Phenylhexyl isothiocyanate inhibits histone deacetylases and remodels chromatins to induce growth arrest in human leukemia cells

XUDONG MA[1,2], YUQIANG FANG[1], ANASTASIA BEKLEMISHEVA[1], WEI DAI[1], JINGYANG FENG[1], TAUSEEF AHMED[1], DELONG LIU[1] and J.W. CHIAO[1]

[1]Department of Medicine, New York Medical College, Valhalla, NY 10595, USA;
[2]Department of Hematology, Zhangzhou Municipal Hospital, Zhangzhou, Fujian, P.R. China

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Abstract. Natural isothiocyanates, present in cruciferous vegetables and synthetic phenylhexyl isothiocyanate (PHI) are chemopreventive agents which act by blocking the initiation of carcinogen-induced tumors in rodents. We have demonstrated that isothiocyanates are also growth regulators, inhibiting cell cycle cdk activity and up-regulating inhibitor $p21^{WAF1}$ (p21) in cancer cells. The up-stream mechanism to modulate cell cycle progression remained to be elucidated. Here, we have demonstrated that exposure of HL-60 leukemia cells to PHI induced G1 arrest and apoptosis. The hypothesis that PHI inhibits cell growth via chromatin remodeling was investigated. PHI mediates the complex cross talk between chromatin and DNA, and it was demonstrated for the first time as an inhibitor of histone deacetylases (HDAC). Thus, the HDAC activity in PHI-exposed HL-60 cells was reduced. Additionally, PHI reduced the expression of HDAC and increased the level of acetyl transferase p300, in favor of accumulation of acetylated histones. Within hours, global acetylation of histones was enhanced. PHI further mediated selective alterations of histone methylation, with a pattern consistent to the marks of transcription competent chromatins. The relationship between acetylated histones and p21 was examined by chromatin immunoprecipitation (ChIP) assay. Chromatins from cells exposed to PHI contained more p21 DNA in the precipitates of hyperacetylated histones, indicating more accessibility of transcription machinery to the p21 promoter after chromatin unfolding. The cell cycle inhibitors were activated as a result. In contrast to the PHI-induced apoptosis in HL-60 cells, which was mediated by caspase-9 up-regulation and bcl-2 reduction, PHI did not induce significant apoptosis in the mononuclear cells from normal peripheral blood and bone marrow. The results revealed a potential selective effect of isothiocyanates to inhibit the growth of malignant cells.

Correspondence to: Drs Delong Liu and J.W. Chiao, Department of Medicine, New York Medical College, Valhalla, NY 10595, USA
E-mail: ldl1997@yahoo.com
jen-wei_chiao@nymc.edu

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Introduction

The local chromatin architecture is an important factor in the regulation of gene expression. The nucleosome, the fundamental subunit of chromatin, is composed of four core histones, i.e. a H3/H4 tetramer and two H2A/H2B dimmers, surrounded by 146 bp of DNA. The highly charged histones may bind tightly to the phosphate backbone of the DNA, preventing it from being decoded and expressing genes. Modification of the amino acid residues in the histone tails is one mechanism whereby chromatin condensation is controlled. Chemical modifications, such as acetylation of lysine side chains, neutralize the charge interaction with the DNA, thus allowing chromatin unfolding. The different modifications of amino acids in histones, and their combinations, have been suggested to be the 'histone code' that predetermines the functionally distinct effects on the nuclear processes (1,2). The level of histone acetylation is controlled by a pair of opposing enzymes, acetyltransferases and deacetylases (HDAC). Some HDAC inhibitors have been considered as targeted new anti-leukemia agents to induce growth arrest and promote apoptosis in tumor cells (3).

We have investigated isothiocyanates as growth regulators in cancer cells. Natural isothiocyanates occur as thioglucoside conjugates, i.e. glucosinolate, in a wide variety of cruciferous vegetables including broccoli, cabbages, watercress, and Brussel's sprouts. The isothiocyanates are released when the vegetables are cut or masticated (4,5). Natural and synthetic isothiocyanates are potent cancer chemopreventive agents in a number of carcinogen-induced cancer models in rodents (5,6). The primary mechanism is the blocking of initiation of carcinogenesis via inhibiting cytochrome P450s, and inducing detoxifying enzymes to remove carcinogens (7).

Our lab has reported that phenethyl isothiocyanate and sulforaphane induce growth arrest and apoptosis in prostate cancer cells in culture, as well as in xenografted tumors in immunodeficient mice, suggesting that they act also at the

post-initiation progression stages (8,9). Isothiocyanates have been further reported to induce JNK-MAP kinases, p53-mediated apoptosis and cdk-mediated cell cycle arrest in different types of cancer cells (10-13). Natural isothiocyanates, including benzyl, phenethyl, and allyl isothiocyananates, have been reported to induce growth arrest and apopotosis in leukemia cells (14-20). We have since examined the growth regulatory effects of a synthetic isothiocyanate, phenylhexyl isothiolcyanate (PHI), on leukemia cells. PHI was selected because it is considered one of the most potent isothiocyanates for preventing carcinogen-induced lung tumors in mice (21,22) and it has not been investigated regarding growth regulation in leukemia cells. In this report, we demonstrated for the first time that PHI is an inhibitor of HDAC, which induces histone modifications in favor of chromatin unfolding for transcriptional activation of cell cycle inhibitors to mediate cell cycle arrest in leukemia cells.

Materials and methods

Chemicals and cells. Phenylhexyl isothiocyanate (PHI), >98% pure, was purchased from LKT Lab (St. Paul, MN). The HL-60 promyelocytes were seeded at $0.3x10^6$ per ml of RPMI-1640 medium supplemented with 15% heat-inactivated fetal calf serum, penicillin and strepto-mycin, and maintained at 37˚C in a humidified atmosphere containing 5% CO_2. The cells in exponential growth were exposed to various concentrations of PHI using a stock PHI solution of 75% methanol. The PHI preparation was evaluated as stable for more than 2 weeks. The control cultures were supplemented with the methanol medium as a vehicle control. Cell viability was determined from at least triplicate cultures by the trypan blue exclusion method. Cell density was calculated by viable cell counts per ml. Normal mononuclear cells were isolated from peripheral blood and bone marrow specimens by Ficoll-Hypaque density gradient centrifugation. These specimens were discarded samples without direct or indirect identifiers, after being determined to be normal in mononuclear cell numbers and cell morphology. The mononuclear cells were cultured with or without the addition of PHI using the same conditions as the HL-60 cell assay.

Apoptosis and cell cycle phases. Apoptotic cells were determined by their characteristic morphology and by the presence of DNA strand breaks with terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick-end labeling (TUNEL). The TUNEL detection of apoptosis *in situ* was performed with cytospin preparations using a detection kit (Roche Diagnostics, Indianapolis, IN) as described previously (23). The percentage of apoptotic cells was calculated by counting at least 500 cells from multiple fields. Analysis of cell cycle phases was performed using a Becton-Dickinson FACScan flow cytometer (23). The cells were stained with propidium iodide solution (50 μg/ml) on ice.

Protein expression. The protein levels of HL-60 cells were determined by Western blot analyses using standard procedures as described previously (23). Total proteins were prepared from each culture condition with a lysis buffer containing freshly prepared protease inhibitors. The histones were isolated from the fraction of nuclear pellets of cell lysates using an acid extraction procedure (24).

Antibodies against poly(ADP-ribose)polymerase (PARP), bcl-2, caspases-8 or -9, HDAC1, and p27 were purchased from Santa Cruz (Santa Cruz, CA). Antibody against p21 was purchased from Dako (Carpinteria, CA). Antibodies against trimethylated histione H3 lysine 9, mono/di/trimethylated histone H3 lysine 4, acetylated histones H3 (lysines 9 and 14) or H4 (lysines 5, 8, 12, and 16), acetylated histone H3 lysine 14, and acetyl transferase p300/CBP (p300) were purchased from Upstate Biotechnology (Lake Placid, NY). An anti-ß-actin was used as a loading control. The reactive proteins were visualized using the ECL system.

Chromatin immunoprecipitation (ChIP) analyses. The HL-60 cells ($1x10^6$) were cultured for 7 h with PHI at 20 or 40 μM, or with vehicle control medium. ChIP analyses were performed according to the protocol from a ChiP assay kit (Upstate Biotechnology, Lake Placid, NY). Briefly, after the DNA and histones were crosslinked by formaldehyde, cell lysates were prepared for sonication. The lysates were incubated in a ChIP buffer with a rabbit antibody against acetylated histone H3, or a non-specific control Ig. The DNA molecules in the immunoprecipitates were recovered by phenol/chloroform extraction followed by ethanol precipitation. The p21 gene fragments TATA area (-33 to +47; 5' primer cgg ttg tat atc agg gcc gc; 3' primer cag ttc ctt gtg gag ccg ga), down TATA area (+43 to +122; 5' primer gga gct ggg cgc gga ttc gc; 3' primer gca gac aac agg gga ccc cg), C (+269 to +362; 5' primer cac cgg tgt ctc tat aag tg; 3' primer tac aag tac agg aat ccc tg), and D (+3267 to +3366; 5' primer ggg ctc aaa tga ttc tcc ca; 3' primer gtg att gtg atg ggc ctc tc) were selected for amplification by PCR as described (3).

Assay of HDAC activity. A nuclear extract of HeLa cells containing HDAC, from a HDAC1 and 2 activity assay kit (Color de Lys, Biomol, Plymouth, PA) was incubated with PHI at various concentrations for 10 min at 37˚C. The mixture was added with a substrate of acetylated peptides, incubated for 30 min, followed by the addition of a color developer according to the manufacturer's instructions. Incubation of HDAC with trichostatin A (TSA) was used as a positive control of inhibition. A separate test was performed by adding PHI to a solution of standard HDAC activity to evaluate any interference of PHI with the developer. For determining the HDAC activity from HL-60 cells, the cell lysates (15 μg) that had been exposed to PHI or not were added with the substrate to determine the HDAC activity as described. The absorbance of triplicate analyses was assayed at 405 nm with a Bio-Tec microtiter-plate reader. The difference in the absorbance between PHI supplementation and without PHI was calculated as % changes. The changes were evaluated by Student's t-test for statistical difference and $P<0.05$ was considered to be significant.

Results

Growth inhibition and apoptosis. The effects of PHI on the growth of human leukemia cells were examined using the HL-60 cell line. Exposure of HL-60 cells to PHI induced a

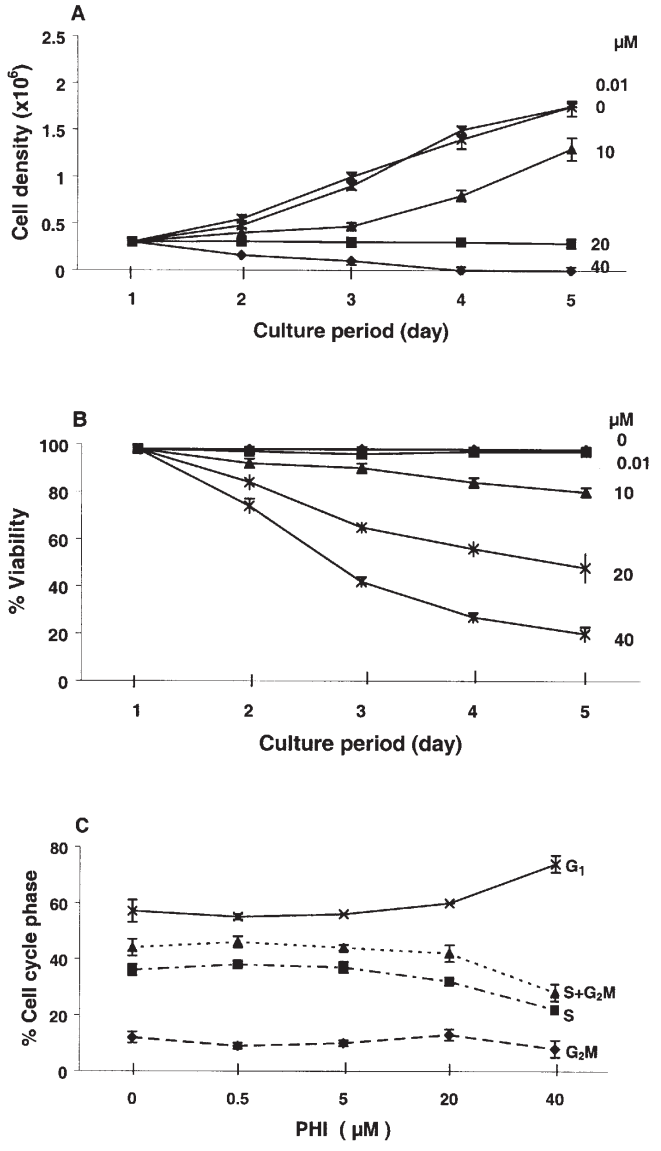

Figure 1. Growth modulation by PHI. A, concentration-related decrease of cell density after exposure of HL-60 cells to PHI during a 5-day period. Vertical bars are means ± SD of 3 independent experiments. B, the reduction of cell viability using trypan blue exclusion. C, a concentration-related decrease of S-phase cells concomitant with an increase of G1 cells as determined by flow cytometry. Values are means ± SD of 3 independent experiments, 3 h after PHI exposure.

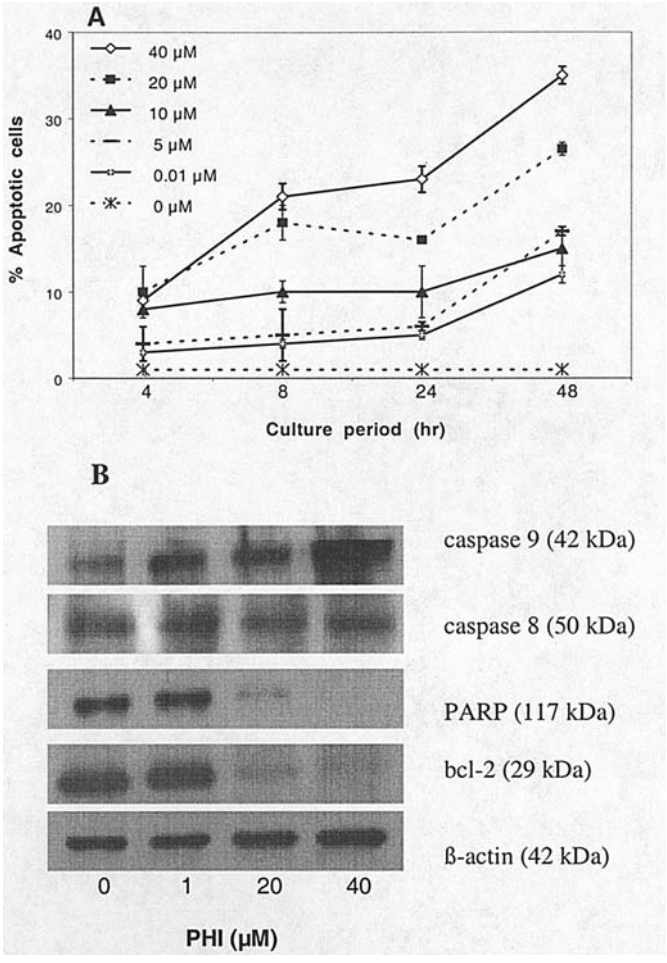

Figure 2. Induction of apoptosis by PHI. HL-60 cells were exposed to PHI at indicated concentrations. A, concentration- and time-related induction of apoptotic cells, determined by the DNA strand breaks using TUNEL assay with three independent assays. Vertical bars represent the means ± SD. Western blots in B show the altered expression of caspase-9, PARP, and bcl-2 during apoptosis, exposure to PHI for 14 h.

concentration-dependent decrease of cell density and viability. Approximately 55% and 70% reduction in cell density was seen with PHI at 10 and 20 μM after 3-day incubation (Fig. 1A). The cell viability was reduced to approximately 70% with 20 μM PHI. The cultures became static after exposure to higher concentrations of PHI for three days. The cell growth was evaluated with cell cycle phase distribution, determined by the DNA frequency histogram of cells using the flow cytometric method. A significant decrease of S-phase was evident after exposure to PHI for 7 h, at concentrations greater than 5 μM (Fig. 1C). A concomitant accumulation of cells in G1-phase was observed, indicating that the transition from G1- to S-phase was blocked.

Morphologically, some of the PHI-exposed cells displayed the condensed chromatin, plasma membrane blebbing and cell shrinkage characteristic of apoptosis. To determine the presence of apoptosis, cells with DNA strand breaks were measured by the TUNEL method. The presence of apoptotic cells could be detected within 4 h of PHI exposure. By 48 h, approximately 15% of apoptotic cells were seen with 10 μM PHI, and 35% with 40 μM (Fig. 2A). An increased quantity of apoptotic cells were detected after a longer culture period with PHI at lower dosages, indicating a time-dependent effect.

The cleavage of enzyme PARP is a hallmark of apoptosis. A concentration-dependent proteolytic cleavage of PARP was detected in PHI exposed cells (Fig. 2B). At 20 μM PHI, a >5-fold decrease was detected, and PARP became undetectable at higher concentrations. Caspases are known to play a central role in mediating apoptosis responses. Western blotting demonstrated a concentration-related increase of caspase-9, approximately 3-fold at 1 μM of PHI. In contrast, alteration of caspase-8 was not significant at those concentrations. In addition, the bcl-2 protein was significantly reduced at ≥ 20 μM PHI (Fig. 2B).

PHI inhibits HDAC and induces histone modifications. The inhibitors of cell cycle progression, such as the p21 gene, have

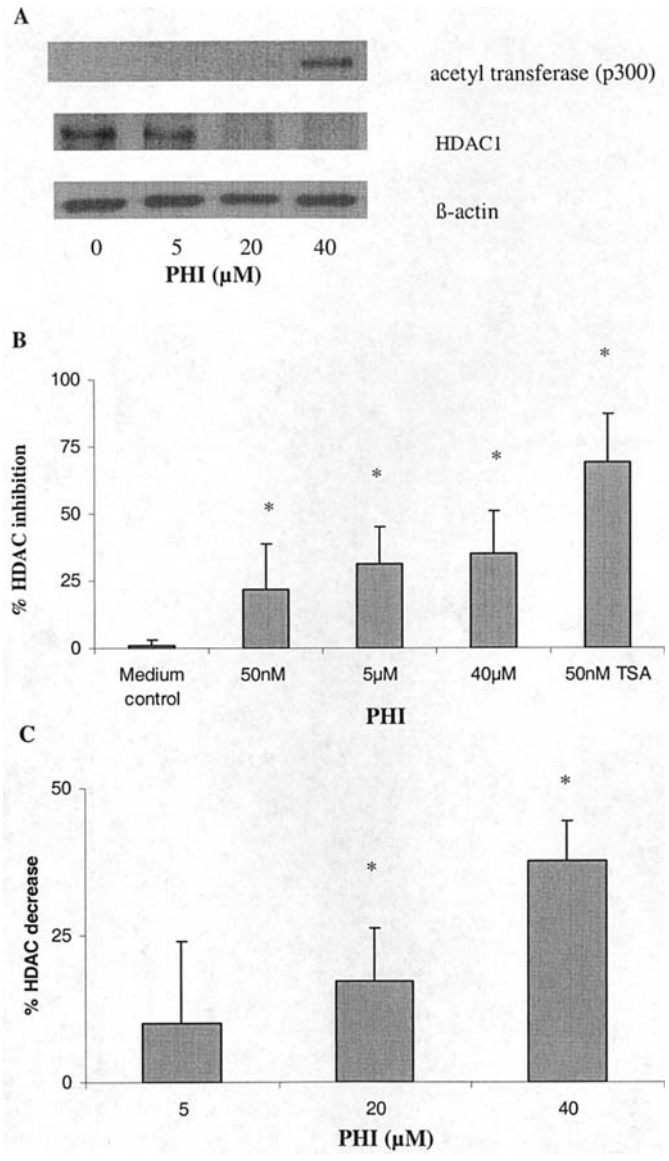

Figure 3. Altered levels of enzymes regulating histone acetylation and blocking of histone deacetylase (HDAC) activity by PHI. A, after HL-60 cells were exposed to PHI for 7 h, Western blots revealed an increased expression of acetyl transferase p300, and decreased HDAC1. B, concentration-related inhibition of the activity of HDAC1 and 2 from nuclear extract of HeLa cells by the addition of PHI, described in Materials and methods. TSA was included as a control of positive inhibitor. Data depicted are means ± SD of 3 independent experiments. C, % decrease of the activity of HDAC1 and 2 in HL-60 cell lysates, as compared with cells without PHI. The HDAC activity was determined using a deacetylase activity assay kit. Vertical bar values are means ± SD of 3 independent experiments. *Statistically significant changes ($P<0.05$).

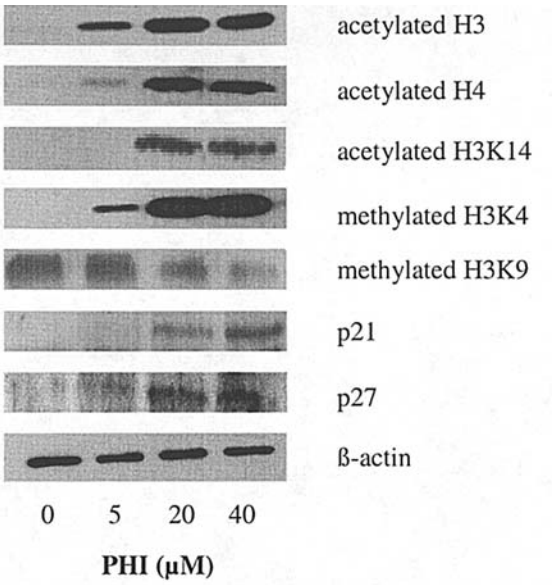

Figure 4. Histone acetylation and methylation and induction of cell cycle regulators by PHI. HL-60 cells were exposed to PHI for 7 h and the status of histone acetylation and methlation was analyzed. Western blotting revealed a significant concentration-related enhancement of acetylated histones H3 and H4, and methylated histone H3 lysine 4. The level of methylated histone H3 lysine 9 was decreased. The expression of cell cycle regulators p21 and p27 was increased during the time period.

been described to be activated by the inhibitors of HDAC due to histone acetylation and chromatin unfolding (25). To investigate the relation of PHI-mediated growth regulation to histone modifications, the effects of PHI on the expression of the pair of enzymes that acetylate and deacetylate histones were measured by Western blotting. Fig. 3A demonstrates that exposure to PHI for 7 h down-regulated HDAC1, starting at 5 μM PHI and becoming almost undetectable at higher concentrations. The expression of acetyl transferase p300 was up-regulated at the higher PHI concentrations, in contrast to being barely detectable in cells without PHI.

Experiments were then performed to determine whether PHI inhibits the activity of HDAC. PHI was incubated with HDAC from the nuclear extract of HelLa cells in a cell-free assay. An HDAC inhibitor, TSA, was used as a positive control. As demonstrated in Fig. 3B, PHI inhibited the activity of HDAC1 and 2 while the vehicle control of PHI showed no effect. A separate test showed that PHI alone did not interfere with the developer of the assay. Statistically significant inhibition was achieved with 50 nM PHI and approximately 33% inhibition with 5 μM PHI.

To further determine whether PHI reduced the HDAC activity in HL-60 cells, the cellular extracts of HL-60 cells, exposed to PHI or not, were determined for HDAC activity using an activity assay kit for HDAC1 and 2. Fig. 3C shows that, after exposure to PHI for 7 h, the HDAC activity was significantly diminished compared to HL-60 cells without PHI. A statistically significant effect was found with 20 and 40 μM PHI that had respectively a 17 and 35% reduction of HDAC activity compared to HL-60 cells without PHI, indicating a reduced HDAC activity in PHI-exposed HL-60 cells.

Since PHI reduces HDAC activity, we postulated that PHI could induce specific modifications of core histones. Experiments were performed to examine the status of acetylation of histones from PHI-exposed HL-60 cells. Fig. 4 demonstrates that PHI significantly induced the accumulation of acetylated histones H3 and H4 and histone H3 lysine 14, with ≥ 5 μM PHI after 7-h exposure. PHI further mediated selective mono/di/trimethylation at lysine 4 of H3 (Fig. 4). Methylation of this lysine on the histones was barely detectable without PHI. There was also a significant concentration-related decrease, 3-fold at 20 μM, of trimethylated lysine 9 of H3 after a 7-h period of culture.

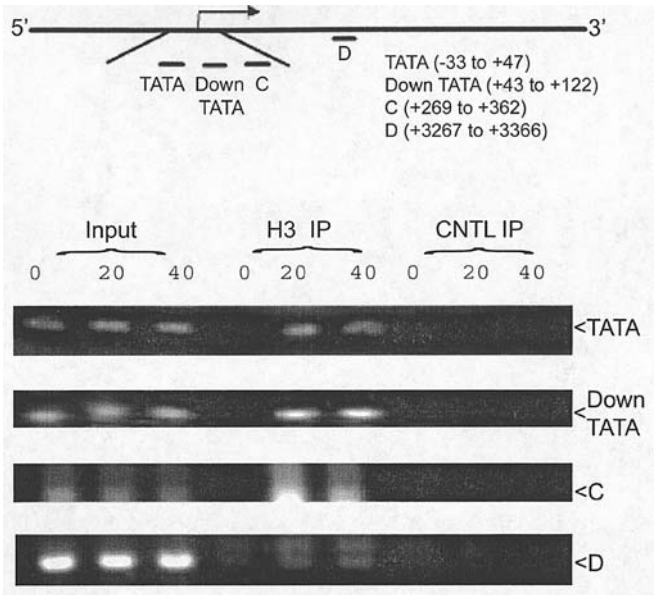

Figure 5. Association of p21 gene with acetylated histones accumulated by PHI induction. Schematic representation of PCR primer sets TATA, down TATA, C, and D for the p21 gene in the upper graph. Lower figure: chromatin fragments from HL-60 cells after exposure for 7 h to medium without PHI (0), 20 μM PHI (20), or 40 μM PHI (40) were precipitated with an anti-acetylated histone H3 antibody (H3 IP), or with a non-specific Ig as controls (CNTL IP). Indicated primers for areas of the p21 gene were used for PCR amplification of DNA isolated from the immunoprecipitated chromatin. Input: DNA extracted from an equal volume of cell lysates were used as template and the PCR product served as input.

Table I. Lack of apoptotic effect of PHI on normal mononuclear cells from bone marrow and peripheral blood.

	% viability (mean ± SD)		% apoptotic cells (mean)	
	Culture period (day)			
PHI (μM)	1	2	1	2
BM (n=3)				
0	95.3±0.5	92.6±0.5	0	0
10	94.3±1.1	90.3±1.1	0	0
20	93.6±1.1	89.6±2.3	0	0
40	93.3±0.5	87.3±6.3	0	0
PBL (n=3)				
0	98.6±1.1	98.3±0.5	0	0
10	99.6±0.5	98.3±1.1	0	0
20	99.3±0.5	98.3±1.0	0	0
40	96.6±2.8	93.0±1.0	0	0

Mononuclear cells from bone marrow (BM) and peripheral blood (PBL) were cultured in RPMI-1640 medium with 15% FCS and exposed to PHI at indicated concentrations.

Acetylated histones associate with the p21 gene. Since it is known that highly acetylated histones, as induced by HDAC inhibitors, are associated with the activation of cell cycle inhibitors, we examined the expression of p21 and p27, the cdk kinase inhibitors. Fig. 4 shows clearly that the levels of p21 and p27 were significantly enhanced in HL-60 cells exposed to ≥ 5 μM PHI. To further determine the association of p21 gene transcription with acetylated histones by PHI, ChIP analyses were performed. DNA from the immunoprecipitates was amplified according to four regions of p21 gene fragments, including the promoter region as schematically presented in Fig. 5. As demonstrated in Fig. 5, the chromatin from cells exposed to PHI clearly contained p21 DNA after precipitation with the antibody against acetylated H3, as compared with cells exposed to control medium without PHI. Immunoprecipitation using non-specific Ig were used as controls to show negative background (Fig. 5). The p21 sequence was detected in the promoter areas TATA, down TATA, and C, as compared with cells not exposed to PHI where p21 was nearly undetectable. The results show that more p21 DNA was in the precipitates of highly acetylated histones, indicating an association between the p21 gene and the acetylated histones mediated by PHI.

Minimal PHI effects on normal cells. Normal mononuclear cells from human peripheral blood and bone marrow were exposed in culture to various concentrations of PHI; the presence of apoptotic cells was evaluated during a two-day incubation period. Table I shows that there were no apoptotic cells induced by 10-40 μM PHI among normal mononuclear cells, as measured by TUNEL assay. The cell viability was not significantly reduced as compared to the control cells not exposed to PHI. These observations indicate that PHI may not induce significant apoptosis and cell death in normal mononuclear cells, as compared to that induced in HL-60 leukemic cells (Fig. 1).

Discussion

Evidence is presented that PHI induces G1 arrest in HL-60 leukemic cells. Analyses of the mechanism revealed activation of cell cycle cdk inhibitors, including p21, by PHI. To investigate how PHI induces p21, we demonstrated for the first time that PHI reduces the level and activity of HDAC and alters the status of acetylation and methylation of histones. The association of acetylated histones with the p21 gene was confirmed by ChIP analyses, indicating that the highly acetylated histones allow chromatin unfolding and accessibility of regulators in the p21 promoter for transcriptional activation.

Hyperacetylation of histones, particularly at certain lysine residues of histone tails is associated with transcription competent chromatin. Hypoacetylation of histones, on the other hand, is associated with the formation of heterochromatin and gene silencing (26-28). Despite the modest activity of PHI to reduce the activity of HDAC, the two-pronged actions of PHI on the activity and level of HDAC, as well as the enhancement of acetyl transferase could effectively induce acetylation of core histones. The induction of chromatin unfolding and transcriptional activation of p21 could represent the cumulative effects of hyperactylation of histones and selective histone methylation. The latter includes an increase of methylated histone H3 lysine 4, and a decrease of methylated

H3 lysine 9. Methylation of histone H3 lysine 9 plays an active role in the establishment and maintenance of pericentric and telomeric heterochromatin (29,30). Methylation of H3 lysine 4 on the other hand, occurs predominantly in euchromatin (31,32). The di- and trimethylation of histone H3 lysine 4 in yeast by the SET1 complex marks transcriptionally active genes (33,34). The patterns of the PHI-mediated acetylation and methylation of histones are consistent with the marks of chromatin unfolding and increased accessibility to the DNA regulators. The mechanism of how PHI alters histone methylation remains to be clarified. Whether it includes a regulation of histone methyl transferases and/or demethylase, such as LSD1 (35), needs to be investigated. The possibility that PHI may mediate other cross talk between DNA and chromatin, serving as epigenetic regulation is currently being investigated.

As an HDAC inhibitor, PHI may be different from another isothiocyanate, sulforaphane. Sulforaphane, itself, has only a borderline inhibitory effect on the activity of HDAC but its metabolite, the cysteine conjugate of sulforaphane, is more effective as an inhibitor (36). Its activity, in comparison, is similar to that of PHI. Based on molecular modeling studies, Myzak *et al* (36) suggested a plausible interaction for the sulforaphane conjugate within the active site of the deacetylase-like protein. The longer carbon chain length of PHI could, in part, be responsible for a more optimal interaction with the active site of the HDAC protein, compared to sulforaphane.

At the G1-phase of the cell cycle, cells can have different destinations. They may proceed to replicate, to differentiate, or exit the cell cycle and undergo apoptosis. Our experiments revealed that PHI induced growth reduction and apoptosis in the leukemia cells, indicating that the cells most likely underwent apoptosis after G1 arrest as an integral part of PHI's effects. Regarding apoptosis, PHI did not induce significant apoptosis among normal mononuclear cells, as compared to HL-60 cells based on the same PHI concentrations. One reason could be that the normal cells are non-replicating and less sensitive to PHI's effects on cell growth. This observation is significant since there have been no reports regarding the selective effects of isothiocyanates to induce apoptosis and cell death in transformed but not in normal cells. Further analyses are needed to elucidate the basis of cellular response to PHI and other isothiocyanates.

Acknowledgments

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