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# Evaluation of Isothiocyanates as Potent Inducers of Carcinogen-Detoxifying Enzymes in the Urinary Bladder: Critical Nature of In Vivo Bioassay

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***Abstract:*** *Deficiency of carcinogen-detoxifying phase 2 enzymes, such as glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1), increases bladder cancer risk in humans. We report that several isothiocyanates (ITCs) that have not been previously examined, 1-methylbutyl ITC in particular, potently and preferentially induce both GST and NQO1 in the rat bladder. Comparison of 25 ITCs that are closely related in chemical structures showed that a 3–5-carbon aliphatic side chain with a methyl group attached to the alpha carbon was crucial for maximal inducer activity in the bladder. Surprisingly, cell-based bioassays failed to predict the phase 2 enzyme-inducing activity of the ITCs in the bladder. Furthermore, although ITCs are principally metabolized in vivo to dithiocarbamates (DTCs), which are believed to serve as the carriers of ITCs and are rapidly eliminated and concentrated in the urine, the total urinary levels of ITC plus DTC did not correlate with the degree of GST and NQO1 induction by the ITCs in the bladder of rats. Thus, several underappreciated ITCs are exceedingly potent inducers of GST and NQO1 in the rat bladder but were predicted neither by in vitro bioassays of phase 2 enzyme induction nor by their appearance or concentration in urine in vivo.*

## Introduction

It is now well established that carcinogen-detoxifying phase 2 enzymes, such as NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutathione S-transferase (GST), play a critical role in cellular defense against chemical carcinogens and oxidants. Deficiencies of these enzymes lead to increased DNA damage and cancer risk, and increased expression of these enzymes suppresses cancer development (1,2). Indeed, individuals who are deficient in NQO1 or GST show a significantly increased risk of developing urothelial or urinary bladder cancer (3–7), whereas oltipraz, a strong inducer of phase 2 enzymes, has been shown to inhibit nitrosamine-induced bladder tumorigenesis in mice (8,9). Moreover, genes encoding these enzymes are transcriptionally up-regulated upon stimulation of the Nrf2-ARE signaling pathway, and many chemical agents stimulate Nrf2-ARE signaling and cause a coordinate induction of multiple phase 2 enzymes (10).

Organic isothiocyanates (ITCs or R-N=C=S), many of which occur in plants, especially in cruciferous vegetables, are promising cancer chemopreventive agents. Studies with several ITCs in cultured cells, animal models, and human subjects have shown cancer-preventive activities (11–14). These compounds stimulate Nrf2-ARE signaling and induce phase 2 enzymes besides triggering other chemopreventive mechanisms (13–15). We have previously shown that the degree of induction of both NQO1 and GST by several ITCs in a wide range of rat tissues was greatest in the urinary bladder (16,17), suggesting that the most promising chemopreventive utility of ITCs may be bladder cancer prevention. The selective effect of ITCs on phase 2 enzymes in the bladder could well be a reflection of the nature of ITC metabolism and disposition in vivo, as explained below.

ITCs are metabolized in vivo principally by the mercapturic acid pathway. Initial conjugation of the –N=C=S group with the cysteine thiol of glutathione (GSH) forms the corresponding conjugates, which are then metabolized sequentially to the cysteinylglycine-, cysteine-, and *N*-acetylcysteine (NAC)-conjugates, which are excreted and concentrated in the urine (14,18). NAC conjugates and the intermediate metabolites of ITCs, known collectively as dithiocarbamates (DTCs), are believed to facilitate the delivery of ITCs to the bladder, because they are unstable and readily dissociate to their parent ITCs (19). Available evidence suggests that urinary concentrations of ITC plus DTC are considerably higher than those in the blood after ITC in-

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gestion so that the bladder is the organ that may receive the greatest exposure to ITCs (20). For example, Bollard et al. reported that the $^{14}C$ contents in the bladders of male F344 rats were 14–18 times that in the liver and kidney after a single oral dose of [$^{14}C$] allyl ITC (21).

Although ITCs seem to be selectively delivered to the bladder, it was not known if the urinary ITC plus DTC level is a biomarker of induction of phase 2 enzymes by these compounds in the bladder in vivo. Moreover, cell-based assays, which are more rapid and less expensive than animal studies, have been widely used to screen inducers of phase 2 enzymes (22–25). However, it was not known if the activity of an ITC in inducing phase 2 enzymes in cultured cells in vitro predicts its activity in the bladder in vivo. Answers to these questions are of critical importance to our effort to identify and develop ITCs for bladder cancer prevention. In the present study, we have attempted to address these questions by examining 25 ITCs, which are closely related in chemical structure. During the course of our study, we also identified a number of exceedingly potent inducers of phase 2 enzymes in the bladder.

## Materials and Methods

### Chemicals

Ethyl-, *n*-propyl-, *iso*-propyl-, allyl-, *n*-butyl-, *iso*-butyl-, *tert*-butyl-, *n*-pentyl-, *n*-hexyl-, *n*-heptyl-, *n*-octyl-, *n*-nonyl-, *n*-decyl- and *n*-dodecyl-ITC were purchased from Lancaster Synthesis (Morecambe, UK). Methyl-, *sec*-butyl-, 1-methyl butyl-, 3-methylbutyl-, and 1-ethylpropyl-ITC were synthesized by the general method of Li et al. (26). 2-Methylbutyl- and 1,3-dimethylbutyl-ITC were synthesized by the thiophosgene method as described by Drobnica et al. (27). 1-Methylallyl- and but-2-enyl-ITC were synthesized by the method of Kjaer et al. (28), 2-methylallyl ITC by the method of Bruson and Eastes (29), and but-3-enyl ITC by the method of Kjaer and Jensen (30).

### Cell Culture, ITC Treatment, and Assays of GST and NQO1

Three cell lines were used: rat bladder carcinoma NBT-II cells, human bladder carcinoma UM-UC-3 cells, and murine hepatoma Hepa1c1c7 cells. The source and culture of these cells have been previously reported (31–33). All ITCs were dissolved in acetonitrile (ACN), and the final ACN concentration in the culture medium was 0.1% or less (vol/vol).

To determine the effect of an ITC on GST and/or NQO1 in NBT-II cells or UM-UC-3 cells, approximately $1 \times 10^6$ cells were grown in a 10-cm dish with 10 ml medium for 48 h and then incubated in fresh medium with an ITC at the desired concentrations for 24 h. At the end of ITC treatment, cells were harvested as pellets by trypsinization and centrifugation. Cell lysates were prepared by mixing each cell pellet with 200 µl digitonin solution [0.08% in 2 mM ethylenediamine tetra-acetic acid (EDTA), pH 7.8] and incubating the mixture at 37°C for 10 min, followed by gentle shaking for 15 min at room temperature. In the case of NBT-II cells, the lysates were further sonicated to complete cell lysis. All lysates were then centrifuged at 9,500 *g* at 4°C in a microfuge, and the supernatant portions were used for enzyme analysis. The GST activity (1-chloro-2,4-dinitrobenzene as substrate) and NQO1 activity (menadione as substrate) in cell lysates were determined as previously reported (34).

Only NQO1 was measured in Hepa1c1c7 cells, which were grown in 96-well plates ($1 \times 10^4$ cells/well) for 24 h and then exposed to an ITC in fresh medium (200 µl/well) at the desired concentrations for 48 h. At the end of ITC treatment, NQO1 activity (menadione as substrate) was determined as previously reported (22,33).

### Animals

Female Sprague-Dawley rats (11–12 weeks of age) from the Ruakura (New Zealand) colony were randomly allocated to treatment groups. For the study on the effect of ITCs on tissue enzyme activities, the rats were housed in solid bottomed cages containing bedding of softwood shavings, and for the experiment on urinary excretion of ITCs, they were maintained in metabolism cages. In both situations, the rats were allowed free access to food (Laboratory Chow, Sharpes Animal Feeds, Carterton, NZ) and water. The room temperature was maintained at 21–23°C under a 12-hr light/dark cycle.

### Assay of Tissue NQO1 and GST Activities of ITC-Treated Rats

Freshly prepared solutions of the 25 ITCs in soybean oil were administered by oral intubation to groups of 5 rats at a dose of 250 µmol/kg/day for 5 days. A further group of rats received vehicle alone. On the sixth day, the rats were killed by carbon dioxide inhalation. Tissues were dissected out and stored at –80°C before analysis. The tissue samples were weighed and then homogenized in ice-cold 0.2% Triton X-100 using a Polytron tissue homogenizer. 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 (35) and for GST by the method of Habig et al. (36), using 1-chloro-2,4-dinitrobenzene as substrate. Enzyme activities were calculated as international units per gram of tissue. The NQO1 activities in the rat tissues and the cell lines were assayed with different substrates for purpose of comparison with previously obtained results of ITCs.

### Measurement of Urinary Excretion of ITC Plus DTC in Rats

Groups of 5 rats were given a single dose of 25 or 250 µmol/kg of 1-methylbutyl ITC (MBITC), allyl ITC (AITC), or *n*-decyl ITC (DITC) by oral intubation as a solution in soybean oil. Urine was collected over two consecutive 24-h peri-

ods and stored frozen at –80°C until analysis. Total urinary concentrations of ITC plus DTC were determined using the cyclocondensation assay (reaction with 1,2-benzenedithiol) (37), as described by Shapiro et al. (38). This assay measures the sum total of ITCs and their DTC metabolites, but provides no information on the identity of individual compounds. Because urine is the principal ITC disposal route, this assay has been found to be an excellent tool for determining human exposure to ITCs based on the measurement of total urinary ITC plus DTC levels (38–40).

### Statistical Analysis

Statistical significance was tested by analysis of variance, followed by the Student-Newman-Keuls multiple comparisons test using Instat 2.04a software (GraphPad, San Diego, CA).

## Results

### ITCs Displayed Considerable Variability in Inducing Phase 2 Enzymes in the Bladder In Vivo

We recently showed that oral administration of AITC (compound 11) led to dose-dependent induction of both GST and NQO1 in the rat bladder (16). In the present study, we compared the inducer activity of AITC with 24 analogs that are closely related to one another in chemical structure (Table 1). The rats were dosed by oral intubation with each ITC at 250 μmol/kg body weight daily for 5 days. This relatively high dose was selected to obtain a clear distinction of the inductive activity of the different compounds. There were no significant differences among the test and control groups with regard to body weight or bladder weight, and the animals remained in good health throughout the experiment. It is also noteworthy that when AITC was previously tested at up to 200 μmol/kg in the same strain of rats, the induction of both GST and NQO1 in rat bladder and other tissues increased dose dependently (16). The results with the 25 ITCs are shown in Fig. 1 and can be summarized as follows: 1) every ITC induced NQO1 to higher levels than GST, but the ITC that was a better inducer of NQO1 was not necessarily a better inducer of GST; 2) the induction of NQO1 and GST by different ITCs differed by factors of nearly 8 and 6 respectively, with MBITC (compound 1) and *tert*-butyl ITC (compound 25) being the most and least effective inducers respectively; 3) ITCs that contain a side chain of 3–5 carbons with a methyl group attached to the alpha carbon, including MBITC, 1,3-dimethylbutyl ITC, 1-methylallyl ITC, and *sec*-butyl ITC, were the most potent inducers, and the inducer activity of an ITC decreased as the carbon number of its side chain decreased or increased, or when two methyl groups were attached to the alpha carbon; and 4) the presence of an alkene group did not affect the inducer activity of the compound.

**Table 1.** Name and Chemical Structure of Isothiocyanates (ITCs)

| | |
|---|---|
| 1. 1-Methylbutyl ITC (MBITC) | $CH_3-(CH_2)_2-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-N=C=S$ |
| 2. 1-Methylallyl ITC | $CH_2=CH-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-N=C=S$ |
| 3. *sec*-Butyl ITC | $CH_3-CH_2-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-N=C=S$ |
| 4. 1,3-Dimethylbutyl ITC | $CH_3-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-CH_2-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-N=C=S$ |
| 5. *n*-Pentyl ITC | $CH_3-(CH_2)_4-N=C=S$ |
| 6. 3-Methylbutyl ITC | $CH_3-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-(CH_2)_2-N=C=S$ |
| 7. *iso*-Butyl ITC | $CH_3-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-CH_2-N=C=S$ |
| 8. 2-Methylbutyl ITC | $CH_3-CH_2-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-CH_2-N=C=S$ |
| 9. 1-Ethylpropyl ITC | $CH_3-CH_2-\underset{\displaystyle CH_2CH_3}{\underset{\vert}{CH}}-N=C=S$ |
| 10. *n*-Butyl ITC | $CH_3-(CH_2)_3-N=C=S$ |
| 11. Allyl ITC (AITC) | $CH_2=CH-CH_2-N=C=S$ |
| 12. 2-Methylallyl ITC | $CH_2=\underset{\displaystyle CH_3}{\underset{\vert}{C}}-CH_2-N=C=S$ |
| 13. *iso*-Propyl ITC | $CH_3-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-N=C=S$ |
| 14. But-3-enyl ITC | $CH_2=CH-(CH_2)_2-N=C=S$ |
| 15. *n*-Propyl ITC | $CH_3-(CH_2)_2-N=C=S$ |
| 16. But-2-enyl ITC | $CH_3-CH=CH-CH_2-N=C=S$ |
| 17. Ethyl ITC | $CH_3-CH_2-N=C=S$ |
| 18. Methyl ITC | $CH_3-N=C=S$ |
| 19. *n*-Heptyl ITC | $CH_3-(CH_2)_6-N=C=S$ |
| 20. *n*-Hexyl ITC | $CH_3-(CH_2)_5-N=C=S$ |
| 21. *n*-Octyl ITC | $CH_3-(CH_2)_7-N=C=S$ |
| 22. *n*-Decyl ITC (DITC) | $CH_3-(CH_2)_9-N=C=S$ |
| 23. *n*-Nonyl ITC | $CH_3-(CH_2)_8-N=C=S$ |
| 24. *n*-Dodecyl ITC | $CH_3-(CH_2)_{11}-N=C=S$ |
| 25. *tert*-Butyl ITC | $CH_3-\underset{\displaystyle CH_3}{\underset{\vert}{CH}}-N=C=S$ |

### Cell-Based In Vitro Bioassays of ITCs Did Not Predict Their Activity in Inducing GST and NQO1 in the Bladder In Vivo

Cultured cells have frequently been used to screen inducers of phase 2 enzymes, as these assays are less time-consuming and much less expensive than animal experiments. We wondered how the ITCs previously described might affect these enzymes in cultured cells. We first evaluated the 25 ITCs in NBT-II cells, a rat bladder epithelial cancer cell line. Cells were treated with each ITC at 3.75, 7.5, and 15 μM for 24 h and then assessed for induction of GST and NQO1. Higher ITC concentrations were not used due to cytotoxicity (results not shown). As shown in Fig. 2: 1) both GST and NQO1 in these cells had a very limited response range, that is, maximal enzyme induction by an ITC ranging from increases of 16% to 79% over control GST and 17% to 120% over control NQO1; 2) there was little correlation between

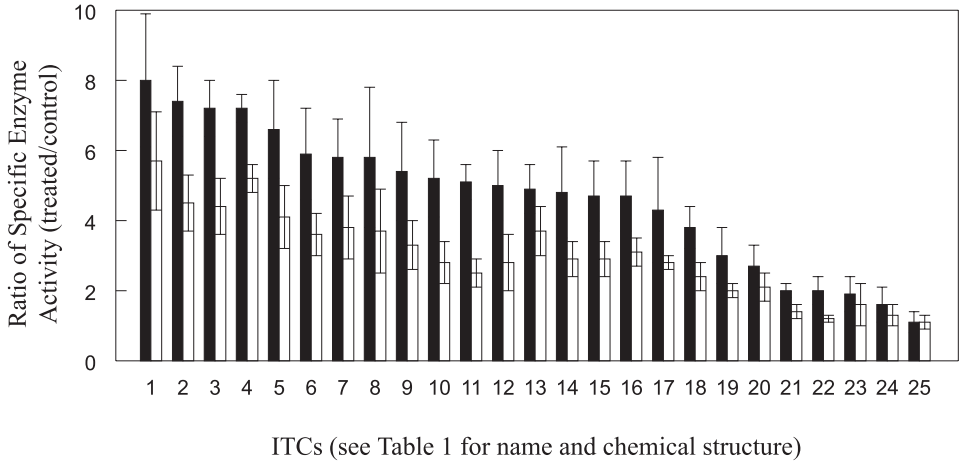


**Figure 1.** Induction of glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1) in rat bladder by isothiocyanates (ITCs). Each rat was dosed by oral intubation with an ITC at 250 μmol/kg body weight daily for 5 days. The rats were killed 24 h after the last dose, and the bladders were removed and assessed for specific activity of NQO1 (■) and GST (□). The results are expressed as the ratio of specific enzyme activity (the activity in ITC-treated bladder is divided by that in vehicle-treated bladder). Each value is a mean ± SD ($n$ = 5).

the rank order of the inducer activities of the ITCs in the bladder (Fig. 1) and the rank order of those in NBT-II cells; and 3) the ITCs that were the most effective inducers of GST and NQO1 in the bladder in vivo, including MBITC, 1,3-dimethylbutyl ITC, and 1-methylallyl ITC, behaved at best as mediocre inducers in NBT-II cells, whereas but-2-enyl ITC, but-3-enyl ITC, and methyl ITC, which were only moderate inducers in vivo, were the most effective inducers in cultured NBT-II cells.

To rule out a cell-specific effect, 14 ITCs, which span the range of activities reported in Fig. 1 and Fig. 2, were also tested for induction of NQO1 at the same concentrations in both UM-UC-3 cells (a human bladder epithelial cancer cell line) and Hepa 1c1c7 cells (a mouse hepatoma cell line). The latter cells have been widely used to screen for inducers of phase 2 enzymes (25). The inducer activities of the majority of the 14 ITCs were similar between the two cell lines and to those in NBT-II cells, except for DITC (compound 22) and *n*-dodecyl ITC (compound 24), both of which were particularly effective inducers in Hepa 1c1c7 cells, elevating NQO1 more than 3 and 4 times, respectively (Fig. 3).

We must thus conclude that 1) cell-based (in vitro) bioassays are not reliable predictors of the potencies of ITCs to induce phase 2 enzymes in the bladder (in vivo) and 2) the ITC inducer potency may be cell line-specific when evaluated using in vitro bioassays.

## Urinary ITC Excretion Levels Did Not Correlate With the Degree of Phase 2 Enzyme Induction in the Bladder

As mentioned earlier, ITCs are almost exclusively excreted and concentrated in the urine as DTCs, and urinary excretion levels of ITC plus DTC, as measured by the cyclocondensation assay, have been shown to be an excellent biomarker of human exposure to ITCs from cruciferous vegetables. We measured the urinary excretion levels of 3 representative ITCs, MBITC, AITC, and DITC, which differed sharply in their ability to induce GST and NQO1 in the bladder in vivo (Fig. 1). The rats received a single dose of ITC by oral intubation at 25 or 250 μmol/kg body weight, and two consecutive 24-h urine samples were collected from each rat immediately thereafter. There was a dose-dependent urinary excretion of ITC plus DTC for each of the 3 ITCs evaluated (Table 2), and urinary elimination was largely completed within the first 24 h after dosing. However, the three ITCs showed markedly different urinary excretion levels of ITC plus DTC, in the following order: AITC > MBITC > DITC. For example, when the rats were dosed with each ITC at 250 μmol/kg body weight, 33.6% of MBITC dosed, 83.0% of AITC dosed, and 7.2% of DITC dosed were detected in the first 24 h urine, respectively. Moreover, the most striking finding was that the total urinary ITC plus DTC concentrations during the first 24 h, especially in the case of AITC, were astonishingly high. After dosing the rats with 25 and 250 μmol ITC/kg, total ITC plus DTC concentrations in the first 24 h urine samples were 250.8 and 1,388.8 μM (MBITC), 360.3 and 4,212.5 μM (AITC), and 47.1 and 483.0 μM (DITC), respectively. These results confirm that the bladders, in particular their lumenal epithelia, were exposed to very high concentrations of ITC plus DTC when the rats were dosed at 250 μmol ITC/kg.

More importantly, our results showed that the total urinary ITC plus DTC levels of the three ITCs were not correlated with their ability to induce GST and NQO1 in the bladder. The activities of GST and NQO1 in the bladder were increased 437% and 686% respectively by MBITC, but only 181% and 301% respectively by AITC (Fig. 1). However, the 24-h urinary ITC plus DTC concentration following oral administration of 250 μmol MBITC/kg was 1,388.8 μM, only 33% of that (4,212.5 μM) following the same AITC dose. Moreover, the total 24-h urinary ITC plus DTC following an oral dose of 250 μmol DITC/kg was 483.0 μM, which was 11.5% of that of AITC, but the induction of GST and NQO1 by DITC was 38.8% and 48.4% of those by AITC, respec-

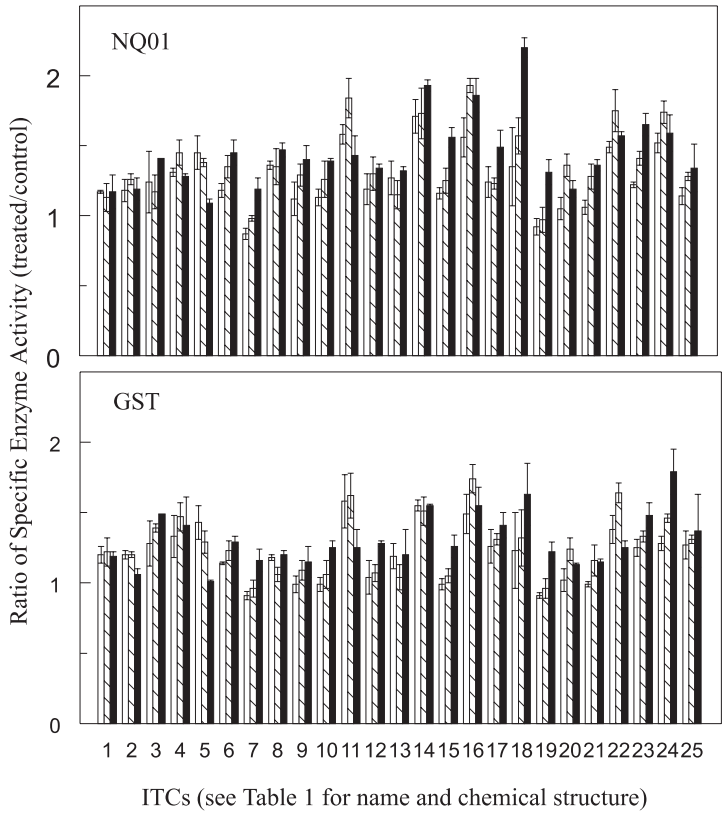


**Figure 2.** Induction of glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1) in NBT-II cells. Approximately $1 \times 10^6$ cells were grown in a 10-cm dish for 48 h and then incubated with each isothiocyanate (ITC) at 3.75 μM (□), 7.5 μM (▨), or 15 μM (■) for 24 h. Cells were then harvested and lysed for determination of GST and NQO1 activities. The results are expressed as mean ± SD ($n$ = 3).

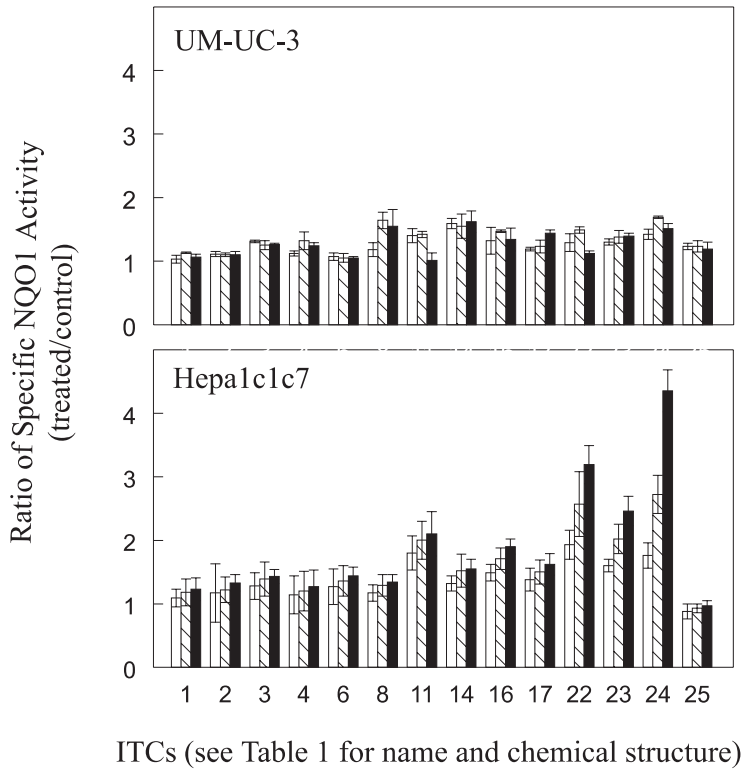


**Figure 3.** Induction of NAD(P)H:quinone oxidoreductase 1 (NQO1) by isothiocyanates (ITCs) in UM-UC-3 cells and Hepa1c1c7 cells. The culture of UM-UC-3 cells, their treatment with ITCs, and enzyme analysis are the same as with NBT-II cell (Fig. 2). Hepa1c1c7 cells were grown in 96-well plates ($1 \times 10^4$ cells/well) for 48 h and then incubated with each ITC for 24 h at 3.75 μM (□), 7.5 μM (▨), or 15 μM (■) before determination of NQO1 activity. Each value is a mean ± SD ($n$ = 3).

**Table 2.** Urinary Excretion of Orally Administered ITCs in Rats[a,b]

| ITC | ITC Dose (μmol/kg b.w.) | Urine Collection Period Post-ITC Dosing (h) | Total Urine Volume (ml)[c] | Urinary ITC Plus DTC Concentration (μM)[c] | Urinary ITC Plus DTC % Recovery |
|---|---|---|---|---|---|
| MBITC | 25 | 0-24 | 13.6 ± 2.7 | 250.8 ± 32.4 | 53.9 |
| | | 25-48 | 14.0 ± 2.8 | 5.5 ± 2.6 | 1.1 |
| | 250 | 0-24 | 15.5 ± 2.4 | 1388.8 ± 245.9 | 33.6 |
| | | 25-48 | 15.2 ± 2.9 | 53.0 ± 16.9 | 1.3 |
| AITC | 25 | 0-24 | 12.9 ± 2.6 | 360.3 ± 125.9 | 69.9 |
| | | 25-48 | 12.9 ± 3.0 | 2.6 ± 1.6 | 0.6 |
| | 250 | 0-24 | 11.5 ± 2.8 | 4212.5 ± 581.6 | 83.0 |
| | | 25-48 | 12.1 ± 3.6 | 29.9 ± 8.8 | 0.6 |
| DITC | 25 | 0-24 | 15.0 ± 3.8 | 47.1 ± 13.4 | 10.8 |
| | | 25-48 | 14.9 ± 3.1 | 1.4 ± 0.4 | 0.3 |
| | 250 | 0-24 | 10.0 ± 2.1 | 483.0 ± 197.2 | 7.2 |
| | | 25-48 | 10.1 ± 2.8 | 33.5 ± 21.7 | 0.5 |

*a:* Abbreviations are as follows: ITC, isothiocyanate; MBITC, 1-methylbutyl ITC; AITC, allyl ITC; DITC, *n*-decyl ITC.
*b:* $n = 5$. Each Sprague-Dawley rat received a single dose of ITC by oral intubation.
*c:* Values are mean ± SD.

tively. These results strongly suggest that total urinary ITC plus DTC levels cannot serve as a biomarker of induction of phase 2 enzymes by ITCs in the bladder.

We also compared the activities of both GST and NQO1 in a number of other tissues of rats, after oral dosing with MBITC, AITC, or DITC at 250 μmol/kg daily for 5 days. In rats dosed with MBITC and AITC, all the tissues examined, including colon, kidney, liver, lung, and glandular stomach, showed markedly lower induction levels of GST and NQO1 than did the bladder (Table 3), which underscores the fact that the bladder is by far the most responsive organ to ITC treatment. DITC appears to show the same tissue specificity, but the results are less revealing due to its poor inducer activity in all tissues. It should also be noted that in no tissues were the urinary excretion levels of the three ITCs correlated with the induction levels of GST and NQO1.

## Discussion

### Identification of Important ITCs for Bladder Cancer Prevention

Our study of the 25 ITCs in rat bladder in vivo suggests that ITCs with a 3–5-carbon aliphatic side chain and a methyl group attached to the alpha carbon are highly potent inducers of GST and NQO1. Hence, MBITC, 1,3-dimethylbutyl ITC, 1-methylallyl ITC, and *sec*-butyl ITC are all exceedingly potent inducers (Fig. 1). To the best of our knowledge, no ITC has been previously shown to display such a potent inducer activity in the bladder. Sulforaphane (SF), a well known cancer chemopreventive ITC that is present in broccoli and other cruciferous vegetables, is an extremely potent inducer of NQO1 in Hepa1c1c7 cells (41,42). However, the levels of NQO1 and GST induction in the rat bladder after oral administration of SF were similar to that of AITC (17), which was only moderately potent in the current study. The majority of the 25 ITCs examined in the current study occur in plants, including methyl-, ethyl-, *n*-propyl-, *n*-butyl-, *n*-pentyl-, *n*-hexyl-, *iso*-propyl-, *sec*-butyl-, *iso*-butyl-, 1-methylbutyl-, 2-methylbutyl-, 3-methylbutyl-, allyl-, 2-methylallyl- and but-3-enyl-ITC, and *sec*-butyl ITC (43). Although we only measured the effect of the ITCs on GST and NQO1, it is likely that other phase 2 enzymes in the bladder will be induced by these compounds as well, because ITCs have been shown to stimulate the Nrf2-ARE signaling pathway (15,44,45), which up-regulates a variety of phase 2 enzymes (10), through interaction with the –N=C=S group (46,47). It is also noteworthy that the GST activity measured represents the total activity of GST isoforms, as multiple GST isoforms may exist in a given tissue, and 1-chloro-2,4-dinitrobenzene is a substrate of many isoforms, including alpha, mu, pi, sigma, and theta (48). Interestingly, ITCs are also substrates of GST, but the effect of GST on urinary disposition rate of ITCs remains undefined (14). In this context, our current study does not show a correlation between GST induction levels and urinary disposition rates of ITCs.

### The Induction of Both GST and NQO1 in Rat Organs, in Response to Orally Administered ITCs, Is by Far the Greatest in the Bladder

As discussed earlier, previous studies have shown that ITCs in vivo are primarily disposed of and concentrated in the urine as DTCs, which serve as carriers of ITCs. Indeed, we found that the bladder was the single most responsive organ, in terms of induction of GST and NQO1, to orally administered MBITC and AITC. We have previously shown similar results with AITC (16). Moreover, when [$^{14}$C]AITC was administered orally to rats, it was found that the radioac-

**Table 3.** Induction of GST and NQO1 in Rat Organs by Orally Administered ITCs

| ITC (250 μmol/kg, daily × 5) | Enzyme | Ratio of Specific Enzyme Activity (treated/control) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Bladder | Colon | Kidney | Liver | Lungs | Stomach |
| MBITC | GST | 5.70 ± 1.4* | 1.22 ± 0.1 | 1.06 ± 0.1 | 1.21 ± 0.2 | 1.25 ± 0.1 | 1.27 ± 0.1 |
| | NQO1 | 7.95 ± 1.9* | 2.07 ± 0.7 | 1.52 ± 0.3 | 1.52 ± 0.5 | 1.51 ± 0.1 | 1.70 ± 0.3 |
| AITC | GST | 2.50 ± 0.4* | 1.04 ± 0.1 | 1.22 ± 0.2 | 1.09 ± 0.1 | 1.07 ± 0.1 | 1.44 ± 0.1 |
| | NQO1 | 5.10 ± 0.5* | 1.63 ± 0.2 | 1.68 ± 0.8 | 1.48 ± 0.4 | 1.58 ± 0.7 | 2.33 ± 0.3 |
| DITC | GST | 1.20 ± 0.1 | 1.15 ± 0.1 | 1.17 ± 0.1 | 1.15 ± 0.1 | 1.11 ± 0.1 | 1.02 ± 0.2 |
| | NQO1 | 2.00 ± 0.1* | 2.20 ± 0.7 | 1.19 ± 0.5 | 1.57 ± 0.5 | 1.06 ± 0.1 | 1.25 ± 0.2 |

*a:* Abbreviations are as follows: GST, glutathione S-transferase; NQO1, NAD(P)H:quinone oxidoreductase 1; ITC, isothiocyanate; MBITC, 1-methylbutyl ITC; AITC, allyl ITC; DITC, *n*-decyl ITC.

*b:* Each rat was dosed by oral intubation with an ITC at the indicated dose for 5 days. The rats were killed 24 h after the last dose, and the organs were removed and assessed for specific activity of GST and NQO1. The results are expressed as the ratio of specific enzyme activity (the activity in ITC-treated organ is divided by that in vehicle-treated organ). In the case of stomach, only the glandular portion was examined. The results of bladder are presented in Fig. 1, but are shown here again for purpose of comparison.

*c:* Each value indicated by an asterisk (*) is significantly different from other values in the same row ($P < 0.05$), except for the NQO1 value in the bladder of DITC-treated rats, which is only significantly different from those in the kidney, lung, and stomach ($P < 0.05$).

tivity in the bladder was markedly higher than that in other organs (21). These findings therefore indicate that the cancer chemopreventive activity of ITCs is selectively delivered to the bladder through urinary disposition. Because the induction of both GST and NQO1 is dramatically higher in the bladder than in any other organ, especially with MBITC, it is likely that the ITC dose may be reduced to a level that still allows significant induction of phase 2 enzymes in the bladder but avoids potential systemic toxicity of the compound. This consideration is important because chemopreventive agents are typically administered to humans over a prolonged period of time, and the potential toxicity of such agents must be minimized.

### Cell-Based In Vitro Bioassays Failed to Predict the In Vivo Phase 2 Enzyme Inducing Activity of ITCs in the Bladder

Although cell-based bioassays have been widely used to screen and identify inducers of phase 2 enzymes, much to our surprise, assays utilized in the current study failed to predict the relative phase 2 enzyme-inducing activity of ITCs in the bladder in vivo. This was true in all of the three cell lines examined: rat bladder NBT-II cells, human bladder UM-UC-3, and mouse liver Hepa1c1c7 cells (Fig. 2 and 3). The microtiter plate-based assay using NQO1 in Hepa1c1c7 cells as a representative of phase 2 enzymes has been widely used to screen for inducers of phase 2 enzymes (25). Moreover, our studies also show that the response range of GST and/or NQO1 to the ITCs in the cultured cells was much smaller than that in the rat bladder and that relative ITC potency may differ somewhat among cell lines. Our results therefore reveal the limitation of cell-based bioassays in identifying ITCs as inducers of phase 2 enzymes in vivo and question the relevance of prior studies describing the effects of ITCs using cultured cells.

### Total Urinary Excretion Level of ITC Plus DTC Is Not a Biomarker of Phase 2 Enzyme Induction by Orally Administered ITCs In Vivo

In the current study, urinary excretion of ITC metabolites, expressed as total ITC plus DTC levels, were measured by the cyclocondensation assay, which detects both free ITCs and their metabolites (DTCs) formed in the mercapturic acid pathway. Many previous studies have shown that ITCs are excreted in the urine principally as NAC conjugates in humans and rats (18,49,50). NAC-ITCs themselves are not believed to directly cause induction of phase 2 enzymes, because cellular uptake of intact NAC-ITCs appears to be very limited (51). Rather, they can dissociate to the parent ITCs (19) and therefore act as carriers of ITCs. In fact, a recent report by Hwang and Jeffery (52) showed the NAC conjugate of SF was slightly more potent than SF itself in inducing NQO1 in Hepa1c1c7 cells, suggesting that NAC-ITCs may potentiate the chemopreventive activity of ITCs by suppressing their degradation (ITCs are reactive electrophiles) and prolonging their interaction with cells. We have previously shown that total intracellular accumulation levels of ITC plus DTC predicts the phase 2 enzyme inducer activity of ITCs in a number of cultured cell lines (34,53). Our current finding that total urinary excretion levels of ITC plus DTC do not correlate with the induction of GST and NQO1 in the bladder in these rats may suggest that the dissociation rates of NAC-ITCs to free ITCs in the urine vary, and consequently the potency of induction by NAC conjugates may not parallel that of their cognate ITCs because urine storage time in the bladder is limited.

In addition, the lack of association between the urinary ITC plus DTC levels and the induction of GST and NQO1 in the bladder may also be related to the urinary excretion kinetics of ITC plus DTC. Previous studies suggest that more lipophilic ITCs are eliminated more slowly and to a

lesser extent in the urine (38,40,54). Although we have not experimentally compared the lipophilicities of MBITC, AITC, and DITC, a reasonable assumption is that they rank in the following order, based on their chemical structures: DITC > MBITC > AITC. This correlates well with total urinary excretion levels of ITC plus DTC (Table 2). It is therefore possible that the urinary excretion rates of ITC plus DTC may follow the order of AITC > MBITC > DITC, which could mean that the overall exposure of the bladder to AITC might be lower than to MBITC when each compound is administered orally at the same dose. It is also likely that highly lipophilic ITCs such as DITC may have low gastrointestinal absorption and high fecal elimination. Further studies are needed to clarify the reason for differential potency of ITCs in the bladder.

It is also noteworthy that urinary ITC plus DTC concentrations were extremely high when the rats were dosed with 250 μmol ITC/kg, especially in the cases of AITC and MBITC (Table 2). Even when the ITC dose was reduced to 25 μmol/kg, the average total urinary ITC plus DTC concentrations in the first 24 h post-dosing were 360 μM (AITC) and 251 μM (MBITC), respectively. Considering that ITCs at 10–100 μM can cause retardation of cell growth and even cell death in cultured cell lines (14,55), future studies are warranted to address what urinary ITC plus DTC concentrations are safe and non-toxic to the urinary bladder, particularly the epithelium. However, it is likely that much higher urinary ITC plus DTC concentration may be needed to produce effects in vivo similar to those seen in cultured cells in vitro, because the bladder epithelium is normally impermeable and is covered by a protective layer of glycosaminoglycan. Humans are normally exposed to much lower ITC doses, as daily intake of total ITCs, based on a measurement of urinary ITC plus DTC or a dietary questionnaire, was estimated to be 10–100 μmol per person (38,56,57), although humans are exposed to ITCs over a much longer time period than the rats employed in the present experiments. Whether these dose levels of ITC provide protection in the bladder is unknown, but Michaud et al. (58) reported that consumption of cruciferous vegetables, which are rich in ITCs, was inversely correlated with bladder cancer risk.

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## References

1. Kensler TW: Chemoprevention by inducers of carcinogen detoxication enzymes. *Environ Health Perspect* **4**(Suppl), 965–970, 1997.
2. Kensler TW and Talalay P: Inducers of enzymes that protect against carcinogens and oxidants. In *Cancer Chemoprevention, Vol 1, Promising Cancer Chemoprevention Agents,* Kelloff GJ, Hawk ET, and Sigman CC (eds). Totowa, NJ: Humana Press, 2004, pp 3–20.
3. Schulz WA, Krummeck A, Rosinger I, Eickelmann P, Neuhaus C, et al.: Increased frequency of a null-allele for NAD(P)H:quinone oxidoreductase in patients with urological malignancies. *Pharmacogenetics* **7,** 235–239, 1997.
4. Georgiou I, Filiadis IF, Alamanos Y, Bouba I, Giannakopoulos X, et al.: Glutathione S-transferase null genotypes in transitional cell bladder cancer: a case-control study. *Eur Urol* **37,** 660–664, 2000.
5. Toruner GA, Akyerli C, Ucar A, Aki T, Atsu N, et al.: Polymorphisms of glutathione S-transferase genes (GSTM1, GSTP1 and GSTT1) and bladder cancer susceptibility in the Turkish population. *Arch Toxicol* **75,** 459–464, 2001.
6. Salagovic J, Kalina L, Habalova V, Hrivnak M, Valansky L, et al.: The role of human glutathione S-transferases M1 and T1 in individual susceptibility to bladder cancer. *Physiol Res* **48,** 465–471, 1999.
7. Komiya Y, Tsukino H, Nakao H, Kuroda Y, Imai H, et al.: Human glutathione S-transferase A1 polymorphism and susceptibility to urothelial cancer in the Japanese population. *Cancer Lett* **221,** 55–59, 2005.
8. Moon RC, Kelloff GJ, Detrisac CJ, Steele VE, Thomas CF, et al.: Chemoprevention of OH-BBN-induced bladder cancer in mice by oltipraz, alone and in combination with 4-HPR and DFMO. *Anticancer Res* **14,** 5–11, 1994.
9. Lida K, Itoh K, Kumagai Y, Oyasu R, Hattori K, et al.: Nrf2 is essential for the chemopreventive efficacy of oltipraz against urinary bladder carcinogenesis. *Cancer Res* **64,** 6424–6431, 2004.
10. Zhang Y and Gordon GB: A strategy for cancer prevention: stimulation of the Nrf2-ARE signaling pathway. *Mol Cancer Ther* **3,** 885–893, 2004.
11. Zhang Y and Talalay P: Anticarcinogenic activities of organic isothiocyanates: chemistry and mechanisms. *Cancer Res* **54**(Suppl), 1976–1981, 1994.
12. Hecht SS: Inhibition of carcinogenesis by isothiocyanates. *Drug Metab Rev* **32,** 395–411, 2000.
13. Conaway CC, Yang Y, and Chung F-L: Isothiocyanates as cancer chemopreventive agents: their biological activities and metabolism in rodents and humans. *Curr Drug Metab* **3,** 233–255, 2002.
14. Zhang Y: Cancer-preventive isothiocyanates: measurement of human exposure and mechanism of action. *Mutation Res* **555,** 173–190, 2004.
15. Kaum YS, Jeong WS, and Kong ANT: Chemoprevention by isothiocyanates and their underlying molecular signaling mechanisms. *Mutation Res* **555,** 191–202, 2004.
16. Munday R and Munday CM: Selective induction of phase II enzymes in the urinary bladder of rats by allyl isothiocyanate, a compound derived from *Brassica* vegetables. *Nutr Cancer* **44,** 52–59, 2002.
17. Munday R and Munday CM: Induction of phase II detoxification enzymes in rats by plant-derived isothiocyanates: comparison of allyl isothiocyanate with sulforaphane and related compounds. *J Agric Food Chem* **52,** 1867–1871, 2004.
18. Brusewitz G, Cameron BD, Chasseaud LF, Gorler K, Hawkins DR, et al.: The metabolism of benzyl isothiocyanate and its cysteine conjugate. *Biochem J* **162,** 99–107, 1977.
19. Conaway CC, Krzeminski J, Amin S, and Chung FL: Decomposition rates of isothiocyanate conjugates determine their activity as inhibitors of cytochrome p450 enzymes. *Chem Res Toxicol* **14,** 1170–1176, 2001.
20. Tang L and Zhang Y: Isothiocyanates in the chemoprevention of bladder cancer. *Curr Drug Metab* **5,** 193–201, 2004.

21. Bollard M, Stribbling S, Mitchell S, and Caldwell J: The disposition of allyl isothiocyanate in the rat and mouse. *Food Chem Toxicol* **35,** 933–943, 1997.
22. Prochaska HJ and Santamaria AB: Direct measurement of NAD(P)H:quinone reductase from cells cultured in microtiter wells: a screen assay for anticarcinogenic enzyme inducers. *Anal Biochem* **169,** 328–336, 1988.
23. Zhu M and Fahl WE: Development of a green fluorescent protein microplate assay for the screening of chemopreventive agents. *Anal Biochem* **287,** 210–217, 2000.
24. Brooks JD, Goldberg MF, Nelson LA, Wu D, and Nelson WG: Identification of potential prostate cancer preventive agents through induction of quinone reductase in vitro. *Cancer Epidemiol Biomark Prev* **11,** 868–875, 2002.
25. Fahey JW, Dinkova-Kostova AT, Stephenson KK, and Talalay P: The "Prochaska" microtiter plate bioassay for inducers of NQO1. *Meth Enzymol* **382,** 243–258, 2004.
26. Li G, Tajima H, and Ohtani T: An improved procedure for the preparation of isothiocyanates from primary amines by using hydrogen peroxide as the dehydrosulfurization reagent. *J Org Chem* **62,** 4539–4540, 1997.
27. Drobnica L, Kristián P, and Augustin J: The chemistry of the –NCS group. In *The Chemistry of Cyanates and their Thio Derivatives, Part 2*, Patai S (ed). Chichester, UK: John Wiley, 1977, pp 1003–1221.
28. Kjaer A, Rubinstein K, and Jensen KA: Unsaturated five-carbon/isothiocyanates. *Acta Chem Scand* **7,** 518–527, 1953.
29. Bruson HA and Eastes JW: Action of sulfuric acid upon unsaturated isothiocyanates: mercaptothiazolines. *J Am Chem Soc* **59,** 2011–2013, 1937.
30. Kjaer A and Jensen RB: *iso*Thiocyanates XX. 4-Pentenyl *iso*thiocyanate, a new mustard oil occurring as a glucoside (glucobrassicanapin) in nature. *Acta Chem Scand* **10,** 1365–1371, 1956.
31. Tang L and Zhang Y: Dietary isothiocyanates inhibit the growth of human bladder carcinoma cells. *J Nutr* **134,** 2004–2010, 2004.
32. Li J, Yao S, and Zhang Y: The role of c-Jun in the AP-1 activation induced by naturally occurring isothiocyanates. *Food Chem Toxicol* **43,** 1373–1380, 2005.
33. Fahey JW, Zhang Y, and Talalay P: Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proc Natl Acad Sci USA* **94,** 10367–10372, 1997.
34. Ye L and Zhang Y: Total intracellular accumulation levels of dietary isothiocyanates determine their activity in elevation of cellular glutathione and induction of Phase 2 detoxification enzymes. *Carcinogenesis* **22,** 1987–1992, 2001.
35. Ernster L: DT diaphorase. *Meth Enzymol* **10,** 309–317, 1967.
36. Habig WH, Pabst MJ, and Jakoby WB: Glutathione S-transferases. The first enzymatic step in mercapturic acid formation . *J Biol Chem* **249,** 7130–7139, 1974.
37. Zhang Y, Wade KL, Prestera T, and Talalay P: Quantitative determination of isothiocyanates, dithiocarbamates, carbon disulfide, and related thiocarbonyl compounds by cyclocondensation with 1,2-benzenedithiol. *Anal Biochem* **239,** 160–167, 1996.
38. Shapiro TA, Fahey JW, Wade KL, Stephenson KK, and Talalay P: Human metabolism and excretion of cancer chemoprotective glucosinolates and isothiocyanates of cruciferous vegetables. *Cancer Epidemiol Biomark Prev* **7,** 1091–1100, 1998.
39. Chung FL, Jiao D, Getahun SM, and Yu MC: A urinary biomarker for uptake of dietary isothiocyanates in humans. *Cancer Epidemiol Biomark Prev* **7,** 103–108, 1998.
40. Ye L, Dinkova-Kostova AT, Wade KL, Zhang Y, Shapiro TA, et al.: Quantitative determination of dithiocarbamates in human plasma, serum, erythrocytes and urine: pharmacokinetics of broccoli sprout isothiocyanates in humans. *Clin Chim Acta* **316,** 43–53, 2002.
41. Zhang Y, Talalay P, Cho CG, and Posner GH: A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc Natl Acad Sci USA* **89,** 2399–2403, 1992.
42. Posner GH, Cho CG, Green JV, Zhang Y, and Talalay P: Design and synthesis of bifunctional isothiocyanate analogs of sulforaphane: correlation between structure and potency as inducers of anticarcinogenic detoxication enzymes. *J Med Chem* **37,** 170–176, 1996.
43. Fahey JW, Zalcmann AT, and Talalay P: The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochem* **56,** 5–51, 2001.
44. Dinkova-Kostova AT, Fahey JW, and Talalay P: Chemical structures of inducers of nicotinamide quinone oxidoreductase (NQO1). *Methods Enzymol* **382,** 423–448, 2004.
45. Holtzclaw WD, Dinkova-Kostova AT, and Talalay P: Protection against electrophile and oxidative stress by induction of phase 2 genes: the quest for the elusive sensor that responds to inducers. *Adv Enzyme Regul* **44,** 335–367, 2004.
46. Dinkova-Kostova AT, Holtzclaw WD, Cole RN, Itoh K, Wakabayashi N, et al.: Direct evidence that sulfhydryl groups of Keap1 are the sensors regulating induction of phase 2 enzymes that protect against carcinogens and oxidants. *Proc Natl Acad Sci USA* **99,** 11908–11913, 2002.
47. Wakabayashi N, Dinkova-Kostova AT, Holtzclaw WD, Kang M-I, Kobayashi A, et al.: Protection against electrophile and oxidant stress by induction of the phase 2 response: Fate of cysteines of the Keap1 sensor modified by inducers. *Proc Natl Acad Sci USA* **101,** 2040–2045, 2004.
48. Hayes JD and Pulford DJ: The glutathione S-transferase supergene family: regulation of GST* and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit Rev Biochem Mol Biol* **30,** 445–600, 1995.
49. Mennicke WH, Gorler K, Krumbiegel G, Lorenz D, and Rittmann N: Studies on the metabolism and excretion of benzyl isothiocyanate in man. *Xenobiotica* **18,** 441–447, 1988.
50. Chung FL, Morse MA, Eklind KI, and Lewis J: Quantitation of human uptake of the anticarcinogenic phenethyl isothiocyanate after a watercress meal. *Cancer Epidemiol Biomark Prev* **1,** 383–388, 1992.
51. Zhang Y: Role of glutathione in the accumulation of anticarcinogenic isothiocyanates and their glutathione conjugates by murine hepatoma cells. *Carcinogenesis* **21,** 1175–1182, 2000.
52. Hwang ES and Jeffery EH: Induction of quinone reductase by sulforaphane and sulforaphane N-acetylcysteine conjugate in murine hepatoma cells. *J Med Food* **8,** 198–203, 2005.
53. Zhang Y and Talalay P: Mechanism of differential potencies of isothiocyanates as inducers of anticarcinogenic phase 2 enzymes. *Cancer Res* **58,** 4632–4639, 1998.
54. Conaway CC, Jiao D, Kohri T, Liebes L, and Chung FL: Disposition and pharmacokinetics of phenethyl isothiocyanate and 6-phenylhexyl isothiocyanate in F344 rats. *Drug Metab Dispo* **27,** 13–20, 1999.
55. Zhang Y, Yao S, and Li J: Vegetable-derived isothiocyanates: antiproliferative activity and mechanism of action. *Proc Nutr Soc* **65,** 68–75, 2006.
56. Fowke JH, Hebert JR, and Fahey JW: Urinary excretion of dithiocarbamates and self-reported cruciferous vegetable intake: application of the "method of triads" to a food-specific biomarker. *Public Health Nutr* **5,** 791–799, 2002.
57. Seow A, Shi CY, Chung FL, Jiao D, Hankin JH, et al.: Urinary total isothiocyanates (ITC) in a population-based sample of middle-aged and older Chinese in Singapore: relationship with dietary total ITC and glutathione S-transferase M1/T1/P1 genotypes. *Cancer Epidemiol Biomark Prev* **7,** 775–781, 1998.
58. Michaud DC, Spiegelman D, Clinton SK, Rimm EB, Willett WC, et al.: Fruit and vegetable intake and incidence of bladder cancer in a male prospective cohort. *J Natl Cancer Inst* **91,** 605–613, 1999.