Mechanism of Differential Potencies of Isothiocyanates as Inducers of Anticarcinogenic Phase 2 Enzymes

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ABSTRACT

Isothiocyanates occur in many edible plants and are consumed in substantial quantities by humans. A number of isothiocyanates block chemical carcinogenesis in a variety of animal models by inhibiting Phase 1 enzymes involved in carcinogen activation and by inducing Phase 2 enzymes that accelerate the inactivation of carcinogens. There are large but unexplained potency differences among individual isothiocyanates. When murine hepatoma (Hepa 1c1c7) and several other cell lines were exposed to low concentrations (1-5 μM) of certain isothiocyanates, the intracellular isothiocyanate/dithiocarbamate concentrations (measured by cyclocondensation with 1,2-benzenedithiol) rose rapidly (30 min at 37°C) to very high levels (e.g., 800-900 μM). The intracellular accumulation of isothiocyanates/dithiocarbamates was temperature, structure, and glutathione dependent and could not be saturated under experimentally achievable conditions. When murine hepatoma cells were exposed to nine isothiocyanates (5 μM for 24 h at 37°C) that differed considerably in structure and Phase 2 enzyme inducer potencies, the intracellular concentrations (area under curve) correlated closely and linearly with their abilities as inducers of the Phase 2 enzymes: NAD(P)H:quinone reductase and glutathione S-transferases. Isothiocyanates that did not accumulate to high levels were not inducers. These observations suggest strongly that induction of Phase 2 enzymes depends on intracellular levels of isothiocyanates/dithiocarbamates. Depletion of glutathione by treatment of Hepa cells with buthionine sulfoximine increased the inducer potencies of several isothiocyanates but could not be directly related to changes in intracellular isothiocyanate/dithiocarbamate concentrations, suggesting that glutathione may play several roles in the induction process.

INTRODUCTION

Isothiocyanates have engendered widespread interest in a number of laboratories during the last three decades because of their ability to inhibit tumor formation in several animal models (1, 2). A range of isothiocyanates with quite different structures are chemoprotectors against tumors evoked by a number of chemical carcinogens in a variety of animal organs, suggesting that there is a common mechanism underlying these effects. It is generally accepted that induction of cancer-protective Phase 2 enzymes (e.g., glutathione transferases, quinone reductase, and glucuronosyltransferases) and/or inhibition of cancerogen-activating Phase 1 enzymes play a major role in protection (3-7). Recently, several isothiocyanates have also been shown to induce apoptosis in several cell lines, including HeLa cells (8, 9). Because isothiocyanates are present in edible plants, particularly in the family Cruciferae, and are widely consumed by humans in considerable quantities (up to 100 mg daily; Refs. 10-12), they offer significant promise as chemoprotectors against cancer in humans. A better understanding of the absorption, distribution, metabolism, and mechanism of action of this class of compounds is essential to devise rational strategies for chemoprotection.

Exposure of cultured cells and animal tissues to isothiocyanates leads to coordinate induction of several Phase 2 enzymes, including glutathione transferases, quinone reductase, and glucuronosyltransferases (13-15). From our initial studies, it was clear that there were considerable differences in the potencies of various isothiocyanates in inducing Phase 2 enzymes. Because structure/activity analyses failed to explain the large differences in inducer potencies of various isothiocyanates (13, 14), we undertook a more detailed analysis of the metabolic disposition of these compounds. Our studies were initially hampered by lack of a simple and rapid method to measure isothiocyanates and their metabolites in cells and tissues. We therefore developed a method to measure unlabeled isothiocyanates, which has played a crucial role in analyzing the effects of different compounds of this class. In this report we provide evidence that: (a) certain isothiocyanates are accumulated to very high levels in several cell lines; (b) the degree of accumulation correlates with their ability to induce Phase 2 enzymes; and (c) cellular GSH levels influence their potencies as inducers of Phase 2 enzymes.

MATERIALS AND METHODS

Materials

Sulforaphane [1-isothiocyanato-4-(methylsulfinyl)butane], erysolin [1-isothiocyanato-4-(methylsulfonyl)butane], exo-2-acetyl-exo-6-norbornyln-CNS, and endo-2-acetyl-exo-6-norbornyln-CNS were gifts of Professor G. H. Posner (Department of Chemistry, The Johns Hopkins University). [14C]Phenethyl-NCS was a gift of Dr. F-L. Chung (American Health Foundation, Valhalla, NY). All other isothiocyanates were obtained commercially from Aldrich (Milwaukee, WI) or Trans World Chemicals (Rockville, MD, and many were distilled before use. 1,2-Benzenedithiol and buthionine (R,S)-sulfoximine were also from Aldrich, and the former was distilled before use. H2O and [14C]carboxylated dulcin were obtained from Amersham (Arlington Heights, IL). Dibuty phthalate, diisononyl phthalate, and ethyl glutathione were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

All cells were cultured in 5% CO2 (unless otherwise stated) at 37°C in a humidified incubator. Media and special growth conditions were as follows: for Hepa 1c1c7 cells, α-MEM plus 10% fetal bovine serum (Life Technologies, Inc.) treated previously with 1% charcoal at 55°C for 90 min and then filtered; for LNCaP cells, a human prostate cell line, RPMI 1640 medium containing 1 mm L-glutamine and 5% fetal bovine serum (17); for M1 cells, an endothelial cell line from mouse pancreas, DMEM plus 5% fetal bovine serum (18); for 3T3-L1 cells, a fibroblast cell line from mouse, DMEM plus 10% fetal bovine serum in a 10% CO2 atmosphere (19); and for L6 cells, a rat skeletal muscle myoblast line, DMEM with 4 mm L-glutamine, 1.5 g/liter of sodium bicarbonate, 4.5 g/liter of glucose, 1 mm sodium pyruvate, and 10% fetal bovine serum (20).

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Measurement of Intracellular Accumulation of Isothiocyanates

Cell Exposure to Isothiocyanates and Preparation of Cell Lysates. For these studies, 7 × 10^5 cells were plated in a 10-cm Petri dish with 10 ml of medium. After 3 days, the cell number reached 4–5 × 10^6 cells. This cell density was selected because intracellular accumulations of isothiocyanates did not significantly deplete the isothiocyanate concentration in the medium, yet the accumulated compounds could be accurately measured by the cyclocondensation assay on cells obtained from a single plate. The cell monolayers were then either directly exposed to specified concentrations of isothiocyanates in 10 ml of medium, or suspensions were prepared by treating the cells with 1 ml/plate of 0.05% trypsin and then mixing with 9 ml of medium before exposure. For monolayers, at the end of exposure to the isothiocyanates, the cells in each plate were harvested by scraping with a razor blade into ~0.5 ml of medium and rapidly transferred to a 1.5-ml Eppendorf tube containing 0.8 ml of a water-immiscible oil mixture (1 volume of diisononyl phthalate and 2 volumes of dibutyl phthalate; Ref. 21). For all cell suspension experiments carried out at 0–4°C, the entire suspension (10 ml) was transferred to a 15-ml tube containing 2 ml of the same oils but mixed at a 1:1 ratio. The density of the oil mixture was such that only cells moved through the oil layer upon centrifugation at 4°C for 15 s at 14,000 rpm for Eppendorf tubes or for 2 min at 3000 rpm for 15-ml tubes. After centrifugation, the medium and oil were aspirated, and the tube tip containing the cell pellet was cut off with a razor blade. The entire pellet was resuspended in 160 μl of H_2O and mixed with 20 μl of 10 mm β-mercaptoethanol and 20 μl of 1% aqueous N-lauryl sarcosine. The mixture was then incubated at 37°C for 1 h with occasional vortex mixing to ensure complete cell lysis.

Quantitation of Isothiocyanates and Dithiocarbamates in Cell Lysates by the Cyclocondensation Assay. As shown in Fig. 1, isothiocyanates and dithiocarbamates react quantitatively with 1,2-benzenedithiol by a cyclocondensation reaction to give rise to 1,3-benzodithiole-2-thione, which can be quantified spectroscopically after simple isocratic reverse-phase high-performance liquid chromatography (22, 23). Dithiocarbamates are the conjugation products of isothiocyanates with thiol compounds, and intracellular isothiocyanates may exist partly as dithiocarbamates (e.g., GSH conjugates). The assay was modified for very small quantities as follows. Five or 10 μl of the cell lysate were added to give a 200-μl final volume of reaction solution containing: 50% methanol (v/v), 25 mm potassium phosphate (pH 8.5), 20 mm 1,2-benzenedithiol dissolved in methanol, in a 250-μl glass insert housed in a 4-ml glass vial with compression spring. H-style vial cap, and self-sealing septum (Waters, Milford, MA). The vial was heated to 65°C for 2 h. After the incubation, the solution was centrifuged at low speed to sediment the cell debris, and the supernatant fraction was injected into a reverse phase high-performance liquid chromatography column for quantitation of 1,3-benzodithiole-2-thione (23). This assay can detect as little as several pmol of isothiocyanates or dithiocarbamates.

Quantitation of Cell Volume. The measured isothiocyanate content of each cell lysate was converted to intracellular concentration based on the intracellular fluid volume, which was determined by a calibration curve relating intracellular volume to the protein content of the cell lysate. The protein content of each cell lysate was measured by the bicinchoninic acid assay (24). For volume determination, 2–10 × 10^6 Hepa 1c1c7 cells were incubated in 5 ml of medium containing both ^3H_2O and [14C]inulin for 5 min at 37°C, which was sufficient to establish uptake equilibrium. The specific activities of the incubation medium were 6.7 × 10^4 cpm/ml for ^3H_2O and 3.1 × 10^4 cpm/ml for [14C]inulin (1.75 μg/ml). The cells were centrifuged through a layer of the oil mixture, the cell pellet was retrieved as described above, and lysed in 200 μl of 0.1% N-lauryl sarcosine, and the radioactivity of both ^3H and 14C and the protein content in the lysates were determined. Inulin crosses the cell membrane extremely slowly, and the amount of 14C measured in the lysates was assumed to arise from inulin trapped in the water of the extracellular space. Assuming that the extracellular contamination of inulin was the same as that of water, the intracellular volume was calculated as the total ^3H contained in the lysate minus the total 14C contained in the lysate. The contribution of [14C]inulin to the total radioactivity in the lysate was ~8%. A linear relation (r = 0.99) between cell volume and protein content of the lysate was thus established (Fig. 2).

Calculation of the AUC of Intracellular Isothiocyanate Concentrations. Intracellular isothiocyanate concentrations were measured at multiple time points, and the data were processed by an interactive computer program to determine the AUC. The computer program (LAGRAN method) was developed by Rocci and Jusko (25) and further modified by Ediss and Tam (26).

Induction of Phase 2 Enzymes

Phase 2 enzyme induction was measured in Hepa 1c1c7 cells that were grown either in 96-well microtiter plates or 10-cm plastic Petri dishes. The microtiter plate assay was performed as described (16, 27); only induction of QR was measured by this method. Briefly, 10,000 cells were cultured in each microtiter well with 200 μl of medium for 24 h and then exposed to a series of concentrations of the inducer in 150 μl of medium/well for 24 or 48 h. The inducers were all dissolved in acetone (final concentration of acetone, 0.1% by volume). When BSO was used to deplete cellular GSH, the cells were grown for 24 h in the microtiter plate, treated with 100 μM BSO in 150 μl of...
medium/well for 24 h, and finally exposed to the test inducer in fresh medium (150 µL/well) for another 24 h in the presence of 100 µM BSO.

The 10-cm Petri dish assay was performed as follows. Cells (5 × 10^5) were grown in each plate with 10 ml of medium for 48 h and then exposed to the test inducer in 10 ml of fresh medium/plate for another 24 h. After exposure, the cells were washed with 10 ml of Dulbecco’s buffer, harvested by scraping, and centrifuged at 2000 rpm for 5 min at 4°C. The buffer was removed, the cell pellet was resuspended in 0.25 ml of fresh buffer and homogenized in a glass homogenizer (Kontes, Vineland, NJ), and the homogenates were centrifuged at 10,000 rpm for 30 min at 4°C. The resultant supernatant fraction was assayed for both QR and GST activities, and the amount of protein was determined (28). QR and GST activities were measured as described (13, 14).

Determination of Cellular GSH

Cellular GSH was measured by a fluorometric assay (29). Briefly, cell lysates were diluted with 0.1 M potassium phosphate buffer, pH 8.0, and proteins were precipitated with trichloroacetic acid. A 100-µL sample was then mixed with 100 µL of a-phenylisothiocyanate (10 mg in 10 ml of methanol) and 1.8 ml of 0.1 M potassium phosphate buffer (pH 8.0) containing 5 mm EDTA. The solution was incubated at room temperature for 10 min, and formation of GSH-phenylisothiocyanate conjugate was then measured by a luminescence spectrophotometer (Perkin-Elmer Model LS50). The conjugate was excited at a wavelength of 350 nm, and the fluorescence emission was measured at 420 nm.

Statistics

To measure intracellular concentrations of isothiocyanates or GSH, two separate experiments, each of which was assayed in duplicate, were carried out. To measure cellular enzyme activities (QR and GST), three separate experiments, each of which was assayed in duplicate or quadruplicate, were carried out. Each value represents the mean of these analyses. Replicate analyses routinely had SEs of <10% of the mean.

RESULTS

Validation of the Cyclocondensation Assay for Measurement of Intracellular Isothiocyanate and Dithiocarbamate Concentrations. The cyclocondensation assay is the only available method for the accurate and highly sensitive quantitation of isothiocyanates that does not require their radiolabeling. The procedure for measuring cellular accumulation of isothiocyanates is described under “Materials and Methods.” Because we did not know whether isothiocyanates might be metabolized in cells to products that were not detected by the cyclocondensation assay, we measured the cellular uptake of two isothiocyanates that could be quantitated by other means: radioactive phenethyl-NCS (C_6H_3 CH_2-14C CH_2-NSC) and fluorescein-NCS. In suspensions of Hepa 1c1c7 cells exposed to [14C]phenethyl-NCS in concentrations up to 100 µM in the medium, radioactivity accumulated up to the equivalent of more than 3 nM in just 30 min at 37°C. As shown in Fig. 3, 80–90% of phenethyl-NCS accumulated in cells (measured by radioactivity) was also measured by the cyclocondensation assay, and this percentage remained constant as the intracellular concentration increased from 200 µM to more than 3 nM. Hence, the majority of cellular phenethyl-NCS is measurable by the cyclocondensation assay. Fluorescein-NCS accumulated to much lesser extent in Hepa 1c1c7 cells, but nearly all of the isothiocyanates in the cells (measured by fluorescence) were also detected by the cyclocondensation assay. Moreover, when several other isothiocyanates were incubated with Hepa 1c1c7 cell lysates for 1 h at 37°C, nearly all of the isothiocyanate added was detected by the cyclocondensation assay (data not shown). Therefore, these experiments established that the cyclocondensation assay provides a valid measurement of intracellular isothiocyanates or their dithiocarbamate metabolites. Isothiocyanates are metabolized to dithiocarbamates, i.e., GSH derivatives. The cyclocondensation assay cannot distinguish between these two classes of compounds but measures both species quantitatively (23). However, for reasons of simplicity, we refer to the total amounts of isothiocyanates/dithiocarbamates in cells measured by the cyclocondensation assay as intracellular concentrations of “isothiocyanates,” as indicated in Figs. 3–10.

Isothiocyanates Accumulate to Very Different Concentrations in Hepa 1c1c7 Cells. The accumulation of phenethyl-NCS in Hepa 1c1c7 cells in the above experiment (Fig. 3) was surprisingly high. Therefore, we used the cyclocondensation assay to examine the accumulation of other isothiocyanates. Hepa 1c1c7 cells in monolayers were exposed to 5 µM concentrations of sulforaphane, benzyl-NCS, phenethyl-NCS, phenyl-NCS, or α-naphthyl-NCS. As shown in Fig. 4, sulforaphane and benzyl-NCS accumulated very rapidly to very high concentrations in Hepa 1c1c7 cells, reaching levels of 280 and 370 µM, respectively, in just 30 min, whereas the highest intracellular levels of phenethyl-NCS attained were wider. Although the cellular concentration of benzyl-NCS began to decrease rapidly after 30 min of incubation, that of sulforaphane continued to accumulate for 6 h, reaching 350 µM, and the subsequent decrease was relatively slow. In contrast, very little phenyl-NCS or α-naphthyl-NCS accumulated, with maximal intracellular concentrations rising <5-fold, and none were detected after 3 h of incubation. Thus, it became clear that, whereas some isothiocyanates accumulated rapidly to very high concentrations in Hepa 1c1c7 cells, the degree of the maximal accumulation apparently depended on the specific isothiocyanate. Our
preliminary experiments suggested that isothiocyanate accumulation and removal are likely to be a continuous process, and cellular concentration of an isothiocyanate at a given time during the exposure period may depend on its degradation rate in the medium (data not shown).

**Isothiocyanates Accumulate in Various Types of Cells.** We next examined whether the rapid and concentrative uptake of isothiocyanates was unique to Hepa 1c1c7 cells. We focused on four cell types: mouse pancreatic endothelial cells (MS1); mouse fibroblast cells (3T3-L1); rat skeletal muscle myoblast cells (L6); and human prostate cancer cells (LNCaP). Both sulforaphane and benzyl-NCS rapidly accumulated in all four cell lines, although the degree of accumulation differed. As shown in Fig. 5, when these cells were exposed to either 5 μM sulforaphane or 5 μM benzyl-NCS in the medium at 37°C for 30 min, the intracellular concentrations attained were 40–180 times higher than the initial extracellular isothiocyanate concentration. Hence, these experiments established that cellular accumulation of isothiocyanates is likely to be a common property of many cell types from a variety of species. Interestingly, in all cell lines tested, the maximal accumulation of benzyl-NCS was always higher than that of sulforaphane.

**The Phase 2 Enzyme Inducer Potencies of Isothiocyanates Correlate with Their Levels of Accumulation in Cells.** The above experiments not only showed that various isothiocyanates accumulated to different levels in Hepa 1c1c7 cells but also suggested that the accumulations are correlated with the inducer potencies of these compounds. Thus, the rank order of their potencies in inducing QR (induced for 48 h in the microtiter assay), i.e., sulforaphane (CD, 0.2 μM) > benzyl-NCS (CD, 1.5 μM) > phenethyl-NCS (CD, 4.5 μM), whereas phenyl-NCS and α-naphthyl-NCS were not inducers, matched the rank order of their cellular accumulations. Furthermore, other isothiocyanates, including fluorescein-NCS and p-tolyl-NCS, which did not accumulate to high levels in Hepa 1c1c7 cells, also did not induce QR. To clarify to what degree the inducer potencies of isothiocyanates depended on their cellular accumulations, we examined accumulation of nine isothiocyanates with very different structures and inducer potencies (Fig. 6A). Hepa 1c1c7 cells in monolayers were exposed to each compound at 5 μM in the medium for 24 h. The cells were then harvested, and the activities of QR and GST were assayed. Another set of cells were exposed to the same isothiocyanates at the same concentration but for different time periods. The cells were harvested at 1, 3, 6, 12, or 24 h, at which times the intracellular concentration of each isothiocyanate was determined by the cyclocondensation assay, and the area under the concentration with respect to time curve (AUC) of the intracellular isothiocyanates was computed. As shown in Fig. 6B, the maximal accumulation and the time course of accumulation varied widely among these isothiocyanates. Moreover, both QR and GST were induced by these isothiocyanates but also to very different levels. For example, whereas 5 μM sulforaphane raised QR activity ~10-fold, no induction was observed upon exposure to 5 μM phenyl-NCS. Although the level of GST induction by isothiocyanates was uniformly much lower than that of QR, there was a very close correlation between the induction of the two enzymes, i.e., an isothiocyanate that was a more potent inducer of QR was also a more potent inducer of GST. When the specific activities of both QR and GST of cells treated with isothiocyanates were divided by the values of control cells and compared with the AUC of the intracellular concentrations of these isothiocyanates, a remarkable correlation was found (Fig. 6C); cells that accumulated higher total concentrations of isothiocyanates also induced
higher levels of QR and GST, regardless of the structural differences of these compounds. A similar correlation was also observed when cells were exposed to these isothiocyanates for only 12 h, but the induced levels of both enzymes were lower than those after 24 h of treatment (data not shown). Therefore, we conclude that inducer potencies of isothiocyanates in Hepa 1c1c7 cells depend largely, if not entirely, on their intracellular accumulation.

**Initial Velocity of Uptake of Isothiocyanates into Hepa 1c1c7.** The rates of accumulation of the active isothiocyanates were extremely rapid at 37°C, effectively precluding determination of the initial uptake kinetics. When suspensions of Hepa 1c1c7 cells were exposed to 5 μM sulforaphane, the initial rate of accumulation of sulforaphane was reduced by at least 4-fold when the temperature was lowered from 26°C to 4°C (Fig. 7). Similar temperature effects were also observed for several other isothiocyanates tested (data not shown). At 4°C, the initial uptake of several isothiocyanates in Hepa 1c1c7 cells was linear with time for at least the first 12 min and was directly proportional to extracellular concentration. For sulforaphane, the uptake rate was concentration-dependent up to more than 150 μM but could not be accurately determined at higher concentrations (Fig. 8). The initial rate of sulforaphane accumulation in these cells (2.7 μM/min for each 1 μM extracellular concentration of sulforaphane) was not saturable under experimentally attainable conditions. Moreover, the initial rates of uptake of allyl- and benzyl-NCS (at 1 μM in the medium) over the first 12 min at 4°C were also linear and were considerably faster than those of sulforaphane. Allyl-NCS accumulated 9 times (22 μM/min) and benzyl-NCS 18 times (46 μM/min), respectively, more rapidly than sulforaphane (results not shown), emphasizing that uptake rates are also dependent on structure. Notably, the initial uptake rate of sulforaphane was the slowest of the three compounds, yet the ultimate accumulation of sulforaphane was the highest (Fig. 4).

**Isothiocyanate Accumulation in Cells Is Influenced by Cellular GSH Levels.** How the cell achieves such rapid and high cellular accumulation of isothiocyanates is not yet clear, but the accumulation is apparently influenced by cellular GSH concentrations. There are two reasons for examining the relation between intracellular GSH levels and the uptake of isothiocyanates into cells: (a) isothiocyanates react rapidly with GSH to form dithiocarbamates both nonenzymatically and enzymatically (30–32); and (b) GSH levels have been implicated in the regulation of Phase 2 enzymes (33–36). Hepa 1c1c7 cells normally contain 5 mM GSH, but this level is reduced to 0.8 mM when the cells are treated for 24 h with 100 μM BSO, which is widely used to deplete GSH because it inhibits γ-glutamylcysteine synthetase (37). When cells, with or without BSO treatment, were exposed in suspension for 30 min to either sulforaphane or benzyl-NCS at 0.9, 9.1, or 91 μM in the medium at 37°C, the intracellular accumulation of both compounds was drastically reduced in the BSO-treated cells (Fig. 9). Without BSO treatment, cellular sulforaphane concentrations reached 0.2, 1.2, or 6 mM, respectively, and with treatment the cellular concentrations reached only 0.07, 0.3, or 1.5 mM, respectively, a 3–4-fold decrease. Cellular accumulation of benzyl-NCS was similarly affected. Thus, depletion of cellular GSH reduced the uptake of isothiocyanates. Surprisingly, over longer periods of exposure (1–24 h) to these two isothiocyanates, although the cellular accumulation of sulforaphane was continuously depressed in BSO-treated cells, the cellular accumulation of benzyl-NCS was unexpectedly higher in BSO-treated cells than that in untreated cells (Fig. 10). Two additional isothiocyanates, allyl-NCS and phenethyl-NCS were also examined. Although their accumulations in Hepa 1c1c7 cells were similarly affected by GSH levels in short-term exposure (<1 h), their accumulations were not significantly affected by cellular GSH level when the cells were exposed for longer periods of time (1–24 h; Fig. 10). Thus, the effect of GSH on cellular accumulation is isothiocyanate specific. Although most isothiocyanates are known to react readily with GSH, to what degree dithiocarbamate formation is directly responsible for accumulation of these compounds is not known.

![Graph showing initial velocity of uptake of isothiocyanates into Hepa 1c1c7 cells](image-url)
Cellular GSH Levels Modulate Induction of Phase 2 Enzymes by Isothiocyanates. The observation that cellular GSH level affected accumulation of several isothiocyanates in Hepa 1c1c7 cells led us to examine whether the potency of these compounds as inducers of Phase 2 enzymes was similarly affected. Hepa 1c1c7 cells grown in microtiter plate wells were treated with 100 \( \mu \text{M} \) BSO for 24 h and then exposed in fresh medium to an isothiocyanate for an additional 24 h in the presence of 100 \( \mu \text{M} \) BSO. Parallel controls were not treated with BSO. Four isothiocyanates, sulforaphane, benzyl-NCS, allyl-NCS, and phenethyl-NCS, were examined because their cellular concentrations had been measured in both BSO-treated and control cells. Surprisingly, in BSO-treated cells, all four isothiocyanates showed higher potencies in inducing QR and were from 1.3-fold (sulforaphane) to as much as 3.8-fold (allyl-NCS) more potent than they were in control cells (Table 1). As discussed above, depletion of GSH by BSO in Hepa 1c1c7 cells results in uniformly lower accumulation of all four isothiocyanates after short-term exposure (30 min). Over longer periods of exposure (1–24 h), sulforaphane accumulation in BSO-treated cells was continuously depressed, whereas allyl-NCS and phenethyl-NCS accumulations were similar to those in untreated cells. Benzyl-NCS accumulation was \(~\!) 40\% higher than that in untreated cells. Clearly, the enhanced inducer potency of these four isothiocyanates in GSH-depleted cells cannot be attributed to changes in their cellular accumulations. Changes in GSH levels alone did not appear to affect the enzyme levels, because neither treatment of cells with 100 \( \mu \text{M} \) BSO for 48 h nor treatment with 5 \( \mu \text{M} \) ethyl-GSH for 24 h resulted in any significant elevation of QR activity. Ethyl-GSH is transported intact into cells and raises GSH levels (38). Thus, our experiments clearly suggest that the normally high cellular GSH levels appear to lower the inducer potencies of these isothiocyanates. Although the exact reasons are unknown, it is possible that free isothiocyanates are the important signals for enzyme induction, and the concentration of free isothiocyanates in cells depends inversely on cellular GSH concentrations because isothiocyanates are readily and reversibly converted to dithiocarbamates.

DISCUSSION

Isothiocyanates have been shown to block chemical carcinogenesis against a diverse group of carcinogens in many target tissues of several animal species (1, 2). They also inhibit Phase 1 enzymes (cytochromes P-450) involved in carcinogen activation and induce Phase 2 enzymes that accelerate cellular disposal of activated carcinogens in a variety of cells and animal tissues (1, 2, 15). Blocking carcinogen activation and/or detoxifying activated carcinogens are recognized to be effective strategies in reducing cancer risk in animals (3–7). However, the relative importance of inhibition of Phase 1 enzymes and induction of Phase 2 enzymes by isothiocyanates has been difficult to assess. Efforts to relate the structures of isothiocyanates to their potencies have been only partially successful (13, 14, 39–42). Nevertheless, the anticarcinogenic and the enzyme-regulating activities of isothiocyanates are clearly concentration dependent. For these reasons, the previously unrecognized rapid and concentrative accumulation of isothiocyanate enzyme inducers in cell lines is of special interest. Studies in Hepa 1c1c7 cells clearly indicated that the initial rates of uptake of isothiocyanates are structure-related and are influenced by cellular GSH levels. Because isothiocyanates can undergo conjugation with GSH, nonenzymatically and enzymatically, it is possible that GSH conjugation with isothiocyanates is involved in their concentrative cellular accumulation, and this may be further enhanced by GSTs. In fact, the initial uptake rates of benzyl-NCS, allyl-NCS, and sulforaphane in Hepa 1c1c7 cells appear to be correlated with the second order rate constants for their reaction with GSH (30). However, these observations are in conflict with the fact that other isothiocyanates, such as phenyl-NCS and \( \alpha \)-naphthyl-NCS, which are rather reactive with GSH, were not accumulated. Moreover, the effect of GSH levels on cellular accumulation of isothiocyanates seems unpredictable when cells were exposed to these compounds for longer periods of time (>1 h). For example, although the accumulation of sulforaphane was continuously depressed during the entire exposure period, benzyl-NCS accumulated ultimately to a higher level than that in GSH-undepleted cells. These results raised the possibility that GSH may be involved in both the uptake and export of isothio-
cyanates, and depletion of cellular GSH may decrease the export of cellular isothiocyanates or their metabolites. In this connection, it is well known that elimination of many drugs in cells by drug transporters (e.g., multiple resistance pumps) requires GSH (43, 44).

To understand how cellular concentrations of isothiocyanates might affect their biological potencies, we focused on the induction of two Phase 2 enzymes. Undoubtedly, the rapid and concentricative accumulation of isothiocyanates will also increase the inhibition of Phase 1 enzymes. The finding that inducer potencies of isothiocyanates in Hepa 1c1c7 cells depend closely on the degree to which they are accumulated in cells is significant in that it may provide an understanding of the structure-activity relationship and point to ways to design more effective chemoprotective agents. It is very likely that strategies that increase the levels and prolong accumulation of isothiocyanates in cells will also increase their Phase 2 enzyme inducer potencies. Moreover, because these isothiocyanates differ widely in their chemical structures, our results also suggest that the specific structures of isothiocyanates may have little importance in enzyme induction once these compounds have entered cells.

Our experiments also clearly show that decreasing cellular GSH levels in Hepa 1c1c7 cells increases the inducer potencies of isothiocyanates. This finding is consistent with several previous observations. For example, depletion of cellular GSH also increased the potency of compounds such as mercury chloride in inducing QR in Hepa 1c1c7 cells (36). In a transient gene expression assay in HepG2 cells, lowering cellular GSH levels with BSO stimulated the response of the electrophile responsive element to tert-butylhydroquinone and other compounds (33). Moreover, isothiocyanates that reacted more rapidly with GSH were found to be less potent as inhibitors of lung tumor formation by 4-(methyl nitrosamine)-1-(3-pyridyl)-1-butanone (41). This finding may help us to control isothiocyanate inducer potency and to understand the mechanism of enzyme induction by isothiocyanates and possibly other compounds. However, further study is needed to clarify the inhibitory mechanism of GSH.

In summary, by following cellular accumulation of isothiocyanates with this cyclocondensation assay, we have discovered that many isothiocyanates are rapidly accumulated to high concentrations in a number of cultured mammalian cells and that the magnitude of such accumulation is related to the specific isothiocyanates, incubation temperature, and cellular GSH content. We also found that the Phase 2 enzyme inducer potencies of isothiocyanates correlate with their levels of accumulation in Hepa 1c1c7 cells, thus suggesting a plausible mechanism for the differential potencies of these compounds in inducing Phase 2 enzymes. Moreover, our experiments also indicated that cellular GSH may negatively modulate the potencies of isothiocyanates as inducers of Phase 2 enzymes.

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POTENCIES OF ISOThIOCYANATES AS ENZYME INDUCERS


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