Research Article

Pyrophen isolated from endophytic fungi *Aspergillus fumigatus* strain KARSV04 synergizes the effect of doxorubicin in killing MCF7 but not T47D cells

Endofitik mantarlardan izole edilen pirofen *Aspergillus fumigatus* KARSV04, doksorubisin in MCF7'nin öldürülmesinde sinerji, ancak T47D hücrelerinde bu etkiyi gostermez

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Abstract
Introduction: Pyrophen, an amino acid-pyrone derivative isolated from *Aspergillus fumigatus* strain KARSV04 has been reported to exhibit anticancer effect on T47D cells by inhibiting the growth of the cells and modulate the cell cycle on S phase. Present study, effect of pyrophen in the doxorubicin (Dox) chemotherapy on an *in vitro* model of breast cancers was studied. **Methods:** The cytotoxicity of pyrophen, doxorubicin and in combination were evaluated in T47D and MCF-7 cells using MTT assay. Modulation on 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 concentration of 9.20 µg/mL induced slight increase of 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 due to modulation of cell cycle at 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.

Keywords : *Aspergillus fumigatus*, pyrophen, doxorubicin, T47D, MCF-7

ÖZ

Amaç: *Aspergillus fumigatus* KARSV04'ten izole edilen amino asit-piron türevi olan pirofen, hücrelerin büyümesini engeller ve S fazındaki hücre döngüsünü modüle ederek T47D hücreleri üzerinde antikanser etkisi gösterdiği rapor edilmiştir. Bu çalışmada, doksorubisin (Dox) kemoterapisindeki pirofen meme kanseri modelinde "in vitro" modeli üzerindeki etkisi incelenmiştir. **Metotlar:** Pirofen, doksorubisin ve kombinasyon halindeki sitotoksisite, MTT tahlili kullanılarak T47D ve MCF-7 hücrelerinde değerlendirildi. Hücre döngüsü dağılımı ve apoptozis modülasyonu akış sitometrisi ile incelendi. **Bulgular:** Bulgularımız, pirofen, T47D hücrelerinde Dox kaynaklı sitotoksisiteyi önemli ölçüde artırmadığını gösterdi. Pirofen eklenmesi, 9.20 µg/mL konsantrasyonunda Dox ile muamele edilmiş T47D hücrelerine S-faz hücre popülasyonunda hafif bir artışa neden oldu. Bu bileşik, IC50 70.57 µg/mL olan MCF-7 hücrelerinin sitotoksisitesini tesir etmistir. MCF-7 hücrelerinde pirofen ve Dox'un birlikte işlenmesi, sadece Dox ile karşılaştırıldığında sitotoksisiteyi arttırmıştır; bu, kısmen G2/M fazındaki hücre döngüsünün modülasyonu ve apoptoz nedeniyle önerilmiştir. **Sonuç:** Veriler, pirofen uygulamasına cevap olarak iki farklı hücre çizgisi tarafından hücre ölümünü teşvik etmede farklı düzenleme mekanizmaları olduğunu göstermektedir.

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

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INTRODUCTION

Breast cancer is one of the most common cancer affecting women all around the world with the risk factor increases are influenced by age.[1,2] Doxorubicin, 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, inhibits topoisomerase II as well as generation of reactive oxygen species (ROS), resulting in apoptosis of tumor cells.[4,5,6] Despite of the widely used doxorubicin (Dox) to treat cancer, the side effects of doxorubicin including cardiotoxicity and the development of drug resistance limits its clinical application in cancer therapy.[7,8,9] Many strategies are developed to minimize the side effects of doxorubicin and improve its chemotherapeutic effect, one of which by combining doxorubicin (Dox) with some sensitizing agents.[10,11,12] 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 (PDE-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 alternate*.[13,14] Recently Reber and Burdge reported that this compound was able to be synthesized using commercially available *N*-Boc amino acids.[15] Investigation on its potential as anti-cancer agent is limited. Previous finding demonstrated that pyrophen isolated from *Aspergillus* sp endophytic fungi modulated T47D cell cycle.[16] This study was aimed to examine whether pyrophen enhanced chemotherapeutic effect of doxorubicin in T47D as well as in other type of cells, MCF7.

MATERIALS AND METHODS

Materials

Pyrophen was isolated from the culture of endophytic fungi *Aspergillus fumigatus* strain KARSV04 (Culture collection of Pharmaceutical Biology Department, Faculty of Pharmacy UGM).[16] RPMI 1640, Fetal Bovine Serum, Penicillin - Streptomycin, Fungizon, Sodium bicarbonate, were supplied from Gibco. 4-(2-hydroxyethyl)-1- piperazine ethane sulfonic acid (HEPES) were obtained from Invitrogen; Phosphate-Buffered Saline (PBS), propidium iodide (PI), Annexin – V-FLUOS staining kit, 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazoliumbromide (MTT), RNAse, doxorubicin (Dox) were purchased from Sigma-Aldrich.

Cell culture

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

Cytotoxicity assay

Cell cytotoxicity assay was conducted using modified MTT assay.[17] Briefly, 100 µl of media containing 10^4 cells was added to 96-well plate and incubated for 24 hours. The cells were further grown alone or treated with pyrophen or in combination with Dox for another 24 hours. The treated cells were gently washed with pre-warmed 1X Phosphate-Buffered Saline (PBS), and 100 µl of media containing 0.5 mg/mL MTT was added to the wells. The cells were incubated for 4 hours at 37ºC. Then, 100 µL of 10% sodium dodecyl sulfate (SDS) was added to the cells and incubated overnight at room temperature in the dark. The absorbance in each well was measured using microplate reader (Bio-Rad) at 595 nm. Data generated were used to plot cell viability curve. Each experiment was conducted in triplicates.

Cell cycle distribution and apoptotic cell analysis

Cell cycle distribution and percentage of apoptotic cells were examined by flow cytometry. Briefly, 1.5 x10^5 cells were inoculated in 6 well plates and grown for

24h at 37ºC. For cell cycle analysis, following treatment with pyrophen or in combination with Dox, the cells were harvested and fixed with cold ethanol. After washing with PBS, the cells were re-suspended in buffer containing 1 mg/mL Propidium Iodide and 10 mg/mL RNAse, incubated for 5 minutes and subjected to flow cytometry analysis. For apoptotic cell analysis, following addition of pyrohen or in combination with Dox, annexin-V and PI were added to the cells, incubated in the dark for 15 minutes at 4^0 C and analysed by flow cytometry

Statistical analysis

The results are expressed as mean ± SEM. Normal distribution was analyzed by Shapiro-Wilk test. $p>0.05$ indicated normal distribution. The difference on 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

Previous study reported the cytotoxic effect *in vitro* of pyrophen on T47D breast cancer cells.[16] In order to assess the anti-cancer activity of this compound on other molecular subtype of breast cancer, MCF-7 cell line was used. MCF-7 cells were treated with pyrophen at the indicated 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 dose dependent manner and this was confirmed by morphological changes of the cells following 24 hours treatment (Figure 1). The IC_{50} of pyrophen on MCF-7 cells was observed at 70.57 µg/mL.

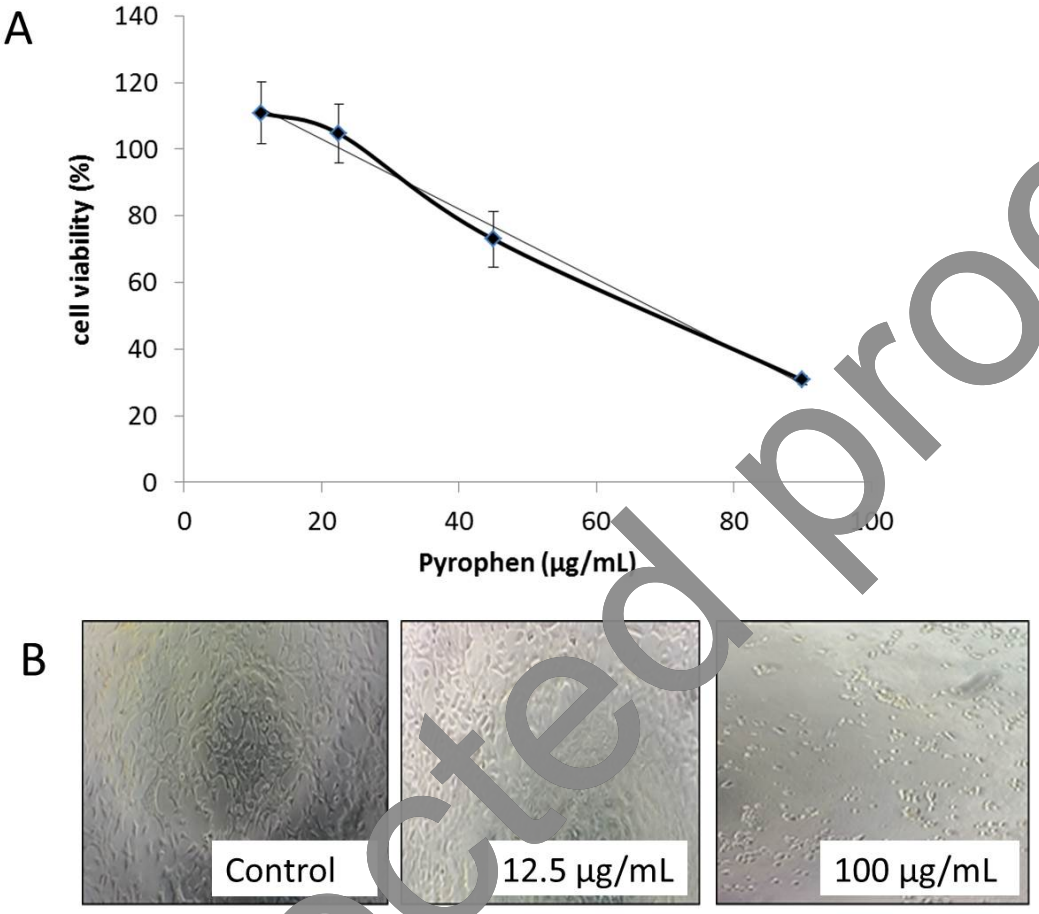

Figure 1: Cell viability and cell cycle profile of MCF-7 cells treated with pyrophen. Dose-viability curve response (A), and morphological changes of MCF-7 cells treated with pyrophen at various concentrations (B).

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

In order to study its potential as adjuvant of existing chemotherapeutic agents, T47D and MCF-7 cells were treated individually with the tested compounds or in combination. In this study, pyrophen at 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 rised to IC_{50} value (9.2 µg/mL), there was a shift in S phase cell population from 17.16 to 22.15% (Figure 3). It was an interesting to note that there was a decrease of MCF-7 cells viability when the cells were treated with a combination of pyrophen – Dox, compared to that of Dox alone. The effect was obvious when the cells were treated with pyrophen at 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 G0/G1 phase population and increased number of cell in G2/M phase. Adding higher concentration of pyrophen up to 90 µg/mL, increased 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 anexin 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).

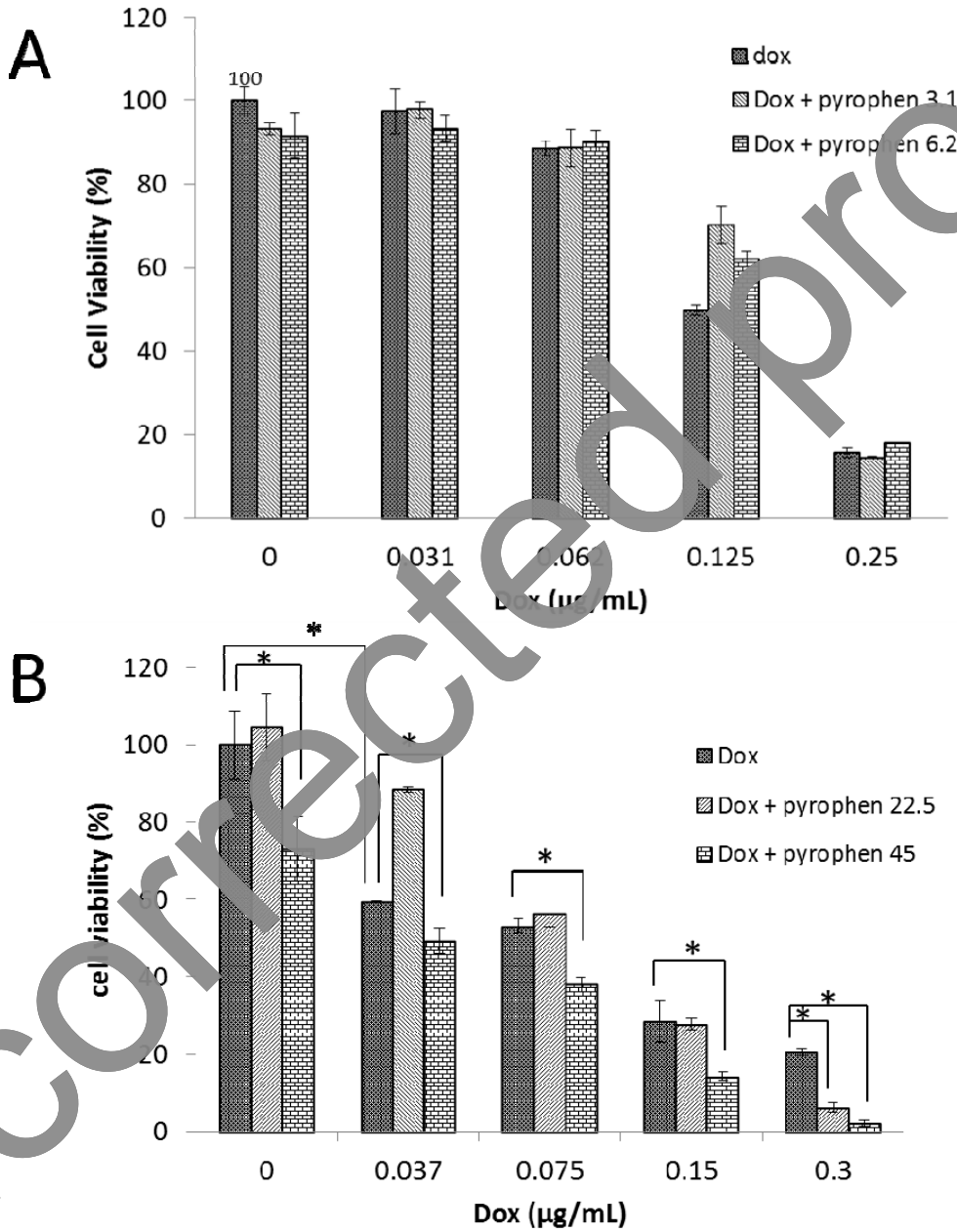

Figure 2. Viability of T47D cells (A) and MCF-7 cells (B) treated with Doxorubicin in combination with pyrophen. * $p<0.05$ compared with the value obtained for Dox alone.

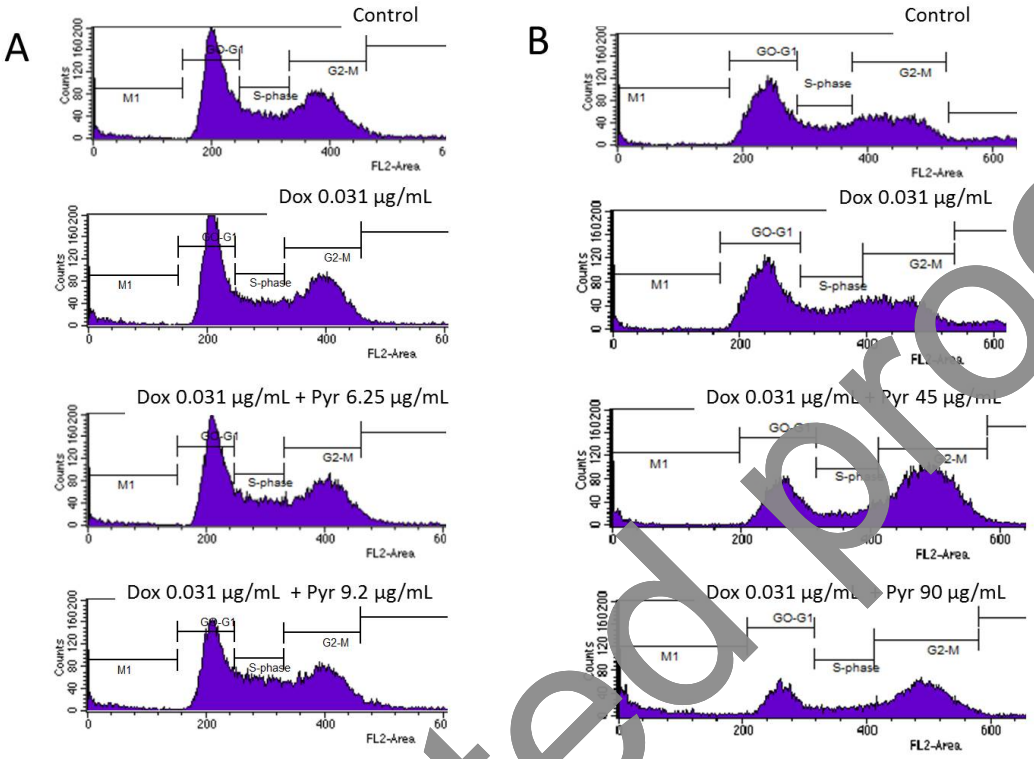

Figure 3. Cell cycle distribution of T47D (A) and MCF-7 (B) cells treated with Doxorubicin and in combination with pyrophen. Cells were inoculated in 6 wells plate followed by 16 hours incubation with Doxorubicin alone or in combination with pyrophen in humidified incubator at 37^0C, 5% $C0_2$. The cells were analyzed for cell cycle distribution by flow cytometry

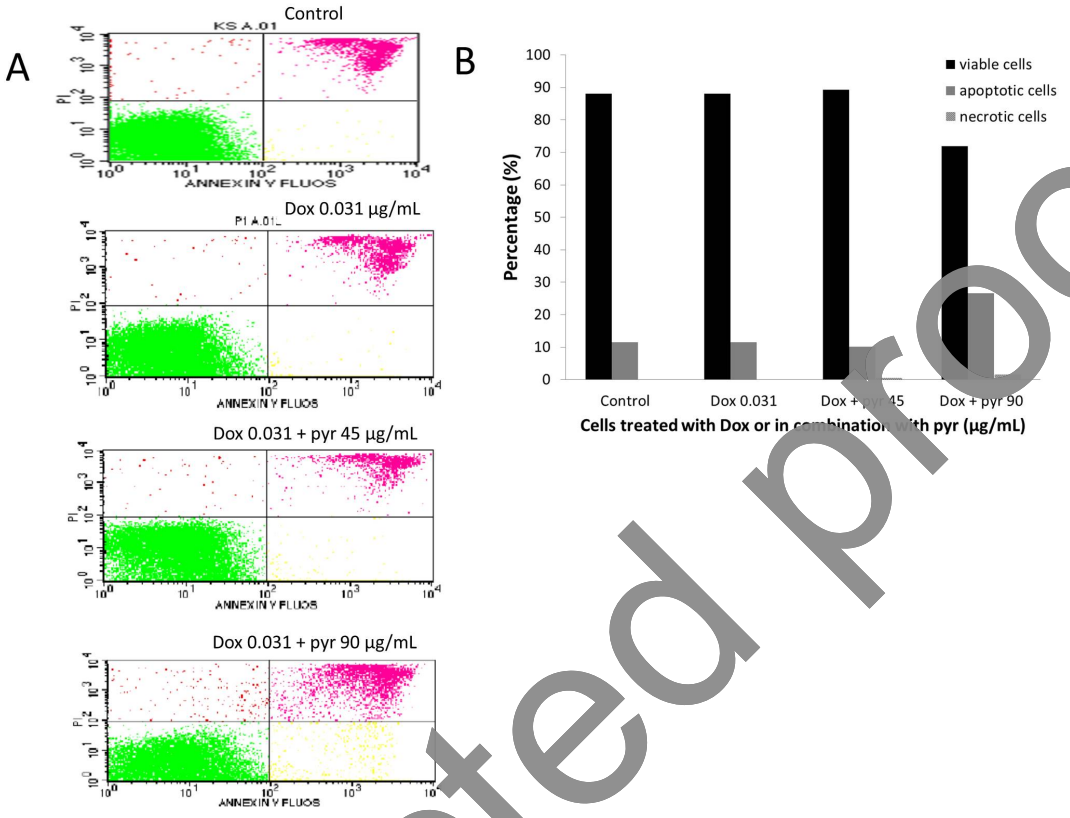

Figure 4. The effect of pyrophen on Dox treated MCF-7 cells. Flow cytometry profiles (A) and apoptotic and necrotic cell distribution (B).

DISCUSSION

Previous study reported that pyrophen isolated from endopytic fungi *Aspergillus fumigatus* KARSY04 showed toxicity towards p53 defective breast cancer cell T47D.[16] Further exploration on its potency to kill other breast cancer cell was examined by observing its toxicity towards p53 competent cells MCF-7 cells. In this study we found that pyrophen showed cytotoxity towards MCF-7. It inhibits the growth of MCF-7 cells in concentration dependent manner. However, its IC_{50} value is higher than that of IC_{50} T47D.

Further examination on its potential as adjuvant with existing conventional anticancer drug Doxorubicin was conducted. Adjuvant therapy of Doxorubicin with novel or bioactive compounds was reportedly exhibited promising approaches to increase the efficacy of this drug in breast cancer.[18,19,20] In this study we found that although pyrophen is more toxic to T47D cells compared to that of MCF-7 cells, combining this compound to Dox-treated cells demonstrated different effects. Pyrophen did not significantly affect the viability of Dox-treated T47D cells whilst synergistic effect was seen on Dox-treated MCF-7 cells in combination with pyrophen. Interesting to note that adding pyrophen at 9.2 µg/mL to Dox-treated cells increased S phase population of T47D cells indicating that this compound may interfere the cell cycle progression though S phase modulation. Similar finding was found upon treating T47D with this compound alone.[16] On the other hand, it is indicated that pyrophen increased G2/M

population of MCF-7 cells. Combination of Doxorubicin with higher concentration of pyrophen showed increase in cell death as examined in increase of sub-G1 population and apoptotic cells. The decrease in the number of MCF-7 cells in G2/M phase may contribute to shift of cell population to sub G1 phase. The difference response upon treatment of T47D and MCF-7 cells with pyrophen might be explained by the fact that these two breast cancer cell line have different characteristics in that T47D cells are p53 defective mutants whilst MCF-7 bearing p53 wildtype.[21] The accumulation of T47D cells in S phase may be in part due to inability of cells to promote p53 dependent cell death and rely on S phase arrest following treatment of pyrophen. Tumor cells which has mutation on p53 has been reported to be resistant to DNA damaging agent and diminish response to apoptosis inducing agents.[22,23] MCF-7 cells, however, arrest on G2/M phase upon treatment of Doxorubicine, and further promote G2/M arrest upon combination with pyrophen. The functional p53 in MCF-7 cells may contribute in the additional increase of 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 a G2 arrest.[24,25] Adding higher concentration of pyrophen to the Dox-treated MCF-7 cells, however, led to the cell death suggested that p53 may shift its function from promoting arrest to induction of apoptosis. Further study is needed to examine the effect of pyrophen in regulating apoptosis.

CONCLUSION

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

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