Turk J Pharm Sci 2020;17(3):280-284
DOI: 10.4274/tjps.galenos.2019.30633

ORIGINAL ARTICLE

Pyrophen Isolated from the Endophytic Fungus *Aspergillus fumigatus* Strain KARSV04 Synergizes the Effect of Doxorubicin in Killing MCF7 but not T47D Cells

Endofitik Mantar *Aspergillus fumigatus* KARSV04 Suşundan İzole Edilen Pirofen Doksorubisinin MCF7 Hücrelerini Öldürme Etkisini Sinerjize Etmiş T47D Hücreleri Üzerindeki Etkisini Değiştirmemiştir

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ABSTRACT

Objectives: Pyrophen, an amino acid-pyrone derivative isolated from *Aspergillus fumigatus* strain KARSV04 has been reported to have an anticancer effect on T47D cells by inhibiting the growth of cells and modulating the cell cycle in the S phase. In the present study, the effect of pyrophen in doxorubicin (Dox) chemotherapy in an *in vitro* model of breast cancers was studied.

Materials and Methods: The cytotoxicity of pyrophen and Dox separately and in combination were evaluated in T47D and MCF-7 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Modulation of cell cycle distribution and apoptosis was examined by flow cytometry.

Results: Our findings showed that pyrophen did not significantly potentiate Dox-induced cytotoxicity in T47D cells. Adding Dox-treated T47D cells with pyrophen at a concentration of 9.20 µg/mL induced a slight increase in the S-phase cell population. This compound induced cytotoxicity of MCF-7 cells with IC_{50} of 70.57 µg/mL. Co-treatment of pyrophen and Dox in MCF-7 cells increased cytotoxicity relative to Dox alone, which was suggested in part to be due to modulation of the cell cycle in the G2/M phase and apoptosis.

Conclusion: The data suggest different mechanisms of regulation in promoting cell death by two different cell lines in response to administration of pyrophen.

Key words: *Aspergillus fumigatus*, pyrophen, doxorubicin, T47D, MCF-7

ÖZ

Amaç: *Aspergillus fumigatus* KARSV04 suşundan izole edilen amino asit-piron türevi olan pirofenin, hücrelerin büyümesini engelleyerek ve S fazındaki hücre döngüsünü modüle ederek T47D hücreleri üzerinde antikanser etki gösterdiği rapor edilmiştir. Bu çalışmada, pirofenin doksorubisin (Dox) kemoterapisindeki olası etkisi *in vitro* meme kanseri modelinde incelenmiştir.

Gereç ve Yöntemler: Pirofen ve Dox için ayrı ayrı ve kombinasyon halinde T47D ve MCF-7 hücrelerinde 3-(4,5- dimetil tiyazol -2-yl)-2,5- difenil tetrazolium bromür ile sitotoksisite deneyi yapılmıştır. Hücre döngüsünün modülasyonu ve apoptozis akış sitometrisi ile incelendi.

Bulgular: Bulgularımız, pirofenin T47D hücrelerinde Dox kaynaklı sitotoksisiteyi önemli ölçüde artırmadığını gösterdi. Dox ile muamele edilmiş T47D hücrelerine, 9,20 µg/mL konsantrasyonunda pirofen eklenmesi, S-faz hücre popülasyonunda hafif bir artışa neden oldu. Bu bileşik, MCF-7 hücrelerinde

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Received: 16.01.2019, Accepted: 21.02.2019

sitotoksisiteyi indüklemiştir (IC_{50} 70,57 µg/mL). MCF-7 hücrelerine pirofen ve Dox'un birlikte uygulanması, tek başına Dox ile karşılaştırıldığında sitotoksisiteyi arttırmıştır; bunun kısmen hücre döngüsünün G2/M fazında modülasyonu ve apoptoz kaynaklı olduğu düşünülmüştür.

Sonuç: Veriler, pirofen uygulamasına yanıt olarak iki farklı hücre hattında indüklenen hücre ölümünün farklı düzenleme mekanizmaları olduğunu düşündürmektedir.

Anahtar kelimeler: *Aspergillus fumigatus*, pirofen, doksorubisin, T47D, MCF-7

INTRODUCTION

Breast cancer is one of the most common cancers, affecting women all around the world, and the risk factor increases are influenced by age.[1,2] Doxorubicin (Dox), an anthracycline antibiotic, is one of the most widely used chemotherapeutic agents for breast cancer treatment.[3] It exhibits anticancer activity by intercalation with DNA and inhibits topoisomerase II as well as generation of reactive oxygen species, resulting in apoptosis of tumor cells.[4-6] In spite of the widely used Dox to treat cancer, its side effects, including cardiotoxicity, and the development of drug resistance limit its clinical application in cancer therapy.[7-9] Many strategies have been developed to minimize its side effects and improve its chemotherapeutic effect, one of which involved combining Dox with some sensitizing agents.[10-12] A proteasome inhibitor, carfilzomib, was reported to increase Dox-induced cytotoxic effects and apoptosis in various subtypes of breast cancer.[10] Similarly, a phosphodiesterase-5 inhibitor, sildenafil, was shown to enhance Dox-induced apoptosis in PC-3 and DU145 prostate cancer cells. A flavonoid, luteolin-7-O-β-D-glucopyranoside, isolated from *Dracocephalum tanguticum* exhibited protective activity against Dox-induced toxicity in H9c2 cardiomyocytes.[12]

Pyrophen is an amino acid-pyrone derivative isolated from various organisms including *Aspergillus niger* and *Alternaria alternata*.[13,14] Recently, Reber and Burdge[15] reported that this compound was able to be synthesized using commercially available *N*-Boc amino acids. Investigations on its potential as an anticancer agent are limited. Previous findings demonstrated that pyrophen isolated from *Aspergillus* sp. endophytic fungi modulated the T47D cell cycle.[16] The aim of the present study was to examine whether pyrophen enhanced the chemotherapeutic effect of Dox in T47D as well as in another type of cells, MCF-7.

MATERIALS AND METHODS

Materials

Pyrophen was isolated from the culture of endophytic fungus *Aspergillus fumigatus* strain KARSV04 (culture collection of Pharmaceutical Biology Department, Faculty of Pharmacy, UGM).[16] RPMI 1640, fetal bovine serum (FBS), penicillin/streptomycin, Fungizone, and sodium bicarbonate were supplied by Gibco. 4-(2-Hydroxyethyl)-1- piperazine ethane sulfonic acid was obtained from Invitrogen. Phosphate-buffered saline (PBS), propidium iodide (PI), Annexin-V-FLUOS staining kit, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), RNAse, and Dox were purchased from Sigma-Aldrich.

Cell culture

T47D and MCF-7 breast cancer cells were grown in RPMI and DMEM media, respectively. Each medium was supplemented with 10% heat inactivated FBS, 1% penicillin/streptomycin, and 1 µg/mL Fungizone. The cultures were incubated in a humidified incubator at 37 ˚C with 5% CO_2.

Cytotoxicity assay

The cell cytotoxicity assay was conducted by modified MTT assay.[17] Briefly, 100 µL of media containing 10^4 cells was added to a 96-well plate followed by incubation for 24 h. The cells were further grown alone or treated with just pyrophen or in combination with Dox for another 24 h. The treated cells were gently washed with prewarmed 1X PBS, and 100 µL of media containing 0.5 mg/mL MTT was added to the wells. The cells were incubated for 4 h at 37 ˚C. Then 100 µL of 10% sodium dodecyl sulfate was added to the cells and they were incubated overnight at room temperature in the dark. The absorbance in each well was measured using a microplate reader (Bio-Rad) at 595 nm. The data generated were used to plot a cell viability curve. Each experiment was conducted in triplicate.

Cell cycle distribution and apoptotic cell analysis

Cell cycle distribution and percentage of apoptotic cells were examined using flow cytometry. Briefly, $1.5x10^5$ cells were inoculated in 6 well plates and grown for 24 h at 37 ˚C. For the cell cycle analysis, following treatment with pyrophen alone or in combination with Dox, the cells were harvested and fixed with cold ethanol. After washing with PBS, the cells were resuspended in buffer containing 1 mg/mL PI and 10 mg/mL RNAse, incubated for 5 min, and subjected to flow cytometry analysis. For the apoptotic cell analysis, following the addition of pyrophen alone or in combination with Dox, Annexin-V and PI were added to the cells, followed by incubation in the dark for 15 min at 4 ˚C and analysis by flow cytometry.

This article contains experimental studies that do not require approval by an ethics committee.

Statistical analysis

The results are expressed as mean ± standard error of the mean. Normal distribution was analyzed by Shapiro-Wilk test. $p>0.05$ indicated normal distribution. The difference in the averages of cell viability between groups was analyzed by one-way ANOVA using SPSS version 23.00. $P<0.05$ indicated a significant difference.

RESULTS

Pyrophen modulates the growth of MCF-7 cells

A previous study reported the cytotoxic effect *in vitro* of pyrophen on T47D breast cancer cells.[16] In order to assess the anticancer activity of this compound on other molecular subtypes of breast cancer, the MCF-7 cell line was used. MCF-7 cells were treated

with pyrophen at concentrations of 11.25-90.00 µg/mL, and the cell viability was determined by MTT assay. The results showed that pyrophen reduced the viability of MCF-7 cells in a dose-dependent manner and this was confirmed by morphological changes in the cells following 24 h treatment (Figure 1). The IC_{50} of pyrophen on MCF-7 cells was observed at 70.57 µg/mL.

The effect of pyrophen on Dox-treated T47D and MCF-7 cells

In order to study its potential as an adjuvant to existing chemotherapeutic agents, T47D and MCF-7 cells were treated with the tested compounds individually or in combination. In the present study, pyrophen at the concentrations used did not reduce the viability of Dox-treated T47D cells (Figure 2). Adding the Dox-treated cells with pyrophen up to 6.25 µg/mL also did not affect the cell cycle distribution. However, when the pyrophen concentration was raised to the IC_{50} value (9.2 µg/mL), there was a shift in the synthesis (S) phase cell population from 17.16% to 22.15% (Figure 3). It was interesting to note that there was a decrease in MCF-7 cell viability when the cells were treated with a combination of pyrophen and Dox compared to Dox alone. The effect was obvious when the cells were treated with pyrophen at a higher concentration (45 µg/mL) and this was observed in all concentrations of Dox-treated cells. Cell cycle analysis of Dox-treated MCF-7 cells administered together with pyrophen showed a decrease in the G0/G1 phase population and increased number of cells in the G2/mitotic (M) phase. Addition of a higher concentration of pyrophen up to 90 µg/mL increased the sub-G1 phase population from 3.5% (Dox-treated MCF-7 cells) to 20% (Dox-treated MCF-7 cells + pyrophen 90 µg/mL) (Figure 3). Further observation using Annexin-V in combination with PI staining showed that the number of apoptotic cells increased from 11.60% (Dox-treated MCF-7 cells) to 26.67% (Dox-treated MCF-7 cells + pyrophen 90 µg/mL) (Figure 4).

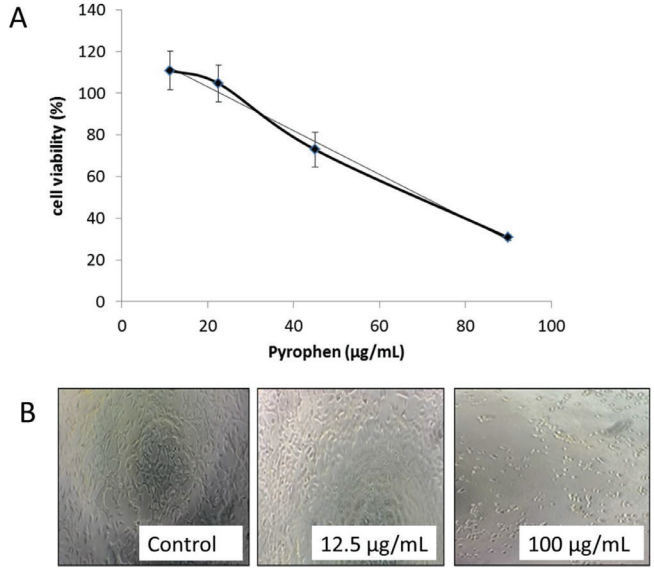

Figure 1. Cell viability and cell cycle profile of MCF-7 cells treated with pyrophen. Dose-viability curve response (A) and morphological changes in MCF-7 cells treated with pyrophen at various concentrations (B)

DISCUSSION

A previous study reported that pyrophen isolated from the endophytic fungus *Aspergillus fumigatus* KARSV04 showed toxicity towards p53 defective breast cancer cell line T47D.[16] Its potency to kill other breast cancer cells was further explored

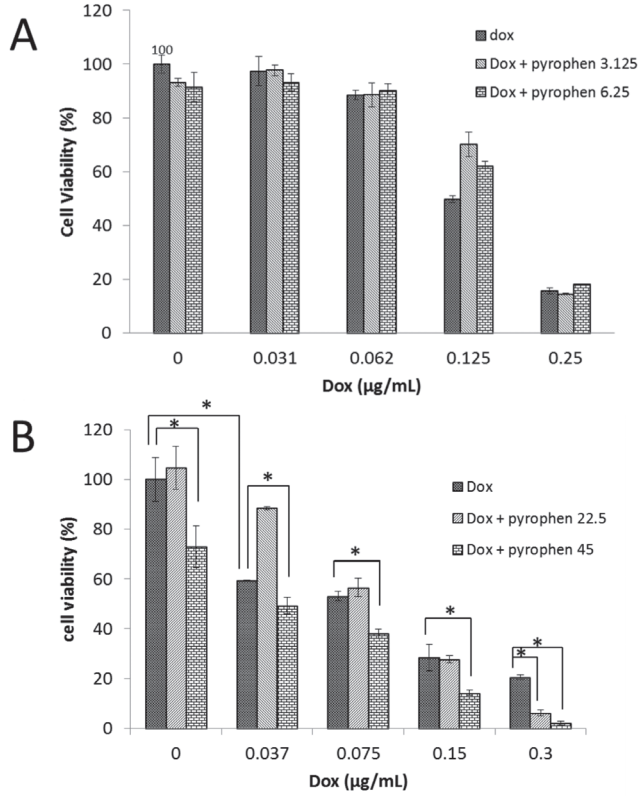

Figure 2. Viability of T47D cells (A) and MCF-7 cells (B) treated with Dox in combination with pyrophen. *p<0.05 compared with the value obtained for Dox alone

Dox: Doxorubicin

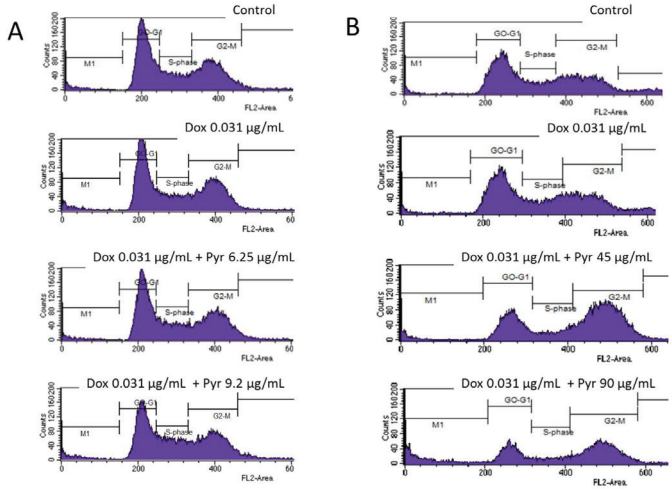

Figure 3. Cell cycle distribution of T47D (A) and MCF-7 (B) cells treated with Dox alone and in combination with pyrophen. Cells were inoculated in a 6-well plate followed by 16 h incubation with Dox alone or in combination with pyrophen in a humidified incubator at 37 °C, 5% CO_2. The cells were analyzed for cell cycle distribution by flow cytometry

Dox: Doxorubicin

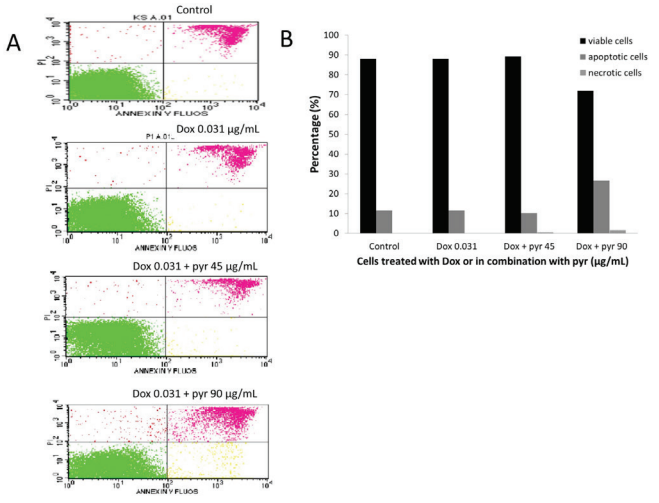

Figure 4. The effect of pyrophen on Dox-treated MCF-7 cells. Flow cytometry profiles (A) and apoptotic and necrotic cell distribution (B)
Dox: Doxorubicin

by observing its toxicity towards p53 competent MCF-7 cells. In the present study we found that pyrophen showed cytotoxicity towards MCF-7 cells. It inhibits the growth of MCF-7 cells in a concentration-dependent manner. However, its IC_{50} value is higher than that of T47D.

Further examination of its potential as an adjuvant to the existing conventional anticancer drug Dox was conducted. Adjuvant therapy of Dox with novel or bioactive compounds is reportedly a promising approach to increase the efficacy of this drug in breast cancer.[18-20] In the present study we found that although pyrophen is more toxic to T47D cells than to MCF-7 cells, combining this compound with Dox-treated cells demonstrated different effects. Pyrophen did not significantly affect the viability of Dox-treated T47D cells, while a synergistic effect was seen on Dox-treated MCF-7 cells in combination with pyrophen. It was interesting to note that adding pyrophen at 9.2 µg/mL to Dox-treated cells increased the S phase population of T47D cells, indicating that this compound may interfere with cell cycle progression though S phase modulation. A similar finding was obtained upon treating T47D with this compound alone.[16] On the other hand, it is indicated that pyrophen increased the G2/M population of MCF-7 cells. Combination of Dox with higher concentrations of pyrophen caused an increase in cell death as shown by an increase in the sub-G1 population and apoptotic cells. The decrease in the number of MCF-7 cells in the G2/M phase may contribute to a shift of the cell population to the sub-G1 phase.

The difference in response upon treatment of T47D and MCF-7 cells with pyrophen might be explained by the fact that these two breast cancer cell lines have different characteristics in that T47D cells are p53 defective mutants while MCF-7 bear p53 wild type.[21] The accumulation of T47D cells in the S phase may be in part due to the inability of cells to promote p53-dependent cell death and rely on S phase arrest following pyrophen treatment. Tumor cells that have a mutation on p53 have been reported to be resistant to DNA-damaging agents and diminish response to apoptosis-inducing agents.[22,23] MCF-7 cells, however, arrest at the G2/M phase upon treatment with Dox and further promote G2/M arrest upon combination with pyrophen. The functional p53 in MCF-7 cells may contribute to the additional increase in the G2/M cell population. Upon DNA damage, ATM and/or ATR kinase are activated, which then phosphorylate Chk1/Chk2, causing the inactivation of cdc25 phosphatase and thus preventing entry into mitosis. The ATM/ATR also phosphorylates p53 on S15, leading to increased transcription of $p21^{Waf/Cip1}$, GADD45, and 14-3-3, proteins, which are suggested to be responsible for maintaining G2 arrest.[24,25] Adding a higher concentration of pyrophen to the Dox-treated MCF-7 cells, however, led to cell death, which suggested that p53 may shift its function from promoting arrest to induction of apoptosis. Further research is needed to examine the effect of pyrophen in regulating apoptosis.

CONCLUSION

Pyrophen induced cytotoxicity towards MCF-7 cells and this effect was synergistic upon treatment with Dox. This compound induced accumulation of Dox-treated MCF-7 cells in the G2/M phase. Dox-treated T47D cells accumulated in the S phase upon treatment with pyrophen, suggesting a different mechanism in regulating cell cycle progression in these two cell lines.

ACKNOWLEDGEMENTS

The authors thank the Indonesian Ministry of Research, Technology and Higher Education under Universitas Gadjah Mada PUPT Grant 2016-2017 for providing funding for this study.

Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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