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Synergistic Combination of Fluoro Chalcone and Doxorubicin on HeLa Cervical Cancer cells by Inducing Apoptosis

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Abstract. Doxorubicin (Dox), a primary chemotherapeutic agent used for cancer treatment is known to have various side effect included multidrug resistance (MDR) phenomenon. Combination chemotherapy is one of some approaches to reduce Dox side effect. Chalcones have been reported to reduce the proliferation of many cancer cells. The research were conducted to investigate the cytotoxic activity and apoptosis induction of a chalcone derivate which is containing fluoro substituent [1 - (4" - fluorophenyl) -3 - (4' - hydroxy - 3' - methoxyphenyl) - 2 - propene - 1 –on] (FHM) and its combination with Dox on HeLa cells line. The observation of the cytotoxic activity was conducted using MTT [3 - (4, 5 - dimethyl thiazol - 2 - yl) - 2.5 - diphenyltetrazolium bromide] assay. Apoptosis induction was determined by flow cytometric. The changes of cell morphology were observed using phase contrast microscopy. The combination index (CI) was used to determine the effect of the combination. The study showed that FHM inhibited the HeLa cell growth with IC_{50} of 34 μM, while the IC_{50} of Dox was 1 μM. The combination had a higher inhibitory effect on cell growth compare to the single treatment of FHM and Dox. All of the combination doses under IC_{50} of FHM and Dox gave synergistic (CI: 0.3 – 0.7) up to strong synergistic effect (CI: 0.1 – 0.3). The synergistic effects of the combination were due to their ability to induce apoptosis in the HeLa cells. According to the result, FHM was potential to be developed as a co-chemotherapeutic agent with Dox for cervical cancer.

INTRODUCTION

Cervical cancer remains a significant threat to women in the world. World Health Organization (WHO) in 2012 [1] reported that cervical cancer is the fourth most common cancer in the women. In developing countries such as Indonesia are many risk factors can lead to cervix cancer, thus causing very high incidence rate. It has been reported that cervical cancer is the first rank (28.66%) of most women cancer cases in Indonesia [2]. One of the cancer treatments is chemotherapy using Doxorubicin (Dox). This chemotherapeutic drug is given in various types of cancer. However, long term used of Dox caused various problems such as heart toxicity, drug resistance, and toxic effect of normal tissue [3]. The combination of Dox and other chemoprevention have been reported to reduce side effect and resistance [4,5,6]. Therefore, the exploration of compounds that can increase the efficacy of Dox is still indispensable.

A natural product from various plants has shown promising anti-cancer activity. Chalcone (1,3-diphenyl-2-propone-1-one (Fig 1a) one of the major classes of a natural product with widespread distribution in spices, tea, beer, fruits, and vegetables, display various interesting activities including anticancer properties [7,8]. Chalcone and its derivates can be synthesized classically by Claisen-Schmidt condensation between benzaldehyde and acetophenone employing strong bases such as NaOH, KOH, $Ba(OH)_2$, hydrotalcite, LiHMDS, calcined

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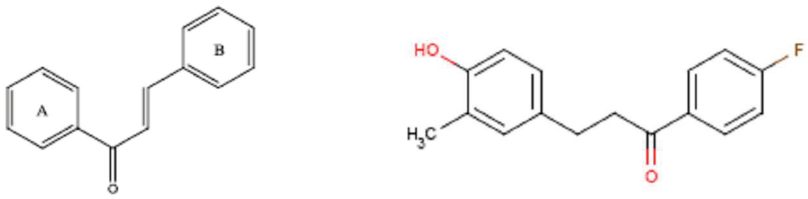
NaNO3/natural phosphate. There are also some reports of acid-catalyzed aldol condensations, e.g. $AlCl_3$, BF_3, dry HCl, $ZrH_2/NiCl_2$ and $RuCl_3$ (for cyclic and acyclic ketones) [9, 10]. Generally, the main target of chalcone and its derivates is to induce apoptosis and inhibit cell cycle [11-16]. Arty *et al*. [17] have synthesized several chalcones derivates, one of them was 1- (4 "-fluorophenyl)-3- (4'-hydroxy-3'-methoxyphenyl) -2-propene-1-on or FHM (Fig 1b). This compound displays the antioxidant activity and inhibited lipid peroxidation and cyclooxygenase. FHM also showed the cytotoxic activity on HeLa, Raji, and T47D cells [18, 19]. The aims of the study were to investigate the cytotoxic activity of FHM alone, Dox alone, and the combination of both in HeLa cells and to study their effects on apoptosis.



(a) (b)

FIGURE 1. The structure of (a) Chalcone and (b) 1 - (4" - fluorophenyl) -3 - (4' - hydroxy - 3' - methoxyphenyl) - 2 - propene - 1 –on or FHM

MATERIALS AND METHODS

Materials

The compound, [1 - (4" - fluorophenyl) -3 - (4' - hydroxy - 3' - methoxyphenyl) - 2 - propene - 1 –on] (FHM), was produced through a cross aldol condensation reaction of *p*-fluoro acetophenone and 4-hydroxy-3-methoxy-benzaldehid in an acidic condition. The compound was used as a stock solution with a concentration of 100 mM in dimethylsulfoxide (DMSO). The final concentration of DMSO in the study wells was kept less than 0.2%. Chemotherapeutic agent doxorubicin (Dox) was produced from Ebewe, Indonesia.

HeLa breast cancer cells from ATCC were obtained from the collection of the Laboratory of Parasitology, Faculty of Medicine, Universitas Gadjah Mada (UGM). Cells were grown in medium culture Rosewell Park Memorial Institute Medium (RPMI) 1640 from Gibco containing 10% FBS (Fetal Bovine Serum, Gibco), 0,5% fungizone, and 2% penicillin-streptomycin (Gibco) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The RPMI medium was prepared by dissolving 10.4 g RPMI; 2.2 g $NaHCO_3$ (Merck); and 2 g Hepes (Gibco) in sterile aquabidest and conditioned at a pH of 7.2 to 7.4, then was added aquabidest until a final volume of 1 liter. Furthermore was sterilized with a 0.2-micron filter. Trypsin-EDTA 0.025% (Gibco) was used to detach cells on the flask.

Methods

Cytotoxicity Assay

Cytotoxicity experiments were performed using MTT assay. HeLa cells were seeded at a density of 10^4 cells per well for cytotoxic assay and allowed to attach for 24 hours in a humidified incubator at 37°C. One day after initial seeding, the cells were treated various concentrations of FHM alone, Dox alone, and their combination. After 24 hours incubation, the culture medium was removed and the cells were washed with 100 µL Phosphate Buffered Saline (Sigma). Then, 100 µL of MTT (Sigma Chemical, St. Louis, MO, USA) solution (0.5 mg/ml diluted with RPMI medium) was added to each well, followed by incubation for 4 h at 37°C. Viable cells react with MTT to produce purple formazan crystals. Then, 100 µL stopper reagent (10% Sodium dodecyl sulfate from Sigma Chemical, St. Louis, MO, USA in 0.01M HCl) was added to dissolve the formazan crystal. The cells were incubated for 12 hours (overnight) at room temperature and protected from light. The absorbance of each well was measured

using ELISA reader (Bio-Rad) at λ 595 nm. The absorbance was converted to a percentage of viable cells (20). The IC_{50} concentration was calculated by the concentration that caused 50% inhibition of cell growth [21]. The potential applications in combination therapy were analyzed using the Combination Index (CI) (Table 1).

TABLE 1. Interpretation of Combination Index (CI) Values

CI value	Interpretation
<0.1	very strong synergistic effect
0.1-0.3	strong synergistic effect
0.3-0.7	synergistic effect
0.7-0.9	moderate synergistic effect
0.9-1.1	nearly additive effect
1.1-1.45	moderate antagonist effect
1.45-3.3	antagonist effect
>3.3	Very strong

The CI was calculated based on equation below [22]:

$$CI = \frac{D1}{Dx1} + \frac{D2}{Dx2} \quad 1)$$

D1 : combination concentration of FHM
D2 : combination concentration of Dox
Dx1 : concentration of FHM in single dose that could inhibit the HeLa cells growth at the same point with combination concentration
Dx2 : concentration of Dox in single dose that could inhibit the HeLa cells growth at the same point with combination concentration

Apoptosis Detection

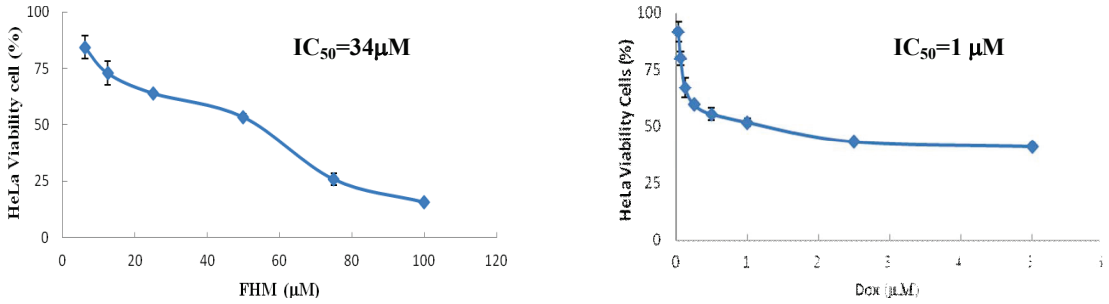
Apoptosis was detected using flowcytometer. HeLa cells were seeded on a six tissue culture well-plate at 5 x 10^5 cells per well then incubated for 24 hours. The cells then were treated with various concentration of FHM alone, Dox alone, and their combination. After 24 hours incubation, the cells were removed using 0.25% trypsin solution, then were centrifuged at 2000 rpm for 3 minutes, and were washed twice with cold PBS. The cells were re-suspended in 500 µl of Annexin V buffer (Roche), then were treated with Annexin V and propidium iodide (PI) for 10 minutes at room temperature and protected from light. The treated cells were subjected to FAC-Scan flow-cytometer.

RESULTS AND DISCUSSION

The work steps in this study were the cytotoxic assay of the single treatment of FHM and Dox, followed by testing of the cytotoxic effect of FHM-Dox treatments, and apoptosis assay. The concentrations applied in combination chemotherapy of FHM-Dox were referred to IC_{50} value of the single compound. The comparison of concentrations for combination test used in this study were 1/8; 1/4; 3/8 and 1/2 of the IC_{50} value.

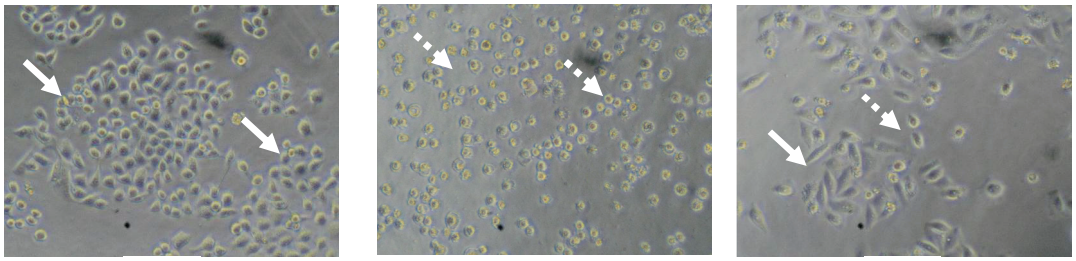
Cytotoxic Effect of FHM, Dox and FHM-Dox Treatments in HeLa cells

The results of the cytotoxic assay of single treatment (Fig. 2) indicated that FHM and Dox had cytotoxic activity in HeLa cells in a dose-dependent manner. FHM concentrations used in the treatment were 6.25; 12.5; 25; 50; 75 and 100 μM, respectively, while Dox concentrations were 0.03125; 0.0625; 0.125; 0,25; 0.5; 1; 2.5; and 5 μM, respectively. Based on a linear regression between the log of concentration and cell viability ($p<0.05$), IC_{50} of FHM was 34 μM, while IC_{50} of Dox was 1 μM. FHM and Dox treatments had also caused cells morphology changing as presented in Fig. 3. Some cells appear became rounded and detached from the bottom flask with a massive morphology. The rounded shape of cells indicated the mortality of HeLa cells. The changes were more apparent in line with increasing concentrations of FHM and Dox. This study showed that FHM has potent cytotoxicity against HeLa cells. We suspect that the presence of OH groups and fluoro groups contained in these compounds contribute to cytotoxic activity against HeLa cells. Some literature showed the substitution of a methoxy group on the A ring and the substitution of fluoro, chloro, bromo and ring B are able to improve the inhibitory activity of NF-κB, a transcription factor that plays a role in the development and progression of cancer [23, 24]. In addition, their unsaturated carbonyl groups α, β (α, β -unsaturated carbonyl) contained in chalcone is extremely electrophilic also contribute to the cytotoxic activity of cells [25].



(a) (b)

FIGURE 2. The effect of FHM and Dox treatments on the viability of HeLa cells. The cytotoxic activity was expressed by cell viability (%) is shown as mean ± SD of 3 experiments. The HeLa cells were incubated with (a) FHM and (b) Dox at various concentrations during 24 hours at 37°C. The viability of the HeLa cells was measured using MTT assay. FHM and Dox decreased the viability of HeLa cells in a dose-dependent manner. The IC_{50} value of FHM and Dox were 34 μM and 1 μM respectively.



(a) (b) (c)

FIGURE 3. The effects of FHM and Dox treatments on the morphology of HeLa cells. The morphologies of HeLa cells were (a) untreated, and treated with (b) FHM and (c) Dox at concentration 25 μM and 2.5 μM respectively. The observation of the cell morphology was performed using inverted microscope with a magnification of 100x. The bold arrows indicate normal living cells, whereas the dashed arrows indicate the cell morphological changes. The data showed that the single treatments of FHM and Dox caused the cells death which is marked with a rounded cell shape.

The FHM concentrations were used in the combination treatments as follows: 4.25; 8.5; 12.75; and 17 μM, while the Dox concentrations were 0.125; 0.25; 0.375; and 0.5 μM. The data showed that the combination treatments more decreased the viability cells compare to that of the single treatment of FHM and Dox (Fig.4). The lowest cells

viability (26.40%) was obtained at combination concentration of FHM and Dox of 17 μM and 0.5 μM respectively. The combination treatments also caused more changes morphology compared to that of the single treatment.

Based on CI value are presented in Table 2, the study showed that all combination concentration of FHM and Dox had a strong synergistic effect (CI: 0.2-0.3), except the combination concentration at 17 μM FHM and 0.125 μM Dox which had CI value of 0.4 (synergistic). The study showed that FHM has the potential to be used as a co-chemotherapeutic agent with Dox. We suspect that the synergy effects were due the contribution of FHM and Dox which have the same mechanism. The cytotoxic effects of each compound could be related to their ability to stimulate apoptosis and inhibit cell cycle.

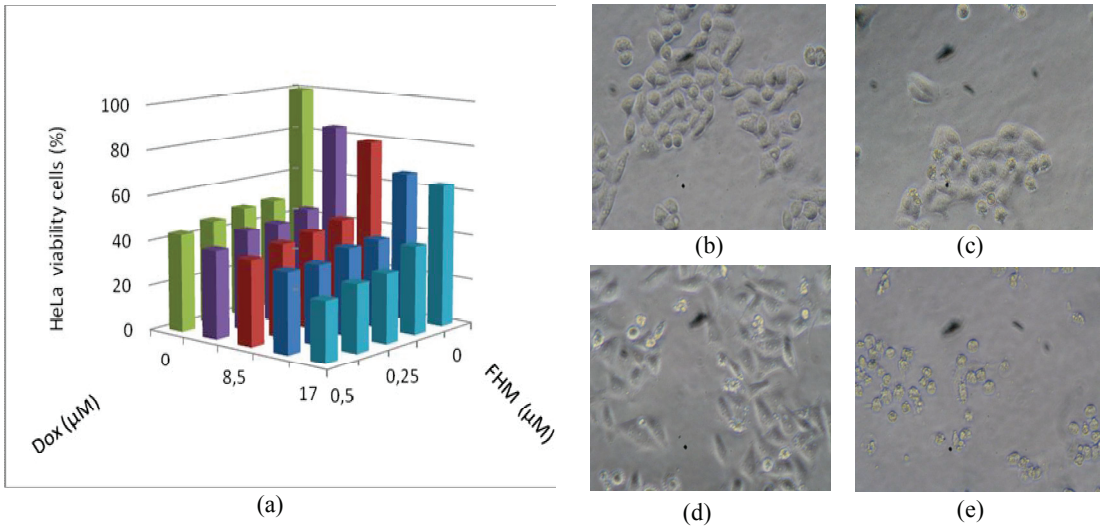


FIGURE 4.The effect of FHM-Dox treatment on the HeLa cell viability**.** The cytotoxic effect was expressed by percent cell viability. HeLa cells ($1x10^4$ cells/well) were seeded in 96 well plates and incubated for 24 hours. The cells were treated with FHM (4.25; 8.5; 12.75; and 17 μM); Dox (0.125; 0.25; 0.375; and 0.5 μM); and their combination for 24 hours. The cells viability was determined by using MTT assay. Graph (a) showed that the FHM-Dox combination decreased cell viability compared to their single treatment. The combination treatment affect the cells morphology: (b) untreated cells (c) 17 μM FHM, (c) 0.5 μM Dox (d), and combination 17 μM FHM and 0.5 μM Dox.

TABLE 2. The combination Index (CI) of FHM-Dox treatments in HeLa cells

FHMH	**Dox (μM)**			
(μM)	**0.125**	**0.25**	**0.375**	**0.5**
4.25	0.2	0.2	0.3	0.2
8.5	0.3	0.3	0.3	0.3
12.5	0.3	0.3	0.3	0.3
17	0.4	0.3	0.3	0.2

The effects of FHM, Dox, and FHM-Dox treatments on the HeLa cells apoptosis

The synergistic effect of the combination treatments of FHM-Dox could be related to apoptosis. Therefore in this study, we were observed the effects of FHM and Dox on apoptosis using flowcytometric. The apoptosis phenomenon was detected using Annexin VFITC. The reagent used as a marker to identify apoptosis (together with DNA staining with propidium iodide, PI). Annexin has a high affinity for negatively charged phosphatidylserine

(PS). In live non-apoptotic cells PS is almost exclusively observed on the inner surface of the membrane, but early in apoptosis, PS undergoes translocation to the external leaflet of the plasma membrane. Non-apoptotic cells are Annexin V-negative and PInegative, early apoptotic cells are Annexin V-positive but PI-negative, and late apoptotic cells, as well as necrotic cells, are stained intensely with PI.

The observation of apoptosis (Figure 5) showed that the FHM treatment for 24 hours at concentration 17 µM caused 8.74% (early and late apoptotic) HeLa cells to undergo apoptosis. This amount is higher compared to untreated cells (2.09%). Similarly, the Dox treatment at concentrations of 0.5 µM caused 11.44% of the cells to undergo apoptosis, while the FHM-Dox combination treatment caused 12.07% of cells undergoing apoptosis. These data showed that the combination treatments more induce apoptosis compared to that the single treatment. Hsu *et al.*(16) indicated that the core structure of chalcone inhibited the cell proliferation of breast cancer cells by inhibiting the cell cycle progression and induced apoptosis through the mitochondrial pathway and death receptor. We suspect FHM which are chalcone derivatives that have hydroxyl, fluoro, and methoxy substituent have the same mechanism in triggering apoptosis, but the mechanism needs to be explored further.

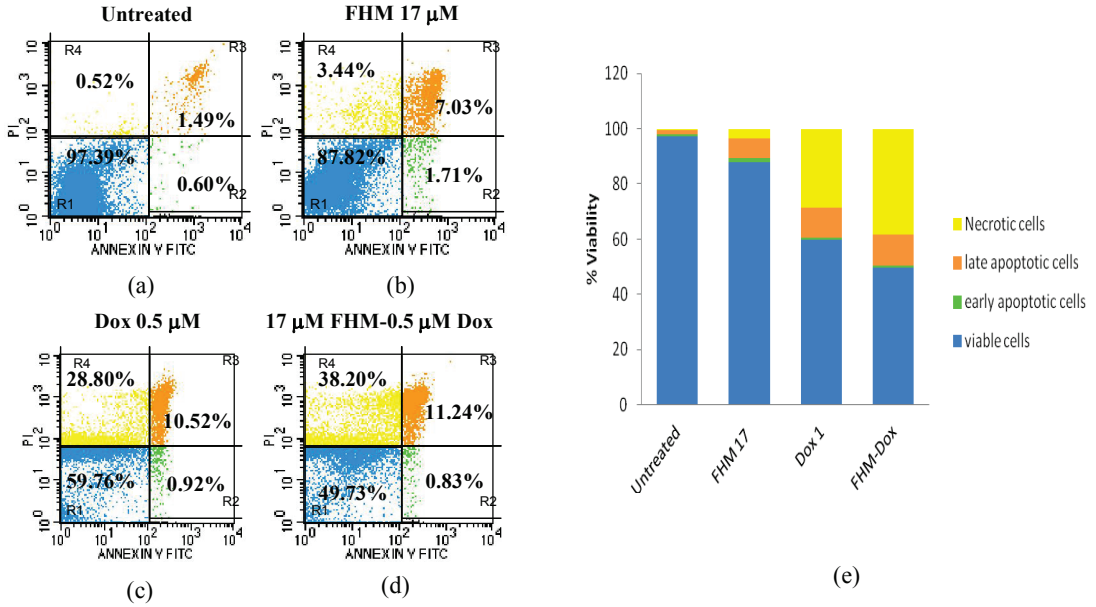


FIGURE 5. The effect of FHM, Dox, and FHM-Dox treatment on the HeLa cells apoptosis. The cells were seeded at $5x10^5$ cells/well on six wells tissue culture plate, then treated with FHM, Dox, and FHM-Dox. After 24 hours incubation, cells were harvested as described in the methodology, added with AnnexinV and PI reagents, were then subjected to FACS flow cytometer. The flowcytometric profiles of cells were treated respectively with (a) 0 µM or control, (b)17 µM FHM, (c) 0.5 µM Dox, (d) FHM-Dox. There are four quadrants: the lower left (R1), marked in blue, indicates viable cells, the lower right (R2), marked in green, indicates early apoptotic cells, the upper right (R3), marked in orange, indicates late apoptotic cells, and the upper left (R4), marked in yellow, indicates necrotic cells. The Graph (e) showed that the combination treatment of FHM-Dox cause the higher apoptosis compared to that the single treatment of FHM and Dox.

CONCLUSIONS

The combination treatments of FHM-Dox resulted in a synergistic up to strong synergistic effect on HeLa cells through a mechanism by inducing apoptosis. The combination treatment is potential to be developed in cervical cancer treatment.

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