Study on the method to avoid infusion-site adverse events following chemotherapeutic treatment with epirubicin and fosaprepitant using immortalized human umbilical vein endothelial cells

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Abstract. The combination of intravenous Proemend® containing fosaprepitant meglumine, a prodrug for fosaprepitant (FAP), and Tween 80 and chemotherapy with anthracyclines, such as epirubicin (EPI), can cause infusion-site adverse events in clinical practice. In immortalized human umbilical vein endothelial (HUEhT-1) cells, the cytotoxic effects of FAP, EPI, diluted Proemend with culture medium and Tween 80 alone, and a combination of FAP and EPI, were evaluated using the WST-1 cell viability assay. FAP, EPI and diluted Proemend exhibited cytotoxicity in a concentration-dependent manner and marked synergic cytotoxicity was observed between FAP and EPI. The washing of the cell surface following incubation with diluted Proemend containing FAP and Tween-80 eliminated the synergic cytotoxicity of EPI applied thereafter. These results indicated that washing of the infusion-site vascular tissue following intravenous Proemend administration via intravenous tube flushing with an efficient amount of saline may reduce the infusion-site adverse events, which are caused by the combined use of FAP and EPI.

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Abbreviations: AP, aprepitant; EPI, epirubicin; FAP, fosaprepitant

Key words: chemotherapy, FAP, EPI, synergic cytotoxicity, HUEhT-1 cells

Introduction

In chemotherapy for breast cancer patients, FEC or EC regimen is widely used, and these regimens contain epirubicin (EPI), an anthracycline-based antineoplastic drug, as follows: FEC regimen consists of fluorouracil, EPI and cyclophosphamide, and EC regimen consists of EPI and cyclophosphamide. Oral aprepitant (AP), which is a neurokinin 1 (NK1) antagonist, is ingested once before and twice after EPI treatment, once/day for 3 days, mostly for inpatients, and intravenous Proemend® containing fosaprepitant (FAP) meglumine, a phosphoryl prodrug for AP, and Tween 80, a non-ionic surfactant, is administered intravenously through an intravenous (IV) tube once by the constant-rate infusion of more than 30 min just before the EPI treatment mostly for outpatients (1). The combined use of FAP and EPI, however, can cause infusion-site adverse events such as primarily edema/swelling, erythema or dermatitis, in addition to individual hypersensitivity systemic reactions (2-5). The risk for the incidence of infusion-site reactions with intravenous FAP before the administration of chemotherapy drugs involves the following three factors: age, location of IV line, and simultaneous maintenance IV fluid rate of <100 ml/h (6). The incidence of infusion-site reactions decreased to 5.74% from 28.7% when the intravenous FAP vial was diluted to FAP 150 mg/250 ml from FAP 150 mg/150 ml, and it was infused for more than 30 min (7). Additionally, the use of HTX-019 130 mg was reported as an alternative to FAP 150 mg. HTX-019 130 mg is a polysorbate 80- and synthetic surfactant-free AP injectable emulsion. It was reported that the number of treatment-emergent adverse events was lower with HTX-019 130 mg (30-min infusion) than with FAP 150 mg (20- or 30-min infusion) (8,9). We also studied the method to avoid adverse events induced by the combined use of intravenous FAP followed by EPI based on the viewpoint of the perivascular tissue distribution of EPI, because the infusion-site adverse events in breast cancer chemotherapy

with the FEC regimen combined with Proemend® for I.V. Infusion containing FAP and Tween 80 were sometimes observed in the hospital (Chugoku Rosai Hospital) (10). In rats, the administration of FAP and EPI using the same IV tube exhibited significantly higher perivascular tissue distribution of EPI compared to that administered from different peripheral veins, and the higher EPI distribution caused more severe infusion-site adverse events. Based on these findings, we suggested that the infusion of FAP and EPI from different peripheral veins (right and left) can avoid the infusion-site adverse events greatly (10). In the present study, we further studied the method to avoid infusion-site adverse events in the chemotherapy with FEC or EC regimen combined with the intravenous infusion of FAP by employing immortalized human umbilical vein endothelial (HUEhT-1) cells.

Materials and methods

Materials. AP, FAP and EPI were obtained from Combi-Blocks (San Diego, USA), Sigma-Aldrich Japan (Tokyo, Japan), and Toronto Research Chemicals (Toronto, Canada), respectively. Separately, Proemend I.V. Infusion containing FAP meglumine 243.3 mg (corresponding to FAP 150 mg) and Tween 80 (polysorbate 80) 78.8 mg was obtained from Ono Pharmaceutical Co., Ltd. (Osaka, Japan) and used by diluting with culture medium appropriately. The diluted Proemend solution containing FAP meglumine and Tween 80 was described as FAP (Proemend) to distinguish it from FAP alone, and the concentration of FAP (Proemend) shown in the figures refers to the concentration of FAP within Proemend. Tween 80 was obtained from MP Biochemicals, LLC (Santa Ana, USA). Regents used for cell culture were obtained as follows: culture medium, Endothelial Cell Growth Medium (EGM) from Takara Bio (Siga, Japan), fetal bovine serum (FBS) from Moregate Biotech (Bulimba, Australia), and Endothelial cell growth SupplementMix from Takara Bio (Siga, Japan). Other chemicals such as acetic acid and acetonitrile used for high-performance liquid chromatography (HPLC) analysis were of the highest grade available.

Cell culture. HUEhT-1 cells, an immortalized human umbilical vein endothelial cell line (HUVEC) with cell No. JCRB1458 established by electroporation of pIRES-hTERT-hygr, between passages 6 and 20 were obtained from JCRB Cell Bank, National Institute of Biomedical Innovation, Health and Nutrition (Osaka, Japan). For cytotoxicity experiments, HUEhT-1 cells were seeded at a density of $10x10^4$ cells/100 μl/well in a 96-well plate (Corning Japan KK, Tokyo, Japan). For the intracellular accumulation study of EPI, HUEhT-1 cells were seeded at a density of $5x10^4$ cells/well on 12 well collagen I coated plates (Corning Japan KK, Tokyo, Japan). These cells were cultured for 72 h in EGM medium supplemented with 10% fetal bovine serum and 2% EGM SupplementMix under 5% CO_2-95% air at 37˚C according to the indication by JCRB Cell Bank of cells as preincubation before experiments. Cell culture experiments were repeated four times for each test sample independently.

Cytotoxicity of AP, FAP, FAP (Proemend), Tween 80, and EPI alone (evaluated by viability). HUEhT-1 cells were incubated with a culture medium containing either AP, FAP, FAP (Proemend), Tween 80, or EPI with different concentrations (AP: 0, 1, 3, 10, 30, 50, 100 μg/ml; FAP: 0, 1, 3, 10, 30, 50, 100 μg/ml; FAP (Proemend): 0, 1.5, 7.5, 15, 30, 45, 75, 150 μg/ml; Tween 80: 0, 0.3, 1, 3, 10, 30, 100, 300, 1,000 μg/ml; EPI: 0, 0.5, 1, 3, 5, 10, 30, 50, 100 μg/ml) to evaluate the cytotoxicity of each test compound. After 24 h-incubation at 37˚C, the cell viability was estimated by WST-1 assay according to the manufacturer's protocol (Dojindo, Kumamoto, Japan) in the same manner as reported previously (11). Briefly, each culture medium containing the test compound was discarded and WST-1 reaction mixture (100 μl) containing WST-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt, a cell proliferation reagent] and 1-methoxy phenazinium methylsulfate was added according to the manufacture's protocol (Dojindo, Kumamoto, Japan). After 60 min-incubation, the ultraviolet (UV) absorbance at 450 nm was measured with a microplate reader (SpectraMax Plus 384, Molecular Devices Japan). The UV absorbance of samples that were cultured with a culture medium alone was regarded as a control (100%).

Cytotoxicity of FAP and FAP (Proemend) (evaluated by LDH leakage). HUEhT-1 cells were incubated for 24 h with a culture medium containing FAP or FAP (Proemend) at different concentrations at follows: FAP; 0, 1, 3, 10, 30, 50, 100 μg/ml; FAP (Proemend); 0, 1.5, 4.5, 7.5, 15, 30, 45, 150 μg/ml. After 24 h-incubation, the concentrations of lactate dehydrogenase (LDH) leaked from the cells into the incubation medium were determined using LDH Cytotoxicity Assay Kit (Nacalai Tesque, Kyoto, Japan). Separately, cells incubated with a culture medium alone were mixed with Triton-X (final concentration, 1%) to estimate the maximal leakage of LDH (100%).

Cytotoxicity of combined use of FAP and EPI (evaluated by viability). HUEhT-1 cells were incubated for 24 h with a culture medium containing EPI alone at different concentrations (0, 0.5, 1, 3, 5, 10, 30, 50, 100 μg/ml) or a combination of EPI at different concentrations and FAP (Proemend) 15 μg/ml (a non-cytotoxic concentration). Similarly, HUEhT-1 cells were incubated for 24 h with a culture medium containing FAP (Proemend) alone at different concentrations (0, 1.5, 4.5, 7.5, 15, 30, 45, 75, 150 μg/ml) or a combination of FAP (Proemend) at different concentrations and EPI 1.0 μg/ml (a non-cytotoxic concentration). Cell viability was estimated after 24 h-incubation by WST-1 assay. In this study, the values of half maximal inhibitory concentration (IC50) of each compound (EPI, FAP (Proemend) and combinations of EPI and FAP (Proemend) for the viability of HUEhT-1 cell were estimated to evaluate the synergic cytotoxicity of EPI and FAP (Proemend) in a quantitative manner. Estimation of IC50 values for isobolographic analysis was made by using concentration-viability curves and ImageJ, a Java program inspired by NIH Image that runs on Windows <https://imagej.nih.gov/ij/> in the same manner as reported previously (12-14).

Effect of FAP, FAP (Proemend) and Tween 80 on the intracellular accumulation of EPI. HUEhT-1 cells precultured on 12 well collagen I coated plates were washed with 1 ml of isotonic, pH 7.4 phosphate-buffered saline (PBS) twice and incubated with PBS for 10 min at 37˚C (preincubation) to

remove the culture medium. Then, to evaluate the effects of FAP, FAP (Proemend) and Tween 80 on the intracellular accumulation of EPI, cells were incubated for 10 min at 37˚C with PBS containing a mixture of EPI 20 μg/ml and FAP (0, 1.5, 15 or 150 μg/ml), a mixture of EPI 20 μg/ml and FAP (Proemend) (0, 1.5, 15 or 150 μg/ml), or a mixture of EPI 20 μg/ml and Tween 80 at a concentration of 0, 7.88, or 78.8 μg/ml, respectively. Solutions of FAP (Proemend) (1.5, 15 or 150 μg/ml) contain 0.788, 7.88, or 78.8 μg/ml of Tween 80, respectively. After 10-min incubation of cells with a medium containing the above compound(s), the culture medium was discarded, cells were washed 3 times with 1-ml PBS and were dissolved with PBS containing 0.1% Triton-X. The concentrations of EPI and protein in cell lysates were determined fluorometrically at 458 nm for excitation and 538 nm for emission and photometrically at 562 nm using the TaKaRa BCA protein assay kit (Takara Bio, Siga, Japan), respectively.

Effect of cell washing on cytotoxicity of combined use of FAP and EPI (evaluated by viability). The effect of cell washing on the cytotoxicity of FAP (Proemend) alone at different concentrations (0, 1.5, 7.5, 15, 30, 45, 75, 150 μg/ml) was evaluated. HUEhT-1 cells were incubated with a culture medium containing FAP at different concentrations for 30 min, the medium was discarded, and then cells were incubated with a fresh culture medium alone for 24 h (cytotoxicity of FAP (Proemend), without washing). For comparison, HUEhT-1 cells were incubated with a culture medium containing FAP (Proemend) at different concentrations (0, 1.5, 7.5, 15, 30, 45, 75, 150 μg/ml) for 30 min, the medium was discarded, the cell surface was washed with culture medium (100 μl each), and then incubated for 24 h with fresh culture medium (cytotoxicity of FAP (Proemend), with washing). Separately, the effect of cell washing on the cytotoxicity of a combination of FAP (Proemend) at different concentrations (0, 1.5, 7.5, 15, 30, 45, 75, 150 μg/ml) and EPI 1.0 μg/ml was evaluated. After HUEhT-1 cells were incubated with FAP (Proemend) at different concentrations (0, 1.5, 7.5, 15, 30, 45, 75, 150 μg/ml) for 30 min, the culture medium was discarded. Then, cells were cultured with fresh medium containing EPI 1.0 μg/ml (a non-cytotoxic concentration) for 24 h (cytotoxicity of combined use, without washing). For comparison, the cell surface was washed with a culture medium (100 μl each) after 30-min incubation of cells with FAP (Proemend) at different concentrations (0, 1.5, 7.5, 15, 30, 45, 75, 150 μg/ml) and then incubated for 24 h with a culture medium containing EPI 1.0 μg/ml (cytotoxicity of combined use, with washing). Cell viability was estimated after 24 h-incubation by WST-1 assay.

Analysis of EPI in cells. The concentrations of EPI accumulated in HUEhT-1 cells after incubation were determined in the same manner as reported previously by high performance liquid chromatography (HPLC) (10). Briefly, the HPLC column used was a YMC-Triart C18 column (YMC Inc., Kyoto, Japan). The mobile phase was a mixture of 1% acetic acid and acetonitrile in a ratio of 7:3 (v/v%), and the flow rate was set at 1.0 ml/min. EPI was detected fluorometrically at an excitation wavelength of 470 nm and an emission wavelength of 585 nm, respectively.

Statistical analysis. The data were presented as the mean ± SE (experiments of cell culture were repeated with four independent repetitions), and statistical analysis was performed by one-way ANOVA, followed by the Tukey-Kramer method for multiple comparisons. The level of significance was set at $P<0.05$.

Results

Cytotoxicity of AP, FAP, FAP (Proemend), Tween 80 and EPI alone on HUEhT-1 cells (viability). HUEhT-1 cells were incubated for 24 h with a medium containing AP, FAP, FAP (Proemend), Tween 80, or EPI at different concentrations and the viability of treated cells was evaluated by WST-1 assay. AP and Tween 80 alone exerted no cytotoxicity in a concentration range from 0 to 50 μg/ml and from 0 to 1,000 μg/ml, respectively. In contrast, FAP, FAP (Proemend) and EPI exhibited cytotoxicity in a concentration-dependent manner (decrease in viability) at more than 30, 15 and 1.0 μg/ml, respectively, indicating the potency of cytotoxicity was in the following order: EPI > FAP (Proemend) > FAP (Fig. 1).

Cytotoxicity of FAP and FAP (Proemend) each on HUEhT-1 cells (LDH leakage). HUEhT-1 cells were incubated for 24 h with either FAP or FAP (Proemend) at different concentrations, and their cytotoxic potencies were evaluated by measuring LDH leakage from cells. The leakage of LDH was induced at more than 30 and 15 μg/ml of FAP and FAP (Proemend), respectively. No significant difference was observed in the potency of cytotoxicity between FAP and FAP (Proemend) when evaluated at a concentration of 50 μg/ml of FAP (Fig. 2).

Cytotoxicity of combined use of FAP (Proemend) and EPI. In HUEhT-1 cells, the co-existence of FAP (Proemend) at a concentration of 15 μg/ml significantly increased the cytotoxicity of EPI (Fig. 3A), although FAP (Proemend) alone at a concentration of 15 μg/ml showed no cytotoxicity (Fig. 1C). Similarly, the co-existence of a non-toxic concentration of EPI (1 μg/ml) significantly increased the cytotoxicity of FAP (Proemend) (Fig. 3B). These results indicate the synergistic cytotoxicity of FAP (Proemend) and EPI. Using these concentration-viability curves, values of IC50 were estimated for isobolographic analysis (Fig. 3C). Estimated IC50 values were 11.4 μg/ml for EPI alone, 0.96 μg/ml for a combination of EPI and FAP (Proemend), 32.6 μg/ml for FAP (Proemend) alone, and 15.4 μg/ml for a combination of FAP (Proemend) and EPI. These results indicate that the cytotoxicity of EPI can be greatly increased by the presence of FAP (Proemend) even at a non-toxic concentration, although the increase in the cytotoxicity of FAP (Proemend) is small even in the co-presence of a non-toxic concentration of EPI.

Effect of FAP, FAP (Proemend) and Tween 80 on the intracellular accumulation of EPI. HUEhT-1 cells were incubated for 10 min with a culture medium containing EPI 20 μg/ml and either FAP, FAP (Proemend) or Tween 80 at different concentrations to evaluate the effect of each compound on the intracellular accumulation of EPI. FAP and FAP (Proemend) at concentrations of more than 15 μg/ml

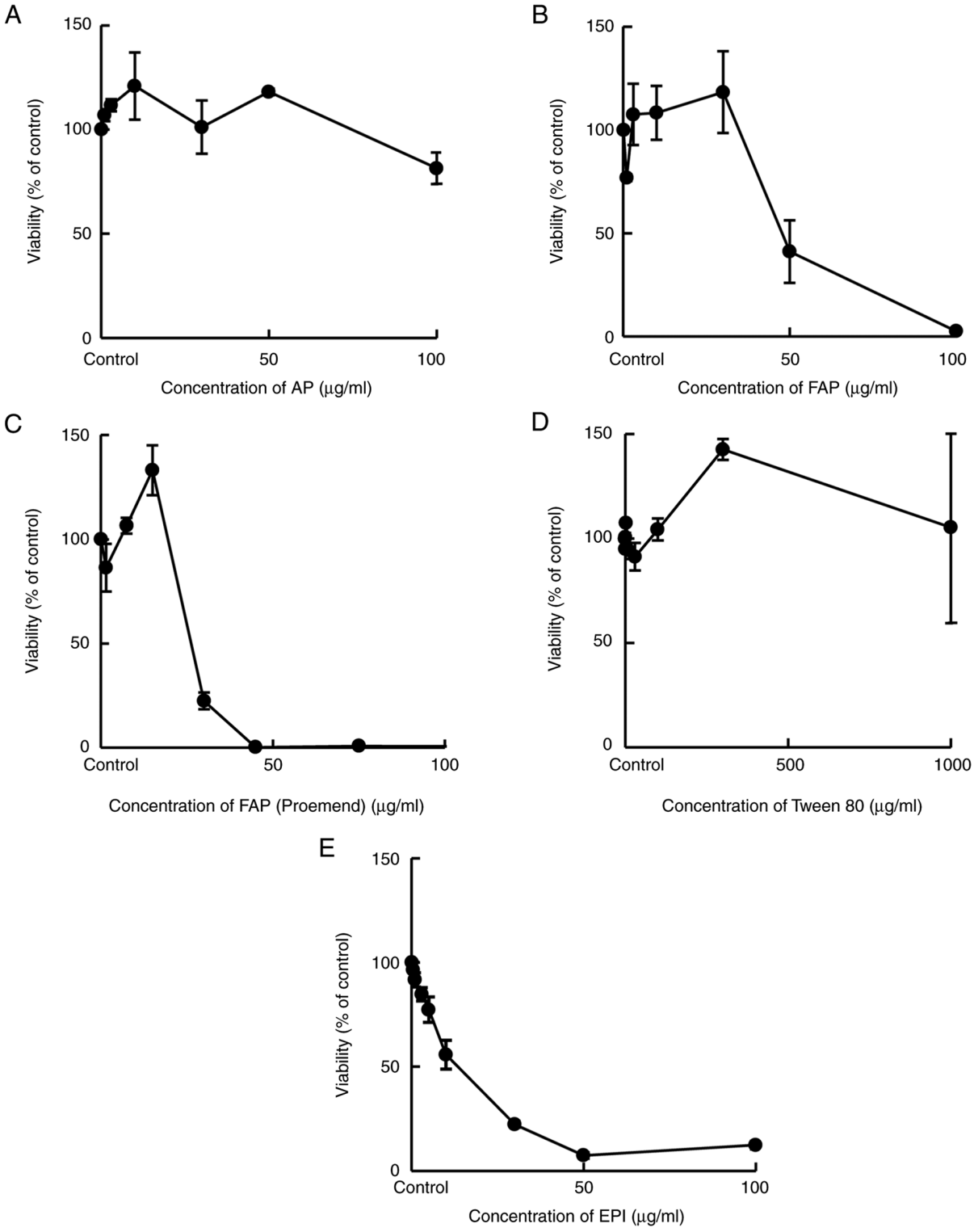

Figure 1. The concentration-dependent cytotoxicity of (A) AP, (B) FAP, (C) FAP (Proemend®), (D) Tween 80 and (E) EPI in HUEhT-1 cells. Incubation was made for 24 h, and cytotoxicity was evaluated by measuring the viability (WST-1 assay) of cells. The concentration of FAP (Proemend) was expressed as the concentration of FAP. Each value represents the mean ± SE of 4 trials. AP, aprepitant; FAP, fosaprepitant; FAP (Proemend), diluted Proemend containing fosaprepitant meglumine; EPI, epirubicin.

and Tween 80 at a concentration of 78.8 μg/ml increased the intracellular EPI concentration significantly (Fig. 4A-C).

Effect of cell surface washing on cytotoxicity of combined use of EPI and FAP (Proemend) (viability). The effect of cell surface washing on the cytotoxicity of FAP (Proemend) alone or a combination of FAP (Proemend) and EPI 1 μg/ml was examined in HUEhT-1 cells. The cytotoxicity of FAP (Proemend) observed at a concentration of 150 μg/ml was eliminated by washing the cell surface with a fresh culture medium after 30-min incubation of HUEhT-1 cells with FAP (Proemend) (Fig. 5A). The viability of washed cells that were

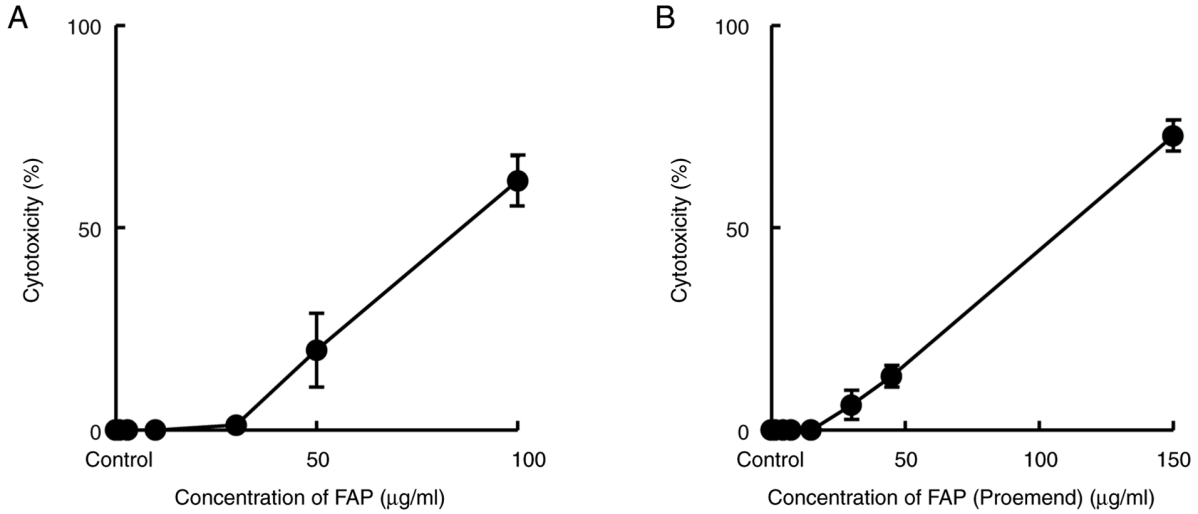

Figure 2. The concentration-dependent cytotoxicity of (A) FAP and (B) FAP (Proemend®) in HUEhT-1 cells. Incubation was made for 24 h, and cytotoxicity was evaluated by measuring the leakage of LDH from cells. The concentration of FAP (Proemend) was expressed as the concentration of FAP. Each value represents the mean ± SE of 4 trials. FAP, fosaprepitant; FAP (Proemend), diluted Proemend containing fosaprepitant meglumine.

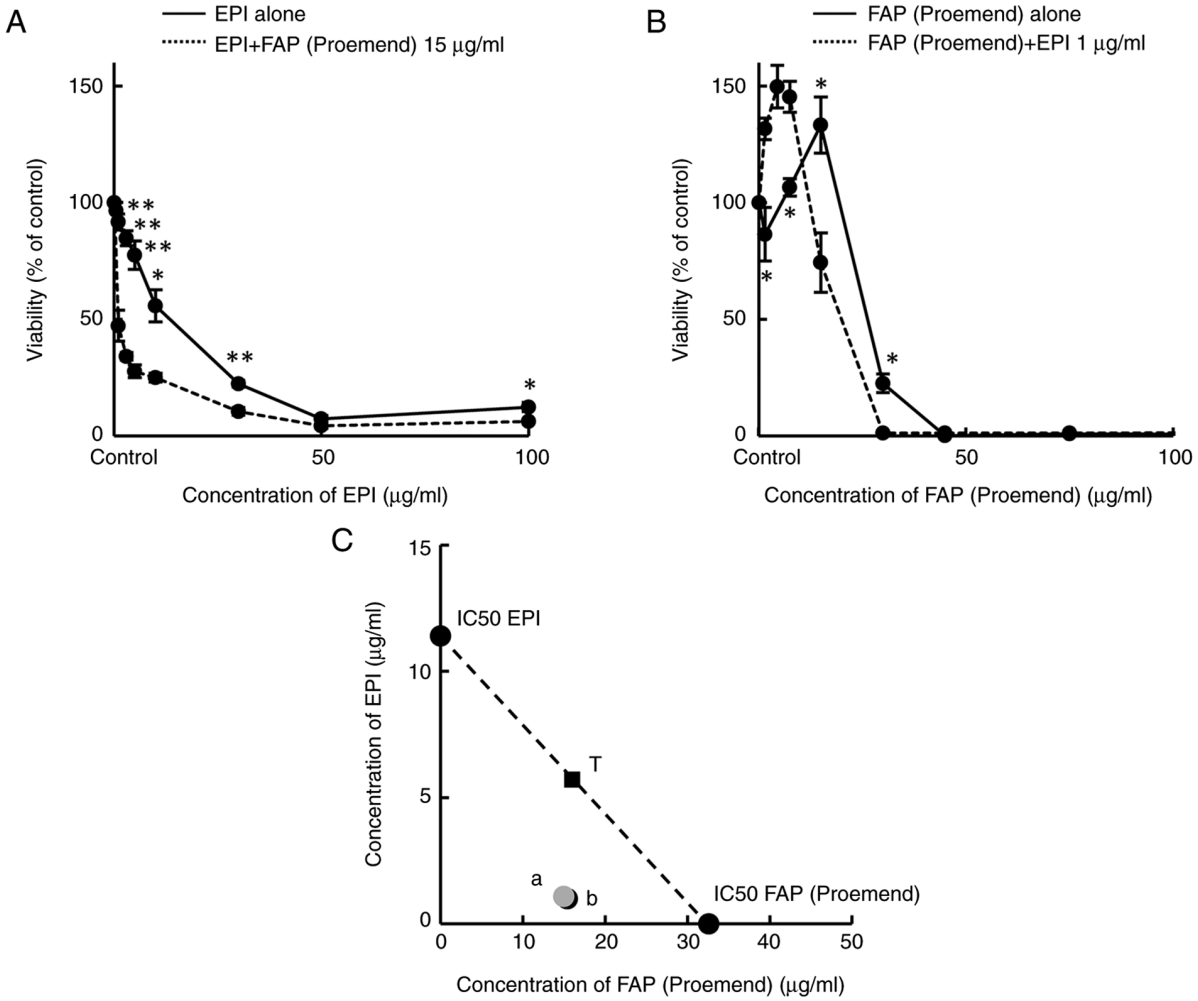

Figure 3. Cytotoxicity of combined use of FAP (Proemend®) and EPI in HUEhT-1 cells. (A) Cytotoxicity of EPI alone at various concentrations and a combination of EPI at various concentrations and FAP (Proemend) 15 μg/ml. (B) Cytotoxicity of FAP (Proemend) alone at various concentrations and a combination of FAP (Proemend) at various concentrations and EPI 1 μg/ml. The concentration of FAP (Proemend) was expressed as the concentration of FAP. A significant difference was detected between the single and combined use at a level of *P<0.05 or **P<0.01, respectively. (C) Graphic representation of isobologram of cell growth-inhibiting effects (IC50 values) of EPI alone, FAP (Proemend) alone, a combination of EPI and 15 μg/ml FAP (a), a combination of FAP (Proemend) and 1 μg/ml EPI (b) and theoretical additive inhibition effect (T). The IC50 values of (a) and (b) were 0.96±0.26 μg/ml and 15.4±0.36 μg/ml, respectively. These experimentally obtained IC50 values (a and b) indicated a synergistic interaction between FAP (Proemend) and EPI. FAP (Proemend), diluted Proemend containing fosaprepitant meglumine; EPI, epirubicin.

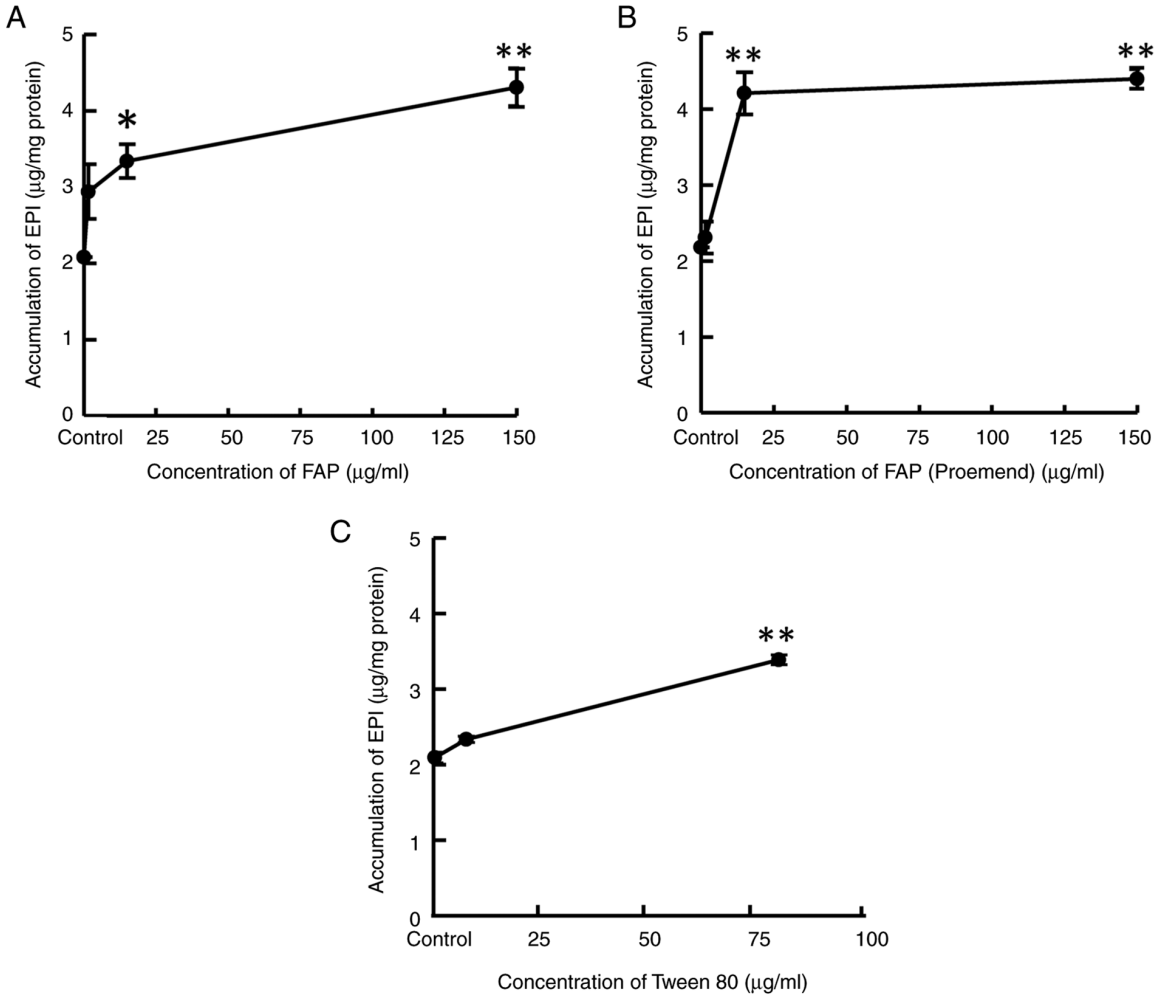

Figure 4. Relationship between the concentrations of (A) FAP, (B) FAP (Proemend®) and (C) Tween 80 and the intracellular accumulation of EPI in HUEhT-1 cells. HUEhT-1 cells were incubated with medium containing EPI 20 μg/ml and either FAP, FAP (Proemend) or Tween 80 at different concentrations for 10 min. The concentration of FAP (Proemend) was expressed as the concentration of FAP. Each value represents the mean ± SE of 4 trials. A significant difference was detected at a level of *P<0.05 or **P<0.01, vs. control, respectively. FAP, fosaprepitant; FAP (Proemend), diluted Proemend containing fosaprepitant meglumine; EPI, epirubicin.

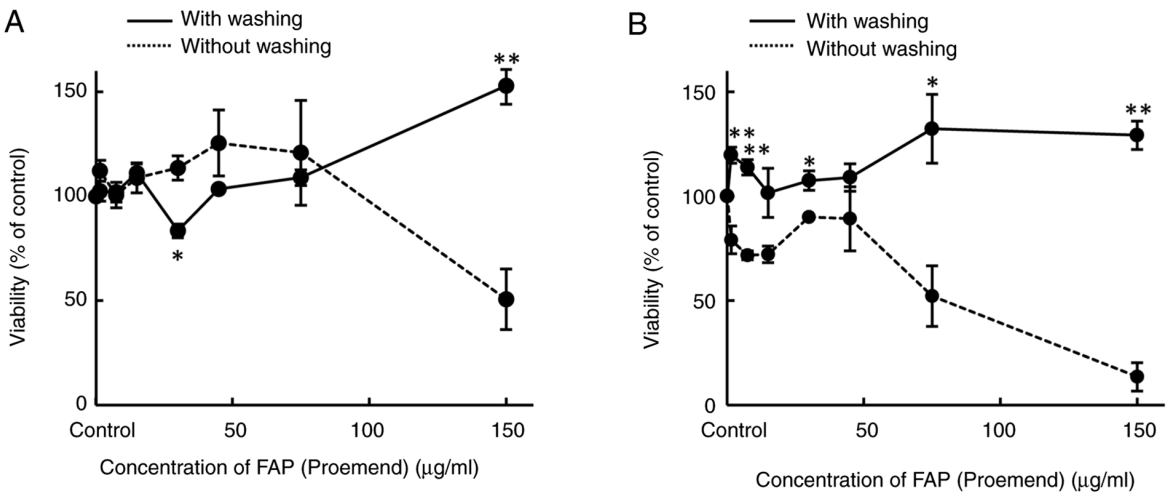

Figure 5. Effect of cell washing on the cytotoxicity of FAP (Proemend®) alone and combined use of FAP (Proemend) and EPI in HUEhT-1 cells. (A) Cytotoxicity of FAP (Proemend) alone at different concentrations, without cell surface washing and with cell surface washing. (B) Cytotoxicity of combined use of FAP (Proemend) at different concentrations and EPI 1 μg/ml, without cell surface washing and with cell surface washing. The concentration of FAP (Proemend) was expressed as the concentration of FAP. Cell surface washing was made with fresh culture medium (100 μl) after 30-min incubation with FAP (Proemend). After liquid used for cell washing was discarded, cells were incubated with fresh culture medium alone (A) or medium containing EPI 1 μg/ml (B) for 24 h. Cytotoxicity was evaluated by WST-1 assay. Each value represents the mean ± SE of 4 trials. A significant difference between without and with washing was detected at a level of *P<0.05 or **P<0.01, respectively. FAP (Proemend), diluted Proemend containing fosaprepitant meglumine; EPI, epirubicin.

incubated with 150 μg/ml FAP (Proemend) rather increased by about 1.5-fold of control (100%) (Fig. 5A). In addition, the washing of the cell surface after incubation of cells with FAP (Proemend) at different concentrations eliminated completely the subsequently evoked synergic cytotoxicity of FAP (Proemend) and EPI (Fig. 5B).

Discussion

FEC and EC regimens in the chemotherapy for breast cancer patients contain EPI, an anthracycline drug with a high emetic risk, and the use of oral AP, intravenous FAP, dexamethasone, or olanzapine, an atypical antipsychotic is recommended (15). The combined use of intravenous infusion of FAP and EPI that is frequently used for outpatients, however, can cause infusion-site adverse events compared with the combination of oral AP and EPI or intravenous FAP and cisplatin (1,15). We also observed the induction of infusion-site adverse events in breast cancer chemotherapy with the FEC regimen with intravenous FAP and studied the mechanism of adverse events based on the viewpoint of the perivascular tissue distribution of EPI by comparing three different treatments groups using rats (10). In the FAP-S group, FAP and EPI were infused into the jugular vein using the same IV tube. In the FAP-D group, FAP and EPI were infused into different jugular veins using two IV tubes (right and left jugular vein), respectively, and in the AP group, AP was administered orally, and EPI was infused into the jugular vein using an IV tube. The concentrations of EPI in plasma and perivascular tissue were compared among the FAP-S, FAP-D, and AP groups at 30 min and 24 h after the 5-min constant-rate infusion of EPI. There was no significant difference in the plasma EPI concentrations among the three groups. However, concentrations of EPI in perivascular tissues of infusion-site at 30 min and 24 h after EPI infusion were scattered greatly as follows: FAP-S group, the mean concentration was 2.30 μg/g at 30 min and 3.86 μg/g at 24 h; FAP-D group, 0.96 and 0.76 μg/g; AP group, 0.66 and 0.28 μg/g, respectively. The magnitude of histological damage at infusion-site adverse events was in the following order: EPI-infusion-site of the FAP-S group >> EPI-infusion-site of the FAP-D group >> EPI-infusion-site of the AP group and FAP-infusion-site of the FAP-D group (no damage). These results suggested that EPI has more potent cytotoxicity than FAP, and the co-existence of FAP at a higher concentration increased perivascular tissue concentrations of EPI infused thereafter and caused severe infusion-site adverse events, indicating the synergic cytotoxicity between FAP and EPI. Based on these results, we previously suggested that the infusion of FAP and EPI from different peripheral veins (right and left) can reduce the infusion-site adverse events greatly (10).

In the present study, the possible synergistic cytotoxicity of FAP and EPI and avoiding method of infusion-side adverse events were further studied employing HUEhT-1 cells. As shown in Fig. 1, the incubation of cells with FAP, FAP (Proemend) and EPI alone showed cytotoxicity of HUEhT-1 cells in a concentration-dependent manner, and the potency of cytotoxicity was in the following order: EPI > FAP (Proemend) > FAP when the cytotoxicity was evaluated by viability with WST-1 assay. These results suggested that Tween 80 contained in Proemend IV Infusion can increase the cytotoxicity of FAP, although Tween 80 alone showed no cytotoxicity in a concentration range from 0 to 1.0 mg/ml (Fig. 1). In contrast, the greater cytotoxicity of FAP (Proemend) compared to FAP was not clearly detected when evaluated by the LDH leakage assay, although FAP (Proemend) induced LDH leakage at a lower concentration compared to FAP (Fig. 2). It was reported that the assay with the neutral red and the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] was the most sensitive in detecting cytotoxic events compared to the LDH leakage and the protein assays when the cytotoxicity of hepatoma cell lines following exposure to cadmium chloride was detected (16). It was also reported that the WST-1 reagent presents several advantages compared to the two other tetrazolium salt-based cell proliferation reagents, MTT and XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide], including water-solubility, rapidity, greater stability and sensitivity (17,18). Thus, in the present study, the WST-1 assay was mainly used to evaluate the viability, or cytotoxicity, of each test compound. In these studies, however, a low concentration of FAP and/or FAP (Proemend) appeared to increase cell viability as shown in Figs. 1C and 3B. Similar phenomena were also observed in cells that were incubated with FAP (Proemend) 150 μg/ml for 30-min and then the cell surface was washed (Fig. 5A and B). In addition, large variability in cell viability was observed at a concentration of Tween 80 1 mg/ml in the medium (Fig. 1D). As shown in Fig. 4C, Tween 80 can increase the EPI distribution into HUEhT-1 cells and would cause cytotoxicity more or less. It may be considered that a slight cytotoxic effect rather stimulates the viability of cells. Further study is necessary to clarify the mechanism of such cell stimulation.

The effect of the combined use of FAP and EPI on cytotoxicity was examined, in which the presence of a nontoxic concentration of FAP (15 μg/ml) and EPI (1.0 μg/ml) significantly increased the cytotoxicity of EPI and FAP (Proemend), respectively (Fig. 3A and B). The synergic cytotoxicity of EPI and FAP (Proemend) was clearly detected by isobolographic analysis, in which the co-presence of nontoxic concentration of FAP (Proemend) greatly increased the cytotoxicity of EPI as evaluated by IC50 values (Fig. 3C), and the co-presence of either FAP or FAP (Proemend) was found to significantly increase the intracellular accumulation of EPI, in which Tween 80 alone also increases the EPI cell distribution depending on the concentration (Fig. 4). To avoid such synergic cytotoxicity of FAP (Proemend) and EPI, the effect of cell-surface washing after application of FAP (Proemend) on the synergic cytotoxicity was examined (Fig. 5). The washing of cell surface with culture medium after incubation with FAP (Proemend) eliminated the cytotoxicity caused by FAP (Proemend) alone and synergic cytotoxicity of FAP (Proemend) and EPI almost completely (Fig. 5).

These findings obtained in *in-vitro* HUEhT-1 cell studies imply the efficacy of washing the infusion in avoiding the infusion-site adverse events in chemotherapy using FEC or EC regimen and Proemend IV Infusion. Detailed preclinical animal studies are necessary to examine the efficacy of infusion-site washing in avoiding infusion-site adverse events in chemotherapy with EPI and intravenous FAP, including the effect of the timing of infusion-site washing and the quantity of saline

for infusion-site washing. Regarding the timing of infusion-site washing, it may be considered as follows: FAP meglumine, a negatively charged phosphoryl prodrug for AP (19), and Tween 80 (polysorbate 80) with HLB 15.0 are both water-soluble compounds, and the adsorption on the cell surface or on the vascular endothelial cells would not be strong, because the surface layer of endothelial cells is covered with a negatively charged, brush-like glycocalyx (20). In contrast, the adsorption to the cell surface, or charged interaction, and the intracellular accumulation of weakly basic drugs, AP with a pKa value of 9.7 (19) and EPI with a pKa value of around 8.5 (21), are considered to be strong, because many weakly basic lipophilic drugs with a pKa value of more than 6.5 bind to acidic phospholipids, especially phosphatidylserine, in the cellular membrane (22,23). In addition, the cytotoxicity of EPI was greatly increased in the presence of FAP (Proemend) even at a nontoxic concentration, compared to the combination of FAP (Proemend) and nontoxic concentration of EPI (Fig. 3C). Taken together, in *in-vivo* preclinical studies, it will be important to administer Proemend at first and wash the vascular infusion-site via IV tube with an efficient amount of saline immediately after the IV infusion of Proemend, and thereafter administer EPI by infusion to avoid infusion-site adverse events in pharmacotherapy with EPI and Proemend. Regarding the washing of IV tube, the flushing of IV tube post administration of medications or between medications in IV administrations of multiple agents is recommended to prevent medicine loss (or flushing of residual medication from the line), to improve cannula patency (prevention of loss of function of peripheral intravenous catheters), or to prevent incompatibility issues between medication (24-27). However, to eliminate the adsorbed FAP and Tween 80 on the infusion-site vascular tissue almost completely, a greater volume of saline than the volume of IV tube flushing, for example, the same volume used for Proemend infusion (100-150 ml), will be necessary. Taking these considerations into account, detailed preclinical animal studies are needed to clarify the interaction mechanism between FAP and EPI and evaluate the efficacy of infusion-site washing in avoiding infusion-site adverse events.

In conclusion, the washing of the cell surface with culture medium after incubation with FAP (Proemend) was found to greatly decrease the synergic cytotoxicity of FAP, EPI and Tween 80 in HUEhT-1 cells. Based on our previous (10) and present studies, we would like to suggest that washing the infusion site after the application of Proemend with saline through an IV tube, or infusion of Proemend and EPI from different peripheral veins (right and left) may avoid or reduce the infusion-site adverse events.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

MY, KOd, KOm, YM and TM designed the study, and KOm approved the study. MY and KOd performed cellular experiments using HUEhT-1 cells, and TI, MM, ST, KOm, NM and TN assisted in the biological assay. MY and KOd analyzed the data. MY, KOd, YM and TM confirm the authenticity of all the raw data. MY and TM wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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