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Structure-activity relationships and organ specificity in the induction of GST and NQO1 by alkyl-aryl isothiocyanates

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Abstract

Purpose—To compare the ability of alkyl-aryl isothiocyanates (ITCs) to increase the activities of the Phase 2 detoxification enzymes NAD[P]H:quinone acceptor oxidoreductase (NQO1) and glutathione *S*-transferases (GST) in rat tissues *in vivo* and in cells *in vitro*.

Materials and Methods—12 alkyl-aryl ITCs and the fully-reduced derivative of benzyl ITC (cyclohexylmethyl ITC) were administered to rats each day for 5 days. The animals were then killed and organs harvested. The ITCs were also evaluated in a bladder cell line in culture. The activities of NQO1 and GST in the organs and cells were measured.

Results—*In vivo*, the organ most susceptible to the inductive activity of the ITCs was the urinary bladder, with α -methylbenzyl ITC and cyclohexylmethyl ITC being the most effective. Inductive activity in the bladder *in vivo* did not, however, correlate with that in bladder cells *in vitro*.

Conclusions—Induction of Phase 2 enzymes increases resistance to chemical carcinogenesis. ITCs could therefore be valuable chemopreventative agents, and the specificity of these substances toward the urinary bladder suggest that they could be particularly useful for protecting against bladder cancer. In this regard, α -methylbenzyl ITC and cyclohexylmethyl ITC could be especially valuable.

Keywords

isothiocyanates; chemoprevention; urinary bladder; Phase 2 enzymes; NAD[P]H:quinone acceptor oxidoreductase; glutathione *S*-transferase

INTRODUCTION

Plants of the Brassica family, used for food throughout the world, contain a variety of glucosinolates of diverse chemical structure. Through the action of the enzyme myrosinase, glucosinolates are hydrolyzed to isothiocyanates (ITCs), which have been shown to afford protection against many chemical carcinogens, including those targeting the oesophagus (1, 2), lung (3), pancreas (4), liver (5), intestine (6,7), and mammary gland (8). This chemopreventative action of ITCs has been suggested to involve inhibition of carcinogen activation through inhibition of Phase 1 enzymes, promotion of carcinogen detoxification through induction of Phase 2 enzymes, inhibition of cell proliferation, and/or destruction of transformed/malignant cells in the target tissue through apoptosis (9).

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We have previously shown that aliphatic ITCs are effective in increasing the activity of NAD [P]H:quinone acceptor oxidoreductase 1 (NQO1, EC 1.6.99.2) and the glutathione *S*-transferases (GST, EC 2.5.1.18) in the urinary bladders of rats (10). Both NQO1 and GST protect cells against carcinogens (11,12) and play an important role in the prevention of cancer in the bladder (13–16). It has recently been shown that the aliphatic ITCs present in an extract of broccoli sprouts (predominantly sulforaphane) afford excellent protection against bladder cancer induced in rats by *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) (17).

Alkyl-aryl ITCs, including benzyl ITC and phenylethyl ITC, which are commonly consumed by humans through consumption of certain cruciferous vegetables (18), also increase Phase 2 enzyme activity in rat tissues, including the bladder (19,20). This suggests that alkyl-aryl ITCs may also be useful for bladder cancer prevention, although no systematic investigation of the relative inductive activity of alkyl-aryl ITCs has been undertaken up to the present. In the present study, the ability of 12 alkyl-aryl ITCs to increase the activities of NQO1 and GST in bladder tissue and bladder cells has been determined both *in vitro* and *in vivo*. Moreover, the effects of these compounds in the bladder have been compared with those in other organs. For further comparison, the inductive activity of the fully-reduced derivative of benzyl ITC, cyclohexylmethyl ITC, has also been examined.

MATERIALS AND METHODS Materials

The structures of the test compounds are shown in Figure 1. Compounds 1, 2 and 4 were purchased from Lancaster Synthesis (Morecambe, Lancs, UK). Compounds 7, 8 and 13 were from Alfa Aesar (Heysham, Lancs, UK) while Compound 3 was from Acros Organics (Geel, Belgium). Compounds 5, 6, 9, 10, 11 and 12 were synthesized from the corresponding amine by reaction with thiophosgene (21). The purity of all the ITCs was 96% or above. They were stored at –20°C before use. Reagents for enzyme assays were from Sigma (St. Louis, MO). The antibodies recognizing GST- α , GST- μ and GST- π were purchased from Diagnostics International (San Antonio, TX). The anti-NQO1 antibody was purchased from Cell Signaling Technology (Danvers, MA). An antibody specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Chemicon International (Billerica, MA).

Animals and housing

Female Sprague-Dawley rats (11–12 weeks of age) from the AgResearch Ruakura colony were randomly allocated to treatment groups. They were housed in solid-bottomed cages containing bedding of softwood shavings and allowed free access to food (Laboratory Chow, Sharpes Animal Feeds, Carterton, NZ) and tap water. The room temperature was maintained at 21–23°C with a 12 h light-dark cycle. The body weights of the animals were recorded daily. Conduct of the animal experiments adhered to the Principles of Laboratory Animal Care (NIH publication #85–23) and were approved by the Institutional Animal Ethics Committee.

Dosing and necropsy

Groups of 6 rats were dosed with freshly-prepared solutions of the ITCs in soya oil at a dose level of 250 μ moles/kg/day for 5 days. This dose was employed in order to permit comparison with results with alkyl isothiocyanates (10). A further group of animals received soya oil alone. On the sixth day, the animals were killed by carbon dioxide inhalation. The liver, kidneys, spleen, heart, lungs and urinary bladder were dissected out and placed in plastic vials. The whole of the gastrointestinal tract was dissected out and separated into forestomach, glandular stomach, duodenum, jejunum, ileum, cecum and colon plus rectum. These tissues were cut longitudinally and the contents washed out under running water. They were gently blotted on absorbent paper and stored, along with the other organs, at –80°C before analysis.

Cell culture and ITC treatment

Rat bladder cancer NBT-II cells (purchased from ATCC, Manassas, VA) were maintained in MEM medium (Invitrogen, Grand Island, NY) with 10% fetal bovine serum (Biosource International, Camarillo, CA) in a humidified incubator at 37°C with 5% CO₂. One million cells were plated in a 10-cm dish for 24 h and then incubated with the ITCs at 3.75 and 7.5 µM for 24 h. The test compounds were dissolved in acetonitrile. The final concentration of the solvent in the culture medium was 0.1% (v/v). Following the ITC treatment, cells were harvested by trypsin treatment and stored at -70°C.

Enzyme assay in rat tissues and cell lysates

Tissue samples were weighed and homogenized in ice-cold 0.2% Triton X-100 using a Polytron tissue homogeniser. The homogenates were centrifuged at 12,000 g for 20 sec, and the supernatants assayed at 25°C for NQO1 by the 2,6-dichlorophenol indophenol method of Ernster (22) and for GST by the method of Habig et al. (23), using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The cell samples were treated as previously described (10). Briefly, cell lysates were prepared by sonication in 0.08% digitonin with 2 mM EDTA, pH 7.8, using a Branson Model 450 sonifier. The lysates were then centrifuged at 9,500 g at 4°C in a microfuge, and the supernatant portions were used for determination of specific activities of GST (CDNB as substrate) and NQO1 (menadione as substrate). The degree of induction of these enzymes by the various ITCs was expressed as the ratio of the specific enzyme activities in the tissues of the treated animals and in the cells to those in controls.

Western blot analysis

Only benzyl ITC was examined for induction of GST and NQO1 using Western blot analysis. Cells were treated with benzyl ITC and then harvested as described above. Cell pellets were washed with ice-cold PBS, and each pellet was suspended in 200 µl cell lysis buffer (Cell Signaling Technology, Danvers, MA), supplemented with 1 mM phenylmethylsulfonyl fluoride, and then sonicated. The protein concentration in each sample was measured using a bicinchoninic acid assay kit (Pierce, Rockford, IL). Each sample (25 µg protein) was resolved by 12% SDS-PAGE and blotted to polyvinylidene difluoride membranes, which were probed by specific antibodies (anti-NQO1, anti-GST-alpha, anti-GST-mu or anti-GST-pi) and visualized using an ECL chemiluminescence system from Amersham Biosciences (Piscataway, NJ). GAPDH was used as a loading control.

Statistical analysis

Statistical significance was tested by analysis of variance, followed by the Student-Newman-Keuls multiple comparisons test using Instat software (GraphPad, San Diego, CA).

RESULTS

All rats remained in good health throughout the experimental period, and no differences in body weight gain were recorded among the various treatment groups. At necropsy, thickening of the wall of the forestomach, together with mucosal erosion, was observed in all rats receiving benzyl ITC. The weights of the forestomachs of these animals were significantly higher than those of the controls. Similar effects were seen with α -methylbenzyl ITC, 4-chlorobenzyl ITC, the methoxybenzyl ITCs and 4-methylbenzyl ITC. Slight thickening of the forestomach wall was observed with 3,4,5-trimethoxybenzyl ITC and cyclohexylmethyl ITC, while no effects were seen in the rats dosed with 2-phenylethyl ITC, 3-phenylpropyl ITC, 1-methyl-3-phenylpropyl ITC or 4-phenylbutyl ITC. No abnormalities were observed at necropsy in the glandular stomach or in any of the other tissue.

Tissue activities of the Phase 2 enzymes are shown in Fig. 2. In all organs, the degree of induction of NQO1 was generally higher than that of GST. All the tested ITCs induced the greatest increases in the activities of both NQO1 and GST in the urinary bladder. α -Methylbenzyl ITC and cyclohexylmethyl ITC were the most effective inducers in this organ. Phenylethyl ITC was somewhat more effective than benzyl ITC, but further increases in the length of the alkyl chain decreased the inductive activity. Substitution with a chloro group in the 4-position of the aromatic ring of benzyl ITC decreased inductive activity, as did substitution with methoxy groups in the 3- or 4-positions. Substitution with a methyl group in the 4-position had little effect.

The next most responsive tissue was the small intestine, with all the tested compounds causing significant induction of both NQO1 and GST in the duodenum, jejunum and ileum. However, the relative effectiveness of the various compounds was quite different to that seen in the bladder. α -Methylbenzyl ITC was only weakly active in all the sections of the small intestine, and while cyclohexylmethyl ITC was a highly effective inducer in the duodenum, it was only weakly active in the jejunum and ileum. 1-Methyl-3-phenylpropyl ITC was a very good inducer in the small intestine, particularly in the jejunum and ileum, while 4-chlorobenzyl ITC was particularly effective in the ileum.

Several ITCs increased the activity of both NQO1 and GST in the forestomach of the rats, with 2-methoxybenzyl ITC being the most effective. Many ITCs, benzyl ITC in particular, also caused significant induction of the enzymes in the glandular stomach, although their rank order of potency in the glandular stomach differed from that in the forestomach (results not shown). Benzyl ITC and the longer-chain alkyl-aryl ITCs (1-methyl-3-propyl, 3-phenylpropyl and 4-phenylbutyl ITCs), together with the 2- and 4-methoxybenzyl derivatives, were most effective in the cecum and large intestine. 4-chlorobenzyl ITC was of relatively low activity in these tissues. In contrast, in the heart, lungs, and spleen, 4-chlorobenzyl ITC showed the highest activity of all the ITCs tested. The inductive activity of this substance was also relatively high in the liver and kidney. The methoxy derivatives were effective inducers in the lung, although the increases in enzyme activity seen in this organ, and in the spleen, liver, kidney and heart, were relatively small.

The effects of the ITCs, at concentrations of 3.75 and 7.5 μ M, on NQO1 and GST in rat bladder NBT-II cells are shown in Figure 3. Higher concentrations of the ITCs were not tested due to cytotoxicity of some of the compounds. It is also of note that in addition to inducing Phase 2 enzymes, ITCs such as benzyl ITC and phenyl ITC also induce apoptosis and cell cycle arrest in cultured cells and animal organs (9). The relative activities of the test compounds in inducing GST and NQO1 were quite different in these cells to those in rat bladder. α -Methylbenzyl ITC and cyclohexylmethyl ITC, which were very active *in vivo*, were weak inducers *in vitro*. Conversely, 4-phenylbutyl ITC, benzyl ITC and 3-phenylpropyl ITC, which were of comparatively low activity in the rat bladder, were the most active in the bladder cells *in vitro*. Using benzyl ITC as an example, we also examined the protein expression levels of GST and NQO1. Under the treatment condition which caused significant increases in GST and NQO1 activities, benzyl ITC significantly elevated the protein levels of GST- μ and NQO1 (Figure 4), but had no impact on GST- α and GST- π (results not shown).

DISCUSSION

In accord with previous results (19,20), alkyl-aryl ITCs were effective inducers of Phase 2 enzymes *in vivo*. Like alkyl ITCs (10), these substances exerted their greatest effect in the urinary bladder, with the most effective compounds showing approximately 8- and 5-fold increases in bladder NQO1 and GST activities respectively. The high activity of ITCs in the bladder is believed to result from the metabolism and disposition of these substances *in vivo*.

The major pathway of ITC metabolism involves initial conjugation with the thiol group of reduced glutathione (GSH). The GSH conjugate is subsequently degraded to the cysteinylglycine, cysteine and N-acetylcysteine conjugates, which are hydrophilic materials that are excreted and concentrated in the urine (9,24). These conjugates are unstable, however, and dissociate within the urine, liberating the original ITC (25). This process constitutes an effective mechanism for delivery of ITCs to the bladder, and high concentrations of these substances and their conjugates have been found in urine after administration of ITCs to animals and to humans (10,26). Variations in the effectiveness of the different ITCs in increasing Phase 2 enzyme activities in the bladder may reflect differences in the extent and location of absorption from the gut, the rate and degree of GSH conjugate formation and metabolism, the rate and degree of elimination of these substances in the urine and the rate and degree of their dissociation therein.

The inductive activity of the most effective alkyl-aryl ITC (α -methylbenzyl ITC) in the bladder was similar to that of the most effective alkyl ITCs (1-methylbutyl ITC, 1-methylallyl ITC, sec-butyl ITC and 1,3-dimethylbutyl ITC) identified in our previous study (10). Interestingly, all these compounds have a methyl group (α to the isothiocyanate moiety, and this appears to enhance inductive activity. The reason for the potentiating effect of the α -methyl group is not known at present, but the value of this structural type should be borne in mind when selecting ITCs for high inductive activity in the urinary bladder.

Several of the alkyl-aryl isothiocyanates caused thickening of the forestomach wall, with erosion of the mucosa. Similar effects were seen with allyl isothiocyanate, and these are most likely attributable to the irritant effect of isothiocyanates (27). Although the bladder epithelium may be exposed to high concentrations of isothiocyanates, there were no macroscopic lesions in this tissue. No histological examination of the bladders of the rats in this experiment was conducted, but in previous experiments with isothiocyanates, at levels sufficient to cause marked increases in Phase 2 enzyme activity, no histological abnormalities were observed in the bladders (28).

Since the throughput of *in vitro* assays is generally much higher than that *in vivo*, it would be very useful if ITCs with high inductive activity in the bladder could be identified on the basis of their ability to increase Phase 2 enzyme activity in cultured bladder cancer cells. This does not, however, appear to be a feasible proposition. While the majority of the ITCs were able to elicit the induction of the enzymes, especially NQO1, in NBT-II cells *in vitro*, there was no correlation between the inductive activity *in vivo* and their activity *in vitro*, possibly reflecting the complexity of absorption, metabolism and disposition in the whole animal. Similar effects were recorded with aliphatic ITCs (10), and it would appear that *in vivo* experiments are required for evaluation of the relative inductive activity of ITCs. Although one cell line was used in the present study, we recently showed that *in vitro* bioassays involving 3 cell lines also failed to predict *in vivo* activities of alkyl ITCs (10). Nevertheless, study of benzyl ITC in NBT-II cells revealed that induction of enzyme activity reflected an increase in protein expression, as the expression level of both GST- μ and NQO1 was significantly elevated after benzyl-ITC treatment (Figure 4). This result is consistent with literature data that ITCs cause transcriptional upregulation of Phase 2 enzymes (9). Moreover, the present study also suggests that GST induction by benzyl ITC and analogs in bladder cell and tissue may result primarily if not entirely from transcriptional upregulation of GST- μ . It is also worth noting that the ITCs in the present studies induced GST and NQO1 at 3.75 and 7.5 μ M in NBT-II cells. Since orally ingested ITCs are believed to be delivered to bladder tissue primarily through urinary excretion (26), it would be interesting in future studies to measure the urinary levels of ITCs in rats dosed with these compounds, which resulted in significant induction of Phase 2 enzymes in the bladder.

It is also impossible to predict the inductive activity of an ITC in one organ from its effect in other organs. For example, the inductive activity of benzyl ITC, 1-methyl-3-propyl, 3-phenylpropyl and 4-phenylbutyl ITCs in the bladder was relatively weak, but these were among the most active compounds in the caecum and large intestine. It is possible that these compounds are not readily absorbed from the small intestine, but remain in the gut to facilitate a response in the distal part of the gastrointestinal tract. Conversely, α -methylbenzyl ITC may be readily absorbed from the upper intestine. This compound was the most effective inducer in the bladder, but showed little activity in either the small or large intestine. It is possible that the effectiveness of 4-chlorobenzyl ITC in the lungs, heart and spleen reflects slow or incomplete conjugation with GSH, permitting it to circulate to the internal organs unchanged. More information on the pharmacokinetics of ITCs is required in order to shed light on the organ-specificity of induction by these substances.

The isothiocyanate sulforaphane has attracted much interest in view of its exceptionally high inductive activity *in vitro* (29). *In vivo*, however, the activity was similar to that of allyl isothiocyanate (30), which was found to be of only moderate activity compared to other alkyl isothiocyanates (10). The experiments with the alkyl isothiocyanates were conducted using an identical protocol to that used in the present experiments, and comparison of the data indicates that the most active alkyl-aryl ITCs are likely to be significantly more effective than sulforaphane in increasing the activity of Phase 2 enzymes in the urinary bladder *in vivo*.

The very high inductive activity of certain alkyl-aryl ITCs in the bladder suggests that these substances could be particularly useful for protecting against bladder cancer, but the effect of such compounds on chemically-induced bladder cancer is equivocal. Benzyl ITC and phenylhexyl ITC were shown to protect against BBN-induced bladder cancer when given simultaneously with the carcinogen (31,32), but benzyl ITC and phenylethyl ITC at high dose levels increased the incidence and multiplicity of bladder tumours induced by carcinogens when given post-initiation (33–35) and may themselves induce bladder cancer (35–37). ITCs are irritant substances, and the detrimental effects in the forestomach seen with some of the test materials in the present study are consistent with the results of previous studies with such compounds (27,38). Their cancer-initiating and promoting-activity in the bladder is believed to result from regenerative proliferation following damage to the bladder epithelium caused by exposure to high doses of these compounds in the urine (33,35,39).

Clearly, if ITCs are to be used as chemopreventative agents in humans, it is important that their protective action is expressed without the detrimental effects of tumor induction or promotion. For this, it would be beneficial to employ dose levels as low as possible, consonant with maintaining good inductive activity. We have shown that the aliphatic ITCs that are present in broccoli sprout extract are effective in preventing chemically-induced bladder cancer *in vivo* (17). The major ITC in broccoli sprout extract is sulforaphane, which is a good, but not outstanding, inducer of bladder Phase 2 enzymes in rats (30). It is possible that more active ITCs could give a good degree of chemoprotection at low dose-levels, which would have the advantage of minimizing the risks involved with the irritant effects of high doses of ITCs. Among the ITCs worth considering in this respect are 1-methylbutyl ITC, 1-methylallyl ITC, sec-butyl ITC and 1,3-dimethylbutyl ITC, identified previously (10), and α -methylbenzyl ITC, which was shown to be particularly effective in the present study. Furthermore, cyclohexylmethyl ITC was found to be an excellent inducer, and in view of the increased effectiveness of ITCs conferred by an α -methyl group, the methyl derivative of this substance, 1-cyclohexylethyl ITC, could be a compound worth evaluating in the future.

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Figure 1.

The chemical structure of ITC. 1) benzyl ITC; 2) phenylethyl ITC; 3) 1-phenylethyl ITC; 4) 3-phenylpropyl ITC; 5) 4-phenylbutyl ITC; 6) 1-methyl-3-phenylpropyl ITC; 7) 4-methylbenzyl ITC; 8) 4-chlorobenzyl ITC; 9) 2-methoxybenzyl ITC; 10) 3-methoxybenzyl ITC; 11) 4-methoxybenzyl ITC; 12) 3,4,5-trimethoxybenzyl ITC; 13) cyclohexylmethyl ITC.

Figure 2.

Induction of GST and NQO1 by ITCs in rat organs. Groups of 6 rats were dosed with each ITC (see Fig. 1 for name and chemical structure) in soya oil at a dose level of 250 μ moles/kg/day for 5 days. A further group of animals received soya oil alone. On the sixth day, the animals were killed, and tissue levels of GST and NQO1 were measured. Each value is a ratio of specific enzyme activity (treated/control) of GST ($\square$) and NQO1 ($\blacksquare$) (mean $\pm$ SD). The specific enzyme activities (I. U./g tissue, mean $\pm$ SD) of NQO1 and GST in the control tissues were, respectively: Bladder, 25.3 ± 8.1 ; 3.93 ± 0.96 . Forestomach, 14.8 ± 3.2 ; 3.32 ± 0.34 . Kidneys, 5.37 ± 1.01 ; 15.3 ± 1.6 . Duodenum, 15.0 ± 2.8 ; 27.0 ± 5.9 . Liver, 42.5 ± 10.2 ; 150 ± 24 . Jejunum, 6.85 ± 1.43 ; 15.7 ± 2.2 . Lungs, 19.2 ± 3.1 ; 7.10 ± 0.81 . Ileum, 20.3 ± 6.3 ; 4.83 ± 0.65 . Spleen, 1.83 ± 0.67 ; 6.25 ± 0.93 . Cecum, 48.9 ± 19.0 ; 4.92 ± 0.50 ; Heart, 2.20 ± 0.76 ; 3.93 ± 0.47 . Colon + rectum, 22.7 ± 6.9 ; 3.28 ± 0.37 . The increase in enzymatic activity was significant in each of the following measurements ($P < 0.05$): All compounds on both GST and NQO1 in the urinary bladder, duodenum, jejunum and ileum; compounds # 1, 8, 10, 11 and 13 on GST and compound # 8 on NQO1 in the kidney; compound # 6 on GST and compounds # 1, 3, 5, 6, 8 and 9 on NQO1 in the liver; compound # 8 on GST and compounds # 1, 7 and 8–12 on NQO1 in the lung; compounds # 6, 8 and 11 on GST and compound # 8 on NQO1 in the spleen; compound # 8 on both GST and NQO1 in the heart; compounds # 2, 4, 6, 7, 9 and 11–13 on GST and compounds # 1, 2, 4–13 on NQO1 in the forestomach; compounds # 1, 3, 8, 9 and 11 on GST and compounds # 1, 3, 4, 6, 9 and 11 on NQO1 in the cecum; compounds # 3 and 6 on GST and compounds # 1, 3, 4–6, 9 and 11 on NQO1 in the colon + rectum.

Figure 3.

Induction of GST and NQO1 by ITCs in NBT-II cells. Cells were treated with each ITC (see Fig. 1 for name and chemical structure) at 0, 3.75 (□) or 7.5 μM (■) for 24 h and then harvested for measurement of GST and NQO1 activities. Each value is a ratio of specific enzyme activity (treated/control) of GST and NQO1 (mean ± SD, n=3). The specific enzyme activities (nmol/min/mg) of GST and NQO1 in control cells were 25.1 ± 0.9 and 1066.9 ± 55.0 (mean ± SD, n=3), respectively. The increase in enzymatic activity was significant in each of the following measurements ($P < 0.05$): At 3.75 μM, compounds # 4, 5, and 6 on GST and compounds # 1–6, 8 and 9 on NQO1. At 7.5 μM, compounds 5, 6 and 8 on GST and compounds # 1–6, 8, 9 and 11 on NQO1.

Figure 4.

Effect of benzyl ITC on expression of GST and NQO1. NBT-II cells were treated with benzyl ITC at the specified concentrations for 24 h and then harvested for measurement of expression of GST-mu and NQO1 by Western blot analysis. GAPDH was used as a loading control. The results are representative of at least two experiments. The expression levels of GST-alpha and GST-pi were not changed after benzyl ITC treatment (results not shown).