RESEARCH ARTICLE

Identification of glucosinolate congeners able to form DNA adducts and to induce mutations upon activation by myrosinase

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Scope: Juices from Brassicales are mutagenic in *Salmonella typhimurium* and characteristic adducts are formed with the endogenous DNA in Brassicales homogenates. These effects require myrosinase activity, suggesting an involvement of breakdown products of glucosinolates (GLs). We aimed to identify GLs congeners producing these effects.

Methods and results: We investigated twelve individual GLs for mutagenicity in *S. typhimurium* TA104 and TA100 and for adduct formation with herring sperm DNA using the 32P-postlabelling/thin-layer chromatography method. All bacteriotoxic and mutagenic effects observed required the presence of myrosinase. Neoglucobrassicin, 4-methoxyglucobrassicin and sinalbin showed mutagenicity over wide concentration ranges, with neoglucobrassicin being the most potent congener. Six other GLs led to modest increases in the number of revertants in a small concentration range, before toxicity overshadowed this effect. The remaining three GLs showed some toxicity, but no mutagenicity. However, all twelve GLs formed DNA adducts. Clearly the highest adduct levels were detected with the indole GLs tested. They matched the major adduct spots formed in Brassicales homogenates.

Conclusion: The observation that GLs are genotoxic demands follow-up studies on possible genotoxic and carcinogenic effects of these common food compounds in animal models and humans. Our study may be used to prioritize the congeners in further studies.

Keywords:
Brassicales / DNA adducts / Glucosinolates / Mutagenicity / ^{32}P-postlabelling

1 Introduction

Glucosinolates (GLs, general structure is shown in Fig. 1) are natural pesticides of plants of the order of Brassicales [1, 2]. They are used as defence against microbial infections and herbivorous animals. The Brassicales contain numerous food and fodder plants, such as cabbage (*Brassica oleracea* var. *capitata*), broccoli (*B. oleracea* var. *italica*), rape (*Brassica napus*), pak choi (*Brassica rapa* var. *chinensis*), radish (*Raphanus sativus*), rock salad (*Eruca sativa*), mustard (*Sinapis alba*) and garden cress (*Lepidium sativum*). Therefore, the biological effects of GLs on humans and production animals are of interest.

GLs have no pesticidal activity as such, but require metabolic activation by glycosidases [3]. Specifically, Brassicales express glycosidases with high activity towards GLs in idioblastic myrosin cells. These enzymes, termed myrosinases or β-thioglucoside glucohydrolases (E.C. 3.2.1.147), and the GLs are stored in separate, but neighbouring cells, in the plant tissue. This GL-myrosinase system is activated

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Abbreviation: GL, glucosinolate

upon mechanical damage of the plant. GLs and myrosinase come together, followed by the formation of various electrophilically reactive breakdown products, such as isothiocyanates, nitriles, thiocyanates and epithioalkylnitriles (scheme is shown in Fig. 1), depending on the side chain structure of the GL and the reaction conditions. Major products act as pesticides by covalent binding to nucleophilic cellular structures. For the genetic toxicologist, electrophilic reactivity is a clear warning lamp indicating a particular risk of genotoxic activity. Therefore, it is surprising that GLs have been tested hardly for genotoxic and carcinogenic activity. In part, this may be due to the fact that most GLs are not commercially available, at least not in the quantities required for toxicological investigations. Therefore, they have to be purified from Brassicales, work that is outside the common experience of most toxicological laboratories. However, some isothiocyanates, known breakdown products of GLs, have demonstrated mutagenic and other genotoxic effects in various *in vitro* models [4–7]. Furthermore, Kassie *et al.* [8] have detected that juices from various Brassicales vegetables are mutagenic in bacterial and mammalian cells in culture. We confirmed that broccoli juice is mutagenic in a his$^-$ *Salmonella typhimurium* strain (Ames test) and that this mutagenicity is mediated by myrosinase [9]. In the same study, we demonstrated that characteristic adducts are formed with the endogenous DNA, used as a surrogate target, in Brassicales homogenates. Again, this adduct formation was dependent on the presence of enzymatically active myrosinase, suggesting an involvement of breakdown products of GLs. The aim of the present study was to identify GLs that can form DNA adducts and induce mutations. Moreover, we aimed to compare the adduct profiles produced by individual GLs with those detected in tissue homogenates of Brassicales.

2 Materials and methods

2.1 Chemicals and enzymes

GLs were obtained from different vegetable-based sources, as summarized in Table 1. Slightly different purification procedures were used in the three laboratories involved.

2.1.1 Laboratory A (Research Centre for Industrial Crops, Bologna)

Seeds were first ground to a fine powder and defatted with hexane. The solvent was removed and the defatted meals were used as starting material. Leaves, vegetables and sprouts were freeze-dried and ground. The samples were treated with boiling 70% ethanol in order to quickly deactivate the endogenous enzyme myrosinase. GLs were extracted using an Ultraturrax homogenizer at a medium speed for 15 min. The resulting homogenate was centrifuged at $17\,700 \times g$ for 30 min. The isolation of GLs from the extract was carried out by one-step anion exchange

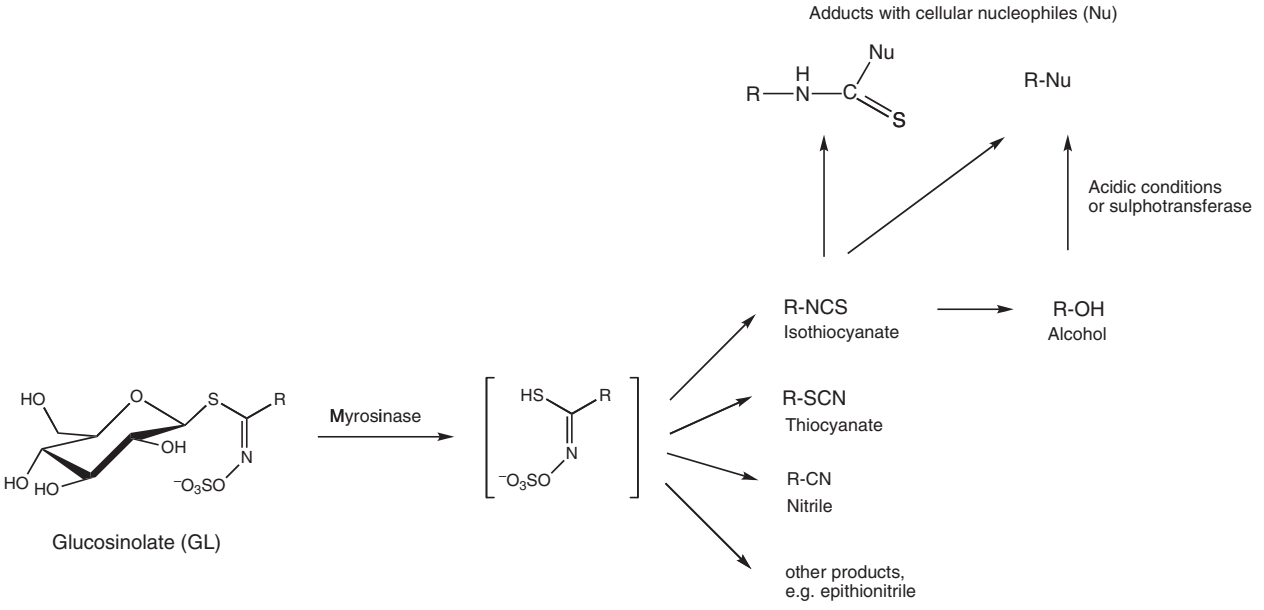

Figure 1. Scheme of myrosinase-mediated breakdown of GLs. Several products are electrophilically reactive. This reactivity has been studied primarily for isothiocyanates. They can undergo addition as well as substitution reactions with nucleophiles [1, 3, 26, 27]. Substitution reactions have been observed primarily with benzylic isothiocyanates, a class that includes indolylmethyl isothiocyanates. These intermediates may also react with water resulting in the formation of benzylic alcohols, which still may react with nucleophiles, especially under acid conditions or upon enzymatic sulpho conjugation [26, 27].

Table 1. GLs used in the present study and plant sources they were isolated from. The isolations were conducted in the Research Centre for Industrial Crops, Bologna (A), the German Institute of Human Nutrition, Potsdam-Rehbrücke (B) and the Leibniz-Institute of Vegetable and Ornamental Crops, Grossbeeren (C)

Trivial name	CAS	Side chain (R in Fig. 1)	Plant source (laboratory)
Glucoiberin	554-88-1	3-Methylsulphinylpropyl	*Iberis amara* seeds (A)
Glucoraphanin	457655-34-4	4-Methylsulphinylbutyl	*B.* var. *acephala* subvar. *laciniata* seeds (A)
Glucoraphenin	28463-24-3	4-Methylsulphinyl-3-butenyl	*R. sativus* seeds (A)
Glucorophasatin	28463-23-2	4-Methylthio-3-butenyl	*R. sativus* sprouts (A)
Sinigrin	3952-98-5	2-Propenyl	*Brassica juncea* seeds (A)
Gluconapin	19041-09-9	3-Butenyl	*B. rapa* seeds (A)
Glucatropaeolin	499-26-3	Benzyl	*L. sativum* seeds (A, C)
Sinalbin	20196-67-2	4-Hydroxybenzyl	*S. alba* seeds (A, C)
Gluconasturtiin	499-30-9	2-Phenylethyl	*Barbarea verna* seeds (A)
Glucobrassicin	4356-52-9	3-Indolylmethyl	*Isatis tinctoria* leaves (A); *B.* var. *italica* mature florets heads and *B. rapa chinensis* leaves (B)
Neoglucobrassicin	5187-84-8	1-Methoxy-3-indolylmethyl	*B. oleracea* var. *botrytis* subvar. *cymosa* mature florets heads (A); *B. oleraceae* var. *italica* mature florets heads and *B. rapa chinensis* leaves (B)
4-Methoxyglucobrassicin	83327-21-3	4-Methoxy-3-indolylmethyl	*B. oleraceae* var. *italica* mature florets heads and *B. rapa chinensis* leaves (B)

chromatography, as previously described [10, 11]. The extract was loaded on a DEAE-Sephadex A-25 (Pharmacia, Milan, Italy) anion-exchange column (150 × 26 mm) conditioned with 25 mM acetate buffer (pH 5.6). After washing with 1 L of distilled water, the GLs were eluted with 500 mL of aqueous K_2SO_4 (0.1–0.2M for aliphatic and aromatic GLs, 0.8M for indole GLs). Eluate fractions were concentrated to dryness using a rotary evaporator at 60–70°C under vacuum. Three subsequent extractions were carried out with 70–100 mL of boiling methanol. Then, the alcoholic extracts were filtered and concentrated to 15–20% of the initial volume. The solution was warmed and slowly added, dropwise, to 200 mL of ethanol that was previously cooled to −20°C. This led to the precipitation of a white powder. After centrifugation, the solid GL (as potassium salt) was dried and sealed under vacuum to prevent moisture uptake by the highly hygroscopic compound. The purity of the alkyl-GLs was further improved by gel-filtration performed using an XK 26/100 column packed with Sephadex G10 (Amersham Biosciences, Milan, Italy) connected to a fast protein liquid chromatograph system (FPLC System, Pharmacia), as previously described with some modification [12–14], whereas the pale yellow substances present in the sample of indole GLs were removed using an XK 16/70 column containing Sephadex LH20 (Amersham Biosciences). A GL sample of 500–1000 μL was loaded onto the column and eluted using a mobile phase of water (alkyl-GLs) or 70% ethanol in water (indole GLs) at a flow rate of 2.0 mL/min. After the void volume was discarded, 5-mL fractions were collected. Individual fractions were analysed by HPLC and those containing pure GL were pooled and freeze-dried. GLs were characterized by ^{1}H and ^{13}C NMR spectroscopy and the purity was assayed by HPLC analysis of the desulpho-derivative according to the ISO 9167-1 method [15]. The purities were in the range of 95–99%.

2.1.2 Laboratory B (German Institute of Human Nutrition, Potsdam-Rehbrücke)

Indole GLs were isolated from mature broccoli florets heads, broccoli sprouts and pak choi sprouts following the procedure of Iori *et al.* [12] with slight modification. Freeze-dried plant homogenate (12 g dry matter) was extracted with 70% boiling methanol. Indole GLs were pre-purified using a DEAE-Sephadex A-25 (Sigma-Aldrich, Munich) anion-exchange column (20 × 2 cm) as described previously [12]. The eluate fractions containing indole GLs were subjected to preparative HPLC using a Prep LC 150 (Waters, Escheborn, Germany) coupled to a 996 photodiode array detector (Waters). First, GLs were purified *via* a semi-preparative column SunFire C18 OBD (150 × 19 mm, 5 μm; Waters) using a 20-min linear gradient starting from 100% solvent A (20 mM ammonium acetate) to 50% solvent A/50% solvent B (methanol) at a flow rate of 20 mL/min. After freeze-drying, the product was further purified on a Delta Pak C4 column (300 × 19 mm, 15 μm; Waters) using a 20-min linear gradient starting from 100% solvent C (5% methanol in water) to 50% solvent C/50% solvent B (methanol) at a flow rate of 20 mL/min. The fractions containing a given

indole GL were combined and freeze-dried. The products were stored at −80°C under argon. The purity was >99% as indicated by LC-MS.

2.1.3 Laboratory C (Leibniz-Institute of Vegetable and Ornamental Crops, Grossbeeren)

Aromatic GLs were extracted from seeds with 80% methanol and then purified on DEAE-Sephadex columns as described [16].

2.1.4 Purification of myrosinase

The enzyme myrosinase was isolated from seeds of *S. alba* L., as described by Pessina et al. [17] with some modification. The specific activity of the stock solution used in the present study was about 60 U/mg of soluble protein. The enzymatic activity was about 30 U/mL and the solution was stored at 4°C in sterile distilled water until use. One myrosinase unit was defined as the amount of enzyme able to hydrolyse 1 μmol sinigrin *per* min at pH 6.5 and 37°C.

2.2 Mutagenicity in bacteria

Mutagenicity was determined using a liquid-preincubation version of the assay described by Maron and Ames [18] with some modification. *Salmonella* strains, obtained from B. Ames (Berkeley, California), were stored at −80°C. After growing in Nutrient Broth No. 2 at 37°C for 8 h, the cultures were centrifuged, suspended in resuspension medium (1.6 mg/mL Bacto Nutrient Broth and 5 mg/mL NaCl), adjusted nephelometrically to a titre of $1–2 \times 10^9$ colony-forming units *per* millilitre and kept on ice for maximally 5 h. Shortly before use they were centrifuged again and suspended at a 5-fold higher density in resuspension medium.

Glass tubes (10 mL) containing 400 μL buffer (150 mM KCl and 10 mM sodium phosphate buffer, pH 6.4), with or without myrosinase (10 mU), and bacterial suspension (100 μL) were warmed to 37°C in a water bath for 2 min. Then, the test compound (dissolved in 100 μL water) was added. After incubation for 60 min at 37°C, 2.0 mL of 45°C warm soft agar (6 mg/mL agar, 6 mg/mL NaCl, 50 μM L-histidine, 50 μM D-biotin and 50 μM L-tryptophan in 25 mM sodium phosphate buffer, pH 7.4) was added, and the mixture was poured onto a Petri dish containing 24 mL minimal agar (15 mg/mL agar in Vogel-Bonner E medium with 20 mg/mL glucose). After incubation for 2 days at 37°C in the dark, the colonies (his^+ revertants) were counted. Incubations were carried out in triplicate.

The amount of myrosinase used *per* incubation (10 mU) is able to hydrolyse 10 nmol sinigrin *per* minute, or 600 nmol in the liquid-preincubation period (60 min), or 29 μmol in the total incubation period (2 days). The last value is clearly above the highest doses of the GLs used (0.5–5 μmol, depending on the congener). Indeed, the mutagenic activity of gluconasturtiin, a relatively weak mutagen, in strain TA104 amounted to 0, 0.2, 1.5, 2.1 and 1.9 revertants *per* nanomole at myrosinase levels of 0.3, 1, 3, 10 and 30 mU, respectively. Thus, the standard level (10 mU) was sufficient for maximal activation.

We used several different bacterial strains (*S. typhimurium* TA1535, TA1537, TA97, TA98, TA100 and TA104, as well as *Escherichia coli* WP2 uvrA) in initial experiments with selected GLs. Strains TA100 and TA104 were most responsive and, therefore, were used in the main study. TA100 is a standard that carries the hisG46 mutation and is normally reverted by substitution mutations at G/C sites [18, 19]. TA104 carries the same hisG428 mutation as the standard strain TA102. Reversions of these strains are often due to substitution mutations at A/T sites of *hisG*, although suppressor mutations in tRNAs are an alternative [18, 19]. The hisG428 gene is situated on the bacterial chromosome in TA104, but on a multi-copy plasmid in TA102. We prefer the former strain, as we often introduce xenobiotic-metabolizing enzymes *via* plasmid-mediated gene-transfer, which is complicated in the presence of other plasmids.

2.3 Adduct analysis in herring sperm DNA

Individual GLs (10 μM) were incubated with herring sperm DNA (0.5 mg/mL) and myrosinase (20 mU) in 100 μL sodium phosphate buffer (20 mM, pH 6.0) at 37°C for 2 h. Then, the reaction mixture was extracted three times with ethyl acetate and the DNA was precipitated by adding 10 μL of NaCl solution (5 M) and 60 μL of isopropanol. It was washed with 75% ethanol and then dissolved in solution B (0.15 mM sodium citrate, 1.5 mM NaCl, pH 7.5). The DNA concentrations were measured spectrophotometrically using a Nanodrop ND – 1000 Spectrophotometer (peQlab, Erlangen, Germany).

DNA adducts were detected using the ^{32}P-postlabelling/thin-layer chromatography, as described by Phillips and Arlt [20] with minor modification. DNA (5 μg) was hydrolysed by incubation with micrococcal nuclease (30 mU/μL) and spleen phosphodiesterase II (0.5 mU/μL) in succinate buffer (16.7 mM sodium succinate, 8.3 mM $CaCl_2$, pH 6.0, total volume 4.8 μL) at 37°C for 1 h. Adducts were enriched by nuclease-P1-mediated hydrolysis of normal nucleotides. This was performed by adding 4.8 μL of a solution containing nuclease P1 (250 mU/μL), sodium acetate buffer (125 mM, pH 5.0) and $ZnCl_2$ (90 μM). The reaction, carried out at 37°C for 1 h, was stopped by adding 1.92 μL Tris base (0.5 M). 3′-Phosphomononucleotides were subsequently converted to radiolabelled 3′,5′-biphosphates by incubation with 50 μCi [γ-^{32}P] ATP and 0.6 μL T4 polynucleotide kinase (6 U) in 1 μL kinase buffer (200 mM bicine, 100 mM $MgCl_2$, 100 mM DTT and 10 mM spermidine, pH 9.0) at 37°C for

30 min. A 9-μL sample of this digest was applied onto the origin (1.5 cm apart from the side, 8.5 cm from the bottom edge) of a polyethylene-imine-cellulose thin-layer chromatography sheet (10 × 20 cm, Macherey-Nagel, Düren, Germany) with a filter paper attached on the top. Normal nucleotides were removed by overnight development in solvent D1 (2.3 M sodium phosphate buffer, pH 5.7). Then, the plates were cut down to 10 × 10 cm, washed in water and dried. Adducts were further resolved in the opposite direction in solvent D3 (2.8 M lithium formate, 6.6 M urea, pH 3.35) and in the perpendicular direction in solvent D4 (0.8 M lithium formate, 0.5 M Tris-HCl, 8.5 M urea, pH 8.0). After each chromatographic step, the plates were washed in water and then dried. DNA adducts were visualized and quantified with an Instant Imager (Canberra Packard, Meriden, USA). The relative adduct levels were calculated from the levels of radioactivity in the nucleotide adduct spots detected on the postlabelling chromatograms and the specific activity of the [γ-^{32}P] ATP used, taking into account the labelling activity (assessed with 3′-phospho-dA) of the T4 polynucleotide kinase batch used and the counting efficiency of the imager [21].

2.4 DNA adduct analyses in *S. typhimurium*

Bacteria were exposed to GLs in the presence of myrosinase for 60 min, as described in Section 2.2. However, the amount of myrosinase was 30 rather than 10 mU *per* incubation unit, as relatively high GL concentrations were used. After the incubation, the bacterial DNA was isolated, as described in detail elsewhere [9], and analysed for the presence of adducts as described in Section 2.3.

3 Results

3.1 Mutagenicity and toxicity in *S. typhimurium*

None of the GLs tested showed any sign of mutagenicity (reflected by an increase in the number of revertants) or cytotoxicity (reflected by a decrease in the number of revertants and/or a thinning of the his$^-$ background lawn) in strains TA104 and TA100 in the absence of myrosinase (Figs. 2 and 3, open squares). However, when myrosinase was added, all GLs became toxic and/or mutagenic in one or both strains used (Figs. 2 and 3, solid circles). Neoglucobrassicin and sinalbin led to the highest increases in the number of revertants in both strains. However, neoglucobrassicin required lower doses and, therefore, was more potent than sinalbin. The slope of the initial part of the dose–response curve (using a linear scale for the dose) amounted to 100 and 50 revertants *per* nanomole in strains TA104 and TA100, respectively, for neoglucobrassicin. The corresponding values for sinalbin were 3.5 and 2.5 revertants *per* nanomole.

4-Methoxyglucobrassicin led to a moderate increase in the number of revertants with both strains (Figs. 2 and 3, last panel). The effect monotonously increased with the dose.

Several other GLs (glucoraphasatin, sinigrin, gluconapin, glucotropaeolin, gluconasturtiin and glucobrassicin) led to a moderate increase in the number of revertant colonies above the spontaneous level in at least one strain in relatively narrow dose ranges, just before toxicity became dominant. All these increases were verified in repeat experiments (data not shown). The remaining GLs tested (glucoiberin, glucoraphanin and glucoraphenin) did not show any indication of mutagenicity, but became toxic at high dose levels. The mutagenic potencies (slopes of the dose–response curves) of the various GLs are listed in Table 2.

3.2 Formation of adducts in herring sperm DNA

In the presence of myrosinase, all GLs formed adducts with herring sperm DNA detected in the ^{32}P-postlabelling assay (Fig. 4). The GLs could be classified into two groups according to the pattern and level of adducts formed. All aliphatic GLs (glucoiberin, glucoraphanin, glucoraphenin, sinigrin and gluconapin) and aromatic GLs (glucotropaeolin, sinalbin and gluconasturtiin) only produced a single, rather faint adduct spot. Moreover, these spots were formed by different aliphatic and aromatic GLs which showed similar chromatographic properties, approximately matching the minor adduct spot (No. 5) in DNA of broccoli homogenate.

Nearly, 100-fold higher adduct levels were found in DNA exposed to indole GLs (glucobrassicin, neoglucobrassicin and 4-methoxyglucobrassicin) as compared to all other GLs. Each indole GLs generated several adduct spots. They matched the major adduct (No. 1–4) occurring in DNA of broccoli homogenate. Spot 1 was formed by glucobrassicin, spot 2 by glucobrassicin and 4-methoxyglucobrassicin, spot 3 was formed by neoglucobrassicin, and spot 4 was formed by all indole GLs. In addition, neoglucobrassicin produced a weak spot that may correspond to spot 5 in DNA of broccoli homogenate.

3.3 Formation of DNA adducts in *S. typhimurium*

Although glucobrassicin and 4-methoxyglucobrassicin formed similar levels of adducts in herring sperm DNA to those produced by neoglucobrassicin, they were the only weak mutagens in *S. typhimurium*. This finding raised the question whether the adducts of these other indole GLs are less mutagenic than those formed by neoglucobrassicin. Alternatively, the toxicokinetics of the active metabolites may have differed between both experimental models used. Therefore, we studied the adduct

formation in *S. typhimurium* under the conditions of the mutagenicity experiment. Neoglucobrassicin and glucobrassicin produced the same patterns of adducts in *S. typhimurium* (Fig. 5) as in herring sperm DNA (Fig. 4), suggesting that these adducts were causal to the mutagenicity. However, adduct levels were approximately

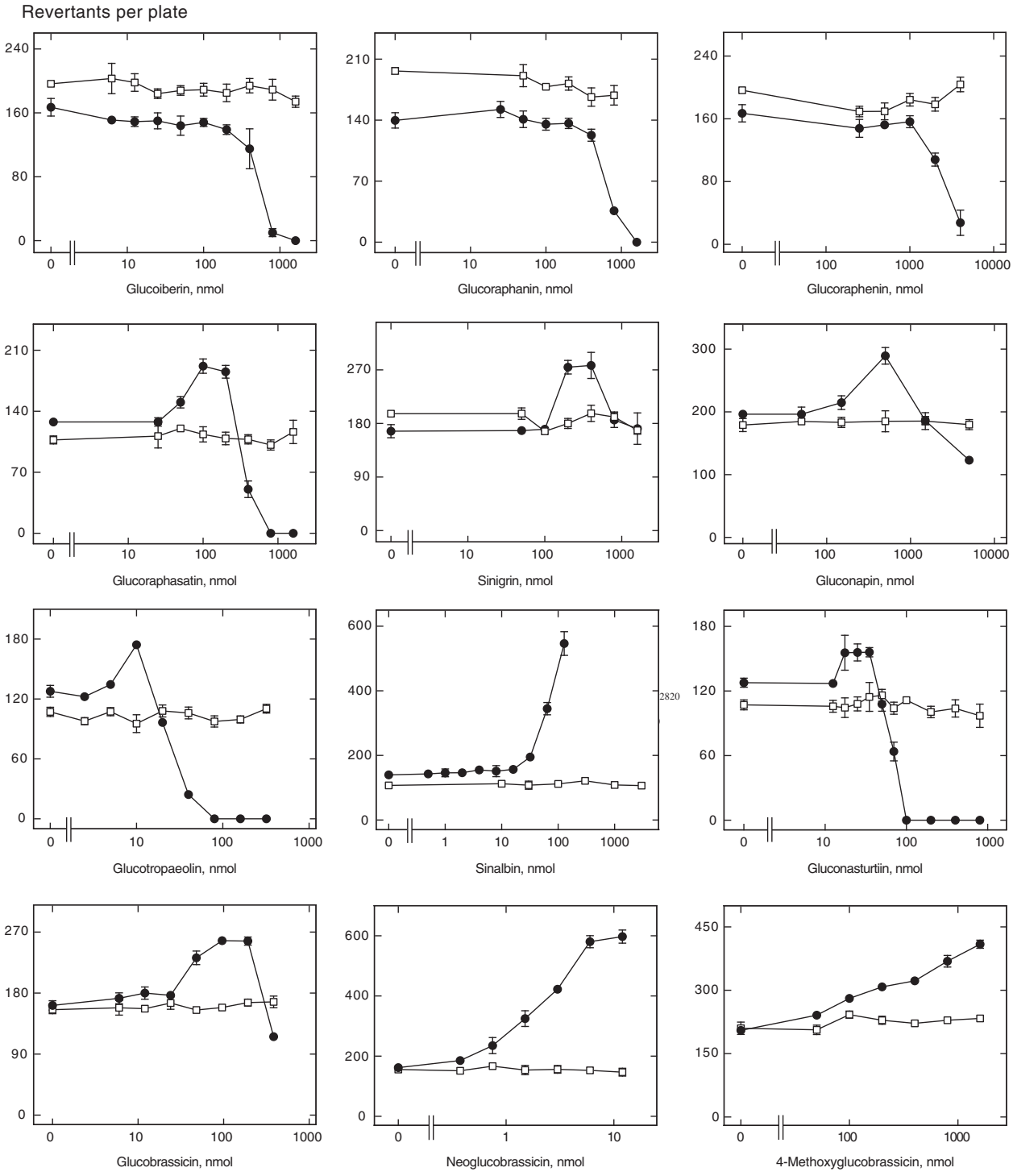

Figure 2. Dose–response curves of the mutagenicity of GLs in *S. typhimurium* TA104 in the presence (solid circles) and absence (open squares) of myrosinase. Decreases in the number of revertants at high dose levels were due to bacteriotoxicity, which also led to a decrease in the his^- background lawn. For the compounds showing mutagenicity over a wide dose range, neoglucobrassicin and sinalbin, only the increasing part of the dose–response curve is presented. Values are mean $\pm$ SE of three plates.

10-fold lower in *S. typhimurium*, although higher concentrations of the test compounds (43 μM neoglucobrassicin and 430 μM glucobrassicin for 1 h) were used than with herring sperm DNA (10 μM of either compound for 2 h). The reduced adduct formation in the cells may be due to limited permeation of the bacteria by the active metabolites, reaction of the metabolites with other cellular nucleophiles, active detoxification and/or DNA repair.

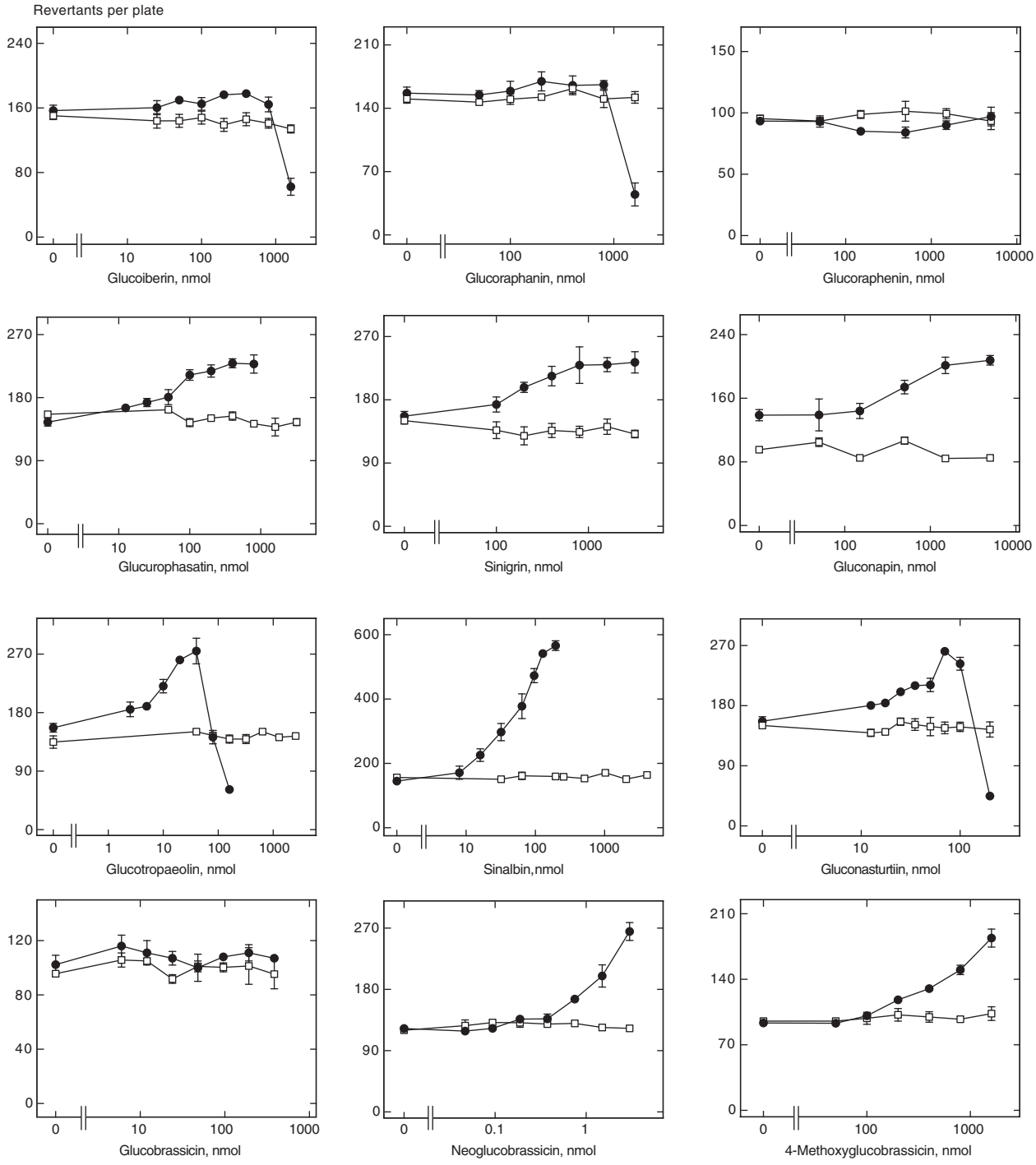

Figure 3. Dose–response curves of the mutagenicity of GLs in *S. typhimurium* TA100 in the presence (solid circles) and absence (open squares) of myrosinase. Decreases in the number of revertants at high dose levels were due to bacteriotoxicity, which also led to a decrease in the his^- background lawn. For the compounds showing mutagenicity over a wide dose range, neoglucobrassicin and sinalbin, only the increasing part of the dose–response curve is presented. Values are mean ± SE of three plates.

Table 2. Mutagenic activity of GLs in *S. typhimurium* strains TA104 and TA100, tested in the presence of myrosinase. Values represent the slope of the initial part of the dose–response curves depicted in Figs. 2 and 3

Test compound	Revertants per nmol	
	TA104	TA100
Glucoiberin	−(<1)	−(<0.4)
Glucoraphanin	−(<0.4)	−(<0.4)
Glucoraphenin	−(<0.2)	−(<0.2)
Glucorophasatin	0.6[a)]	0.6[a)]
Sinigrin	0.5[a)]	0.2[a)]
Gluconapin	0.2[a)]	0.05[a)]
Glucotropaeolin	4[a)]	6[a)]
Sinalbin	3.5	5
Gluconasturtiin	2[a)]	1.5[a)]
Glucobrassicin	1.5[a)]	−(<0.3)
Neoglucobrassicin	100	50
4-Methoxyglucobrassicin	0.2	0.08

a) The number of revertants was only elevated over a small concentration range, just before the compound became toxic, and did not reach a two-fold increase above the spontaneous level.

Interestingly, a clearly lower concentration of neoglucobrassicin, compared to glucobrassicin, was required to produce similar adduct levels in the bacteria (Fig. 5), in agreement with its higher mutagenic activity. 4-Methoxyglucobrassicin, a weak mutagen, did not form detectable adducts in the bacteria.

4 Results and Discussion

We previously reported that characteristic adducts are formed in the endogenous DNA after homogenizing Brassicales plants and that this adduct formation requires myrosinase activity, suggesting a causal involvement of breakdown products of GLs [9]. In the present study we demonstrate that purified GLs can form adducts with the same chromatographic properties when activated by myrosinase. The major adduct spots observed in broccoli homogenate appear to be formed by indole GLs. Adduct spot 3 matches with the major adduct produced by neoglucobrassicin, but with no adduct spot observed with any other GLs studied. Adduct spots 1, 2 and 4 in broccoli homogenate may be predominantly formed by glucobrassicin, which – together with neoglucobrassicin – is the major indole GL in mature broccoli florets heads [1]. Neoglucobrassicin may also contribute to spot 2, and 4-methoxyglucobrassicin may contribute to spots 2 and 4 in homogenates of some Brassicales materials. The minor adduct spot 5 in broccoli homogenate could be formed by many different GLs: neoglucobrassicin as well as all alkyl and non-indole aromatic GLs studied produced adducts with similar chromatographic properties. We did not find any GL that formed

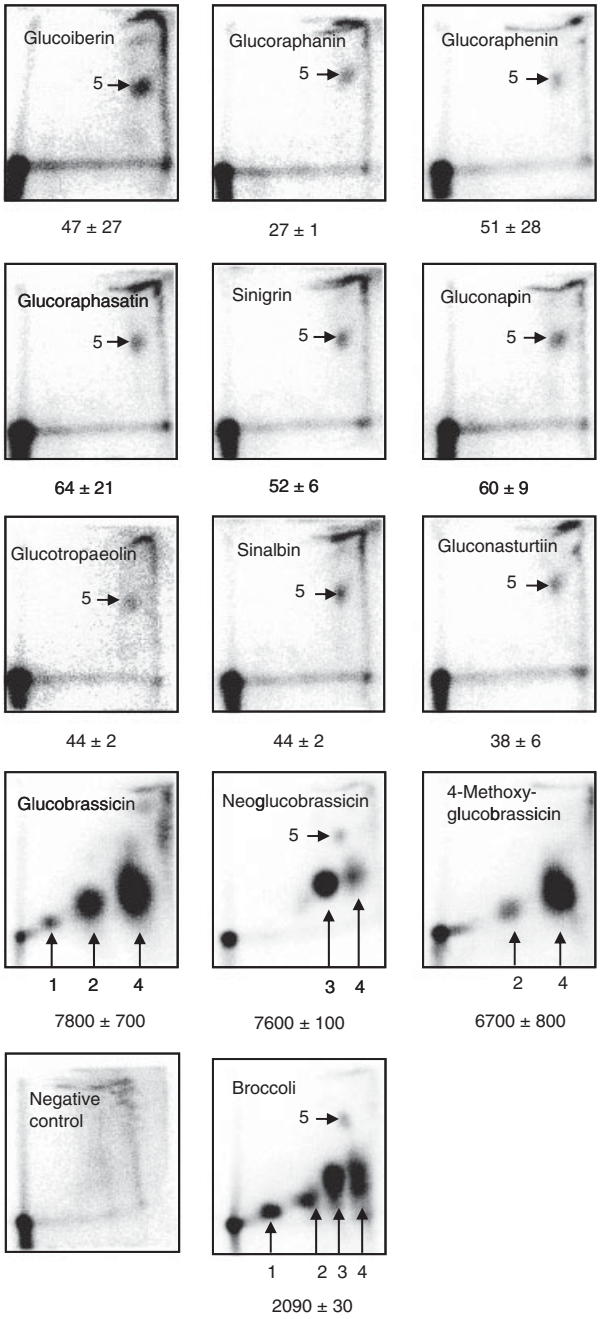

Figure 4. Profiles and levels of DNA adducts detected upon incubation of herring sperm DNA with individual GLs (10 μM) in the presence of myrosinase (37°C, 2 h). DNA treated with myrosinase only was used as a negative control. For comparison, we show adduct spot patterns detected in the endogenous DNA of broccoli homogenate incubated at 37°C for 2 h (panel labelled ''Broccoli''), following a protocol described elsewhere in detail [9]. Adduct spots with similar chromatographic properties were labelled by a common number. The line-like signals parallel to and near the lower and right edges of the autoradiograms contain reagents and side-products of the labelling reaction. The numbers below the chromatograms indicate the adduct level (*per* 10^8 nucleotides), as determined from the radioactivity contained in the marked spots. They are means and ranges of two incubations.

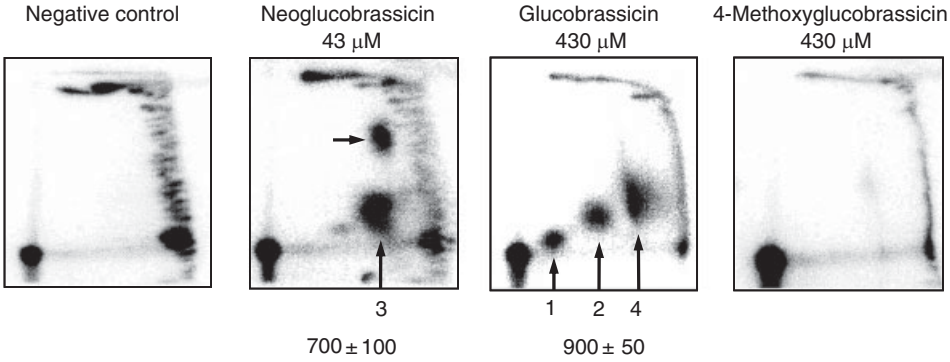

Figure 5. Formation of DNA adducts in *S. typhimurium* TA100 by indole GLs using the same exposure conditions as in the mutagenicity experiment, but elevated concentrations of the GLs – 43 μM (equivalent to 30 nmol *per* mutagenicity assay) of neoglucobrassicin and 10-fold higher levels of the other congeners. Bacteria exposed to myrosinase in the absence of a GL were used as a negative control. The numbers below the chromatograms indicate the adduct level (*per* 10^8 nucleotides), as determined from the radioactivity contained in the marked spots. They are means and ranges of two determinations.

adducts with the chromatographic properties of adduct 6 (a minor adduct spot detected in Brussels sprout [*B. oleracea* var. *gemmifera*] only [9]) and adduct 7 (a minor adduct spot detected in Savoy cabbage [*B. oleracea* convar. *capitata* var. *gemmifera*] only [9]).

Broccoli juice is mutagenic in *S. typhimurium* strains [8, 9]. We found that several individual GLs are mutagenic in the same experimental model. However, neoglucobrassicin was much more potent than any other congener tested, suggesting that it was the dominating mutagen in broccoli juice. This notion was corroborated by the observation that adduct spot 3, which is characteristic for neoglucobrassicin (present study), was by far the strongest adduct spot in *S. typhimurium* exposed to broccoli juice under the conditions of the mutagenicity experiment [9].

Neoglucobrassicin induced 100 and 50 revertants *per* nanomole in strains TA104 and TA100, respectively, and therefore is a rather potent mutagen, similar to the classical carcinogens benzo[*a*]pyrene and 2-acetylaminofluorene, reported to induce 121 and 108 revertants *per* nanomole, respectively, in the most responsive strain after activation by liver postmitochondrial fraction [22].

Sinalbin was the other GL showing increasing numbers of revertants over a wide concentration range and a relatively strong maximal effect in the bacterial mutagenicity assay. However, it only demonstrated relatively weak adduct signals in herring sperm DNA, similar to the other aliphatic and aromatic GLs tested. It is possible that we missed some adducts with the ^{32}P-postlabelling method used. Alternatively, the adduct detected may have been sufficient for a mutagenic effect. Indeed, the mutagenic potency (revertants *per* nanomole) – unlike the maximal mutagenic response – of sinalbin was relatively low, similar to that of other aromatic GLs studied (glucotropaeolin, gluconasturtiin, and glucobrassicin). Thus, the major difference was the higher bacteriotoxicity of the latter congeners, hindering the detection of high numbers of revertants. Slightly lower mutagenic potencies were observed with the alkyl GLs sinigrin and glucorophasatin. The remaining alkyl GLs tested – glucoiberin, glucoraphanin and glucoraphenin – showed bacteriotoxic, but no mutagenic effects, although they formed DNA adducts in the cell-free system. Possibly, breakdown products preferentially react with nucleophiles other than DNA in the bacteria.

Mutagenicity is an important mechanism of carcinogenicity. However, GLs are often associated with anticarcinogenic rather than carcinogenic effects [23]. For example, breakdown products of various GLs activate the NRF2 transcription factor, adjusting the organism to an elevated exposure to reactive metabolites. One of the effects involves the induction of various xenobiotic-metabolizing enzymes able to detoxify certain carcinogens, for example, aflatoxin B_1. However, it is important to notice that not all GLs are equal in their biological effects. For example, isothiocyanate sulphoraphane, the major breakdown product of glucoraphanin, is a particularly potent activator of NRF2 [23, 24]. In the present study, glucoraphanin activated by myrosinase formed the lowest level of DNA adducts in the cell-free system among all GLs tested and it was not mutagenic in bacteria. In contrast, neoglucobrassicin was a potent mutagen. It does not activate NRF2; it even counteracts some inductive effects of sulphoraphane [25].

In the present study we used *in vitro* models to explore genotoxic effects of GLs. Studies are going on in our laboratories to see whether GLs are also genotoxic in animal models and in humans. The *in vitro* data are being used to prioritize the compounds to be investigated *in vivo*.

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5 References

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