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Inhibitory Effects of Resveratrol and Pterostilbene on Human Colon Cancer Cells: A Side-by-Side Comparison

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ABSTRACT: The effects of resveratrol and pterostilbene (two structurally related stilbene compounds) on three human colon cancer cells were systematically compared. Cell viability tests indicated that IC_{50} values of pterostilbene were 2−5-fold lower than those of resveratrol in all three cancer cells. Pterostilbene was also more potent in inhibiting colony formation of all three cancer cells. Annexin V/propidium iodide costaining assay and Western blotting analysis showed pterostilbene had a stronger apoptosis-inducing effect, which was evidenced by the higher percentage of annexin V positive cells and higher levels of cleaved caspase-3 and poly(ADP-ribose) polymerase proteins in cancer cells treated with pterostilbene compared with resveratrol. High-performance liquid chromatography analysis demonstrated that intracellular levels of pterostilbene were 2−4-fold higher than those of resveratrol after treatments with individual compounds at the same concentration. Overall, the results demonstrated that pterostilbene had more potent inhibitory effects on colon cancer cells than resveratrol, which may be associated with the superior bioavailability of pterostilbene to resveratrol.

KEYWORDS: pterostilbene, resveratrol, colon cancer, bioavailability, apoptosis

INTRODUCTION

Colon cancer is one of the deadliest cancers in the United States and was ranked the third cause of cancer death.[1] Moreover, an increase in colon cancer incidence in adults under 50 years old has been reported.[2] Cancer chemoprevention has been considered as a promising strategy to controlling cancer death, especially in colon cancer, because the generally slow progression of colorectal adenomatous polyps of colon cancer allows better opportunities for chemoprevention regimens. Results from epidemiological studies have linked fruit and vegetable consumption with reduced risk of colon cancer, and multiple phytochemicals have been identified as potential cancer-fighting agents from commonly consumed fruits and vegetables.

Stilbenes comprise a class of natural polyphenolic compounds that have been studied for their health-promoting effects including anticarcinogenic activities.[3] The core chemical strucuture of stilbene compounds is 1,2-diphenylethylene (Figure 1). *trans*-Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene; REV) (Figure 1A) is the most widely studied stilbene found in small fruits such as grapes, berries, peanuts, and some medicinal plants.[4] Resveratrol has been considered as a good anticarcinogenic agent because of its low toxicity and capability of modulating multiple molecular pathways involved in cancer progression.[5] These pathways play important roles in cell cycle progression, antiapoptosis, angiogenesis, and tumor invasion. Studies have demonstrated that resveratrol is antiproliferative, proapoptotic, and antiangiogenic in multiple cell culture models.[6−8] Moreover, in vivo studies showed that resveratrol inhibited tumor progression in multiple organ sites such as breast, prostate, lung, and gastrointestinal tract.[9] One potential problem associated with the use of resveratrol in chemoprevention is that resveratrol has low systemic bioavailability,[10,11] which may lower its efficacy in humans. Consequently, more efforts have been exerted to develop resveratrol derivatives with better bioavailability profiles.

Pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene; PTS) (Figure 1B), a dimethylether analogue of resveratrol, has gained increasing attention as a potential cancer chemopreventive agent. It has been reported that pterostilbene is as effective as resveratrol in inhibiting carcinogen-induced preneoplastic lesions in a mouse mammary organ culture model, in inhibiting metastatic growth to the liver, and in antioxidant activities.[12,13] Moreover, studies have demonstrated that pterostilbene had superior effects to resveratrol in inhibiting DNA synthesis and decreasing the expression levels of inflammatory genes in colon cancer cells.[14] Most importantly, pterostilbene has been shown to be more potent than resveratrol in preventing colon carcinogenesis in azoxymethane-treated mice.[15] With the longer half-life in vivo in comparison to resveratrol,[16] pterostilbene is a promising dietary factor for chemoprevention. Herein, we investigated the extent to which the chemical structural differences between resveratrol and pterostilbene affect their inhibitory effects on three human colon cancer cell lines.

MATERIALS AND METHODS

Materials and Cell Culture. Resveratrol and pterostilbene were obtained from Quality Phytochemical LLC (New Jersey). The 100 mM stock was prepared by dissolving the compounds in dimethyl sulfoxide (DMSO). Human colon cancer cells HCT116, HT29, and Caco-2 were obtained from American Type Cell Collection (ATCC, Manassas, VA) and were maintained in McCoy's 5A or RPMI media (ATCC)

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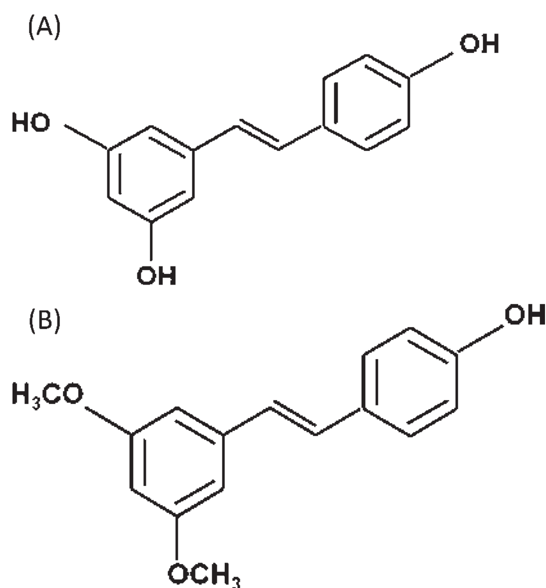

Figure 1. Chemical structures of resveratrol (A) and pterostilbene (B).

supplemented with 5% heat inactivated FBS (Mediatech, Herndon, VA), 100 U/mL of penicillin, and 0.1 mg/mL streptomycin (Sigma-Aldrich) at 37 °C with 5% CO_2 and 95% air. Cells were kept subconfluent, and media were changed every other day. All cells used were within 3−30 passages. DMSO was used as the vehicle to deliver resveratrol and pterostilbene, and the final concentration of DMSO in all culture media was 0.1%.

Cell Viability Assay. HCT116 (1500 cells/well), HT29 (2000 cells/well), or Caco-2 (3000 cells/well) cells were seeded in 96-well plates. After 24 h, media were replaced with 200 μL of media containing serial concentrations of resveratrol or pterostilbene. After suitable treatment periods, cells were subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The treatment media were replaced by 100 μL of fresh media containing 0.5 mg/mL of MTT (Sigma-Aldrich). After 2 h of incubation at 37 °C, MTT-containing media were removed and the reduced formazan dye was solubilized by the addition of 100 μL of DMSO to each well. After gentle mixing, the absorbance was monitored at 570 nm using a microplate reader (Elx800 absorbance microplate reader, BioTek Instruments, Inc., Winooski, VT).

Apoptosis Assay. Apoptosis induction was quantified by annexin V/propidium iodide (PI) double staining followed by flow cytometry. Annexin V/PI double staining was performed using an apoptosis detection kit (Biovision, Mountain View, CA) following the manufacturer's instruction. In short, cells were gently detached by brief trypsinization (any floating cells were also collected) and then washed with ice-cold PBS. After another wash with binding buffer, cells were suspended in 300 μL of binding buffer containing annexin V and PI and incubated for 5 min at room temperature. Early apoptotic cells were identified as annexin V positive/PI negative cells, whereas late apoptotic/necrotic cells were identified as annexin V positive/PI positive cells using a BD LSR II cell analyzer.

Immunoblotting. Human colon cancer cells were seeded in 10-cm cell culture dishes. After 24 h, cells were treated with serial concentrations of resveratrol or pterostilbene. Cells were incubated for another 24 or 48 h, washed with ice-cold PBS, incubated on ice for 10 min in lysis buffer (Cell Signaling Technology, Inc., Beverly, MA) supplemented with cocktails of protease inhibitor (4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF, 50 mM), aprotinin (30 mM), Besstain leupeptin (1 mM) (1:100); phosphatase inhibitor 1 (imidazole sodium

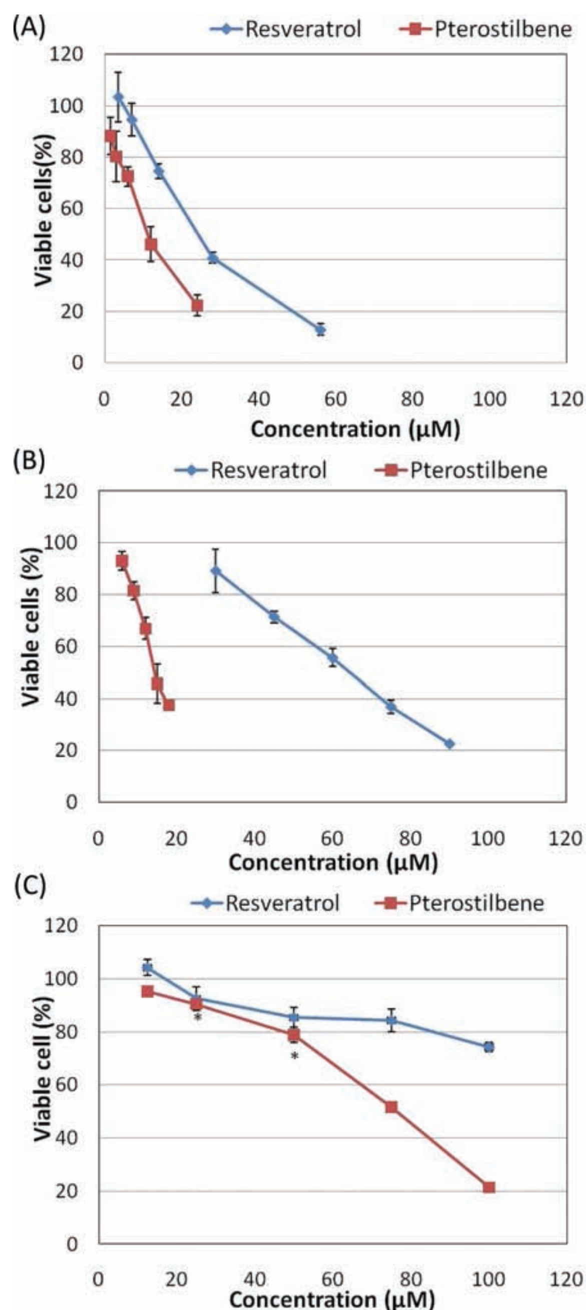

Figure 2. Growth inhibitory effect of resveratrol and pterostilbene on HCT116, HT29, and Caco-2 human colon cancer cells. Colon cancer cells HCT116 (A), HT29 (B), and Caco-2 (C) were seeded in 96-well plates. After 24 h, cells were treated with serial concentrations of resveratrol or pterostilbene. After 48 h of treatment, cell viability was measured by MTT assay as described under Materials and Methods. Data are expressed as the mean ± standard deviation. All results for resveratrol and pterostilbene at the same concentrations were significantly different from each other except as noted (*, $p < 0.01$).

fluoride, sodium molybdate, sodium orthovanadate, sodium pyrophosphate tartate) (1:100), and phosphatase inhibitor 2 (sodium fluoride, sodium orthovanadate, sodium pyrophosphate, β-glycerophosphate) (1:100) (Boston Bioproducts, Ashland, MA). Cell suspensions were subjected to sonication (5 s, three times), followed by incubation for another 20 min on ice. The cells were then centrifuged at 15325 RCF for 20 min at 4 °C, and supernatants were collected. Protein content were

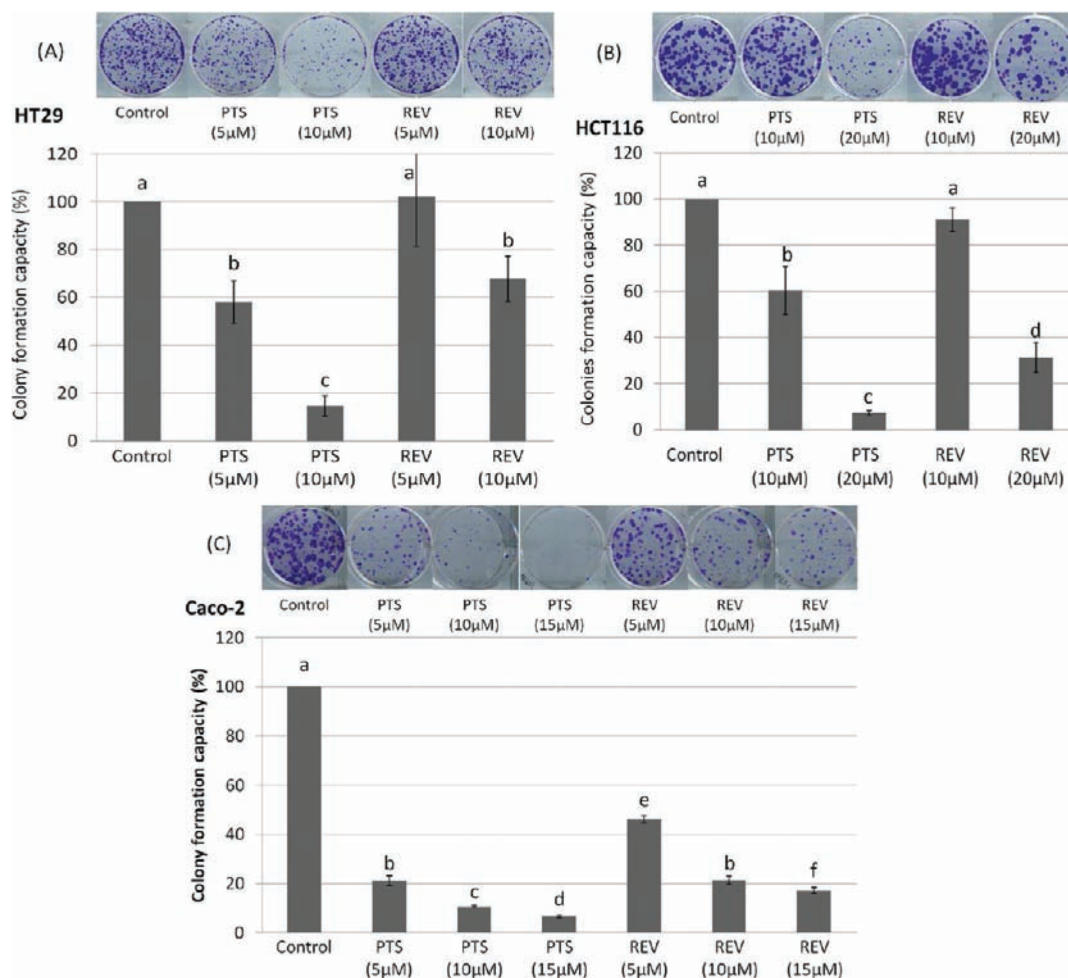

Figure 3. Effects of resveratrol (REV) and pterostilbene (PTS) on colony formation of HT29 (A), HCT116 (B), and Caco-2 (C) human colon cancer cells. The cells were seeded in 6-well plates and then treated with REV or PTS at the concentrations indicated in the figure. The media containing REV, PTS, or vehicle control were changed every other day. After 10 days of incubation, the colonies were stained with crystal violet and quantified as described under Materials and Methods. The amount of colonies formed by control cells was set as 100%. Data are expressed as the mean ± standard deviation. In the bar figures, the different letters indicate statistical difference based on ANOVA analysis ($p < 0.01$).

quantified using a BCA protein assay kit (Thermo Scientific, Rockford, IL), and 20−50 μg of protein was resolved by SDS-PAGE and transferred to a nitrocellulose membrane. After blocking, proteins of interest were probed using different antibodies at the manufacturer's recommended concentrations (1:500−1:1000) and then visualized using enhanced chemiluminescence (Boston Bioproducts). Antibodies for β-actin, cleaved PARP, and cleaved caspase-3 were from Cell Signaling Technology, Inc.

Colony Formation Assay. HCT116 (750 cells/well), HT29 (750 cells/well), and Caco-2 (750 cells/well) cells were seeded in 6-well tissue culture plates. After 24 h of incubation for adhesion, the media were replaced with treatment media containing serial concentrations of resveratrol or pterostilbene. The treatment media were refreshed every other day. After 10 days of incubation, the colonies were stained with crystal violet solution (0.2% crystal violet in 2% ethanol) for 10 min, followed by a gentle rinse with warm tap water to remove any free dye. One milliliter of SDS solution (0.5% SDS in 50% EtOH) was added to each well to solublize the bound dye, and the absorbance of the solution was measured at 570 nm using a microplate reader (Elx800 absorbance microplate reader, BioTek Instrument, Inc.) to quantify colonies formed.[17]

Cellular Uptake Assay. Colon cancer cells (2×10^5 cells) were suspended in 1 mL of culture media containing different concentrations of resveratrol or pterostilbene in glass culture tube and then incubated at 37 °C with 5% CO_2 and 95% air for 0.5, 1, or 2 h. The cell suspensions were centrifuged in a benchtop centrifuge at 491 RCF for 2 min at 4 °C. Supernatant was removed, and cells were suspended in 1 mL of ice-cold PBS and then centrifuged at 491 RCF for 1 min at 4 °C. After the supernatant was removed, 1 mL of buffer (pH 7.5, 10 mM-Tris-HCl, 1 mM EDTA, 1 mM $MgCl_2$) was added to suspend the cells. The cell suspension was kept on ice for 5 min before cells were sonicated with a probe sonicator (5 s, three times). After another centrifugation (15325 RCF, 25 min at 4 °C), the supernatant was collected as cytosol fraction. An appropriate amount of MeOH was added to the cytosol fraction to make the final MeOH concentration 40%. An equal volume of ethyl acetate was then used to extract resveratrol and pterostilbene (twice). The pooled ethyl acetate fractions were evaporated to dryness, reconstituted in 50% MeOH, and then analyzed using a HPLC method reported previously.[18]

Statistical Analysis. All results are expressed as the mean ± standard deviation (SD). The statistical significance of the mean difference between two groups was calculated by using Student's two-tailed t test. An analysis of variance (ANOVA) model was used when more than two groups' differences were compared. A significance level of $p < 0.01$ was used for all tests.

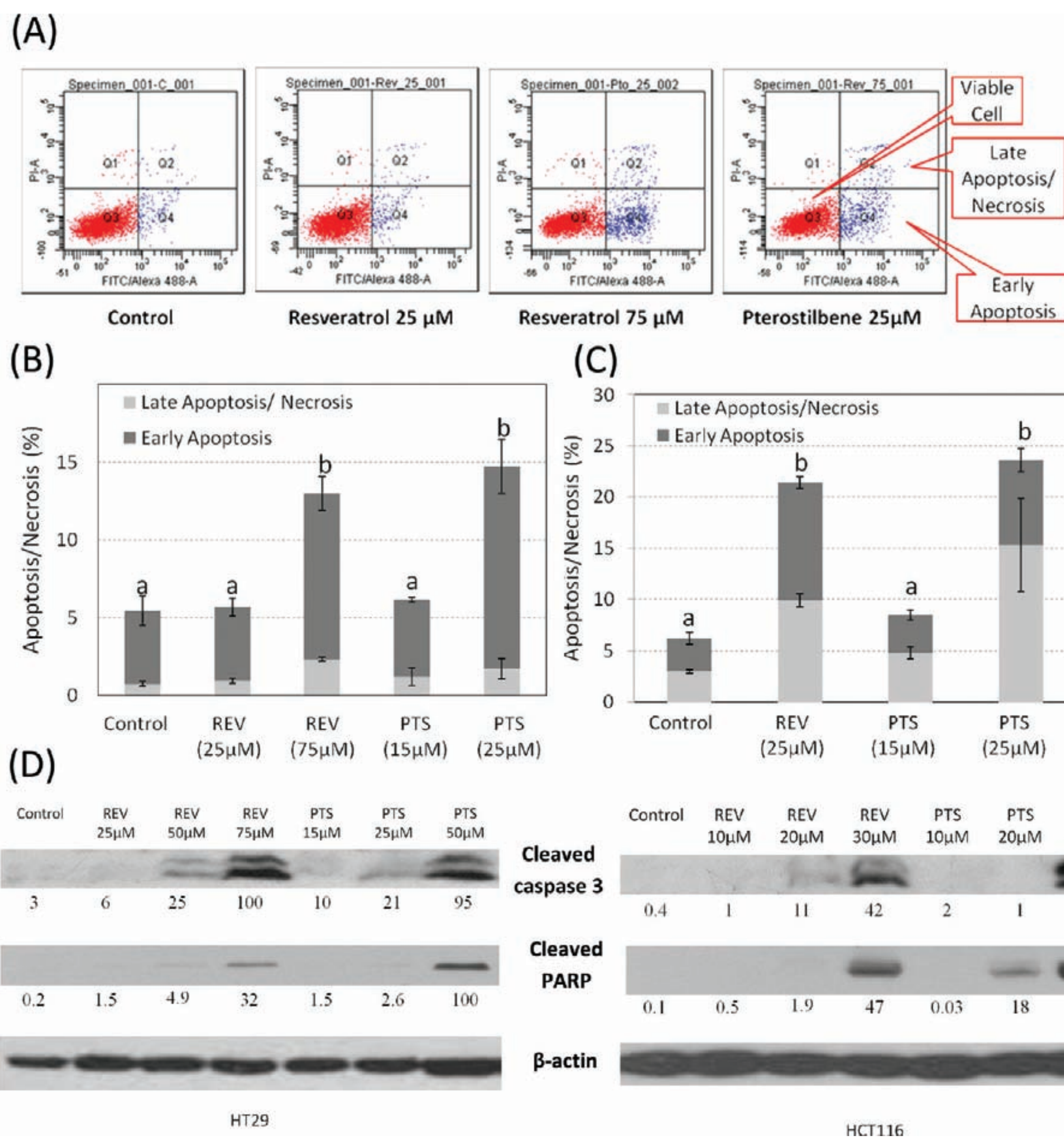

Figure 4. Effects of resveratrol (REV) and pterostilbene (PTS) on apoptosis of colon cancer cells. Cells were treated with serial concentrations of REV and PTS for 48 h and then subjected to annexin V/PI costaining assay. (A) Annexin V/PI costaining dot plots of HT29 after treatments with REV or PTS. Quantification of early and late apoptosis after different treatments is shown in (A) HT29 and (C) HCT116 cells. (D) Immunoblot of cleaved caspase-3 and cleaved PARP in HT29 and HCT116 cells after treatments with REV and PTS. The numbers below the blots represent band intensities (normalized to the loading controls, means of three independent experiments) measured by Image J software. The standard deviations (all within ±15% of the means) are not shown. The experiments were repeated three times. β-Actin were used as equal loading control.

RESULTS

Pterostilbene Is More Potent than Resveratrol in Inhibiting Colon Cancer Cell Growth and Colony Formation. We compared the inhibitory effects of REV and PTS on three colon cancer cell lines using a cell viability (MTT) assay. The results showed that REV and PTS caused dose-dependent inhibition of the cell viability of all three cancer cells (Figure 2). Among these cells, HCT116 cells were the most sensitive to REV and PTS treatments, whereas Caco-2 cells were the least sensitive. All three cancer cells were more sensitive to PTS treatments in comparison with REV treatments. Pterostilbene showed IC_{50} of about 15 μM in HT29 cells, whereas the IC_{50} of resveratrol was 4.3-fold higher (about 65 μM). In HCT116 and Caco-2 cells, the IC_{50} values of PTS were about 12 and 75 μM, respectively, and the IC_{50} values of REV were about 25 and >100 μM, respectively.

Next, we determined the inhibitory effects of pterostilbene and resveratrol on colony formation of colon cancer cells. After 10 days of incubation, all three cancer cells produced substantial colonies in vehicle-treated controls (Figure 3). The sensitivity of three cancer cells to the PTS and REV treatments was in the order Caco-2 > HT29 > HCT116. Pterostilbene showed stronger inhibitory effects on all three cancer cells in comparison with REV. For example, PTS at 10 μM suppressed the colony formation of HT29 and HCT116 cells by 85 and 40%, respectively, whereas REV at the same concentration decreased colony

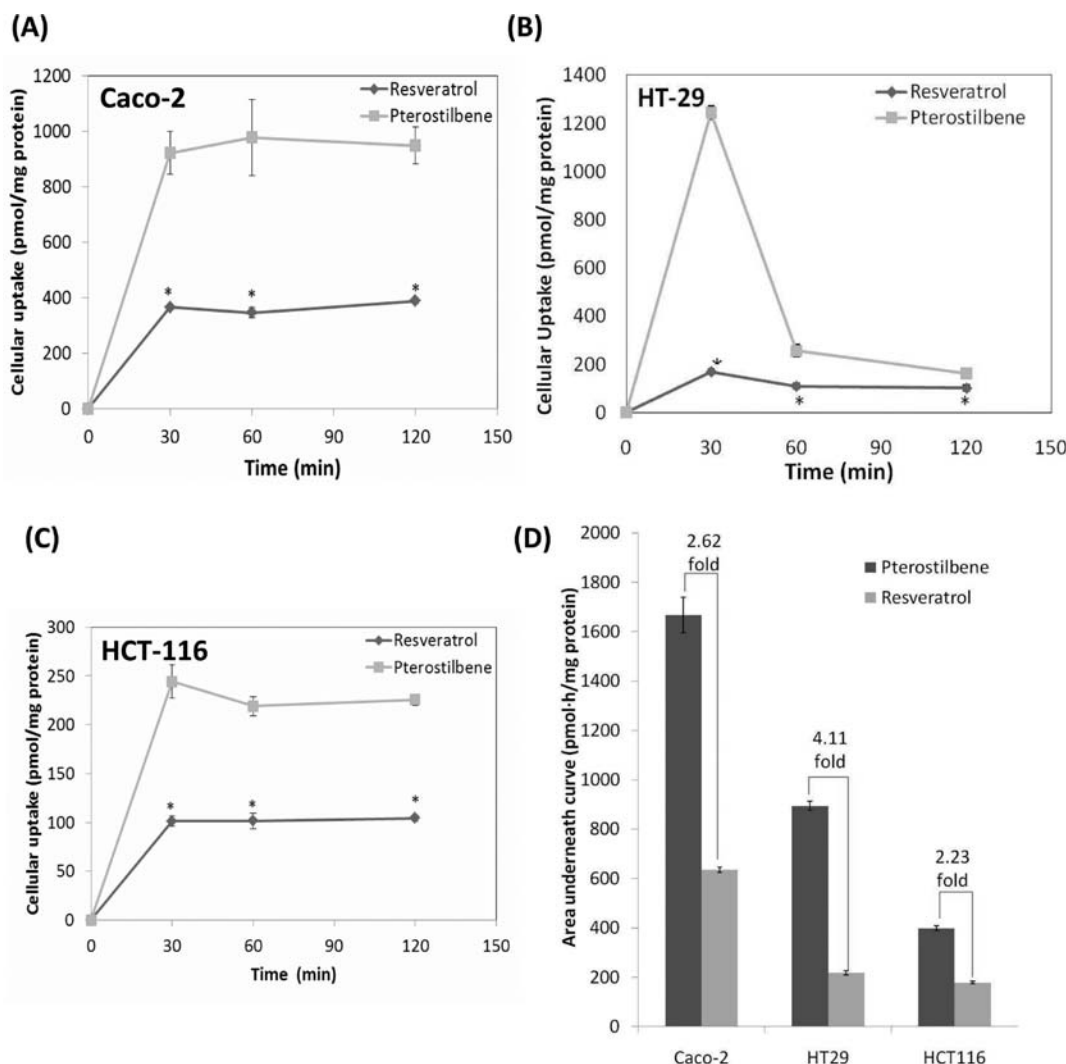

Figure 5. Intracellular levels of pterostilbene and resveratrol in colon cancer cells as indicated by cellular uptake of resveratrol and pterostilbene in the cytosol of Caco-2 (A), HT29 (B), and HCT116 (C) human colon cancer cell lines. Colon cancer cells were incubated with 10 μM resveratrol or pterostilbene for 0.5, 1.0, or 2.0 h. The intracellular concentrations of resveratrol and pterostilbene were measured by the HPLC method in Caco-2 (A), HT29 (B), and HCT116 (C) cells. Data are expressed as the mean ± standard deviation (SD). The experiments were repeated three times. All results for resveratrol and pterostilbene at the same concentrations were significantly different from each other (∗, $p < 0.01$).

formation by only 32 and 9%, respectively. In Caco-2 cells, PTS and REV at 5 μM caused 79 and 54% inhibition in colony formation, respectively.

Pterostilbene Shows Stronger Inducing Effects on Apoptosis in Colon Cancer Cells than Resveratrol. To determine the mechanism by which PTS and REV inhibit the growth of cancer cells, we determined the extent to which treatments of REV or PTS induced apoptosis in colon cancer cells. After 48 h treatments, cancer cells were subjected to double-staining by annexin V and PI to quantify early and late apoptosis by flow cytometry (Figure 4A−C). Representative dot plots from the double-staining assay are shown in Figure 4A. In HT29 cells, REV at 25 μM did not significantly induce apoptosis, whereas PTS at the same concentration was able to increase the level of apoptotic cell population to 14.7% (2.7-fold of the control). This level was even higher than that produced by REV at 75 μM (Figure 4A,B). In HCT116 cells, PTS showed similar level of inducing effects on total apoptotic cell population than resveratrol at the same concentration.

We further determined the effects of pterostilbene and resveratrol on the levels of two key proapoptotic proteins, that is, cleaved caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP) proteins. Western blot analysis demonstrated that PTS had much stronger effects in increasing the levels of cleaved caspase-3 and PARP, especially cleaved PARP, than did REV. For example, in HT29 cells, PTS at 50 μM increased the level of cleaved caspase-3 to an extent that was about 4-fold of that produced by REV at 50 μM and similar to that produced by REV at 75 μM (Figure 4D, left panel). Moreover, PTS at 50 μM is about 20 and 3 times stronger than REV at 50 and 75 μM, respectively, in inducing the cleavage of PARP protein. In HCT116 cells, PTS at 30 μM was about 2-fold more potent that REV at the same concentration in inducing the cleavage of caspase-3 and PARP proteins (Figure 4D, right panel).

Pterostilbene Shows Superior Bioavailability to Resveratrol in Colon Cancer Cells. Bioavailability is an important factor dictating the bioactivity of food components in human. Using an HPLC method, we determined the intracellular levels (bioavailable

fraction to the cancer cell) of PTS and REV in colon cancer cells after incubation with the two compounds at 10 μM for 0.5, 1.0, or 2.0 h. In Caco-2 cells, the intracellular levels of PTS were much higher than those of REV at all three time points, and the levels of PTS and REV remained relatively constant from 0.5 to 2 h (Figure 5A). Similar results were observed in HCT116 cells (Figure 5C). In HT29 cells, the level of PTS at 0.5 h was the highest followed by a drastic drop (5−6-fold lower) at 1.0 and 2.0 h (Figure 5B). Nevertheless, the levels of PTS were much higher than those of REV at all three time points. As shown in Figure 5D, the area under the curve (AUC) was calculated for REV and PTS from 0 to 2.0 h on the basis of the results from Figure 5A−C. The results demonstrated that the accumulated amount of intracellular PTS was much higher than that of REV in all three colon cancer cells. Specifically, the AUC values of PTS were 2.6-, 4.1-, and 2.2-fold higher than those of REV in Caco-2, HT29, and HCT116 cells, respectively.

DISCUSSION

As a stilbene compound, resveratrol has been widely studied because of its various health-promoting potentials, whereas in recent years, pterostilbene has attracted increasing attention in terms of its unique biochemical properties. Resveratrol and pterostilbene share an identical stilbene core structure, but differ in functional group; that is, resveratrol possesses a 3,5-dihydroxy motif in the A ring, whereas pterostilbene has a 3,5-dimethoxy motif instead. Previous papers have suggested that chemical structure modification such as methoxylation could enhance the bioactivities of stilbene compounds.[19,20] Herein, we compared the effects of REV and PTS in multiple human colon cancer cells.

We determined the inhibitory effects of pterostilbene and resveratrol on the growth of three human colon cancer cells, that is, HCT116, HT29, and Caco-2 cells. These cells have different genetic aberrations and gained different growth aggressiveness. For example, HCT116 cells contain wild type APC, mutant β-catenin, and mutant K-RAS; HT29 cells are APC-null but have wild type β-catenin and wild type K-RAS protein, whereas Caco-2 cells contain mutant APC, mutant β-catenin, and wild type K-RAS. Consequently, they showed different sensitivities to the treatments of PTS and REV. However, the results from cell viability assay demonstrated that all three types of cancer cells were more sensitive to PTS treatments than REV treatments. These findings are consistent with a previous study showing that pterostilbene had stronger inhibitory effects on ^{3}H incorporation into the DNA of cancer cells.[21] We further demonstrated that both REV and PTS significantly reduced the capacity of all three types of human colon cancer cells to form colonies. These results indicated that both compounds were able to inhibit anchorage-dependent growth, which is the early process of cancer transformation.[22] More importantly, our results showed that pterostilbene had stronger inhibitory effects on colony formation in comparison to resveratrol in all three cancer cells tested. Overall, our results convincingly demonstrated that PTS is a superior inhibitor to REV on the proliferation and expansion of all three types of human colon cancer cells in culture.

To establish the mechanism by which REV and PTS inhibit colon cancer cell growth, we studied the effects of the two compounds on cellular apoptosis. As a programmed cell death process, apoptosis is a mechanism the organism uses to eliminate unwanted or damaged cells. During cancer development, adequate mutations can allow the mutated cells to evade apoptosis and become cancerous; thus, the induction of apoptosis in precancerous and cancer cells is an effective strategy for cancer treatment and prevention.[23] In the annexin V/PI costaining assay, we observed that both REV and PTS were able to increase apoptotic cell population. Moreover, PTS showed stronger capacity in inducing cellular apoptosis than REV in both HT29 and HCT116 cells. This was evidenced by increased annexin V positive cell population and cleavage of key apoptosis-related proteins, that is, caspase-3 and PARP. Caspase-3 is the key player in both death receptor-mediated and mitochondria-mediated apoptosis. The cleavage of caspase-3 activates the protein and results in proteolytic cleavage of downstream proteins, such as PARP. PARP plays an important role in DNA repair; thus, the inactivation (cleavage) of PARP by caspase-3 results in the accumulation of unrepaired DNA and eventually leads to cell death.[24] Pterostilbene was also found to be more effective than resveratrol in inducing apoptosis in other types of cancer cells such as Fas-ligand resistant lymphoma cells (HUT78B1 and HUT78B3) and multi-drug-resistant leukemia cells (HL60-R and K562-ADR).[25,26]

Bioavailability is an important factor that can dictate the efficacy of bioactive dietary components. Only bioavailable fractions of dietary compounds can be accessible to the target cells and/or tissues and subsequently be bioactive to these cells and/or tissues. We measured the intracellular levels of PTS and REV in colon cancer cells after incubation with the two agents separately. These intracellular levels are indicators of the cellular uptake efficacy of PTS and REV by cancer cells. Our results demonstrated that, in all three colon cancer cells, the intracellular levels of PTS were much higher than those of REV after incubation with PTS or REV at the same concentration. This suggests that cancer cells can take up PTS more efficiently than they can REV and that PTS is more bioavailable to these cells than REV. Recently, the bioavailabilities of PTS and REV were compared in rats after oral gavage at the same doses, and the results showed that plasma levels of PTS were markedly greater than plasma levels of REV.[27] In our study, the higher intracellular levels of PTS than of REV found in colon cancer cells may be associated with the higher lipophilicity of PTS due to substitution of two hydroxyl groups by methoxyl groups in comparison with REV.[14,28] Increased lipophilicity may promote binding of PTS to the plasma membrane, which in turn may increase the uptake of PTS into the cytosol of the cells. It is also possible that colon cancer cells have different preference in taking up PTS and REV. The exact mechanism by which the methoxylation may modulate the bioavailability of PTS is an attractive topic for future investigation. As a consequence of better cellular uptake, the higher intracellular levels of PTS can cause more potent inhibitory effects on colon cancer cells in comparison with REV. Another possible reason for the superior inhibitory effects of PTS as compared with REV on colon cancer cells is that PTS may have higher binding affinity than REV to the same target signaling proteins and/or PTS may have target binding proteins different from those of REV due to the difference in their chemical structures. The interaction between PTS and these target signaling proteins may cause downstream events that eventually lead to apoptosis and growth inhibition. Identification of these target proteins is a challenging yet promising area that warrants more future research.

Altogether, our study reveals the superior anticarcinogenic effects of PTS to REV in three human colon cancer cells in culture. These effects include inhibition of cell viability, inhibition of colony

formation capacity, and induction of apoptosis. Our results also demonstrated that the higher cellular uptake of PTS by cancer cells in comparison to REV may contribute to the superior anticarcinogenic effects of PTS to REV. This study supports the notion that pterostilbene is a promising cancer-fighting dietary component due to its stronger anticarcinogenic effects and better bioavailability in comparison to resveratrol.

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ABBREVIATIONS USED

AEBSF, 4-(2-aminoethylbenzenesulfonyl fluoride hydrochloride; ATCC, American Type Cell Collection; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PTS, pterostilbene; REV, resveratrol.

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