

Cytotoxic activity and apoptosis induction of 8-hydroxyisocapnolactone-2',3'-diol and its combination with Doxorubicin on MCF-7 and T47D cells

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ABSTRACT

The cytotoxic activity and apoptosis induction of 8-hydroxyisocapnolactone-2',3'-diol (HICD) and its combination with doxorubicin (Doxo) on MCF-7 and T47D cells have been evaluated. The cytotoxic assay was performed using MTT method. The IC₅₀ was used to express the cytotoxic potency, while the combination index (CI) was calculated to determine the effect of combination. The apoptotic assay was carried out using acrydine orange - ethidium bromide double staining method, while Bcl-2 and Bax expression were investigated by immunocytochemistry. The HICD exhibited cytotoxic activity on MCF-7 and T47D cells with the IC₅₀ of 8 µg/ml (21.2 µM) and 4 µg/ml (10.6 µM), respectively. The combination of HICD with Doxo showed synergistic effect and increased apoptosis induction on both cell lines. The HICD did not affect Bcl-2 but increased Bax expression on MCF-7 cells, while on T47D cells it suppressed Bcl-2 expression but did not modulate Bax expression

INTRODUCTION

Breast cancer remains a major health problem for women worldwide and is a significant cause of death of women (Jemal *et al.*, 2010). The morbidity and mortality from cancer is continue to increase every year, whereas chemotherapeutic treatment of cancer remains costly and is associated with many side effects (Angela *et al.*, 2013). The efficacy of chemotherapeutic drugs, such as doxorubicin is often decreased due to the emergence of multidrug resistance mechanisms of the cancer cells (Szakács *et al.*, 2006). It is also reported that the long term use of doxorubicin causes cardiotoxicity. A lot of natural compounds or their derivatives have been found and reported to have chemopreventive activity (Meiyanto *et al.*, 2012). These substances are potential to be combined with chemotherapeutic agent to produce a synergistic or additive effect in order to increase the sensitivity of cancer cells and reduce the side effects (Hermawan *et al.*, 2010). In general, these chemopreventive compounds can inhibit tumorigenesis through cell cycle regulation, apoptosis induction, and or inhibit

the expression of multidrug resistance proteins (Meiyanto *et al.*, 2011). The coumarin 8-hydroxyisocapnolactone-2',3'-diol (HICD) (fig. 1) isolated from *Micomelum minutum* was found to be cytotoxic against CEM-SS, HL60, HeLa, HepG2 (Susidarti *et al.*, 2009), and NS-1 (Yasmina *et al.*, 2005) cell lines. In NS-1 cells, the compound induced apoptosis and suppressed Bcl-2 expression. Based on these data, HICD is a promising chemopreventive agent to be combined with chemotherapeutic agents in order to overcome the resistance of cancer cell. However, the activity of HICD on MCF-7 and T47D cells has not been reported. The over-expression of Bcl-2 is a marker for breast cancer prognosis (Fatah *et al.*, 2013).

On the other hand, there is suppression of pro-apoptotic Bax protein expression along with the development of breast cancer (Pluta *et al.*, 2011). Both proteins play an important role in the development of breast cancer. In the present study, the cytotoxic activity, apoptosis induction and modulatory effect of Bcl-2 and Bax expressions of single HICD and its combination with doxorubicin on MCF-7 and T47D cell lines were evaluated.

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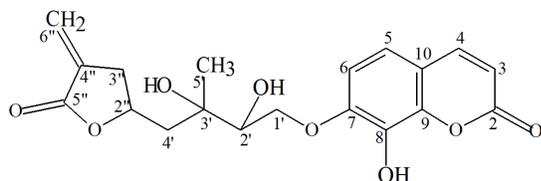


Fig. 1: 8-Hydroxyisocapnolactone-2',3'-diol (HICD).

METHODS

Chemicals

The 8-hydroxyisocapnolactone-2',3'-diol and MTT reagent or 3-[4,5-dimethyl thiazole-2-yl(-2,5-diphenyltetrazoliumbromide)]. Dulbecco's Modified Eagle Medium (DMEM) powder (Gibco), Fetal Bovine Serum (FBS) 10% v/v (Gibco), and Penicillin-Streptomycin 10,000 units/ml-10,000 µg/ml (Gibco) were used for cell culture medium. Cells were prepared using Tripsin EDTA 25% (Gibco).

For cytotoxicity assay Sodium Dodecyl Sulphate (SDS) 10% v/v (Merck) dissolved in HCl 0.1N (Merck) was used as stopper reagent, while Phosphate Buffer Saline (PBS) pH 7.4 containing KCl, NaCl, Na₂HPO₄ and KH₂PO₄ (all were HPLC grade, Sigma) as washing reagent. Etidium bromide and Acridin Orange were used for apoptotic assay, whereas methanol (Merck), normal goat serum (Novocastra), primary antibodies against Bcl-2 (Dako) and Bax (Dako), PBS, streptavidin, biotin, antibody IgG secondary biotinylated (Novocastra), streptavidin conjugate of the enzyme "horse radish" peroxidase (Novocastra), 3,3'-diaminobenzidine kromogen (DAB) (Novo Castra), distilled water, and haematoxylin (Dako) were used for immunocytochemistry.

Cell lines

MCF-7 & T47D cells used were the collection of Cancer Chemoprevention Research Center (CCRC) Faculty of Pharmacy, Gadjah Mada University, which obtained from Prof. Kawaichi, Nara Institute of Science and Technology (NAIST), Japan.

Cytotoxicity assay

MTT assay was used to examine the cytotoxicity of HICD and its combination with Doxo on MCF-7 and T47D cells. Cells were distributed to 96-well plate with the density of 5x10³ cells/well and then incubated at 37 °C with 5% CO₂ for 24 hours. The concentration of HICD used in the single treatment was 1, 10, 50, 100, 250, 500, and 750 µg/ml. After 24-hours incubation, MTT reagent was applied followed by 4-hours incubation. The SDS 10% v/v in HCl 0.1N as stopper reagent was then applied. Plate was kept from the light overnight and the absorbance of the formazan formed was then measured at λ 595 nm using ELISA reader (Bio-Rad) to calculate the IC₅₀. In combination assay, the concentration of HICD and Doxo used was ½, ⅓, ¼ and ⅛ of their IC₅₀. The Combination Index (CI) was then determined.

Analysis

Single Cytotoxicity assay

The absorbance obtained was used to calculate the percentage (%) of cell viability. The plot of log concentration

versus % cell viability gave the equation of y=ax+b, which used to calculate IC₅₀ value, that is the concentration inhibiting 50% cell proliferation.

Combination Cytotoxicity Assay

The effect of combination treatment on MCF-7 and T47D cell lines was evaluated by calculating Combination Index (CI) value (Reynolds and Maurer, 2005) using the formula as follows:

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

D1 and D2 represented the concentrations used in combination treatment, while Dx1 and Dx2 are the single treatment concentration giving the same response as D1 and D2, respectively. Interpretation of the data was based on the classification listed in Table 1.

Table 1: Interpretation of CI value representing potency of combinational application.

CI	Interpretation	CI	Interpretation
< 0.1	Very strongly synergist	0.9-1.1	Closely additive
0.1-0.3	Strongly synergist	1.1-1.45	Middle antagonist
0.3-0.7	Synergist	1.45-3.3	Antagonist
0.7-0.9	Middle synergist	> 3.3	Strongly antagonist

Apoptosis induction assay

Cells with the density of 5x10⁴ cells/well were grown on glass cover-slips in the 24-well plate until 60-75 % confluent and then incubated with HICD, Doxo or the combination of both compounds for 48 hours. In the single treatment, the concentration of HICD or Doxo applied was same as its IC₅₀, while in the combination treatment, the mixed concentration of both compounds giving strongest synergistic effect was used. After that, the cover-slips were washed with phosphate buffered saline and fixed on the object glass. Cells were then stained with 5 µL acridine orange and ethidium bromide and visualized by fluorescence microscope. Apoptotic cells showed orange colour and characteristic morphological features, whereas normal cells appeared green.

Immunocytochemistry

The T47D and MCF-7 cells were grown on cover-slips in 24-well plate (5x10⁵ cells/well) until 80 % confluent (24 hours incubation). The medium was then replaced by fresh medium containing HICD, Doxo or the combination of both. The plate was then incubated at 37°C with 5% CO₂ for 16 hours. After incubation, the cells were washed with PBS and then fixed with cold methanol for 10 minutes at -4°C. The cells were washed with PBS again and then blocked with hydrogen peroxide solution for 10 minutes at room temperature. The cells were incubated with primary antibody of Bcl-2 and Bax for one hour. The cells were washed three times with PBS, incubated with secondary antibody for 10 minutes, and then washed with PBS again. The cells were incubated in 3,3'-diaminobenzidine (DAB) solution for 10 minutes and then washed with aquadest. After that, the cells were counterstained with Mayer-Haematoxylin for 3 minutes. After incubation, the coverslips were taken and washed with aquadest,

and then immersed with xylol and alcohol. The expression of Bcl-2 and Bax proteins was determined using a light microscope (Nikon, Japan) and photographed using a digital camera (Canon, Japan). Expression of Bcl-2 and Bax were marked by the emergence of a dark brown color in cytoplasm, whereas cells with no protein expression appeared purple.

RESULTS

The effect of single treatment of HICD and Doxo on MCF-7 and T47D cells viability

Experimental results showed that HICD exhibited cytotoxic activity towards both MCF-7 and T47D cells in a dose dependent manner. Increasing concentrations resulted in a decreased in the percentage of viable cells. A similar cytotoxicity trend was also observed when cells were treated with Doxo. The T47D cells was more sensitive toward both compounds than MCF-7 cells (Figure 2). Based on the linier regression equation, the IC_{50} of HICD and Doxo on MCF-7 cells was 8 $\mu\text{g/ml}$ (21.2 μM) and 0.19 $\mu\text{g/ml}$ (350 nM), whereas on T47D cells was 4 $\mu\text{g/ml}$ (10.6 μM) and 0.02 $\mu\text{g/ml}$ (40 nM), respectively.

The effect of HICD-Doxo combination treatment on MCF-7 and T47D cells viability

Based on the CI values obtained (Figure 3) it can be seen that most of HICD and Doxo combination were synergist (CI < 0.9), of which the effect of the combination is greater than the sum of each single effect at the same dose. Synergism was apparent on low concentrations of HICD with any Doxo concentrations (Table 3; Figure 3). The strongest synergism (CI 0.35) occurred by the combination of 0.8 $\mu\text{g/ml}$ of HICD ($1/10 IC_{50}$) with 175 nM of Doxo ($1/2 IC_{50}$). Even the combination with lowest HICD concentration caused greater morphological changes of the cancer cells than single HICD treatment at 8 $\mu\text{g/ml}$ (IC_{50}).

The combination treatment of HICD with Doxo was conducted to examine the efficacy of combination in inhibiting the growth of MCF-7 and T47D cells and to determine whether the combination is synergistic, additive, or antagonistic. Based on each IC_{50} values, the ratio of concentration of HICD and Doxo used in the experiment was shown in Table 2.

Differing from MCF-7 cells, T47D cells exhibited greater sensitivity towards HICD and Doxo, of which all combination of HICD with high concentrations of Doxo were almost strongly synergist (Table 4). The strongest synergistic effect (CI 0.21) was given by the combination of 0.4 $\mu\text{g/ml}$ of HICD with 20 nM of Doxo. Morphological changes of T47D cells due to HICD, Doxo, and their combination treatments can be seen in Figure 4. This cytotoxicity assay concluded that of HICD can increase the effectivity of Doxo against MCF-7 and T47D cells.

The effect of HICD, Doxo, and the combination of both on apoptosis induction

All treatments were basically able to induce apoptosis in MCF-7 and T47D (Fig. 5). The ability of Doxo in inducing MCF-7

apoptosis was low, whereas HICD was much stronger. The ability of Doxo in inducing apoptosis was significantly improved when combined with low concentration of HICD. Thus, it strongly supports that HICD has a synergistic effect with Doxo in inducing apoptosis. In T47D cells that are sensitive to Doxo, apoptosis induction of Doxo is greater than that in MCF-7. Low concentration of HICD can also increase apoptosis induction of Doxo on this cell.

Effect of HICD, Doxo, and the combination of both on the expression of Bcl-2

Observation of proteins involved in the apoptosis of MCF-7 and T47D cells gave further information about the molecular mechanism leading to the synergism of HICD and Doxo in inducing apoptosis. In this study, Bcl-2 and Bax proteins were observed to represent anti-apoptosis and pro-apoptosis proteins, respectively. The expression of both proteins was observed by immunocytochemistry. Brown color in the cytoplasm showed the presence of protein expression, while blue color indicated no protein expression. The intensity of the brown color was used as a qualitative parameter for the modulation of proteins expression.

The expression of Bcl-2 in MCF-7 cells can be observed in Figure 6. Doxo demonstrated the ability to significantly reduce the expression of Bcl-2, while single treatment of HICD did not decrease the expression of Bcl-2. The combination of Doxo with low concentration of HICD weakly suppressed the expression of Bcl-2. These findings indicated that the synergism of HICD and Doxo in inducing apoptosis of MCF-7 cells is probably not through down-regulation of Bcl-2 pathway.

On T47D cells, however treatment of single HICD and Doxo at their IC_{50} were able to decrease the expression of Bcl-2. Down-regulation of Bcl-2 expression induced by the combination of both compounds was also observed (Figure 7). Based on this observation it was concluded that the synergism of HICD – Doxo on T47D cells mostly likely through down-regulation of Bcl-2 expression.

Effect of HICD, Doxo, and the combination of both on the expression of Bax

The expression of pro-apoptotic protein Bax were also observed to explore further the possibility of a mechanism that mediates cytotoxicity and synergism of the compounds. Figure 8 shows the modulation of Bax expression in MCF-7 cells. Untreated cells with and without addition anti-Bax primary antibody showed blue cytoplasm, which means that no expression of Bax. Doxo slightly increased Bax expression, but HICD increased Bax expression greater than Doxo. This is supported by the fact that low concentration of HICD in combination with Doxo is also able to increase the expression of Bax. These results indicated that the cytotoxicity of HICD and the possibility of synergistic mechanism with Doxo on MCF-7 were through up-regulation of Bax pathway. However, treated HICD did not effect in BAX expression on T47D (Figure 9).

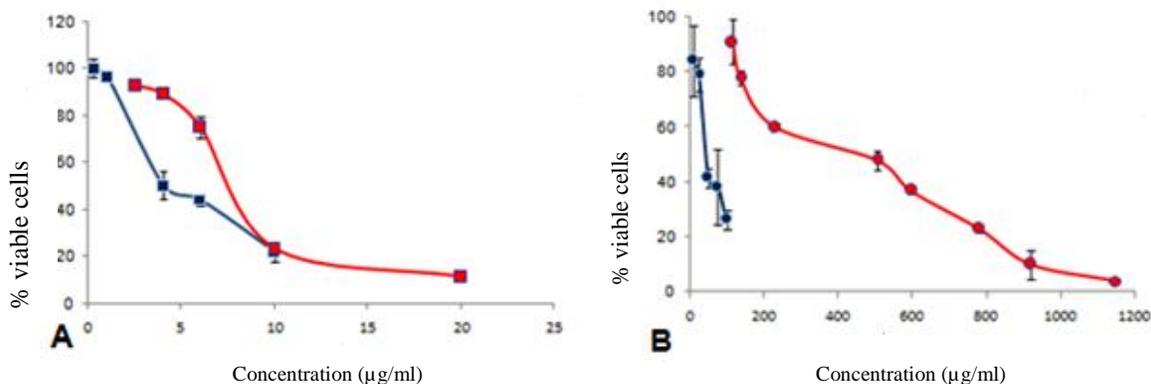


Fig. 2: Cytotoxicity of HICD and Doxo towards MCF-7 and T47D cells. Treatment of both HICD (A) and Doxo (B) reduced the enzymatic activity of succinate dehydrogenase to convert MTT into formazan, which is linear with viable cells. The profile of MCF-7 viability is shown by the red curve while T47D by the blue curve. The plot of log concentration versus % cell viability gave linear regression equation used to determine the IC₅₀ value. Viability profile is presented from the mean ± standard error (SE) from 3 experiments. The treatment of HICD on MCF-7 and T47D cells resulted in reduced cell viability.

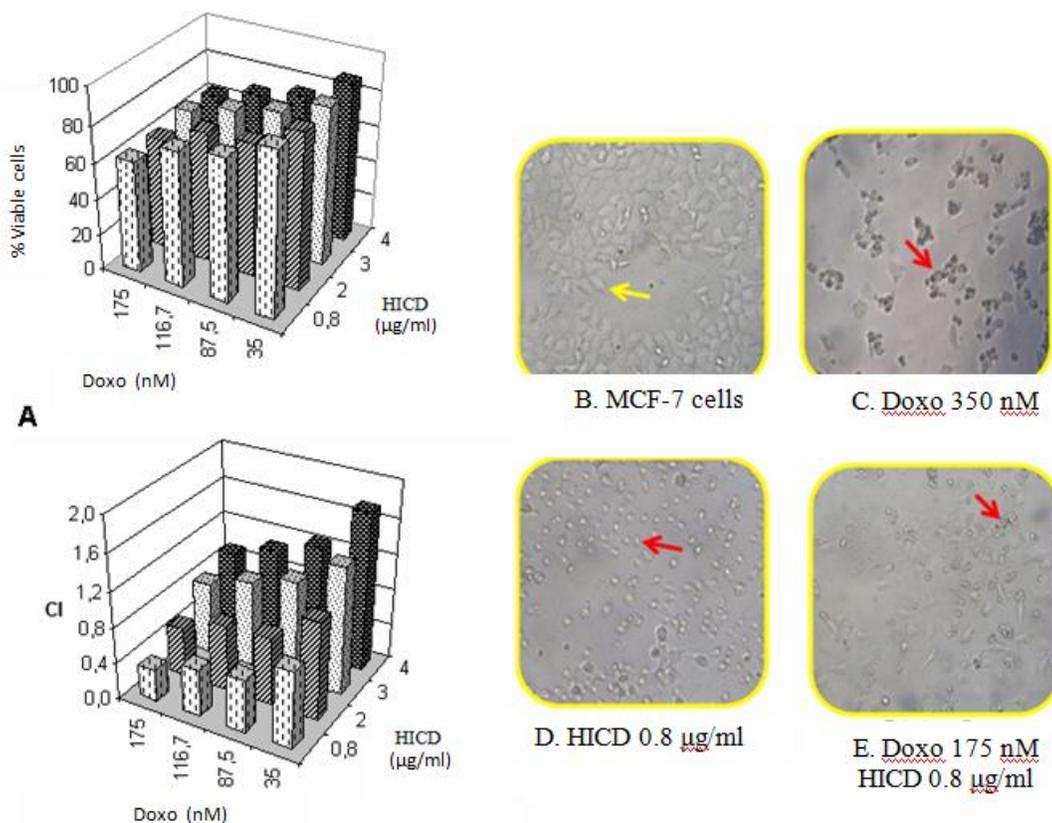


Fig. 3: The inhibition of MCF-7 cells growth because of the HICD-Doxo combination treatment. The histogram depicts the cell viability (A, above) and CI values (A, bottom) of the HICD-Doxo combination treatment of at various concentrations. Combination treatment at the best CI value (E) gave equivalent morphological changes with single Doxo (C), but greater than single HICD (D) and untreated cells (B). The yellow arrow points viable cell, while red arrow points to cells with morphological changes. Cell morphology was observed under an inverted microscope with 400x magnification.

Table. 2: Concentration combinations used in this experiment.

Doxo (IC ₅₀)	HICD (IC ₅₀)			
	1/10	1/4	1/3	1/2
1/10	1/10 : 1/10	1/10 : 1/4	1/10 : 1/3	1/10 : 1/2
1/4	1/4 : 1/10	1/4 : 1/4	1/4 : 1/3	1/4 : 1/2
1/3	1/10 : 1/3	1/3 : 1/4	1/3 : 1/3	1/3 : 1/2
1/2	1/10 : 1/2	1/2 : 1/4	1/2 : 1/3	1/2 : 1/2

Table 3: Combination index (CI) values of HICD – Doxo on MCF-7 cells.

Doxo Concentrations (nM)	HICD Concentrations (µg/ml)			
	0.8	2	3	4
35	0.85	1.09	1.41	1.76
87.5	0.59	0.76	1.11	1.26
116.7	0.52	0.73	0.96	1.09
175	0.35	0.55	0.80	0.92

Table 4: Combination Index (CI) values of HICD–Doxo on T47D cells.

Doxo Concentration (nM)	HICD concentrations (µg/ml)			
	0.4	1	1.5	2
4	0.92	1.38	1.28	7.66
10	0.93	1.64	0.78	4.89
15	0.41	0.85	0.48	2.25
20	0.21	0.33	0.24	0.78

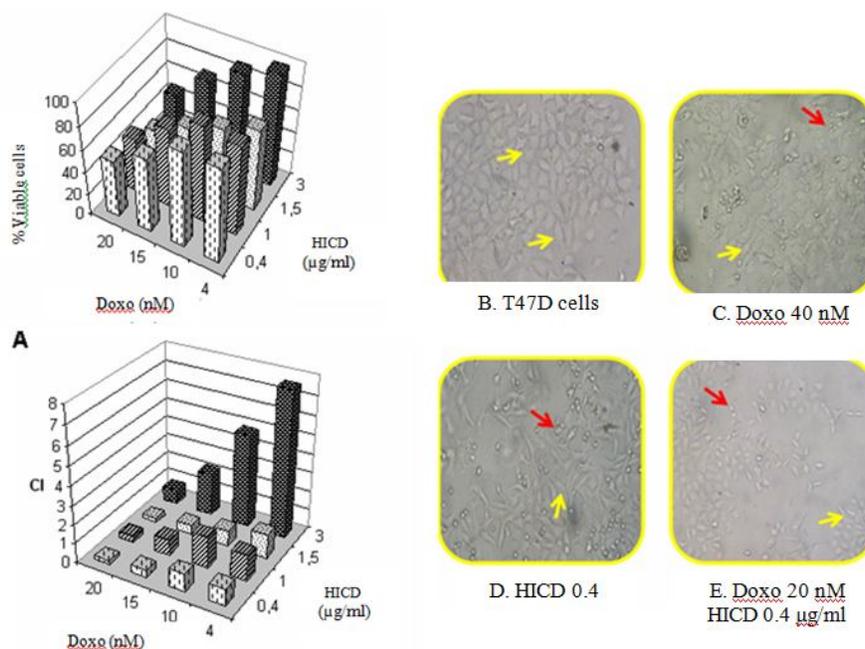


Fig. 4: The inhibition of T47D cells growth because of the HICD-Doxo combination treatment. The histogram depicts the cell viability (A, above) and CI values (A, bottom) of the HICD-Doxo combination treatment of at various concentrations. The morphology of living cells is shown by the untreated group (B). Treatment of single Doxo (C) and HICD (D) and the best combination of both (E) gave morphological changes. The yellow arrow points to living cells, while red arrows points to cells with morphological changes. Cell morphology was observed under an inverted microscope with 400x magnification.

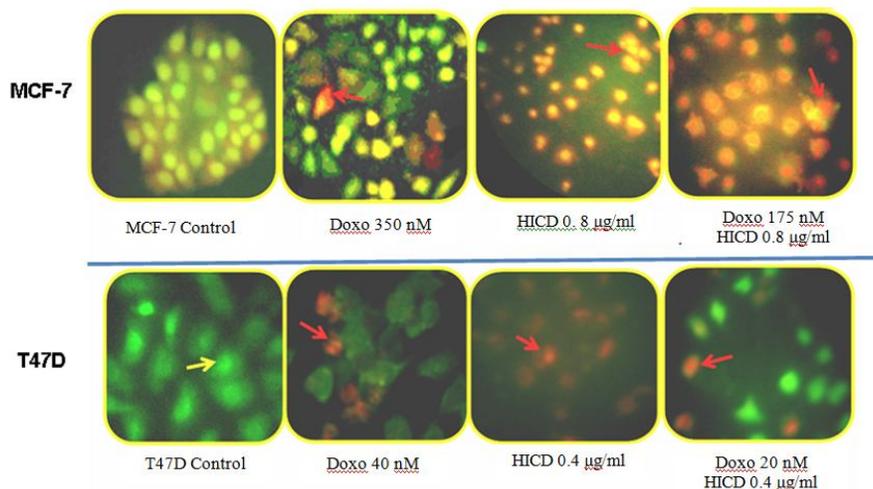


Fig. 5: Apoptosis induction effect of single and combination treatments on MCF-7 and T47D cells. Staining with acridine green – ethidium bromide on cells treated with single Doxo or HICD and the combination of both gave green fluorescence for living cells (yellow arrows) and orange color for apoptotic/dead cells (red arrow). The upper row is the observation results for MCF-7 cells while the lower row is for T47D cells. Single Doxo and HICD were given at IC50 each, while in the combination treatment the combination concentration of both compounds giving the best CI value was given. Cell morphology was observed under an inverted microscope with 400x magnification.

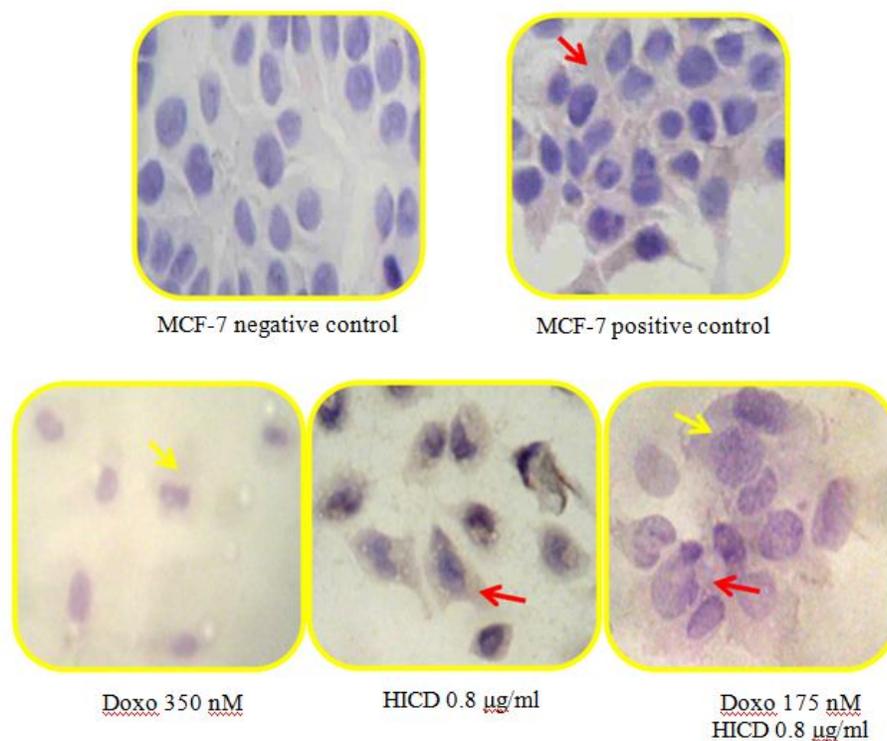


Fig. 6: Effect of HICD, Doxo, and the combination of both on the expression of Bcl-2 on MCF-7 cells. Untreated cell without addition anti-Bcl-2 primary antibody exhibited blue cytoplasm, while untreated cell with anti-Bcl-2 primary antibody gave brown color, which means there is Bcl-2 expression. Treatment of 350 nM of Doxo was significantly able to decrease Bcl-2 expression, but not with the 8 µg/ml of HICD. Combination of both compounds weakly inhibit Bcl-2 expression. Apoptosis induction was observed after 15 hours incubation. Yellow arrows mark a decrease in expression, while red arrows mark the presence of expression. The cell morphology was observed under an inverted microscope with 400x magnification.

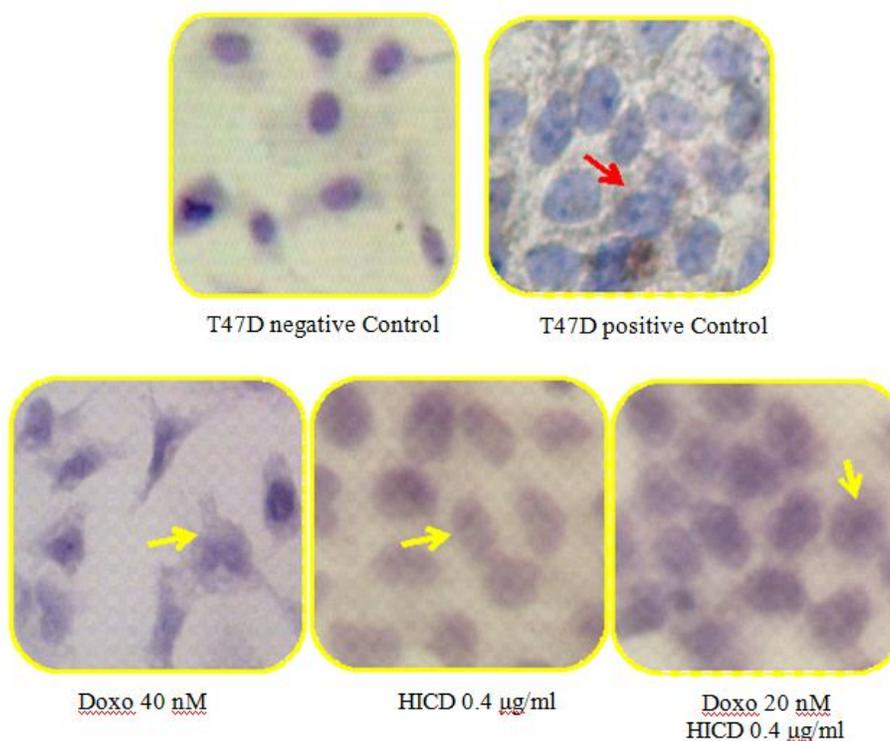


Fig. 7: Effect of HICD, Doxo, and the combination of both on the expression of Bcl-2 on T47D cells. Treatment of 40 nM of Doxo as well as 4 µg/ml of HICD was able to decrease the expression of Bcl-2 significantly. The combination of lower concentrations of HICD and Doxo was also decrease Bcl-2 expression. Apoptosis induction was observed after 15 hours incubation. Yellow arrows mark a decrease in expression, while red arrow marks the presence of expression. Cell morphology was observed under an inverted microscope with 400x magnification.

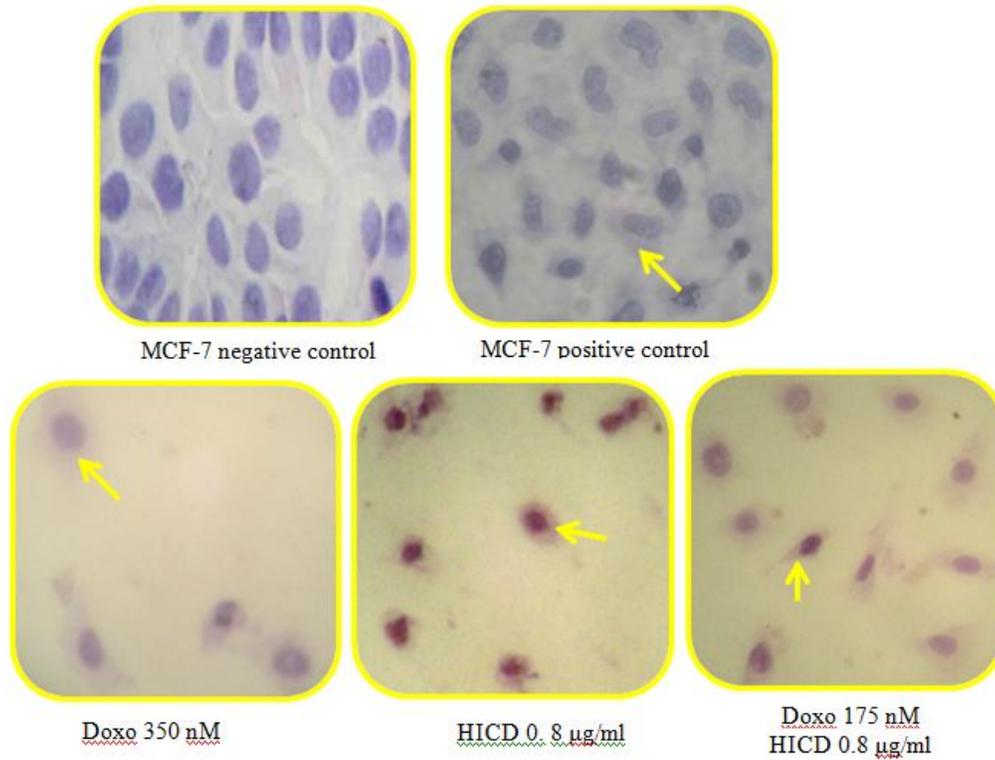


Fig. 8: Effect of HICD, Doxo, and their combination on the expression of Bax on MCF-7 cells. Untreated cell without addition anti-Bax primary antibody exhibited blue cytoplasm, while untreated cell with anti-Bax primary antibody gave brown color, which means there is Bax expression. Single treatment of Doxo and HICD and their combination each at 15 hours of incubation period could not induce the expression of Bax. Yellow arrow marks a decrease of expression, while red arrows mark the presence of expression. Cell morphology was observed under an inverted microscope with 400x magnification.

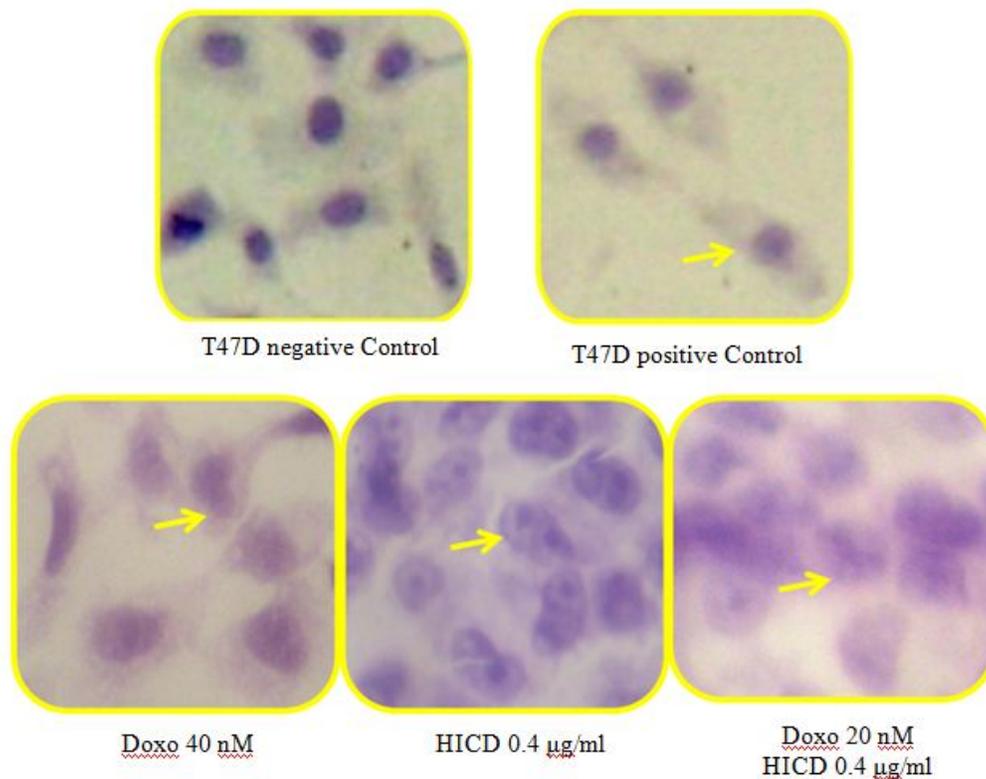


Fig. 9: Effect of HICD, Doxo, and their combination on the expression of Bax on T47D cells. Untreated cell without addition anti-Bax primary antibody exhibited blue cytoplasm, while untreated cell with anti-Bax primary antibody gave brown color, which means there is Bax expression. Single treatment of Doxo and HICD and the combination of both each at 15 hours of incubation period could not induce the expression of Bax. Yellow arrows mark decreases in expression. Red arrows mark the presence of expression. Cell morphology was observed under an inverted microscope with 400x magnification.

DISCUSSIONS

The cytotoxic activity of Doxo, HICD and the combination of both on MCF-7 and T47D cell lines has been studied. The IC₅₀ value of Doxo on MCF-7 was 350 nM (0.19 µg/ml), while on T47D was 40 nM (0.02 µg/ml). The much greater IC₅₀ value of Doxo on MCF-7 cells than on T47D cells indicating that MCF-7 cancer cells are more resistant towards Doxo compared to T47D cells. This is in-line with the characteristics of MCF-7 as a chemo-resistant cell model. The HICD exhibited cytotoxic activity on MCF-7 and T47D cells with the IC₅₀ of 8 µg/ml (21.2 µM) and 4 µg/ml (10.6 µM), respectively. The synergistic effect of HICD-Doxo combination was observed on both MCF-7 and T47D cells (CI < 0.9). This interesting finding served a promising solution to increase the effectiveness of Doxo.

Based on double staining results, it can be proved that the apoptosis induction of HICD-Doxo combination on MCF-7 and T47D cells was better than their single treatments. The mechanism of apoptosis induction can follow the mitochondria independent pathway through the activation of caspase 8 by the Fas receptor or through the mitochondria dependent pathway preceded by the expression of p53 tumor suppressor as a result of cellular stress. The expression of p53 will induce the expression of pro-apoptosis proteins (Bad, Bax, Bid) which in turn will release cytochrome C from the mitochondria. The release of cytochrome C can be inhibited by the anti-apoptosis protein Bcl-2 which is expressed by the activation of the transcription factor NFκB (Simstein *et al.* 2003). Results of this study indicate that treatment of HICD, Doxo, and their combination on MCF-7 cells did not involve the modulation of Bcl-2 or Bax expressions. While on T47D cells, Bcl-2 down-regulation was observed after treatment of HICD even though Bax modulation was not observed. A possible mechanism of Bcl-2 inhibition is through the inhibition of NFκB activation as is generally observed for flavonoids (Sanjeev and Sanjay, 2004). However, the mechanism of inhibition of Bcl-2 expression after HICD treatment need to be studied further.

Treatment of HICD on T47D cells reduced the expression of Bcl-2, thus increased the sensitivity of the cancer cells. This aided the increased efficacy of Doxo in inducing apoptosis through the Fas pathway (Massart *et al.*, 2004). The Fas pathway is inhibited when stimulation of epidermal growth factor (EGF) occurs. The EGF receptor is over-expressed in breast cancers. Stimulation of EGF can also inhibit proteolysis of proteins downstream to EGF, such as Cbl and Akt (Spencer *et al.*, 1999). And thus, activation of EGF receptor can result in proliferative responses and also resistance to chemotherapy. This data is also supported by Xinqun *et al.* (2005), which stated that exposure of T47D cells with Doxo for 24 hours exhibited an increase of phosphorylated Akt kinase. Inhibition of the activation of EGF receptor can increase the activity of Doxo in inducing Fas and/or Fas ligand. Induction of Fas and/or Fas ligand will activate a caspase cascade, which eventually lead to apoptosis. Most likely HICD inhibit EGF receptor activation thus able to increase the activity of Doxo in inducing Fas and/or Fas ligand. The T47D cells

express ER+ (positive estrogen) receptors, thus responsive to estrogen stimulation. Zampieri *et al.* (2002) stated that there are not increasing in P-gp when T47D cells are exposed to estrogen. And so, the synergism of HICD with Doxo on T47D cells most likely did not follow the P-gp pathway. Since Bcl-2 and Bax activation are usually marked in breast cancer cells, thus HICD is potential to be developed further as a specific-targeted co-chemotherapeutic agents for breast cancer through apoptosis induction.

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