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Effects of the sulforaphane analog compound 30, indole-3-carbinol, D-limonene or relafen on glutathione *S*-transferases and glutathione peroxidase of the rat digestive tract

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Abstract

Several dietary compounds have been demonstrated to reduce gastrointestinal cancer rates in both humans and animals. We showed that high human gastrointestinal tissue levels of glutathione *S*-transferase (GST), a family of detoxification enzymes consisting of class Alpha, Mu, Pi and Theta isoforms, were inversely correlated with cancer risk. We now investigated whether the sulforaphane analog compound 30, indole-3-carbinol, D-limonene or relafen, supplemented in the diet for two weeks at 1450, 250, 10 000, and 200 ppm, respectively, influenced (i) GST activity, (ii) GST isoenzyme levels, (iii) GSH levels, or (iv) glutathione peroxidase (GPx) activity in the gastrointestinal tract of male Wistar rats. Sulforaphane analog compound 30 enhanced GST activity in all organs studied (1.2–2.4 ×). It induced GST Alpha levels in small intestine and liver, GST Mu levels in stomach and small intestine, GST Pi levels in stomach and small and large intestine, and GSH levels in stomach and proximal and middle small intestine. Indole-3-carbinol induced gastric GST Mu and hepatic GST Alpha levels. D-limonene induced hepatic GST Alpha, colonic GST Pi levels and proximal small intestinal GST enzyme activity and GST Pi levels. Relafen induced hepatic GST Alpha levels, distal small intestinal and gastric GST Pi levels, and oesophageal and proximal small intestinal GSH levels. GPx activity was enhanced by relafen in oesophagus, and in distal small intestine by sulforaphane analog compound 30. Enhancement of GSTs and to a lesser extent GPx and GSH, resulting in a more efficient detoxification, may explain at least in part the anticarcinogenic properties of sulforaphane analog compound 30, and to a much lesser extent of indole-3-carbinol and D-limonene. © 1998 Elsevier Science B.V.

Keywords: Anticarcinogen; Chemoprevention; Glutathione; Glutathione *S*-transferase; Nonsteroidal anti-inflammatory drug; (Wistar rat)

Abbreviations: DSI, distal small intestine; GST, glutathione *S*-transferase; I3C, indole-3-carbinol; MSI, middle small intestine; NSAID, nonsteroidal anti-inflammatory drug; PSI, proximal small intestine; Se-GPx, selenium-dependent glutathione peroxidase; t-GPx, total glutathione peroxidase

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1. Introduction

The discovery of naturally occurring anticarcinogenic compounds from edible plants has been a very productive area for obtaining potential chemopreventive agents. A number of natural products isolated from these plants have shown anticarcinogenic properties in several animal models [1–6].

Compound 30 ((±)-exo-2-acetyl-6-isothiocyanatonorbornane) is a ketoisothiocyanate, synthesized as a structural analog of sulforaphane, a component of broccoli [7,8]. Compound 30 is relatively easily synthesized and it may be more stable metabolically than sulforaphane [7]. Sulforaphane and several natural and synthetic isothiocyanates were demonstrated to: (i) have anticarcinogenic properties in rodents [8–12], (ii) induce phase II enzymes both in vivo and in cells in culture [3,8,13], and (iii) inhibit activation of certain carcinogens. Sulforaphane analog compound 30 is an equally potent inducer of quinone reductase as sulforaphane, and is capable of in vivo enhancement of GST in several mouse organs [8].

Indole-3-carbinol (I3C), present as glucosinolate precursor in cruciferous vegetables (cabbage, broccoli, Brussels sprouts, cauliflower, etc.), is able to inhibit chemically induced neoplasia in forestomach [14], mammary gland [14,15], liver [16], colon [17], lung [18] and tongue [19] in rodents. It is noteworthy that I3C, given in capsules at 400 mg/day for three months to female human volunteers, produced no toxic or untoward effects and resulted in a change in oestrogen metabolism indicative of a potential chemopreventive effect against breast cancer [20]. Such chemopreventive effects are well established in rodent mammary tumour models [14,21].

D-limonene, a monocyclic terpene, is consumed by humans predominantly as an ingredient of traditional food, e.g. citrus fruit, carrots, coffee, orange, and nutmeg [1,22]. D-limonene was able to reduce tumour incidence in forestomach, lung and mammary tissue [1,2,22–25] and caused regression of mammary tumours in rodents [1,26].

Relafen is a nonsteroidal anti-inflammatory drug (NSAID). Nonsteroidal anti-inflammatory drugs are among the most prescribed drugs worldwide. In addition to their therapeutic use, there is strong evidence that NSAIDs may be anticarcinogenic in humans. Epidemiological studies suggest that regular, prolonged use of aspirin-based NSAIDs may reduce the incidence and mortality rates of oesophageal, gastric, colonic and rectal cancer ([27–29], and refs. therein). Many animal studies have revealed NSAIDs to significantly protect against chemically induced cancers ([27,29], and refs. therein). In clinical trials, relafen was as effective as aspirin, diclofenac, indomethacin, ibuprofen, and naproxen in the treatment of patients with osteoarthritis or rheumatoid arthritis [30]. However, relafen may be preferred because of its lower incidence of abdominal pain, dyspepsia, gastritis, gastrointestinal distress, ulcer, and clinically meaningful decreases in haemoglobin [30,31]. Furthermore, if necessary, the dose of relafen can be increased without a concordant increase of adverse effects [32].

The exact mechanism for the chemopreventive potential of above mentioned compounds has not been clearly defined yet. However, inhibitors of carcinogenesis often have an enhancing effect on carcinogen detoxification systems such as glutathione *S*-transferases (GSTs; EC 2.5.1.18) [33,34]. The soluble GSTs are a family of dimeric enzymes comprised of four classes: Alpha, Mu, Pi and Theta [34,35]. They catalyze the nucleophilic attack of the sulphur atom of glutathione (GSH) on the electrophilic centres of a seemingly endless variety of xenobiotics. Since most reactive ultimate carcinogenic forms of chemical carcinogens are electrophiles, GSTs take considerable importance in carcinogen detoxification [34,35]. Enhanced GST activity may result in a more efficient elimination of carcinogens and ultimately lead to cancer prevention.

Another important parameter in inhibiting carcinogenesis is prevention of oxidative damage by glutathione peroxidases (GPxs). GPxs are enzymes that catalyze the reduction of organic hydroperoxides and hydrogen peroxide [36]. Two major types of GPx have been identified [36], a selenium-dependent form (Se-GPx) which is active with both organic hydroperoxide and hydrogen peroxide, and a selenium-independent form (t-GPx) which has no activity towards hydrogen peroxide and mainly comprises of GSTs [36].

The present study was designed to investigate the effects of dietary administration of compound 30, indole-3-carbinol, D-limonene and relafen on glutathione, glutathione *S*-transferases and glutathione

peroxidases in rat oesophagus, intestine, stomach and liver.

2. Materials and methods

2.1. Animal treatment

Forty male Wistar rats (177 ± 1 g; Central Laboratory Animal Centre, University of Nijmegen, The Netherlands) were housed in pairs on wooden shavings in macrolon cages, maintained at 20–25°C and 30–60% relative humidity. A ventilation rate of seven air cycles/h and 12 h light/dark cycles were used. The rats were randomly assigned to one of the dietary treatment groups. All groups were fed powdered RMH-TM lab chow (Hope Farms, Woerden, The Netherlands) from the same batch. After acclimatization for seven days the animals were fed either the basal diet (control group) or one of the four experimental diets. Food and water were available ad libitum. Food cups were replenished every 2–3 days. Food consumption and gain in body weight were recorded daily.

2.2. Diets

Compounds and dose levels used were selected on antimutagenic and/or anticarcinogenic properties [9,10,13,22] or induction of phase II enzymes in rodents [1,7,37–40]. Relafen was selected based on results of a previous study by us, showing enhancing effects of other nonsteroidal anti-inflammatory drugs on GSTs in male Wistar rats [41], and the dose used was based on data on gastrointestinal toxicity [31,42]. The following five diet groups (8 animals per group) were studied: (a) RMH-TM lab chow only, and lab chow supplemented with (b) 1450 ppm sulforaphane analog compound 30 (synthesized as described before, see Ref. [7]), (c) 250 ppm indole-3-carbinol (Sigma, St. Louis, MO, USA), (d) 10 000 ppm D-limonene (Aldrich), and (e) 200 ppm relafen (Sigma). A food processor was used to obtain a homogenous mixture of test compound and powdered lab chow. After receiving the diets for two weeks the rats were killed by decapitation. The study protocol was approved by the local ethical committee for animal experiments of the University of Nijmegen.

2.3. Tissue preparation

All handlings were performed on ice. After decapitation, oesophagus, stomach, intestine (proximal, middle, and distal small intestine and colon) and liver were excised immediately. Intestine and stomach were slit longitudinally and the contents were removed by washing with cold buffer A (0.25 M saccharose, 20 mM Tris/HCl, 1 mM dithiothreitiol, pH 7.4). The organs were directly frozen in liquid nitrogen and stored at −20°C until use. For preparation of the cytosolic fraction the tissue was thawed quickly using cold running water. The mucosal surface of stomach and intestine was collected by scraping with a scalpel and was homogenized in buffer A (4 ml/gram tissue) in a glass/glass Potter–Elvehjem tube. The liver was homogenized in buffer A (4 ml/gram tissue) with ten strokes at 1000 rpm of a motor-driven glass/Teflon homogenizer (Braun, Germany). The homogenate was centrifuged at 9000 × *g* (4°C) for 30 min. The resulting supernatant fraction was transferred to an ultracentrifuge tube and spun at 150 000 × *g* (4°C) for 60 min. The oesophagus was homogenized in 5 ml buffer A per gram tissue in a glass/glass Potter–Elvehjem tube. These homogenates were centrifuged at 150 000 × *g* for 60 min (4°C). Aliquots of the 150 000 × *g* supernatant, representing the cytosolic fraction, were frozen in liquid nitrogen and stored at −20°C.

2.4. Assays

Protein concentration was assayed in quadruplicate by the method of Lowry et al. [43] using bovine serum albumin as the standard. GST activity was determined in triplicate according to Habig et al. [44], using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. GST isoenzyme levels were determined as described before [33]. In short, cytosolic fractions were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (11% acrylamide, w/v), and subsequently to Western blotting, using a semidry blotting system (Novablot II, Pharmacia, Uppsala, Sweden). Western blots were incubated with monoclonal antibodies against human GST class Alpha [45], Mu [46], and Pi [47]. Class Alpha antibodies react with rat GST subunit 1, class Mu antibodies recognize rat GST subunits 3 and 4, and class Pi antibodies react with rat GST subunit 7 [33]. The

Table 1
Daily food consumption, anticarcinogen-intake and gain in body weight of male Wistar rats receiving diets supplemented with compound 30, indole 3-carbinol, D-limonene or relafen

Treatment group ($n = 8$)	Dose (ppm)	Food consumption (g/day)	Total anticarcinogen-intake (mg/day.kg body weight)	Gain in body weight (g/day)
Control	–	21.1 ± 0.9	–	3.5 ± 0.3
Compound 30	1450	18.0 ± 0.8 [b]	131 ± 6	2.2 ± 0.1 [b]
Indole 3-carbinol	250	18.1 ± 0.3 [c]	22.6 ± 0.4	3.4 ± 1.5
D-limonene	10 000	20.0 ± 0.2	1001 ± 11	3.9 ± 0.2
Relafen	200	20.3 ± 1.1	20.3 ± 1.1	3.8 ± 0.3

Values given are means ± SEM. The one-tailed Wilcoxon rank sum test was used to assess statistical significance of differences between control and treated groups.
[b] $P < 0.01$.
[c] $P < 0.005$.

specific binding of the monoclonal antibodies to the isoenzymes was demonstrated by incubation with peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark) and subsequent peroxidase staining with 4-chloro-1-naphthol and hydrogen peroxide. Staining intensity on the immunoblots was quantified using a laser densitometer (Ultroscan XL, LKB, Bromma, Sweden). Known amounts of purified GSTs were run in parallel with the experimental samples and served as standards for the calculation of absolute amounts of the isoenzymes. Glutathione was quantified by high performance liquid chromatography after reaction with monobromobimane, as described before [33]. In this assay, total glutathione is measured since oxidized glutathione present is reduced by adding sodium borohydride to the reaction mixture. Glutathione peroxidase enzyme activity was measured using both hydrogen peroxide and *t*-butylhydroperoxide as substrates, essentially as described by Howie et al. [48].

2.5. Statistical analyses

The Wilcoxon rank sum test was used to assess statistical significance of differences between experimental and control groups: [a] $p < 0.05$, [b] $p < 0.01$ and [c] $p < 0.005$.

3. Results

Daily food consumption, intake of the anticarcinogens and gain in body weight are given in Table 1. D-limonene and relafen did not show any significant impact on food consumption and gain in body weight, as compared to the control group. In the sulforaphane

Table 2
Effects of compound 30, indole 3-carbinol, D-limonene or relafen on rat alimentary tract glutathione *S*-transferase activities

Treatment group ($n = 8$)	Glutathione *S*-transferase activity (nmol/min.mg protein)						
	Oesophagus	Stomach	PSI	MSI	DSI	Colon	Liver
Control	69 ± 6	142 ± 10	360 ± 29	140 ± 7	70 ± 6	101 ± 6	1549 ± 233
Compound 30	117 ± 8 [c]	206 ± 14 [c]	870 ± 77 [c]	169 ± 13 [a]	220 ± 22 [c]	144 ± 8 [c]	2396 ± 343 [a]
Indole 3-carbinol	77 ± 7	133 ± 15	350 ± 14	146 ± 6	71 ± 4	99 ± 4	2498 ± 435
D-limonene	74 ± 8	132 ± 23	525 ± 30 [c]	157 ± 10	75 ± 2	106 ± 6	2318 ± 494
Relafen	73 ± 6	179 ± 18	425 ± 26	137 ± 6	74 ± 4	112 ± 6	1493 ± 201

PSI, proximal small intestine; MSI, middle small intestine; DSI, distal small intestine.
[a] $P < 0.05$.
[c] $P < 0.005$.

Table 3
Effects of compound 30, indole 3-carbinol, D-limonene or relafen on rat alimentary tract glutathione *S*-transferase Alpha levels

Treatment group ($n = 8$)	Glutathione *S*-transferase Alpha level (ng/mg protein)						
	Oesophagus	Stomach	PSI	MSI	DSI	Colon	Liver
Control	ND	197 ± 40	4992 ± 975	3801 ± 606	203 ± 43	ND	11583 ± 671
Compound 30	ND	261 ± 55	21416 ± 2052 [c]	8234 ± 976 [c]	2254 ± 410 [c]	ND	19037 ± 827 [c]
Indole 3-carbinol	ND	233 ± 47	4142 ± 990	3767 ± 473	200 ± 24	ND	18265 ± 2221 [b]
D-limonene	ND	240 ± 56	5258 ± 1039	3582 ± 280	209 ± 41	ND	14499 ± 1271 [b]
Relafen	ND	232 ± 75	7077 ± 1455	2722 ± 384	292 ± 52	ND	15929 ± 1778 [b]

ND, Not detectable.
[b] $P < 0.01$.
[c] $P < 0.005$.

analog compound 30 and indole-3-carbinol groups a reduced food intake (approximately 15%) was found, resulting in a significant reduction in body weight gain (37%) in the sulforaphane analog compound 30 group.

Table 2 shows the effects of the anticarcinogens on GST activity in the organs investigated. Sulforaphane analog compound 30 enhanced GST enzyme activity in all organs studied, ranging from 1.2× in middle small intestine to 3.1× in distal small intestine. D-limonene induced GST enzyme activity only in the proximal small intestine (1.5×).

In Tables 3, 4 and 5 the effects of the anticarcinogens on GST class Alpha, Mu and Pi isoenzyme levels are given. In control animals GST Alpha (Table 3) was undetectable in oesophagus and colon, low in stomach (197 ± 40 ng/mg protein) and distal small intestine (203 ± 43 ng/mg protein) and high in liver (11583 ± 671 ng/mg protein) and proximal and middle small intestine. None of the diets significantly influenced gastric GST Alpha expression. Sulforaphane analog compound 30 induced GST Alpha levels in proximal, middle and distal small intestine (4.3, 2.2 and 11.1×, respectively) and in liver (1.6×). In liver GST Alpha levels were induced by indole-3-carbinol (1.6×), limonene (1.3×) and relafen (1.4×).

GST Mu (Table 4) was expressed at high levels in all tissues examined. Sulforaphane analog compound 30 induced GST Mu levels in the stomach (1.2×) and small intestine (1.7–2.9×), and indole-3-carbinol induced gastric GST Mu levels (1.2×).

GST Pi (Table 5) was undetectable in oesophagus and liver, and relatively low in all other organs studied, ranging from 171 ± 15 ng/mg protein in distal small intestine to 1088 ± 62 ng/mg protein in stomach in control animals. All compounds tested, except for indole-3-carbinol, increased GST Pi levels at one or more sites: sulforaphane analog compound 30 induced gastric (1.5×) and intestinal (2.3–5.4×)

Table 4
Effects of compound 30, indole 3-carbinol, D-limonene or relafen on rat alimentary tract glutathione *S*-transferase Mu levels

Treatment group ($n = 8$)	Glutathione *S*-transferase Mu level (ng/mg protein)						
	Oesophagus	Stomach	PSI	MSI	DSI	Colon	Liver
Control	4711 ± 392	12620 ± 760	3748 ± 543	4735 ± 605	2429 ± 337	4665 ± 657	31180 ± 3387
Compound 30	4726 ± 265	14972 ± 425 [a]	10931 ± 908 [c]	7964 ± 1015 [a]	5869 ± 750 [b]	5444 ± 915	33784 ± 7701
Indole 3-carbinol	4650 ± 407	15704 ± 1261 [a]	3554 ± 628	5815 ± 843	2555 ± 482	5842 ± 419	36405 ± 5484
D-limonene	5154 ± 420	13072 ± 2466	3850 ± 559	6153 ± 886	2396 ± 318	4345 ± 652	31746 ± 4871
Relafen	4698 ± 365	15521 ± 1852	4695 ± 679	4824 ± 614	3045 ± 333	4749 ± 399	32357 ± 7160

[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.005$.

GST Pi levels. D-limonene induced GST Pi levels in proximal small intestine (1.3 ×) and colon (1.6 ×), and relafen enhanced GST Pi levels in stomach (1.3 ×) and distal small intestine (1.7 ×).

Table 6 shows the effect of the anticarcinogens on the GSH content in the organs studied. Distal small intestinal, colonic and hepatic GSH contents were not influenced by any of the compounds tested. Elevations of GSH were only seen by sulforaphane analog compound 30 in the stomach (1.3 ×) and proximal and middle small intestine (2.4 × and 1.4 ×, respectively), and by relafen in oesophagus and proximal small intestine (1.3 × and 1.8 ×, respectively).

In Tables 7 and 8 the data of glutathione peroxidase (GPx) enzyme activity measurements are presented. Total (t-GPx) and selenium-dependent GPx (Se-GPx) activities were measured using *t*-butyl-hydroperoxide and hydrogen peroxide as substrates,

Table 5
Effects of compound 30, indole 3-carbinol, D-limonene or relafen on rat alimentary tract glutathione *S*-transferase Pi levels

Treatment group ($n = 8$)	Glutathione *S*-transferase Pi level (ng/mg protein)						
	Oesophagus	Stomach	PSI	MSI	DSI	Colon	Liver
Control	ND	1088 ± 62	437 ± 45	449 ± 54	171 ± 15	698 ± 99	ND
Compound 30	ND	1609 ± 147 [c]	1532 ± 186 [c]	1029 ± 110 [c]	929 ± 200 [c]	1888 ± 213 [c]	ND
Indole 3-carbinol	ND	1103 ± 131	365 ± 39	336 ± 55	241 ± 49	774 ± 96	ND
D-limonene	ND	972 ± 82	584 ± 48 [a]	352 ± 47	177 ± 29	1139 ± 135 [a]	ND
Relafen	ND	1401 ± 174 [a]	519 ± 56	426 ± 49	283 ± 26 [c]	843 ± 84	ND

ND, Not detectable.
[a] $P < 0.05$.
[c] $P < 0.005$.

Table 6
Effects of compound 30, indole 3-carbinol, D-limonene or relafen on rat alimentary tract glutathione levels

Treatment group ($n = 8$)	Glutathione (nmol/mg protein)						
	Oesophagus	Stomach	PSI	MSI	DSI	Colon	Liver
Control	35.4 ± 1.6	19.8 ± 0.9	29.2 ± 6.3	20.8 ± 2.6	12.4 ± 0.7	25.8 ± 3.1	20.9 ± 3.2
Compound 30	40.4 ± 4.0	26.7 ± 1.8 [c]	69.3 ± 16.8 [a]	28.5 ± 1.3 [a]	14.1 ± 1.4	29.0 ± 3.5	22.5 ± 2.5
Indole 3-carbinol	39.6 ± 4.0	22.4 ± 1.8	40.2 ± 9.3	21.2 ± 1.9	11.6 ± 1.1	28.4 ± 1.4	24.6 ± 2.6
D-limonene	34.5 ± 2.0	22.4 ± 1.4	34.2 ± 6.5	23.2 ± 2.4	12.4 ± 1.1	29.4 ± 3.5	22.7 ± 4.4
Relafen	46.9 ± 2.5 [c]	26.1 ± 2.9	52.8 ± 9.4 [a]	22.1 ± 1.7	11.7 ± 1.5	29.3 ± 2.1	20.6 ± 2.4

[a] $P < 0.05$.
[c] $P < 0.005$.

Table 7
Effects of compound 30, indole 3-carbinol, D-limonene or relafen on rat alimentary tract total glutathione peroxidase activities

Treatment group ($n = 8$)	Total glutathione peroxidase activity * (nmol/min mg protein)						
	Oesophagus	Stomach	PSI	MSI	DSI	Colon	Liver
Control	320 ± 15	988 ± 65	113 ± 8	136 ± 7	122 ± 23	119 ± 13	1236 ± 113
Compound 30	295 ± 25	869 ± 131	116 ± 7	135 ± 8	196 ± 23 [a]	144 ± 17	1242 ± 135
Indole 3-carbinol	301 ± 26	679 ± 84	112 ± 8	122 ± 16	129 ± 14	107 ± 4	1489 ± 241
D-limonene	271 ± 18	865 ± 100	120 ± 5	129 ± 6	97 ± 12	138 ± 9	1699 ± 363
Relafen	289 ± 22	1032 ± 189	108 ± 8	152 ± 25	120 ± 10	133 ± 14	1128 ± 175

* *t*-Butyl hydroperoxide was used as substrate.
[a] $P < 0.05$.

Table 8
Effects of compound 30, indole 3-carbinol, D-limonene or relafen on rat alimentary tract selenium-dependent glutathione peroxidase activities

Treatment group ($n = 8$)	Selenium-dependent glutathione peroxidase activity * (nmol/min mg protein)						
	Oesophagus	Stomach	PSI	MSI	DSI	Colon	Liver
Control	336 ± 15	1201 ± 125	100 ± 11	135 ± 5	108 ± 15	79 ± 5	1592 ± 172
Compound 30	373 ± 29	1085 ± 146	107 ± 13	142 ± 16	224 ± 28 [c]	104 ± 11 [a]	1530 ± 237
Indole 3-carbinol	394 ± 40	1301 ± 286	122 ± 6	130 ± 16	95 ± 10	107 ± 25	1859 ± 221
D-limonene	369 ± 29	1202 ± 113	101 ± 17	123 ± 8	96 ± 14	94 ± 11	2109 ± 410
Relafen	401 ± 27 [a]	1836 ± 335	110 ± 11	177 ± 30	114 ± 10	83 ± 6	1302 ± 207

* Hydrogen peroxide was used as substrate.
[a] $P < 0.05$.
[b] $P < 0.01$.

respectively. GPx activities are highest in liver and stomach, and lowest in small and large intestine. Indole-3-carbinol and limonene had no effect on GPx enzyme activity, whereas sulforaphane analog compound 30 induced both t-GPx and Se-GPx enzyme activity in distal small intestine (1.6× and 2.1×) and Se-GPx enzyme activity (1.3×) in colon. Relafen slightly enhanced oesophageal Se-GPx enzyme activity (1.2×).

4. Discussion

Consumption of fruits and vegetables reduces the risk of gastrointestinal cancer. The discovery of naturally occurring anticarcinogenic compounds in edible plants is a very productive area for obtaining potential chemopreventive agents. A number of natural products with great diversity in chemical structure, showing inhibitory activity in several types of animal tumorigenesis assays, were isolated from fruits and vegetables [1–6]. Although the mechanisms of this protection are unclear, feeding of fruit and vegetables induces enzymes involved in the xenobiotic metabolism, thereby accelerating the metabolic disposal of xenobiotics ([9,13,33,34,49], and refs. therein). Phase II detoxification enzymes like GST, NAD(P)H:quinone reductase, UPD-glucuronosyltransferase, and epoxide hydrolase [41,50,51] can modulate the response of animal cells to carcinogen exposure. Recent data, mostly obtained from animal studies, have indicated that many naturally occurring dietary anticarcinogens are able to elevate the levels of GSTs ([3,7,8,13,33,34,38–40], and refs. therein). In human organs at relatively high risk for cancer development such as colon, low glutathione *S*-transferase (GST) levels were measured [52]. Enhancement of GSTs was found in humans after consumption of cruciferous vegetables such as broccoli and Brussels sprouts [49,53]. In addition, a reduction of oxidative DNA damage was measured after consumption of Brussels sprouts [54]. In view of these results, we studied the effect of several dietary anticarcinogens on GSTs.

Compound 30 is a structural analog of sulforaphane, which is present in broccoli [7,8]. Sulforaphane has chemopreventive properties against mammary carcinogenesis in rats [55]. In the sulforaphane analog compound 30 group reduced food consumption coincided with reduced body weight gain, which was not reported in an earlier study using the same dose [7]. During the course of the experiment, no changes in behavioral pattern of the animals were observed. In addition, none of the organs studied showed any macroscopical sign of toxicity. Therefore we believe that the lower body weight gain in the sulforaphane analog compound 30 group may be the result of diminished absorption of macronutrients in the gastrointestinal tract, and not due to any kind of toxicity.

In our study sulforaphane analog compound 30 induced GST activity throughout the gastrointestinal tract, ranging from 1.2 in MSI to 3.1 in DSI. This result is in agreement with an earlier study in mice, showing that daily administration of 7.5, 15 or 30 μmol of sulforaphane analog compound 30 for 5 days induced GST activity in liver (2–4×), forestomach (2–4×), glandular stomach (2–4×)

and PSI (14–16 ×) in a dose-dependent manner [7]. Using a concentration of 15 μmol we now find slightly lower induction rates in rats, which may be due to species differences. Interestingly, 15 μmol sulforaphane daily for 5 days induced GST activity in liver (1.9 ×), forestomach (2.0 ×), glandular stomach (3.0 ×), PSI (2.1 ×) and lung (1.2 ×) of mice as well [8].

No data were available on the changes in the levels of GST isoenzymes as a result of feeding sulforaphane analog compound 30. The isoenzymes showed a tissue specific distribution. Class Alpha GSTs are abundant in liver and small intestine, whereas class Pi GSTs are present in stomach, small and large intestine [13,33,41]. GST Alpha was not detectable in the oesophagus and colon, and GST Pi was undetectable in both oesophagus and liver and none of the compounds tested had any effect on this. In contrast, class Mu enzymes seem to be less organ specific since they were detected at high levels in all tissues examined. In the sulforaphane analog compound 30 group, enhancement of GST activity was parallelled by a rise in GST Alpha levels in small intestine and liver, a rise in GST Mu levels in stomach and small intestine, and a rise in GST Pi levels in stomach and intestine. In the oesophagus an enhanced GST activity was found without any effect on the GST isoenzyme levels measured here, which may suggest that other classes of GSTs, e.g. Theta class, or other isoenzymes of the GST Alpha, Mu and Pi classes are involved.

The second compound tested was I3C, present as glucosinolate precursor in cruciferous vegetables. It has anticarcinogenic effects in several organs [14–21]. Rats receiving I3C had a lower daily food consumption as compared to the control group. However, body weight gain in these rats was similar to the control group. In addition, Bradfield and Bjeldanes [40] showed that 50 to 500 ppm I3C did not effect food intake or body weight gain [40]. We found no effect of I3C on rat gastrointestinal GST activity, which is in contrast to results by others, showing an induction of hepatic and proximal small intestinal GST activity in both mice [56,57] and rats [58,59]. However, Bradfield and Bjeldanes [40], in accordance with our results, showed that 250 and 500 ppm of I3C did not change GST activity in liver and gastrointestinal tract.

In our study I3C incidently induced GST isoenzymes such as gastric GST Mu and hepatic GST Alpha levels. Few data on the effect of I3C on GST isoenzymes are available in the literature. Stresser et al. [39] showed a 4-fold induction of rat hepatic subunit 10 by 0.2% I3C, and Kim et al. [38] noticed GST Pi positive foci in the liver after 0.25% I3C in the diet for 2 weeks. In male CD-1 mice, hepatic GST Mu levels were elevated by I3C (750 mg/kg body weight), whereas 250 and 500 mg/kg did not show any effect [60].

The monocyclic monoterpene D-limonene is a natural product constituting up to 95% of orange peel oil and some other essential oils. D-limonene has significant chemopreventive and chemotherapeutic activity without toxicity in rodents [22–26,61,62]. We only found an enhancing effect of D-limonene on GST activity and GST Pi levels in the proximal small intestine, and on GST Alpha and GST Pi levels in liver and colon, respectively. Previously, induction of hepatic GST activity was demonstrated in mice after feeding 1% of D-limonene for 10 days [63] or of 20 mg/animal for 2 days [64], and in liver of rats after feeding 5% (2.4 ×) or 1% (1.6 ×) of D-limonene [65]. No effect of D-limonene on GST activity was found in small intestine and forestomach of the mouse [64,66]. In female Wistar–Furth rats, Elegbede et al. [65] showed that 5% D-limonene induced hepatic class Alpha rat GST subunit 1. They also demonstrated an increase in rat liver subunits 3 and 4, and an induction of GST activity, which was not noticed in our study, possibly due to a lower dose given by us.

The NSAID relafen induced GST Alpha levels in liver and GST Pi levels in stomach and DSI, without showing an effect on GST activity. GST Mu is the most abundant class of GSTs in the rat gastrointestinal tract with the highest specific enzyme activity. It is possible that the minor increases in GST Alpha and Pi levels do not give rise to a significant increase in GST activity. NSAIDs may protect against both human and animal oesophageal, gastric, and colonic cancer. We recently demonstrated that the NSAIDs indomethacin, ibuprofen, piroxicam and sulindac induced GST activity and isoenzyme levels in the rat gastrointestinal tract [41], which could be a common working mechanism for cancer prevention by NSAIDs. Since the NSAIDs mentioned above [41]

may have severe adverse effects, we now studied relafen, which is as effective as aspirin, diclofenac, indomethacin, ibuprofen, and naproxen in the treatment of patients with osteoarthritis or rheumatoid arthritis [30], but may have less side effects [30,31], even at higher doses [32]. No anticarcinogenic properties of relafen have been demonstrated so far, and therefore it is striking that relafen only has minor effects on GSTs.

GSH is an important tripeptide thiol which in addition to being the substrate for GSTs, maintains cellular oxidation–reduction balance and protects cells against free radical species [67]. A number of inducers of GST also enhanced GSH levels in a variety of tissues [13,33,41]. Thus, determination of tissue GSH levels in combination with GST activities was used to evaluate the detoxifying potential of anticarcinogens. Until now, no data on the effects of sulforaphane analog compound 30, I3C and relafen on GSH levels were published. Sulforaphane analog compound 30 induced GSH in stomach, PSI and MSI, and relafen induced GSH levels in oesophagus and PSI. D-limonene and I3C did not influence GSH levels. In Swiss Webster mice, 1% D-limonene slightly induced hepatic GSH levels (1.2 ×) (63), whereas in female A/J mice, 20 mg D-limonene per day for three days resulted in a reduction of GSH in forestomach (0.49 ×), whereas no effect was seen in liver, small intestine, lung and colon [66]. In female Wistar–Furth rats, 1% D-limonene did not change hepatic GSH levels [65].

GPx, which utilizes GSH to catalyze the reduction of hydroperoxides, is a cellular defence system against the deleterious effects of hydroperoxides [68,69]. Compound 30 induced Se-GPx in DSI and colon and t-GPx in DSI, and relafen induced oesophageal Se-GPx activity. Increase of GPx levels may therefore also contribute to the overall anticarcinogenic effect of these compounds, however probably to much lesser extent as compared to the GSTs. Again, there is little information available in the literature. In male Wistar rats, 50 mg of I3C/kg body weight per day for 10 consecutive days resulted in a 19% reduction of GPx activity [58].

In conclusion, our data demonstrate that dietary administration of sulforaphane analog compound 30 and to a lesser extent indole-3-carbinol and D-limonene may exert their chemopreventive effect in the digestive tract of the rat by enhancing GST and, to a lesser extent, GPx and GSH levels.

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