Original Article

Myricetin and methyl eugenol combination enhances the anticancer activity, cell cycle arrest and apoptosis induction of cis-platin against HeLa cervical cancer cell lines

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Abstract: Drug combination therapies are common practice in the treatment of cancer. In this study, we evaluated the anticancer effects of myricetin (MYR), methyl eugenol (MEG) and cisplatin (CP) both separately as well as in combination against cervical cancer (HeLa) cells. To demonstrate whether MYR and MEG enhance the anticancer activity of CP against cervical cancer cells, we treated HeLa cells with MYR and MEG alone or in combination with cisplatin and evaluated cell growth and apoptosis using MTT (3 (4, 5 dimethyl thiazol 2yl) 2, 5 diphenyltetrazoliu bromide) assay, LDH release assay, flow cytometry and fluorescence microscopy. The results revealed that, as compared to single drug treatment, the combination of MYR or MEG with CP resulted in greater effect in inhibiting cancer cell growth and inducing apoptosis. Cell apoptosis induction, Caspase-3 activity, cell cycle arrest and mitochondrial membrane potential loss were systematically studied to reveal the mechanisms of synergy between MYR, MEG and CP. Combination of MYR or MEG with CP resulted in more potent apoptosis induction as revealed by fluorescence microscopy using Hoechst 33258 and AO-ETBR staining. The combination treatment also increased the number of cells in G0/G1 phase dramatically as compared to single drug treatment. Mitochondrial membrane potential loss (ΔΨm) as well as Caspase-3 activity was much higher in combination treatment as compared to single drug treatment. Findings of this investigation suggest that MYR and MEG combined with cisplatin is a potential clinical chemotherapeutic approach in human cervical cancer.

Keywords: Cervical cancer, combination therapy, apoptosis, myricetin, methyl eugenol, cell cycle

Introduction

Cervical cancer is a cancer arising from the cervix. It is due to the uncontrolled growth of cells that have the capability to attack or spread to other parts of the body. Initially there are typically no symptoms. Later symptoms may include: abnormal vaginal bleeding, pelvic pain or pain during sexual intercourse. Cervical cancer is the second most common malignancy in women with an estimated 493,000 new cases and 274,000 deaths in 2002 [1]. Although radiotherapy represents effective treatment modality, up to one third of patients will grow progressive or recurrent tumors, the pelvis being the most common site of failure [2-4]. The relapse rate of cervical cancer ranges between 11 and 22% in FIGO stages Ib-Ila and between 28 and 64% in FIGO stages IIb-Iva [5]. Human papillomavirus (HPV) infection seems to have a key role in the development of more than 90% of cases. However, most people who have had HPV infections do not develop cervical cancer. Other risk factors include: smoking, a weak immune system, birth control pills. [6-8]. Cervical cancer is of different types including squamous cell carcinomas which constitute about 90% of the cases followed by adenocarcinoma constituting 10% of the cases. Cervical cancer treatment consists of a combination of surgery, chemotherapy and radiotherapy. In the United States, there are around 68% of the five year survival rates and this rate greatly depends on how early the cancer is diagnosed. Larger
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early stage tumors may be treated with radiation therapy and cisplatin-based chemotherapy, hysterectomy (which then usually requires adjuvant radiation therapy), or cisplatin chemotherapy followed by hysterectomy [9-11]. Recently FDA has approved the use of a combination of two chemotherapy drugs namely hycamtin and cisplatin for women with late stage cervical cancer. However, this combination treatment is accompanied with serious side effects including neutropenia, anemia, and thrombocytopenia [12]. Keeping in view this fact, the objective of the current study was to identify some natural products from plant sources which can enhance the anticancer activity of cisplatin against cervical cancer (HeLa) cells. In this study we found that myricetin and methyl Eugenol enhanced the anticancer activity of HeLa cancer cells in vitro along with enhancing the induction of apoptosis, cell cycle arrest and mitochondrial membrane potential loss. To investigate the mechanism further by which the combination of cisplatin with myricetin and methyl Eugenol induces apoptosis, effect on Caspase-3 was studied which indicated that these combinations enhanced activation of caspase-3 remarkably.

Materials and methods

Cell culture and myricetin and methyl eugenol treatment

HeLa (cervical cancer cells) were procured from the Shanghai Institute of Cell Biology (Shanghai, China). MTT was purchased from Sigma Chemical Co., (St. Louis, MO, USA). The cells were cultured in Dulbecco’s modified Eagle’s media supplemented with 10% fetal bovine serum (Lonza Biologics, Singapore) and 100 U/mL penicillin and 100 μg/mL streptomycin (Vega Pharma Limited, Zhejiang, China). The cells were kept at 37°C in a humidified atmosphere containing 5% CO₂. In cell proliferation experiments, HeLa cells were treated with either cisplatin or myricetin and methyl eugenol alone, or vehicle alone for 12, 24 and 48 h. For apoptosis assay, cells were treated with cisplatin or myricetin and methyl eugenol either alone or combined, or vehicle alone for 48 h. Myricetin and methyl eugenol and cisplatin was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in DMSO (Sigma Chemical Co.) (Final concentration 0.2% in medium). Cytotoxicity Detection Kit (LDH) was purchased from Roche Chemical Co.

Cell proliferation assay

MTT assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was used to measure the inhibition of cell proliferation. MTT was added in cells exposed to either cisplatin or myricetin and methyl eugenol or their combinations. Three hours later, the formazan precipitate was dissolved in 100 μL dimethyl sulfoxide, and then the absorbance was measured in an ELISA reader (Thermo Molecular Devices Co., Union City, USA) at 570 nm.

The cell viability ratio was calculated by the following formula:

Inhibitory ratio (%) = (OD control - OD treated)/ OD control × 100%.

Cytotoxicity was expressed as the concentration of capillarisin inhibiting cell growth by 50% (IC₅₀ value).

Lactate dehydrogenase (LDH) leakage assay for assessing cell cytotoxicity

Leakage of enzymes such as LDH into the culture medium is a well-known indicator of damage or injury to the cell membrane. Briefly, 1 × 10⁶ cells/well of HeLa cells was transferred to 96-well plates. The plates were incubated overnight at 37°C to allow the cells to attach and proliferate. On the next day, 300 μl of fresh medium containing drug concentrations (myricetin, methyl eugenol and cisplatin or their combinations) were added to each well, and the plates were incubated at 37°C in 5% CO₂. All drug concentrations were tested at least in triplicate wells and the assays were repeated independently three times. After 48 h, the plates were removed from the incubator and then 100 μl of medium from each well was carefully transferred to new plates. 100 μl of LDH substrate prepared according to the manufacturer’s direction (Cytotoxicity Detection Kit, Roche Chemical Co.) was added to each well. After 20 min shaking at room temperature, the enzymatic reaction was arrested by adding 50 μl of 1 M hydrochloric acid.

Lactate dehydrogenase activity was determined by change in absorbance at 490 nm. For the purpose of calculating percent cytotoxicity values, background LDH release from culture cells was considered as low control and triton-X 100 (0.01%) treated cells as high control.
Leakage (%) = \left[ \frac{A_{490} \text{(sample)} - A_{490} \text{(low control)}}{A_{490} \text{(high control)} - A_{490} \text{(low control)}} \right] \times 100\% 

**Hoechst 33258 staining to detect apoptosis**

HeLa cervical cancer cells were fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 20 min before staining with 5 μg/mL of Hoechst 33258 (Shanghai Hanhong Chemical Co., Ltd., China) at 37°C for 20 min. HeLa cells were treated with cisplatin (1 μM), myricetin (60 μM) and methyl eugenol (60 μM) or their combinations for 48 hours. Then, the cells were washed once with PBS, and then observed under a fluorescence microscope (Nikon). The condensed DNA of apoptotic cells was recognized by intense local staining in the nucleus, in contrast to diffused staining of DNA in normal
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![Graph showing % inhibition for different treatments]

Figure 3. Combined effect of Myricetin (MYR 60 µM), methyl eugenol (MEG 60 µM) and cisplatin (CP 1 µM) on HeLa cell proliferation. *P < 0.05 vs. control group. **P < 0.01 vs. control group.

AO-ETBR staining

After incubation of HeLa cells for 3 hours, a staining method using acridine orange (AO) and ethidium bromide (EB) (Sigma, St. Louis, MO, USA) was done. HeLa cells were treated with cisplatin (1 µM), myricetin (60 µM) and methyl eugenol (60 µM) or their combinations for 48 hours. Then, cells on coverslips were collected, washed with PBS twice, stained by AO/EB solution (30 µg/ml both), and then photographed using a fluorescence microscope (Nikon Tokyo Japan).

Cell cycle analysis by flow cytometry

Cervical cancer cells (HeLa) (1 × 10⁵) in a 60 mm dish were subjected to cisplatin (1 µM), myricetin (60 µM) and methyl eugenol (60 µM) or their combinations for 48 hours. The cells were collected by trypsinization and washed twice with PBS (Sigma Chemical Co.). Cells were incubated in 50% ethanol at -20°C overnight and then treated with 40 µg/ml RNase A (Guangzhou Geneshun Biotech Ltd. China), then stained with 10 µg/ml of propidium iodide (PI) (Guangzhou Geneshun Biotech Ltd. China). Finally, the stained cells were analyzed by using FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Cell apoptosis and quantification of apoptosis assay

To verify the synergistic enhancing effect of MYR and MEG combinations, the induction of apoptosis caused by MYR + CP and MEG + CP HeLa cancer cells was carried out. Apoptosis detection was performed using the Annexin V-FITC and PI apoptosis kit (Bestbio, Shanghai, China). HeLa cells were plated at a density of 2 × 10⁵ cells/well into 12-well plates and incubated overnight. Apoptosis was induced by treating cells with MYR, MEG, CP or combinations of MYR and MEG with CP solution (final concentrations of MYR, MEG and CP were 60, 60 and 1 µM, respectively). Cells grown in media containing an equivalent amount of DMSO without any drug were served as control. After 48 h of incubation, the cells with different treatments were harvested and the final samples were measured on a FACS Calibur flow cytometry (Becton Dickinson, San Jose, CA, USA).
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Effect on mitochondrial membrane potential loss (ΔΨm)

Mitochondrial membrane potential (ΔΨm) in cervical cancer cells (HeLa) was measured by Rhodamine-123 dye (Zouping Mingxing Chemical Co., Ltd.). HeLa cells (5 × 10⁶) were treated with cisplatin (1 µM), myricetin (60 µM) and methyl eugenol (60 µM) or their combinations for 48 hours and mitochondrial membrane potential was measured by flow-cytometry. Rhodamine-123 (20 mM) was added 2 h before the end of experiment. After it, the cells were washed with PBS and incubated with PI (10 µg/ml) for 30 min. Finally the cells were analyzed by a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Caspase-3 activity analysis

HeLa cells were collected by trypsinization and lysed with lysis buffer (1% Triton X-100, 0.40 M sucrose, 10 mM EDTA, 5 mM Tris-HCl, pH 7.5, 4 mM DTT, 2 mM PMSF, 2 µg/ml aprotinin, 1 mg/ml leupeptin) (Guangzhou Geneshun Biotech Ltd. China). Thereafter, the lysates were transferred to wells in a 96-well flat-bottom plate. A peptide with the caspase-3 target motif DEVD bound to the chromophore p-nitroanilide (Santa Cruz, CA, USA) was added and incubated at 37°C for 1 h. The intensity of the developed color was read at 405 nm in a microplate reader (Thermo Molecular Devices Co., Union City, USA).

Statistical analyses

All statistical analyses were done using SPSS® software (version 19.0) and were conducted by one-way analysis of the variance (ANOVA) and Tukey test. Data were expressed as the mean ± SEM and P values < 0.05 was considered significant.

Results

Effects of myricetin (MYR), methyl eugenol (MEG) and cisplatin (CP) combination on cell proliferation (MTT assay)

The results of the cell viability with different treatments (MYR, MEG, and CP or their combi-
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Figures 1-3. For making combinations of MYR and MEG with CP, 60 µM each of MYR and MEG were combined with 1 µM concentration of CP. Comparing with MYR, MEG or CP, their combinations (MYR + CP and MEG + CP) showed much lower cell viabilities at all given concentrations, indicating a strong potential for combination treatment. MEG + CP combination showed higher potent growth inhibition of HeLa cells as compared to MYR + CP combination.

Evaluation of the cytotoxic effect of myricetin (MYR), methyl eugenol (MEG) and cisplatin (CP) combination by lactate dehydrogenase (LDH) leakage assay

The effects of MYR, MEG, CP and their combinations on lactate dehydrogenase release are shown in Figure 4. As compared to the control treatment, MYR, MEG or CP treatments induced a significant release of LDH from the HeLa cells. However, the effect of the combinations of MYR, MEG with CP was much more pronounced and induced significant cytotoxic effects against cervical cancer cells. Control wells were treated with equivalent amount of media alone. This assay along with MTT assay confirm that the combinations of MYR and MEG with CP produce effects which is much more potent as compared to the effects produced by these compounds alone.

Hoechst 33258/AO-ETBR staining to detect apoptosis in HeLa cells

Apoptosis is a highly organized biochemical process to eradicate injured or abnormal cells in multicellular organisms. In order to establish whether cell death induced by MYR, MEG, CP or their combination is mediated through apoptosis, HeLacells were treated with myricetin (MYR, 60 µM), methyl eugenol (MEG, 60 µM) or their combinations (MYR + CP and MEG + CP) for 48 hours, and the characteristic morphological features of apoptosis were examined under an inverted light fluorescence microscope. Using Hoechst 33258 to stain the nucleus, it was observed (Figure 5) that as compared to control cells, MYR, MEG, CP and their combination treatment resulted in the appearance of cell shrinkage along with membraneblebbing which are characteristic features of cell apoptosis. As compared to the separate treatments, the combinations of MYR (60 µM) + CP (1 µM) and MEG (60 µM) + CP (1 µM) exhibited remarkable apoptotic features.

Acridine orange and ethidium bromide (AO/ETBR) double staining of the HeLa cells in order to observe cell apoptosis with the help of a fluorescence microscope after staining with a mixture of acridine orange and ethidium bromide, control cells (no drug treatment) (Figure 6) exhibited large green nuclei, which indicated
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the fact that the cell membranes were undamaged. However when treated with 60 μM each of MYR and MEG, the number of cells with large green nuclei reduced significantly (Figure 6).

Figure 6. Cell apoptosis observed by AO-ETBR staining. HeLa cells were treated with (B) 60 μM myricetin (MYR), (C) 60 μM methyl eugenol (MEG), (D) 1 μM cisplatin (CP), (E) combination of MYR + CP and (F) combination of MEG + CP for 48 h. (A) Compared with the control group, treated cells exhibited chromatin condensation, nuclear fragmentation and apoptotic body formation.

Figure 7. Effect of myricetin, methyl eugenol, cisplatin and their combinations on the cell cycle phase distribution in cervical (HeLa) cancer cells. A. Represents control cells. B-F. Represent effect of 60 μM myricetin (MYR), 60 μM methyl eugenol (MEG), 1 μM cisplatin (CP), combination of MYR + CP and combination of MEG + CP respectively. Compared to control cells and MYR and MEG treated cells, combination of MYR + CP and MEG + CP indicate a significant increase in the sub-G1 population of cells which is a hallmark of apoptosis and DNA damage caused by the combination treatment.
However, the effect of the combination of MYR (60 µM) and MEG (60 µM) with CP (1 µM) induced a significant reduction of cells with large green nuclei which points that the number of apoptotic cells grew significantly with combination treatment. Myricetin, methyl eugenol and cisplatin augment each other in inducing apoptosis in HeLa cancer cells as their combination is much more potent in inducing apoptosis as compared to the compounds alone.

**Effect of MYR and MEG combinations with CP on cell cycle**

In order to establish whether combination treatment of MYR, MEG with CP induces cell cycle disturbances in cervical cancer (HeLa) cells, flow cytometric analysis using propidium iodide (PI) as a staining agent was performed after MYR, MEG and their combination treatment with CP for 48 hours. The results revealed that the proportions of the treated cells at G0/G1 were increased in a dose-dependent manner (Figure 7). Control treated cells showed 4% cells in G0/G1 phase which got increased to 9% and 15% on treatment with MYR and MEG respectively. The increase in the G0/G1 cells was slight in case of MYR or MEG treatment alone. However, the combination treatment MYR (60 µM) + CP (1 µM) or MEG (60 µM) + CP (1 µM) produced a much more potent effect because the number of G0/G1 cells, MYR + CP and MEG + CP showed 45% and 49% of cells in the G0/G1 phase which was quite higher as compared to separate treatments by any of the compounds or the drug (cisplatin).

**Effect of the MYR, MEG and their combination with CP on cell apoptosis**

To verify the synergistic effects of MYR and MEG with CP on HeLa cells, the apoptotic effects of MYR + CP and MEG + CP were tested using Annexin V-FITC and PI apoptosis kit. Annexin V staining can detect phosphatidyl serine and as such can be used for its analysis. After cells are stained with annexin V in tandem with propidium iodide (PI), this reagent enters the cell only when the plasma cell membrane is deteriorated. The representative dot-plots illustrating apoptotic status are shown in Figure 8. The percentage of apoptotic cells (early apoptotic plus late apoptotic cells) treated with MYR (60 µM) + CP (1 µM) and MEG (60 µM) + CP (1 µM) combination solutions was 63.85% ± 4.12% and 85.47 ± 6.42% respectively, which was significantly higher in comparison with MYR (60 µM) (17.82% ± 2.16%, P < 0.01), MEG (60 µM) (48.93 ± 3.26%, P < 0.01) or CP (1 µM)
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Figure 9. Effect of myricetin, methyl eugenol, cisplatin and their combinations on the mitochondrial membrane potential loss in cervical (HeLa) cancer cells. A. Represents control cells. B-F. Represent effect of 60 μM myricetin (MYR), 60 μM methyl eugenol (MEG), 1 μM cisplatin (CP), combination of MYR + CP and combination of MEG + CP respectively. Compared to control cells and MYR and MEG treated cells, combination of MYR + CP and MEG + CP indicate a significant loss of mitochondrial membrane potential. (58.34% ± 5.32%, P < 0.01) treatment alone. These results indicated the potential synergistic enhancement of cancer therapy using MYR + CP and MEG + CP combinations.

Effect of the MYR and MEG combinations with CP on mitochondrial membrane potential loss (ΔΨm) in HeLa cervical cancer cells

The fluorescent dye, rhodamine-123 (Rh-123) is a specific probe for the detection of alterations in mitochondrial membrane potential in living cells. Our results revealed that the combination treatment of MYR and MEG with CP induced a significant reduction in the number of cells with intact membrane potential and increased the number of cells with low ΔΨm after 48 h. As can be seen from Figure 9, as compared to the control cells (no drug treatment), the MYR (60 μM), MEG (60 μM) and MYR (60 μM) + CP (1 μM) and MEG (60 μM) + CP (1 μM) treated cells indicated a much higher loss of mitochondrial membrane potential.

Activation of Caspase-3 HeLa cells by MYR + CP and MEG + CP combination therapy

Caspases-3 is one of the vital mediators of apoptosis, being critical for certain processes associated with the dismantling of the cell and the formation of apoptotic bodies [13]. To confirm the probable pathways of synergistic apoptotic effect of MYR + CP and MEG + CP, Caspase-3 activities of cervical cancer cells were evaluated by a Caspase-3 Activity Assay Kit. As shown in Figure 10, significantly higher Caspase-3 activity of HeLa cells was observed in cells treated with MYR + CP (60 μM MYR + 1 μM CP) and MEG + CP (60 μM MEG + 1 μM CP) comparing with that of cells treated with MYR (60 μM), MEG (60 μM) or CP (1 μM), alone (P < 0.01). These results were in agreement with the study of apoptosis tests mentioned in other assays here. Taking these results together, it was obvious to believe that the synergistic enhancement of apoptosis caused by MYR + CP and MEG + CP was probably mediated by the significant activation of Caspase-3.

Discussion

Combination therapy plays a more and more important role in the field of anti-cancer therapy. At present, cisplatin is the most active chemotherapeutic agent for the treatment of different cancers and is usually combined with other agents such as docetaxel, gemcitabine...
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and paclitaxel [14]. However, its use is limited due to severe side effects such as anemia, neurotoxicity, nephrotoxicity and the attainment of drug resistance [15]. To address these problems, attention has been focused on identifying novel agents that can be combined with cisplatin to increase the therapeutic efficacy and decrease side effects. The toxic side effects of currently used anticancer drugs can be reduced by using non-toxic potentiating agent or synergistic compound in combination with anticancer drugs which may considerably potentiate their effectiveness while minimizing their toxicity by reducing the dose. Such non-toxic potentiating agents which can possibly solve many problems of the clinical cancer treatment include low molecular weight terpenoids present in essential oils [16, 17]. Myricetin and methyl eugenol are also found naturally in many plants and essential oils. Myricetin is a naturally occurring flavonoid found in many grapes, berries, fruits, vegetables, herbs etc. Walnuts are a rich dietary source. Trace amounts can be found as glycosides. It is one of the phenolic compounds present in red wine [18, 19]. Myricetin has been reported to possess antioxidant properties. A study correlated high myricetin consumption with lowered rates of prostate cancer [20]. Another clinical study revealed that intake of three flavonoids (kaempferol, quercetin, and myricetin) were connected with a reduced occurrence of pancreatic cancer in people [21]. Methyleugenol (is a phenylpropene, a type of phenylpropanoid compound,) is a natural constituent of a large number of essential oils of plant origin including plants like in Artemisia dracunculus French type (tarragon), Syzygium aromaticum (clove), Daucus carota (carrot), Myristica fragrans (nutmeg) and Rosmarinus officinalis (rosemary) [22, 23].

In the present study, we evaluated these two naturally occurring compounds for their anticancer activity and their effect in enhancing the anticancer activity of cisplatin against HeLa cervical cancer cells. The results indicated that these compounds individually exhibit cancer growth inhibitory activity and when combined with cisplatin in specific dose form produce synergistic enhancement of the anticancer effect of cisplatin against these cells. Their
combination with cisplatin was found always to be more active as compared to separate treatment. We further evaluated the effect of the combination of MYR and MEG with CP on apoptosis, cell cycle phase distribution, mitochondrial membrane potential as well as Caspase-3 activity. All these experiments indicated that the combination of MYR and MEG with CP was more effective as compared to the compound or drug alone.

The mechanism of action of cisplatin includes the inhibition of cell proliferation and induction of cell apoptosis [24]. To determine whether MYR and MEG enhance the anticancer effect of cisplatin, HeLa cells were treated with MYR or MEG alone or in combination with cisplatin and cell growth and apoptosis were evaluated using MTT assay, flow cytometry assay and fluorescence microscopy. We found that in comparison with single agent treatment, the combination of MYR or MEG with cisplatin produced greater effectiveness in growth inhibition and apoptosis induction, suggesting that MYR and MEG play a combination role in cisplatin-induced apoptosis and growth inhibition in cervical cancer cells.

In conclusion, this study made a reasonably comprehensive investigation on the potential application of MYR, MEG in combination with anti-cancer drugs like cisplatin (CP), making up for the deficiency of the previous research in the field of combination therapy. Our results indicated that MYR and MEG synergistic combinations with cisplatin are more active than separate treatment and could be developed further for effective cancer treatments against cervical cancers. Further in vivo studies will establish whether these combinations hold true for animal studies as well. The severe side effects associated with cisplatin therapy could be reduced by such combinations because it leads to dose-reduction which has a direct relation with side effects.

Disclosure of conflict of interest

None.

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