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NATURAL DRUGS

THE INDUCTION OF CYTOTOXICITY BY PTEROSTILBENE IN VARIOUS HUMAN CANCER CELL LINES

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Abstract: Pterostilbene is a naturally occurring compound found primarily in blueberries and grapes. It has been found to possess several biological activities such as antioxidative and anti-inflammatory. The study evaluated the cytotoxic activity of pterostilbene in various human cancer cell lines, i.e., melanoma (A2058, C32), colon carcinoma (HT-29, SW1116), breast adenocarcinoma (MCF-7, SKBR3) and ovary adenocarcinoma (SKOV3). The cells were treated with pterostilbene concentrations ranging from 5 to 75 μM for 72 h. The cytotoxicity of pterostilbene was evaluated using the Sulforhodamine B assay and expressed as a percentage of that of untreated control cells. The concentration of pterostilbene required for 50% reduction of cell viability (IC_{50}) was calculated from log dose-response curves. The results of this study showed that pterostilbene exerted dose-dependent cytotoxic effect on cancer cells. Among the all cell lines tested, the C32 melanoma cells were the most sensitive to the cytotoxic effect of pterostilbene (IC_{50} ~10 μM) and SW1116 colon cancer cells showed the lowest sensitivity (IC_{50} ~70 μM).

Keywords: pterostilbene, cytotoxic activity, SRB assay, cancer cell lines

Cancer is one of the major public health problems all over the world, as it is one of the leading causes of death worldwide (1, 2). Epidemiological studies have consistently shown that consumption of a diet including fruits and vegetables is strongly associated with reduced risk of cancer (3). A large number of plants and their isolated constituents have been shown to possess potential anticancer activity (4). Stilbenes are phytochemicals present in berries, grapes, peanuts, and red wine. Recently, these compounds have attracted increasing attention and interest due to their wide range of health-beneficial effects (5). A widely studied stilbene, resveratrol, has been shown to exert antioxidant, anti-inflammatory, chemopreventive and anti-aging effects in many biological systems. Resveratrol is potentially capable of inhibiting carcinogenesis at the stages of initiation, promotion and progression (6). Pterostilbene (trans-3,5-dimethoxy-4'-hydroxystilbene) is a natural analog of resveratrol showing higher bioavailability and longer half-life *in vivo* than the latter (7) which makes it a promising dietary factor for chemoprevention (8). Due to its close structural similarity to resveratrol pterostilbene possesses resveratrol-like health benefits (9). Recent studies showed that pterostilbene exhibited the hallmark characteristics of a valuable anticancer agent including modulation of expression of phase II detoxifying enzymes, regulation of aberrant cell cycle or induction of cell death (10, 11, 12). Despite the reports on the biological activity of pterostilbene, data on its cytotoxicity against cancer cells are still limited. Therefore, the main goal of this study was to investigate the cytotoxicity of pterostilbene in human cancer cells of different origin, i.e., tumor cells derived from colon, breast and ovary as well as malignant melanoma cells and to compare their sensitivity to pterostilbene. Various cell lines may differ in their sensitivity towards this stilbene, so the use of a broader spectrum of cell lines was considered to ensure better insight into its cytotoxic activity. In addition, the study aimed at evaluating whether the sensitivity of breast and ovarian cancer cells to pterostilbene could be correlated to their ER and HER2 status.

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EXPERIMENTAL

Cell lines and culture conditions

The seven selected cancer cell lines used in this research were derived from human: ovary adenocarcinoma (SKOV3, ATCC No. HTB-77) breast adenocarcinoma (SKBR3 ATCC No. HTB-30; MCF-7 ATCC No. HTB-22) colon carcinoma (HT-29 ATCC No. HTB-38; SW1116 ATTC No. CCL-233) and melanoma (A2058 ATCC No. CRL-11147; C32 ATCC No. CRL-1585). All cell lines were obtained from LGC Promochem (Łomianki, Poland). The five cancer cell lines were cultured in MEM medium (Sigma Aldrich) supplemented with 10% fetal bovine serum (PanBiotech), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma Aldrich), while the two cell lines (SKBR3 and SKOV3) were cultured in McCoy's medium (Sigma Aldrich) containing 10% fetal bovine serum (PanBiotech), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma Aldrich). The cell cultures were cultivated as monolayers at 37°C in a humidified atmosphere containing 5% CO_2.

Preparation of pterostilbene stock solution

Pterostilbene was purchased from Sigma Aldrich (Cat. number P1499, purity = 97%). The stock solution of pterostilbene was prepared in dimethyl sulfoxide (DMSO) and further diluted in sterile culture medium to desired concentrations immediately before use. The final DMSO concentration in the working solutions was 0.1%.

Cytotoxicity assay

The cytotoxic effect of pterostilbene on cancer cells was analyzed by using an *in vitro* toxicology assay kit, which is Sulforhodamine B (SRB) based (Sigma Aldrich). The SRB is a dye that binds electrostatically to cellular proteins under mild acidic conditions. The amount of dye incorporated into protein and extracted under basic conditions was measured colorimetrically. The absorbance value is related proportionally to the total biomass and consequently, cell number (13). Cells were seeded in 96-well plates at an initial density of 2 000–5 000 cells (depending upon the cell line) in 200 µL of culture medium and allowed to adhere and grow for 24 h. The medium was then removed from each well and replaced with fresh medium containing pterostilbene (5, 10, 20, 40, 50, 60, 75 µM) and the cells were cultured for 72 h. After removal of culture media from the wells, the cells were washed with phosphate buffered saline (PBS) and fixed in 10% trichloroacetic acid, followed by 5 washes with deionized water. Cells were stained with 0.4% SRB for 30 min. Afterwards, plates were washed with 1% acetic acid and air-dried. After the liberation of incorporated SRB with 10 mM Tris-HCl, absorbance was measured at 570 nm and 690 nm (reference wavelength) using the multiplate reader Labtech LT-5000. The viability of treated cells was expressed as a percentage of untreated control cells. The drug concentration that reduced the viability of cells by 50% (IC_{50}) compared to untreated controls was determined by fitting a four-parameter logistic model (Hill equation) using computer curve-fitting software (GraphPad Prism ver. 7, San Diego, USA) to the experimental data.

Statistical analysis

Statistical analysis was performed with the use of Statistica PL ver. 12.0 Software (StatSoft). The examined parameters were first evaluated for normal distribution (Shapiro-Wilk test). One-way analysis of variance (ANOVA) with Tukey's *post-hoc* test was used to evaluate significances between examined groups. Results were expressed as the means ± standard deviation (SD). Differences with a probability (p) value less than 0.05 were considered statistically significant.

RESULTS

Seven different human cancer cell lines were used to screen for the cytotoxic activity of pterostilbene *in vitro*. Melanoma (A2058, C32), colon carcinoma (HT-29, SW1116), breast adenocarcinoma (MCF-7, SKBR3) and ovary adenocarcinoma (SKOV3) cells were treated with increasing concentrations of pterostilbene (5-75 µM) for 72 h and viable cells were detected with SRB assay. Pterostilbene reduced cell viability in all cell lines tested here and in a concentration-dependent fashion (Fig. 1). In addition, Figure 1 shows the respective IC_{50} value for pterostilbene in each cell line, calculated from these concentration-response curves.

The cytotoxic effect of pterostilbene on HT-29 and SW1116 colon carcinoma cells is shown in Figures 1A and 1B. At a concentration of 5 µM, it did not affect colon carcinoma cell viability. A significant viability suppression of both cell lines was observed at higher concentrations (≥ 10 µM) of pterostilbene. Furthermore, the much stronger inhibitory effect was observed in HT-29 cells than SW1116 cells. At 75 µM pterostilbene, the viability of HT-29 and SW1116 cells was reduced by 95.4% and 50%, respectively. The IC_{50} values of about 20 µM and 70 µM for HT-29 and SW1116 cells respectively, reflected their different sensitivity to pterostilbene.

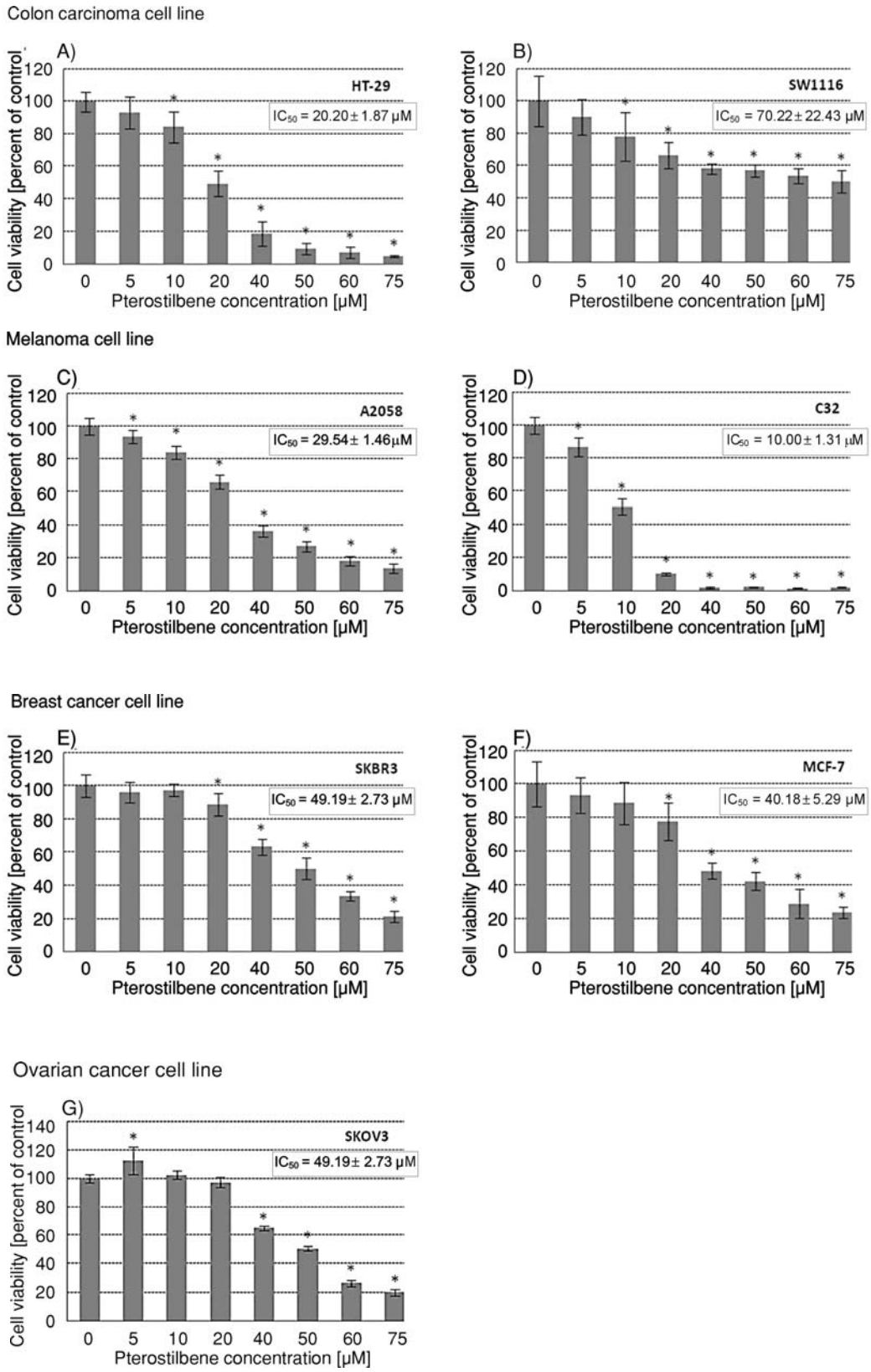

Figure 1. Cytotoxic effect of pterostilbene on human cancer cells after 72 h treatment. The results are expressed as percentage of untreated control (the means ± SD; *$p < 0.05$ vs. control)

The A2058 and C32 cell lines were used as an experimental *in vitro* model of skin *melanoma malignum*. These cell lines originate from different melanoma types: melanotic (A2058) and amelanotic (C32). Pterostilbene at the all concentrations used (5-75 μM) significantly suppressed the viability of both A2058 (Fig. 1C) and C32 (Fig. 1D) cells in a concentration-dependent manner. Amelanotic C32 cells were more sensitive to pterostilbene than melanotic A2058 cells, showing above 80% inhibition at relatively low concentration of 20 μM pterostilbene, whereas in A2058 cell cultures comparable cytotoxicity was achieved with its higher concentrations (60 and 75 μM). The IC_{50} value for pterostilbene was almost 3-fold higher in A2058 cells than in C32 cells.

Cytotoxicity of pterostilbene in SKBR3 and MCF-7 adenocarcinoma cells is demonstrated in Figures 1E and 1F. Pterostilbene at the concentrations up to 10 μM did not affect SKBR3 and MCF-7 cell viability. A substantial cell viability reduction was observed in cells incubated with higher concentrations (≥ 20 μM). The maximum decrease of SKBR3 and MCF-7 cell viability versus corresponding control cultures (78.6% and 76.5% respectively) was evoked by the highest dose of pterostilbene. For SKBR3 cell line, the IC_{50} was 49.19 μM. The comparable value was obtained for MCF-7 cells (40.18 μM).

The SKOV3 cell line was used as an experimental *in vitro* model of ovary adenocarcinoma. The experimental data presented in Figure 1G indicate that the exposure of SKOV3 cells to pterostilbene exhibited a dual effect. Pterostilbene at a concentration of 5 mM induced an increase in cell viability, while at higher concentrations (≥ 40 μM) it was cytotoxic. The most pronounced reduction of cell viability by pterostilbene was observed in cell cultures treated with 75 μM of pterostilbene. The IC_{50} for stilbene was found to be 47.03 μM.

DISCUSSION

Pterostilbene is a natural methoxylated resveratrol derivative. It has higher biostability due to slower metabolism and lower excretion rate in comparison to other stilbenes, which renders it a better potential health-promoting nutraceutical (14). Recent studies have shown that pterostilbene could be a novel promising chemopreventive and chemotherapeutic agent (15). The mechanisms underlying its anticancer activity have not been fully elucidated and are believed to include e.g. anti-oxidative, anti-proliferative and cytotoxic effects. The antioxidant properties of pterostilbene are assumed to enable this compound to protect cells from oxidative damage. Pterostilbene may exhibit cancer cell death via apoptosis and autophagy and apoptosis rather is postulated as the main mechanism of its cytotoxic activity (10, 16, 17). Prooxidant properties of pterostilbene, particularly at higher concentrations, are held likely to be responsible for its pro-apoptotic effects. Pterostilbene has been shown to induce the intrinsic apoptotic pathway in cancer cells by increasing intracellular ROS production, mitochondrial depolarization, regulation of mitochondrial proteins Bcl-2, Bax, cytochrome c and caspase cascade activation. Proapoptotic mechanism of pterostilbene also may include extrinsic pathway (10, 18). Moreover, pterostilbene-induced cell death may also occur via the caspase-independent mechanism. Recent studies revealed that pterostilbene induced lysosomal membrane permeabilization leading to the activation of caspase-independent lysosomal cell death program (19). Other studies have shown the cytotoxic and growth inhibitory effect of pterostilbene on cancer cells such as lymphoma (18), liver (20), lung (21) and glioma (22) cancer cells. The present experiments were aimed at comparing the cytotoxic activity of pterostilbene among several types of human cancer cell lines. The concentration range of 5 to 75 μM was selected for testing based on a report of plasma levels of pterostilbene after iv administration in mice wherein plasma levels of pterostilbene were observed to vary from 1 to 116 μM over the range of 5 min to 480 min (23). Detectable concentrations of pterostilbene and its glucuronidated metabolite (0.05-100 μg/mL) were also detected in rat's serum up to six hours after oral consumption (24). It was also reported that the levels of pterostilbene consumed with the diet exceeded 20 μM in mice colonic mucosa (25). Only a few studies evaluated the safety of pterostilbene administration to humans thus far (14), but to our knowledge, there is no report on serum levels of pterostilbene in humans. Currently, it is not known whether oral consumption of pterostilbene might be sufficient to elicit its levels commensurate with cancer chemopreventive efficiency. However, it is important to note that metabolites of pterostilbene may also contribute to biological effects of this stilbene (25).

Pterostilbene reduced cell viability in all tested cell lines in a concentration-dependent manner. Cells from different origins showed different sensitivities to pterostilbene. The statistically important decrease of cell viability in melanoma cells (C32 and A2058) was evoked by pterostilbene at concentration 5 μM whereas in ovarian cancer cells (SKOV3) by 40 μM.

Since pterostilbene may easily be included in the human diet, its potential activity against human colon cancer cells is worth closer investigation. The current study demonstrates the cytotoxic activity of pterostilbene (at concentrations = 10 μM) on two colon cancer cell lines with IC_{50} values of 20.20 μM (HT-29) and 70.22 μM (SW1116). Other literature has confirmed the cytotoxic activity of pterostilbene on colon cancer cells HT-29 (IC_{50} = 23.8 μM) after 72 h treatment (26). The weaker cytotoxic effect of pterostilbene on HT-29 cells (higher IC_{50} values) was observed by Mena et al. (19) and Sun et al. (25) which could likely be due to a shorter incubation period of treatment (48 h). Studies carried out by Harun and Ghazali (27) did not support the cytotoxic effect of pterostilbene at concentrations up to 100 μM against HT-29 cells after 24 treatment. Pterostilbene was also reported to reduce the viability of other colon cancer cell lines such as HCT1116 (25) and Caco-2 (26). As observed in this study, a more pronounced cytotoxic effect of pterostilbene was observed against HT-29 cells presenting low expression of enzyme cyclooxygenase-2 (COX-2) than against SW1116 cells with its high expression. COX-2 plays a crucial role in the inflammation process, cell proliferation and colon carcinogenesis (28). Since Chiou et al. (11) reported marked decrease of azoxymethane-induced COX-2 expression in mice following dietary pterostilbene intake, determination of the possible COX-2 pathway involvement in cytotoxic activity of pterostilbene in SW1116 and HT-29 cells is warranted.

Thus far, limited studies have investigated cytotoxic and antitumor effects of pterostilbene on skin cancer including melanoma (21). The current study showed that pterostilbene at all concentrations exerted cytotoxic activity against both amelanotic and melanotic melanoma cells after 72 h treatment. The most pronounced effect was exerted by pterostilbene against the C32 amelanotic melanoma cells. The strongest cytotoxicity was observed with 40 to 75 μM of pterostilbene and IC_{50} for C32 cell line was 10 μM and it was 2.9-fold lower than that for A2058 cell line. Other research supports cytotoxic effects of this stilbene against highly invasive SK-MEL-2 and MeWo melanoma cells and over a wide range of concentrations (10-100 μM) (29). On the other hand, Benlloch at al. (23) reported that pterostilbene at low concentrations (1-5 μM) does not alter melanoma cell growth and viability after 72 h.

In the present experiment, the cytotoxic effect of pterostilbene on breast and ovarian cancer cells with different expression of estrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2) was also evaluated. ER and HER2 play pivotal role in those cancer cells and their expression is an important factor in determining the therapy effectiveness of breast and ovarian cancer (30, 31, 32). Pterostilbene significantly decreased viability of MCF-7 ($ER^+/HER2^-$; responsive to estradiol) and SKBR3 ($ER^-/HER2^+$; nonresponsive to estradiol) breast cancer cells as well as SKOV3 ($ER^-/HER2^+$, nonresponsive to estradiol) ovarian cancer cells. The obtained experimental IC_{50} values for all these cells were similar, thus, the cytotoxic effect of pterostilbene appears not to be associated with their ER/HER2 status. These data concur with the results published by Alosi et al. (33) who demonstrated that pterostilbene (20-100 μM) induced a significant concentration and time-dependent growth decrease of MDA-MB-231 and MCF-7 breast cancer cells. In the other studies, pterostilbene offered cytotoxic effect against SKOV3 cells at a concentration of IC_{50} about 55 μM (34).

It is worth mentioning that pterostilbene exhibited a dual effect on the viability of SKOV3 cells. At a lower concentration (5 μM) it stimulated while at higher concentration (≥ 40 μM) it reduced SKOV3 cell viability. A similar biphasic activity of pterostilbene was also observed towards primary macrophages (35). This biphasic dose-response model (characterized by a low-dose stimulation and a high-dose inhibition) on human tumor cell lines has also been demonstrated for other stilbenes: resveratrol (36, 37) and piceatannol (38). Based on those reports it is important to perform more detailed studies of biological activity of stilbenes in a wide range of concentration.

The present results indicate that pterostilbene may be a potential anti-cancer agent because it decreases the viability of all tested colon cancer cell lines. Melanoma C32 cells, followed by colon carcinoma HT-29 cells and melanoma A2058 were the most sensitive cells to pterostilbene, with IC_{50} values lower than 30 μM, while colon carcinoma SW1116 cells were the least sensitive. Although we did not determine whether it was selectively cytotoxic for neoplastic cells, Sun et al. (25) demonstrated that the growth of normal human colon fibroblasts was not altered following 24 and 48-hour exposure to lower concentrations (5-40 μM) of pterostilbene. This result is also consistent with the findings of Dewi et al. (39) who did not observe the antiproliferative activity of pterostilbene (12.5-100 μM) against normal myofibroblasts and skin fibroblasts.

In conclusion, the present results showed the cytotoxic effect of pterostilbene against cancer cells derived from four of the most prevalent cancers: colon, melanoma, breast and ovary cancer. It can

also be concluded that cytotoxicity induced by this stilbene in breast and ovarian cancer cells is not associated with their ER and HER2 status. However, further studies must be done to seek for the molecular mechanisms of its pterostilbene activity.

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