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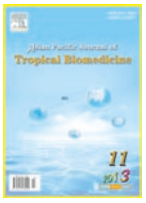

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Combination of low-concentration of novel phytoestrogen (8,9)-furanyl-pterocarpan-3-ol from *Pachyrhizus erosus* attenuated tamoxifen-associated growth inhibition on breast cancer T47D cells

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PEER REVIEW

Peer reviewer

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Comments

The paper has a good scientific quality. It was conducted on the basis of a current topic of vital interest to the health with promising results. The paper is acceptable for publication.
Details on Page 851

ABSTRACT

Objective: To investigate the estrogenic effect of (8,9)-furanyl-pterocarpan-3-ol (FPC) on growth of human breast cancer T47D cells and the interactions between the FPC and tamoxifen (TAM), on the growth of estrogen receptor-dependent breast cancer T47D cells.
Methods: The proliferation effect of FPC were conducted on T47D cells *in vitro* by MTT test. T47D cells were treated with FPC alone (0.01-200 μmol/L) or in combination with TAM 20 nmol/L. Furthermore, the expression of ERα or c-Myc were also determined by immunohistochemistry.
Results: The results indicated that administration of an anti-estrogen TAM showed growth inhibitory effect on T47D cells, wheraes co-administered with low concentration (less than 1 μmol/L) of FPC attenuated to promote cell proliferation. In contrast, the combination of TAM with higher doses (more than 20 μmol/L) of FPC showed growth inhibitory. This result was supported by immunocytochemistry studies that the administration of 20 nmol/L TAM down-regulated ER-α and c-Myc, but the combination of 20 nmol/L TAM and 1 μmol/L FPC robustly up-regulated expression of ER-α. Thus, the reduced growth inhibition of TAM 20 nmol/L by FPC 1 μmol/L on T47D cells may act via the modulation of ER-α.
Conclusions: The findings indicate and suggest that FPC had estrogenic activity at low concentrations and anti-estrogenic effect that are likely to be regulated by c-Myc and estrogen receptors. We also confirm that low concentration of FPC attenuated the growth-inhibitory effects of TAM on mammary tumor prevention. Therefore, the present study suggests that caution is warranted regarding the consumption of dietary FPC by breast cancer patients while on TMA therapy.

KEYWORDS
Phytoestrogen, (8,9)-furanyl-pterocarpan-3-ol, Tamoxifen, Estrogenic effect, T47D cells

1. Introduction

Breast cancer is a leading cause of death and disability among women, especially young women, in low and middle income countries. In many developing countries, the incidence of breast cancer is now rising sharply due to changes in reproductive factors, lifestyle, and increased life expectancy[1]. Recently, more than half of incident cases occured in the developing countries[2,3]. The recent finding described that the mortality from breast cancer is a leading cause of death among adult women in developing countries as well as the developed world[1]. Many factors contribute to the development and progression of breast cancer. Among these factors, estrogens play a crucial role. Estrogens bind to estrogen receptors (ERs),

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resulting in an activated complex that acts as a transcription factor through binding to target genes, therefore promoting cell proliferation and tumor growth. Agents that modify ER associated signaling are typical selective estrogen receptor modulators (SERM). SERMs exhibiting mixed antagonist-agonist tissue-specific activities and modulating ER expression may alter ER conformation and ligand binding, and change the expression or binding of co-regulator proteins[4,5]. Anti-estrogens agents have been widely studied and used for their roles in the prevention and treatment of breast cancer, especially estrogen-dependent breast tumors. Anti-estrogenic strategies have been remarkably efficacious for breast cancer treatment and prevention. Among these, tamoxifen (TAM) is the most widely prescribed SERM worldwide[5,6]. TAM is one of anti-estrogens that blocks activity of estrogens in most tissues that are sensitive to estrogens. TAM has become the standard treatment for breast cancer at all stages of the disease in both pre-menopausal and post-menopausal women[7]. Factors that might interfere with the efficacy of TAM, by either direct or indirect mechanisms, may modify its efficacy for breast cancer treatment or prevention[8].

Plant-derived phytoestrogens (or isoflavones) are also SERMs because they show mixed estrogen agonist-antagonist activities. Phytoestrogen (or soy) consumption has been associated with reducing risk of breast cancer[9,10], improving cardiovascular health, and increasing in bone density at higher levels. Plant phytoestrogens are increasingly used as an unregulated drug supplement in cosmetics, a food additive to enhance dietary protein, or a food texture modifier. Intentional dietary enrichment or supplement use is common in women seeking an alternative to hormonal replacement therapy and a preventive agent against breast cancer, osteoporosis, or cardiovascular disease[11-13]. More than 50% of breast cancer survivors use alternative medicine for hormone replacement therapy[14], and isoflavones are one of their favorable choices[11,15,16]. On the other hand, the interactive effect of soy components and TAM on breast cancer was not well defined experimentally, and the results have been conflicting[17,18]. Previous study reported that *in vitro* studies of human and mouse mammary tumor cell lines confirmed that co-administration of TAM with low dose of genistein promoted cell proliferation[17], while other study reported that combination of TAM with genistein or soy phytochemical concentrate, especially at the lower dose of TAM, had synergistic effects on delaying the growth of MCF-7 tumors[18]. (8,9)-furanyl-pterocarpan-3-ol (FPC) is a novel compound which is found and isolated from *Pachyrhizus erosus* (L). (*P. erosus*). The structure of this compound is similar to soy isoflavones[19]. On the basis of structure similarity, it is interesting to investigate the estrogenic effect of other phytoestrogens.

When TAM is widely used for breast cancer prevention and treatment, and consumption of plant phytoestrogens supplements by women with increased breast cancer risk is increasing, it is interesting to investigate the interactive effect between FPC and TAM on breast cancer prevention. This study is to evaluate the interactive effects of TAM and the novel phytoestrogen, FPC on preventing the growth of estrogen-dependent human breast cancer (T47D) cells *in vitro* and in a clinically relevant tumor model of breast cancer.

2. Materials and methods

2.1. Materials

FPC was extracted and purified from *P. erosus* (L) (Fabaceae) in Institut of Pharmacy, Wuerzburg University, under supervision by Prof. Dr. Ulrike Holzgrabe, who kindly supplied the substance for the research. TAM was obtained from Nacalai Tesque, Japan. All other chemicals used were in high analytical grade.

2.2. Cell lines

Human breast carcinoma T47D cells were kindly provided by Prof. Masashi Kawaichi from Nara Institute of Science and Technology (NAIST), Nara, Japan. T47D cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing fetal bovine serum (10% v/v) (Gibco, Invitrogen Corp, NY, USA) and penisillin-streptomisin (1% v/v) (Gibco, Invitrogen Corp, NY, USA).

2.3. Cell proliferation assay

Cell viability was determined by the MTT [3-(4,5 dimethylthiazol-2-yl]-2,5-dipheniltetrazolium bromide) (Sigma-Aldrich Co., St. Louis, MO, USA) colorimetric assay. Cells were cultured in an appropriate medium at 37 °C in a 5% CO_2 atmosphere. After cells reached 80% confluence and good viability, cells were seeded at a concentration of 1.0×10^4 cells/well. After 48 h of attachment, culture medium was discarded and the cells were treated with various concentrations of FPC (0.01-200 μmol/L) or a combination of FPC and TAM (20 nmol/L) in 100 μL serum-free and phenol red-free DMEM. T47D cells were treated with FPC alone or combination for 48 h incubation. After treatment, cells were added with 10 μL of MTT (5 mg/mL) and incubated for 6 h at 37 °C. After 6 h, stopper sodium dodecyl sulphate (10%) (Sigma-Aldrich Co., St. Louis, MO, USA) in 0.01 mol/L HCl were added to dissolve formazan crystal. Cells were incubated over night and protected from light. Cells were shaken for 10 min before read by ELISA reader at λ=595 nm. The absorbance of each well was converted to percentage of viable cells.

2.4. Immunocytochemistry of ERα and cMyc

Breast cancer T47D cells (5×10^4 cells/well) were seeded in cover slips in 24-well plates (Iwaki, Japan) until 80% confluence. Cells was treated with test compound, low concentration (1 μmol/L) of FPC or combination with TAM (20 nmol/L) for 10-15 h. Then the culture medium was removed and the cells were washed

by 500 μL phosphate buffere saline (PBS), added with trypsin/EDTA (100 μL) and incubated for 2 min at 37 °C. After incubation cells were added into the culture medium, homogenized, and filled in Eppendorf centrifugal machine. Subsequently, cells were centrifuged at 3 000 r/min for 5 min, and supernatant was removed, culture medium were added and cells were suspended in the medium. Cells then was smeared on poly-l-lysine slide at room temperature and then fixed in acetone for 10 min and washed with PBS for 5 min. Slide was incubated with endogenous H_2O_2 (Lab Vision Plus Co., CA, USA) for 10 min and washed with distilled water and PBS for 5 min. Slide then was incubated with Ultra V Block (Lab Vision Plus Co., CA, USA) for 5 min at room temperature to prevent non-specific staining. Afterward, slide was dropped by primary antibody of anti-estrogen ERα (F-10): sc-8002 and c-Myc (C-19) (sc-788) (Santa Cruz Biotechnology, Inc., CA, USA) with 1:100 dilution for 60 min and washed with PBS for 3-5 min. Further, slide was incubated with secondary antibody Biotinylated Goat Anti-Polyvalent Plus (Lab Vision Plus Co., CA, USA) for 5 min at room temperature and rinsed with PBS for 3-5 min. Finally, slide was incubated with streptavidin-peroxidase complex reagent for 10 min, removed, and washed with PBS and incubated with chromogen 3,3-diaminobenzidin (Novocastra Lab. Ltd., UK) for 5-15 min and washed with distilled water. Slide was soaked in haematoxylin (Sigma-Aldrich Corp, St. Louis, MO, USA) for 2-4 min to counter-stain and washed with distilled water and closed the cover slip. ERα or cMyc expression was observed using a light microscope. Cells with positive ERα or cMyc expression appear in dark brown color, while cells with no expression appear in blue or violet color.

2.5. Statistical analysis

Data from the *in vitro* experiments were expressed as the mean±SEM. Calculation of the statistical significances was performed with a One-way ANOVA, followed by Bonferroni post-hoc test analysis using rel 15.0 software SPSS (Chicago, IL, USA). Values of $P<0.05$ were considered to indicate significant differences.

3. Results

3.1. The growth inhibiton of TAM or FPC alone on T47D cells

The result demonstrated that TAM administration alone very effectively inhibited the growth of T47D cells with very low concentrations in the range of 1-50 nmol/L. Results of linear regression calculations indicated the IC_{50} value of 27.48 nmol/L (Figure 1A). In contrast, FPC have estrogenic effects which promote cell proliferation with low concentrations lower than 1 μmol/L (Figure 1B). Increasing concentration of FPC demonstrated the concentration-dependent inhibition of growth of T47D cells with IC_{50} value of 104.14 μmol/L (Figure 1B).

3.2. Co-administration of FPC on TAM-associated growth inhibition on T47D cells

When these cells were exposed to TAM alone, cell growth was inhibited in T47D cells. When culture cells were exposed to TAM plus low doses of FPC (0 to 1 μmol/L), TAM-associated growth inhibition was attenuated by FPC. When higher concentrations of FPC were added to TAM (>20 μmol/L), growth inhibition occurred in cell lines. This inhibition was little bit lower than that observed with TAM alone, suggesting that the combination of TAM and higher doses of FPC reduced its ability to inhibit T47D cell growth (Figure 2).

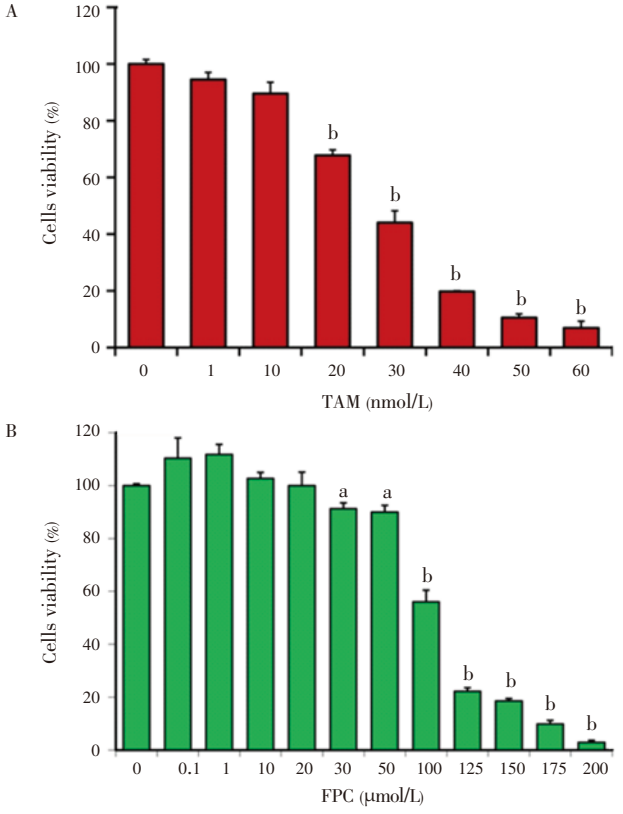

Figure 1. The inhibitory of TAM (A) or FPC (B) alone on growth of breast cancer T47D cells.
Data represent mean of triplicate independent assay±SEM. The *in vitro* experiment was carried out for 48 h incubation. a: $P<0.05$ and b: $P<0.01$ significantly different with control.

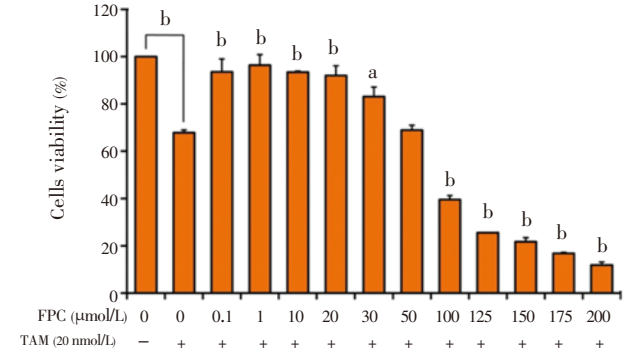

Figure 2. Effect of combination of TAM (20 nmol/L) and FPC on growth of human breast cancer T47D cells.
Data represent mean of triplicate independent assay±SEM. The *in vitro* experiment was carried out for 48 h incubation. a: $P<0.05$ and b: $P<0.01$ significantly different with TAM 20 nmol/L alone.

3.3. Immunohistochemistry analysis

In the immunocytochemical study, we found that TAM 20 nmol/L down-regulated ERα *in vitro* (Figure 3B), but the combination of TAM 20 nmol/L and FPC 1 μmol/L robust up-reguated the expression of ERα (Figure 3D). Thus, the reduced growth inhibition of TAM 20 nmol/L in combination with FPC 1 μmol/L may act via the modulation of ERα expression. In the present study we also examined cMyc expression induced by TAM alone or in combination with FPC. The results demonstrated that TAM 20 nmol/L also down-regulated cMyc *in vitro* (Figure 4B), but the combination of TAM 20 nmol/L and FPC 1 μmol/L also up-reguated the expression of cMyc (Figure 4D).

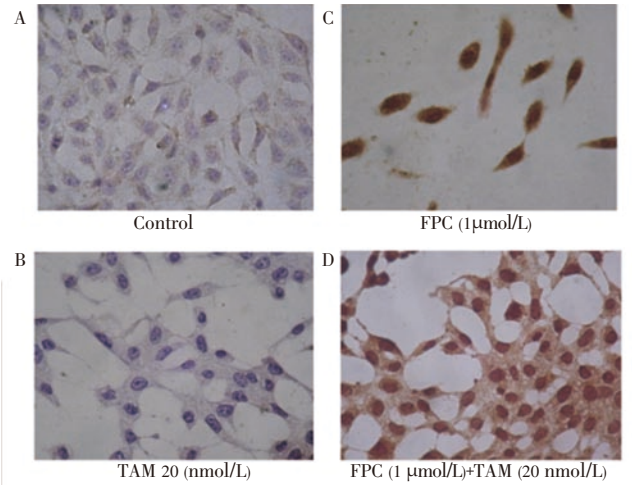

Figure 3. Effect of FPC and TAM, alone and combination, on expression of ERα by immunocytochemistry.

(A) Control treatment; (B) TAM 20 nmol/L; (C) FPC 1 μmol/L; (D) FPC 1 μmol/L+TAM 20 nmol/L. Cells with positive ERα expression appear in dark brown color, while cells with no expression appear in blue or violet color.

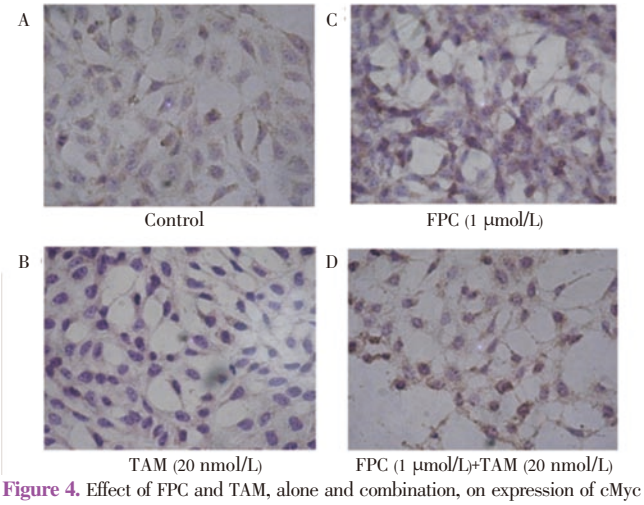

Figure 4. Effect of FPC and TAM, alone and combination, on expression of cMyc by immunocytochemistry.

(A) Control treatment; (B) TAM 20 nmol/L; (C) FPC 1 μmol/L; (D) FPC 1 μmol/L + TAM 20 nmol/L. Cells with positive cMyc expression appear in dark brown color, while cells with no expression appear in blue or violet color.

4. Discussion

The study revealed that TAM, one of agent SERMs, has a very effective activity against breast cancer T47D cells. TAM is a drug that is widely used for the treatment of breast cancer survivals in clinical researches[20-22]. For latest decades, TAM has been still standard treatment for breast cancer especially for premenopausal women[23]. T47D cells are ER-positive human breast cancer as evidenced by the response to increased cell proliferation as a results of exposure to 17-estradiol[24]. TAM is one of the estrogen antagonist which inhibits the activity of estrogen in most tissues that are sensitive to estrogen. TAM has become the standard treatment for all stages of breast cancer in both pre- and post menopausal women[7]. The potential benefits of the use of TAM as a preventive has been widely studied and reported that TAM has ability to reduce the risk of breast cancer in approximately 50% of most women who have a high risk of breast cancer[6]. The result of TAM on inhibitory cell proliferation of T47D was support by previous studies.

In addition, we also determined the estrogenic effects of FPC using human breast cancer T47D cells which is a positive ER model. The present results suggested that FPC had estrogenic effects of cell proliferation at concentrations lower than 20 μmol/L and shown growth inhibition above 20 μmol/L on T47D cells. The biphasic effect FPC are consistent with previous study in which the other phytoestrogens, genistein and its synthetic analogs could inhibit the growth of cancer cells *in vitro*, including hormone-dependent breast cancer or not with IC_{50} in the range between 10-50 μmol/L (more than 10 μmol/L)[25,26]. While at concentrations lower than 10 μmol/L or low to moderate dose (6.25 and 12.50 g/kg body weight), the growth of MCF-7 (estrogen receptor-positive cell line) or human breast cancer cells (MCF-7) implanted into athymic mice were stimulated by genistein or soy isoflavone extracts[27-29]. The biphasic effect of phytoestrogens genistein or FPC is most likely related to estrogen-like effects at low concentrations, while at high concentrations, these phytoestrogens act not only on estrogen receptors, as for example is the inhibition of one or more cellular signaling molecules that control cell growth and cell apoptosis.

The interaction between FPC and TAM as a standard treatment for all stages of breast cancer in pre- and post-menopausal women was never studied in more detail. As the use of alternative dietary phytoestrogen supplements is increasing, it is very important to examine the estrogenic effects of novel phytoestrogen, FPC and its interactions with TAM as the standard treatment of breast cancer. It is important to note that even though FPC is weakly estrogenic, the estrogenic effects of FPC was still sufficient to attenuate or reduce the growth inhibition effect of TAM on T47D cells. This result was supported by previous study low-dose dietary genistein negates the therapeutic effect of TAM in athymic nude mice[17].

Estrogen plays the important role in the development and growth of estrogen-dependent breast cancer. Estrogen act the estrogenic effect via ERα and ERβ that initiates the cascade of events, leading the modulation of hormon-responsive gene and cell proliferation. ERs especially ERα play an important role inmediating estrogen for the estrogenic effect[30,31]. The molecular mechanism behind these interactions may be multifactorial. However, a weak estrogenic FPC can compete with TAM and its metabolites for binding to ER-α. In this interaction, activation of ER-mediated processes occurs resulting in up-regulation of estrogen-responsive element and cell cycle progression-regulated gene expressions, and attenuated TAM growth inhibitory effect on T47D cells. It is also possible that another cellular mechanisms may involved in which FPC may act through non transcriptional pathways such as growth factors, protein tyrosine phosphorylation and activation of the mitogen-activated protein kinase pathway. cMyc protein is a specific regulator of cell growth rate in G0-S phase by affecting the expression of the G1 phase, cyclin D thus able to pass the restriction point in the cell cycle, including in breast cells[32]. Previous study reported that almost all of acutely estrogen-regulated genes in cell growth are also cMyc targets, and that estrogen activation of rRNA synthesis and protein synthesis depends on cMyc[33]. The results indicated that the regulation of cMyc on cell proliferation may associate with estrogen reseptors. The results showed that TAM act as anti-estrogen and FPC modulate cMyc at least in part via ERs.

In conclusion, these findings indicated and suggested that FPC was a novel phytoestrogen and had an estrogenic activity at low concentrations (lower than 20 μmol/L) and anti-estrogenic (more than 20 μmol/L). In the present study, we confirmed that low-concentration of FPC attenuated the growth-inhibitory effects of TAM on breast cancer T47D cells. Therefore, the results from this study raise the suspicion that low-concentration phytoestrogens FPC may interfere and attenuated the prevention or treatment benefits of TAM in clinical setting.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors wish to thank Prof. Dr. Ulrike Holzgrabe (Institut of Pharmacy, Wuerzburg University, Germany), who kindly supplied the FPC for this study. This work was supported in part by Hibah Competition Grant Research of Faculty of Pharmacy, Gadjah Mada University (Grant No.UGM/FA/851.d/M/05/01).

Comments

Background

Worldwide, the breast cancer is a public health problem that causes death among women. This study shows that there are products which inhibit the growth of cancer cells. It's therefore very useful as this study demonstrates that FPC may interfere and attenuated the prevention or treatment benefits of TAM in clinical setting

Research frontiers

This work was undertaken for the assessment of the effect of phytoestrogen substance FPC, extracted from *P. erosus* (L) (Fabaceae) on the growth of breast cancer cells in T47D cells alone and in combination with an anti-estrogenic product called tamoxifen.

Related reports

The toxicity assessment *in vitro* of natural products studies uses human cells from cancer cells by MTT method. It is obvious that this study as part of the search for anti-estrogenic produced by the MTT technique and T47D cells gives good results.

Innovations and breakthroughs

There are many studies on breast cancer cells, but the peculiarity of this study is to provide for the first time information about a molecule from plant used by the people in consumption of dietary. Based on the results, not only confirms the anti-estrogenic effect, but also advises on the use of the molecule by patients

Applications

As many studies that show the existence of substances which reduce the breast cancer cells proliferation, this study shows that it's now possible to rely on the molecule in the fight against breast cancer.

Peer review

Biochemical and pharmacological studies have shown that plants have active molecules against several diseases. This study has shown that *P. erosus* (L) (Fabaceae) has anti-estrogenic properties therefore has a molecule that can be used to fight against breast cancer. This is an investigational study which reinforces pharmacological research.

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