



Genistein enhances cytotoxic and antimigratory activities of doxorubicin on 4T1 breast cancer cells through cell cycle arrest and ROS generation

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ABSTRACT

Genistein (Gen) demonstrates growth induction and inhibition on several cancer cells depending on the dose. By using triple-negative breast cancer metastatic cells, 4T1, this study aimed to evaluate the cytotoxic and anti-metastatic effects of Gen in combination with doxorubicin (Dox). Gen exhibited cytotoxic effects in dose- and time-dependent manners with an IC₅₀ value of 50 μM. Interestingly, Gen in combination with Dox enhanced the growth inhibitory effect in 48 hours and caused increases in the G2/M phase arrest, as well as apoptosis. Gen alone increased cyclin B expression that may prevent the cells to enter anaphase. Moreover, the single treatment of Gen inhibited cell migration up to 45% in 24 hours. Under gelatin zymography and immunoblotting experiments, a combination of Gen and Dox subsided the activities of matrix metalloproteinase-9 and the expression level of Rac1, thereby showing the potency of Gen as an antimigratory agent. In accordance with the growth inhibitory effect, Gen also considerably stimulated the cellular reactive oxygen species production and autophagy activity. In conclusion, Gen increased the cytotoxic activity of Dox in 4T1 cells and inhibited cell migration.

INTRODUCTION

As an isoflavone robustly discovered in soy (*Glycine max*), genistein (Gen) has been mainly explored as an antioxidant (Chen *et al.*, 2018) and potential chemoprevention and anticancer agent (Ganai and Farooqi, 2015; Mukund *et al.*, 2017; Nabavi *et al.*, 2015). However, the specific mechanism as cancer chemoprevention as well as anti/pro-oxidant benefits of Gen remains unclear in the reported evidence. The common phenomena show that Gen induces physiological changes in the cells based on

its concentration. Gen exhibits a biphasic effect on the estrogen receptor (ER)-positive cells, such as breast cancer cells, thereby showing that a low concentration of Gen tends to induce cell proliferation; however, a high concentration of Gen inhibits cell growth (Marik *et al.*, 2011). Special emphasis should be given to this phenomenon during the administration of Gen in the case of breast cancers. In addition, we should pay attention to the critical dose in this transition effect.

Gen also reveals an inconsistency effect on the malignant cancer cells. At a low concentration (< 10 μM), Gen induces metastasis caused by 4T1 cell xenograft on mice (Yang *et al.*, 2015). In the prostate cancer cells, Zhang *et al.* (2008) showed that Gen induces the epithelial-to-mesenchymal transition (EMT) by E-cadherin upregulation. In contrast, Gen attenuates the metastatic evidence of malignant prostate cancer induced by doxorubicin (Dox) (Wang *et al.*, 2018). Gen inhibits cell proliferation and migration by downregulating the transforming growth factor

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(TGF) β -signaling, resulting in an EMT regulation (Kim *et al.*, 2015), a decrease in the matrix metalloproteinase-2 (MMP-2) activity (Tsai *et al.*, 2017), and inhibition of nuclear factor (NF)- κ B and Akt-signaling activities that are critical regulators of cell survival (Cui *et al.*, 2017). These downregulation effects of Gen seem to correlate with the inhibitory effect of some protein kinases.

Hence, Gen shows a pleiotropic effect in the molecular events on the cell and is most likely to be proper in specific cell types, especially in cancer cells with ER-positive or ER-negative expressions. A study on *ER α* -negative MDA-MB 231 cells revealed that Gen at the concentration of 25 μ M can restore the *ER α* expression, which increases the cell sensitivity to tamoxifen treatment comparably to that examined in *ER α* -positive MCF-7 cells (Li *et al.*, 2013). In another study, Gen modulates the expression of ER at the mRNA levels by decreasing *ER α* but enhances *ER β* expression on ER-positive breast cancer cells (MCF-7, 21PT, and T47D cells), which further inhibits cell proliferation (Marik *et al.*, 2011).

Alongside, a study on MDA-MB 231 triple-negative breast cancer (TNBC) cells by using the quantitative phosphoproteomics proved that 40- μ M Gen promotes G2/M arrest and DNA damage that suppresses cell proliferation (Fang *et al.*, 2016). Interestingly, Gen also demonstrates a dose-dependent effect on the 4T1 cell migration (Yang *et al.*, 2015). Gen also exhibits a synergistic effect with Dox on MCF7/Adr cells via downregulation of multidrug resistance protein 1 (MDR1) expression (Xue *et al.*, 2014). This mechanism is still unknown; however, Gen inhibits NF- κ B activation, a transcription factor of PgP/MDR1. This phenomenon may correlate with the inhibiting action of Gen on the prostate cancer cell migration induced by Dox (Wang *et al.*, 2018).

Dox is a first-line chemotherapeutic agent for breast cancers. However, Dox raises the resistance of cancer cells, especially in the metastatic type of breast cancers (Lovitt *et al.*, 2018). Therefore, the development of complementary agent against this issue is still vital. Subsequently, it is intriguing to comprehend the effect of Gen in combination with Dox on TNBC metastatic cells. Thus, in this study, we aimed to examine the effect of Gen in enhancing the cytotoxicity and inhibiting migration induction of Dox on the 4T1 TNBC metastatic cells, by analyzing cell cycle profiles, apoptosis evidence, autophagy activities, and the intracellular level of reactive oxygen species (ROS).

MATERIALS AND METHODS

Cell culture

The murine mammary carcinoma 4T1 cell line was provided by Dr. Masashi Kawaichi, Nara Institute of Science and Technology (NAIST), Japan. Briefly, the cells were cultured in complete media comprising a high glucose Dulbecco's Modified Eagle Medium (Gibco, Gaithersburg, MD) with the addition of 10% (*v/v*) fetal bovine serum (FBS) (Sigma, St. Louis, MO), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and sodium bicarbonate for controlling pH, antibiotics [150 IU/ml penicillin and 150 μ g/ml streptomycin (Gibco, Gaithersburg, MD)], and antifungal [1.25 μ g/ml amphotericin B (Gibco, Gaithersburg, MD)]. The cells were kept at 37°C in a humidified incubator with 5% CO₂ aeration.

Cytotoxicity assay

The cytotoxic activity of Gen and Dox was measured by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as modified from the previous work (Ikawati *et al.*, 2018). The cells (2×10^3 /well) were seeded in a 96-well microplate. After 24 hours, the cells were treated with a serial concentration of Gen (G6649, Sigma-Aldrich), Dox (44583, Sigma-Aldrich), or its combination at several concentrations for 24, 48, or 72 hours. Cells in at least three wells were kept untreated to serve as control. After treatment, 0.5 mg/ml MTT (Biovision) in the complete medium was administered. After 2 hours incubation, the reaction was terminated with a stopper reagent solution [0.01 N HCl in sodium dodecyl sulfate (SDS)], followed by incubation at room temperature and protected from light for overnight. The absorbance at 595 nm was recorded using a microplate reader (Corona SH-1000). The percentage of cell viability was obtained by comparing the absorbance of treated groups with the untreated one. The IC₅₀ value, a concentration that inhibits cell growth by 50% as a parameter of cytotoxicity, was calculated by a linear regression equation $y = Bx + A$, in which y is the percentage of cell viability and x is the concentration. The cytotoxic combination assay to obtain the combination index (CI) value was performed as formerly reported (Ikawati and Septisetiyani, 2018; Kusumastuti *et al.*, 2019).

Cell cycle analysis

The cell cycle analysis was carried out by flow cytometry using propidium iodide (PI) staining according to Kim and Sederstrom (2015). The cells (2×10^5 /well) were grown in a 6-well plate. After 24 hours, the cells were treated with Gen, Dox, or its combination and incubated for another 24 or 48 hours. The media were discarded and the cells were harvested by trypsinization [trypsin-EDTA 0.25% (Gibco)] and centrifuged at 2,000 rpm for 3 minutes. The cell pellets were fixed with ethanol for 30 minutes, centrifuged again for 3 minutes, and washed twice with cold phosphate-buffered saline (PBS). The cells then were being resuspended with the PI-staining solution (50 mg/ml PI/1% Triton X-100 (Merck)/PBS) and treated with DNase-free RNase A (20 μ g/ml) for 30 minutes at 37°C and protected from light. After staining, the cells were then introduced to the flow cytometer (FACSCalibur, BD Biosciences). The red fluorescence signal was captured by using the FL-1 setting (log mode) after electronically gating out the cell debris. In total, 200,000 events were obtained for a subsequent analysis by using Cell Quest software (BD Biosciences).

Apoptosis assay

The flow cytometry was also used to examine the apoptosis induction by the Annexin-V-FITC/PI staining as previously described (Wlodkowic *et al.*, 2009). Following similar steps as the cell cycle analysis, after the treatment, media were discarded and the cells were trypsinized and stained with Annexin-V-FLUOS-staining kit (Roche) (consisting of 2 ml Annexin-V and 2 ml PI in 100 ml binding buffer). According to the manufacturer's instruction, the cells were then incubated for 10 minutes in the dark. After the staining procedure, the cells were detected by flow cytometer for further analysis using Cell Quest.

Migration assay

The migration of 4T1 cells under Gen, Dox, or its combination treatment was determined by the scratch wound healing assay as amended from Liang *et al.* (2007). The cells (7.5×10^4 /well) were grown in a 24-well plate for 24 hours in a 37°C incubator to reach an 80% confluence. The cells were washed with PBS and the medium was changed into a starvation medium containing 0.5% (v/v) FBS and incubated for 24 hours. A scratch was made in each of the bottom centers of the well by using the tip of sterile yellow tip. To remove unattached cells, the well was washed with PBS. The tested compounds at the designated concentration dissolved in the starvation medium were administered to the cells. The cell was kept in the 37°C incubator and images were captured at different time points under an inverted microscope. The cell migration was calculated by measuring the decrease of the “wound” from time to time by using ImageJ software and defined as the percentage of closure.

Gelatin zymography

To determine the activity of MMPs, gelatin zymography was performed by using SDS-polyacrylamide gel (PAGE) (Hibbs *et al.*, 1985). After treating the cells with tested compounds, the culture medium was recovered as the samples. The samples without the reducing agent were electrophoresed on an 8% PAGE containing 0.1% gelatin. Following electrophoresis, the gel was washed and incubated in 2% Triton X-100/distilled water for 30 minutes at room temperature. After washing, the gel was incubated in 100 ml gel development buffer that consists of 40 mM Tris-HCl pH 8, 10 mM CaCl₂, and 0.02% NaN₃ for 24 hours at 37°C. The development buffer was discarded and the gel was stained in Coomassie Brilliant Blue R-250 solution, followed by incubation in the destaining solution that consists of methanol, acetic acid, and water in a 2:1:7 ratio to clear the band in the dark blue background. The zymogram was documented and the clear bands representing gelatinolytic activity were measured by densitometry using ImageJ. The relative activity of MMPs was compared to untreated bands.

Immunoblotting

Approximately, 4×10^5 cells/well were grown in a 10-scm tissue culture dish for 24 hours. After treating the cells with Gen, Dox, or its combination for 24 hours, the proteins were extracted from the cells by using radioimmunoprecipitation assay buffer containing Tris-HCl pH 7.6, NP40, sodium deoxycholate, NaCl, SDS, phenylmethylsulfonyl fluoride, NaF, and protease inhibitor cocktail. The proteins were separated in a 10% acrylamide gel (for cyclin B, tubulin, and β -actin) or a 14% acrylamide gel (for Rac-1) by SDS-PAGE electrophoresis (Schnelzer *et al.*, 2000), followed by a transfer to polyvinylidene fluoride membranes. The membranes were incubated in the specified antibodies overnight at 4°C with the following: mouse monoclonal antibody cyclin B (#4135, Cell Signaling), Rac-1 (ab33186, Abcam), tubulin (t9026, Sigma), or β -actin (sc-47778, Santa Cruz), followed by the incubation with anti-mouse secondary antibody (sc-516102, Santa Cruz) for 1 hour at the room temperature. The bands were identified by using electrochemiluminescence (ECL) reagent (#1705062, Biorad or ECL Prime, Amersham Bioscience) and

were visualized by using Luminescent Image Analyzer LAS4000 (Fujifilm) or Lumino Graph (Atto). The relative protein level was calculated as the ratio with the amount of the housekeeping protein.

Autophagy assay

The 4T1 cells (2×10^5) grown in a 6-well plate were treated with Gen for 24 hours and then stained with the green detection reagent from the Autophagy Detection Kit (Abcam) according to the manufacturer’s instructions. The cells then were analyzed by BD Accuri C6 flow cytometer (BD Bioscience).

Intercellular ROS assay

The 4T1 cells (5×10^4) were seeded on a 24-well plate, incubated overnight, and trypsinized. Five hundred microliters 1x supplemented buffer [10% (v/v) FBS in PBS] was added and the cells were stained using 25- μ M 2,7-dichlorofluorescein diacetate (DCFDA) (Sigma). After incubation at 37°C 5% CO₂ for 30 minutes, the cells were treated with Gen or Dox and were incubated for another 4 hours. The ROS level was measured by the flow cytometer using BD Accuri C6 as previously described (Angraini *et al.*, 2019).

RESULTS AND DISCUSSION

Results

Gen increases the cytotoxic effect of Dox on the 4T1 cells

According to the MTT assay carried out to evaluate Gen effects toward 4T1 cells, Gen demonstrated cytotoxic effects in dose- and time-dependent manners. We observed the cell viability in 24, 48, and 72 hours after the administration with Gen. The lower concentration of Gen was not found to be toxic, but the higher concentration and longer incubation time caused a decrease in the viability of cells, hence providing the IC₅₀ value of Gen after 24 and 48 hours of observation as 50 and 27 μ M, respectively (Fig. 1A). In the combination treatment, we used a low concentration of Dox as it was reported to induce the formation of lamellipodia and cell migration (Amalina *et al.*, 2017; Bandyopadhyay *et al.*, 2010). Meanwhile, we used Gen at low and high concentrations (1 and 50 μ M) for further experiments. Gen enhanced the cytotoxicity of Dox at a low concentration on 4T1 cells for 24 and 48 hours (Fig. 1B). The cytotoxic effects of Gen, Dox, and its combination on 4T1 cells might be regulated through the modulation on cell cycle, apoptosis induction, or migration inhibition.

Gen and its combination with Dox arrest the cell cycle at the G2/M phase

Gen exhibited cytotoxic effects on 4T1 cells with the IC₅₀ values of 50 and 27 μ M in 24 and 48 hours, respectively. In addition, when combined with Dox, the cytotoxic activity increased. Then, we evaluated the cell cycle profile under the treatment of Gen and its combination with Dox in 24 and 48 hours. The results revealed that the single treatment of 1 μ M Gen did not show any effect; however, Gen at 50 μ M showed an increase in cell accumulation at the G2/M phase for 24 and 48 hours (Fig. 2A). After concurrent treatment with Dox, 1- μ M

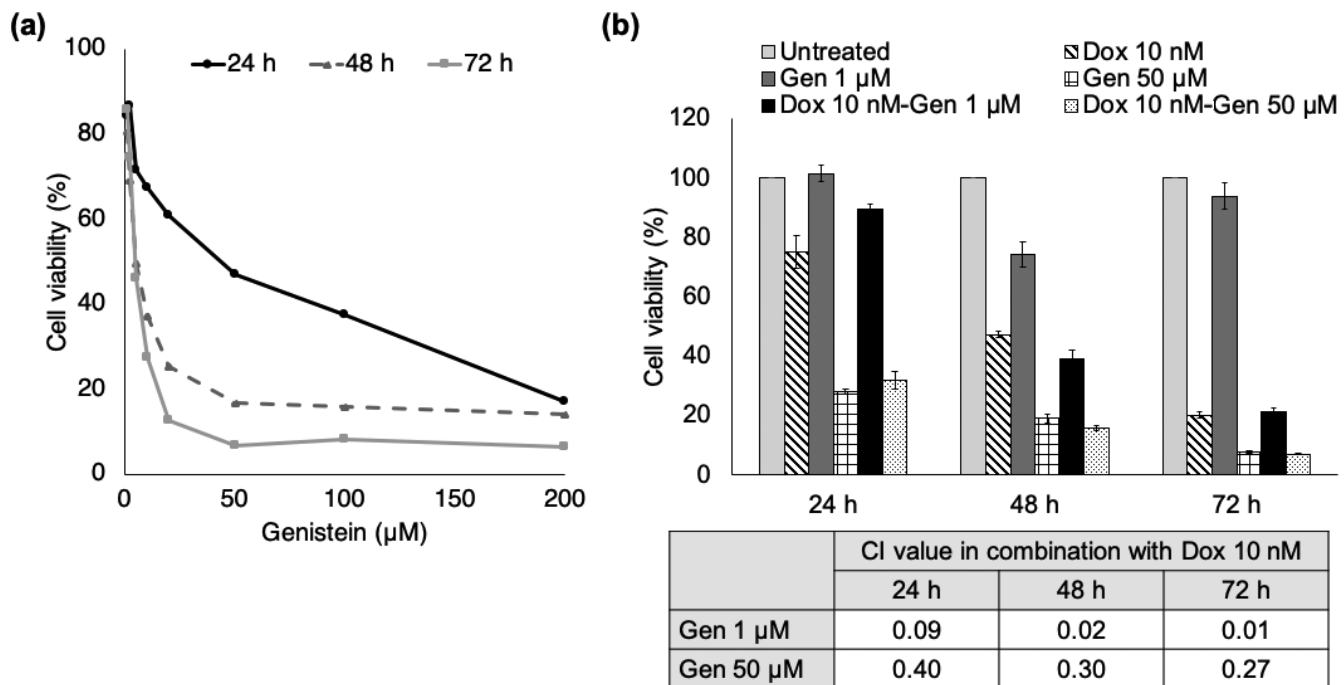


Figure 1. Gen improved the cytotoxic effect of Dox toward 4T1 cells. The 4T1 cells were treated with (a) Gen at a serial concentration and (b) Dox 10 nM in combination with Gen at the concentrations of 1 and 50 μM for the indicated time periods. Cell viability was analyzed at the end of treatments with MTT assays. The experiments were carried out more than once in triplicate for each experiment and representative results are shown. The CI values of Gen-Dox combination on 4T1 cells were calculated and are displayed in the lower right table. The graph represents means \pm SE ($n = 3$).

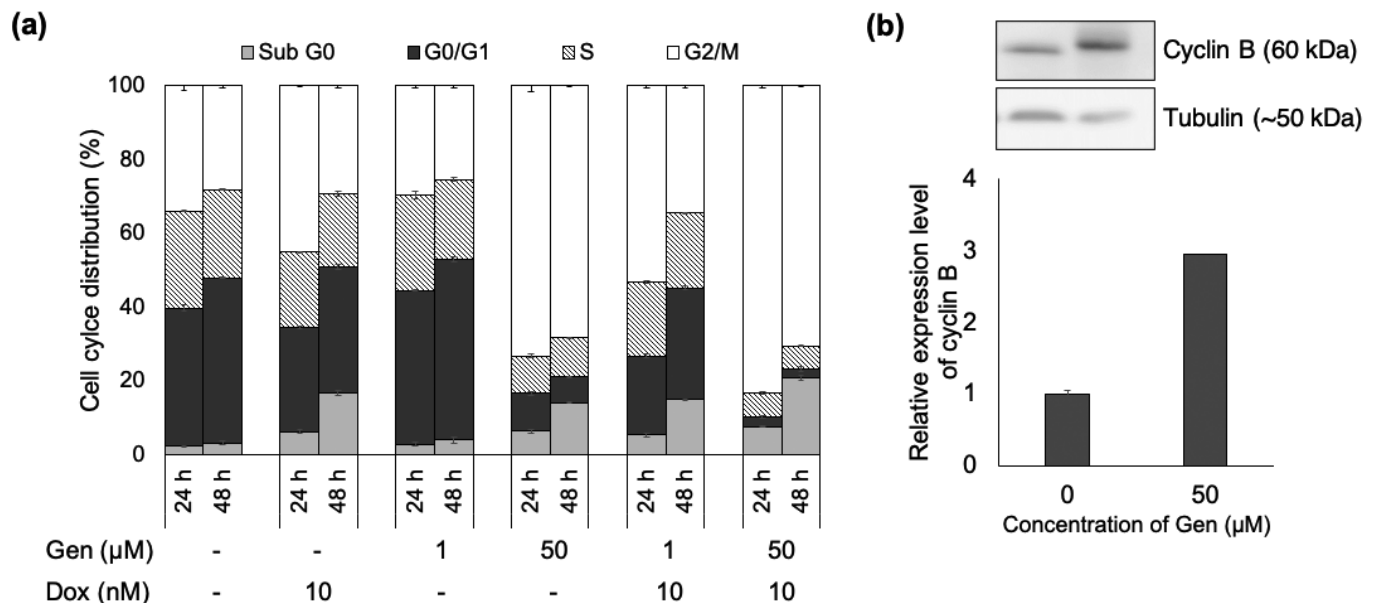


Figure 2. Gen and its combination with Dox modulated the 4T1 cell cycle. The cells were treated with high or low concentrations of Gen and its combination with 10 nM of Dox for 24 and 48 hours, respectively. (a) 4T1 cell cycle profiles were generated by the PI staining, followed by recording the DNA cell contents by flow cytometry in triplicate. The bar represents means \pm SE ($n = 3$). (b) The expression of cyclin B after treatment with 50 μM Gen. The protein expressions were assayed by immunoblotting (upper panel). The band density was analyzed by densitometry using ImageJ in triplicate. After normalization with tubulin, the expression level of cyclin B was calculated and the relative expression level is shown (lower panel). The bar represents means \pm SE ($n = 3$).

Gen still did not affect the cell cycle profile, regardless of a slight arrest at the G2/M phase caused by 10 nM Dox. Interestingly, the combination of 50 μM Gen and 10 nM Dox increased the cell

cycle arrest at the G2/M phase. Additionally, the combination induced an accumulation of cell population in the sub-G0/G1 phase. We confirmed that the cells arrested at the G2/M phase

showed the stabilization of cyclin B as indicated by a thicker band in immunoblotting (Fig. 2B). Taken together, a high concentration of Gen increased the cytotoxic effect of Dox that led to the G2/M phase cell cycle arrest.

Gen enhances Dox to induce apoptosis on 4T1 cells

The combination treatment of Gen–Dox showed cell accumulation at the sub-G0/G1, which probably represents apoptosis induction. According to this result, we further analyzed the apoptosis effect by performing Annexin-V-PI staining under flow cytometry for exploring the effects of single and combination treatments. Our data indicated that 50- μ M Gen in both single and combination with Dox correlates with apoptosis induction (Fig. 3). This result confirmed that Gen increased cell apoptosis.

Gen inhibits cell migration

Cancer metastasis was initiated with migration, invasion, intravasation, and extravasation for the development of a secondary cancer on the target. The 4T1 cells are known as a TNBC model and for their metastatic ability. The scratch wound healing assay was executed to comprehend the 4T1 cell migration under the treatments of Gen and its combination with Dox. The concentrations of 1 and 50 μ M Gen and 10 nM of Dox were observed for 0, 18, and 24 hours (Fig. 4A). The inhibition of migration was quantified by closure width, which indicated the potency of the compound as an antimigratory agent.

The migration assay after 18 and 24 hours showed that 50- μ M Gen inhibited the migration of the cells as compared to the control and other treatments. The single treatment of Dox after 18 and 24 hours did not show any inhibitory effect and showed a relatively same percentage of closure with the untreated sample (Fig. 4B). Meanwhile, the combination of 50 μ M Gen and 10 nM Dox showed an inhibition effect after administering for 18 hours with higher inhibition percentage as compared to the single treatment of Dox (Fig. 4B). Hence, the inhibitory effect of Gen needs to be observed for the MMP-9 and MMP-2 activity and Rac-1 protein expression as an initiation process in cell metastasis.

The observations on MMP-9 and MMP-2 activity and Rac-1 expression were conducted to understand the ability of

the compound in inhibiting migration. Subsequently, the levels of metastasis-associated protein in the 4T1 cells after treatment with Gen and Dox were determined and quantified by the gelatin zymography assay for the gelatinolytic activity of MMPs and immunoblotting for Rac-1. Our results showed that single and combination treatments of Gen and Dox could suppress the MMP-9 activity and Rac-1 expression in 4T1 cells, with a higher suppression found at the combination of high concentration of Gen (50 μ M) (Fig. 5). This result indicated that Gen was able to

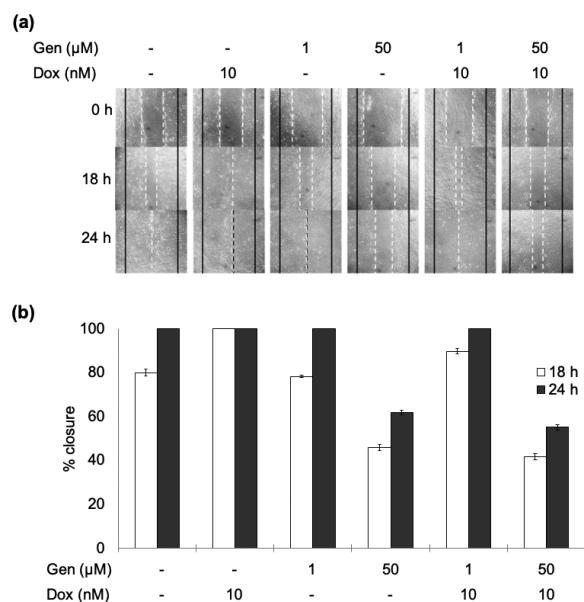


Figure 4. The effect of Gen and its combination with Dox on 4T1 cell migration. The cell migration was assessed by a scratch wound-healing assay, as described in the Methods section, under different treatments (Gen, Dox, or its combination) as indicated. (a) The scratch areas of each treatment at indicated time points were photographed under an inverted microscope at the 100 \times magnification and representative figures are depicted. (b) The wound areas of each treatment were analyzed using ImageJ in triplicate to quantify the percentage of closure and are summarized in the graph. The bar represents means \pm SE ($n = 3$).

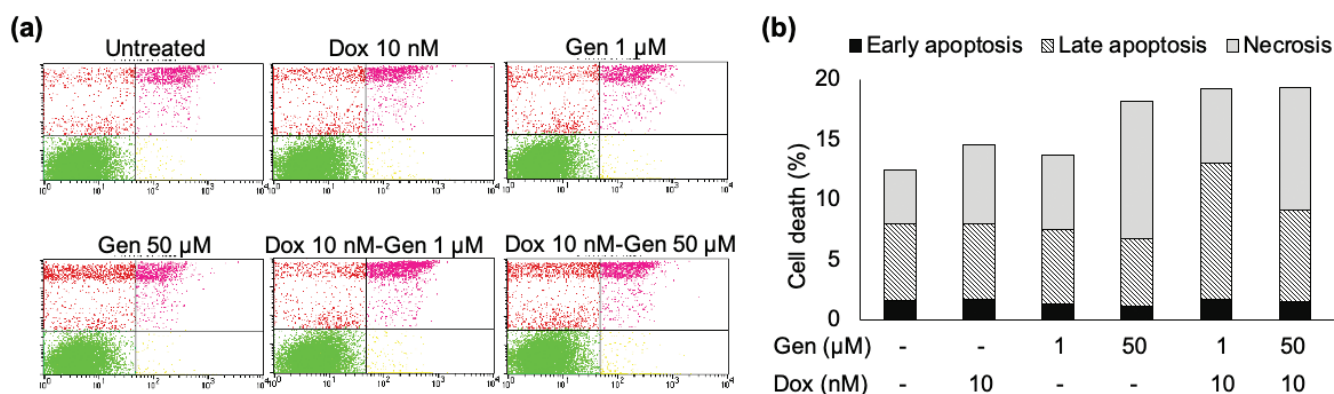


Figure 3. The effect of Gen and its combination with Dox on the apoptosis induction in 4T1 cells. The cells were treated with high or low concentrations of Gen and its combination with 10 nM Dox for 24 hours. Following the Annexin-V-PI staining, the cells were subjected to a flow cytometry. (a) The representative distribution profiles of living cells (green in bottom-left), early apoptotic (yellow in bottom-right), late apoptosis (pink in upper-right), and necrosis (red in upper-left) are shown in each treatment as indicated. (b) The graph represents the percentage of cells undergoing apoptosis and necrosis in each treatment.

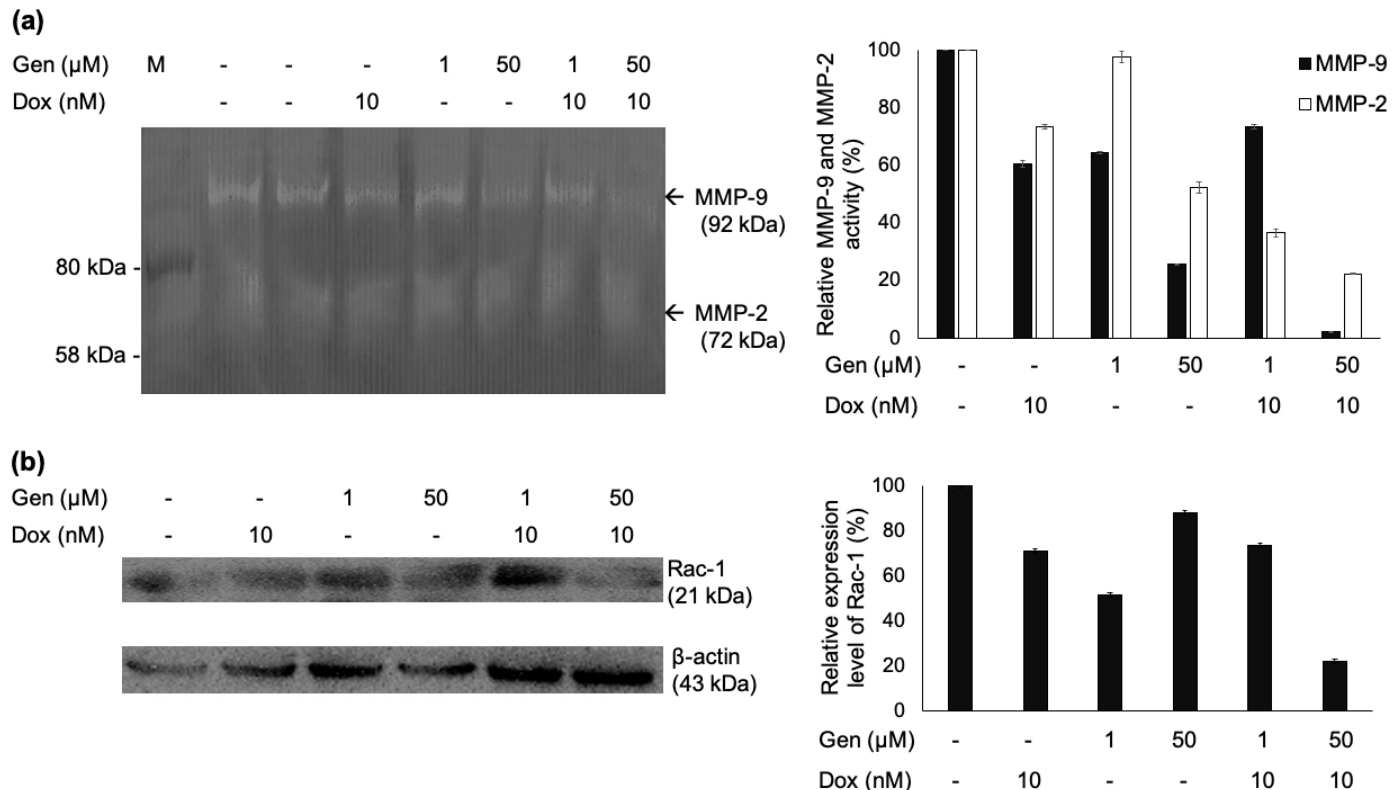


Figure 5. The effect of Gen and its combination with Dox on MMP-9 and MMP-2 activities and Rac-1 expression in 4T1 cells. The cells were incubated with Gen or its combination with Dox as indicated. After 24 hours, the cell medium was recovered for the gelatin zymography, while the cell lysates were also further analyzed by immunoblotting, as described in the Methods section, respectively. (a) The zymogram showed the activity of MMP-9 and MMP-2. The bands were quantified with ImageJ and their relative activities compared to the control are presented in the graph (right panel). MMP-9 and MMP-2 activities of the untreated control group were set to 100%. The bar represents means \pm SE ($n = 3$) from triplicate quantification. M = marker. (b) The immunoblot showed the expression of Rac-1. The band density was analyzed by densitometry using ImageJ in triplicate. The expression level of Rac-1 was normalized with β -actin, and the relative expression levels compared to the control are shown in the graph (right panel). The expression level of Rac-1 in untreated control group was set to 100%. The bar represents means \pm SE ($n = 3$).

inhibit the cell migration and eventually inhibit the metastasis in breast cancer cells, which shows a promising result for Gen to be developed as an antimigratory agent via decreasing the MMP-9 activity and Rac-1 protein expression.

Gen induces the autophagy activity of 4T1 cells

The modulation effects of Gen on the physiological process of the cells appear to be a unique phenomenon that may include the intracellular changes as observed at the cell size distribution. Afterward, we evaluated the effect on the autophagy evidence. Under flow cytometry analysis based on the LC-3 fluorescent detection, we found that Gen at the low and high concentrations positively induced autophagy activity of the cells (Fig. 6). The higher concentration of Gen showed that higher autophagy activity occurs in the cells. The increased autophagic activity might be induced by the intracellular damage that may appear due to the oxidative stress inside the cell.

Effect of Gen on cellular ROS level in the 4T1 cells

The cytotoxic activity of drugs that generate cell damage could be correlated with the increase in the ROS level. Here, we demonstrated that Gen alone or in combination with Dox induced cellular ROS level (Fig. 7). However, the increasing level of

intracellular ROS only occurred in higher concentrations of Gen or in combination with Dox at both low and high concentrations. These phenomena suggest that the increase in the ROS level under Gen contributes to the cytotoxic synergistic effect of the combination.

Discussion

The purpose of this study is to evaluate the contribution of Gen in enhancing the cytotoxic effect of Dox because Gen itself actually is not strong enough to counter the cancer cells and has a relatively much lower cytotoxic activity than anticancer agent Dox. We confirmed that Gen strongly enhances the cytotoxic effect of Dox, even at a very low concentration (1 μM) for a longer time exposure. It is interesting to underline this phenomenon that Gen may increase the cytotoxic-based mechanism of Dox, such as increase in the production of ROS (Fig. 7). It was already revealed that the mechanism of action of Dox is to intercalate into DNA and instantly affect transcription and replication and elevate ROS, resulting in the disruption of cell macromolecules including DNA (Minotti *et al.*, 2004). Moreover, ROS also activates IKK that activates NF- κ B transcription factor (Sethi *et al.*, 2015), which in turn could be inhibited by Gen.

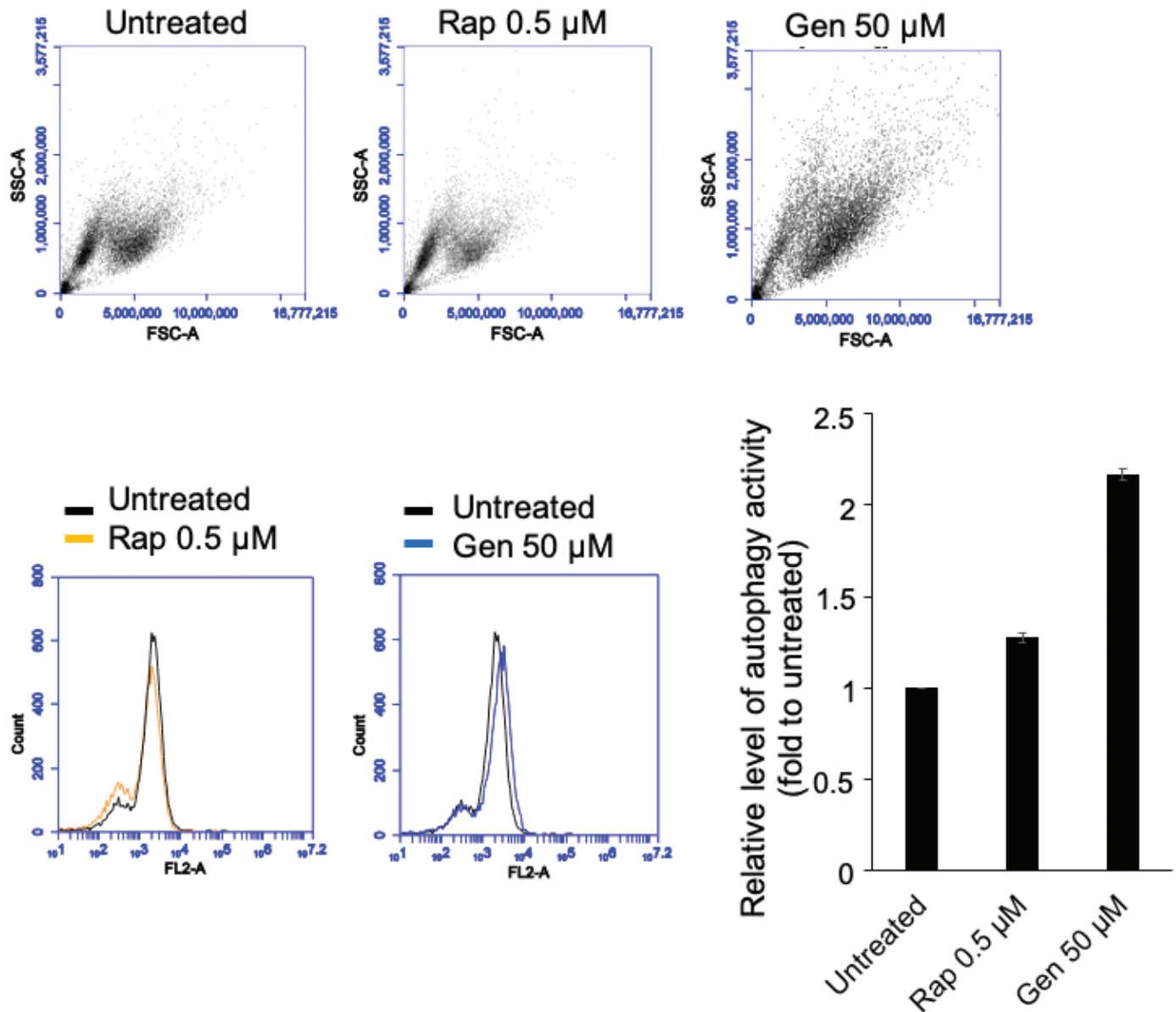


Figure 6. The effect of Gen on the autophagy activity of 4T1 cells. The cells were incubated with Gen or rapamycin as a positive control at the indicated concentration, stained with the green fluorescent, as described in the Methods section, and subjected to a flow cytometry. The representative flow cytogram is shown and the mean fluorescence of untreated control group was set to 1. The relative level of autophagy activity under different treatments is shown as means \pm SE ($n = 3$), as summarized in the graph (lower right panel).

To clarify the cytotoxicity mechanism of Gen combined with Dox on the 4T1 cells, we conducted apoptosis assay using flow cytometry and Annexin-V-PI staining. Compared to the untreated control, the single treatment of Dox did not induce apoptosis (Fig. 3). Likewise, the combination of Gen and Dox showed a similar phenomenon that was allegedly because of low concentration of Dox (10 nM) and did not induce apoptosis yet. The high concentration treatment of Dox has been proved to induce apoptosis via the p53-dependent pathway (Wang *et al.*, 2004); meanwhile, 4T1 cell has a very poor p53 expression (Yerlikaya *et al.*, 2012). Deficient p53 expression cells are vulnerable toward genetic damage (Sugimoto *et al.*, 2002). Thus, the cytotoxicity

mechanism possibly resulted from the cell cycle arrest pathway rather than the apoptosis/necrosis pathway.

Cell cycle modulation through G2/M arrest is induced by the high concentration of Gen (50 μM), despite the fact that 1- μM Gen also modulates the cell cycle in several cancer cell lines. The MCF-7/Adr cell is known to arrest the cell cycle at the G2/M phase (Xue *et al.*, 2014). Gen also increases the p38 MAPK activation on the RAW 264.7 cells, resulting in a cell cycle progression through G2/M arrest (Cui *et al.*, 2014). It is also understood that the G2/M arrest is caused by DNA damage induction on the HO-8910 ovary cancer cell line (Ouyang *et al.*, 2009). Putri *et al.* (2016) also found that the low concentration of Dox (below the IC_{50} value) induces

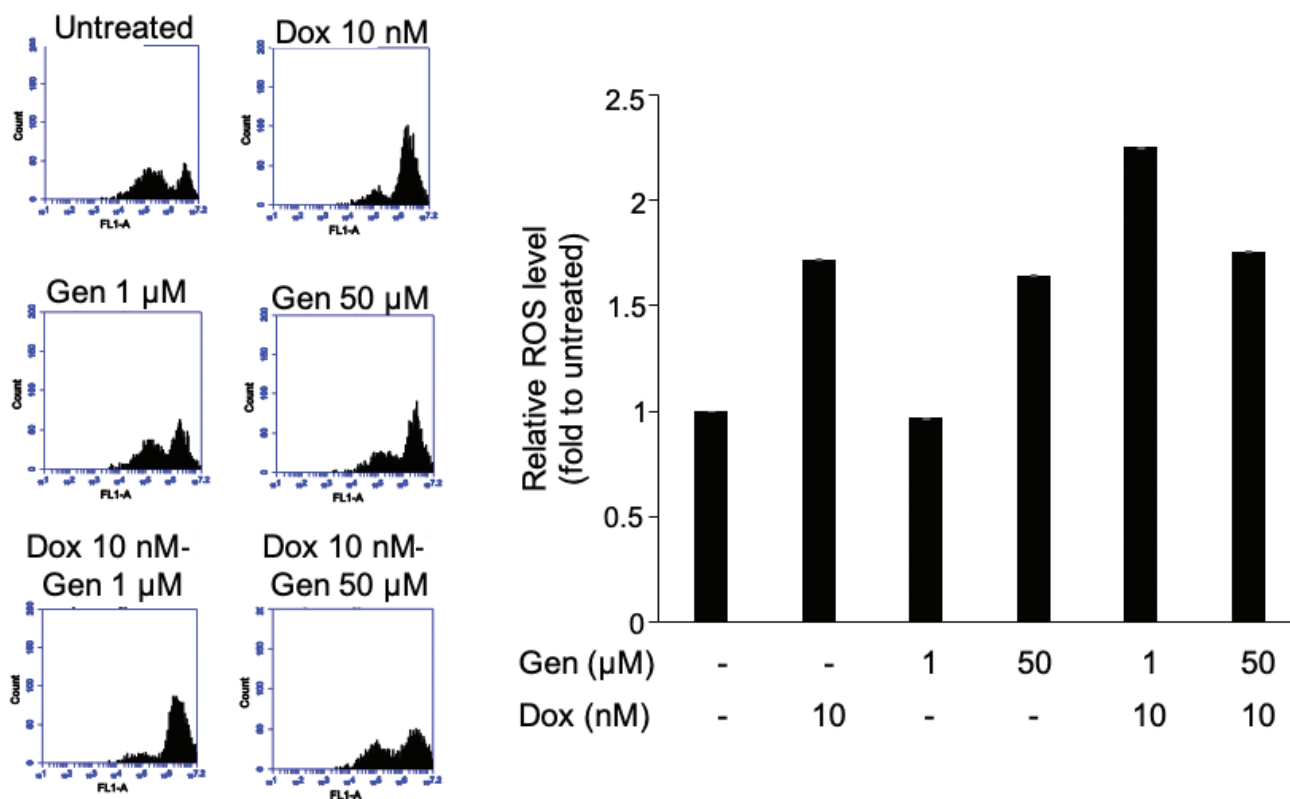


Figure 7. The effect of Gen and its combination with Dox on the cellular ROS level of 4T1 cells. The cells were incubated with Gen, Dox, or its combination at the indicated concentration, stained with DCFDA fluorescent, as described in the Methods section, and subjected to a flow cytometry. The representative flow cytogram is shown and the mean fluorescence of untreated control was set to 1. The relative level of cellular ROS under different treatments is shown as means \pm SE ($n = 3$), as summarized in the graph (right panel).

G2/M arrest on 4T1 cells. The cell cycle arrest on the G2/M phase is regulated by Chk1 and Chk2 along with the involvement of Cdc25C protein as a part of anaphase-promoting complex (APC), which in turn induces phosphorylation and degradation of cyclin B (de Gooijer *et al.*, 2017). Activity of topoisomerase II involved in the transcription process is also inhibited by Dox (Nitiss, 2009). This process ceases the DNA replication resulting in DNA damage. DNA damage is one aspect that triggered the activity of Chk1 through ATM signaling or Chk2 through ATR signaling. Both enzyme activities inhibit the phosphorylation process of Cdc25C, causing a cyclin B accumulation and Cdk1 inactivation. In this regard, we confirmed that Gen stabilizes cyclin B (Fig. 2B). This process resulted in a cell cycle arrest at the G2/M phase, eventually failing the cells to enter anaphase (Donzelli and Draetta, 2003). More interestingly, all of the physiological phenomena may correlate with the autophagy activity caused by Gen and the induction of ROS level. Increasing intracellular ROS levels in 4T1 cells may induce apoptosis, as presented by PGV-1, a curcumin analog (Larasati *et al.*, 2018). These phenomena should be clarified as a further research topic in future studies.

Gen was also acknowledged to downregulate the activity of proteins involved in metastasis (Tsai *et al.*, 2017). Cancer cell motility was affected by protein activity through the Rac pathway that induced cell migration and Rho/EOCK signaling that in turn

induced cell contraction (Donzelli and Draetta, 2003). The Rac-1 protein induces PAK activity that activates myosin light chain kinase (MLCK), which induces phosphorylation of myosin light chain resulting in active and dynamic actins. Based on our observation, the single treatment of Gen inhibits the cell migration in dose- and time-dependent manners. The capability of Gen in inhibiting migration is related to the suppression of Rac-1 expression (Fig. 5). Even though the high concentration of Gen (50 μM) also inhibits the proliferation of cells that may affect the migratory process, we should realize that the migration of cells possesses different mechanisms as compared to the proliferation. Thus, the appearance of a wider closure of the wound suggested the occurrence of the inhibition of cells migration. Based on this result, Gen in combination with Dox shows a more effective inhibitory effect on the 4T1 cell migration.

The increasing evidence of autophagy evidence caused by Gen together with the elevating ROS level gives more proof that Gen in a high concentration or in combination with Dox suppresses the migratory effect of Dox despite enhancing cytotoxicity. The results of this study contrast the study by Yang *et al.* (2015) on the 4T1-induced mouse, which showed that the administration of soy isoflavone-containing Gen combined with daidzein at a concentration of 750 mg/kg BW induces the migration of cancer cells from bones to lungs. A study from our group also reported that Gen also displays a biphasic effect on CHO-K1 epithelial

cells treated with estrogen: at low concentrations, Gen induces senescence and apoptosis, but at high concentrations, Gen regulates the cell cycle (Jenie *et al.*, 2019). These phenomena prove that the concentration is an important key variable of Gen in exhibiting different effects on the progression of cancer metastasis. These data bring us to a better understanding of Gen by featuring its role in the cytotoxic activity to the antimigration effect and the potency to lessen the chemoresistance phenomenon of Dox.

CONCLUSION

Our study confirmed that Gen enhances the growth inhibitory effects of cytostatic agent Dox. The combination of Gen and Dox results in increases in the cell cycle arrest and inhibitions of cell migration, accompanied by stimulations of cellular ROS production and autophagy activity. Thus, Gen and natural products containing Gen have a potency to be developed as a combination therapy (cochemotherapeutic) agent that could overcome the cancer metastasis.

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AUTHORS' CONTRIBUTIONS

RIJ and EM contributed to the conception of the work. MI, RYU, NDA, and GNPI contributed to the acquisition of the work. MI, RIJ, RYU, NDA, GNPL, and EM contributed to the analysis and interpretation of data. MI, RYU, NDA, and GNPI contributed to drafting the work. MK and EM contributed to revising the work critically. MI contributed to the revising of the manuscript. EM is responsible for giving the final approval of the manuscript.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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