Identification of pinostilbene as a major colonic metabolite of pterostilbene and its inhibitory effects on colon cancer cells

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Abbreviation

PTE, pterostilbene; PIN, pinostilbene; p-Rb, phospho-Retinoblastoma protein; c-PARP, cleaved poly ADP-ribose polymerase; AOM, azoxymethane.

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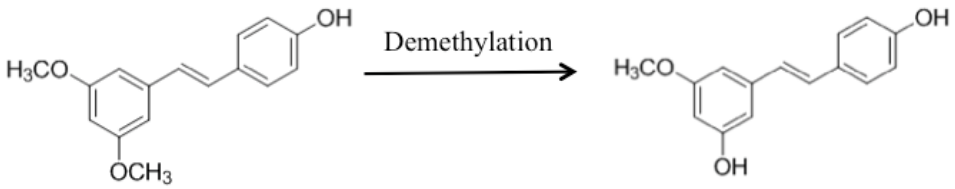
Abstract

Scope: Pterostilbene (PTE) is a resveratrol derivative mainly found in blueberries, and it has been shown to inhibit colon carcinogenesis in multiple animal models. To shed light on the mechanism of PTE in inhibiting colon carcinogenesis, we investigated the PTE metabolites in the mouse colon and in the human colon cancer cells.

Methods and results: CD-1 mice were fed PTE-containing diet for 3 weeks, and colonic content and colonic mucosa were collected and subjected to LC-MS analysis. Pinostilbene (PIN) was identified as a major metabolite of PTE in the mouse colon. Importantly, the level of PIN was found to be approximately equivalent to that of PTE in the colonic mucosa. PIN significantly inhibited the growth of human colon cancer cells, i.e., HCT116 and HT29. These inhibitory effects were similar to those produced by PTE. Moreover, under physiologically relevant conditions, 20 and 40 μM of PIN caused cell cycle arrest at S phase and induced apoptosis in colon cancer cells. These effects were associated with profound modulation of signaling proteins related with cell proliferation and programmed cell death.

Conclusion: Our results demonstrated that PIN is a major metabolite of PTE in the colon of mice fed with PTE, and PIN may play important roles in the anti-colon cancer effects elicited by orally administered PTE.

Key Words: pinostilbene, pterostilbene, metabolite, colon cancer, apoptosis, cell cycle arrest.



Pterostilbene (PTE) **Pinostilbene (PIN)**

Pterostilbene (PTE) is a resveratrol derivative mainly found in blueberries, and has been shown to inhibit colon cancer in animal models. Our results shown that orally administered PTE was transformed to pinostilbene (PIN) via demethylation in mice. The level of PIN in the colonic mucosa of PTE-fed mice was relatively high, and at this level range, PIN inhibited the growth of multiple human colon cancer cells by inducing cell cycle arrest and apoptosis. These results provided insights on the anti-colon cancer effects of PTE.

1. Introduction

According to the World Cancer Report 2014, colon cancer is the third most common type of cancer globally. The development of colon cancer is closely associated with unhealthy dietary habits and lifestyle [1]. Colon cancer chemoprevention has been proposed as a promising strategy to inhibit, retard or even reverse the process of colon carcinogenesis. Epidemiological studies have suggested that fruit and vegetable consumption may contribute to the decreased risks of multiple cancers, including colon cancer [2]. The protective effects of fruit and vegetable against colon cancer have been attributed to the content of bioactive phytochemicals such as phenolic compounds. These dietary phenolic compounds with potential cancer preventive effects have been under intensive investigations.

Resveratrol (trans-3,5,4’-trihydroxystilbene) is one of the most studied dietary phenolic compounds for its potential health effects, including anti-cancer effects. However, the poor oral bioavailability of resveratrol has been a concern for its utilization as an effective nutraceutical [3]. Pterostilbene (4'-hydroxy-3,5-dimethoxystilbene, PTE, Fig. 1) is a dimethylether analogue of resveratrol, and has gained increasing attention as a health-promoting nutraceutical [4]. For example, we and others have reported that PTE had more potent antioxidant, anti-inflammation, and anticancer effects than resveratrol [5-8]. It is noteworthy that pterostilbene has been shown to have greater oral bioavailability than resveratrol, which renders pterostilbene higher potential for human application [9, 10].

Animal studies have demonstrated that orally administered PTE effectively inhibited chemically induced colon carcinogenesis in multiple animal models [6, 7, 11]. For example, dietary treatment of PTE (400 ppm in diet) for 45 weeks was shown to inhibit azoxymethane (AOM)-induced colon tumorigenesis [6]. It was also demonstrated that dietary treatment of PTE (50-250 ppm in diet) was more effective than resveratrol in reducing AOM-induced formation of aberrant crypt foci (ACF), lymphoid nodules (LNs), and tumors [7]. However, the mechanism of anticancer actions of PTE in the colon is poorly understood, especially regarding the role of bioavailability and biotransformation. Dietary compounds undergo complex physiological and biochemical processes before reaching the colon. The availability of the parent compounds and their bioactive metabolites in the colon plays critical role in the overall biological effects in the colon. In this study, for the first time, we identified pinostilbene (PIN) as a bioactive metabolite of PTE in the colon of mice fed PTE, and determined its inhibitory effects on human colon cancer cells.

2. Materials and methods

2.1 Materials

PIN and PTE were synthesized and authenticated as previously reported [12, 13]. Their purity was determined by HPLC-MS. PIN and PTE with purity greater than 98% were used in this study. All other chemicals and solvents were of HPLC grade and were purchased from Fisher Scientific (Pittsburgh, PA).

2.2 Animal experiment

The protocol (#2011-0066) for the animal experiment was approved by the Institutional Animal Care and Use Committee of the University of Massachusetts. Ten male CD-1 mice (5 week of age) were obtained from Charles River Laboratories (Wilmington, MA, USA). After one week of acclimation with AIN93G diet, mice were randomly divided into two groups (5 mice/group). The mice in the control group were fed AIN93G diet, while the PTE-treated group was fed with AIN93G diet containing 0.1% (w/w) PTE. Both groups were allowed to eat and drink ad libitum during the whole experiment period. After three weeks of treatment, the colons were harvested to collect colonic content and colonic tissue. Half of the colon tissue was fixed in 10% buffered formalin (pH 7.4) for 24 h and then processed for paraffin-embedding, sectioning, haematoxylin and eosin (H&E) staining,

and histological analysis as we previously described[14]. The other half was used to collect colonic mucosa.

2.3. Sample preparation and LC-MS conditions

Aliquots of colonic mucosa and colonic content sample were homogenized with 50% methanol using a Bead Ruptor homogenizer (Omni International, Kennesaw GA, USA). The homogenates were then extracted with equal volume of ethyl acetate for three times. The combined ethyl acetate extracts were dried using a vacuum concentrator (Model: SVC 100H, Thermo Fisher Scientific Inc., USA), and then dissolved in 50% methanol for LC-MS analysis.

Liquid chromatographic separation and mass spectrometric detection were performed using a single-quad LC-MS system (Model 2020, Shimadzu, Kyoto, Japan). Optimized mass spectra were acquired with an interface voltage of 4.5 kV, a heat block temperature of 400°C and a desolvation gas temperature of 250°C. Data acquisition and processing were accomplished using Shimadzu Labsolutions software. Quantitation was performed by selected ion monitoring (SIM), using ESI mode. Liquid chromatographic separation was achieved using a Zorbax SB-Aq C18 column (150 mm × 4.6 mm, 5 µm, Agilent Technologies, USA). Mobile phase A comprised 5% acetonitrile/water, mobile phase B comprised 100% acetonitrile. Gradient elution was performed starting at 80% A/20% B, progressing to 80% B/20% A over 30 min, held at 80% B for 5 min then reduced to 80% A/20% B in 5 min, then kept for the remainder of the run to re-equilibrate. Total flow rate and injection volume were 1 mL/min and 10 µL, respectively.

2.4. Analysis of cell viability, cell cycle and apoptosis

Assays for cell viability, cell cycle and apoptosis were conducted as we previously described [15-17]. In brief, human colorectal cancer cells, HCT116 and HT29 (ATCC, Manassas, VA, USA), and normal human colon diploid fibroblasts CCD-18Co (ATCC, Manassas, VA, USA) were seeded in 96-well plates. After 24 h, cells were treated with serial concentrations of PTE and PIN, and the cell viability was quantified by MTT method [15-17]. Cells were seeded in 6-well plates for cell cycle and apoptosis analysis as we previously reported [15-17]. In brief, after cell attachment, cells were treated with 20 or 40 µM of PIN in serum complete media for 24 or 48 h, then the cells were harvested for cell cycle and apoptosis analysis by flow cytometry. DMSO was used as vehicle to deliver PTE and PIN to the cells. The final concentration of DMSO in all experiments was 0.1% v/v in cell culture media.

2.5. Immunoblot analysis

HCT116 cells were seeded in 150 mm culture dishes. After cell attachment, cells were treated with PIN (20 or 40 μM) for 24 or 48 h, and then used for Western blotting analysis as we previously reported [15-18]. Antibodies for Bax, cleaved caspase-3 (Asp175), cleaved poly ADP-ribose polymerase (c-PARP), cyclin E, p21, p53, phospho-Retinoblastoma protein (p-Rb) were obtained from Cell Signaling Technology (Beverly, MA, USA). β-Actin was used as a loading control. Anti-β-actin antibody was from Sigma-Aldrich (St. Louis, MO, USA).

2.6. Statistical analysis

All data were expressed as mean ± SD, the statistical significance between two groups was assessed by Student's *t*-test. A p value <0.05 was considered to be statistically significant.

3. Results

3.1 PIN was a major metabolite of PTE in the colon after oral administration of PTE in mice

In order to better understand the biological effects of dietary administration of PTE in the colon, it is important to determine the identity and abundance of metabolites in the colon. Herein, PTE was orally administered to the mice as part of regular diet (0.1% w/w) for three weeks. This dose was equivalent to about 380 mg of PTE oral dose per day in human (60 kg body weight). Compared to the control mice that were fed basal AIN93G diet without PTE, among the parameters measured, PTE-treated mice showed no sign of adverse effects from PTE treatment. This was evidenced by that no difference was found between PTE-treated group and the control groups in terms of diet consumption, body weight, liver weight or spleen weight, and no behavioral or appearance difference was observed either. Moreover, there is no difference was observed in the morphology of colonic mucosa of control mice and PTE-fed mice (Figure 2A and B). During the three weeks of treatment

period. We analyzed the colonic content samples from both groups of mice by LC-MS to identify the metabolites of PTE. We identified substantial amount of PTE (SIM: m/z 255 $[M-H]^-$, retention time = 20.3 min, Figure 2C) in the colonic content of PTE-treated mice using synthesized PTE as chemical standard. Furthermore, using LC-MS, we successfully identified multiple metabolites in the colonic content. Among these metabolites, one metabolite (SIM: m/z 241 $[M-H]^-$, retention time = 16.9 min, Figure 2D) showed highest abundance. Based on the molecular weight, it was hypothesized that this metabolite was PIN (Figure 1). We chemically synthesized and authenticated PIN according to the previous reports [12, 13]. Using synthesized PIN standard, we confirmed that the metabolite (SIM: m/z 241 $[M-H]^-$, retention time = 16.9 min, Figure 2D) was PIN. Using PTE and PIN standards, we determined the levels of PTE and PIN in the colonic content and colonic mucosa. As shown in the Figure 2E and 2F, the levels of PTE and PIN in colonic mucosa were 27.44 and 21.36 nmol/g, respectively, while, the levels of PTE and PIN in the colonic content were 296.4 and 201.9 nmol/g, respectively.

3.2. In comparison with PTE, PIN showed similar inhibitory effects on the growth of human colon cancer cells.

To establish the anti-cancer effects of PIN, we compared the inhibitory effects of PTE and PIN on the growth of two human colon cancer cell lines, HCT116 and HT29, using a cell viability (MTT) assay. HT29 and HCT116 were treated with a series concentration of PTE and PIN for 24 and 48 h. As shown in Figure 3A and B, both compounds showed dose-dependent and time-dependent inhibition on the growth of HCT116 and HT29 cells. Overall, PTE and PIN showed similar potency in inhibiting the growth of two human colon cancer cells. The effects of PTE and PIN on normal colon cells were determined in the CCD-18Co cells. As shown in figure 3C, PTE or PIN (up to 40 μM) did not cause significant inhibition on the growth of normal colon cells.

3.3. PIN caused S phase cell cycle arrest in human colon cancer cells

In order to further investigate the mechanism by which PIN inhibited colon cancer cell growth, cell cycle analysis was conducted on the HCT116 and HT29 cells treated with PIN for 24 h. As shown in Fig. 4A, PIN at 20 μM and 40 μM caused a significant and dose-dependent increase in the percentage of cells in S phase in both HCT116 and HT29 cells compared to the control cells ($p < 0.001$). Treatments with PIN at 40 μM increased the percentage of S phase cell population to 2.3-fold and 2.05-fold of that of the control HCT116 and HT29 cells, respectively. PIN at 20 μM also induced a modest increase of cell population in G2/M phase in HT29 cells ($p < 0.01$).

3.4. PIN induced apoptosis in human colon cancer cells

In order to determine the extent to which apoptosis contributed to the growth inhibition caused by PIN, the effects of PIN on cellular apoptosis of human colon cancer cells were investigated. After 48 hours of treatments with PIN at 20 and 40 μM, the early and late apoptotic cells were quantified by the flow cytometry with Annexin V/Propidium iodine double staining assay as previously described [15, 19]. As shown in Fig. 4B, extensive apoptosis was caused by PIN in HCT116 and HT29 cells in a dose-dependent manner. In HCT116 cells, PIN at 20 and 40 μM increased the early apoptotic cell population by 2.7-fold (from 0.78 to 2.1%) and 6.9-fold (from 0.78 to 5.4%), respectively, compared to the control group. Moreover, in HCT116 cells, PIN at 20 and 40 μM also increased late apoptotic cell population by 1.7-fold (from 1.32 to 2.3%) and 5.6-fold (from 1.32 to 7.37%), respectively, compared to the control. Similar apoptosis-inducing effects of PIN were also observed in the HT29 cells (Fig. 4B). For example, in HT29 cells, PIN treatment (at 40 μM) increased the total apoptotic cell population by 4.6-fold in comparison with the control.

3.5. PIN modulated expression of key signaling proteins related to cell proliferation and apoptosis

To further elucidate the molecular mechanism underlying the inhibitory effects of PIN on cancer cells, several key protein markers related to the cell cycle and apoptosis pathways were examined by immunoblotting analysis in HCT116 cells. Cell cycle-related proteins $p21^{Cip1/Waf1}$, cyclin E and p-Rb were quantified after 24 hours of treatment (Fig. 5, left panel), and pro-apoptotic proteins p53, Bax, cleaved caspase-3 and cleaved PARP were evaluated after 48 hours of treatment (Fig. 5, right panel). Immunoblotting results showed that PIN treatments led to significant changes on the expression levels of these signaling proteins. PIN significantly increased the expression levels of p53, Bax, cleaved caspase-3, cleaved PARP and $p21^{Cip1/Waf1}$, while decreased the expression levels of cyclin E and p-Rb. Generally, these effects were dose dependent. For example, PIN at 40 μM increased the

levels of $p21^{Cip1/Waf1}$, p53, Bax, cleaved caspase-3 and cleaved PARP by 1.3-, 3.2-, 2.2-, 1.7- and 4.5-fold, respectively, in comparison with those observed in the control cells. In the meantime, 40 μM of PIN caused 0.6- and 0.7-fold reduction of the expression levels of cyclin E and p-Rb, respectively.

4. Discussion

Epidemiological studies have provided accumulating evidence that bioactive phytochemicals from certain fruits and vegetables can lower the risk of multiple human cancers. In recent years, PTE has attracted growing attention due to its unique beneficial health effects. For example, PTE has shown various protective effects against different cancers, such as bladder, breast, colon, leukemia, lung, and prostate cancers [20]. As a methylated analog of resveratrol, PTE share the same stilbenoid core structure as resveratrol, but differ in functional groups, i.e., two methoxy groups in the A ring (Fig. 1). This structure difference was suggested to be the reason that PTE has a higher biostability due to slower metabolism and lower excretion, thus greater bioactivities in comparison with resveratrol [21]. However, after oral consumption, PTE still undergo extensive metabolism to produce various metabolites [22]. The identity and abundance of these metabolites played important roles in the biological effects elicited by oral consumption of PTE. For example, if ingested PTE was mainly transformed to inactive metabolites in the body, it was likely that the biological activities of PTE would be decreased after metabolism.

Previous studies have demonstrated potent inhibitory effects of orally administered PTE on colon carcinogenesis in rodents [6, 7, 11]. However, the identity and abundance of PTE metabolites in the colon is unknown, which greatly limited our ability to elucidate the mechanism of action of PTE in inhibiting colon carcinogenesis. In this study, for the first time, we identified PIN as a major metabolite of PTE in the colon of the mice fed PTE for 3 weeks. Sharing a same stilbenoid core structure as PTE, PIN is a demethylated derivative of PTE (Fig. 1) and naturally exists in *Soymida febrifuga* (Myanmar), *Dracaena loureiri* (Thailand), *Dranaena cochinchinensis* (China), *Rumex bucephalophorus* (Israel) and *Muscari comosum* (Mediterranean area) [23-27]. PIN has been previously found as a urinary metabolite of PTE in mice after oral gavage of PTE [22]. Importantly, our results showed that the abundance of PIN in the colonic content and colonic mucosa was relatively high, and was even comparable to that of PTE (Fig. 2). Therefore, it is reasonable to postulate that PIN may significantly contribute to the biological effects in the colon observed after the oral consumption of PTE, such as inhibition on colon carcinogenesis.

To establish the bioactivity of PIN in the colon, we determined its inhibitory effects on human colon cancer cells. Our results showed that PIN had a dose-dependent inhibition on the growth of HCT116 and HT29 cells (Fig. 3). It is noteworthy that the inhibitory effects of PIN were similar to those of PTE. This finding reinforced the notion that as a PTE metabolite, PIN may significantly contribute to the anti-colon cancer effects of PTE. It is noteworthy that PTE or PIN (up to 40 μM) showed no significant inhibition on the growth of normal human colon cells, whereas, at the same concentration PTE and PIN significantly inhibited the growth of human colon cancer cells. These results consistent with that dietary treatment with PTE did not cause any change in the morphology of normal colon mucosa (Fig. 2A and 2B). Our results supported the notion that cytotoxic effects of PTE and PIN were cancer cell specific.

We further investigated the mode of action of PIN in inhibiting cancer cell growth by determining the effects of PIN on cell cycle progression and cellular apoptosis. An important consideration for cell culture experiments is to make sure the conditions are of physiological relevance. Our results showed that the tissue level of PIN in the colonic mucosa was about 21 nmol/gram tissue (Fig. 2). This is approximately equivalent to 21 μM if we consider 1 gram of tissue is about 1 mL in volume. It is noteworthy that this level of PIN was achieved by ad libitum feeding of AIN93G diet containing PTE throughout the feeding period of the mice (overnight), and it reflected the sustained and stabilized PIN concentration in the colonic mucosa after chronic dietary exposure to PTE for 3 weeks. This situation is different from that achieved by one time single dose oral administration such as via taking dietary supplements in capsules, by which much higher tissue level of PIN are likely be achieved. Therefore, two concentrations of PIN were used to conduct mechanistic study on cancer cells, i.e., 20 μM of PIN was used to reflect the tissue level achieved through ad libitum feeding in regular diet, and 40 μM of PIN was used to reflect the tissue level achieved through taking dietary supplement capsules.

Uncontrolled cell proliferation is one of hallmarks of cancer cells. Therefore, induction of cell cycle arrest is an effective strategy to control cancer development and progression. Our results showed PIN at 20 or 40 μM caused cell cycle arrest in S phase in both colon cancer cells (Fig. 4A). Several other stilbene compounds, such as resveratrol, polydatin and piceatannol, had also been reported to trigger S-phase cell cycle arrest in several different colon cancer cells [28-30]. It is of future interests to determine the structure-function relationship of different stilbene compounds in inducing cell cycle arrest in cancer cells. Cyclins, cyclin dependent kinases (CDKs) and CDK inhibitors are key regulators of cell cycle progression. Cyclin/CDK complexes promote cell cycle progression while CDK inhibitors induce cell cycle arrest. Cyclin/CDK complexes can bind to CDK inhibitors, such as

$p21^{Cip1/Waf1}$ and p27, which interferes their kinase activities and therefore promote cell cycle progression [31]. For example, cyclin E/CDK2 complex is required for the cell cycle progression [32]. Cyclin E is also a critical component of the Rb pathway, in which progressive phosphorylation of Rb by cyclin E/CDK2 releases E2F. E2F is a transcription factor that regulates the expression of genes involved in S phase [33]. We found that PIN treatment significantly and dose-dependently increased the expression level of $p21^{Cip1/Waf1}$, as well as decreased the expression level of cyclin E (Fig. 5). In addition, PIN also caused down-regulation of p-Rb. Altogether, these modulations of cell cycle regulators by PIN treatment resulted in cell cycle arrest.

Another principal approach to control cancer cell growth is to induce cellular apoptosis (programmed cell death). During the development of cancer, mutated cells can evade apoptosis and survive due to aberrations of the apoptotic signaling pathway, and eventually become cancerous [34]. Nutraceuticals that can induce apoptosis by re-regulating these signaling proteins have the potential for preventing cancers [35]. It was observed that PIN treatments significantly increased the apoptotic cell population in both HCT116 and HT29 cells, and the effects were dose dependent (Fig. 4B). These findings were consistent with previous reports showing that stilbene compounds such as resveratrol and PTE induced cellular apoptosis in various cancer cells, including gastric, leukemia, bladder and hepatocellular cancer cells [36-39]. Our results demonstrated that PIN treatment dose-dependently increased the levels of cleaved caspase-3 and cleaved PARP in colon cancer cells (Fig. 5). Activation (cleavage) of a critical executioner of apoptosis, caspase-3 and its downstream target PARP mediates apoptosis through interfering chromatin condensation and DNA fragmentation. These results were consistent with that from Annexin-V/PI double staining assay showing PIN treatments induced both early and late apoptosis in colon cancer cells (Fig. 4B). We also observed that PIN treatment increased the expression levels of p53 and Bax (Fig. 5). Tumor suppressor p53 plays essential roles in tumor suppression, and it exerts tumor inhibitory effects by inducing cell growth arrest and triggering apoptosis. Moreover, the p53 protein, as a transcription factor, regulates many downstream target genes, including Bax and $p21^{Cip1/Waf1}$. Loss or aberration of p53 function has been found in many human cancers that can lead to elevated cell proliferation, resistance to cell death signal, genomic instability and metastasis [40]. Bax, a Bcl-2 family proapoptotic protein, is responsible for the mitochondrial damage that can result in the activation of caspase cascade. Our results suggested that mitochondria-mediated intrinsic apoptosis was involved in the apoptosis induced by PIN treatment.

5. Conclusions

This study explored the role of biotransformation/metabolism in the biological effects of orally administered PTE. For the first time, we identified PIN as a major PTE metabolite in the colon of mice fed PTE. Importantly, PIN showed similar inhibitory effects on the growth of human colon cancer cells as PTE, and these effects were associated with modulation of multiple key cellular proteins related with cell cycle arrest and cellular apoptosis. Further investigation is warranted to elucidate the biological effects of PIN in the colon and its implication in the colon cancer prevention through oral consumption of PTE-containing food products.

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Y.S. and H.X. conceived and designed experiments. Y.S., X.W., X.C., M.S., J.Z., performed experiments. P.Q., L,Z., S.Z., Z.T. provided technical consultation. Y.S. and H.X. analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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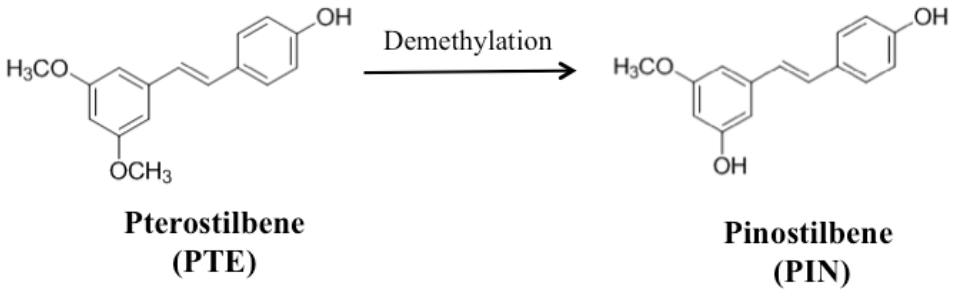
Figure 1. Metabolic production of pinostilbene (PIN) from pterostilbene (PTE) in mice.



Pterostilbene (PTE) **Pinostilbene (PIN)**

Figure 2. Morphology of colonic mucosa of mice treated with control diet (A) or diet containing PTE (B). LC/ESI-MS (negative ion) chromatograms of PTE (C) and PIN (D) in colonic content samples obtained from PTE-fed mice and control mice. The levels of PTE and PIN in the colonic mucosa (E) and colonic content (F) of PTE-fed mice. * represents $p < 0.001$, compared to PTE. All data represent mean ± SD (n = 5). Ten microscopic fields were assessed for each colonic tissue samples for histological analysis. All tissue samples were blinded during analysis.

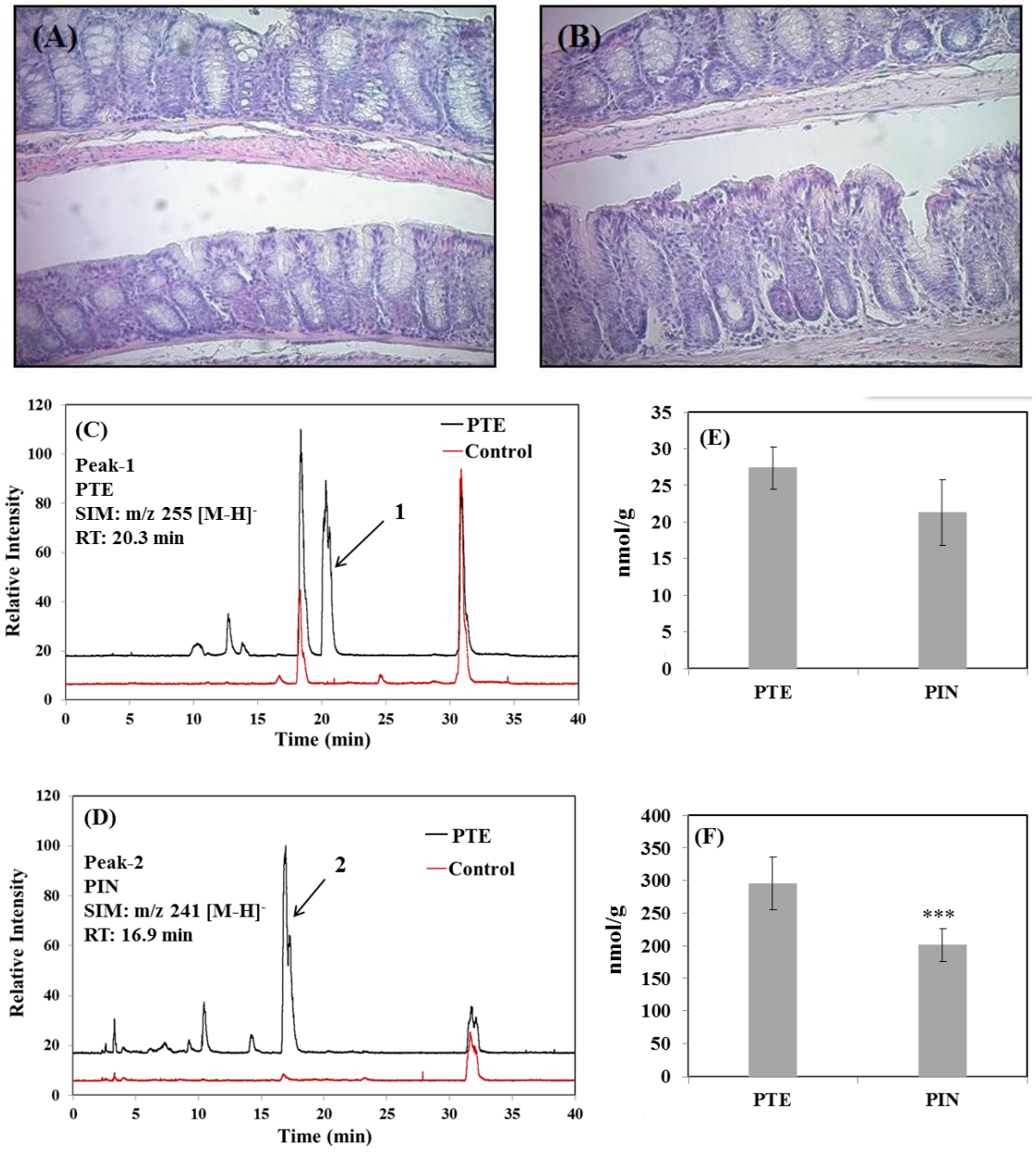


Figure 3. Growth inhibitory effect of PTE and PIN on human colon cancer cells HCT116 (A) and HT29 (B), and normal human colon cells CCD-18Co (C). Cells were seeded in 96-well plates, and after 24 hours of incubation, cells were treated with a series concentration of PTE or PIN as indicated in the figures for 24 and 48 hours. Growth inhibition was measured by MTT assay as mentioned in the method section. Data represent mean ± SD (n = 6, from three independent experiments).

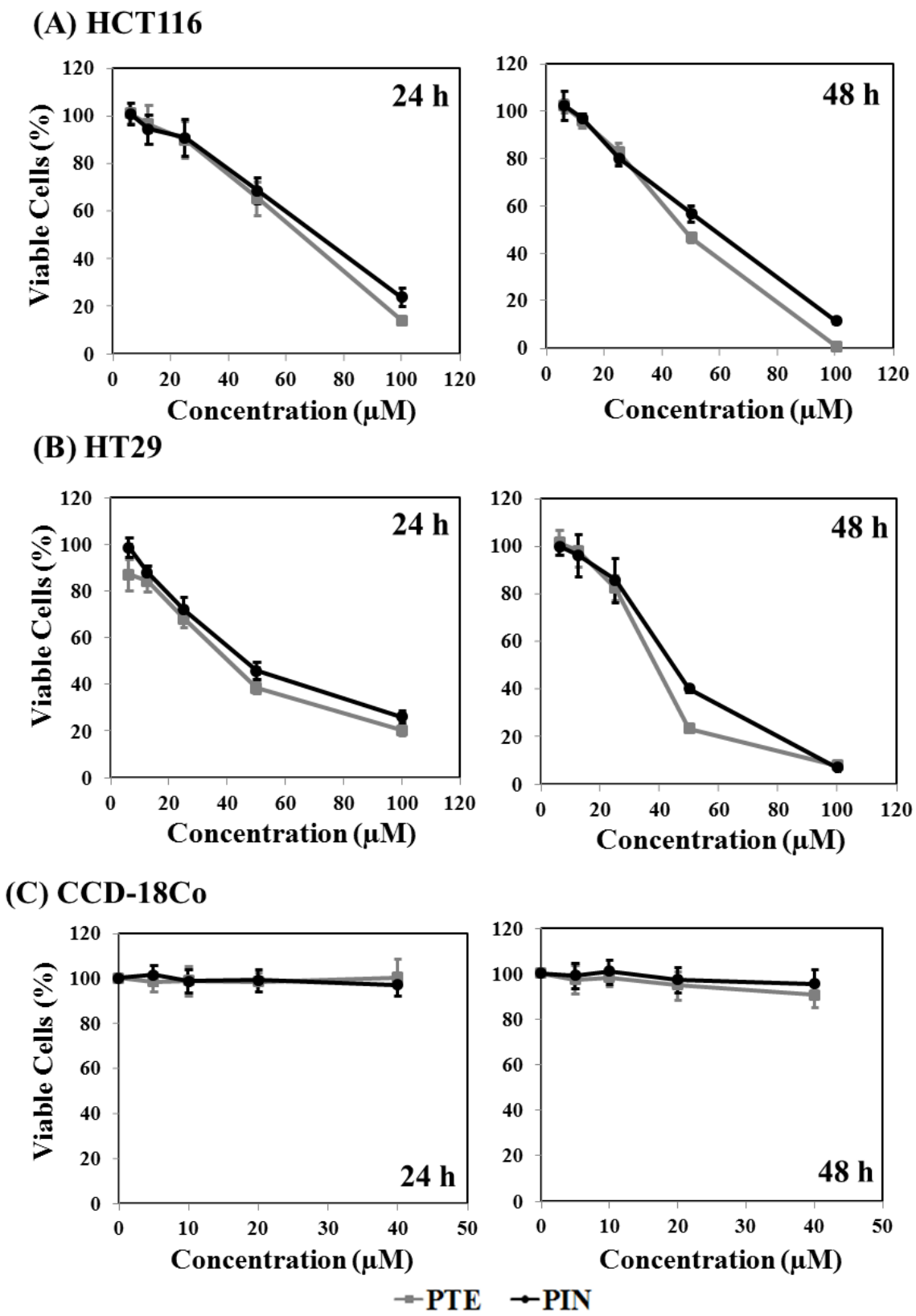


Figure 4. Effects of PIN on cell cycle progression (A) and apoptosis (B) of HCT116 and HT29 human colon cancer cells. The cells were seeded in 6-well plates for 24 hours, and then treated with PIN. After 24 and 48 hours of treatment, cells were collected and subjected to cell cycle and apoptosis analyses, respectively. All data represent mean ± SD (n=3, from three independent experiments). *** indicates $p < 0.001$ and * indicates $p < 0.01$ in comparison with the control.

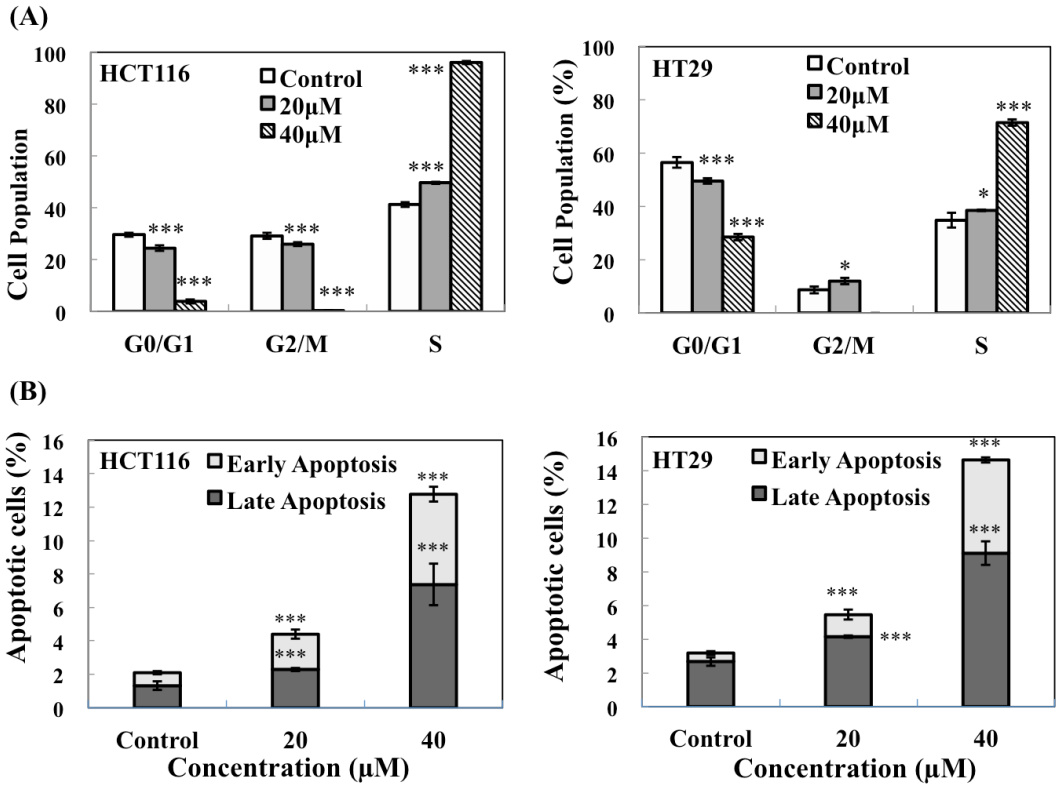


Figure 5. Effects of PIN on cell cycle and apoptosis related signaling proteins in HCT116 human colon cancer cells. HCT116 cells were seed into 15-cm culture dishes for 24 hours, and cells were then treated with different concentrations of PIN. After another 24 or 48 h of incubation, cells were collected for immunoblotting analysis as described in methods section. The numbers underneath of the blots represent band intensity (normalized to β-Actin loading control, means of three independent experiments) measured by densitometry and Image J software. The standard deviation (all within ±15% of the means) were not shown. β-Actin was served as an equal loading control. * indicates statistical significance in comparison with the control ($p < 0.05$, n = 3, from three independent experiments).