1 | 44.3 | 28 |
| | | 55.2 | 10 |
| GA | 88.2 | 44.3 | 16 |
| | | 45.3 | 13 |

based on external calibration curves obtained by spiking blank cytosol with eight different concentrations of the synthesized analytical standards. Spike ranges from $2.85 \times 10^{-3}$ up to $3.6 \times 10^{-1}$ μg/mL for GSH-AA, $1.65 \times 10^{-3}$ to $2.1 \times 10^{-1}$ μg/mL for GSH-SFN and $2.34 \times 10^{-3}$ to $3.0 \times 10^{-1}$ μg/mL for SFN-GSH-AA were carried out.

The LOD was determined to be 5 nM for GSH-AA (*m/z* 379/250), 5 nM for GSH-SFN (*m/z* 485/136), 18 nM for SFN-GSH-AA (*m/z* 556/104) and 100 nM GA (*m/z* 88.2/44.3).

### 2.9 Statistical analysis

The unpaired Student's *t*-test was used to identify statistically significant differences ($p \leq 0.05$).

## 3 Results and discussion

SFN has been shown to induce the enzyme activity of GST [13, 14], an enzyme that primarily functions as a detoxification enzyme by catalysing the conjugation of GSH with a wide range of carcinogens and other toxic chemicals [20]. AA is one of these toxins that is conjugated to GSH through the enzyme activity of GST [8]. Here, we report the effect of SFN on the enzymatic and non-enzymatic formation of the GSH-AA adduct.

### 3.1 Non-enzymatic formation of GSH adducts in the presence of SFN and AA

In a first set of experiments, the effect of reagent concentrations, pH and reaction time on adduct formation was studied *in situ* after chemical synthesis of GSH-AA, GSH-SFN and SFN-GSH-AA. Structures were confirmed by MS and NMR analyses as reported in the previous section (Fig. 2, Table 1), and the yields obtained for GSH-AA, GSH-SFN and SFN-GSH-AA were 47, 41 and 62%, respectively.

At equimolar concentrations of GSH, SFN and AA, the formation of GSH-SFN was favoured over that of GSH-AA, independently of the reaction time (2 h *versus* 10 h) and pH (7 *versus* 10) (Table 2a). Interestingly, also the ternary adduct SFN-GSH-AA was detected and quantified. However, this reaction occurred only at alkaline conditions (pH 10) and reaction times substantially longer than 2 h. This indicates that, for a biological system such as enteral cells for which the physiological pH is around 7, the formation of this SFN-GSH-AA adduct is likely to be negligible.

### 3.2 Effect of AA and SFN on GST activity and GSH concentrations in Caco-2 cells

In order to prove the enzymatic and non-enzymatic formation of these GSH adducts in enteral cells, human Caco-2 cells were treated with either AA or SFN solely or with a combination of both compounds. First, time course experiments were performed according to exposure times applied for studying GST induction in Caco-2 cells before [21–23]. The first set of cell culture experiments was designed to identify optimum conditions for GST activation. Cells treated with AA at concentrations ranging from 0.65 to 2.5 mM showed a maximum increase in GST activity of $20 \pm 3\%$ ($p \leq 0.05$ *versus* non-treated controls) after a 24 h treatment (Fig. 3). In contrast, no difference *versus* non-treated control cells was found in the presence of 5 mM AA. Although the viability of AA treated cells was not different from that of non-treated controls in our own experiments (data not shown), an increase in DNA strand breaks as reported by Puppel *et al.* after a 24 h treatment of Caco-2 cells with 6 mM AA [24] might have contributed to a slight, yet statistically non-significant, viability affecting the AA potential to increase GST activity at the high concentration of 5 mM.

In the work presented here, time course experiments ranging from 24 h up to 96 h of AA treatments at concentrations of 2.5 mM revealed the highest increase in GST activity of $23 \pm 4\%$ ($p \leq 0.05$ *versus* non-treated control cells) after 24 h of incubation (Fig. 4). Treatment of the cells with 2.5 mM AA for 96 h resulted in a 61% decrease in GST activity ($p \leq 0.05$ *versus* controls), which was not associated with decreased cell viability (data not shown). A dose-dependent effect of AA on GST activity has been reported by Srivastava *et al.* [25], who exposed rats to AA at daily doses of 50 mg/kg body weight for 10 days and observed a decrease in GST activity. In contrast, administration of single doses ranging from 100 to 200 mg/kg body weight did not affect the GST activity in the brain. Moreover, the authors demonstrated that AA in concentrations of 2–10 mM caused a concentration-dependent decrease in GST activity in rat brain under *in vitro* conditions.

The dose response experiments on the effect of SFN on GST activity in this work (Fig. 5) revealed a statistically

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**Table 2.** Chemically synthesized amounts of GSH-AA, GSH-SFN and SFN-GSH-AA upon the reaction between AA, SFN and GSH at different pH values and concentrations

| Precursor concentration | Reaction time | pH | GSH-AA (μM) | GSH-SFN (μM) | SFN-GSH-AA (μM) |
|---|---|---|---|---|---|
| **Equimolar (a)** | 2 h | 7 | 0.009 ± 0.002 | 0.082 ± 0.001 | n.d. |
| 100 μM AA | | 10 | 0.240 ± 0.010 | 2.118 ± 0.213 | n.d. |
| 100 μM GSH | 24 h | 7 | 0.022 ± 0.002 | 0.147 ± 0.002 | n.d. |
| 100 μM SFN | | 10 | 0.612 ± 0.035 | 2.353 ± 0.257 | 0.057 ± 0.013 |
| **Cell culture representative (b)** | 2 h | 7 | 0.021 ± 0.002 | 0.076 ± 0.001 | n.d. |
| 2500 μM AA | | 10 | 0.804 ± 0.029 | 0.075 ± 0.001 | n.d. |
| 30 μM GSH | 24 h | 7 | 0.028 ± 0.026 | 0.091 ± 0.011 | n.d. |
| 10 μM SFN | | 10 | 0.820 ± 0.015 | 0.075 ± 0.001 | n.d. |

n.d. not detected (LOQ = 0.054 μM).
Data are presented as mean values ± SD.

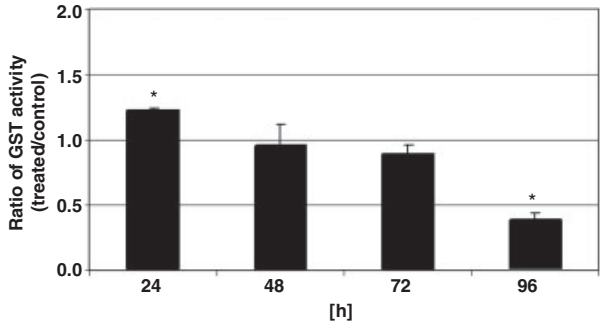


**Figure 4.** Relative activity of GST in Caco-2 cells treated with 2500 μM AA for different times (non-treated control cells = 1). Data are presented as mean values ± SD from ≥ three biological replicates (*$p < 0.05$ *versus* control cells).

significant increase ($p \leq 0.05$ *versus* non-treated control cells) in GST activity of 32 ± 5% and 36 ± 7% after 24 h of incubation with SFN at concentrations of 5 and 10 μM, respectively. This result is consistent with those reported by Zhang [26] who showed that the initial cellular uptake of isothiocyanates and conjugation to GSH is strongly dependent on cellular GST activity. From experiments in which Caco-2 cells were treated with 20 μM SFN for 72 h, Svehlikova *et al.* [27] demonstrated an about twofold increase in protein expression of GSTA1-1 compared with non-treated cells. However, at a higher concentration of 50 μM applied in this work, a 15 ± 4% ($p \leq 0.05$ *versus* non-treated control cells) decrease of GST activity was observed, which was in line with a reduced cell viability of 22 ± 3% ($p \leq 0.05$ *versus* non-treated control cells). These results are in accordance with those reported by Svehlikova *et al.* [27], who showed a decrease of GST protein expression after treatment with 40 μM SFN in Caco-2 cells.

Results obtained in this study from dose response experiments on SFN are in agreement with literature data, which show that SFN is a very potent inducer of phase II enzymes, doubling the activity of quinone reductase at a concentration of 0.58 μM in the Hepa1c1c7 (murine hepatoma) cell line [28] and inducing the GST gene transcription in Caco-2 cells 2.8-fold [27]. Similar results were obtained by Basten *et al.* [29], who studied the effect of SFN on induction of GSTA1 protein expression in HepG2 cells (human hepatoma cell line). Basten *et al.* [29] treated cells with a concentration of 0.3–30 μM SFN for 18 h and observed a significant induction of GSTA1 activity, reaching a maximum (2.3-fold induction) with 15 μM SFN. Ye and Zhang [30] also reported that SFN increased the GST activity about 1.5-fold in papilloma (PE) cells (mouse skin PE cells) after treatment with 5 μM SFN for 24 h. Moreover, *in vivo* experiments demonstrated that treatment of rats and mice with a high dose of SFN (up to 1000 μmol/kg/day for 4–5 days) increased phase II enzyme activities in liver, lung, mammary gland, pancreas, stomach, small intestine and colon [14, 31–34].

The decrease of GST activity demonstrated in our own experiments at a concentration of 50 μM can be explained by cytotoxicity of SFN (cell viability was slightly decreased by 23%). Zhu and Loft [35] also reported a dose-dependent enzyme activation. Treatments of Hepa1c1c7 cells with SFN significantly enhanced the activity of the phase II enzyme quinone reductase at a concentration of 5.6–140 mM, whereas exposure of the cells to higher concentrations of SFN decreased quinone reductase activity due to cytotoxicity.

In a second set of cell culture experiments presented in this work, Caco-2 cells were treated with either 10 μM SFN or 2.5 mM AA, or a combination of both compounds for 24 h since these experimental conditions revealed the highest increase in GST activity.

As demonstrated in the preceding cell culture experiment, an increase in GST activity compared with non-treated control cells was observed after exposure to SFN (38 ± 6%, $p \leq 0.05$) or AA (25 ± 4%, $p \leq 0.05$) solely (Fig. 6). However, cells treated with the combination of both compounds did not show any different GST activity from non-treated controls (Fig. 6). The observed increase in GST activity was accompanied by a reduction of GSH concentrations in cells treated with AA (−23 ± 6%, $p \leq 0.05$) or SFN (−39 ± 8%, $p \leq 0.05$).

Interestingly, GSH concentrations were also lowered by 40 ± 7% after combined treatment of AA and SFN, although

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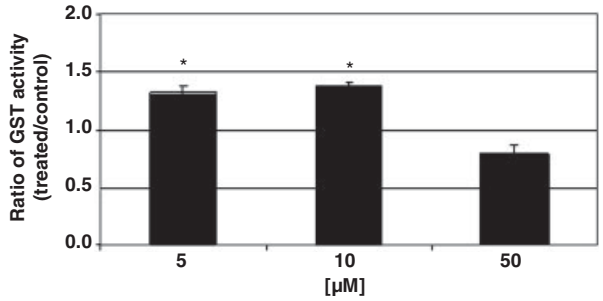


**Figure 5.** Relative activity of GST in Caco-2 cells treated with different concentrations of SFN for 24 h (non-treated control cells = 1). Data are presented as mean values ± SD from ≥ three biological replicates (*$p < 0.05$ *versus* control cells).

the GST activity was not different from non-treated controls (Fig. 6).

The inducing effect of AA and SFN on the GST activity and the depletion of GSH in the presence of either of those compounds demonstrated here are in accordance with data already reported in the literature [27, 36, 37]. Svehlikova *et al.* [27] showed that, also in Caco-2 cells, the induction of GSTA1 transcription by SFN concentrations ranging from 1 to 40 μM was mediated by activation of the nuclear transcription factor NF-κB. Pre-treatment with specific inhibitors of NF-κB or PI3-kinases abolished the induction of GST by SFN.

The GSH-depleting effect of AA in Caco-2 cells has also been shown by Puppel *et al.* [24], who observed an induction of DNA strand breaks at AA concentrations ≥1.25 mM. Zhang [38] reported that SFN accumulated rapidly to high intracellular concentrations in murine hepatoma cells with dithiocarbamates being the major products resulting from conjugation with GSH. The intracellular concentration reached 6.4 mM when cells were exposed to 100 μM SFN for 30 min and 95% of the accumulated product was the GSH conjugate. Besides, cellular accumulation of SFN was accompanied by a profound reduction in cellular GSH levels [38].

Taken together, the increased GST activity in Caco-2 cells treated with AA or SFN in this study might have resulted in the formation of AA-GSH and SFN-GSH adducts, which ultimately results in decreased concentrations of free GSH as long as GSH *de novo* synthesis had either not been initiated or had not been efficient enough to compensate for its consumption. Kim *et al.* [39] studied the role of GSH in HepG2-C8 cells being exposed to SFN (up to 4 h) and showed that after an initial reduction in GSH concentrations there is a marked increase in GSH levels at a longer exposure of 24 h, which is the exposure time applied in the work presented here.

The most striking result in this study is the GST activity that was not different in cells treated with the combination of AA and SFN, although GSH concentrations in these cells were reduced by 40 ± 5% ($p \leq 0.05$ *versus* non-treated control cells). This result indicates a non-enzymatic, GST-independent formation of GSH adducts in the presence of SFN and AA. Ye and Zhang [30] showed that incubating mouse skin PE cells with 5 μM SFN for 24 h resulted in an increase in GST activity of 54%, which is comparable to the results presented herein. However, Ye and Zhang [30] reported not only the GST activity, but also GSH concentrations being higher in SFN-treated cells compared with controls due to an ARE gene activation. Interestingly, repeated exposure of HepG2 cells to 5 μM SFN enriched medium did not result in a further increase of GSH concentrations, indicating a dose-dependent effect. In the here presented work, the SFN concentration of 10 μM was twice as high as that applied by Ye and Zhang [30] and might have led to a down-regulation of GST enzyme expression in Caco-2 cells.

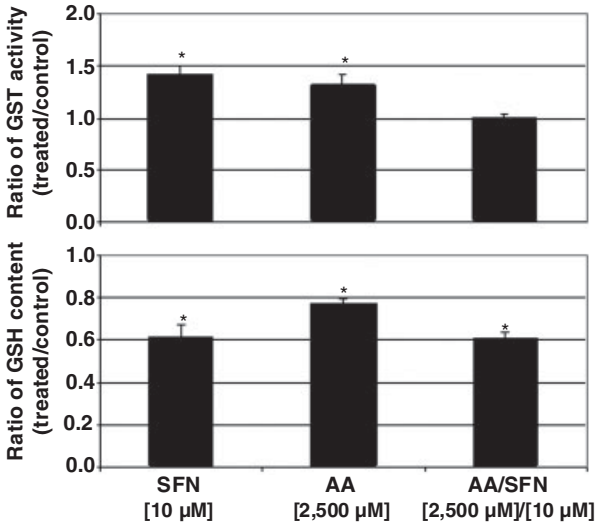


**Figure 6.** Relative GST activity and GSH concentrations in Caco-2 cells treated with SFN, AA or with the combination of both for 24 h compared with non-treated cells (non-treated control cells = 1). Data are presented as mean values ± SD from ≥ three biological replicates (*$p < 0.05$ *versus* control cells).

### 3.3 Effect of AA and SFN on GSH adduct formation in Caco-2 cells

The formation of GSH adducts in AA and SFN treated cells was confirmed by quantitation of GSH-AA, GSH-SFN and SFN-GSH-AA. Prior to the analysis of treated cells, the formation of these conjugates was studied in chemical *in situ* experiments where AA, SFN and GSH were used in concentrations representative of those applied to Caco-2 cells (2.5 mM, 10 μM and 30 μM, respectively). After a reaction time of 24 h and a pH of 7, GSH-AA and GSH-SFN were quantified, with GSH-SFN being the quantitatively dominating adduct (Table 2b). The ternary SFN-GSH-AA adduct was not detected (LOQ = 0.054 μM), even when the pH of the reaction was adjusted to 10. In line with these results, exposure of Caco-2 cells to the combination of AA and SFN resulted in the formation of GSH-SFN, GSH-AA

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(Table 3), but SFN-GSH-AA could not be detected (data not shown). Results displayed in Table 3 show that the combined treatment of Caco-2 cells with AA (2.5 mM) and SFN (10 μM) led to decreased mean concentrations of free AA, SFN and GSH of 46, 84 and 40%, respectively. Interestingly, cells exposed to the same concentration of SFN (10 μM) solely showed the same extent of SFN and GSH depletion, indicating: (i) that the reaction between GSH and SFN is favoured over that between GSH and AA and (ii) a substantially higher reactivity of the ITC (SFN) as compared with the double bond of AA *versus* amines. The latter was also demonstrated by the results from the chemical adduct synthesis (Table 2).

When the cells were incubated with 2.5 mM AA or 10 μM SFN solely, resulting concentrations of the respective adduct with GSH were very different among them. GSH-SFN was quantified in the cells at a concentration of 0.100 μM, which is quite similar to 0.091 μM analysed after chemical synthesis upon the *in situ* reaction between 2.5 mM AA, 10 μM SFN and 30 μM GSH (pH 7, reaction time: 24 h, Table 2b). This result supports the hypothesis that GSH-SFN in cells is preferentially formed non-enzymatically. In contrast, GSH-AA concentration in AA and SFN treated cells was about 1000-fold higher (17.7 μM *versus* 0.028 μM, respectively) than that analysed after *in situ* chemical synthesis using comparable concentrations of the precursors AA and SFN. This result indicates that GSH-AA in cells is preferentially formed enzymatically.

Next to the higher reactivity of SFN towards GSH compared with AA, different uptake rates into the cells and further metabolism reactions might play a role. Schabacker *et al.* [40] already showed that AA monomers are highly bioavailable, passing the cell monolayer *via* concentration-dependent passive diffusion. As presented in Table 3 of this work, GSH-AA formation was reduced by about 50% after treatment of the cells with AA and SFN as compared with cells exposed to AA solely (17.5 μM *versus* 37 μM) whereas concentrations of free AA were not different. Thus, the presence of SFN may either decrease the cellular uptake of AA or may favour its metabolism into GA due to its competing reaction with GSH.

SFN is taken up by mammalian cells *via* passive diffusion and its transport is favoured over that of AA due to its higher hydrophobicity ($\log p$ (octanol/water) = 0.72 for SFN and −1.24 for AA) [41]. Therefore, a simultaneous incubation of the Caco-2 cells with SFN and AA might have led to a decreased absorption of AA and, as consequence, a reduced concentration of the adduct GSH-AA in the cells compared with the cells incubated with AA solely.

Finally, GSH depletion demonstrated for cells treated with the combination of AA and SFN as compared with cells exposed solely to AA might not only be caused by the formation of GSH adducts. Jakubíková *et al.* [37] reported a 50% increase in the formation of reactive oxygen species and a GSH reduction by 20% compared with non-treated control cells for Caco-2 cells exposed to 10 μM SFN for 1 h.

**Table 3.** Percentage recovery and concentrations of AA, SFN, GSH, and GSH-AA as well as GSH-SFN in Caco-2 cells treated with either AA or SFN or a combination of both compounds for 24 h

| Initial concentration (μM) | | | 24 h post-incubation | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Adduct formed (μM) | | Free compound (μM) | | | Compound recovery of concentrations administered (%) | | |
| AA | SFN | GSH | GSH-AA | GSH-SFN | AA | SFN | GSH | AA | SFN | GSH |
| 2500 | – | 30 | 37.0 ± 8.73 | – | 1411 ± 83.1 | – | 24.0 ± 0.93 | 56.4 | – | 77 |
| – | 10 | 30 | – | 0.08 ± 0.00 | – | 1.98 ± 8.73 | 19.1 ± 1.83 | – | 19.8 | 61 |
| 2500 | 10 | 30 | 17.5 ± 2.00 | 0.10 ± 0.00 | 1359 ± 9.86 | 1.55 ± 8.73 | 19.0 ± 0.95 | 54.4 | 15.5 | 60 |

Data are reported as means of triplicate analyses ± SD.

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Thus, GSH might also be consumed to counteract reactive oxygen species [42].

In conclusion, the presence of SFN in AA exposed human intestinal Caco-2 cells impaired the non-enzymatic and GST-catalysed enzymatic detoxification of AA. SFN efficiently competes with AA for GSH, resulting in a reduced formation of GSH-AA conjugates over GSH-SFN adducts.

## 4 Concluding remarks

Knowing that GST induction by SFN plays a role in chemoprevention, we studied the effect of SFN on GST activity and GSH adduct formation in Caco-2 cells in the presence of AA, which is also known to be at least partially detoxified *via* GST-induced GSH conjugation. We could show that GST activity increased after treatment of the cells with SFN or AA, but not after co-incubation of the cells with both SFN and AA. However, GSH concentrations decreased after all treatments. At the same time, quantitative data of GSH adduct formation *in situ* showed that the reaction between GSH and SFN is favoured over that between GSH and AA. We also demonstrated the formation of an adduct between SFN, AA and GSH for the first time. However, this metabolite was not detected in Caco-2 cells.

In conclusion, data suggest that SFN might affect the GSH dependent detoxification of AA by SFN-GSH adduct formation, due to the fact that GSH is the rate-limiting compound in this detoxification pathway.

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*The authors have declared no conflict of interest.*

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