Future Journal of Pharmaceutical Sciences

RESEARCH Open Access

Hesperetin alleviates doxorubicin-induced migration in 4T1 breast cancer cells

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Abstract

Background: Hesperetin (Hst), a citrus flavanone, is widely distributed among citrus fruits, including lemons. Hst has been shown to possess bioactivity as an antioxidant, anti-inflammatory, anti-allergic, hypolipidemic, vasoprotector, and anticancer agent. This study aimed to identify potential combinations of Hst and the chemotherapeutic agent doxorubicin (Dox) as co-chemotherapy agents against 4T1 murine metastatic breast cancer cells.

Results: MTT assay results showed that Hst exhibited cytotoxic effect in 4T1 cells, and its combination with Dox showed a synergistic effect based on the CI value. The combination of Hst and Dox increased G2/M phase cell cycle arrest and apoptosis induction. The combination of Hst and Dox inhibited migration and decreased MMP-9 expression in 4T1 cells.

Conclusion: In conclusion, the results of this study show that Hst has potential as a Dox co-chemotherapy against 4T1 cells by inducing G2/M phase cell cycle arrest and apoptosis. More importantly, Hst reduces Dox-induced migration and decreases MMP-9 expression.

Keywords: Hesperetin, 4T1, Cell cycle, Apoptosis, Metastasis, MMP-9

Background

Breast cancer is the second most common cancer in the world after lung cancer, with an incidence rate of 11.9%. In 2012, about 30% of the 1.7 million patients diagnosed in 140 countries died from breast cancer [1]. The main cause of death in patients with breast cancer is cancer metastasis, which is a series of complex events that characterize malignancy in cancer cells [2].

Doxorubicin (Dox) is the most common chemotherapy agent used for breast cancer therapy [3, 4]. Despite its excellent anti-tumor activity, its clinical utility is limited by acute and chronic toxicity [5, 6]. Dox stimulates TGF-β signaling and thus increases metastasis in breast cancer cells [3]. Moreover, the sensitivity of breast cancer to Dox is reduced due to resistance mechanisms [7]. Therefore, combination treatment with new effective nontoxic drugs and doses of chemotherapy agents should be developed.

Hesperetin (Hst) is a citrus flavanone mostly contained in citrus fruits and lemons [8]. Hst has potential anticancer activity against a specific target for either single-use or as a combination agent [9–13]. Sivagami et al. demonstrated that Hst might cause apoptosis in HT-29 colon cancer cells [9]. Another study showed that Hst inhibits cell proliferation in human gastrointestinal carcinoma [11] and triggers apoptosis in SiHa cervical cancer cells [14]. The combination of Hst and Dox may increase apoptosis and decrease Pgp expression in Dox-resistant breast cancer cells [13]. Nevertheless, the effect of Hst in metastatic breast cancer is poorly understood.

This study aims to identify the potential of Hst and Dox as a combinatorial chemotherapeutic agent in

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metastatic breast cancer cells. In this study, the bioactivity of Hst was assessed in combination with Dox on 4T1 murine breast cancer cells, in terms of cytotoxicity, cell cycle and apoptotic modulation, anti-migratory capacity, and MMP-9 expression. The results of this study highlighted the potential of Hst in inhibition of migration, that is induced by Dox, in metastatic breast cancer cells.

Methods

Cell culture

The 4T1 murine breast cancer cell line was kindly provided by Prof. Masashi Kawaichi, M.D., Ph.D. (Nara Institute of Science and Technology, NAIST, Japan). Cells were cultured in high-glucose Dulbecco's modified Eagle medium (Gibco, New York, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, USA) and 1% v/v penicillin-streptomycin (Gibco, New York, USA). Cells were incubated at 37 °C with 5% CO_2.

MTT assay for cytotoxicity

4T1 cells were seeded in a 96-well microplate at a density of 2×10^3 cells in 100 μL/well and incubated for 24 h at 37 °C with 5% CO_2. Cells were treated with Hst (Sigma-Aldrich, St. Louis, USA), alone and in combination with Dox (Sigma-Aldrich, St. Louis, USA), and incubated for 24 h at 37 °C with 5% CO_2. Then, 0.5 mg/mL MTT reagent (Biovision, CA, USA) was added to each well, and the cells were incubated for 4 h. The reaction was stopped by adding an SDS stop solution containing 0.01 N HCl and incubated overnight. Absorbance was then measured by using a microplate reader (Bio-Rad, California, USA) at 595 nm [15]. Cell viability was calculated from the absorbance data and will be used to calculate the IC50 value. The combination index was calculated and interpreted (Table 1) using Chuo-Talalay method [16, 17] as follows:

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

$(D)1$, $(D)2$: concentration of drug 1 or drug 2.

Dx: concentration of a single drug that produces a similar effect in combinatorial treatment.

Cell cycle analysis

Cell cycle analysis was performed by using propidium iodide (PI) staining flow cytometry-based on a previous study [18]. Briefly, 4T1 cells (5×10^4 cells/well in a 6-well plate) were seeded and treated with various concentrations for 24 h. The cells were harvested on the next day, washed with PBS, and fixed in 70% cold ethanol. The cells were stained with a staining solution containing 1 mg/mL PI, protease inhibitor, 10 mg/mL RNase (Sigma-Aldrich, St. Louis, USA), and 0.1% (v/v) Triton X-100 (Merck); incubated for 5 min in a dark room; and analyzed with a BD FACS Calibur (BD Bioscience, USA).

Apoptosis assay

The effect of Hst and its combination with Dox on 4T1 cells was measured as in a previous study [18]. 4T1 cells were cultured at a density of 1×10^5 cells/mL, distributed into 6-well plates, and incubated for 24 h at 37 °C with 5% CO_2. Cells were treated at various concentrations of Hst, alone and in combination with Dox, and incubated for 24 h at 37 °C with 5% CO_2. Then, the cells were harvested trypsinization with trypsin-EDTA (Gibco, New York, USA) 0.25%. The cells were stained by using the Annexin-V-FLUOS Staining Kit (Roche), consisting of 100 mL of binding buffer and 2 mL of Annexin-V and PI, and incubated for 10 min in the darkroom. Then, the cells were analyzed by using a FACS Calibur flow cytometer (BD Bioscience).

Scratch wound-healing assay

Scratch wound-healing assay was performed as a previous study [19]. Briefly, the 4T1 cells (density 7×10^4 cells/well) were seeded in a 24-well plate and incubated for 24 h. The cells were then starved by replacing the medium with a culture medium supplemented with 0.5% FBS for 24 h. After starvation, the cells were scratched using a sterile pipet tip and treated with 1 mL of a sample dilution series for 24 h. The cells were captured at 0, 18, 24, and 42 h after treatment by a digital camera (Nikon, Japan). Starvation was done to slow down cell growth; therefore, we can be sure that wound closure was due only to cell migration. The results were analyzed by using the ImageJ software, presented as percentage closure.

Gelatin zymography

The 4T1 cells (3×10^5 cells) were seeded in a 6-well plate and treated with a series of concentrations. Each medium was collected as a protein lysate. The samples were then subjected to electrophoresis in 10% SDS-PAGE supplemented with 0.1% gelatin to determine the activity of MMP-9 in the culture medium. After electrophoresis, the gels were washed and incubated with distilled water containing 2% Triton X-100 (Merck) for 30 min at room temperature. The solution was removed from the gels, and then 100 mL of incubation buffer (40 mM Tris-HCl, pH 8, 10 mM $CaCl_2$, 0.02% NaN_3) was added and incubated for 45 min. After that, the incubation buffer was replaced, and the gels incubated for 24 h at 37 °C. After incubation, the gels were stained with Coomassie Brilliant Blue R-250 solution and destained with a destaining solution (20% methanol, 10% acetic

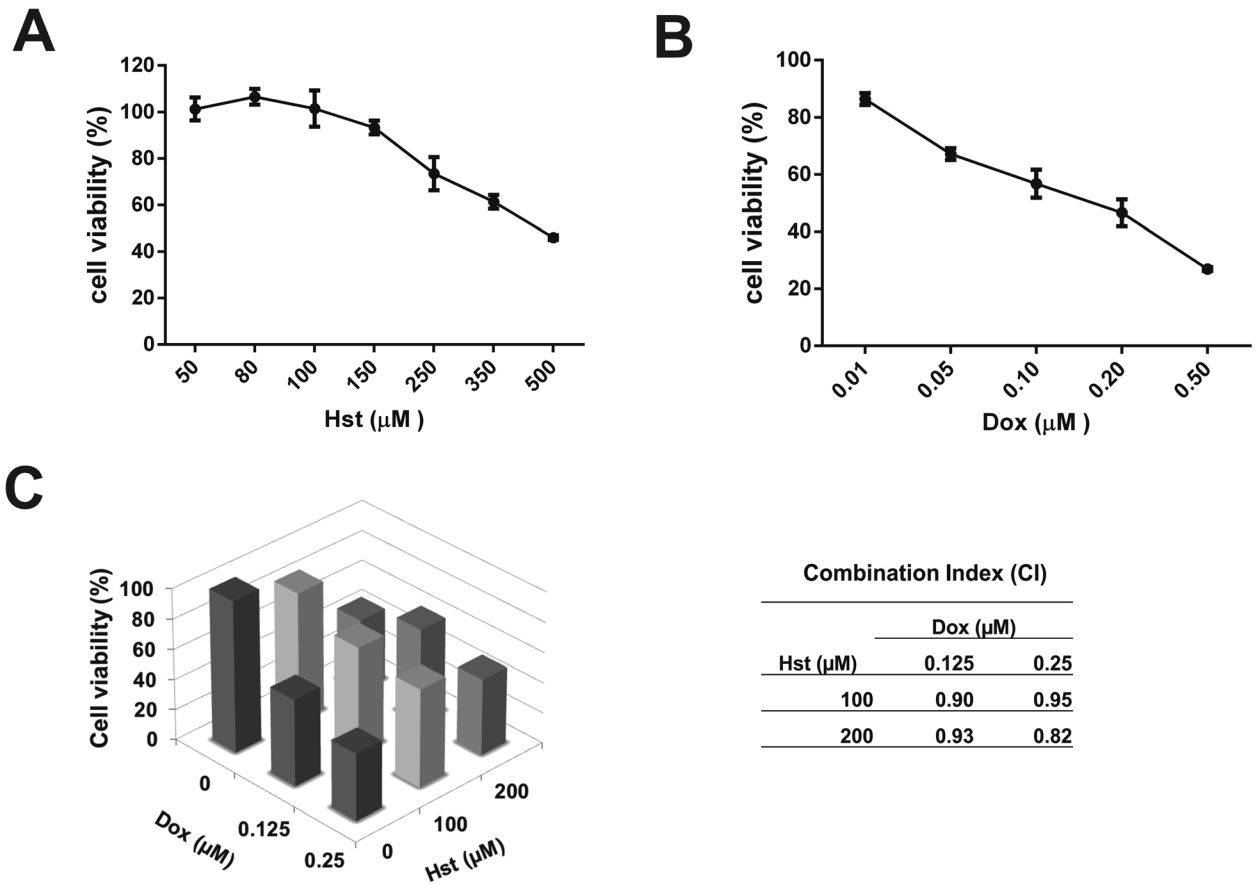

Fig. 1 **a** Single cytotoxicity of Hst and Dox against 4T1 cells. **b** Combination cytotoxicity of Hst and Dox on 4T1 cells, and its combination index (CI) value. The 4T1 cells were grown in 96-well plates with a density of 2×10^3 cells/well. The cells were incubated for 24 h and treated with a series of Hst, Dox, and their combination. Cell viability was determined by using the MTT assay, as described in the "Methods" section. Results are presented as mean ± SD ($n = 3$)

acid, and 70% water). The results were documented and analyzed with the ImageJ software.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) of triplicates. Two-way ANOVA with post hoc Tukey's multiple comparison test was used to analyze the effect of Hst and combined with Dox on the cell cycle, apoptosis, and migration, while one-way ANOVA with post hoc Tukey's multiple comparison test was performed to determine the effect of Hst and combined with Dox on MMP-9 expression. All statistical analyses were conducted with Prism 5.0 Software GraphPad at a significance level of 0.05.

Table 1 Interpretation of the Combination index [16, 17]

CI value	Interpretation
< 0.1	Very strong synergism
0.1–0.3	Strong synergism
0.3–0.7	Synergism
0.7–0.9	Moderate to slight synergism
0.9–1.1	Nearly additive
1.1–1.45	Slight to moderate antagonism
1.45–3.3	Antagonism
> 3.3	Strong to very strong antagonism

Results

Cytotoxicity of Hst and Dox in 4T1 cells

The cytotoxicity of Hst and Dox was determined with the MTT assay. We observed a decrease in the percent cell viability as the concentrations of Hst and Dox were increased (Fig. 1a). The IC50 values for Hst and Dox were 400 μM and 0.5 μM, respectively. These IC50 values were used as a reference in dosing for the combined cytotoxic test. The combination of Hst and Dox showed an additive effect (Fig. 1b) as indicated by the combination index (CI) value based on Chou and Talalay method, which was between 0.9 and 1.1. In the present study, for the following experiment, we treated cells with a low concentration of Dox, which is 10 nM,

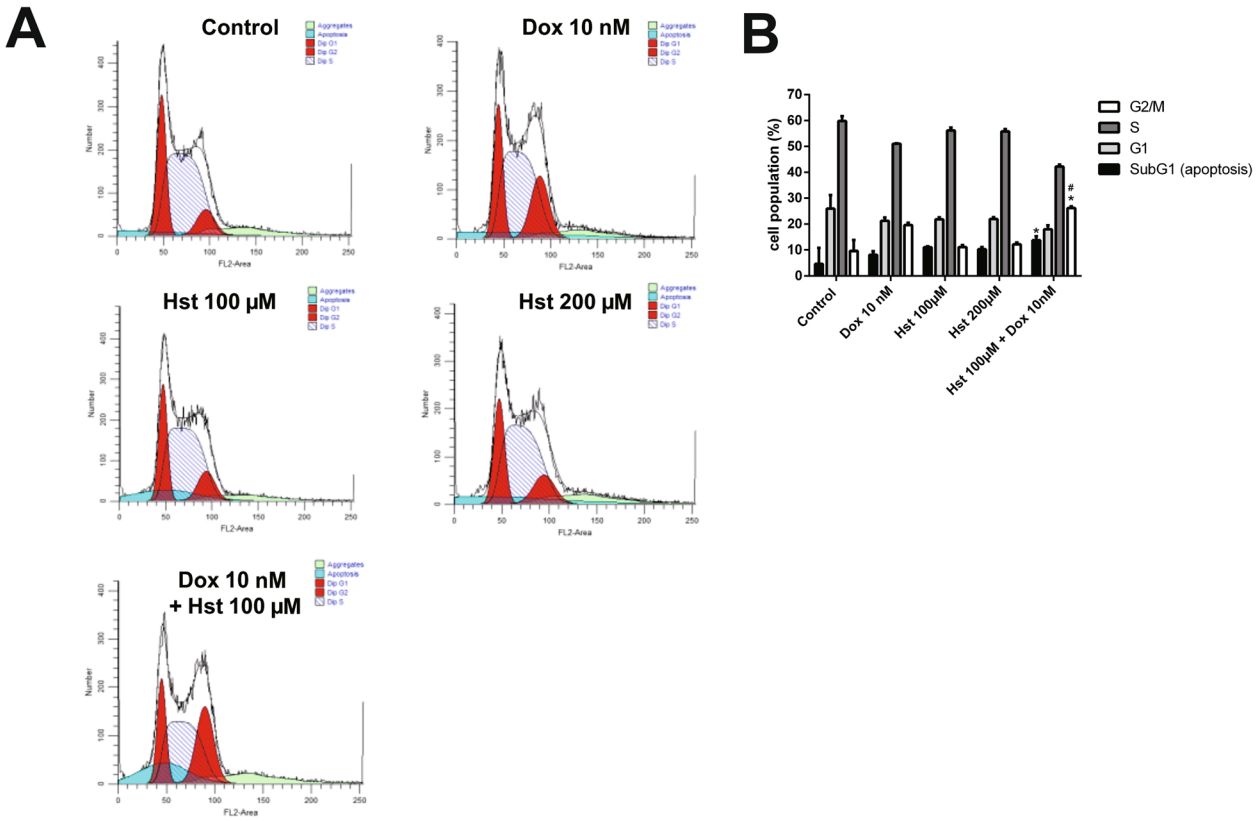

Fig. 2 The 4T1 cell cycle profile upon single treatment with Hst and Dox, as well as their combination, with PI-staining-based flow cytometry. A total of 1.5×10^5 cells were grown on 6-well plates and incubated for 24 h. A concentration series of Hst, Dox, and their combination were administered for the following 24 h. Cells were then collected, fixed with cold ethanol, and stained with PI staining solution before measurement with a flow cytometer. The percent cell distribution was analyzed using the ModFit application. **a** Histogram of cell cycle analysis. **b** Bar chart of cell cycle distribution. PI = propidium iodide. Asterisk and number sign indicate significance to Dox and Hst, respectively ($p < 0.05$)

because we focused on the anti-migratory effect of Dox and Hst rather than cell death induced by Dox.

Effect of Hst, Dox, and their combination on cell cycle modulation in 4T1 cells

The cell cycle assay with flow cytometry was performed to confirm the ability of Hst and Dox to modulate the cell cycle. Hst, Dox, and their combination modulated the cell cycle and caused a cell cycle arrest in certain phases (Fig. 2a). In each treatment group, the cells were mainly distributed in the S phase (Fig. 2b). The percent cell distribution in the G1 phase in the untreated group was higher than the percent distribution in the G2/M phase. Dox treatment increased SubG1 and G2/M arrest compared with the untreated group. The cell cycle profile of the Hst single-treatment group was similar to that of the untreated group, with slightly higher accumulation in the subG1 phase. The combined treatment of Hst and Dox significantly increased SubG1 compared with Dox, and G2/M arrest compared with their single treatment. In summary, combined treatment with Hst and Dox induced SubG1 and G2/M arrest.

Hst and Dox apoptosis test in 4T1 cells

Based on the results of cytotoxicity of Hst, Dox, and their combination, it was necessary to confirm the related cell death pathway mechanism. A single treatment with Dox slightly increased the cell death population, i.e., early and late apoptosis, compared with the untreated group (Fig. 3a, b). Hst single treatment increased apoptosis induction compared with single Dox treatment. Combination treatment with Hst and Dox significantly increased apoptosis compared with their single treatment.

Antimigratory activity of combined Hst and Dox on 4T1 cells

In the present study, the anti-migratory capacity of combined Hst and Dox was measured with a wound-healing assay over a time course, i.e., 18 h, 24 h, and 42 h. The untreated and Dox-treated groups had a large area closure percentage, whereas the single-Hst-treated group and the combined Hst and Dox group showed a smaller closure percentage than the untreated group (Fig. 4a). The percentage closure with Hst single treatments of

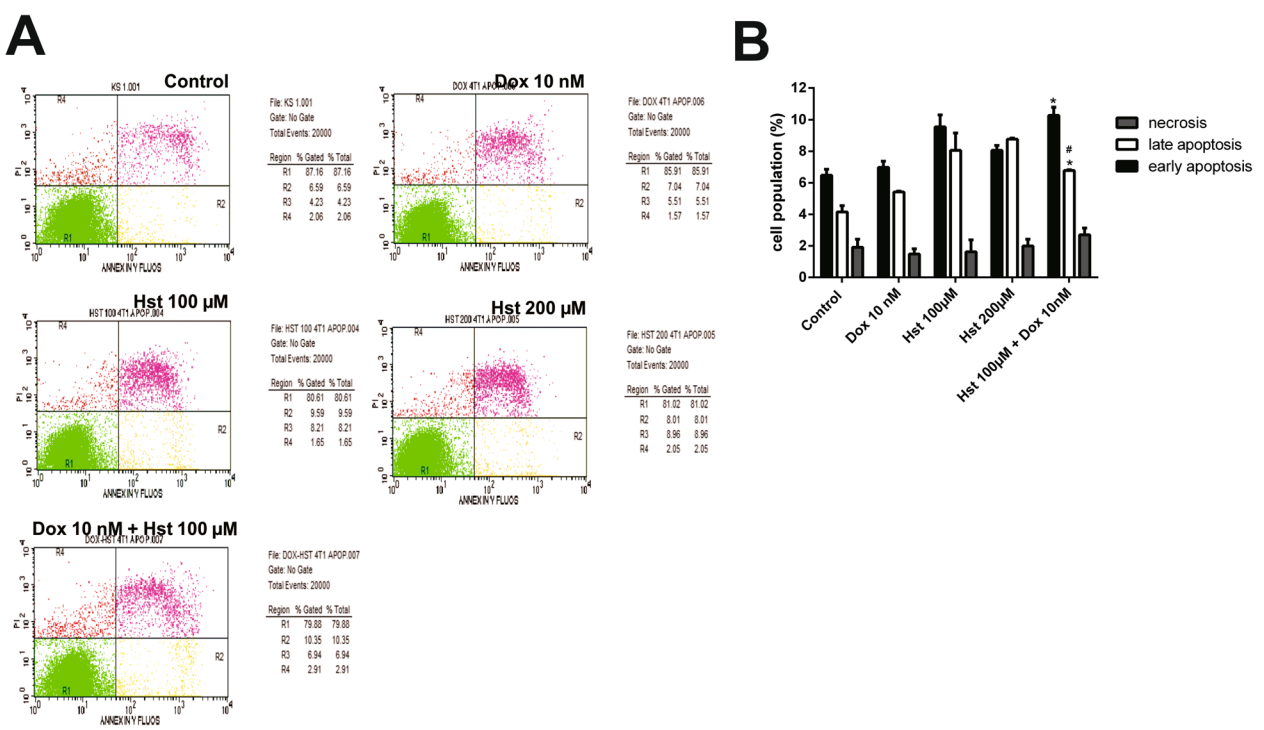

Fig. 3 Induction of apoptosis by Hst, Dox, and their combination in 4 T1 cells. A total of 1.5×10^5 cells were grown on 6-well plates and incubated for 24 h. A concentration series of Hst, Dox, and their combination was administered for the following 24 h. Cells were then collected and stained with an Annexin-V-FLUOS Staining Kit before measurement with a flow cytometer. **a** Histogram of cell death profile, i.e., living cells, early apoptotic cells, late apoptosis, and necrosis. **b** Bar chart of cell death distribution with early and late apoptosis, as well as necrosis. Asterisk and number sign indicate significance to Dox and Hst, respectively ($p < 0.05$)

50 μM and 100 μM at 18 h were 33% and 22%, respectively; this result was not significantly different from the untreated and Dox-treated groups (Fig. 4b). However, at 24 h, there was a significant difference between the single Hst 50 μM and 100 μM groups compared with the untreated group, with closure percentages of 44% and 28%, respectively. Combined treatment with Hst and Dox significantly reduced Dox-induced migration at 18, 24, and 42 h.

MMP-9 is one of the regulatory proteins that control migration and invasion. We performed gelatin zymography in order to determine the effects of Hst, Dox, and their combination on MMP-9 activity (Fig. 5a). Dox slightly increased the activity of MMP-9 compared with the untreated group (Fig. 5b). The MMP-9 activity of the combination of Hst and Dox was significantly decreased compared to Dox. Collectively, Hst could prevent Dox-induced MMP-9 expression, and thus could prevent migration and metastasis in 4T1 cells.

Discussion

The present study aimed to identify the potential combination of Hst and Dox as co-chemotherapy agents against 4T1 murine metastatic breast cancer cells. We tested the hesperetin cytotoxicity in several types of breast cancer cells, both human and murine. Among other cells, 4T1 is the cells with the highest migratory capacity (data not shown). The IC50 value of Hst on 4T1 cells is 400 μM. A compound is considered to have weak cytotoxicity when the IC50 > 20 μM [20]. To determine whether the combination of Hst and Dox has a synergistic, additive, or antagonistic effect, the CI was calculated based on Chou and Talalay method, which was between 0.9 and 1.1. The CI of Hst and Dox at an Hst concentration of 200 μM and Dox at 0 and 250 μM was 0.83 (Fig. 1b), indicating that the combination of Hst and Dox exhibited moderate to slight synergism. This phenomenon was probably caused by antiproliferative activity rather than apoptosis induction by Hst on 4T1 cells. A recent review article discussed that the bioactivity of Hst against cancer cells is predominantly by cell cycle modulation and apoptosis [21].

The results of the present study showed that the combination of Hst and Dox increased the percent cell distribution in the SubG1 and G2/M phase (Fig. 2b). A single treatment with Hst showed a similar cell cycle profile to the untreated group, which contradicts a previous study, which showed that Hst induces the G2/M phase in AGS human gastric cancer cells [22] and SiHa cervical cancer cells [14]. The results of the present

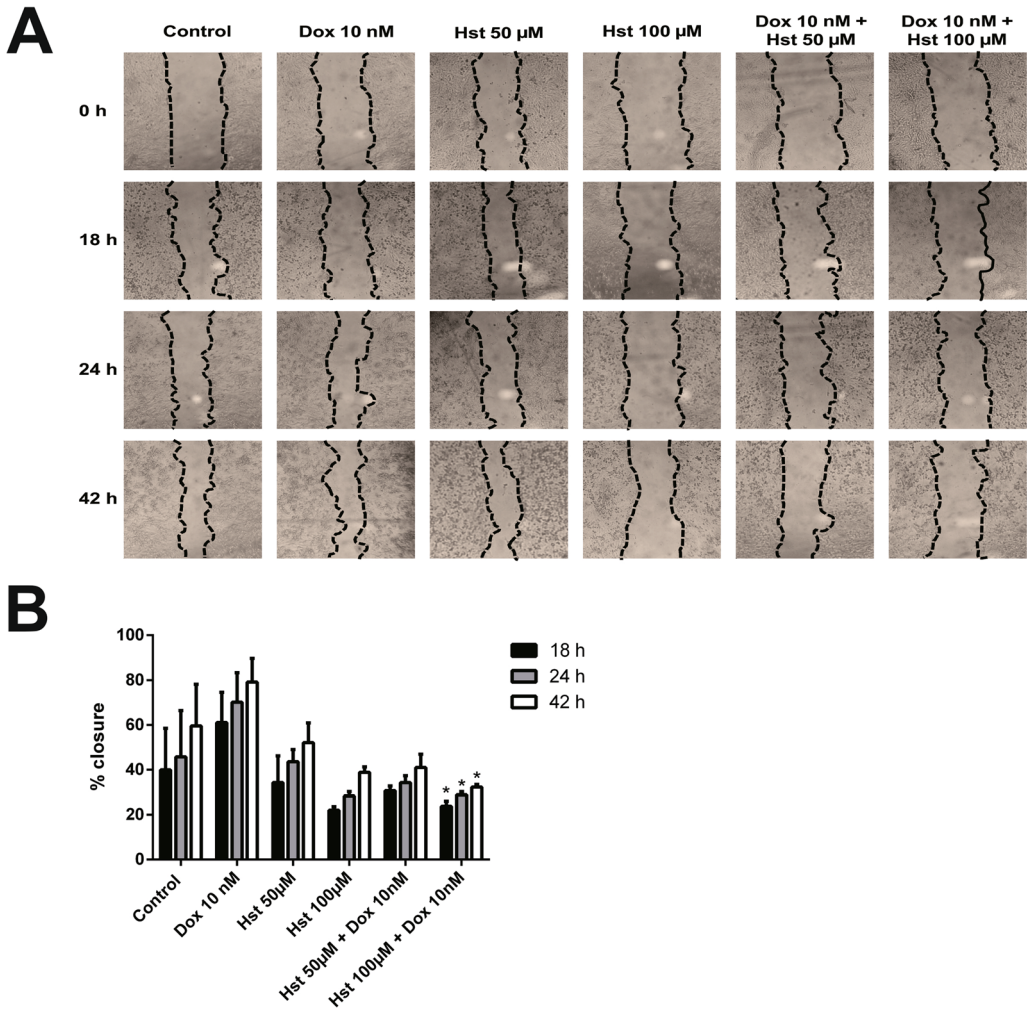

Fig. 4 Hst inhibits Dox-induced migration on 4T1 breast cancer cells. **a** Cell morphology after the scratch; observations were made at 0, 18, 24, and 42 h. **b** Bar chart of wound closure percent; observations were made at 18, 24, and 42 h. Results presented as mean ± SD ($n = 3$). Asterisk and number sign indicate significance to Dox and Hst, respectively ($p < 0.05$)

study indicate that modulation of the cell cycle by Dox is predominant when combined with Hst. A previous study revealed that low concentrations of Dox (below the IC50 value) could induce G2/M arrest in 4T1 cells [19]. G2/M arrest may occur as a result of the deregulation of certain proteins, such as Chk1 and Chk2, involving the Cdc25C protein [23]. Dox can inhibit the activities of Topoisomerase II, which plays a role in the transcription process, thus resulting in DNA damage and apoptosis [24]. A combination of Hst and Dox significantly increased SubG1 (apoptosis) and G2/M arrest. A previous study showed that G2/M arrest could also induce apoptosis [25]. We speculated that combined Hst and Dox increased G2/M arrest and G2/M arrest-mediated apoptosis; nevertheless, the molecular mechanism requires further clarification.

In the present study, the combination of Hst and Dox significantly increased apoptosis induction compared with Dox alone. However, the number of apoptotic cells with Hst alone and in combination with Dox was not significantly different (Fig. 3b). A previous study demonstrated that Hst induces apoptosis via a p53-independent pathway in H522 human lung cancer cells [26]. Accordingly, the mechanism of apoptosis induction by the combination of Hst and Dox needs to be explored in the next study.

Results of the present study showed that single treatment inhibited cell migration, while Dox in low concentrations stimulated cell migration. The combination of Hst and Dox significantly reduced the migratory capacity of Dox. A metastatic form of cancer was produced from cancer progenitor cells through epithelial to mesenchymal transition (EMT) [27]. EMT is a phenomenon whereby epithelial cells decide to become mesenchymal-like cells [28] and is a critical step in the development of cancer metastasis [29]. Dox induces EMT in MCF-7

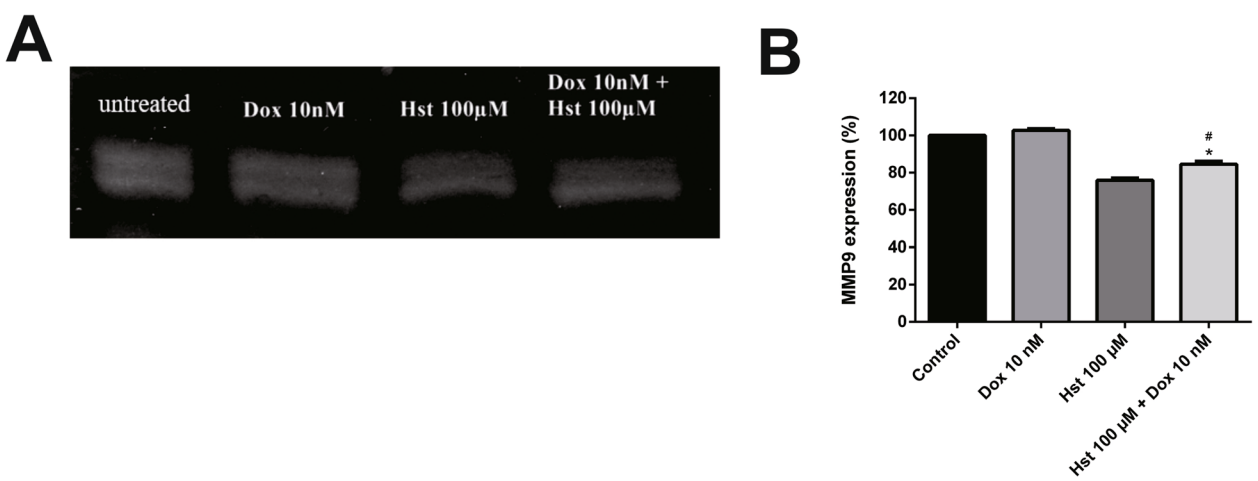

Fig. 5 Hst inhibits Dox-induced MMP-9 activity on 4T1 breast cancer cells. **a** MMP-9 activity by gelatin zymography. **b** Quantification of MMP-9 activity analyzed with ImageJ. Results presented as mean ± SD ($n = 3$). Asterisk and number sign indicate significance to Dox and Hst, respectively ($p < 0.05$).

breast cancer cells [30]. Moreover, a low concentration of Dox (10 nM) can induce lamellipodia extension in 4T1 cells [31]. Extension of lamellipodia causes cells to migrate [32]. The scratch wound-healing assay is one test for measuring the level of cell migration upon treatment with certain compounds or growth perturbation [33].

Activation of TGF-β signaling induces EMT and migration of breast epithelial cells [34]. Blocking of TGF-β signaling leads to inhibition of invasion in breast cancer cells [35]. Moreover, NFκB transactivation is involved in migration and invasion in human hepatocellular carcinoma [36], non-small cell lung carcinoma [37], and breast cancer [38]. Hst inhibits transforming growth factor-β (TGF-β)-induced cancer cell migration and invasion [10]. Hst inhibits the NFκB signaling pathway in mice post-myocardial infarction [39], as well as in cardiac inflammation and cardiac fibrosis in streptozotocin-induced diabetic rats [40]. Accordingly, the mechanism by which Hst inhibits migration in 4T1 cells is probably due to the inhibition of TGF-β and NFκB signaling; however, this hypothesis requires future clarification.

One of the critical steps in the migration of cancer cells involves matrix metalloproteinases (MMPs), including MMP-9 and MMP-2, which destroy the extracellular matrix [41]. Dox can cause increased activity of MMP-9 through activation of TGF-β [3]. Results of the present study showed that Dox stimulates the activity of MMP-9 compared with the untreated group, while the combination of Dox and Hst could significantly reduce Dox-induced MMP-9 activity.

Conclusions

In summary, the present study highlights the potential of Hst as a combinatorial agent with Dox in metastatic breast cancer cells. The combination of Hst and Dox induced G2/M phase cell cycle arrest and apoptosis. More importantly, combined Hst and Dox inhibit Dox-induced migration and MMP-9 expression in 4T1 murine metastatic breast cancer cells. Further study on the molecular mechanism responsible for this phenomenon is warranted.

Abbreviations

Hst: Hesperetin; Dox: Doxorubicin; CI: Combination index; MMP: Matrix metalloproteinase; EMT: Epithelial to mesenchymal transition

Acknowledgements

The authors thank Ms. Sonia Meta Angraini for her writing assistance.

Authors' contributions

EY—acquisition, analysis and interpretation of data, drafting and revising the article and final approval of the version to be published
HAM and GPNI—acquisition, analysis, and interpretation of data
EM—conception and design of the study, analysis of data, drafting the article
AH—conception and design of the study, drafting and revising the article, and final approval of the version to be published
All authors have read and approved the final manuscript.

Funding

This work was financially supported by Hibah penelitian Penunjang Tesis from Faculty of Pharmacy UGM 2017 (granted to Prof. Edy Meiyanto) and Penelitian Unggulan Perguruan Tinggi (PUPT) 2017 contract no. 2398/UN1.P.III/DIT-LIT/LT/2017, from Ministry of Research, Technology and Higher Education, Republic of Indonesia (granted to Dr. Adam Hermawan) to purchase the chemicals for the study.

Availability of data and materials

All data and material could be available from the corresponding author upon request.

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests
The authors declare no competing of interest.

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Received: 3 December 2019 Accepted: 14 May 2020
Published online: 22 June 2020

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