

RESEARCH ARTICLE

Antioxidant Effects of Gamma-oryzanol on Human Prostate Cancer Cells

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Abstract

Background: To assess the antioxidant effects of gamma-oryzanol on human prostate cancer cells. **Materials and Methods:** Cytotoxic activity of gamma-oryzanol on human DU145 and PC3 prostate cancer cells was determined by proliferation assay using 3-(4,5-dimethylthiazol,2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent. mRNA levels of genes involved in the intracellular antioxidant system, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GSR) were determined by reverse transcription-polymerase chain reaction (RT-PCR). Cancer cell lysates were used to measure lipid peroxidation using thiobarbituric acid reactive substance (TBARS). Glutathione contents of the cell lysates were estimated by the reaction between sulfhydryl group of 5, 5'-dithio (bis) nitrobenzoic acid (DTNB) to produce a yellow-color of 5-thio-2-nitrobenzoic acid using colorimetric assay. Catalase activity was also analysed by examining peroxidative function. Protein concentration was estimated by Bradford's assay. **Results:** All concentrations of gamma-oryzanol, 0.1-2.0mg/ml, significantly inhibited cell growth in a dose- and time-dependent fashion in both prostate cancer cell lines, DU145 and PC3. Gene expression of catalase in DU145 and PC3 exposed to gamma-oryzanol at 0.5mg/ml for 14 days was down regulated, while mRNA of GPX was also down regulated in PC3. The MDA and glutathione levels including catalase activity in the cell lysates of DU145 and PC3 treated with gamma-oryzanol 0.1 and 0.5mg/ml were generally decreased. **Conclusions:** This study highlighted effects of gamma-oryzanol via the down-regulation of antioxidant genes, catalase and GPX, not cytotoxic roles. This might be interesting for adjuvant chemotherapy to make prostate cancer cells more sensitive to free radicals. It might be useful for the reduction of cytotoxic agents and cancer chemoprevention.

Keywords: Gamma-oryzanol - cytotoxicity - superoxide dismutase - catalase - glutathione peroxidase

Asian Pac J Cancer Prev, 14 (9), 5421-5425

Introduction

Gamma-oryzanol is the major component, 2%, in the extract from rice bran. Rice bran oil contains a mixture of several phytosteryl ferulates. Several reports mention the rice bran oil health beneficial activities, reduction of plasma cholesterol, inhibition of platelet aggregation, and particular antioxidant functions. In Japanese traditional medicine, rice bran oil has been used to ameliorate the effects of menopause, to accelerate growth, to enhance skin capillary circulation, and to treat gastrointestinal problems. Potent antioxidant activity was frequently reported for the radioprotective effect, cancer and chemotherapy (Ismail et al., 2010; Valantina et al., 2010).

To date, many scientific papers reported the benefit of gamma-oryzanol and rice bran oil in treating dyslipidemia through its antioxidant activity. The daily consumption of rice bran oil modified milk significantly decreases total serum cholesterol concentrations and tends to decrease cholesterol associated to low density lipoprotein in type 2

diabetes patients (Lai et al., 2012). Gamma-oryzanol and rice bran oil decrease serum total cholesterol by inhibiting lipid absorption from intestine in both human and animal models. Inhibition of nuclear factor- κ B activation via the antioxidant activity is the mechanism that explain the anti-inflammatory effect (Sakai et al., 2012). Gamma-oryzanol rich extracts acts as a radical scavenger and possesses potent anti-inflammatory activities (Saenjum et al., 2012). Moreover, it up-regulates antioxidant genes and down-regulates the oxidative stress gene markers, possibly due to the many potent antioxidants (Ismail et al., 2010; Valantina et al., 2010). It might be possible that gamma-oryzanol attenuates the carcinogenic process via the antioxidant pathway.

Ghatak and Panchal investigated the immunomodulatory potential of oryzanol isolated from crude rice bran oil in experimental animal models and found the sufficient potential for augmenting immune activity by cellular and humoral mediated mechanisms (Ghatak and Panchal, 2012). In tumor-bearing mice, gamma-oryzanol can

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reduce tumor mass associated with pro-angiogenic biomarkers. Vascular endothelial growth factor (VEGF), cyclooxygenase-2 (COX-2), and 5-lipoxygenase-5 (5-LOX) were significantly reduced in mRNA and protein expression leading to the down regulation of VEGF and neoangiogenic inhibition inside the tumors with the reduction in the number of blood vessels in the tumor mass (Kim et al., 2012). These observation suggest the application of gamma-oryzanol in cancer.

However, very few reports showing the anticarcinogenic effect of gamma-oryzanol has been published. Prostate cancer cell lines were chosen as a model for cancer treatment since previous reports mentioned a strong relation between prostate cancer and oxidative stress. The modulation of the redox status could be a potential therapeutic strategy on prostate cancer cells, including the cancer-initiating cells, migration, and invasion. The elimination of excessive reactive oxygen species (ROS) may be very effective to prevent prostate cancer formation and metastasis (Tangjitjaroenkun et al., 2012). The present study attempted to determine the antioxidative potential of gamma-oryzanol on prostate cancer cells through the key genes regulating intracellular antioxidant enzymes in prostate cancer cell lines, DU145 and PC3. mRNA expression of genes regulating intracellular antioxidant capacity, e.g., superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GSR) was assessed for a protective mechanism.

Materials and Methods

Cell cultures

Human prostate adenocarcinoma cell lines, DU145 and PC3, were grown as a monolayer in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin, and 100µg/mL streptomycin in 10cm diameter tissue culture dishes. They were maintained at 37°C in a tissue culture CO₂ incubator within humidified atmosphere containing 5% CO₂ until 80% confluency, and then subcultured twice a week (Tangjitjaroenkun et al., 2012). All chemicals used for cell culture were purchased from Gibco.

Cell proliferation assay

The cytotoxic effects of gamma-oryzanol on the proliferative capability of the human prostate adenocarcinoma cell lines, DU145 and PC3, were determined by using a modification of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described (Supabphol and Supabphol, 2012). Briefly, the prostate cancer cells at a density of 3.5×10⁴ cells/well in serum-containing medium were seeded in 24-well microplates and grown to 80-90% confluency. Then, they were treated with gamma-oryzanol (Naturalin Bio-Resources) at various concentrations in a humidified atmosphere of 5%CO₂ at 37°C for 24h. After the incubation, cells were washed twice and 150µl of culture medium containing 1mg/ml of MTT dye was added to each well and further incubated for 4h. The medium containing MTT dye was then replaced with 150µl of DMSO. The blue color of the oxidized MTT (formazan)

Table 1. Primers Used for RT-PCR Analysis (Supabphol and Supabphol, 2013)

Gene	Primer sequence (5'-3')	Product size (bp)
SOD	GAGACTTGGGCAATGTGACTG TTACACCACAAGCCAAACGA	201
CAT	AGTTCGGTCTCCACTGTTCG CTTGGGTCTGAAGGCTATCTGT	681
GSR	CTTGCGTGAATGTTGGATGT GACCTCTATTGTGGGCTTGG	257
GPX	GCCGTGCTGATTGAGAATG AGGTAGGCGAAGACAGGATG	269
β-actin	AGAGCTACGAGCTGCCTGAC ACATTGTGAACCTTGGGGGA	622

was determined by measuring the absorbance at 570nm using ELISA microplate reader (Biotex-synergy-HT). The percentage of proliferation was calculated and compared to that of the untreated cells. All chemicals for the cell proliferation assay were obtained from Sigma.

RNA extraction and RT-PCR

Total RNA was extracted from treated and non-treated prostate cancer cells using Trizol reagent (Invitrogen, USA). The purity and concentration of the isolated RNA were determined by reading the absorption at 260 and 280nm. Same amounts of RNA from each sample were mixed in a 20µl reaction mixture and single-stranded complementary DNA (cDNA) was synthesized by SuperScript RT kit (Invitrogen) according to the manufacturer's instructions. The primers in Table 1 were used to amplify the target genes using i-Taq kit (iNtRON Biotechnology). Amplified PCR products obtained by PCR (Bio-Rad C1000) were electrophoretically separated on a 2% agarose gel, stained by 2% ethidium bromide and photographed.

Lipid peroxidation measurement

Whole tumor cell lysates were obtained by repeated freeze-thaw procedures and followed by lipid peroxidation assay according to the protocol previously described by Tangjitjaroenkun et al. (2012). Prostate cancer cells pretreated with various concentrations of gamma-oryzanol were trypsinized, harvested, washed and subjected to repeated freeze-thaw procedures in ice-cold bath and centrifuged. Homogeneous lysates from cancer cells were used for lipid peroxidation measurement using thiobarbituric acid reactive substance (TBARS). Briefly, a 500µL aliquot of prostate cell lysates was combined with a reaction mixture containing 75µl of 8.1% SDS, 565µl of 20% acetic acid, and 565µl of 0.8% thiobarbituric acid (TBA). The resulting mixture was vigorously mixed, incubated at 95°C for 1 h and cooled to room temperature. MDA-TBA complex was extracted with n-butanol and pyridine (15:1, v/v) mixture. The supernatant fraction with the pink color was isolated and the absorbance at 532 nm was measured against mixture of n-butanol and pyridine as a blank. Malonaldehyde bis (dimethyl acetal) was used as a standard control. The content of lipid peroxidation was expressed as nM MDA per mg protein by interpolation in a standard curve in water covering a concentration range of 0-200mM. Protein concentration was estimated by Bradford's method. All samples were

conducted independently in triplicate. All chemicals for the lipid peroxidation measurement were obtained from Sigma.

Glutathione assay

Glutathione in cell lysates was analysed by using the reaction between sulfhydryl group of sulfhydryl group of 5, 5'-dithio (bis) nitrobenzoic acid (DTNB) to produce a yellow-color of 5-thio-2-nitrobenzoic acid (TNB) which is directly proportional to the glutathione level in cell lysates. Total glutathione was determined by reading the absorbance 410nm after 30min incubation (Tangjitjaroenkun et al., 2012). The results were expressed in μ mole per mg protein. All experiments were done in triplicate. All chemicals for the cell proliferation assay were obtained from Sigma.

Catalase activity assay

Basic reaction of catalase to decompose H_2O_2 molecules into water and oxygen was used to determine catalase activity as previously described (Tangjitjaroenkun et al., 2012). The reaction kinetics of catalase activity was performed at room temperature using 50mM phosphate buffer of pH 7.0 containing H_2O_2 as a substrate. After the 1-min incubation with cell lysates, the decrease of absorbance at 240nm was monitored as the catalase activity to decompose hydrogen peroxide molecules. A molar extinction coefficient of $43.6M^{-1}cm^{-1}$ for H_2O_2 was used. The results are expressed in μ mole per μ g protein per min and calculated as percentage to compare them with control. All chemicals in catalase activity assay were ordered from Merck.

Statistical analysis

All numeric data are expressed as the mean of three independent experiments \pm standard deviation compared to control. Determination of statistical significance was performed by analysis of variance using SPSS IBM Singapore Pte Ltd (Registration No.1975-01566-C). Statistical comparisons between groups were performed by use of the Student's t-test. A level of p value <0.05 was considered significant in all statistical tests.

Results

Effect of gamma-oryzanol on prostate cancer cell proliferation

Data in Table 2 shows the inhibitory effect of gamma-oryzanol on two human prostate cancer cell lines, DU145 and PC3. All concentrations of gamma-oryzanol significantly inhibited cell growth in a dose- and time-dependent fashion in both cell lines ($p < 0.05$).

Effect of gamma-oryzanol on the expression of antioxidant genes

The gene expression of CAT, SOD, GPX and GSR in DU145 exposed to gamma-oryzanol at 0.1 and 0.5 mg/ml for 1, 4 and 7 days were not significantly different (data not shown). After 14-day exposure, no significant difference of SOD, GPX or GSR expression was found, while an observation down regulation of CAT expression

was obviously observed at the concentration of 0.5 mg/ml (Figure 1A).

A similar treatment as with DU145 was done using PC3 cells and similar results were found, except that the down regulation of CAT occurred at concentrations of 0.1 and 0.5mg/ml. The down regulation of GPX expression was also found at the concentration of 0.5mg/ml (Figure 1B).

Effect of gamma-oryzanol on the MDA level

The MDA level in the cell lysates of DU145 treated with gamma-oryzanol 0.1 and 0.5mg/ml were 95.41 ± 3.03 and $89.21 \pm 3.58\%$, respectively (Figure 2A). For lysates

Table 2. Growth Inhibition of Prostate Cancer Cells, DU145 and PC3, Exposed to the Increasing of Gamma-Oryzanol Concentration

Gamma-oryzanol concentration (mg/ml)	Percentage of growth inhibition			
	1 day	4 days	7 days	
DU145	0.0	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00
	0.1	5.89 \pm 1.42*	23.86 \pm 3.63*	30.61 \pm 3.53*
	0.5	10.43 \pm 2.69*	38.45 \pm 2.51*	47.73 \pm 1.98*
	1.0	20.85 \pm 2.90*	48.52 \pm 2.92*	68.51 \pm 3.13*
	1.5	29.70 \pm 2.38*	62.02 \pm 2.65*	91.78 \pm 2.99*
	2.0	47.17 \pm 1.84*	72.27 \pm 1.79*	95.32 \pm 2.46*
PC3	0.0	0.00 \pm 0.00	0.00 \pm 0.00	0.00 \pm 0.00
	0.1	6.44 \pm 1.81*	34.52 \pm 2.79*	32.01 \pm 3.42*
	0.5	11.29 \pm 2.25*	42.03 \pm 2.20*	53.69 \pm 3.39*
	1.0	15.79 \pm 2.63*	51.43 \pm 3.76*	57.45 \pm 2.67*
	1.5	23.04 \pm 2.94*	61.92 \pm 2.55*	66.61 \pm 5.05*
	2.0	31.98 \pm 2.70*	81.55 \pm 2.72*	96.23 \pm 3.19*

*Significant from normal control, $p < 0.05$

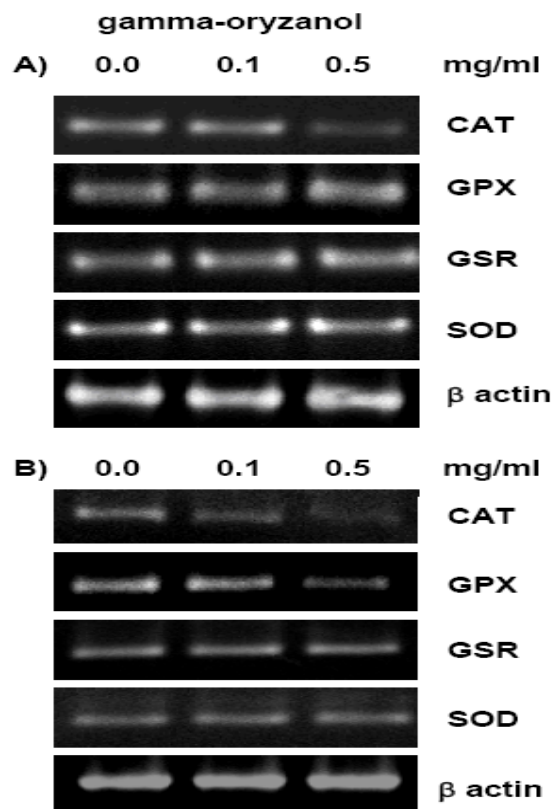


Figure 1. The Expression of Antioxidant Genes, CAT, SOD, GPX and GSR in A) DU145 and B) PC3 Exposed to Gamma-Oryzanol for 14 Days

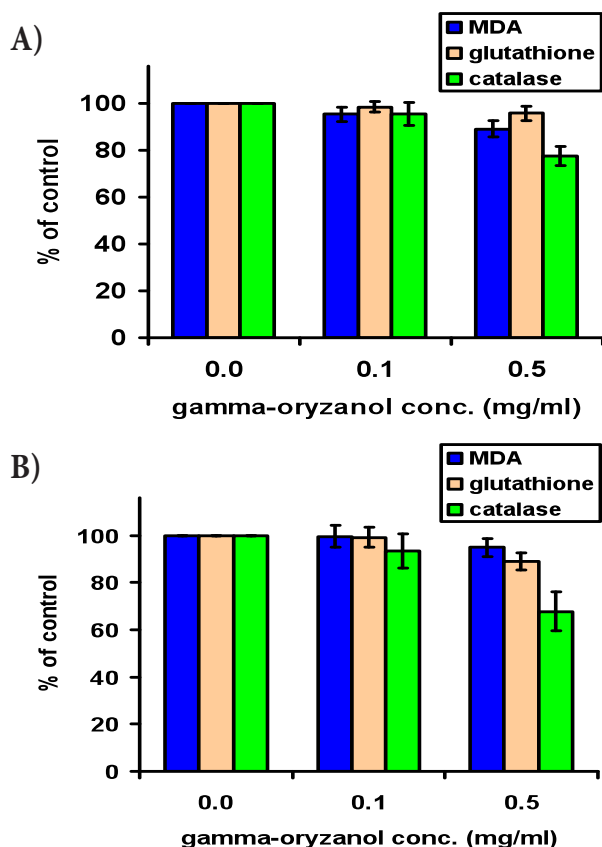


Figure 2. The MDA, Glutathione Content, and Catalase Activity of A) DU145 and B) PC3 Exposed to Gamma-Oryzanol for 14 days

from PC3 cells exposed to gamma-oryzanol 0.1 and 0.5mg/ml contained MDA 99.59±4.65 and 94.91±3.82%, respectively (Figure 2B).

Effect of gamma-oryzanol on the glutathione content

The glutathione content in the cell lysates of DU145 treated with gamma-oryzanol 0.1 and 0.5mg/ml were 98.59±2.18 and 95.82±3.14%, respectively (Figure 2A). For PC3, lysates from cells exposed to gamma-oryzanol 0.1 and 0.5mg/ml contained glutathione 99.25±4.20 and 89.14±3.65%, respectively (Figure 2B).

Effect of gamma-oryzanol on the catalase activity

The catalase activity in the cell lysates of DU145 treated with gamma-oryzanol 0.1 and 0.5mg/ml were 95.54±4.91 and 77.42±4.07%, respectively (Figure 2A). For PC3, lysates from cells exposed to gamma-oryzanol 0.1 and 0.5mg/ml possessed catalase activity 93.31±7.19 and 67.78±8.37%, respectively (Figure 2B).

Discussion

Gamma-oryzanol was reported to contain antioxidant activity that reduced cholesterol oxidation stronger than vitamin E (Xu et al., 2001). Recent reports examined to demonstrate the possibility to using gamma-oryzanol to treat cancer including prostate cancer (Ng and Figg, 2003). The imbalance of oxidant-antioxidant system resulting in increased oxidative stress was reported as an important feature of this type of cancer (Battisti et al.,

2011; Tangjitjaroenkun et al., 2012). The present study chose to assess the antioxidative effect of gamma-oryzanol on prostate cancer cell line, DU145 and PC3. Proliferation assay was firstly done for the appropriate concentration for further experiments. Then mRNA expression of genes regulating intracellular antioxidant capacity, SOD, GPX, GRX and CAT were assessed for antioxidant mechanism.

From the proliferation assay using MTT reagent, the effect of gamma-oryzanol on DU145 and PC3 were slightly different with an IC_{50} after 7 days of exposure at 0.52 ± 0.06 and 0.40 ± 0.08 mg/ml, respectively. For in vitro condition in proliferation assay, IC_{50} values seem to be high in the scale of mg/ml. We believe that IC_{50} values should be markedly lower in the body with the dynamic condition of consistent blood circulation. Complete or nearly complete cell death can be found when prostate cancer cells were exposed to 2.0mg/ml gamma-oryzanol for 7 days. Our previous data have shown that death of prostate cancer cell, DU145 and PC3, exposed to gamma-oryzanol is p53-independent (Klongpityapong et al., 2013). The concentration at 0.1 and 0.5mg/ml were chosen for further experiment due to the low effect to proliferation.

For PCR reaction, 2% FBS was added in 14 day-experiment to maintain cell integrity. The observations in this study showed non-significant down regulation of all four antioxidant genes in both prostate cancer cells exposed to gamma-oryzanol 0.1 and 0.5mg/ml for 3 and 7 days (data not shown). The obvious down-regulation of catalase was observed in DU145 and PC3 cells when the exposure time to gamma-oryzanol was extended to 14 days. A strong down regulation of GPX was also found in PC3 pretreated with gamma-oryzanol for 14 days. These means that gamma-oryzanol is capable of modifying at least one or two antioxidant genes in prostate cancer cells.

Free radicals are harmful and cause cell damage referred as oxidative stress, including modification of lipids, membranes, proteins and genetic materials. The damage caused by free radicals has been implicates in many diseases, particularly cancer. ROS may be considered as a carcinogen. Normally cell has an intracellular antioxidant defense, composed of both enzymatic and non-enzymatic mechanisms, to counteract this damage. Catalase, the hydrogen-peroxide detoxifying enzyme, promotes the conversion of hydrogen peroxide to water and molecular oxygen. This antioxidant enzyme has a very high efficiency with one catalase molecule turning approximately 6 million molecules of hydrogen peroxide to water and oxygen per minute (Rahman, 2007). The decrease in catalase expression leads to an elevation of reactive oxygen species and the cell then become susceptible to free radicals. Our studies have shown the down-regulation of catalase expression in both prostate cancer cells. Moreover catalase activity was also lower. It might be possible that the cell death found in prostate cancer exposed to gamma-oryzanol might be due to the more sensitive situation to free radical than untreated cells.

GPX is also an important antioxidant enzyme and responsible for reducing various hydroperoxides. It is present in high intracellular concentration and accelerates the conversion of hydrogen peroxide or organic peroxides

to water or to a corresponding alcohols, respectively, and the simultaneously oxidizing glutathione to glutathione disulfide. GPX-1-deficient mice have shown an increase in the oxidative stress marker, F₂-isoprostanes, and modulate the endothelial dysfunction. In contrast, the over expression of GPX-1 can decrease the tissue damage after cerebral or myocardial ischemia/reperfusion injury, and protect against angiotensin II-mediated vascular dysfunction. These observations point to the crucial role of GPX in redox cycle (Lubos et al., 2011). We found that GPX expression was down-regulated in PC3 cell only while glutathione decrease in DU145 and PC3. The function of GPX is to reduce lipid hydroperoxides and hydrogen peroxide to their corresponding alcohols and water, respectively. The reduction of GPX expression in PC3 might make PC3 more sensitive to free radical than DU145. The decrease in MDA is interpreted as a decrease in free radical in DU145 and PC3 cells treated with gamma-oryzanol due to glutathione.

The depletion in catalase expression and activity including of glutathione may account for the susceptibility to extracellular free radical and lead to cell death. The mechanism of some current cytotoxic drug, paclitaxel (Taxol[®]), was found to increase cell death via free radical production (Varbiro et al., 2001). The inhibition of the function of catalase may postulated to sensitize cells to conventional cytotoxic agents currently using in cancer therapies, and then the dose of cytotoxic drugs could be reduced. The effect of gamma-oryzanol on catalase and GPX in prostate cancer did not occur in acute condition, but took about 2 weeks to develop. A nonsignificant difference of antioxidant gene expression was found after 3- and 7-day treatment. The gradual effect would be the another advantage since it does not abruptly or seriously affect other vital organs or tissues such as bone marrow.

In conclusion, in summary, our results showed that gamma-oryzanol may affect to prostate cancer cells through the down regulation of some antioxidant genes, CAT and GPX. Furthermore, its action on antioxidant genes is gradually and the main effect is not cytotoxic role to DU145 and PC3. This might be of interest for adjuvant chemotherapy to make cancer cells more sensitized to cytotoxic agent and also useful in cancer chemoprotection. This might provide an interesting target for further investigation in cancer research.

Acknowledgements

We would like to thank Professor Thompson EW, Department of Surgery, St. Vincent's Institute, the University of Melbourne, Australia, for the prostate cancer cell lines, DU145 and PC3. We wish to thank Ms. Waraporn Yahayo for the technical assistance. This research was supported by the annual fiscal budget of Eastern Asia University.

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