

RESEARCH ARTICLE

Potential Chemoprevention Activity of Pterostilbene by Enhancing the Detoxifying Enzymes in the HT-29 Cell Line

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Abstract

Detoxifying enzymes are present in most epithelial cells of the human gastrointestinal tract where they protect against xenobiotics which may cause cancer. Induction of examples such as glutathione S-transferase (GST) and its thiol conjugate, glutathione (GSH) as well as NAD(P)H: quinoneoxidoreductase (NQO1) facilitate the excretion of carcinogens and thus preventing colon carcinogenesis. Pterostilbene, an analogue of resveratrol, has demonstrated numerous pharmacological activities linked with chemoprevention. This study was conducted to investigate the potential of pterostilbene as a chemopreventive agent using the HT-29 colon cancer cell line to study the modulation of GST and NQO1 activities as well as the GSH level. Initially, our group, established the optimum dose of 24 hours pterostilbene treatment using MTT assays. Then, effects of pterostilbene (0-50 μ M) on GST and NQO1 activity and GSH levels were determined using GST, NQO1 and Ellman assays, respectively. MTT assay of pterostilbene (0-100 μ M) showed no cytotoxicity toward the HT-29 cell line. Treatment increased GST activity in the cell line significantly ($p < 0.05$) at 12.5 and 25.0 μ M. In addition, treatment at 50 μ M increased the GSH level significantly ($p < 0.05$). Pterostilbene also enhanced NQO1 activity significantly ($p < 0.05$) at 12.5 μ M and 50 μ M. Hence, pterostilbene is a potential chemopreventive agent capable of modulation of detoxifying enzyme levels in HT-29 cells.

Keywords: Pterostilbene - detoxifying enzymes - glutathione S-transferase - glutathione - HT-29 cell line

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Introduction

Cancer is one of the leading causes of death in Malaysia (National Cancer Registry, 2007). Early cancer screening allows it to be diagnosed at an early stage and may provide a good prognosis (Ott et al., 2009). However, prevention remains the best way to deal with cancer (Shukla and Pal, 2004) due the fact that cancer treatments reduce the life quality of the cancer patients, with side effects accompanying as well as it is reported to be costly (Kroenke et al., 2010). Although cancer is often associated with genetic factors, but other risk factors such as diet, lifestyle, exposure to carcinogens and radiation, viral infections and diseases are modifiable (Świątkowska, 2007; Dossus, 2008). For example, there are a lot of studies have shown a correlation between the consumption of a diet rich in fruits and vegetables can prevent the formation of certain types of cancer.

Phytochemicals are chemicals produced by plants through a specific response to protect plants from pathogen or environmental exposure, such as excessive ultra violet (UV) radiation. Phenolic compounds are one of the phytochemicals that act as phytoalexin in plants and these compounds also have valuable medicinal value.

Resveratrol is a phenolic compound that has been the most studied stilbene for its various biological activities including antioxidant, anti-inflammatory, anticancer and other biological activities (Fremont, 2000). It can be found naturally in grapes, berries and red wine (Bhat and Pezzuto, 2002). Pterostilbene, the natural dimethyl ether analogue of resveratrol has similar biological activity to resveratrol (Remsburg et al., 2008). It is also found in fruits such as grapes and berries. Studies have shown that pterostilbene also has a variety of beneficial biological activities such as antioxidant activity (Pari and Satheesh, 2006) and anti-inflammatory (Paul et al., 2009). In addition, pterostilbene showed anticancer activity against several cancer cells such as breast cancer (Alosi et al., 2010), lung cancer (Schneider et al., 2010) and pancreatic cancer (Mannal et al., 2010). Its potential as a cancer preventive agent has also been found through inhibition of drug metabolizing enzyme cytochrome P450 1A1, 1A2 and 1B1 (Mikstacka et al., 2007).

Thus, this study was conducted to determine the effect of pterostilbene treatment on the modulation of detoxifying enzymes in the colon cell line. Detoxifying enzymes involves in the conjugation or reduction of reactive species into more polar compounds that are easily

excreted (Sheweita and Tilmisany, 2003). Reactive species has been proved to induce oxidative stress resulting in the cellular damage or it eventually can alter the genetic material of a normal cell become cancerous (Ioannides and Lewis, 2004). Thus induction of detoxifying enzymes will give protection to the cell from the action of the reactive species and this can prevent carcinogenesis. Glutathione (GSH), the tripeptide molecule is a substrate that is widely conjugated the electrophilic species intracellularly. Conjugation reaction of the GSH is catalysed by glutathione-S-transferase (GST) enzyme (Sheweita and Tilmisany, 2003; Ionescu and Caira, 2005). In conjunction with GST, the NADPH:quinone oxidoreductase (NQO1) also functions as protective enzyme by catalysing the reduction of reactive species such as quinone, quinonamine and nitro compounds (Siegel et al., 2004).

Materials and Methods

Chemicals

HT-29 cell line was a gift from Dr. Ooi Mei Lee, UKM Molecular Biology Institute (UMBI), Malaysia. The MacCoy's 5A growth media was purchased from Gibco® (USA). The fetal bovine serum (FBS) and Penicillin/Streptomycin were obtained from PAA (Austria). Compounds used in this study including pterostilbene and resveratrol were obtained from Calbiochem® (USA). Other chemicals including Vitamin C, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), β -nicotinamide adenine dinucleotide phosphate (β -NADPH), 2,6-dichlorophenolindolphenol (DCPIP) and dicoumarol was bought from Sigma (USA). The dimethyl sulfoxide (DMSO) and [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) salts were obtained from Fisher Scientific (UK) while ethylenediaminetetraacetic acid (EDTA) was obtained from AJAX Chemicals (Australia). Other chemicals were of the analytical grade.

Cell culture

The HT-29 cell line was cultured in 75 cm² culture flask using modified MacCoy's 5A growth media at 37°C and 5%CO₂. The media was supplemented with 1% of penicillin/streptomycin and 10% Fetal Bovine Serum (FBS). Subculture was performed when cells reached confluent and usually it was done twice a week.

Preparation of sample (whole cell lysate)

About 11 ml of cells suspension (1x10⁵ cells/ml) were seeded onto the culture dish and allowed to attach overnight. Then, the cells were treated with the compounds for 24 hours before harvesting. Finally, the preparation of the sample was done according to method by Inayat-Hussain et al. (2010) for determination of GSH level and GST activities. While, to study NQO1 activity, the sample preparation employed the method by Yakovlev et al. (2004) with slight modification.

Cell viability assay (MTT assay)

The MTT assay was done according to Mossman

(1983). About 200 μ l cells were seeded at concentration of 5x10⁴ cells/ml per well in 96-well plate and allowed to attach overnight at 37°C in 5%CO₂. The cells were then treated with pterostilbene (0-100 μ M) for 24 hours. About 20 μ l MTT solution (5 mg/ml) was added to each well and the plates were incubated at 37°C in the CO₂ incubator for 4 hours. Then, the culture media was removed and finally dimethylsulphoxide (DMSO) (200 μ l) was added to each well to solubilize the purple formazan formed. Absorbance was read at 570 nm with ELISA microplate reader (Bio-Rad, USA).

Ellman assay

Determination of glutathione level was done using method by Ellman (1959). DTNB is a soluble material that is used to determine the sulfhydryl group quantity in a compound. Compounds containing the substance will produce acid 5-thio-2-nitrobenzoic (TNB), which is colored yellow when acting with sulfhydryl. Firstly, the glutathione standard curve was prepared using a serial dilution of 0-10 mM glutathione with two times dilution factor in 96-well plate. Glutathione was diluted in 50 μ l reaction buffer (0.1 M Na₂HPO₄·7H₂O and 1 mM EDTA) pH 6.5. The amount of sample used in this assay is about 50 μ l. The samples were pipetted into empty well. About 50 μ l of reaction buffer (0.1 M Na₂HPO₄·7H₂O and 1 mM EDTA) pH 8.0 was added into well containing glutathione and samples. Finally, 10 μ l DTNB was added to all wells and the microplate was read at 415 nm of wavelength using microplate reader (Bio-Rad, USA). Determination of protein concentration of the samples were done using Bradford assay (1976).

Glutathione S-transferase (GST) assay

The GST activity of the samples was determined using GST assay (Habig and Jakoby, 1981). GST activity was defined as the rate of formation of product (GS-DNB conjugate). The reaction mixture of the GST assay consists of 840 μ l of phosphate buffer (100 mM K₂HPO₄ and 100 mM KH₂PO₄) pH 6.5, 100 μ l GSH (1 mM) and 50 μ l of the sample that were added into a 1 ml cuvet. Approximately about 10 μ l CDNB (75 mM) was added into the reaction mixture to start the reaction. The rate of the reaction (abs/min) was read using UV spectrophotometer (Secomam, Perancis) at 340 nm wavelength for three minutes. The protein concentration of the samples was measured using Bradford assay (1976). The extinction coefficient of 9.6 mM⁻¹ was used to calculate the GST activity of the samples.

NAD(P)H: quinone oxidoreductase (NQO1) assay

Method by Siegel (2000) was employed to study the NQO1 activity. In this assay, the rate of 2,6-dichlorophenolindolphenol (DCPIP) reduction over a period of time is the key principle in determining the NQO1 activity. In addition, dicoumarol was used as the chemical that exerts the competitive inhibition towards the NADPH binding to NQO1 enzyme thus stop the catalytic reaction of the enzyme. The reaction mixture of this assay consisting of 970 μ l reaction buffer (25 mM Tris, 1 mg/ml BSA and 200 μ M NADPH) pH 7.4, 20 μ l of DCPIP solution (2 mM) and 10 μ l samples that was pipetted into

1 ml cuvet. The absorbance was read over one minute using spectrophotometer (Secomam, France) at 600 nm of wave length. On the other hand, for reactions containing dicumarol, about 960 μ l reaction buffer (25 mM Tris, 1 mg/ml BSA and 200 μ M NADPH) pH 7.4, 20 μ l DCPIP solution (2 mM), 10 μ l sample and 10 μ l dicumarol (2 mM) were prepared as a mixture of reactions. Total protein concentration of the samples was determined by Bradford assay (1976).

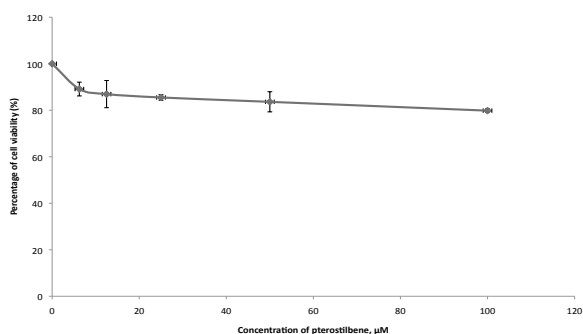


Figure 1. The Cell Viability of the HT-29 Cell Line with Pterostilbene Treatment (0-100 μ M)

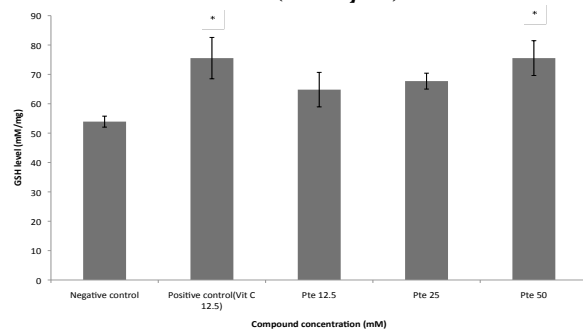


Figure 2. The Effect of Pterostilbene on GSH Level in HT-29 Cell Line

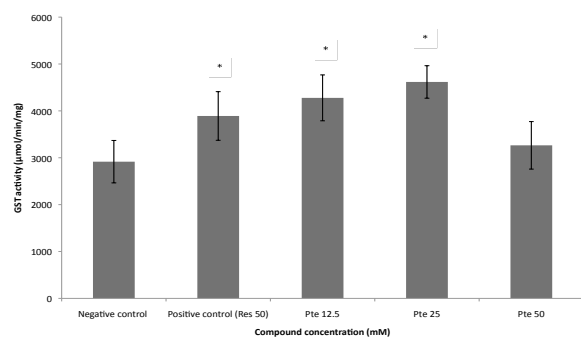


Figure 3. The Effect of Pterostilbene on GST Activity in HT-29 Cell Line

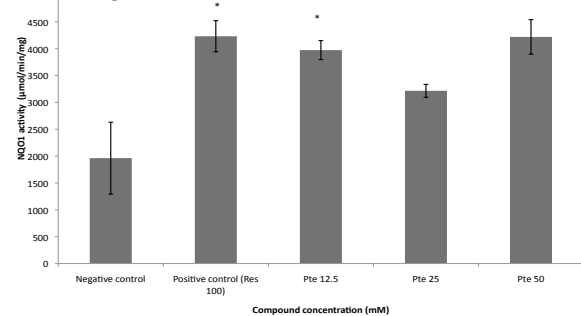


Figure 4. The Effect of Pterostilbene on NQO1 Activity in HT-29 Cell Line

Statistical analysis

Data were analysed statistically using IBM SPSS statistic 20 software (SPSS Inc. Chicago, USA) and were presented as mean \pm SEM.

Results

Result showed that treatment of the pterostilbene in the range of 0-100 μ M did not give the IC₅₀ value (Figure 1). Small reduction of the cell viability was reported when the cells were treated in this range of treatment dose in which there was only 20.17 \pm 0.82 % of cell viability reduction for the highest dose, whereas for 50 μ M, 25 μ M, 12.5 μ M, 6.25 μ M and 0 μ M of the cell viability reduced about 16.38 \pm 4.34%, 14.51 \pm 1.20%, 13.06 \pm 5.84 and 10.88 \pm 2.98% in a dose dependent manner respectively. This showed that treatment of pterostilbene with the chosen range of treatment for 24 hours did not toxic to the cell. Thus the doses were suitable to be used to evaluate the potential of this compound. Hence we have chosen treatment of pterostilbene up to 50 μ M because it retained 80% of the cell viability.

Figure 2 showed the effect of pterostilbene treatment on GSH level in HT-29 cell line. There was an increased of the GSH level compare to the negative control for all the treatment concentration with a significant of increment reported at 50 μ M of pterostilbene treatment with the value of 75.54 \pm 5.92mmol/mg protein. This value is closely similar with the significant increment of GSH level with vitamin C that was used as positive control with the value of 75.54 \pm 7.05 mmol/mg protein. From the result we also could see a slight increasing of the GSH level was in the dose dependent manner but unfortunately the value of pterostilbene treatment at 12.5 μ M and 25 μ M is not significant (p <0.05) compare to negative control.

The effect of pterostilbene on the GST activity was summarized in the Figure 3. The result showed that treatment of pterostilbene can also induce the GST activity of the HT-29 cell line. From the result there were significant (p <0.05) increase of GST activity at 12.5 μ M pterostilbene treatment with the value of 4277.24 \pm 489.32 nmol/min/mg protein and 25 μ M pterostilbene treatment with the value of 4617.12 \pm 345.95 nmol/min/mg protein compare to negative control.

In addition, pterostilbene treatment also induced the NQO1 activity in the cell line. This was shown from the result (Figure 4) in which treatment of pterostilbene at 12.5 μ M significantly (p <0.05) increase the NQO1 activity with the value of 3973.82 \pm 177.33 nmol/min/mg protein. Meanwhile, at 50 μ M of pterostilbene, the NQO1 activity was reported as 4219.60 \pm 321.18 nmol/min/mg protein which was significantly increase compare to negative control.

Discussion

Before the potential of pterostilbene was evaluated, the optimum dose of the compound on HT-29 cell line was evaluated. This was to ensure the compound did not causing damage or induce any stress towards the cells. From the result, the treatment of this compound (0-100

μM) for 24 hours did not cause cytotoxicity to the cells. This result differs from findings by Nukula et al. (2011) in which there was an IC_{50} reported at $15 \mu\text{M}$ of pterostilbene treatment in HT-29 cell line. This might be due to the long incubation period of pterostilbene treatment in the study which was 48 hours. Study by Mannal et al. (2010) on the effect of pterostilbene on cell viability on two types of pancreatic cells showed a concentration and time-dependent manner of pterostilbene treatment. Similarly, pterostilbene was reported to reduce the cell viability of the lung cancer cell (Schneider et al., 2010) and breast cancer lines (Alosi et al., 2010) in the concentration and time-dependent manner.

In this study, we found that pterostilbene, has reported the same ability with resveratrol in which it can induce the detoxifying enzymes. In addition our result was also demonstrated the similar significant effect with regard to the dose with the study by Ghazali et al. (2012). In that study, they used the HepG2 cell line to investigate the effect of pterostilbene on GST activity and GSH level. For the GST activity, the study reported a significant increase in post pterostilbene treatment at $25 \mu\text{M}$ and $100 \mu\text{M}$ but not $50 \mu\text{M}$. This study also found the same findings in which the significant increase in GST activity was reported at $12.5 \mu\text{M}$ and $25 \mu\text{M}$ but not $50 \mu\text{M}$ of pterostilbene treatment. Ghazali et al. (2012) also showed a significant increase of GSH level at $25 \mu\text{M}$ and $50 \mu\text{M}$ that also reported in our study in which at $50 \mu\text{M}$ the GSH level also increase significantly compare to negative control. This suggests that at $25 \mu\text{M}$ pterostilbene treatment, GST activity increase thus there were a slight GSH level depleted, masking the induction effect by pterostilbene. But when there was no significant increase of GST activity at $50 \mu\text{M}$ of pterostilbene treatment, the increase of GSH level are apparent.

In addition, epidemiological studies had correlated the risk of colorectal cancer with the dietary factor (Giovannucci et al., 1994) especially the intake of certain food such as the cooked meat that contain high level of polyaromatic hydrocarbon (PAH) (Martorell et al., 2010). Reactive species generated from metabolism of PAH can initiate a cascade of oxidation that produced more reactive oxygen species (ROS) which can lead to oxidative stress. Oxidative stress is one of the pathways that might contribute to carcinogenesis (Goetz and Luch, 2008). Previous study has demonstrated chemoprevention of pterostilbene on colon cancer by suppressing the formation of tumorigenesis, inhibit the cell proliferation and exert the anti-inflammation on the colon cancer cell (Paul et al., 2010). Induction of GST activity and GSH level in this cell line indicate that, colon could also acquire cellular protection to prevent carcinogenesis through the conjugation reaction to eliminate the reactive species.

Moreover, protection from reactive quinone species also can be achieved via the induction of NQO1 activity. Study by Yang and Liu (2009) reported that resveratrol contributed to a high NQO1 activity in the hepatoma cell. Our study also revealed that pterostilbene was able to increase the NQO1 activity significantly in HT-29 cell line. This provides a promising effect towards prevention of cellular damage caused by reactive quinone species.

Metabolism of environment pollutant such as benzene (Ross et al., 2000) and pentachlorophenol (PCP) (Vaidyanathan et al., 2007) or endogenous quinones produce reactive quinone species that can induce oxidative stress or forming the DNA adduct. Hence, by induction of NQO1 activity, the quinone reactive species can be reduced into a nontoxic quinone derivatives such as hydroquinone via a two electron reduction, by passing the formation of semiquinone (Ross et al., 2000).

Cell protection by means of detoxification always involves the conjugation or reduction of the reactive species to become more polar compound and less reactive. In the basal state, the cell might contain low activity of detoxifying enzymes, but the activity will increase when there is an induction. There are several inducers that have been recognized to cause the induction of detoxifying enzymes and among them are phytochemicals such as resveratrol that was reported to activate the antioxidant response element (ARE) via Nrf2-ARE pathway. *Nuclear factor erythroid 2-related factor 2* (Nrf2) is a transcription protein that translocates from cytoplasm to nucleus to activate ARE sequences that induce the transcription of the detoxifying enzyme such as GST and NQO1 (Lee and Johnson, 2004; Surh et al., 2008). Besides, resveratrol has been reported to increase the expression of Glutamate-cysteine-Ligase (GCL) via the same pathway that leads to increase of the GSH level (Kode et al., 2008). Thus this study postulated that, pterostilbene might also be able to activate the Nrf2-ARE pathway, leading to transcription of detoxifying enzymes.

In conclusion, pterostilbene is a potential chemoprevention agent through its modulation of detoxifying enzymes.

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