# The principal urinary metabolites of dietary isothiocyanates, *N*-acetylcysteine conjugates, elicit the same anti-proliferative response as their parent compounds in human bladder cancer cells

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**Isothiocyanates (ITCs) are a class of well-known cancerpreventive phytochemicals, but are primarily disposed of and concentrated in the urine as *N*-acetylcysteine conjugates (NAC-ITCs) *in vivo*. Because human urinary bladder cancers occur almost exclusively in the bladder epithelium, which is directly exposed to the urine stored in the bladder, we undertook to examine the anti-cancer activity of NAC-ITCs in cultured human bladder cancer cells. In this paper, we report that the NAC conjugates of four naturally occurring ITCs, including allyl ITC, benzyl ITC (BITC), phenethyl ITC and sulforaphane, potently inhibited the growth of cells derived from both low-grade superficial and high-grade invasive human bladder cancers and drug-resistant bladder cancer cells. Moreover, the growth-inhibitory potencies were similar between the conjugates and their parent compounds. Further study of NAC-BITC and BITC as model compounds showed that both compounds accumulated in cells predominantly as the glutathione conjugate of BITC, but the accumulation of the former was slower. Moreover, both compounds also demonstrated the same anti-proliferative mechanisms: causing the cleavage of the same set of caspases (caspase − 3, − 8 and − 9) in apoptosis induction, arresting cells in the same phases (S and $G_2$/M) and targeting the same cell cycle regulator (Cdc25C), although a longer treatment time or slightly higher doses were needed for NAC-BITC to achieve the same effect as BITC, presumably due to slower cellular uptake of NAC-BITC. These data show that the NAC-ITCs are biologically similar to their parent compounds and are highly effective against human bladder cancer cells. *Anti-Cancer Drugs* 17:297–305 © 2006 Lippincott Williams & Wilkins.**

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## Introduction

Several population-based studies have shown an inverse relationship between consumption of cruciferous vegetables and cancer risk, including bladder cancer [1–4]. Glucosinolates (GS; β-thioglucoside *N*-hydroxysulfates), a family of phytochemicals that are abundant in these vegetables, are widely believed to account at least in part for the cancer-preventive effects of cruciferous vegetables. While there is little evidence to show that GS themselves possess cancer-preventive activities, they are precursors of isothiocyanates (ITCs), many of which have been shown to inhibit cancer development in various animal models [5–9]. GS are converted to ITCs when plant cells are injured, such as during cooking, and the conversion is catalyzed by myrosinase (thioglucoside glucohydrolase), an enzyme that coexists with, but is physically separated from, GS under normal conditions [10,11]. Interestingly, GS that escape plant myrosinase may be converted to ITCs *in vivo*, as intestinal microflora are known to possess myrosinase activity [12–14].

Although ITCs display their cancer-chemopreventive activities in a variety of cultured cells and animal organs [8,15], they may be most useful for bladder cancer prevention [16]. The rationales in support of this view include: (i) ITCs are metabolized principally via the mercapturic acid pathway *in vivo*, giving rise to *N*-acetylcysteine conjugates (NAC-ITCs), which are almost exclusively excreted and concentrated in the urine [16–18], and storage of urine containing NAC-ITCs in the bladder likely further enhances the exposure of bladder to these compounds. (ii) As a result, the bladder, especially the bladder epithelium that gives rise to the majority of bladder cancers (90–95% in the US) [19], is likely the most exposed tissue to ITCs and their metabolites *in vivo*. Indeed, Bollard *et al.* [20] reported that the $^{14}C$ content in the urinary bladder was 14–18 times that in the liver and kidney after a single oral dose of [$^{14}C$]allyl ITC (AITC) to male F344 rats. (iii) A number of NAC-ITCs have shown chemopreventive activity in lung and prostate cancer cell lines *in vitro*

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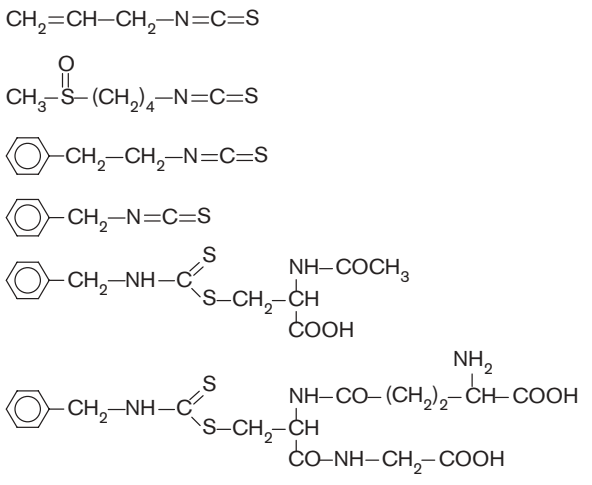
[21,22], and the colon and lungs of rodents *in vivo* [23–26]. If these compounds exert chemopreventive effects in the bladder, a potentially highly favorable strategy for bladder cancer prevention and treatment may be in order – selective delivery of NAC-ITCs through urinary excretion to the bladder target tissue (the epithelium) after oral ingestion of ITCs.

At initial presentation, 80% of bladder cancers are low-grade superficial lesions, which are confined to the epithelium and the subepithelial layer, and are typically removed by transurethral resection [27,28]. Unfortunately, more than half of the patients will experience recurrence in less than 5 years and this number increases to 88% at 15 years post-surgery [29,30]. Although most recurrent tumors show histological grade and stage similar to the primary tumors, 20–40% of recurrent tumors become muscle invasive [27,28,30–32]. Consequently, a major challenge for patients after surgical removal of primary superficial bladder tumors is to prevent recurrence. However, if the recurrence can be delayed for only 5–10 years, the incidence of bladder cancer recurrence may drop dramatically, because the average age at diagnosis is 68–69 years [33], but the average life expectancy for men and women in the US is 74.6 and 79.9 years, respectively [34].

In this study, we synthesized and examined the anti-proliferative activities of the urinary metabolites (NAC conjugates) of four naturally occurring ITCs, including AITC, benzyl ITC (BITC), phenethyl ITC (PEITC) and sulforaphane (SF) (see Fig. 1 for representative chemical structures). All the ITCs have been previously shown to inhibit the proliferation of human bladder cancer cells in culture [35]. The NAC-ITCs were evaluated in cells derived from both low-grade superficial and high-grade invasive human bladder cancers as well as cells that are resistant to doxorubicin and overexpress P-glycoprotein. We then selected BITC and NAC-BITC as model compounds to compare the underlying mechanisms of their anti-proliferative activities. Our results show that the NAC-ITCs not only are potent anti-proliferative agents against various types of human bladder cancer cells, but also almost fully retain the activity of their parent compounds.

**Fig. 1**




Chemical structures of AITC (1), SF (2), PEITC (3), BITC (4), NAC-BITC (5) and GS-BITC (6).

## Materials and methods

### Chemicals

AITC, BITC, PEITC, SF (1-isothiocyanato-[*4R,S*]-[methylsulfinyl]butane) and the cysteine conjugate of BITC (Cys-BITC) were purchased from either LKT Laboratories (St Paul, Minnesota, USA) or Aldrich (Milwaukee, Wisconsin, USA). The antibodies specific for caspases and poly(ADP-ribose) polymerase (PARP) were purchased from Cell Signaling (Beverly, Massachusetts, USA). The antibodies specific for cell cycle regulators and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA) and Chemicon (Temecula, California, USA), respectively. All other reagents were purchased from Sigma (St Louis, Missouri, USA).

### Cell culture

Human bladder cancer RT4 cells were purchased from the ATCC (Manassas, Virginia, USA) and were grown in McCoy's 5A medium with L-glutamine, supplemented with 10% FBS. The medium and FBS were purchased from Cellgro (Herndon, Virginia, USA) and Biosource international (Camarillo, California, USA), respectively. Human bladder cancer UM-UC-6 cells and their doxorubicin-resistant derivative UM-UC-6/dox cells were generously provided by Dr Hebert B. Grossman (MD Anderson Cancer Center, Houston, Texas, USA) [36]. UM-UC-6 cells were grown in MEM NEAA Earle's salts (Irvine Scientific, Santa Ana, California, USA) supplemented with 10% FBS, 10 ml/l L-glutamine and 1% penicillin/streptomycin. UM-UC-6/dox cells were grown in the above medium with the addition of 0.1 μg doxorubicin/l to maintain their resistance phenotype. All cells were maintained in 75-$cm^2$ flasks in a humidified incubator at 37°C with 5% $CO_2$.

### Synthesis of ITC-thiol conjugates

NAC conjugates of AITC, BITC and PEITC were synthesized according to a protocol by Vermeulen *et al.* [37]. To prepare NAC-SF, SF was mixed with a large excess of NAC in the presence of sodium bicarbonate. After all SF was fully converted to NAC-SF, the remaining NAC was converted to NAC-PEITC by adding PEITC to the solution, which was then removed by washing the solution with ethyl acetate. The resultant NAC-SF was

not soluble in ethyl acetate and stayed in the water layer. A large amount of acetonitrile (ACN) was then added to the water fraction to precipitate NAC-SF, which was washed with ACN and dried under high vacuum, yielding a white powder. Synthesis of glutathione (GSH) and cysteinylglycine (Cys-Gly) conjugates of BITC (GS-BITC and Gly-Cys-BITC) followed a similar strategy: BITC was mixed with GSH or Cys-Gly in a sodium bicarbonate solution and each conjugate precipitated (white solids) after HCl addition, which was then washed with water and dried as described above. The chemical structure and the purity of each compound were confirmed by $^{1}$H-NMR and mass spectrometry. The conjugates were stored at $-20^{\circ}$C.

#### Cytotoxicity assay

Five thousand cells were seeded in each well of a 96-well plate with 150 µl medium for 24 h. An ITC or NAC-ITC, originally dissolved in DMSO, was then added to each well in 50 µl medium. The final concentration of the agent in each well ranged between 0.39 and 100 µmol/l (1-fold serial dilution) with no more than 0.025% DMSO (v/v) in medium. Cells were grown in the presence of the test compound for 72 h. At the end of the incubation, the cell density in each well was measured by the MTT assay [38]. The growth curve of agent-treated cells was then constructed to determine the concentration of ITC or NAC-ITC required to inhibit cell growth by 50% ($IC_{50}$).

#### Measurement of total intracellular accumulation of ITCs and their NAC conjugates

The procedures involving cell exposure to ITCs or NAC-ITCs, cell harvest, preparation of cell lysates, and quantitative analysis of ITCs and their metabolites in cell lysates by the cyclocondensation assay [39] were performed as previously described [40]. Since the cyclocondensation assay (reaction with 1,2-benzenedithiol) detects both free ITCs and their metabolites formed in the mercapturic acid pathway, including GSH conjugates, Cys-Gly conjugates, Cys conjugates and NAC conjugates, all of which are generally termed dithiocarbamates (DTCs), the intracellular accumulation levels reported in this paper refer to the total levels of ITCs plus DTCs in cells (ITC/DTC). Measurement of intracellular fluid volume, determination of protein content in cell lysates and calculation of total intracellular ITC/DTC concentration were the same as previously described [40,41].

#### Measurement of intracellular accumulation of individual metabolites of ITCs and NAC-ITCs

In order to limit the scope, we only studied BITC and NAC-BITC in RT4 cells. Five million cells in 10 ml medium were incubated with BITC or NAC-BITC at 10 µmol/l for 1 h, and then harvested and sonicated as previously described [40]. The lysates were centrifuged at 17 000 *g* for 10 min at 4°C, and the supernatant portions were analyzed by the cyclocondensation assay for the total content of ITC/DTC, and by liquid chromatography-tandem mass spectrometry (LC/MS/MS) for the concentration of each compound and its metabolites. An Agilent 1100 LC system (Palo Alto, California, USA), consisting of a vacuum degasser, a binary pump and an autosampler, as well as a Cyano 250 × 4 mm column (5 µm particle size) from Waters (Milford, Massachusetts, USA), were used to separate BITC and its metabolites formed in the mercapturic acid pathway. The compounds were well separated from one another, using the isocratic mobile phase of 0.1% formic acid, 40% water and 60% acetonitrile at a flow rate of 0.6 ml/min. The LC system was coupled to an Applied Biosystems MDS Sciex API 3000 Triple Quadrupole Mass Spectrometer (Concord, Ontario, Canada) through a TurboIonSpray ion source interface for the detection of the compounds. The LC/MS/MS system was controlled by Analyst 1.3 software (Applied Biosystems). The detection of each compound was accomplished in multiple reactions monitoring mode by monitoring a transition pair of *m/z* 310.9 (molecular ion)/161.9 (fragment ion) for NAC-BITC, and 455.5/306.3, 269.4/120.2 and 326.1/177.1 for GS-BITC, Cys-BITC, and Gly-Cys-BITC, respectively, at unit resolution (FWHM 0.6–0.8) with a scan time of 500 ms for each pair. The samples prepared from the cells were kept at 4°C until the moment of injection. The quantification of those compounds was achieved using a calibration curve established at identical LC/MS/MS conditions. To prepare the calibration standards, each compound was mixed with a blank cell lysate supernatant to obtain a final concentration of 0, 0.008, 0.04, 0.1, 0.2, 0.5, 1, 2.5 and 5 µg/ml, each in triplicate. The calibration curves had good linearity with correlation coefficients of 0.999. The detection limit for GS-BITC, Gly-Cys-BITC, Cys-BITC and NAC-BITC, determined from the calibration standards, was 0.13–0.19 ng/ml.

#### Measurement of stability of NAC-BITC

NAC-BITC was dissolved in DMSO and diluted into PBS (pH 7.4) in glass vials. The final concentration of NAC-BITC and DMSO in PBS was 0.1 mmol/l and 0.1% (v/v), respectively. The NAC-BITC solution was incubated at 37°C, and an aliquot was taken at 0, 1, 2, 3 and 24 h to measure the remaining NAC-BITC, based on absorbance change of the solution. NAC-BITC displays a characteristic absorbance spectrum, which shows absorbance maxima at 250 nm ($a_m$ of 11 470 $M^{-1}cm^{-1}$) and a shoulder at 275 nm ($a_m$ of 9390 $M^{-1}cm^{-1}$).

#### Western blot analysis

One million cells were grown in each 10-cm dish with 10 ml medium for 48 h and then treated with BITC or NAC-BITC at the desired concentration. Each compound was dissolved in DMSO. The final concentration of DMSO in medium was 0.1% (v/v). At the end of treatment, cells in each dish were harvested by trypsini-

zation and centrifugation at 500*g* for 5 min at 4°C. The cell pellet from each dish was washed with 10 ml ice-cold PBS and was centrifuged again. The pellet was then suspended in 200 µl cell lysis buffer (Cell Signaling Technology) supplemented with 1 mmol/l PMSF and sonicated by a Branson Model 450 sonifier. The lysates were centrifuged at 10 000*g* for 5 min at 4°C and the supernatant was used for analysis. Protein contents in each sample were measured using the BCA assay. Each sample (25–50 µg protein) was resolved by SDS–PAGE (8–15%) and blotted to PVDF membranes, which were probed by specific antibodies and visualized using an ECL chemiluminescence system (Amersham Biosciences, Piscataway, New Jersey, USA).

### Analysis of cell cycle arrest

Cells were treated and pelleted as above. For each sample, $1 \times 10^6$ cells were suspended in 1 ml modified Krishan buffer containing 1 g/l sodium citrate, 20 mg/l RNase, 0.3% NP-40 and 50 mg/l propidium iodide [42], and incubated on ice in the dark for at least 1 h. Cell cycle distribution was then determined by flow cytometry and 10 000 cells per sample were analyzed.

### Statistics

Results are expressed as mean ± SD (at least three measurements). Data were analyzed by one-way ANOVA, followed by Dunnett's *t*-test for separate comparisons with the control group or followed by Sidak's *t*-test for multiple-group comparisons. Differences were considered significant at $P < 0.05$.

## Results

### NAC-ITCs inhibit the proliferation of human bladder cancer cells

The four NAC conjugates of ITC, i.e. NAC-AITC, NAC-BITC, NAC-PEITC and NAC-SF, were examined in RT4 cells (which were derived from a low-grade superficial human bladder cancer [43]), in UM-UC-6 cells (which were derived from an invasive human bladder cancer) and in UM-UC-6/dox cells (which were derived from UM-UC-6 cells, and were doxorubicin-resistant and over-expressed P-glycoprotein [36]). The latter two cell lines allowed us to evaluate the impact of multidrug resistance on the anti-proliferative effects of NAC-ITCs in human bladder cancer cells. In all experiments, cells were grown for 72 h after addition of a test compound to the culture medium. As shown in Table 1, the three cell lines were similarly sensitive to a given NAC-ITC, as the inter-cell line $IC_{50}$ values of each conjugate varied by less than 1-fold: 5.6–7.2 (NAC-AITC), 7.0–12.7 (NAC-BITC), 9.5–12.5 (NAC-PEITC) and 12.3–17.4 µmol/l (NAC-SF). These results suggest that neither degree of malignancy nor P-glycoprotein-associated drug resistance has an important impact on the sensitivity of human bladder cancer cells to NAC-ITCs. Moreover, the data presented above also show that all four NAC-ITCs inhibited the growth of RT4 cells, and the $IC_{50}$ values varied by only 0.9–2.1-fold between the most and least potent compounds (NAC-AITC versus NAC-SF), indicating that the anti-proliferative activity is a shared property of various NAC-ITCs. We further compared the growth-inhibitory activity of each NAC-ITC with that of the parent ITC in RT4 cells and found that the conjugate in each pair was either slightly more potent (NAC-AITC and NAC-BITC) or slightly weaker (NAC-PEITC and NAC-SF) than the corresponding ITC in inhibiting cell growth, as indicated by their $IC_{50}$ values (Table 1), suggesting that conjugation of ITCs with NAC does not significantly alter the anti-proliferative activity.

**Table 1 Inhibition of cell growth by ITCs and their NAC conjugates**

| | $IC_{50}$ (µmol/l) | | |
|---|---|---|---|
| | RT4 | UM-UC-6 | UM-UC-6/dox |
| AITC | $7.3 \pm 1.0^{a}$ | | |
| NAC-AITC | $5.6 \pm 0.3^{b,e}$ | $6.5 \pm 0.4^{a,f}$ | $7.2 \pm 0.5^{a,g}$ |
| BITC | $8.3 \pm 0.7^{a}$ | | |
| NAC-BITC | $7.0 \pm 0.4^{a,b,e}$ | $10.0 \pm 0.5^{b,f}$ | $12.7 \pm 0.1^{b,g}$ |
| PEITC | $8.5 \pm 1.2^{a}$ | | |
| NAC-PEITC | $12.5 \pm 1.2^{c,e}$ | $9.5 \pm 0.4^{b,f}$ | $12.0 \pm 1.2^{b,c,e}$ |
| SF | $11.8 \pm 0.7^{c}$ | | |
| NAC-SF | $17.4 \pm 1.2^{d,e}$ | $12.3 \pm 1.3^{c,f}$ | $14.2 \pm 1.3^{c,g}$ |

Cells were grown in 96-well plates for 24 h and then exposed to a series of concentrations of an ITC or its NAC conjugate for 72 h. Cell density in each well was then determined using the MTT assay and the $IC_{50}$ value was calculated based on the cell growth curve. Values were tested by one-way ANOVA followed by Sidak's *t*-test for multiple-group comparisons. Each value is a mean ± SD ($n = 6$). Values with different superscript letters within a column (a–d) or within a row (e–g) were significantly different from one another at $P < 0.05$.

### Both NAC-BITC and BITC accumulate in bladder cancer cells primarily as GS-BITC

NAC-BITC and BITC were then selected as model compounds to compare the anti-proliferative mechanisms of NAC-ITCs and ITCs using RT4 cells. Although NAC-AITC was somewhat more potent than NAC-BITC (Table 1), we selected the latter compound for further study because the anti-proliferative mechanism of AITC was not as well known as that of BITC in human bladder cancer cells [35,41]. We first compared the intracellular accumulation levels of NAC-BITC and BITC. ITCs, including BITC, have been shown to rapidly accumulate in cells [44,45], but there is little information on cellular uptake of NAC-ITCs. Thus, RT4 cells were exposed to either BITC or NAC-BITC at 10 or 30 µmol/l for 1 or 3 h and then harvested for measurement of total intracellular accumulation of the compound by the cyclocondensation assay. As mentioned in Materials and methods, the cyclocondensation assay detects ITCs and their metabolites (DTCs) formed in the mercapturic acid pathway. Therefore, the results represent the total amount of ITCs plus DTCs (ITC/DTC). As shown in Fig. 2(a and b), both BITC and NAC-BITC accumulated in cells in a dose-dependent manner, but the uptake of NAC-BITC was slower than BITC. For example, whereas total

intracellular uptake level reached 1.49 ± 0.37 mmol/l (149-fold accumulation) after 1 h exposure to 10 µmol/l BITC and declined thereafter, intracellular uptake of NAC-BITC under the same condition reached only 0.25 ± 0.01 mmol/l (25-fold accumulation) at 1 h exposure, but continued to rise at 3 h (48-fold accumulation) and likely rose further with time. The intracellular accumulation patterns of the other three ITCs and their NAC conjugates were very similar to those of BITC and NAC-BITC (results not shown).

Previous studies in non-bladder cells have shown that ITCs accumulate in cells primarily by rapid conjugation with cellular GSH and the high GSH concentrations in cells (1–10 mmol/l) provide the basis for the high levels of accumulation of ITCs [44,45]. When RT4 cells were incubated with BITC at 10 µmol/l for 1 h and its intracellular identity was examined by LC/MS/MS, BITC was found to be accumulated almost exclusively as a GSH conjugate (94–95%) (Fig. 2b; see Fig. 1 for its chemical structure). The Cys conjugate detected in the cells (4.8–6.5%) is likely converted from the GSH conjugate through the mercapturic acid pathway. Interestingly, NAC-BITC also accumulated mainly as the GSH conjugate of BITC (73–100%) with a small amount of Cys conjugate (1.1–2.8%) and NAC conjugate (1.6–2.1%). The remaining species if any were not identified, but neither free BITC nor Cys-Gly conjugate (downstream metabolite of GS-BITC via the mercapturic acid pathway) were detected. These results show that the main intracellular chemical species is the same (a GSH derivative) whether cells are exposed to BITC or NAC-BITC.

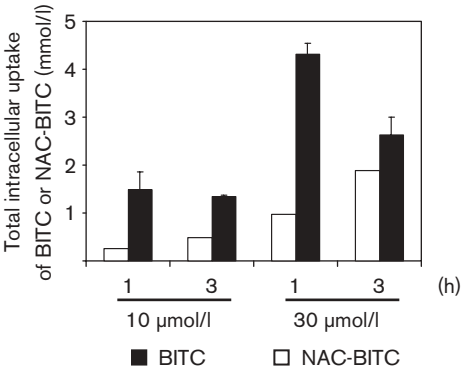
ITCs conjugated to various thiols as DTCs are unstable and dissociate to ITCs [46]. As shown in Fig. 2(c), there was a rapid and significant dissociation of NAC-BITC in PBS at 37°C. Nearly 20% of the conjugate dissociated at the end of the 3-h incubation. This result, combined with our observations that cellular uptake of NAC-BITC is slower than BITC, but both compounds are accumulated mainly as GS-BITC and very little NAC-BITC is found in cells exposed to NAC-BITC, strongly indicate that the cellular uptake of NAC-BITC largely depends on liberation of BITC. This is consistent with our previous finding that intact NAC-BITC is largely unable to enter murine hepatoma Hepa1c1c7 cells [44].

### NAC-BITC and BITC have a similar impact on apoptosis and cell cycle progression in RT4 cells

Because both NAC-BITC and BITC accumulated primarily as GS-BITC in RT4 cells, we hypothesized that their anti-proliferative mechanisms were the same. We examined two mechanisms that have been shown to mediate BITC-induced inhibition of cell proliferation in cancer cells: induction of apoptosis and arrest of cell cycle progression. RT4 cells were treated with BITC or NAC-

**Fig. 2**

(a)

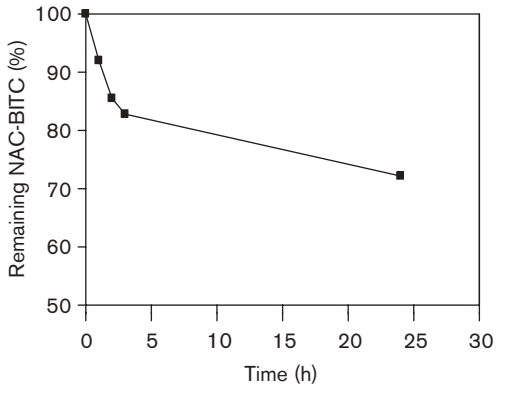



(b) The chemical identity of intracellularly accumulated BITC and NAC-BITC

| | Cell exposure to each compound at 10 µmol/l for 1 h | |
|---|---|---|
| | BITC | NAC-BITC |
| Total intra cellular accumulation (mmol/l) | 1.49 ± 0.37* | 0.25 ± 0.01* |
| GS-BITC (%) | 94–95%# | 73–100%# |
| Cys-BITC (%) | 4.8–6.5%# | 1.1–2.8%# |
| NAC-BITC (%) | 0# | 1.6–2.1%# |

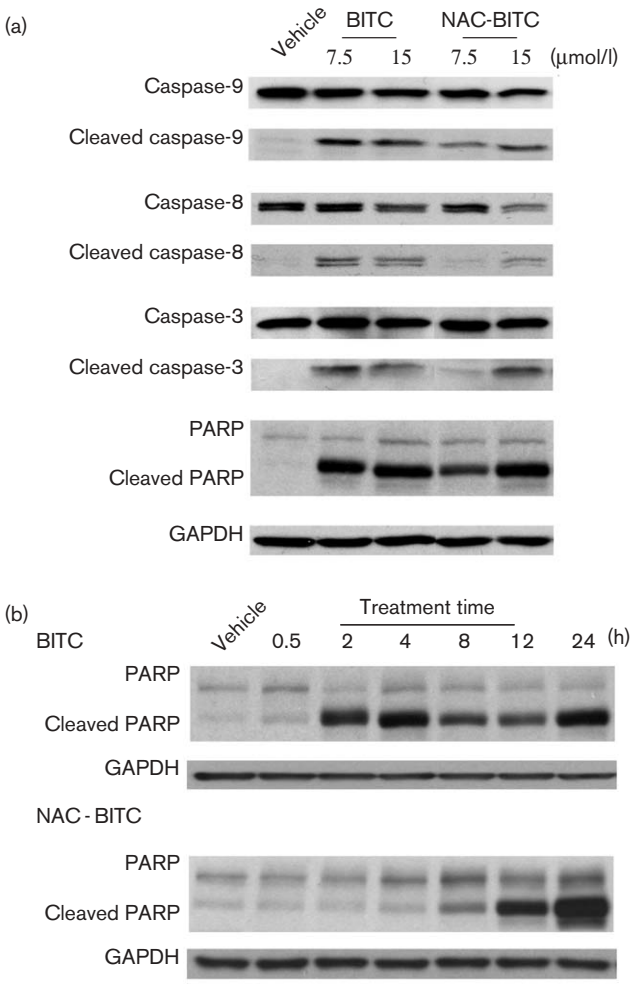
*Each value is a mean ± SD, $n=3$;
#Three independent determinations

(c)




Intracellular uptake of BITC or NAC-BITC in human bladder RT4 cells. (a) Five million cells were suspended in 10 ml medium and incubated with each compound at the indicated concentration for 1 or 3 h at 37°C. Cells were harvested and total intracellular accumulation levels of the compounds were determined by the HPLC-based cyclocondensation assay as described in Materials and methods. Each value is a mean ± SD ($n=3$). (b) Five million cells were suspended in 10 ml medium and incubated with 10 µmol/l BITC or NAC-BITC for 1 h at 37°C. Cells were then harvested and measured for both total intracellular accumulation of the compound as mentioned above and the chemical identity of the accumulate using LC/MS/MS (see Materials and methods). (c) NAC-BITC (100 µmol/l) was incubated in PBS at 37°C for the indicated times and the remaining NAC-BITC was measured by UV spectroscopy.

**Fig. 3**




BITC and NAC-BITC induced apoptosis in human bladder RT4 cells. (a) Activation of caspases and PARP cleavage in BITC- or NAC-BITC-treated RT4 cells. Cells were seeded in 10-cm dishes for 48 h and then treated with DMSO (vehicle) or the compound at 7.5 and 15 μmol/l for 24 h before analysis by Western blotting. (b) Time-dependent increase of PARP cleavage in BITC- or NAC-BITC- treated RT4 cells. Cells were incubated with each compound at 15 μmol/l for an indicated period of time before analysis by Western blotting. GAPDH served as a loading control. An antibody recognizing both uncleaved and cleaved PARP was used, and the relatively weak band intensity of uncleaved PARP in comparison with that of cleaved PARP is likely due to differential antibody affinity toward the proteins.

BITC at 7.5 and 15 μmol/l for 24 h. As shown in Fig. 3(a), both compounds caused the cleavage of caspase −9, −8 and −3 (an executioner caspase activated by both caspase-8 and -9), and PARP (a marker of apoptosis). However, NAC-BITC appeared to be slightly less potent than BITC, as BITC had a maximum effect at 7.5 μmol/l, whereas 15 μmol/l NAC-BITC was needed to reach a similar effect. The weaker effect of NAC-BITC was also reflected in the time course of PARP cleavage. While significant PARP cleavage was detected in cells after 2 h incubation with BITC at 15 μmol/l, a similar change was not detected in NAC-BITC-treated cells until 8 h after incubation with the compound (Fig. 3b). These results could well be a reflection of the fact that intracellular accumulation of BITC was more rapid than NAC-BITC. Indeed, when RT4 cells were exposed to BITC or NAC-BITC as well as other ITCs and their NAC conjugates for 3 h (cells then grew in fresh medium without the test compound for 69 h), the $IC_{50}$ value of each conjugate was approximately 1-fold higher than that of its parent ITC (results not shown). Interestingly, BITC-induced PARP cleavage displayed a biphasic phenomenon (PARP cleavage increased at 2–4 h, decreased at 8–12 h and then increased again at 24 h after BITC treatment started). The exact reason is unknown, but the decrease in PARP cleavage coincided with cell doubling at 8–12 h (data not shown), suggesting that the newly formed cells have yet to respond to BITC-induced PARP cleavage.

Both BITC and NAC-BITC also had similar effects on cell cycle progression of RT4 cells (Table 2). Both compounds caused cell cycle arrest in S and $G_2$/M phases. For example, the combined number of cells in S and $G_2$/M phases increased from 30.8% in the control to 58.3 (BITC) and 49.3% (NAC-BITC) when the cells were incubated with each compound at 15 μmol/l for 24 h. The two compounds also had similar effects on the formation of sub-$G_1$ phase cells. Because cells were arrested in both S and $G_2$/M phases, we examined the corresponding cell cycle regulators, including cyclin A and Cdk2 (S phase), and cyclin B1 and Cdc2 ($G_2$/M phase). No significant changes in the levels of these proteins were detected in cells treated by either BITC or NAC-BITC (Fig. 4a). Interestingly, both BITC and NAC-BITC downregulated the level of Cdc25C (Fig. 4b), but not other Cdc25 family members (Cdc25A and Cdc25B, results not shown). Cdc25C is involved in activating the cyclin/Cdk complex and inactivation/degradation of Cdc25C is known to cause cell cycle arrest [47]. However, as in the case of PARP cleavage, compared with BITC, a longer treatment time was needed for NAC-BITC to cause down regulation of Cdc25C (Fig. 4b).

## Discussion

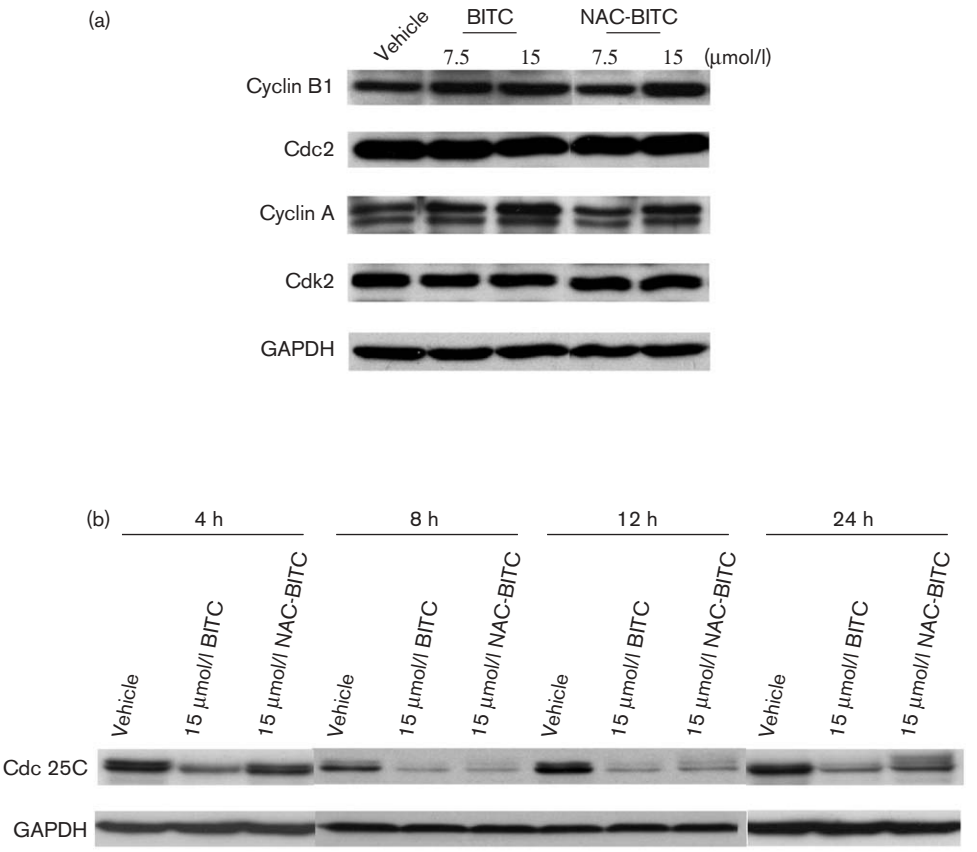
The studies presented herein demonstrate for the first time that the principle urinary metabolites (NAC-ITCs) of four naturally occurring ITCs, including AITC, BITC, PEITC and SF, can potently inhibit the growth of cells derived from both low-grade superficial human bladder cancer (RT4 cells) and high-grade invasive human bladder cancer (UM-UC-6 cells) as well as human bladder cancer cells that are resistant to doxorubicin and overexpress P-glycoprotein (UM-UC-6/dox cells). The growth-inhibitory potencies among the conjugates were similar to one another across the cell lines (Table 1), suggesting that the anti-proliferative activity is a shared property of these compounds. Moreover, comparison of

**Table 2 Effect of BITC and NAC-BITC on RT4 cell cycle progression**

| ITC | Concentration (μmol/l) | Cell cycle distribution (%) | | | |
|---|---|---|---|---|---|
| | | $G_1$ | S | $G_2$/M | Sub-$G_1$ |
| Vehicle | | 68.6 ± 0.9 | 17.9 ± 0.4 | 12.9 ± 0.5 | 0.6 ± 0.0 |
| BITC | 7.5 | 52.4 ± 0.6 | 16.5 ± 0.1 | 27.0 ± 0.2[a] | 4.1 ± 0.5[a] |
| | 15 | 33.8 ± 1.7 | 30.2 ± 1.8[a] | 28.3 ± 0.5[a] | 7.7 ± 0.6[a] |
| NAC-BITC | 7.5 | 63.8 ± 0.8 | 14.0 ± 0.6 | 18.2 ± 0.4[a] | 4.0 ± 0.2[a] |
| | 15 | 44.5 ± 1.5 | 20.3 ± 0.8[a] | 29.0 ± 0.8[a] | 6.1 ± 0.1[a] |

Cells were grown in 10-cm plates for 48 h and then exposed to BITC or NAC-BITC at indicated concentrations for 24 h. The cells were then harvested and analyzed by flow cytometry (10 000 cells per sample). Values were tested by one-way ANOVA followed by Dunnett's *t*-test for separate comparisons with the control group within each column. Each value is a mean ± SD ($n$=3).
[a]Different from the control, $P < 0.05$.

**Fig. 4**




Effect of BITC and NAC-BITC on select cell cycle regulators in human bladder RT4 cells. Cells were treated with either vehicle (DMSO) or the test compound at indicated concentrations for a desired period of time and then harvested for Western blot analysis of cyclins and Cdks (a) and Cdc25C (b). GAPDH served as a loading control.

the conjugates with their parent ITCs in RT4 cells indicate that the conjugates and their parent ITCs are similarly potent in inhibiting the growth of these cells (Table 1). These results show that the main urinary metabolites of ITCs retain the anti-cancer activity of their parent ITCs. This finding is significant, because both primary and recurrent bladder cancers originate in the epithelium that is directly exposed to the urine, and orally ingested ITCs are almost exclusively disposed of and concentrated in the urine as NAC-ITCs. It is not known at the present time how the compounds may affect the growth of normal bladder epithelial cells, as cultured normal human bladder epithelial cells are not available. However, some of the ITCs have been shown to be significantly more toxic to cancer cells than to the corresponding normal cells [48–51]. Our present study shows that the concentrations required for the NAC-ITCs to inhibit the proliferation of bladder cancer cells

are at low micromolar concentrations (7.5–15 μmol/l). These concentrations appear to be readily achievable *in vivo*. For example, when BITC (14.4 mg or 96.5 μmol) was administered orally to each human subject, 54% of the dose (52.1 μmol) was recovered in the urine as NAC-BITC in 10–12 h [17]. If one assumes that an adult person normally produces 1–1.5 l urine in 10–12 h, the average urinary concentration of BITC-NAC after taking 14.4 mg BITC is 34.7–52.1 μmol/l. The finding that only low ITC doses may be needed for bladder cancer prevention is significant, because previous studies reported toxic response in the bladder when rats were fed high doses of ITCs (100–1000 p.p.m. in the diet), which is believed to raise urinary concentrations of ITC metabolites up to mmol/l levels [16].

Further study of BITC and NAC-BITC showed that both compounds were accumulated primarily through reaction with GSH in RT4 cells, but cellular accumulation of NAC-BITC was slower than BITC. This fits well with our subsequent observation that the anti-proliferative mechanisms of BITC and NAC-BITC are identical, but relatively longer treatment time or slightly higher doses are needed for the latter compound to exert the same effect. Moreover, our study also identifies the GSH conjugate of BITC as the principal intracellular chemical species in cells exposed to BITC and NAC-BITC and suggests that the GSH conjugate is likely the actual solicitor of anti-cancer response. In this context, previous studies have shown that GSH conjugates of ITCs are capable of undergoing an exchange reaction with thiols [52], perhaps with protein sulfhydryl groups as well. Certain ITC-thiol conjugates are also known to be unstable and dissociate to ITCs [46,53,54]. Our results indicate that cellular uptake of NAC-BITC, perhaps other NAC-ITCs as well, depends on liberation of the cognate ITC.

Treatment of RT4 cells with BITC and NAC-BITC led to induction of apoptosis, which was associated with cleavage of caspase −3, −8 and −9. We have recently shown in another human bladder cancer cell line (UM-UC-3 cells) that BITC activated the same set of caspases [35], but apoptosis induction resulted primarily from mitochondrial damage and subsequent activation of caspase −9 and −3 [41]. The role of caspase-8 in BITC- and NAC-BITC-induced apoptosis is unclear, as is the signaling mechanism leading to its cleavage by these compounds. Exposure of RT4 cells to BITC and NAC-BITC also causes arrest in S and $G_2$/M phases with down regulation of Cdc25C. Cdc25C is a nuclear phosphatase and activates cyclin B1/Cdc2 complex by removing a phosphate in Cdc2 [47]. Inactivation/degradation of Cdc25C renders Cdc2 inactive. Although cyclinB1/Cdc2 is known as a $G_2$/M phase regulator, several studies have shown that down regulation of Cdc25C by chemopreventive agents such as resveratrol, PEITC, and vitamin C is associated with arrest of cells in $G_2$/M, S or both $G_2$/M and S phases [55–57]. We did not further examine the mechanism of BITC- and NAC-BITC-induced down-regulation of Cdc25C and alteration in Cdc2 phosphorylation status, since we are primarily interested in comparing the chemopreventive property of ITCs with that of NAC-ITCs. However, SF, an analog of BITC, was shown in human prostate cancer cells to activate Chk2, which causes the phosphorylation of Cdc25C at Ser216, leading to its translocation from the nucleus to the cytoplasm due to increased binding with 14-3-3 protein, and subsequent degradation of phosphorylated Cdc25C (inactive) [58].

In summary, the urinary metabolites (NAC-ITCs) of dietary ITCs show potent anti-proliferative activity against human bladder cancer cells. The biological activity of the NAC-ITCs, at least in the case of NAC-BITC, depends on liberation of cognate ITCs and is therefore similar to their parent compounds. These data provide further evidence that ITCs are highly promising chemopreventive/therapeutic agents against bladder cancer, perhaps both primary and recurrent bladder cancers.

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