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TOXICOKINETICS AND METABOLISM

# Induction of epoxide hydrolase and glucuronosyl transferase by isothiocyanates and intact glucosinolates in precision-cut rat liver slices: importance of side-chain substituent and chirality

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**Abstract** The potential of three isothiocyanates, namely *R,S*-sulforaphane, erucin and phenethyl isothiocyanate, of two naturally occurring glucosinolates, namely glucoerucin and glucoraphanin, and of the enantiomers of sulforaphane to modulate glucuronosyl transferase and epoxide hydrolase, two major carcinogen-metabolising enzyme systems, was investigated in precision-cut rat liver slices. Following exposure of the slices to the isothiocyanates (0–25 μM), erucin and phenethyl isothiocyanate, but not *R,S*-sulforaphane, elevated glucuronosyl transferase and epoxide hydrolase activities and expression, determined immunologically. Of the two enantiomers of sulforaphane, the *R*-enantiomer enhanced, whereas the *S*-enantiomer impaired, glucuronosyl transferase activity and only the former increased protein expression; furthermore, *R*-sulforaphane was more effective than the *S*-enantiomer in up-regulating microsomal epoxide hydrolase. When precision-cut rat liver slices were exposed to the same concentrations of glucoerucin and glucoraphanin, both glucosinolates caused a marked increase in the activity and expression of the microsomal epoxide hydrolase but had no effect on glucuronosyl transferase activity. It may be inferred that the ability of isothiocyanates to enhance hepatic microsomal epoxide hydrolase and glucuronosyl transferase activities is dependent on the nature of the side chain. Moreover, in the case of sulforaphane, the naturally occurring *R*-enantiomer increased both activities, whereas, in contrast, activities were impaired in the case of the S-enantiomer. Finally, intact glucosinolates are potent inducers of epoxide hydrolase and can thus contribute directly to the chemopreventive potential associated with cruciferous vegetable consumption.

**Keywords** Isothiocyanates · Glucosinolates · Sulforaphane · Glucoraphanin · Phenethyl isothiocyanate · Erucin

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## Introduction

The well-documented chemopreventive activity of cruciferous vegetables (Ambrosone et al. 2004; Tang et al. 2008) has been attributed to glucosinolates, a class of chemicals encountered in substantial amounts in these vegetables (Steinbrecher and Linseisen 2009; Verkerk et al. 2009). When these vegetables are disturbed, the enzyme myrosinase (*β*-thioglucoside glucohydrolase) comes into contact with the glucosinolates facilitating their degradation to products, such as the isothiocyanates, which are believed to mediate their chemopreventive activity (Hayes et al. 2008); microbial myrosinase, present in the human intestine, may contribute to the formation of these bioactive chemicals (Getahun and Chung 1999). Indeed, in the animal models of cancer, isothiocyanates displayed chemopreventive activity against established chemical carcinogens, suppressing tumour formation in a variety of tissues (Zhang 2004).

Modulation of carcinogen metabolism so as to alter the balance of bioactivation and detoxification, favouring the latter, is considered to be a major mechanism of the chemopreventive activity of isothiocyanates. In in vivo studies, isothiocyanates could suppress the activity of cytochrome

P450, undoubtedly the most important enzyme in the bioactivation of chemical carcinogens to their genotoxic intermediates, and stimulate enzyme systems catalysing the detoxification of these intermediates (Yoxall et al. 2005; Hanlon et al. 2008a; Konsue and Ioannides 2008). Indeed, isothiocyanates prevent the formation of DNA adducts following exposure to carcinogens (Dingley et al. 2003; Singletary and MacDonald 2000; Bacon et al. 2003). In in vitro studies utilising precision-cut liver slices, isothiocyanates such as erucin, *R,S*-sulforaphane and phenethyl isothiocyanate perturbed carcinogen-metabolising enzymes at concentrations as low as 1 μM (Konsue and Ioannides 2010; Hanlon et al. 2009a) that may be attained following dietary intake (Konsue et al. 2010).

Most attention has focussed on quinone reductase and the glutathione *S*-transferases, and the modulation of other major carcinogen-metabolising hepatic enzyme systems by isothiocyanates still remains to be evaluated. The current studies were undertaken to evaluate whether naturally occurring isothiocyanates have the potential to modulate microsomal phase II enzyme systems that contribute to carcinogen metabolism. In order to evaluate whether the substituent plays a major role in the modulation of these enzyme systems by isothiocyanates, five compounds were employed, namely *R,S*-sulforaphane, *R*-sulforaphane, *S*-sulforaphane, erucin and phenethyl isothiocyanate (Fig. 1). A marked difference in the up-regulation of quinone reductase and glutathione *S*-transferases was observed between enantiomers of sulforaphane, with only the naturally occurring *R*-enantiomer being effective (Abdull Razis et al. 2010a). Moreover, as glucosinolates per se can up-regulate cytochrome P450 and phase II enzymes in rat liver and lung (Abdull Razis et al. 2010b, c), the ability of glucoerucin and glucoraphanin (Fig. 1), the precursors of erucin and *R*-sulforaphane, respectively, to modulate these enzyme systems was also investigated.

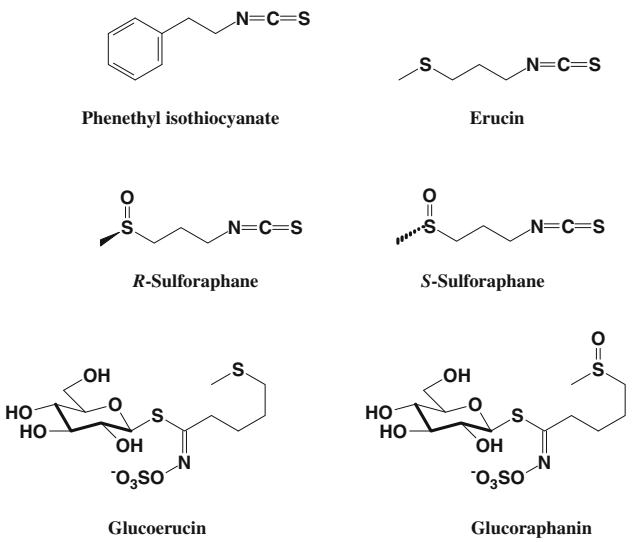


**Fig. 1** Structure of isothiocyanates and glucosinolates used in the current study

## Materials and methods

*R,S*-Sulforaphane, *S*-sulforaphane, phenethyl isothiocyanate (PEITC) and erucin (LKT Laboratories, Minnesota, USA), benzo[a]pyrene 4,5-epoxide and benzo[a]pyrene 4,5-diol (Mid-West Research Institute, Kansas, USA) and 1-naphthol (Sigma Co. Ltd., Poole, Dorset, UK) were all purchased. Antibodies to the microsomal epoxide hydrolase and glucuronosyl transferase (UGT1A6) as well as donkey anti-goat and goat anti-rabbit antibodies were purchased from Santa Cruz Biotechnology (CA, USA) and antibodies against *β*-actin from abcam (Cambridge, UK).

Glucoraphanin and glucoerucin were isolated and purified from *Brassica oleracea* L. var. acephala sabellica (Cavolo nero di Toscana) and *Eruca sativa* ripe seeds, respectively, according to a procedure developed at CRACIN of Bologna that we have previously described (Visentin et al. 1992; Abdull Razis et al. 2010b). *R*-sulforaphane was generated in situ by myrosinase-catalysed hydrolysis of natural glucoraphanin (Abdull Razis et al. 2010b).

Male Wistar albino rats (200–250 g) were obtained from B&K Universal Ltd (Hull, East Yorkshire, UK). The animals were housed at 22 ± 2°C, 30–40% relative humidity, in an alternating 12-h light: dark cycle with light onset at 07.00 h. Rat liver slices (200–300 μm) were prepared from 8-mm cylindrical cores using a Krumdieck tissue slicer (Alabama Research and Development Corporation, Munsford, AL, USA) as previously described (Hashemi et al. 1999). The multiwell plate procedure, using 12-well culture plates, was used to culture the slices in the presence of glucosinolates or isothiocyanates (0–25 μM); isothiocyanates were dissolved in DMSO so that the final concentration was 1.5 μl/ml of incubation medium. One slice was placed in each well, in 1.5 ml of culture medium. Slices were incubated under sterile conditions for 24 h on a reciprocating plate shaker housed in a humidified incubator, at a temperature of 37°C and under an atmosphere of 95% air/5% $CO_2$. The slices were initially pre-incubated for 30 min in order to slough off any dead cells due to slicing. Three different slice pools, each comprising 10 rat liver slices, were used per concentration.

Following incubation, slices were removed from culture media and homogenised, and post-mitochondrial supernatants were prepared and stored at −80° C. When required, microsomes were isolated by centrifugation (105,000g × 1 h). The following assays were carried out on isolated

microsomes: glucuronosyl transferase (UDP-GT) using 1-naphthol as substrate (Bock and White 1974), epoxide hydrolase (EH) using benzo[a]pyrene 4,5-epoxide (Dansette et al. 1979); protein concentration was determined in both cellular subfractions using bovine serum albumin as standard (Bradford 1976). Finally, in order to monitor changes in enzyme protein expression, Western blot analysis was performed. Hepatic microsomal or cytosolic proteins from pooled slices were loaded on to 10% (w/v) SDS–PAGE and then transferred electrophoretically to Hybond-P polyvinylidene difluoride membrane. The immunoblot analysis of rat proteins was carried out by exposure to the primary antibodies followed by the appropriate peroxidase-labelled secondary antibody. $\beta$-Actin was used as the housekeeping protein to normalise protein loading. Immunoblots were quantitated by densitometry using the GeneTool software (Syngene Corporation, Cambridge, UK), with the control band designated as 100%.

Results are presented as mean ± standard deviation of three pools, each comprising 10 slices. Statistical evaluation was carried out by one-way ANOVA followed by the Dunnett's test.

## Results

Exposure of precision-cut rat liver slices to erucin and phenethyl isothiocyanate, but not *R,S*-sulforaphane, resulted in at least a doubling in the glucuronidation of 1-naphthol at an isothiocyanate concentration of 2.5 μM (Fig. 2). Similarly, at the protein level, a modest rise in the expression of this enzyme was only seen in the case of phenethyl isothiocyanate and erucin (Fig. 2). Exposure of the liver slices to phenethyl isothiocyanate caused a very marked increase in microsomal epoxide hydrolase activity; a much less pronounced, but statistically significant, increase was noted in the case of erucin, whereas *R,S*-sulforaphane had no effect; a similar picture emerged at the protein level (Fig. 3).

When the enantiomers of sulforaphane were compared, the *R*-isomer doubled glucuronosyl transferase activity whereas the *S*-isomer, under the same conditions, impaired the activity (Fig. 4). In concordance, *R*-sulforaphane elevated protein levels by about 40%, whereas the *S*-isomer decreased the levels at concentrations of 2.5 μM and higher (Fig. 4). Although both enantiomers enhanced microsomal epoxide hydrolase activity, in the case of the *R*-enantiomer a maximal 65% rise was achieved at the 2.5 μM concentration, whereas a 50% maximal rise was noted following incubation of the slices with *S*-sulforaphane, but was observed at the 10 μM concentration; this is also reflected in the protein levels monitored immunologically (Fig. 4).

Both glucosinolates caused a marked increase in microsomal epoxide hydrolase activity in rat liver slices, which was manifested at 1 μM, the lowest concentration employed, and was accompanied by a parallel rise in protein levels (Fig. 5). Neither compound caused a statistically significant change in glucuronosyl transferase activity but a very modest rise in protein levels was seen at the low levels of exposure (Fig. 5).

## Discussion

Although the effects of isothiocyanates on quinone reductase and glutathione *S*-transferases have been well documented both in vivo and in vitro, their ability to modulate other phase II enzyme systems involved in carcinogen metabolism has received scant attention. UDP-glucuronosyl transferases are a very important phase II biotransformation enzyme system participating in the metabolism of major classes of chemical carcinogens including polycyclic aromatic hydrocarbons and aromatic amines (Bock 2006). A major isoform is UGT1A6, which has been monitored in the present studies immunologically as it is the most important in carcinogen metabolism (Bock 2006). The current studies established that isothiocyanates have the potential to up-regulate glucuronosyl transferase, but the nature of the side chain is very important; erucin was a potent inducer, whereas the structurally related *R,S*-sulforaphane, being its sulphoxide analogue, was inactive in this respect. The rise in activity was the consequence of increased enzyme availability, as indicated in the Western blot studies. These findings are commensurate with in vivo studies where exposure of rats to sulforaphane failed to increase the activity of this enzyme in the liver (Yoxall et al. 2005). At the mRNA level, however, sulforaphane increased UGT1A1 expression in HepG2 cells (Bacon et al. 2003). The present studies support previous findings where cruciferous vegetable diet consumption led to enhanced glucuronidation of the heterocyclic amine PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) in human volunteers, although other components in the vegetables are likely to have also contributed to the up-regulation of this enzyme (Walters et al. 2004). Similarly, in recent studies, consumption of diets supplemented with cruciferous vegetables lowered the levels of serum bilirubin, the glucuronidation of which is catalysed by UGT1A1 (Navarro et al. 2009). Moreover, consumption of watercress, a major source of phenethyl isothiocyanate, increased the metabolism of nicotine as a result of increased glucuronidation on smokers (Hecht et al. 1999), in concordance with the present studies where exposure to of rat liver slices to phenethyl isothiocyanate led to an increase in the glucuronidation of 1-naphthol.

Epoxide hydrolase is involved in the detoxification of many epoxides, which are the reactive intermediates of

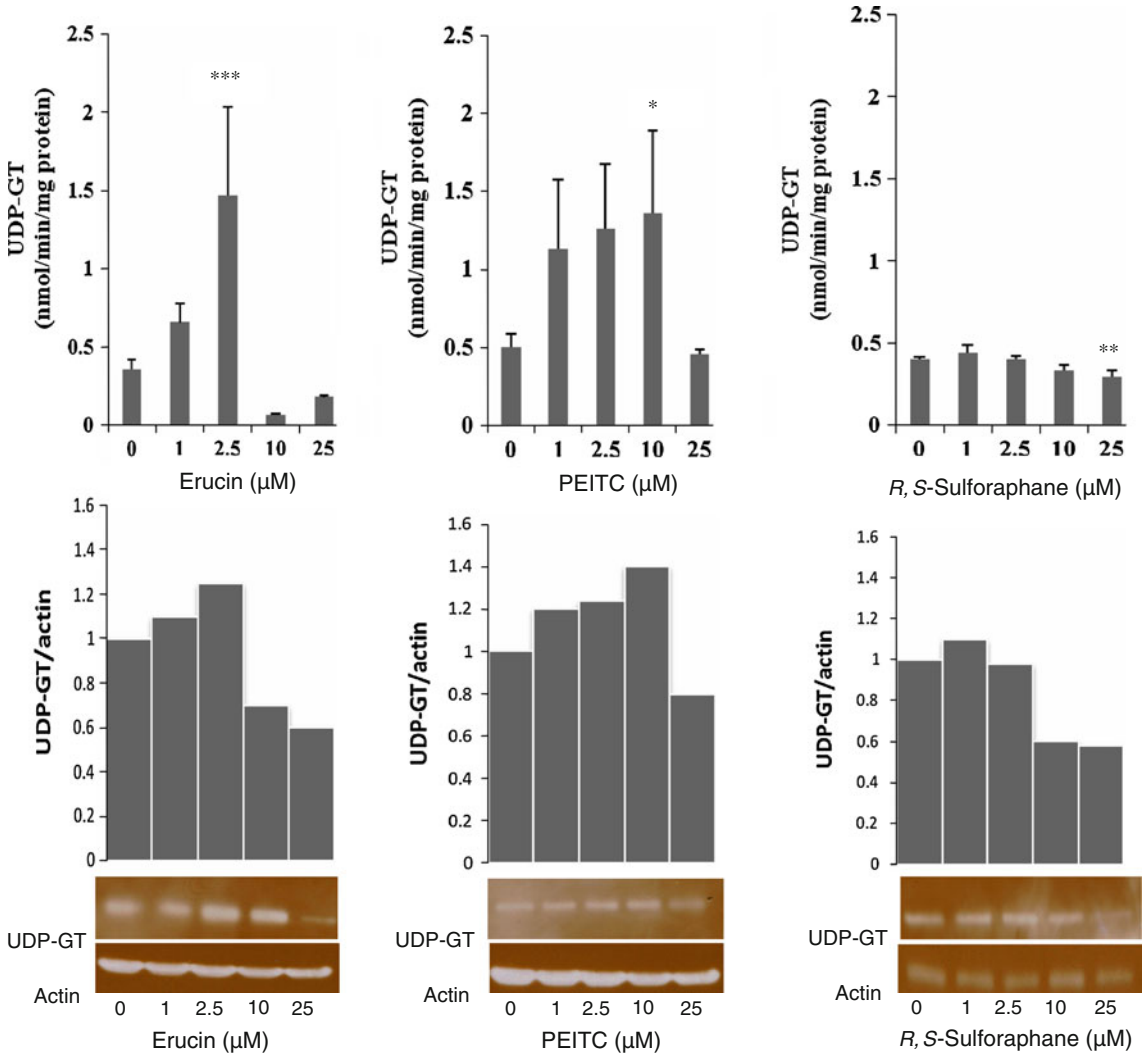


**Fig. 2** Effect of isothiocyanates on the activity and expression of UDP-glucuronosyl transferase in precision-cut rat liver slices. Precision-cut rat liver slices were incubated with isothiocyanates (0–25 μM), for 24 h; slices were pooled and microsomes isolated and UDP-glucuronosyl transferase activity determined. Results are presented as Mean ± SD for three pools of slices, each comprising ten slices. The immunoblot analysis was carried out by exposure to antibodies against glucuronosyl transferase (UGT1A6) followed by the appropriate peroxidase-labelled secondary antibody. Each *lane* was loaded with 30 μg of total protein. The blots were stripped and re-probed with anti-*β*-actin antibody to normalise for differences in protein loading. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

many structurally diverse chemicals including polycyclic aromatic hydrocarbons, mycotoxins such as aflatoxin $B_1$ and halogenated aliphatic compounds such as vinyl chloride (Decker et al. 2009). By far the most effective inducer of epoxide activity was the aromatic phenethyl isothiocyanate, which caused an 8-fold rise in activity, rendering it one of the most potent inducers of this enzyme. Of the two aliphatic isothiocyanates, erucin was a weak inducer, whereas *R,S*-sulforaphane was inactive. Clearly, the nature of the substituent is critical in determining the ability of isothiocyanates to enhance epoxide hydrolase activity.

We have previously reported marked differences in the modulation of cytochrome P450 and of the phase II enzymes quinone reductase and glutathione *S*-transferases by the enantiomers of sulforaphane, with the naturally occurring *R*-isomer being effective (Abdull Razis et al. 2010a). This difference appears to extend to glucuronosyl transferase activity and expression, which were up-regulated only by the *R*-isomer whereas, in contrast, *S*-isomer impaired the activity and decreased protein levels. UGT1 is regulated by the Ah receptor, and it is possible that the mechanism for this difference in the up-regulation of this enzyme may be related to the fact that, although both isomers are poor ligands to the Ah receptor, the *R*-enantiomer is relatively a better ligand than the *S*-enantiomer (unpublished observations). Both enantiomers, however,

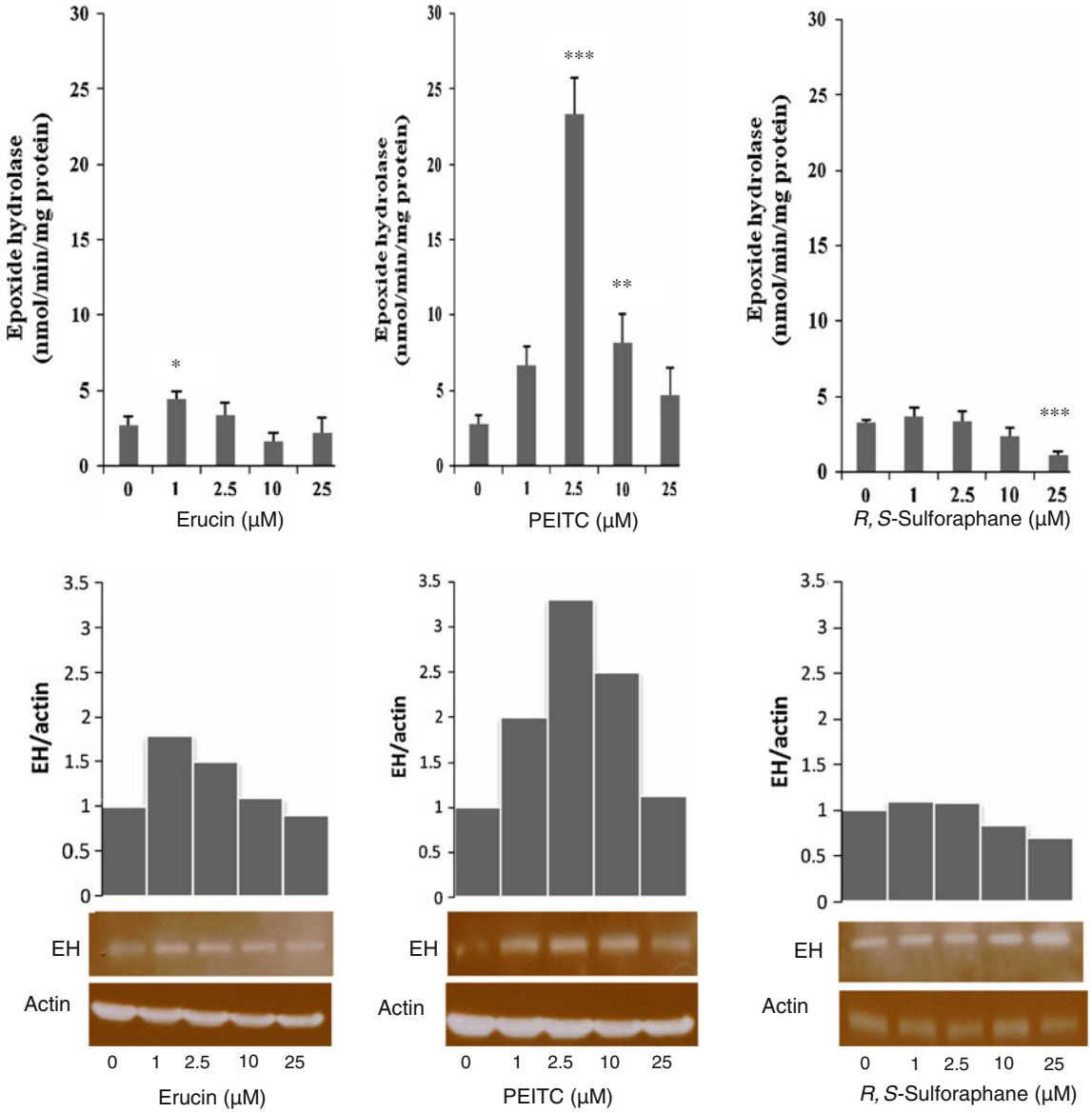


**Fig. 3** Effect of isothiocyanates on the activity and expression of microsomal epoxide hydrolase in precision-cut rat liver slices. Precision-cut rat liver slices were incubated with isothiocyanates (0–25 μM), for 24 h; slices were pooled and microsomes isolated, and epoxide hydrolase activity was determined. Results are presented as Mean ± SD for three pools of slices, each comprising ten slices. The immunoblot analysis was carried out by exposure to antibodies against epoxide hydrolase followed by the appropriate peroxidase-labelled secondary antibody. Each *lane* was loaded with 30 μg of total protein. The blots were stripped and re-probed with anti-$\beta$-actin antibody to normalise for differences in protein loading. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

increased epoxide hydrolase activity, with the *R*-enantiomer being somewhat more potent.

In recent studies, it was demonstrated that, at least in rats and dogs, glucosinolates such as glucoraphanin, encountered in broccoli and being the precursor of the isothiocyanate *R*-sulforaphane, could be absorbed intact following oral intake (Bheemreddy and Jeffery 2007; Cwik et al. 2010) and could conceivably modulate carcinogen-metabolising enzyme systems and thus contribute to the chemopreventive activity associated with the consumption of cruciferous vegetables. In the present studies, the intact glucosinolates glucoerucin and glucoraphanin were more potent than their corresponding isothiocyanates, erucin and *R*-sulforaphane, respectively, in stimulating epoxide hydrolase activity. These observations concord with our previous studies where it was demonstrated that, in both liver and lung, glucosinolates per se can up-regulate enzyme systems that are active in the metabolism of chemical carcinogens (Abdull Razis et al. 2010b, c). The increase in enzyme protein levels indicates that the rise in activity is due, at least partly, to a higher enzyme concentration.

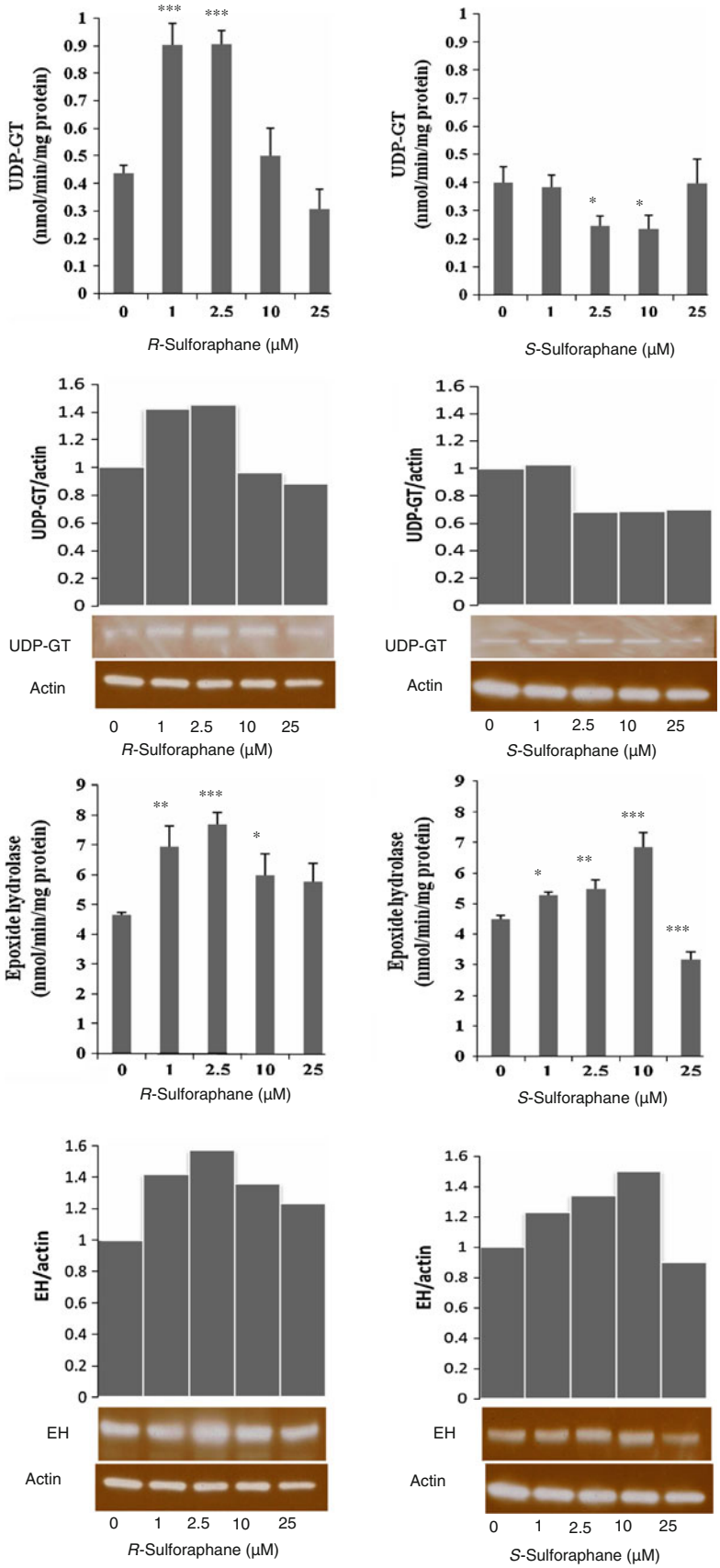


**Fig. 4** Modulation of UDP-glucuronosyl transferase and epoxide hydrolase activities and expression in precision-cut rat liver slices by *R*- and *S*-sulforaphane. Precision-cut rat liver slices were incubated with *R*- and *S*-sulforaphane (0–25 μM), for 24 h; slices were pooled and microsomes isolated, and epoxide hydrolase and UDP-glucuronosyl activities were determined. Results are presented as Mean ± SD for three pools of slices, each comprising ten slices. The immunoblot analysis was carried out by exposure to antibodies against epoxide hydrolase or glucuronosyl transferase (UGT1A6) followed by the appropriate peroxidase-labelled secondary antibody. Each *lane* was loaded with 30 μg of total protein. The blots were stripped and re-probed with anti-*β*-actin antibody to normalise for differences in protein loading. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

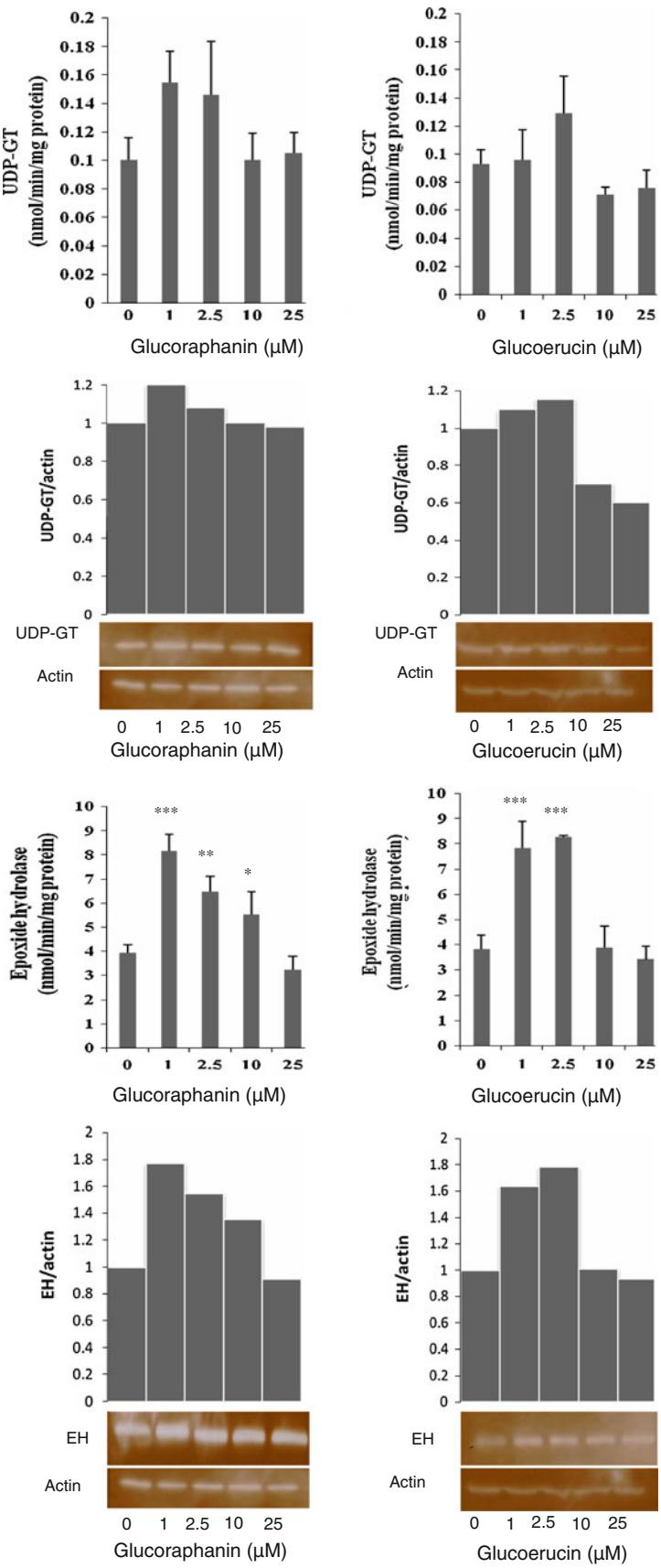


**Fig. 5** Modulation of UDP-glucuronosyl transferase and epoxide hydrolase activities and expression in precision-cut rat liver slices by glucosinolates. Precision-cut rat liver slices were incubated with glucoerucin and glucoraphanin (0–25 μM), for 24 h; slices were pooled and microsomes isolated, and epoxide hydrolase and UDP-glucuronosyl activities were determined. Results are presented as Mean ± SD for three pools of slices, each comprising ten slices. The immunoblot analysis was carried out by exposure to antibodies against epoxide hydrolase or glucuronosyl transferase (UGT1A6) followed by the appropriate peroxidase-labelled secondary antibody. Each *lane* was loaded with 30 μg of total protein. The blots were stripped and re-probed with anti-$\beta$-actin antibody to normalise for differences in protein loading. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

It is important to emphasise that at these concentrations neither glucosinolates nor isothiocyanates display any toxicity to the slices when assessed using as marker the leakage of lactate dehydrogenase into the media (Hanlon et al. 2009a; Konsue and Ioannides 2008; unpublished observations).

In order to extrapolate the effects of the isothiocyanates and glucosinolates to the in vivo situation, it is essential that plasma/liver concentrations of these compounds in humans after dietary intake are established. We are not aware of any studies where the concentrations of glucosinolates in plasma and/or their pharmacokinetic behaviour have been reported. However, there have been a few studies concerned with sulforaphane and phenethyl isothiocyanate. In studies conducted in rat, a single oral dose of phenethyl isothiocyanate, equivalent to the human dietary intake, achieved a plasma $C_{max}$ of about 2 μM (Konsue et al. 2010), but was much lower in the case of sulforaphane following similar treatment (Hanlon et al. 2008b). In a human study conducted in our laboratory, a single oral dose of 300 ml of liquidised broccoli, obtained from a local supermarket and containing low levels of sulforaphane, achieved plasma levels of about 0.1 μM (Hanlon et al. 2009b). However, hepatic intracellular concentrations may be higher than those in the plasma; it has been demonstrated in in vitro studies that peak intracellular concentrations of isothiocyanates are attained within 3 h of exposure, and intracellular concentration may be as much as 200-fold higher than extracellular concentration (Ye and Zhang 2001; Zhang and Callaway 2002).

The present studies allow us to conclude that isothiocyanates enhance rat hepatic epoxide hydrolase and glucuronosyl transferase activities, but the effect depends on the nature of the side chain. When the ability of the enantiomers of sulforaphane to influence these enzymes was compared, the naturally occurring *R*-enantiomer was more effective in increasing both activities compared with the S-enantiomer. The intact glucosinolates glucoerucin and glucoraphanin are potent inducers of epoxide hydrolase but have no effect on glucuronosyl transferase. Finally, these studies emphasise the usefulness of the precision-cut tissue system in assessing and comparing the ability of xenobiotics to modulate carcinogen-metabolising enzyme systems.

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**Conflict of interest** None of the authors has any conflict of interest to declare.

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