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RESEARCH ARTICLE

Gynura procumbens modulates the microtubules integrity and enhances distinct mechanism on doxorubicin and 5-flurouracil-induced breast cancer cell death

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Abstract Recent studies both in vitro and in vivo of *G. procumbens* exhibits chemopreventive properties for tumor inhibition on several types of cancer. Our study was carried out to observe the anticancer property of ethyl acetate fraction of *G. procumbens* leaves (FEG) on breast cancer cells as well as the co-chemotherapeutic potential, and to investigate its molecular mechanisms. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure the growth inhibitory effect of FEG, doxorubicin (DOX), and 5-fluorouracil (5-FU) and their combination. Flowcytometry, 4′,6-diamidino-2-phenylindole (DAPI) staining, and immunobloting were used to explore the mechanism of cell cycle arrest and apoptosis. FEG inhibited cell proliferation, induced G_1 phase arrest and apoptosis. The inhibitory effect of FEG was enhanced when combined with Dox and 5-FU. The apoptosis induction was related to the increase of c-PARP expression after combination treatment of FEG and Dox or 5-FU on MCF-7 cells. However, treatment of DOX, 5-FU, and FEG on T47D cells, resulting no significance DNA fragmentation and nuclei condensation evidance. Only combination treatment of 5-FU+FEG showed c-PARP expression in T47D cells. In T47D cells, The FEG treatment also caused the decrease of microtubule expression as shown by Western blotting assay. The decreasing level of microtubul expression might be caused by protein aggregation, as shown by immunostaning using α-tubulin antibody. All these results suggest that FEG potentiates the DOX and 5-FU efficacy on MCF-7 and T47D cells. FEG induces T47D cell death through different mechanism than MCF-7 that proposed to be mitotic catastrophe. The FEG may have specific targeted on microtubule integrity modulation leading to the cell cycle arrest and proliferation inhibition. Further FEG could be developed as a co-chemotherapeutic agent for reducing side effect and have specific molecular target for breast cancer.

Keywords *Gynura procumbens* · Microtubules · Apoptosis · Breast cancer · MCF-7 · T47D · Combination effect

Nunuk Aries Nurulita has made conception and design of this study, acquisition of data, data collection, analysis and interpretation and statistical data, and drafted the manuscript. Edy Meiyanto and Sugiyanto have made conception and design of this study, analysis and interpret data, and drafted the manuscript. Eishou Matsuda and Masashi Kawaichi have interpreted data and reviewed the manuscript. All author have already read and approved the final revision of this manuscript.

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Introduction

Breast cancer is the most frequently diagnosed cancer and cause of most death from cancer among women worldwide yearly. About 1.3 million new cases of invasive breast cancer are expected to occur. Breast cancer incidence has been rising in many developing countries including Asian and African countries. There have been significant advances in breast cancer treatment that have been improved patient survival and quality of life (Garcia et al. 2007). There are many drugs that already established for breast cancer chemotherapy. Two or more chemotherapeutic agents are used in combination as attempt to increase drug efficacy and reduce the side effects. Unfortunately, these combinations often produce other unacceptable side effect on the patient, such as heart failure. The second problem arises after long terms exposure of these agents. Anticancer administration often triggers somatic, genetic and epigenetic alteration leading to multi drug resistance (MDR) (Devarajan et al. 2002). The increasing dose regimen could not overcome this problem because it will generate worse side effect.

It is widely known that the use of naturally occurring dietary substances from vegetables, fruits and some plants have anti-inflammatory, antioxidant and anticancer properties. The intervention of multistage cancer stadium by modulating intracellular signaling pathways may provide a molecular basic for dietary phytochemical chemoprevention (Surh 2003). *Gynura procumbens* (*G. procumbens*), also known as *sambung nyawa* or *kecam akar*, is widely found in Africa and South-East Asia, especially Indonesia, Malaysia, and Thailand, and extensively used in folk medicine as remedy for eruptive fevers, rash, kidney disease, migrains, constipation, hypertention, diabetes mellitus, and cancer (Perry 1980). Sugiyanto, et al. (2003) have been identified at least three flavon/flavonol as the active compounds in ethanolic extract. Ethyl acetate fraction of *G. procumbens* may contain β-sitosterol and stigmasterol, kaempferol-3-O-Rutinoside, and quercetin (Sadikun et al. 1996; Rosidah et al. 2008). Many studies of this plant show several pharmacologic properties such as anti-inflammatory (Iskander, et al. 2002), antihypertension (Kim et al. 2006) and antioxidant (Rosidah et al. 2008). The toxicity study of the methanol extract of *G. procumbens* did not produce mortality or significant changes in the vital organ and other important parameter on rats (Rosidah et al. 2009).

Recent studies on cancer prevention and therapy show *G. procumbens*'s chemo-preventive properties to inhibit tumor development on benzo(a)pyren-treated mice and breast cancer on DMBA-treated rat (Sugiyanto et al. 2003; Meiyanto et al. 2007). *G. procumbens* ethanolic extract was reported had antimutagenic property against lung cancer on benzo(a) pyren-treated mice. The ethanolic extract of Gynura procumbens leaves could inhibit the progression of 4 nitroquinoline 1-oxide (4NQO) induced rat tongue carcinogenesis in the initiation phase (Agustina et al. 2006). The phenolic fraction suppresses cell proliferation and induces apoptosis on HeLa (Meiyanto and Septisetyani 2005) and also has antiangiogenesis potency (Jenie et al. 2006). *G. procumbens* decreased COX-2 expression and increase p53 and Bax expression on breast cancer cells. Co-chemotherapeutic study of *G. procumbens* ethyl acetate fraction shown the sinergism effect of its combination with doxorubicin on T47D and MCF-7 breast cancer cell lines (Jenie and Meiyanto 2007). Aqueous extract of *G. procumbens* had antiproliferative and inhibited DNA synthesis properties on human mesangial cells (Lee et al. 2007). Proposed mechanism of *G. procumbens* should be through several ways, such as blocking carcinogenesis and suppressing cell proliferation through cell cycle arrest and apoptosis induction. Both mechanisms are suggested by its phenolic and flavonoid contents.

Apoptosis were contemplated as a major mechanism of cancer chemotherapy-induced cell death. Many study of preclinical drug discovery tend to focus on elucidating the molecular modulating during apoptosis induction and its mediating pathway. However accumulating evidence proposes other modes of cell death such as necrosis, autophagy, mitotic catastrophe and senescence, as tumor cell response to chemotherapy treatment (Ricci and Zong 2006). It is important to make deep understanding of nonapoptotic cell death mechanisms.

In this study, we used ethyl acetate fraction of *G. procumbens* (FEG) to examine its combination chemotherapeutic effect with doxorubicin and 5-fluorouracil on MCF-7 and T47D breast cancer cells as models. Our data exhibit that co-treatment with FEG potentiates the DOX and 5-FU efficacy on both cells. FEG induces apoptosis cell death on MCF-7 cells and causes necrosis-like cell death proposed tobe mitotic catsatrophe on T47D cells. FEG also modulates the cells microtubules that may lead to intracelullar signaling perturbation and disrupt on cell cycle progression.

Materials and method

Plant material

The leaves of *G. Procumbens* were obtained from Balai Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (BP2TO2T) Indonesia, and was determined at Laboratorium of Pharmacognocy, Faculty of Pharmacy Universitas Gadjah Mada, Indonesia. Dried powdered leaves were first extracted with ethanol 96%, and concentrated by evaporation under reduced pressure and the temperature was kept below 40°C. The extract was diluted in hot water and then fractionated with n-hexane. The aqueous fraction then was fractionated again with ethyl acetate. The ethyl acetate fraction was concentrated by evaporation under reduced

pressure and the temperature was kept below 40°C. The extract was stored at 4°C prior to use.

Cells and chemicals

MCF-7 and T47D cells were kindly provided by Prof. Masashi Kawaichi (Laboratorium of Gene Function in Animal, Nara Institute of Science and Technology (NAIST), Nara, Japan. The cells were routinely grown and maintained in DMEM (Nacalay, Japan) supplemented with 10% FBS (PAA), 1% v/v Penicillin-streptomycin, and L-glutamine (1 mM) at 37°C in 5% CO2. Doxorubicin (DOX) and 5-Fluorouracil (5-FU) (Ebewe) was purchased from PT. Ferron Par Pharmaceutical Cikarang, Indonesia, Dimethyl sulfoxide (DMSO) (Sigma, Aldrich, Germany), penicillin and streptomycin (Gibco), Tripsin (Sigma), 3-(4,5-dimethyltiazol-2-il)-2,5-diphenyltetrazolium bromide (MTT) (Sigma), triton X-100, propidium iodide (PI), RNaseA (Sigma) are at analytical degree.

Antibodies

Primary antibodies used in western blotting and immunostaining were anti c-PARP (Cell Signaling), anti mSin3A (Santa Cruz), anti α-Tubulin (Santa Cruz), anti GAPDH (Cell Signaling). Primary and secondary antibodies were diluted in PBS containing 5% skim milk, 0,05% Tween (blocking buffer). For c-PARP detection, the anti-c-PARP and its secondary antibody were diluted in Can Get Signal (CGS) solution (Cosmo Bio).

Cytotoxicity assay

Cells (10^4 cells/well) were cultured in 96-well plate (100 uL/well). After 24 h incubation, the medium was replaced with DOX, 5-FU, and FEG-containing medium. After incubation for 24 h, the medium was discarded and replaced with MTT-containing medium (0.5 mg/mL) and incubated for further 4 h at 37°C, 5% CO_2. The reaction was stopped with 10%SDS in 0.1 N HCl solution and was incubated for overnight in light protected chamber, to dissolve formasan salt. The absorbance of each well was measured with ELISA reader at 495 nm. The ratio between treated and control cells absorbance referred to percentage (%) of cells viability. The combination effect was expressed as the combination index (CI) calculated using formula:

$$CI = D_1/Dx_1 + D_2/Dx_2$$

Where,

D_1, D_2 = concentration of each agent that used in combination

Dx_1, Dx_1=the concentration of single compound single compound producing the same effect with combination treatment (Ricci and Zong 2006). CI value interpretation: < 0.1 very strong synergism, 0.1–0.3 strong synergism, 0.3–0.7 synergism, 0.7–0.9 mild to moderate synergism, 0.9–1.1 almost additive, 1.1–1.45 mild to moderate antagonism, 1.45–3.3 antagonism, >3.3 strong to very strong antagonism

Cell proliferation assay

The cells were inoculated in 96-well plates at a density of 10^4 cells/well (100 μL). After 24 h incubation, cells were treated of DOX, 5-FU, FEG as single or combination in indicated concentration. Then, cells were incubated for 0, 24, 48, and 72 h and cells viability was determined using MTT assay. The data are presented as average of triplicate wells.

Fluorescence-activated cell sorting (FACS) analysis

The cells (5.10^5 cells/well) were cultured in 6-well plate and were treated as indicated concentration after 24 h growth. After 24 h treatment, all cells were recovered (both attached and detached cells) using tripsin 0.025%. The cell were washed with cold PBS and fixed in cold 70% ethanol (−20°C) carefully. The fixed cells were washed twice with PBS and incubated with PBS containing propidium iodide (PI) (50 μg/mL), RNaseA (100 μg/mL), and tritonX-100 at 37°C, for 30 min. The mixture solution was analyzed using FACSCalibur (BD Biosciences). The percentage of cells was determined based on DNA content using ModFit Lt. 3.0 to quantify cell distribution on each phase of cell cycle.

DAPI staining and immunoflourescence

Cells were seeded on cover slips and incubated for 24 h then treated with DOX, 5-FU, and FEG as single and combination for additional 24 h. Cells were fixed with in ice-cold 70% ethanol. Permeabilization and blocking non specific binding antibodies were performed by incubation cells in 1% BSA in PBS buffer, were performed at room temperature for 30 min. Cells were washed with PBS 3 time, then incubated with anti-α-Tubulin (1:1000) and fluorescence secondary antibody directed to mouse (Alexa 488) (1:1000), diluted in BSA at room temperature for 60 min. Counterstaining of nuclei was carried out by 10 min incubation with DAPI at room temperature. Cells were washed for 3 X with PBS at room temperature. The number of condensed nuclei was measured by assessing the percentage of cells displaying fragmented or condensed nuclei. Approximately 300 nuclei were counted per sample.

DNA fragmentation assay

The cells (10^6 cells/well) were resuspended with lysis buffer (Qiagen) after treated with DOX, 5-FU, and FEG as single

or combination for 24 h then added with protein precipitation solution and incubated for 1 h at 55°C and centrifuged at 15,000 rpm for 20 min. Supernatant was moved to new microtube and DNA was precipitated by adding an equal volume of isopropanol. RNAse (1 mg/mL) was added to degrade RNA. DNA pellet was obtained after centrifuged and the supernatant was discarded. The DNA pellet was dissolved in 50 μL TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and incubated at 37°C for 24 h. DNA samples were mixed with loading dye solution (4:1), then separated at 50 mA in 1.5% agarose gels and visualized by UV light after incubation in ethidium bromide solution. The Hind λ III served as marker.

Gel electrophoresis and immunoblotting

Cells were recovered, washed in PBS, and lysed for 30 min on ice using lysis buffer (20 mM Tris-HCl, pH 8,0, 5 mM EDTA, 1% NP40, 25 mM NaCl, and complete inhibitors of protease. Cells extract were centrifuged at 15.000 rpm for 20 min at 4°C to separate insoluble material. The protein concentration was determined using Bradford assay. Equal amount of each sample were mixed with SDS loading buffer, boiled for 3 min and subjected to SDS-PAGE at 120 Volt followed by electroblotting to Polyvinylidene fluoride (PVDF) membranes for 2 h at 100 Volt. Membranes were blocked with 5% skim milk in PBS at room temperature for 1 h and subsequently probed with the primary antibody of interest. Blots were exposed by ChemiluminoneSuper (Nacalay Tesque).

Result

FEG potentiated the cells growth inhibition by DOX and 5-FU on MCF-7 and T47D cells

To determine the potency of FEG as co-chemotherapy agent, the cytotoxicity of combination treatments were examined in MCF-7 and T47D cells. FEG (0–500 μg/mL) inhibites cells growth in a dose dependent manner (IC_{50} 270 μg/mL), while Dox (0–1000 nM) and 5-FU (0–500 μM) gave IC_{50} at 441 nM and 405 μM, respectively on MCF-7 cells,. Respectively. On T47D cells FEG (0–200 μg/mL) resulted IC_{50} value of 64 μg/mL, while DOX (0–125 nM) and 5-FU (0–500) had IC_{50} of 56 nM and 222 μM (data not shown). We used combination of subtoxic concentration for both chemotherapy agent (DOX and 5-FU) and FEG to determine the combination effect on the growth of MCF-7 and T47D cells. Combination of FEG with DOX or with 5-FU on both type of cells produced synergistic effect (CI<0,8) (Fig. 1a). Combination of 5-FU+ FEG showed stronger effect (CI<0,7) compared with combination of DOX + FEG on both cells (Fig. 1a and b). FEG enhanced the inhibition of cell growth by DOX and 5-FU treatment (Fig. 1c). DOX, 5-FU and FEG treatment triggered the morphological changing on MCF-7 and T47D cells and the changing become more apparent after combination treatment of DOX+FEG and 5-FU+FEG (Fig. 2c). Thus, FEG had moderate cytotoxicity properties on MCF-7 and T47D cells. FEG potentiated DOX-induced cell growth inhibition on both cells.

Combination treatment DOX + FEG and 5-FU + FEG increased sub G1 accumulation and modulated cell cycle progression

As it has been reported previously, FEG inhibited cell proliferation and induced cell death supposed to be apoptosis. The FEg effect on cell cycle modulation was observed using flocytometry. FEG increased sub G1 accumulation on DOX and 5-FU-treated MCF-7 and T47D cells (Fig. 2). Sub G1 accumulation referred to cell death induction through apoptosis or necrosis. FEG treatment caused cell cycle arrest on G1 phase on MCF-7 and T47D cells. Combination treatment of DOX + FEG decreased G2/M phase arrest caused by DOX (Fig. 2a). This effect was probably resulted from different target of FEG and DOX on cell cycle progression. Combination treatment of 5-FU + FEG augmented G1 phase arrest on both cells compare to single treatment (Fig. 2b). FEG caused breast cancer arrest at G1 phase on both cells. FEG induced cell death through necrosis or apoptosis shown by sub G1 accumulation at MCF-7 cells.

FEG enhances DOX and 5-FU-induced apoptosis on MCF-7 cells, but not on T47D cells

To determine whether FEG could enhance DOX and 5-FU-induced cell death through apoptosis mechanism, we performed immunoblotting assay to observe c-PARP as the main indicator of apoptosis. For further investigation on apoptosis induction, we predominantly focused on the combined treatment with DOX + FEG and 5-FU + FEG compare with single one. The chromatin condensation and membrane shrinkage were shown at MCF-7 cells after treated with DOX, 5-FU and FEG as single or combination while at control cell (DMSO) no condensed nuclei were found. But on T47D cells those phenomenon could not be found (Fig. 3a). The chromatin condensation, membrane shrinkage, and also DNA fragmentation were shown more extensively after cell treated by combined treatment of DOX + FEG and 5-FU + FEG on MCF-7 cells but not on T47D (Fig. 3a and b). Expression of c-PARP referred to cell death induction through apoptosis mechanism. FEG treatment in combination with DOX or 5-FU showed enhancement of c-PARP expression level on MCF-7 cells. c-PARP expression

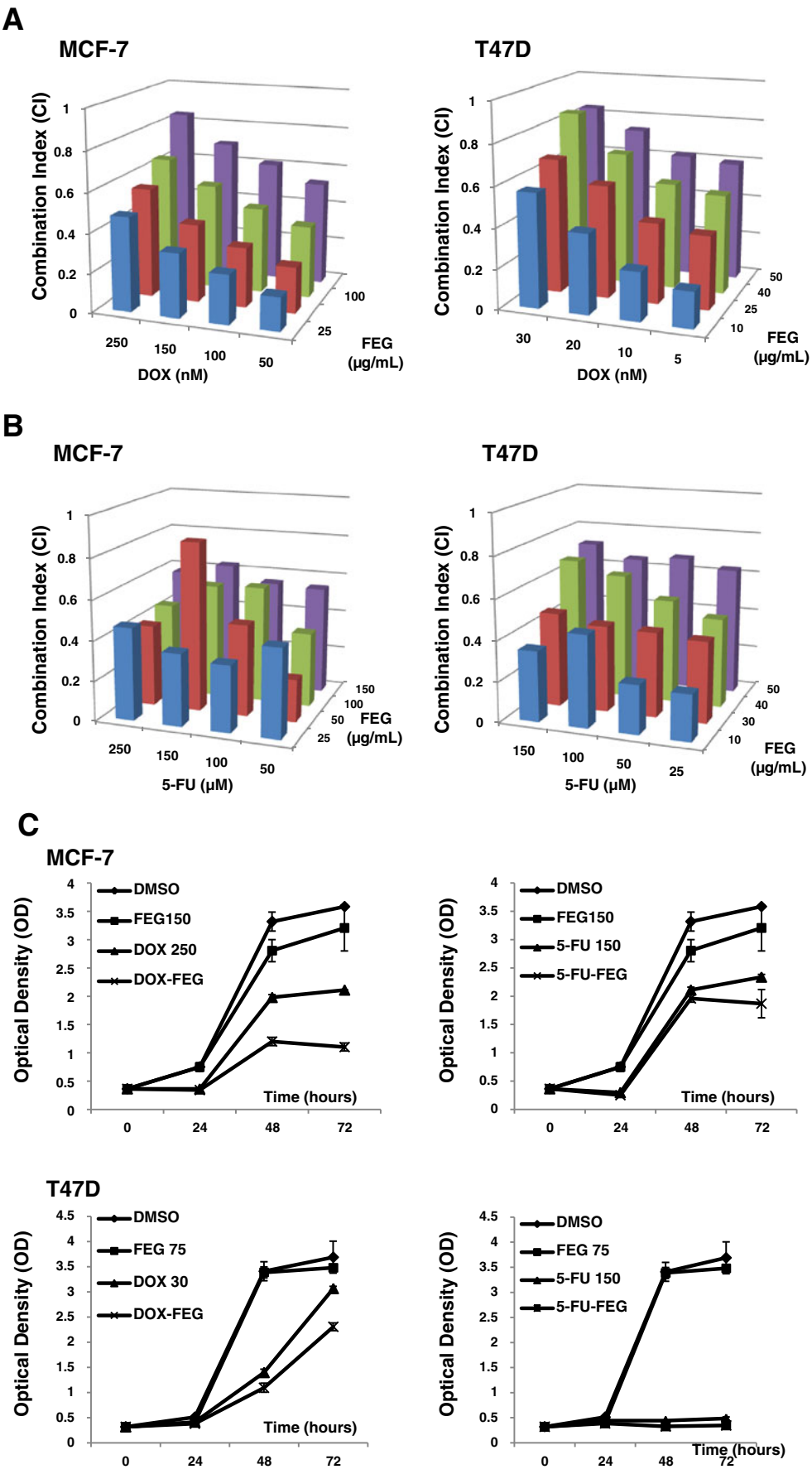


Fig. 1 Combination treatment of DOX or 5-FU and FEG produces synergistic effect on cell viability and increases the inhibition of cells proliferation on MCF-7 and T47D. Cells (A. 10^4 cells/well and B. 3.10^3 cells/well) were seeded in 96 well plate, and incubated in DMEM supplemented with 10% heat-inactivated FBS, without or with DOX, 5-FU, or FEG as single of combination for 24 hs (**a**) or 0, 24, 48, and 72 hs (**b**). Cells viability was determined by MTT assay. The present data represented two independent experiments with similar result

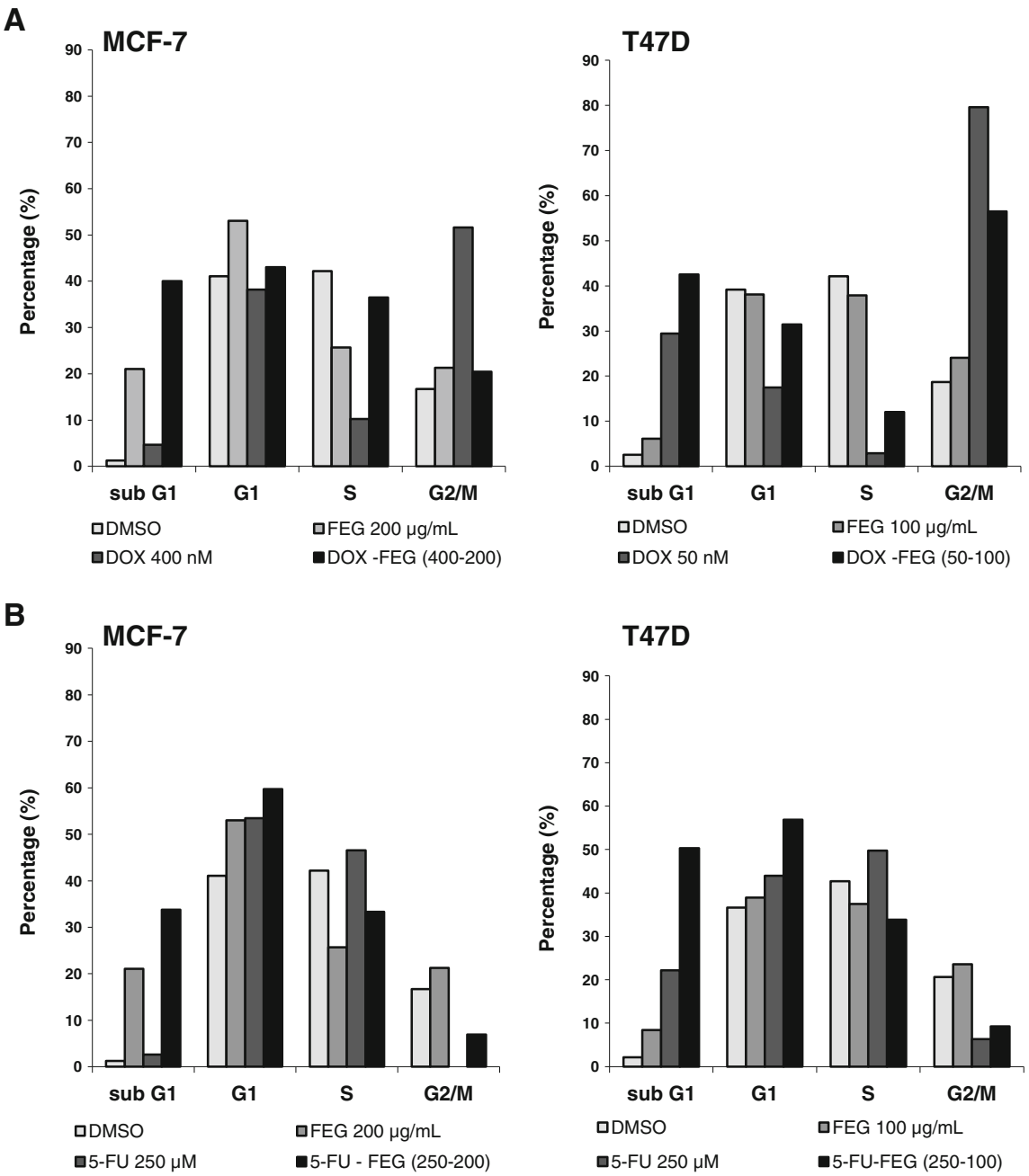


Fig. 2 **a** The effect of DOX, 5-FU and FEG as single or combination treatment on cell cycle and cell death induction on MCF-7 and T47D cells. Cells (5.10^5 cells/well) were incubated with indicated concentration of DOX, 5-FU, and FEG single or combination treatment for 24 hs. Cell cycle prolfile was analyzed by Flowcytometry with Propidium Iodide (PI) as DNA content probe. Su G1 accumulations refer to cell death induction. **b** Morphologycal changing after DOX, 5-FU and FEG as single or combination treatment on MCF-7 and T47D cells. Cells were observed after 24 hs treatment under inverted microscope with 200x magnification

was only revealed after 5-FU + FEG treatment on T47D cells. This protein expression was not detected after the increasing concentration of FEG (Fig. 6). The distinct mechanism of cell death induction on MCF-7 and T47D cells may be related with the different on morphological changing on both cells (Fig. 7).

FEG treatment decreased mSin3A expression levels and induced α-Tubulin aggregation on MCF-7 and T47D cells

FEG treatment lead to decrease α-Tubulin and mSin3A levels as shown the immunoblotting result (Fig. 4a). While DOX and 5-FU alone gave no effect on both of protein

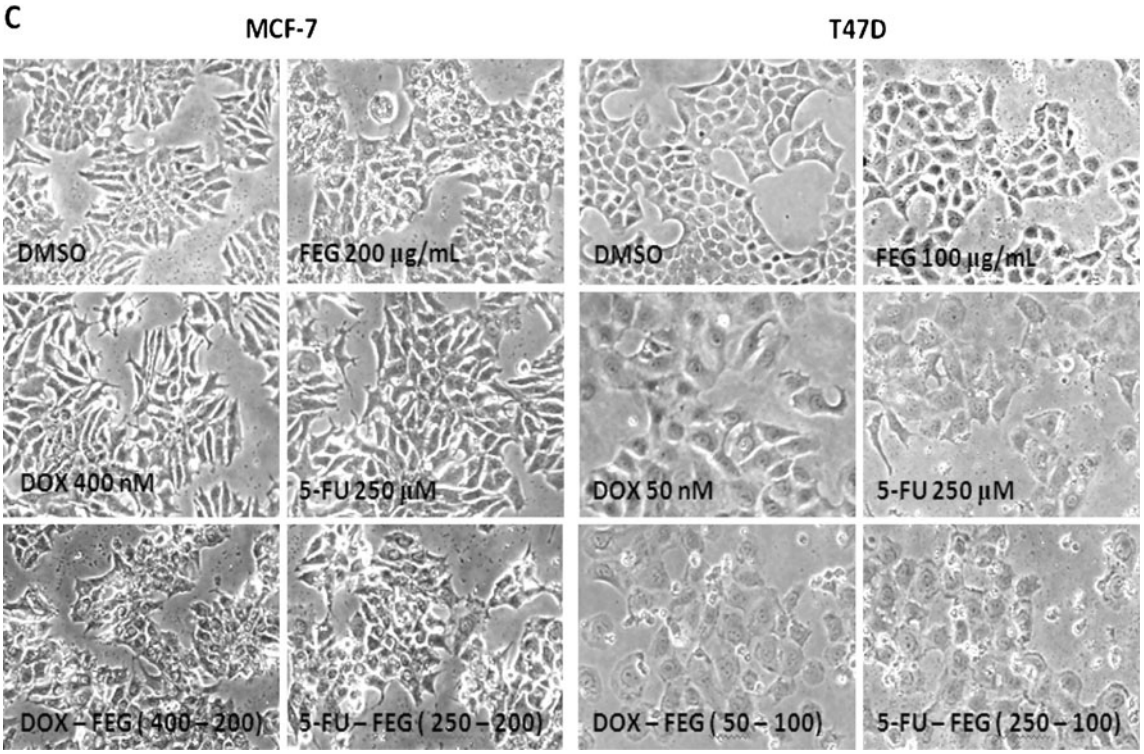


Fig. 2 (continued)

expression levels. The decreasing level of these protein possessed positive corellation with the increasing of FEG concentration. FEG decreased these protein level in concentration dependent manner (Fig. 4b). FEG treatment modulates the microtubule integrity as shown by immunostaining with α-Tubulin and visualized by Alexa 488 (green fluorescence). FEG interacted with this protein and caused microtubule aggregation as shown as cells shape changing and tertiary structure formation. This aggregation was shown by localized high intensity fluorescence of microtubule. This unexpected phenomenon could explain the decreasing level of this protein. The substantial amounts of tubulin were rapidly precipitated during cell lysate preparation and become insoluble. This protein aggregation and precipitation may be as a unique response of MCF-7 and T47D cells to FEG treatment suggesting a result of the structural misfolding caused by interaction or binding with FEG (Fig. 5).

Discussion

MCF-7 and T47D cells exhibit different sensitivity to FEG treatment, shown by IC_{50} value. MCF-7 cells have lower sensitivity than that of T47D cells on DOX and 5-FU treatment. T47D cells have mutated *p53*, as missense mutation at 194 residue (in zinc-binding domain, L2). This mutation leads to disrupt the p53 binding to response element on target genes (Schafer et al. 2000). Although MCF-7 cells have wild type *p53*, but it contain deletion on *CASP-3*, result in the failure of Caspase-3 expression (Onuki et al. 2003; Prunet et al. 2005). MCF-7 cells also have *MDR* and *BCRP* genes generate ATP-binding Cassette family member of drug transporter, such as P-glycoprotein (P-gp), multidrug resistance protein (MRP), and breast cancer resistance protein (BCRP) (Faneyte et al. 2002). This molecular characteristic of MCF-7 cells suggest as the reason of this cells resistance to many anticancer agent. While *p53* status on both cells seem have no correlation with their sensitivity (Figs 6 and 7).

Combination treatment of DOX + FEG and 5-FU + FEG produce strong synergism effect on MCF-7 and T47D cells growth inhibition (CI value<0,8). Parallel with these results, the significance increasing of sub G1 accumulations which were shown after the combination treatment, refer to cell death induction. FEG treatments cause arrest on G1 phase, but when it was combined with DOX showed the decreasing cells accumulation on G2/M phase as caused by DOX treatment alone. This may be caused of the different target of FEG and DOX on cell cycle. The combination treatment of 5-FU + FEG on both cells present cell accumulation on G1 and S phase, especially on T47D cells. 5-FU as single treatment cause G1 and S phase arrest, while FEG alone leads to arrest on G1 phase. So, the combination effect was the resultance of the single treatment.

Apoptosis induction on cancer cells is one main strategy on cancer therapy. There are some indicators refer to

apoptosis induction such as membrane blebing, sitoplasmic shrinkage, chromatin condensation and DNA fragmentation. In molecular level, one of important indicators on apoptosis induction is the appearance of cleaved-PARP (c-PARP) as a sign of effectors Caspase activity.

We found the different of the MCF-7 and T47D cells response to DOX, 5-FU, and FEG as single or combination treatment. Apoptosis was detected by DNA fragmentation, chromatin condensation, and apoptotic bodies formation in MCF-7 cells after 24 h treatment. We also observed the increasing c-PARP level on MCF-7 cell after combination treatment of DOX + FEG and 5-FU + FEG. MCF-7 cells could not express Caspase-3, but still have Caspase-7 as effector Caspase with the PARP as one main target of digestion (Prunet et al. 2005). In contrast, T47D cells after treatment exhibited the different appearance compare with MCF-7 cells. We found the different morphological changes such as larger cells with multiple micronuclei, flattened cells shape, and decondensed chromatin. No DNA fragmentation found after FEG treatment as single and combination on T47D cells. Only combination treatment of 5-FU + FEG on these cells reveals c-PARP expression. All of these results suggested that except apoptosis there is other mechanism on cell death induction at T47D cells.

The distinct mechanism of cell death on T47D cells may be related with its molecular characteristic, especially on *p53* status. Protein p53 plays a central role on apoptosis induction after chemotherapy agent exposure. The decreasing of this protein function could disrupt apoptosis initiation and lead to necrosis-like cell death as define as mitotic catastrophe. Cells with *p53* mutation will precede the delayed cell death, observed as mitotic catastrophe occurrd after ionizing radiation and trigger by treatment with anti-microtubule agents. These agents resulted cell cycle checkpoint disturbance (Jonathan et al. 1999; Vakifahmetoglu et al. 2008). At cells with functionally P53, These agents activated cell cycle checkpoint-regulator protein through two main protein kinase pathways: ATM/Chk2 or ATR/Chk1. This checkpoint activation caused the failure entry to M phase and cell arrest at G2 phase as consequence (Vakifahmetoglu et al. 2008). The lack of p53 directed to premature entry to M phase as the effect of inactivated cell cycle checkpoint. Further, cells will die during metaphase, or at the case mitotic slippage and as consequence of cytokinesis failure. It will generate tetraplolidy and aneuploidy cells accumulation, then lead to cell death (Castedo et al. 2004).

Microtubules are the important component of cytoskeletal in eukaryotic cells and plays a central role on several cell functions, involving such as, cells polarity, mitosis process, intracellular trafficking and maintain the cell shape integrity (Nogales 2001). Microtubules have dynamic structure that can be polymerized and depolimerized through reversible association and dissociation α- and β-Tubulin as its monomer (Zhou and Giannakakou 2005). Disturbance of the microtubules formation and destabilization were reported leading to arrest on G1 phase at several mammalia cell lines (Uetake and Sluder 2007). Microtubule integrity disruption leads to celluler perturbation impacting inhibition of cell cycle progression and proliferation and further cause cell death.

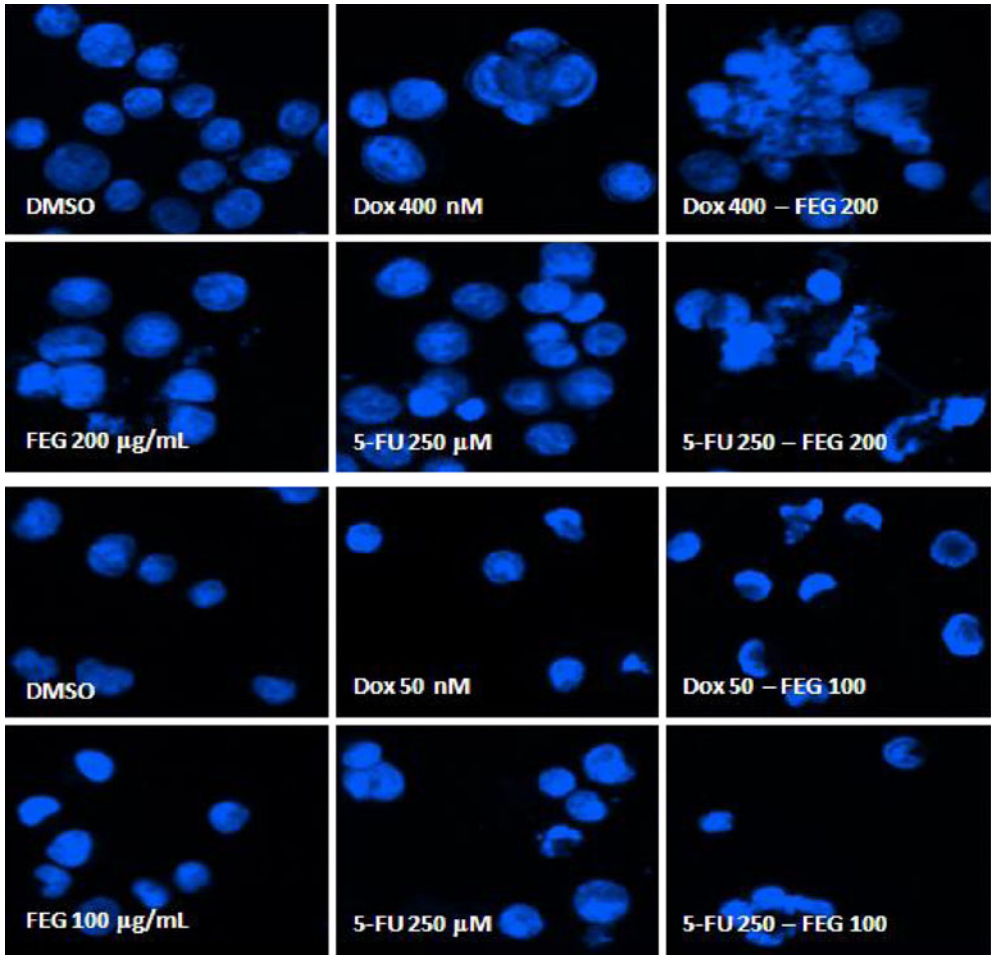
Fig. 3 Combination treatment FEG with DOX or 5-FU causes chromatin condensation and DNA fragmentation on MCF-7 but produces no significance effect on T47D. **a** Cells were recovered after 24 hs treatment, then stained with DAPI. Cells were obcerved under flouresensce microscope with 400x magnification. **b** Cells (10^6 cells/well) were harvested after 24 hs treatment then the DNA was isolated with protocol as described previous

Several compounds have been reported having different target on microtubule compared with well-known microtubule inhibitors. Nuclear transcription factor peroxisome proliferator-activated receptor-gamma (PPARγ) inhibitor, T0070907, could decrease tubulin levels through its post-translational regulation without affected on microtubule dynamic (Schaefer et al. 2006; Schaefer 2008). The decreasing level of tubulin after T0070907 treatment was prevented by proteasome inhibitors (Harris and Schaefer 2009).

Isothiocyanates (ITCs) forms covalent binding with cystein on tubulin leading to cell growth inhibition and apoptosis induction (Mi et al. 2008). ITCs trigger agresome complex formation consists of α and β-Tubulin, chaperone protein, proteasome, and ubiquitination protein. This protein complex initiates the proteasome activation to degrade α and β-Tubulin (Mi et al. 2009). Meanwhile ITCs also act as proteasome inhibitor and causes accumulation of p53 and IkB, affecting significance inhibition of cell growth (Mi et al. 2010). These mechanisms are not correlated with tubulin degradation trough proteasome pathway. Quercetin, a flavonoid one identified component of FEG, resulted cell cycle arrest at early mitosis (M) phase and modulates tubulin polimerisation. The microtubules depolimerisation after quercetin treatment generates unproportional cytokinesis and formed multinuclei cells, then lead to cell death (Jackson and Venema 2006). These compounds modulate the microtubule integrity through different mechanisms such as: 1) disruption on post transcriptional regulation; 2) induction on protein degradation through proteasomal activation; 3) stabilization or destabilization of microtubule. Thus, microtubule become important target for developing anticancer.

In this study we found, FEG treatment decreased α-Tubulin level both on MCF-7 and T47D cells. This phenomenon was different with the effect of common microtubule inhibitors (Taxane, Vinca alkaloids, and ephothylon) that have no effect on this protein level but just affected destabilization and stabilization of microtubul. We also found that level of microtubules after immunostaining had not changed. FEG treatment affected the structural changing

A



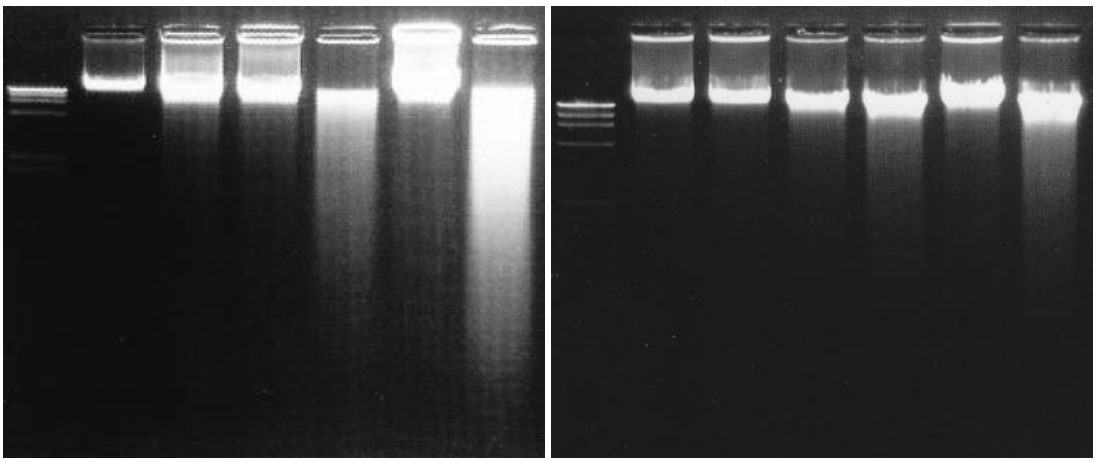
B

MCF-7

M*	DMSO	FEG	DOX	DOX-FEG	5-FU	5-FU-FEG
		200	400	400-200	250	250-200

T47D

M*	DMSO	FEG	DOX	DOX-FEG	5-FU	5-FU-FEG
		100	50	50-100	250	250-100



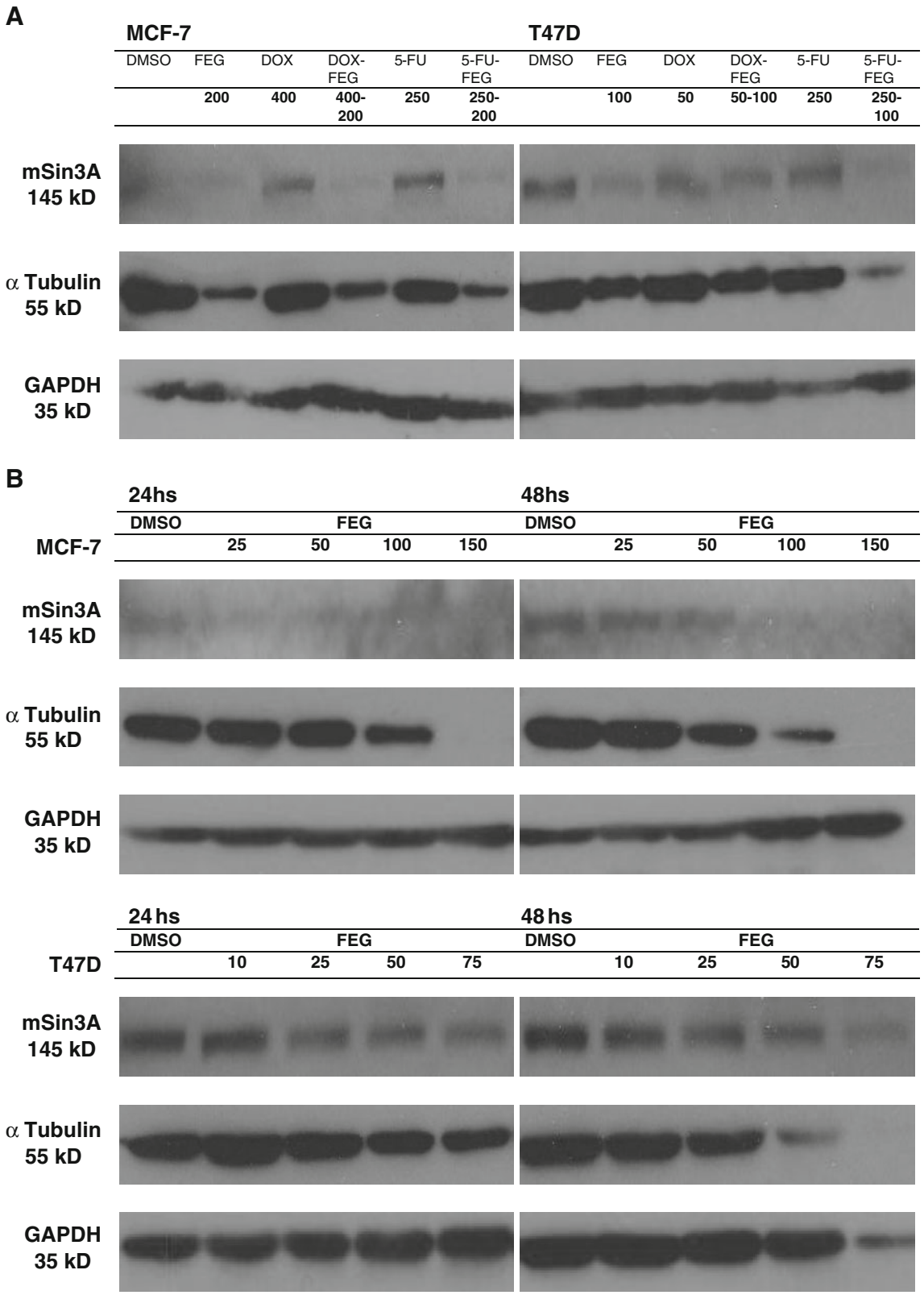


Fig. 4 FEG treatment decrecases the α-Tubulin and mSin3A expression level in concentration and time dependent manner. **a** Cells were exposed to DOX, 5-FU, and FEG as single or combination for 24 hs then α-Tubulin and mSin3A expression were evaluated using specific antibodies. **b** Various concentration of FEG (0–75 μg/mL) lead to decreasing α-Tubulin and mSin3A expression both on MCF-7 and T47D cells. GAPDH was used as a loading control

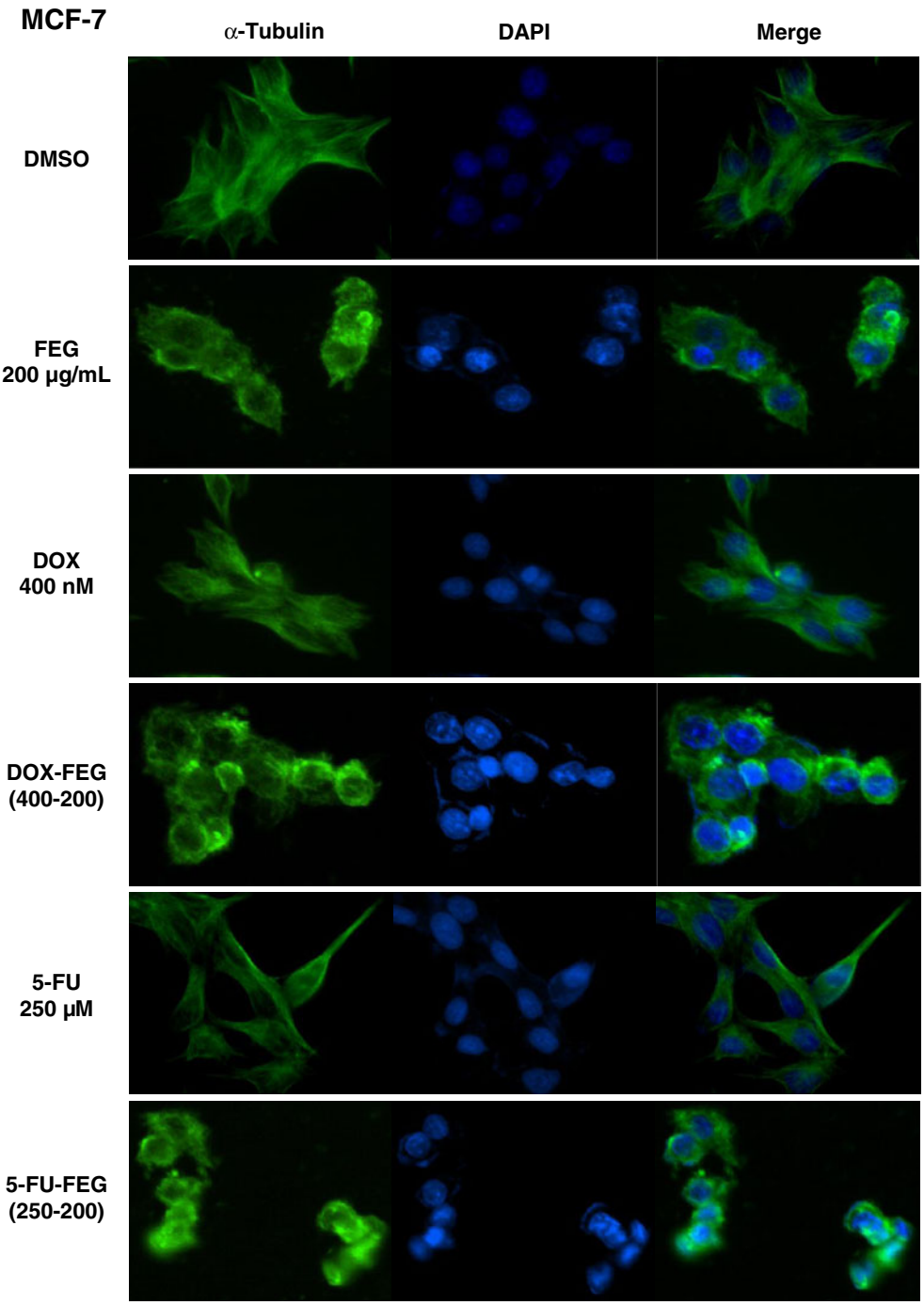
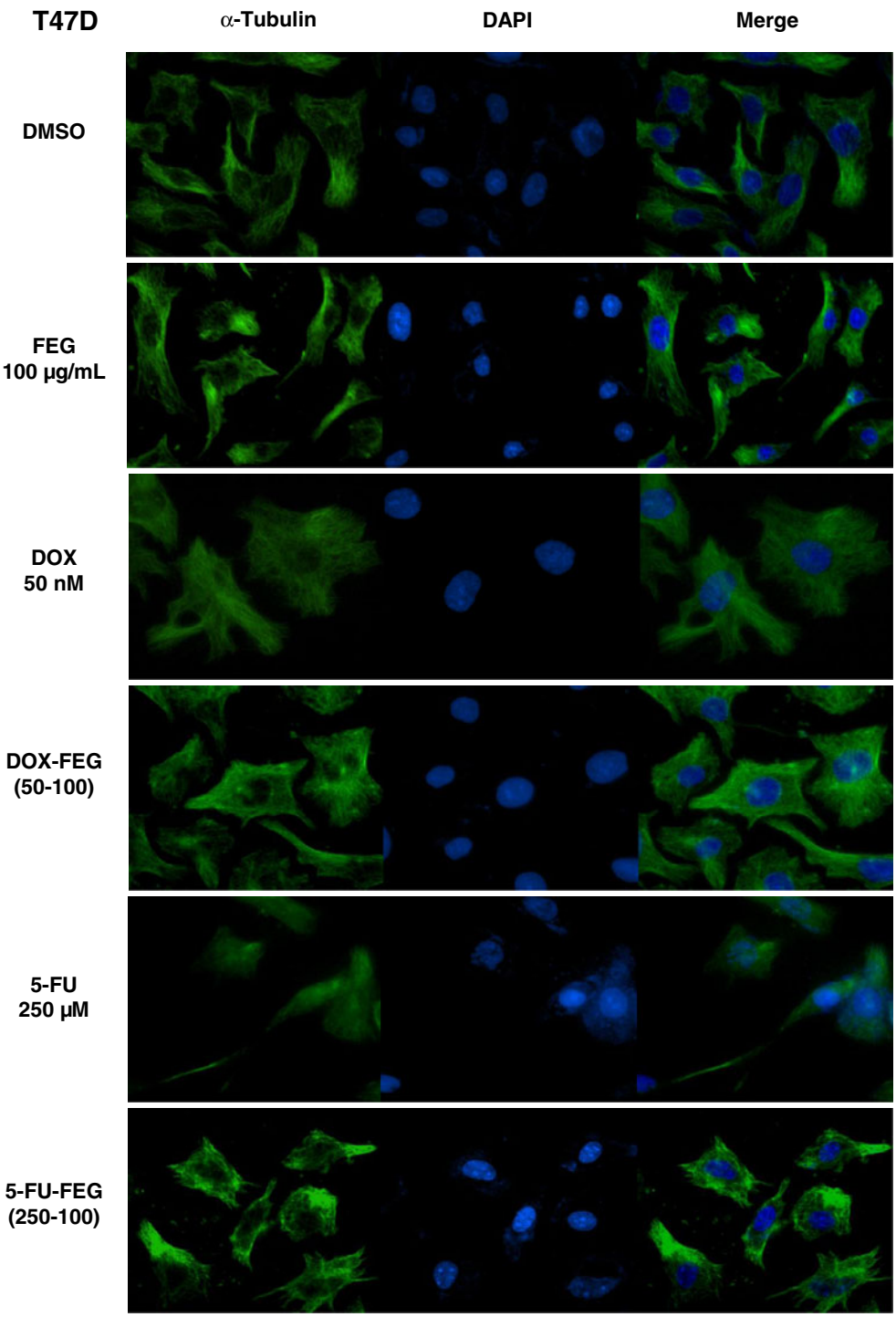


Fig. 5 FEG treatment causes Tubulin aggregation both on MCF-7 and T47D. cells (10^4 cells/well) were seeded on coverslips in 24 well plate. After 24 hs treated with DOX, 5-FU, and FEG as single or combination, cells were stained using antibodies directed against α-Tubulin (Green). Co-staining of nuclei was performed using DAPI. Representative image of the cells were obtained using fluorescence microscope with 200x magnification

of microtubule and morphological changes of the whole cell. We suggest that FEG probably interact and make structural modification through binding with tubulin. The FEG-tubulin binding triggers the conformational changes, protein misfolding and aggregation. This phenomenon suggested the possible explanation of the decreasing level of tubulin in Immunoblotting results. In which aggregated Tubulin became insoluble and will be precipitated during cell lysate preparation.

Aberration on microtubules integrity caused intracellular signaling perturbation, and cells become stack on G1 phase. The signaling perturbation influenced transcriptional activation signal of many genes especially that regulated cell cycle and proliferation. mSin3A is a core component of protein co-repressor histon deacetylase (HDAC) (Silverstein and Ekwall 2004). This protein has important role on transcriptional regulation and cell proliferation as well as development of normal and neoplasma cells (Dannenberg et al.

Fig. 5 (continued)



2005). The high expression of mSin3A and mSin3A/HDAC complexes has high correlation with carcinogenesis and tumor development. These complexes also interact with tumor suppressor protein, p53 and pRb (Murphy et al. 1999; Lai et al. 2001). The microtubule disruption may affect to many protein that have pivotal function on transcriptional regulation, such as mSin3A. Both of MCF-7 and T47D cells expressed mSin3A, and in this case T47D cells have higher expression level of mSin3A than MCF-7 cells. FEG treatment caused the decreasing level of this protein in dose-dependent manner, while DOX and 5-FU alone gave no effect on mSin3A protein expression. The decreasing level of mSin3A may be related with disruption of microtubule integrity.

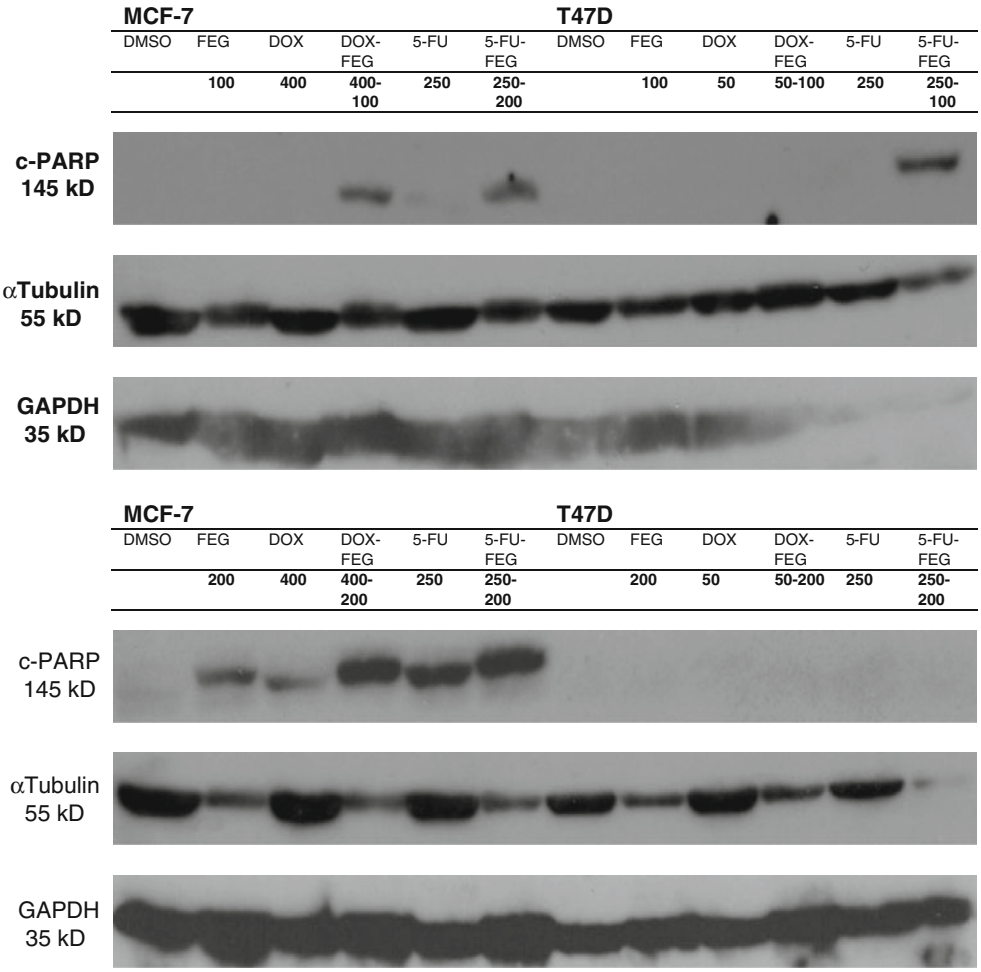


Fig. 6 FEG treatment enhances the DOX and 5-FU-induced apoptosis on MCF-7 cells, but found another cell death mechanism on T47D cells. Cells were treated and lysed as described under material and method. Membranes were probed for c-PARP and α-Tubulin. Blots were re-probe for GAPDH to confirm an equal loading of the samples

Conclusion

FEG potentiates the DOX and 5-FU efficacy on MCF-7 and T47D cells. FEG induces cell death through apoptosis mechanism on MCF-7 cells while on T47D cells, FEG causes necrosis-like cell death proposed as mitotic catastrophe. The FEG may have specific targeted on microtubule integrity modulation. The FEG interacts with microtubule and causes protein aggregation leading to cell cycle arrest and proliferation inhibition. Further FEG potentially could be developed as a co-chemotherapeutic agent for reducing side effect and have specific molecular target.

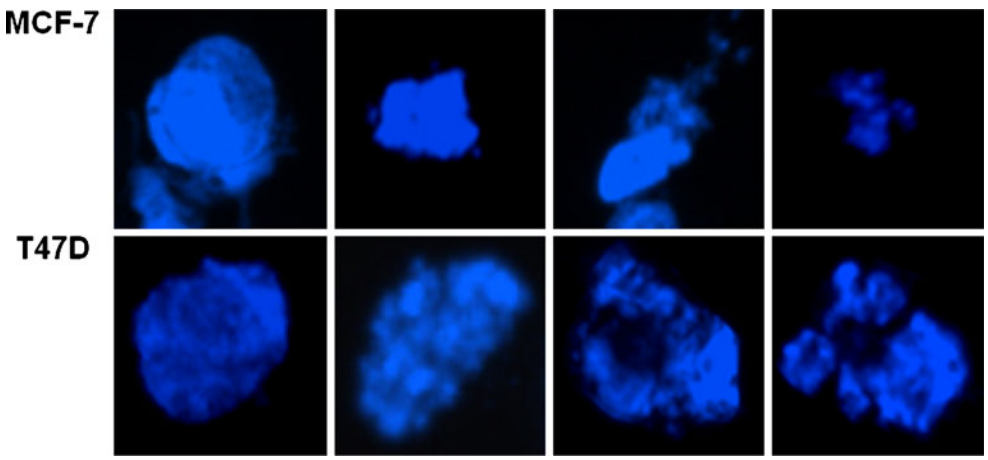


Fig. 7 The distinct of morphological changing on MCF-7 and T47D cells after FEG treatment. Cells were stained with DAPI and observed under fluorescence microscope with 400x magnification. The cells picture were represented three independent experiments

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