The effect of co-treatments of chemotherapeutic drugs and curcumin on cytotoxicity and FLT3 protein expression in leukemic stem cells

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Abstract

This study aims to enhance efficacy and reduce toxicity of the combination treatment of a drug and curcumin (Cur) on leukemic stem cell and leukemic cell lines, including KG-1a and KG-1 ($FLT3^+$ LSCs), EoL-1 ($FLT3^+$ LCs), and U937 ($FLT3^-$ LCs). The cytotoxicity of co-treatments of Dox or Ida at concentrations of the IC_{10} – IC_{80} values and each concentration of Cur at the IC_{20}, IC_{30}, IC_{40}, and IC_{50} values (conditions 1, 2, 3, and 4) was determined by MTT assays. Dox–Cur and Ida-Cur increased cytotoxicity in leukemic cells. Dox–Cur co-treatment showed additive effects in several conditions. The effect of this co-treatment on FLT3 expression in KG-1a, KG-1, and EoL-1 cells was examined by Western blotting. Dox–Cur decreased FLT3 protein levels and total cell numbers in all the cell lines. By contrast, the FLT3 protein levels and total cell number after Cur treatment did not show significant differences as a result of the co-treatments. Dox–Cur decreased FLT3 protein expression in a dose dependent manner. In summary, Cur was the effective compound in inhibiting FLT3 protein expression. Co-treatment with Dox–Cur could enhance the cytotoxicity of Dox by inhibiting the proliferation of AML leukemic stem cells.

Introduction

Leukemia is among the top 10 cancers diagnosed globally. It is a group of cancers of early blood-forming cells, which are characterized by the uncontrolled production and accumulation of blast or immature abnormal blood cells in the peripheral blood and bone marrow. Leukemia can be divided into four major types according to the stage and cell of origin: acute myeloid leukemia (AML), acute lymphoid leukemia (ALL), chronic myeloid leukemia (CML), and chronic lymphocytic leukemia (CLL). AML is the most common type of acute leukemia in adults, with the highest incidence and death rate in both sexes. It can be distinguished by clonal expansion of abnormal myeloid blasts in bone marrow, peripheral blood, or other tissues. According to recent data, 15–25% of AML patients fail to achieve complete remission (CR) due to chemotherapy resistance and may show relapse, with the overall 5-year survival rate of approximately 40%[1,2]. Moreover, between 10 and 40% of newly diagnosed AML patients do not achieve CR with intensive induction therapy, and such patients are categorized as primary refractory or resistant[3]. Hence, AML is defined as an aggressive malignant myeloid disorder.

One theory of resistance and relapse in AML patients involves the presence of subpopulations of leukemic stem cells (LSCs)[4]. LSCs have been described as the human AML-initiating cell with a self-renewal capacity and the ability to give rise to heterogeneous lineages of cancer cells[2,5]. They can be identified by the cell surface phenotype $CD34^+$ hematopoietic stem cell and $CD38^-$ subpopulation[6].

The used traditional chemotherapeutic drugs are incapable of defeating the LSC population due to many reasons. First, these drugs have been designed to eliminate fast-dividing cells by inhibiting cell cycle progression[7]; thus, they cannot it did not affect LSCs which mostly remain in stage G_0 of the cell cycle[8]. Second, the expression of P-glycoprotein (MDR1), a multidrug resistance efflux pump protein, in LSCs potentially removes cytotoxic agents from cancer cells[9]. In addition, LSCs may sustain some mutations

and epigenetic changes, resulting in conventional drug toxicity reduction and resistance[10,11]. Thus, LSCs are considered to play a fundamental role in AML pathogenesis and have become the main targeted therapies of AML.

Although drug resistance in AML patients usually occurs, the traditional chemotherapy remains a popular method for leukemia treatment due to its high ability to destroy cancer cells that can spread throughout the whole body. Anthracycline antibiotics, such as doxorubicin (Dox (14-hydroxydaunorubicin)) and idarubicin (Ida (4-demethoxydaunorubicin)), are generally used as standard chemotherapeutic agents for AML treatment[12]. These drugs function by inhibiting topoisomerase II activity in DNA transcription and also trigger apoptosis or autophagy in cells[13]. The combination of anthracyclines and cytarabine in the initial treatment is capable of inducing complete remission (CR) in approximately 45–70% of patients[14]; however, more than 40% of CR cases eventually experience relapse within 2 years[15]. The previous studies on AML leukemic stem cells demonstrated that anthracycline is less effective in killing LSCs ($CD34^+/CD38^-$ cells) than committed leukemic cells ($CD34^+/CD38^+$ cells)[16], and the co-treatment of cytarabine and anthracyclines is less effective against primitive AML cells than against leukemia blasts[17,18]. Furthermore, with high dose administration, anthracyclines are able to cause the development of side effects in patients in relation to its chemical structure, including nausea, vomiting, hair loss, and myelosuppression[19]. Several reports expressed their concern about the presence of cardiac, renal, and liver toxicity in patients treated with Dox[20,21]. Thus, combination therapy with natural substances with chemosensitizing and chemoprotective activities may be a promising strategy to overcome LSCs and reduce the side effects of anthracyclines.

Curcumin (Cur) is a natural polyphenol constituent of turmeric (*Curcuma longa* Linn.). It exhibits a wide range of pharmacological activities, such as antioxidant, anti-cancer, anti-inflammatory, and antimicrobial effects[22–24]. Previous studies reported that Cur exhibited an excellent cytotoxic effect; induced cell death in several types of leukemic cell lines[25,26]; and showed inhibitory effects on WT1 and FLT3 protein expression, which are associated with cell proliferation[26,27]. Moreover, it inhibited the activity of P-glycoprotein (MDR1)[28] and exhibited cancer chemopreventive properties, especially in myocardial protection[29] by inhibiting ROS generation[30]. Consequently, it may be possible to manipulate the combination of Cur and anthracyclines for reduction in anthracycline toxicity and to overcome drug efflux via Pgp-mediated MDR in leukemia on AML leukemic cells and LSCs. Although Dox and Cur exhibit synergistic cytotoxic effects on cancer cell models, the combination of free Dox and free Cur shows a slightly obvious synergistic effect on the animal model[31].

The aims of this study were to study the cytotoxicity of co-treatment with anthracycline drugs and curcumin for FLT3-overexpressing leukemic stem cells (KG-1a and KG1), FLT3-overexpressing leukemic cells (EoL-1), and non FLT3-expressing leukemic cells (U937). Moreover, the effect of co-treatments on FLT3 protein expression and total cell numbers were determined.

Results

Determination of cytotoxicity of doxorubicin (Dox), idarubicin (Ida), and curcumin (Cur) on leukemic cell viability by MTT assay. From the cell viability curve, it can be seen that Dox (Fig. 1A) and IDa (Fig. 1B) showed the highest cytotoxicity for EoL-1 cells, followed by U937, KG-1, and KG-1a cells. The cytotoxicity of all the treatments was assessed using an inhibitory concentration at a 50% growth (IC_{50}) value. Ida demonstrated the greatest cytotoxic effects on KG-1a, KG-1, EoL-1, and U937 cells with IC_{50} values of 19.82 ± 1.80, 5.45 ± 0.89, 2.57 ± 0.32, and 4.73 ± 2.38 ng/mL, followed by Dox with IC_{50} values of 0.69 ± 0.12, 0.21 ± 0.02, 0.02 ± 0.01, and 0.08 ± 0.02 µg/mL, respectively. The IC_{50} values of all the chemotherapeutic drugs for the leukemic cell line models are shown in Table 1.

Table 1
The IC_{50} values of chemotherapeutic drugs and curcumin on KG-1a, KG-1, EoL-1, and U937 cells.

Treatment	IC_{50} value			
	KG-1a	KG-1	EoL-1	U937
Idarubicin (Ida) (ng/mL)	19.82 ± 1.80$^{###}$	5.45 ± 0.89***	2.57 ± 0.32***	4.72 ± 2.38***
Doxorubicin (Dox) (µg/mL)	0.69 ± 0.12$^{###}$	0.21 ± 0.02***	0.02 ± 0.01***,$^{##}$	0.08 ± 0.02***,$^{#}$
Curcumin (Cur) (µg/mL)	9.19 ± 0.49$^{#}$	7.31 ± 1.45*	5.55 ± 0.46**,$^{#}$	3.55 ± 0.54***,$^{##}$
The significance of mean differences was assessed using one-way ANOVA. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to KG-1a cells. $^{#}p < 0.05$, $^{##}p < 0.01$, and $^{###}p < 0.001$ compared to KG-1 cells.				

Cur was chosen to study the combination effect in order to improve the efficacy of Dox and Ida in AML treatment. For single treatment, Cur exhibited the highest cytotoxicity for EoL-1 cells, followed by U937, KG-1, and KG-1a cells (Fig. 1C). The IC_{50} values of Cur for KG-1a, KG-1, EoL-1, and U937 cells were 9.19 ± 0.49, 7.31 ± 1.45, 5.55 ± 0.46, and 3.55 ± 0.54 µg/mL, respectively (Table 1).

The IC_{50} values of Dox in leukemic stem cells (KG-1a and KG-1) were found to be significantly higher than for leukemic cells, EoL-1 and U937 cells. However, in the group of the leukemic stem cell line, KG-1 cells were substantially more responsive to Dox and Ida than KG-1a cells were, indicating that a high number of LSCs affected the chemotherapeutic treatment's sensitivity. Furthermore, the IC_{50} values of Cur in KG-1a cells were considerably higher than those in the other cells. These findings demonstrated the drug resistance in LSCs against LCs and suggest that it might be possible to use to improve the potency of AML treatment.

Determination of cytotoxicity of combined doxorubicin–curcumin (Dox–Cur) and idarubicin–curcumin (Ida–Cur) on leukemic cell viability by MTT assay. In this study, various doses of Dox and Ida (ranging from 0 to IC_{80} values), and Cur from the previous results, were used to investigate the combination effects on the cell viability of AML cell lines. Dox was used to treat KG-1a (0–1.30 µg/mL), KG-1 (0–0.84

μg/mL), EoL-1 (0–0.08 μg/mL), and U937 (0–0.16 μg/mL) cells, and Ida was used to treat KG-1a (0–40.0 ng/mL), KG-1 (0–20.0 ng/mL), EoL-1 (0–8.0 ng/mL), and U937 (0–9.2 ng/mL) cells; these cells were cotreated with Cur at concentrations of the IC_{20} (condition 1), IC_{30} (condition 2), IC_{40} (condition 3), and IC_{50} (condition 4), respectively. The co-treatments of Dox–Cur and Ida–Cur exhibited higher cytotoxicity for KG-1a, KG-1, EoL-1, and U937 cells than single drug treatments did in dose dependent manners, according to the cell viability (see supplementary Fig. S1, S2). The IC_{50} value of each co-treatment demonstrated that curcumin did not only enhance the efficacy of doxorubicin and idarubicin in leukemic stem cells and non-leukemic stem cells, but it also decreased the doses of drugs in co-treatment when compared to a single treatment due to the decrease in IC_{50} values of drugs used in each treatment. The IC_{50} values of Dox-Cur and Ida-Cur at different conditions in each cell line are shown in the supplementary Table S1 – S4.

Synergistic effects of combination treatment. From the combination index (CI) calculation, formulations 3 and 5 of Dox–Cur showed a synergistic effect (CI < 1) on KG-1a and EoL-1 cells and an additive effect (CI = 1) on U937 cells at the IC_{50} values, while most of the Ida–Cur exhibited an antagonist effect (CI > 1) in all the leukemic cell lines as shown in Table 2. These data might have resulted from the fact that curcumin could not achieve high cytotoxicity for idarubicin, which had a very low usage dose. Thus, most of the Ida–Cur-treated samples exhibited an antagonist effect in AML leukemic cell lines.

Table 2
IC_{50} values of co-treatment of Dox-Cur and Ida–Cur on KG-1a, KG-1, EoL-1, and U937 cells

Cell line	Dox–Cur	CI value	Ida–Cur	CI value
KG-1a	Dox + Cur 1 (4.5 μg/mL)	1.08	Ida + Cur 1(4.5 μg/mL)	1.16
	Dox + Cur 2 (5.5 μg/mL)	1.12	Ida + Cur 2 (5.5 μg/mL)	1.27
	Dox + Cur 3 (7.0 μg/mL)	0.97	Ida + Cur 3 (7.0 μg/mL)	1.21
	Dox + Cur 4 (9.0 μg/mL)	1.02	Ida + Cur 4 (9.0 μg/mL)	1.09
KG-1	Dox + Cur 1 (3.5 μg/mL)	1.36	Ida + Cur 1 (3.5 μg/mL)	1.44
	Dox + Cur 2 (4.5 μg/mL)	1.07	Ida + Cur 2 (4.5 μg/mL)	1.55
	Dox + Cur 3 (6.0 μg/mL)	1.04	Ida + Cur 3 (6.0 μg/mL)	1.28
	Dox + Cur 4 (7.5 μg/mL)	1.07	Ida + Cur 4 (7.5 μg/mL)	1.11
EoL-1	Dox + Cur 1 (3.0 μg/mL)	1.23	Ida + Cur 1 (3.5 μg/mL)	1.27
	Dox + Cur 2 (4.0 μg/mL)	1.12	Ida + Cur 2 (4.0 μg/mL)	1.24
	Dox + Cur 3 (4.5 μg/mL)	0.92	Ida + Cur 3 (4.5 μg/mL)	0.85
	Dox + Cur 4 (5.5 μg/mL)	1.03	Ida + Cur 4 (5.5 μg/mL)	1.03
U937	Dox + Cur 1 (2.0 μg/mL)	1.46	Ida + Cur 1 (2.0 μg/mL)	1.35
	Dox + Cur 2 (2.5 μg/mL)	1.55	Ida + Cur 2 (2.5 μg/mL)	1.40
	Dox + Cur 3 (3.0 μg/mL)	1.42	Ida + Cur 3 (3.0 μg/mL)	1.26
	Dox + Cur 4 (3.5 μg/mL)	1.00	Ida + Cur 4 (3.5 μg/mL)	1.04

Effects of various conditions of combined treatment of Dox–Cur at concentration value of IC_{20} on cell number and cell viability in FLT-3 protein expressing leukemic cells. Due to the synergistic and additive effect of Dox–Cur, all the conditions of co-treatment were chosen to investigate the effects on cell number and viability of FLT-3 protein- expressing AML leukemic cells, including KG-1a, KG-1 and EoL-1 cells. The IC_{20} values of the Dox treatments obtained from the previous section were used to cotreat for 48 h with Cur. Dox–Cur conditions 1, 2, 3, and 4 (Dox (ng/mL) + Cur (μg/mL)) of KG-1a cells were 15.0 + 4.5, 16.0 + 5.5, 12.0 + 7.0, and 8.0 + 9.0, respectively. The conditions for KG-1 cells were 22.0 + 3.5, 10.0 + 4.5, 7.0 + 6.0, and 6.0 + 7.5, while the conditions of EoL-1 cells were 3.0 + 3.0, 0.7 + 4.0, 0.5 + 4.5, and 0.4 + 5.5, respectively. The results show that Dox concentrations at IC_{20} values and all co-treatment conditions consistently reduced the cell number of all leukemic cell lines (see supplementary Fig. S3A, S4A, S5A). The total cell number of KG-1a cells (control group) was 2.59×10^5 cells/mL, and they gradually decreased to 1.85×10^5, 1.59×10^5, 1.42×10^5, 1.24×10^5, and 0.98×10^5 cells/mL in response to Dox and Dox–Cur treatment conditions 1, 2, 3, and 4, respectively. The total cell number of KG-1 cells

decreased from 3.39×10^5 cells/mL (control group) to 2.58×10^5, 2.18×10^5, 2.00×10^5, 1.70×10^5, and 1.44×10^5 cells/mL in Dox and Dox–Cur treatment conditions 1, 2, 3, and 4, respectively. According to the cell number of EoL-1 cells after Dox and Dox–Cur conditions 1, 2, 3, and 4, the treatments reduced to 6.62×10^5, 5.77×10^5, 4.69×10^5, 4.01×10^5, and 3.27×10^5 cells/mL, respectively, compared to 12.07×10^5 cells/mL (control group). Cell viability of each sample was higher than 80% of the total cell count (see supplementary Fig. S3B, S4B, S5B).

The total cell number of Cur treatments at the IC_{20}, IC_{30}, IC_{40}, and IC_{50} values were observed to gradually decrease in all the cell lines in a dose dependent manner (see supplementary Fig. S3C, S4C, S5C). Moreover, these data also corresponded to the decline in the cell numbers during the Dox–Cur treatment. The cell viability for each concentration of Cur was also higher than 80% of the total cell count (see supplementary Fig. S3D, S4D, S5D).

Effects of combined treatments of Dox-Cur at concentration value of IC_{20} on FLT3 protein expressions in FLT-3 protein expressing leukemic stem cells and leukemic cells. FLT3 protein is a member of the class III receptor tyrosine kinase (RTK) family[32]. It is mostly found to overexpress on the cell surface of AML leukemic stem cells and leukemic cells and plays an important role in cell survival and proliferation of leukemic cell blasts[33]. Cur has previously been shown to have an inhibitory effect on FLT3 protein expression in many types of FLT-3 expressing leukemic cell lines, such as EoL-1 and MV4-11[27]. Thus, it was selected as a target protein for Dox–Cur treatment.

In this study, KG-1a, KG-1, and EoL-1 cells were treated with Dox and all conditions of co-treatment at a concentration value of IC_{20}, and FLT3 protein expression levels were detected by Western blotting. The results show that Dox and Dox–Cur co-treatment could decrease FLT-3 protein expression. In KG-1a cells, the FLT3 protein levels of Dox and Dox–Cur treatment conditions 1, 2, 3, and 4 were decreased by 20.4 ± 8.8%, 51.6 ± 14.5%, 54.6 ± 12.2%, 80.2 ± 5.7%, and 92.2 ± 8.2%, respectively (Fig. 2A, 2C). For KG-1 cells, FLT3 proteins were gradually decreased by 2.7 ± 6.5%, 42.3 ± 2.9%, 55.0 ± 5.4%, 52.0 ± 7.3%, and 57.9 ± 11.5% in respond to Dox and Dox–Cur conditions 1, 2, 3, and 4, respectively (Fig. 3A, 3C). Similarly to KG-1a and KG1 cells, FLT3 protein expression in EoL-1 cells was reduced to 2.7 ± 5.6%, 10.7 ± 4.2%, 29.9 ± 6.8%, 35.2 ± 6.4%, and 43.7 ± 15.7% in response to Dox and Dox–Cur conditions 1, 2, 3, and 4, respectively, compared to the control group (100% expression level) (Fig. 4A, 4C). Additionally, the ability of each concentration of Cur in the combination treatment to suppress FLT3 protein expression was evaluated. The results demonstrate that all concentrations of single Cur treatments were able to decrease the protein expression levels compared to the control group in a dose-dependent manner (Fig. 2B, 2D, 3B, 3D, 4B, 4D).

Effects of combination treatments of various concentrations of Cur and a fixed concentration of Dox on cell number and viability in leukemic stem cells and leukemic cells. According to the results presented in previous section, Cur and Dox–Cur treatments could inhibit AML LSC and LC cell proliferation more effectively than Dox treatment alone. Three non-toxic concentrations within the range of Cur's IC_{20} value

and a fixed concentration of Dox from Dox–Cur condition 1 were tested with KG-1a, KG-1, and EoL-1 cells for 48 h to confirm the impact of Cur on cell proliferation inhibition of Dox. The results demonstrate that the co-treatments of Dox–Cur significantly decreased the cell number of both cell lines in a dose dependent manner when compared to a single Dox treatment and control (Fig. 5). The cell number of KG-1a cells in the control group was $3.44 \cdot 10^5$ cells/mL, and decreased to 2.96×10^5, 2.21×10^5, 1.91×10^5, and 1.46×10^5 cells/mL in response to Dox, Dox + Cur at 4 µg/mL, Dox + Cur at 4.5 µg/mL, and Dox + Cur at 5 µg/mL, respectively (Fig. 5A). In addition, the cell number of KG-1 cells decreased from 4.18×10^5 cells/mL in the control group to 3.48×10^5, 2.93×10^5, 2.47×10^5, and 2.22×10^5 cells/mL in response to Dox, Dox + Cur at 3 µg/mL, Dox + Cur at 3.5 µg/mL, and Dox + Cur at 4 µg/mL, respectively (Fig. 5C). Moreover, the number of EoL-1 cells also decreased from 10.43×10^5 cells/mL in the control group to 8.99×10^5, 6.82×10^5, 6.08×10^5, and 4.94×10^5 cells/mL in response to the treatments of Dox, Dox + Cur at 2.5 µg/mL, Dox + Cur at 3 µg/mL, and Dox + Cur at 3.5 µg/mL, respectively (Fig. 5E). All samples demonstrated percentage of viable cells higher than 80% of the total cell count (Fig. 5B, 5D, 5F).

Effects of combination treatments of various concentrations of Cur and a fixed concentration of Dox on FLT3 protein expressions in leukemic stem cells. From the previous section, it can be observed that Cur and Dox-Cur co-treatments were more effective in suppressing FLT3 protein expression in all AML leukemic cell lines than single Dox treatment was. Moreover, the percentages of FLT3 protein in all conditions of co-treatments were also similar to those of Cur treatment alone. As the result, to confirm the effect of Cur in increasing the inhibitory effect of Dox on FLT3 expression, the co-treatment of Dox–Cur condition 1 was used. From the Western blotting, it was observed that all the non-toxic concentrations of Cur used in the study remarkably increased the efficacy of Dox in reducing the FLT3 protein expression in all three AML cell lines. The FLT3 expression level of KG-1a cells after treatment with Dox (15 ng/mL), Dox + Cur (4 µg/mL), Dox + Cur (4.5 µg/mL), and Dox + Cur (5 µg/mL) were decreased by 14.1 ± 5.2%, 35.8 ± 8.5%, 38.2 ± 3.3%, and 37.8 ± 7.0%, respectively, compared to the vehicle control (100% FLT3 protein expression level) (Fig. 6A, 6B). For KG-1 cells, the FLT3 protein levels were decreased by 17.9 ± 7.6%, 38.2 ± 13.0%, 39.7 ± 11.4%, and 47.2 ± 5.4% in response to Dox (22 ng/mL), Dox + Cur (3 µg/mL), Dox + Cur (3.5 µg/mL), and Dox + Cur (4 µg/mL), respectively (Fig. 6C, 6D) while the FLT3 protein expression levels in EoL-1 cells were reduced to 3.5 ± 8.9%, 10.2 ± 8.1%, 15.6 ± 7.1%, and 34.6 ± 8.9% in response to the treatments of Dox (2.8 ng/mL), Dox + Cur (2.5 µg/mL), Dox + Cur (3 µg/mL), and Dox + Cur (3.5 µg/mL), respectively, from 100% protein expression level of the vehicle control (Fig. 6E, 6F).

Discussion

In this experiment, doxorubicin (Dox) and idarubicin (Ida), standard chemotherapy for AML patients, were chosen as chemotherapeutic substance models to be studied. They can destroy leukemic cells by becoming incorporated into the DNA single strand, inhibiting topoisomerase II activity in DNA transcription, and triggering apoptosis or autophagy[13,34,35]. The cytotoxic activity of these anthracyclines was determined in each leukemic cell line by MTT assays. Both drugs showed the greatest cytotoxicity for EoL-1 cells, followed by U937, KG-1, and KG-1a cells. The inhibitory concentrations at cell growth

(IC_{50}) values of 50 of idarubicin on KG-1a and KG-1 cells were 19.82 ± 1.80 and 5.45 ± 0.89 ng/mL, respectively. In contrast, doxorubicin showed lower cytotoxicity than idarubicin with IC_{50} values of 0.65 ± 0.13 and 0.21 ± 0.02 µg/mL, respectively. This may have resulted from the absence of the methoxyl group at position 4 of idarubicin's structure which increased the lipophilicity and rate of cellular uptake, leading to greater toxicity than that of daunorubicin or doxorubicin[36].

In addition, the cytotoxicity of curcumin (Cur), a natural substance with chemosensitizing and chemoprotective activities[23], was also examined with four leukemic cell lines by MTT assay. The results showed that Cur demonstrated the highest cytotoxic effect on EoL-1 cells, followed by U937, KG-1, and KG-1a cells. Thus, Cur was selected as a supplementary substance for enhancing the efficiency and decreasing the toxicity of anthracycline drugs in this study.

From the preliminary experiment, the appropriated concentrations of Dox, Ida, and Cur were chosen for the combination effect study. The co-treatments of Ida and Dox at concentrations in the range of from IC_{10} to IC_{50} values and low concentrations of Cur at 1, 2, and 3 µg/mL did not show a different effect on cell viability of KG-1a and KG-1 cells, and compared to a single treatment (data not shown). Thus, for the new conditions, KG-1a, KG1-a, EoL-1, and U937 cells were treated with Dox and Ida at the concentration values of IC_{10}–IC_{80} combined with Cur at concentrations of IC_{20}, IC_{30}, IC_{40}, and IC_{50} values, respectively, and cytotoxicity of co-treatment was examined by MTT assay. To investigate the combination effect, the percentage of cell viability of each treatment was calculated and compared to that for the single drug and vehicle control, and the inhibitory concentrations at 20% (IC_{20}) and 50% (IC_{50}) were determined.

According to the cell viability curve, the co-treatment of Dox–Cur and Ida–Cur tended to increase the cytotoxicity for KG-1a, KG-1, EoL-1, and U937 cells in dose-dependent manners as compared to single drug treatment. Moreover, Cur also enhanced the cytotoxic efficacy on both chemotherapeutic drugs in dose dependent manners due to the lower IC_{50} values of anthracyclines used in co-treatment in each cell line.

Dox and Ida are usually ineffective due to an increase in LSCs, drug resistance, and relapse in AML patients. In this study, Cur which is a natural supplementary substance, was found to improve the cytotoxicity of Dox and Ida in all the cell lines due to its anti-leukemic (apoptotic induction)[37] and chemosensitizing (decreasing *MDR-1* gene expression)[28] activities. For these reasons, it could decrease the toxicity of both chemotherapies, resulting in lower IC_{50} values for drugs in co-treatments when compared with single drug treatments.

It is notable that effective doses of the co-treatments used to treat KG-1a cells were higher than those for KG-1, EoL-1, and U937 cells. KG-1a and KG-1 cells are leukemic stem cell lines with a high percentage of leukemic stem cells (~95% and ~55%, respectively). These cells are well-known for their chemotherapy resistance, which includes primary rest in the stage G_0 of the cell cycle and high expression of the drug efflux pump. Since the EoL-1 and U937 cells lack a stem cell population, they were more vulnerable to the co-treatments.

The combination treatment of Dox–Cur showed synergistic and additive cytotoxic effects on both AML leukemic stem cell lines (KG-1a and KG-1 cells) and AML leukemic cell lines (EoL-1 and U937 cells). Despite the fact that only Dox–Cur condition 3 showed synergism on KG-1a and EoL-1 cells, Cur was able to be used as a supplement to lower chemotherapeutic agent doses. The combination treatment also reduced the concentration at the IC_{50} value of Dox in each cell line which could be a useful formulation to decrease the cytotoxicity of Dox on normal cells. However, the poor solubility and short biological half-life of Cur, as well as the non-specific activity of Dox, resulted in low absorption and cytotoxicity of these drugs in tumor cells[13,24].

FLT3 is a key driver of AML, and its mutations are associated with the development of high risk of relapse in patients. Previous studies demonstrated that Cur has an inhibitory effect on FLT3 protein expression in leukemic cells[27]. Thus, the combination of Dox and Cur for AML treatment may lead to FLT3 protein expression reduction, which is involved in the proliferation process of leukemic cells.

In this study, the non-toxic doses at IC_{20} of four conditions of Dox–Cur co-treatments could decrease the cell number and showed a higher inhibitory effect on FLT3 protein expression than single Dox in both stem cell and leukemic cell lines. However, when compared to Cur alone, none of the co-treatments showed any differences. To confirm, Dox–Cur condition 1 was selected for the study of the inhibitory effect of various concentrations of Cur with a fixed concentration of Dox on cell number and FLT3 protein expression. The results showed the Dox–Cur treated samples exhibited the lower cell number and FLT3 protein level than single Dox samples in all cell lines. It is possible that Cur was the main compound in co-treatment that could suppress the FLT3 protein expression in a dose-dependent manner, leading to a decrease in cell proliferation, while the main functions of Dox, in order to eradicate tumor cells, involves inhibiting cell cycle progression, producing oxidation; and inducing apoptosis which is unrelated to the inhibition of FLT3 protein expression[13]. In addition, these functions of Dox could affect cell proliferation, resulting in the decrease in cell number in single Dox-treated samples. The co-treatment had an inhibitory effect on FLT3 protein expression. Notably, the IC_{20} values of Dox in Dox–Cur condition 1 (Dox 15 ng/mL + Cur 4.5 µg/mL) and 2 (Dox 16 ng/mL + Cur 5.5 µg/mL) in KG-1a cells were not reduced in a dose-dependent response to Cur increase. This might be because the chosen Cur concentration was too low, making the lowering of the Dox concentration in the reaction impractical, as shown by the cell number and FLT3 protein expression level.

This co-treatment likely assists in enhancing the cytotoxic effects of Dox by inhibiting the cell proliferation activity of AML leukemic stem cells and leukemic cells as a result of the decrease in the cell proliferation rate of each co-treatment condition compared to the single treatment and vehicle control. These findings are consistent with a previous study in which the combination of Dox with SU11657, a FLT3 inhibitor, increased the survival rate of APL mice and overcame resistance to traditional chemotherapies in AML[38].

In the future, the effects of Dox-Cur on cell cycle progression and apoptosis induction will be assessed to validate the mechanism of co-treatment's effect on cell proliferation inhibition and cell death.

Conclusion

Overall, anthracyclines (Dox and Ida) and Cur, a natural phenolic compound with anti-tumor activity, were shown to be effective AML chemotherapeutic agents. Our results show that the combination of Dox and Cur had a synergistic effect and could improve Dox anti-tumor activity in AML cells, particularly leukemic stem cells, by inhibiting cell proliferation through FLT-3 protein suppression. This finding presents an alternative choice that may be useful in the development of a promising regimen for the treatment of AML relapse in the future.

Methods

Reagents and Chemicals. Curcumin (Cur) was purchased from Thai-China Flavours and Fragrances Industry Co., LTD (Nonthaburi, Thailand). MTT dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), trypan blue, and DMSO were purchased from Sigma-Aldrich (St Louis, MO, USA). IMDM, RPMI-1640, penicillin-streptomycin, L-glutamine, and fetal calf serum were purchased from Invitrogen™ Life (Carlsbad, CA, USA). Rabbit polyclonal anti-GAPDH was purchased from Santa Cruz Bitechnology (Santa Cruz, CA, USA). HRP-conjugated goat anti-rabbit IgG was purchased from Promega (Madison, WI, USA). Rabbit polyclonal anti-FLT3 and Luminata™ Forte Western HRP Substrate were purchased from Merck Millipore Corporation (Billerica, MA, USA).

Cell Culture. KG-1a (acute myeloblastic leukemic cell line; ATCC® CCL-246.1™), KG-1 (acute myeloblastic leukemic cell line; ATCC® CCL-246™), and EoL-1 (acute myeloblastic leukemic cell line) were used as human leukemic cell line models in this study. KG-1a and KG-1 cells were cultured in IMDM medium (Invitrogen™, CA, USA) supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. EoL-1 (Eosinophilic leukemic cell line), a model of FLT3 overexpressing leukemic cells, was purchased from RIKEN BRC Cell Bank (Ibaraki, Japan). U937 (monoblastic leukemic cell line) was purchased from ATCC®. These were cultured in RPMI-1640 medium containing 10% fetal calf serum, 1 mM L-glutamine, 100 units/mL penicillin, and 100 mg/mL streptomycin. All the leukemic cell lines were cultured at 37°C in a humidified incubator with 5% CO_2.

Cytotoxicity of single doxorubicin, idarubicin, and curcumin (curcuminoid mixture) on leukemic stem cell and leukemic cell viability by MTT assay. KG-1a and KG-1 cell lines were adjusted to 1.5 ´ 10^4 cells, while EoL-1 and U937 cells were adjusted to 3.0 ´ 10^4 and 1.0 ´ 10^4 cells in 100 µL of complete medium, and then seeded into flat-bottom 96-well plate and incubated at 37°C under 5% CO_2 atmosphere for 24 h. Following that, doxorubicin (Dox), idarubicin (Ida), and curcumin (Cur) were diluted in 100 µL of medium with the 2-fold dilution technique and applied to the cells to obtain the final concentrations from 0.001 to 2 µg/mL for Dox and Ida and 1.56 to 50 µg/mL for Cur for 48 h. Complete medium and DMSO were used as cell control and vehicle control, respectively. Afterwards, 100 µL of medium was removed and 15 µL of MTT dye solution was added, and further incubated for 4 h. After removing the supernatant, 200 µL of DMSO were added to dissolve the formazan crystals (cell viability indication). The optical density was

measured using an ELISA plate reader at 578 nm with the reference wavelength at 630 nm. The percentage of cell survival was calculated from the absorbance of test and control wells using the equation (1), and the inhibitory concentration at 20% (IC_{20}) and 50% (IC_{50}) growth of Dox, Ida, and Cur were determined.

$$\% \text{ Cell viability} = \frac{\text{Absorbance of test}}{\text{Absorbance of vehicle control}} \times 100 \qquad (1)$$

Assessing cytotoxic effects of combination of the chemotherapeutic drug and curcumin on leukemic stem cell and leukemic cell viability by MTT assay. KG-1a, KG-1, EoL-1, and U937 cells were seeded into flat-bottom 96-well plate and incubated at 37°C under 5% CO_2 atmosphere for 24 h. Then, various concentrations of Dox and Ida in the range of IC_{10}-IC_{80} (from the cytotoxic effects of single treatment) were mixed with each concentration of Cur at IC_{20}, IC_{30}, IC_{40}, and IC_{50} values, as well as DMSO, to prepare the combination and single drug treatments, respectively. All the treatments were added to the cells and incubated for 48 h. The cell viability in each treatment was determined by the MTT assay.

Synergistic effects of combination treatment[39]. The combination index (CI) is used to quantitatively define the synergistic (CI < 1), additive (CI = 1), and antagonist effect (CI > 1) of a drug-drug interaction. It can be calculated by using the following equation (2):

$$CI = \frac{\text{Dose of drug in combination at ICx}}{\text{Dose of single drug at ICx}} \times \frac{\text{Dose of Cur in combination at ICx}}{\text{Dose of single Cur at ICx}} \qquad (2)$$

ICx = The concentrations required to produce the given effect, such as IC_{50}.

Cell number and cell viability of FLT3-exprssing cells determined by the trypan blue exclusion method. KG-1a, KG-1, and EoL-1 cells were adjusted to 1.5×10^5 and 3.0×10^5 cells/ mL, respectively, and incubated with non-toxic concentrations (IC_{20}) of Dox, Cur, and combination treatment at 37°C under 5% CO_2 atmosphere for 48 h. Then, the treated cells were collected, and their cell number and percent of cell viability were estimated using the trypan blue exclusion method by mixing the cells and the 0.4% trypan blue solution in a ratio of 1:1; following this, the cells were counted in a hemacytometer under a light microscope.

Western Blotting. KG-1a, KG-1, and EoL-1 cells were prepared and treated with Dox, Cur, and the co-treatment. After that, the cells were harvested after 48 h of incubation, and the whole proteins were extracted using RIPA buffer. The protein concentra-tion was measured with the Folin-Lowry method. The protein lysates were separated through 12% SDS-PAGE and then transferred to PVDF membranes. The membranes were blocked in 5% skim milk and probed by rabbit polyclonal anti-FLT3 and rabbit polyclonal anti-GAPDH antibody at a dilution of 1:1,000. The reaction was followed by HRP-conjugated goat anti-rabbit IgG at 1:15,000 dilution. The proteins were visualized using LuminataTM Forte Western HRP substrate. Finally, the protein band signal was quantified using a scan densitometer (Bio-Rad, CA, USA) or Fluorchem E Western blot and gel imager (ProteinSimple, CA, USA).

Statistical Analysis. The average of triplicate experiments and standard derivation (SD) were used for quantification. The levels of target protein expressions were compared to those of the vehicle control in each experiment. The results are shown as mean ± SD. The differences between the means of each sample were analyzed by one-way analysis of variance (one-way ANOVA). Statistical significance was considered at $p < 0.05$, $p < 0.01$, and $p < 0.001$.

Declarations

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Author contributions statment

Conceptualization, S.A. and C.B.; methodology, F.C.; validation, F.C. and S.T.; investigation, F.C. and S.T.; writing—original draft preparation, F.C. and S.A.; writing—review and editing, S.A., C.B., S.T., and S.C.; supervision, S.A. and C.B.; project administration, S.A.; funding acquisition, S.A. and C.B. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https//doi.org/

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References

1. Juliusson, G. *et al*. Age and acute myeloid leukemia: real world data on decision to treat and outcomes from the Swedish Acute Leukemia Registry. *Blood* **113**, 4179-4187. https://doi.org/10.1182/blood-2008-07-172007 (2009).
2. Roboz, G. J. & Guzman, M. Acute myeloid leukemia stem cells: seek and destroy. *Expert Rev. Hematol.* **2**, 663-672. https://doi.org/10.1586/ehm.09.53 (2009).
3. Dohner, H. *et al*. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood* **115**, 453-474. https://doi.org/10.1182/blood-2009-07-235358 (2010).
4. Sperr, W. R. *et al*. Human leukaemic stem cells: a novel target of therapy. *Eur. J. Clin. Invest.* **34** *Suppl 2*, 31-40. https://doi.org/10.1111/j.0960-135X.2004.01368.x (2004).
5. Dick, J. E. Acute myeloid leukemia stem cells. *Ann. N. Y. Acad. Sci.* **1044**, 1-5. https://doi.org/10.1196/annals.1349.001 (2005).
6. Bonnet, D. & Dick, J. E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat. Med.***3**, 730-737 (1997).
7. Tallman, M. S., Gilliland, D. G. & Rowe, J. M. Drug therapy for acute myeloid leukemia. *Blood* **106**, 1154-1163. https://doi.org/10.1182/blood-2005-01-0178 (2005).
8. Guan, Y., Gerhard, B. & Hogge, D. E. Detection, isolation, and stimulation of quiescent primitive leukemic progenitor cells from patients with acute myeloid leukemia (AML). *Blood* **101**, 3142-3149. https://doi.org/10.1182/blood-2002-10-3062 (2003).
9. Dean, M., Fojo, T. & Bates, S. Tumour stem cells and drug resistance. *Nat. Rev. Cancer* **5**, 275-284. https://doi.org/10.1038/nrc1590 (2005).
10. Zhou, S. *et al*. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat. Med.* **7**, 1028-1034. https://doi.org/10.1038/nm0901-1028 (2001).
11. Ravandi, F. & Estrov, Z. Eradication of leukemia stem cells as a new goal of therapy in leukemia. *Clin. Cancer Res.* **12**, 340-344. doi:10.1158/1078-0432.CCR-05-1879 (2006).
12. Blasiak, J. *et al*. Genotoxicity of idarubicin and its modulation by vitamins C and E and amifostine. *Chem. Biol. Interact.* **140**, 1-18. https://doi.org/10.1016/s0009-2797(02)00012-1 (2002).
13. Tacar, O., Sriamornsak, P. & Dass, C. R. Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. *J.Pharm. Pharmacol.* **65**, 157-170 (2013).
14. Szakacs, G., Paterson, J. K., Ludwig, J. A., Booth-Genthe, C. & Gottesman, M. M. Targeting multidrug resistance in cancer. *Nat. Rev. Drug Discov.* **5**, 219-234. https://doi.org/10.1038/nrd1984 (2006).
15. Yagi, T. *et al*. Identification of a gene expression signature associated with pediatric AML prognosis. *Blood* **102**, 1849-1856. https://doi.org/10.1182/blood-2003-02-0578 (2003).
16. Costello, R. T. *et al*. Human acute myeloid leukemia CD34+/CD38- progenitor cells have decreased sensitivity to chemotherapy and Fas-induced apoptosis, reduced immunogenicity, and impaired dendritic cell transformation capacities. *Cancer Res.* **60**, 4403-4411 (2000).

17. Ayton, P. M. & Cleary, M. L. Molecular mechanisms of leukemogenesis mediated by MLL fusion proteins. *Oncogene* **20**, 5695-5707. https://doi.org/10.1038/sj.onc.1204639 (2001).
18. Armstrong, S. A. *et al*. Inhibition of FLT3 in MLL. Validation of a therapeutic target identified by gene expression based classification. *Cancer Cell* **3**, 173-183. https://doi.org/10.1016/s1535-6108(03)00003-5 (2003).
19. Carvalho, C. *et al*. Doxorubicin: the good, the bad and the ugly effect. *Curr. Med. Chem.* **16**, 3267-3285. https://doi/10.2174/092986709788803312 (2009).
20. Vergely, C., Delemasure, S., Cottin, Y. & Rochette, L. Preventing the cardiotoxic effects of anthracyclines: from basic concepts to clinical data. *Heart Metab.* **35**, 1-7 (2007).
21. Mohan, M., Kamble, S., Gadhi, P. & Kasture, S. Protective effect of Solanum torvum on doxorubicin-induced nephrotoxicity in rats. *Food Chem. Toxicol.* **48**, 436-440. https://doi.org/10.1016/j.fct.2009.10.042 (2010).
22. Jobin, C. *et al*. Curcumin blocks cytokine-mediated NF-kappa B activation and proinflammatory gene expression by inhibiting inhibitory factor I-kappa B kinase activity. *J. Immunol.* **163**, 3474-3483 (1999).
23. Qian, H., Yang, Y. & Wang, X. Curcumin enhanced adriamycin-induced human liver-derived Hepatoma G2 cell death through activation of mitochondria-mediated apoptosis and autophagy. *Eur. J. Pharm. Sci.* **43**, 125-131. https://doi.org/10.1016/j.ejps.2011.04.002 (2011).
24. Anand, P., Sundaram, C., Jhurani, S., Kunnumakkara, A. B. & Aggarwal, B. B. Curcumin and cancer: an "old-age" disease with an "age-old" solution. *Cancer Lett.* **267**, 133-164. https://doi.org/10.1016/j.canlet.2008.03.025 (2008).
25. Anuchapreeda, S., Tima, S., Duangrat, C. & Limtrakul, P. Effect of pure curcumin, demethoxycurcumin, and bisdemethoxycurcumin on WT1 gene expression in leukemic cell lines. *Cancer Chemother. Pharmacol.* **62**, 585-594. https://doi.org/10.1007/s00280-007-0642-1 (2008).
26. Tima, S., Anuchapreeda, S., Ampasavate, C., Berkland, C. & Okonogi, S. Stable curcumin-loaded polymeric micellar formulation for enhancing cellular uptake and cytotoxicity to FLT3 overexpressing EoL-1 leukemic cells. *Eur. J. Pharm. Biopharm.* **114**, 57-68. https://doi.org/10.1016/j.ejpb.2016.12.032 (2017).
27. Tima, S. *et al*. Development and Characterization of FLT3-Specific Curcumin-Loaded Polymeric Micelles as a Drug Delivery System for Treating FLT3-Overexpressing Leukemic Cells. *J. Pharm. Sci.* **105**, 3645-3657. https://doi.org/10.1016/j.xphs.2016.09.010 (2016).
28. Shukla, S. *et al*. Curcumin inhibits the activity of ABCG2/BCRP1, a multidrug resistance-linked ABC drug transporter in mice. *Pharm. Res.* **26**, 480-487. https://doi.org/10.1007/s11095-008-9735-8 (2009).
29. Chen, T. H., Yang, Y. C., Wang, J. C. & Wang, J. J. Curcumin treatment protects against renal ischemia and reperfusion injury-induced cardiac dysfunction and myocardial injury. *Transplant. Proc.* **45**, 3546-3549. https://doi.org/10.1016/j.transproceed.2013.09.006 (2013).

30. Cohly, H. H., Taylor, A., Angel, M. F. & Salahudeen, A. K. Effect of turmeric, turmerin and curcumin on H2O2-induced renal epithelial (LLC-PK1) cell injury. *Free Radic. Biol. Med.* **24**, 49-54. https://doi.org/10.1016/s0891-5849(97)00140-8 (1998).
31. Wang, J., Ma, W. & Tu, P. Synergistically Improved Anti-tumor Efficacy by Co-delivery Doxorubicin and Curcumin Polymeric Micelles. *Macromol. Biosci.* **15**, 1252-1261. https://doi.org/10.1002/mabi.201500043 (2015).
32. Matthews, W., Jordan, C. T., Wiegand, G. W., Pardoll, D. & Lemischka, I. R. A receptor tyrosine kinase specific to hematopoietic stem and progenitor cell-enriched populations. *Cell* **65**, 1143-1152. https://doi.org/10.1016/0092-8674(91)90010-v (1991).
33. Zheng, R. *et al*. FLT3 ligand causes autocrine signaling in acute myeloid leukemia cells. *Blood* **103**, 267-274. https://doi.org/10.1182/blood-2003-06-1969 (2004).
34. Hope, K. J., Jin, L. & Dick, J. E. Human acute myeloid leukemia stem cells. *Arch. Med. Res.* **34**, 507-514. https://doi.org/10.1016/j.arcmed.2003.08.007 (2003).
35. Luo, L. & Han, Z. C. Leukemia stem cells. *Int. J. Hematol.* **84**, 123-127. https://doi.org/10.1532/IJH97.A10503 (2006).
36. Hollingshead, L. M. & Faulds, D. Idarubicin. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in the chemotherapy of cancer. *Drugs* **42**, 690-719. https://doi.org/10.2165/00003495-199142040-00010 (1991).
37. Reuter, S., Eifes, S., Dicato, M., Aggarwal, B. B. & Diederich, M. Modulation of anti-apoptotic and survival pathways by curcumin as a strategy to induce apoptosis in cancer cells. *Biochem. Pharmacol.* **76**, 1340-1351. https://doi.org/10.1016/j.bcp.2008.07.031 (2008).
38. Lee, B. D., Sevcikova, S. & Kogan, S. C. Dual treatment with FLT3 inhibitor SU11657 and doxorubicin increases survival of leukemic mice. *Leuk. Res.* **31**, 1131-1134. https://doi.org/10.1016/j.leukres.2006.11.012 (2007).
39. Chou, T. C. Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res.* **70**, 440-446 (2010).

Figures

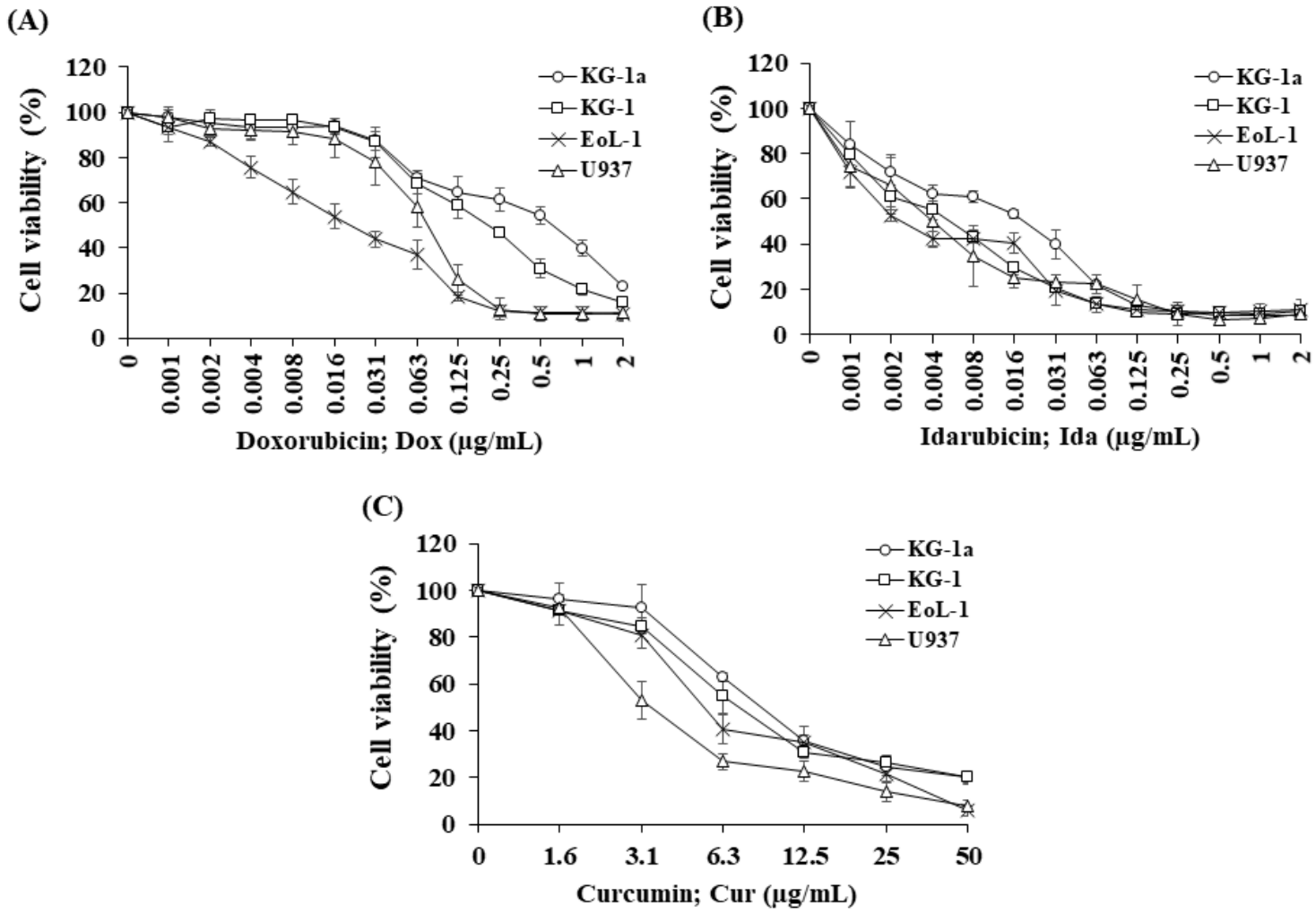

Figure 1

Cytotoxicity of (A) doxorubicin, (B) idarubicin, and (C) curcumin on KG-1a, KG-1, EoL-1, and U937 cells. The data are shown as mean ± SD from 3-time independent experiments.

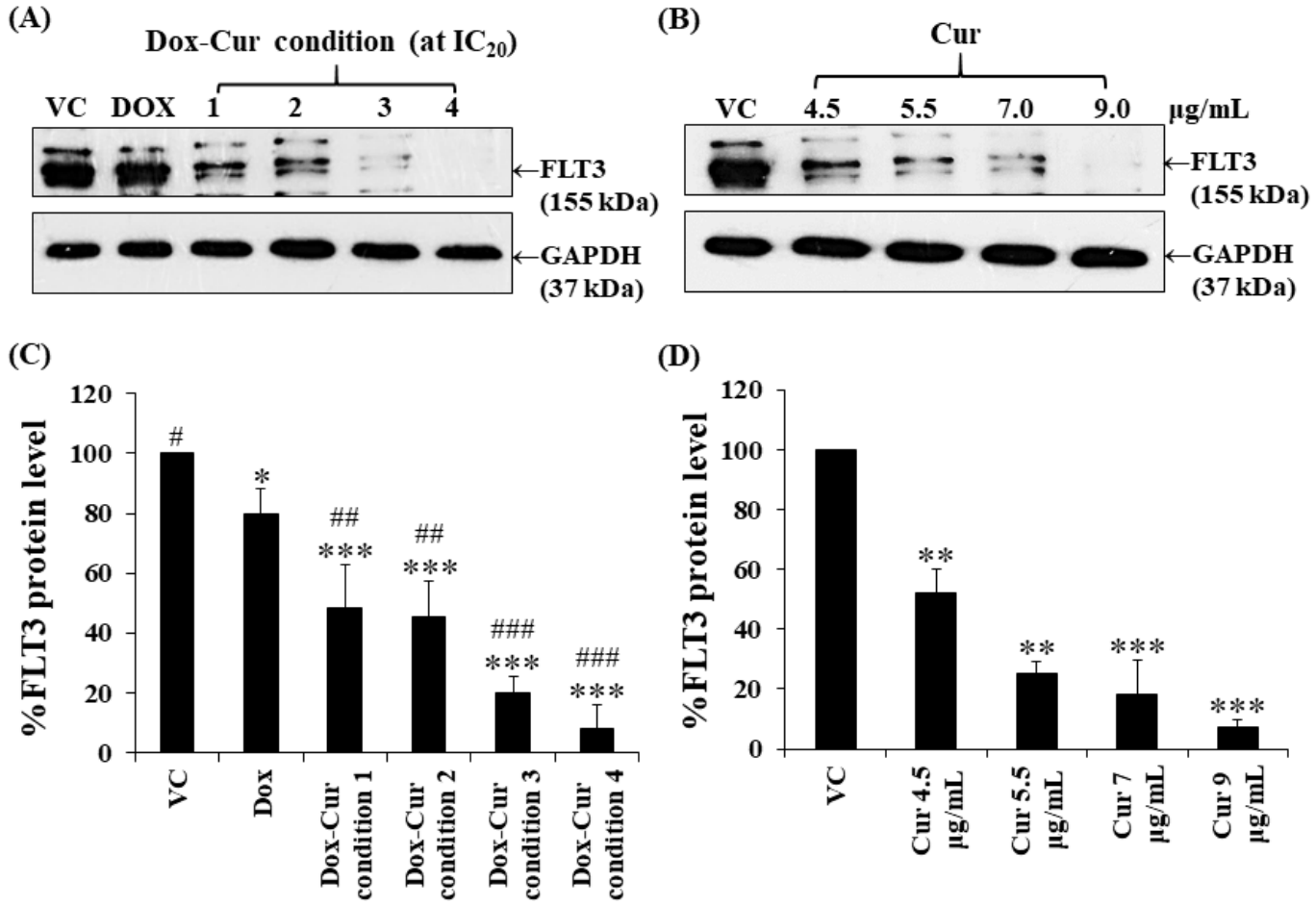

Figure 2

FLT3 protein expression of KG-1a cells after treatment with Dox and combined treatment of Dox–Cur at concentration value of IC20 for 48 h. Protein bands (A) and percentage (C) of FLT3 protein expression level of KG-1a cells treated with DMSO (VC),), Dox at concentration of 60 ng/mL, Dox–Cur condition 1 (15 ng/mL Dox + 4.5 µg/mL Cur), Dox–Cur condition 2 (16 ng/mL Dox + 5.5 µg/mL Cur), Dox–Cur condition 3 (12 ng/mL Dox + 7 µg/mL Cur), and Dox–Cur condition 4 (8 ng/mL Dox + 9 µg/mL Cur) for 48 h. Protein bands (B) and percentage (D) of FLT3 protein expression level of KG-1a cells treated with DMSO (VC), Cur (4.5 µg/mL), Cur (5.5 µg/mL), Cur (7 µg/mL), and Cur (9 µg/mL) for 48 h. The data are shown as mean ± SD from 3 independent experiments. The significance of mean differences was assessed using one-way ANOVA. *p < 0.05, **p < 0.01, and ***p < 0.001 compared to VC. #p < 0.05, ##p < 0.01, and ###p < 0.001 compared to single Dox treatment. Full-length blots are presented in Supplementary Figure S6.

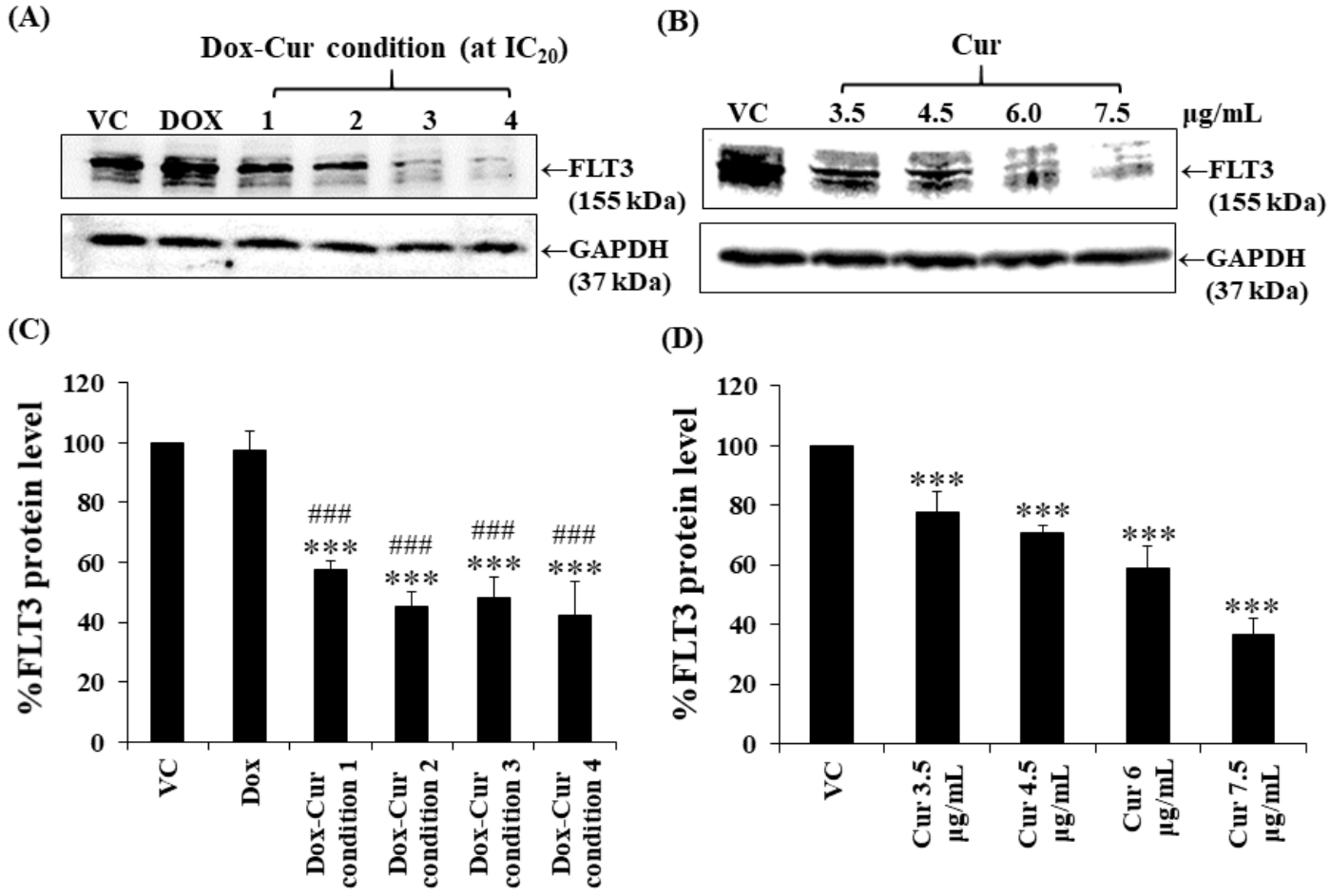

Figure 3

FLT3 protein levels of KG-1 cells after treatment with Dox and Dox–Cur at concentration value of IC20 for 48 h. Protein bands (A) and percentage (C) of FLT3 protein levels of KG-1 cells were from DMSO (VC), Dox (58 ng/mL), Dox–Cur condition 1 (22 ng/mL Dox + 3.5 µg/mL Cur), condition 2 (10 ng/mL Dox + 4.5 µg/mL Cur), condition 3 (7 ng/mL Dox + 6 µg/mL Cur), and condition 4 (6 ng/mL Dox + 7.5 µg/mL Cur). Protein bands (B) and percentage (D) of FLT3 protein level of KG-1 cells were from DMSO (VC), Cur (3.5 µg/mL), Cur (4.5 µg/mL), Cur (6 µg/mL), and Cur (7.5 µg/mL) for 48 h. The data are shown as mean ± SD from 3 independent experiments. The significance of mean differences was assessed using one-way ANOVA. *p < 0.05, **p < 0.01, and ***p < 0.001 compared to VC. #p < 0.05, ##p < 0.01, and ###p < 0.001 compared to single Dox treatment. Full-length blots/gels are presented in Supplementary Figure S7.

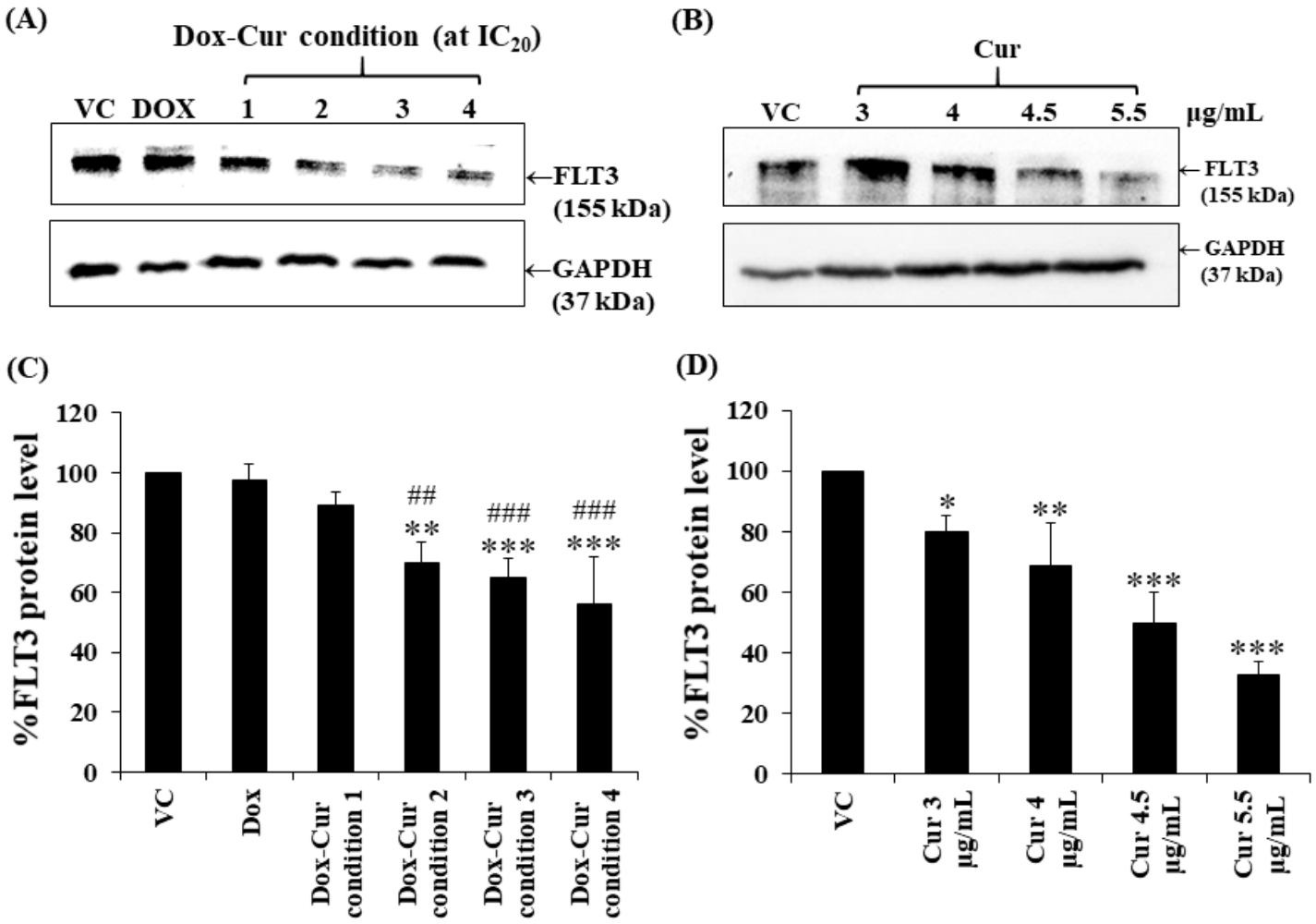

Figure 4

FLT3 protein expression of EoL-1 cells after treatment with Dox and combined treatment of Dox–Cur at concentration value of IC20 for 48 h. Protein bands (A) and percentage (C) of FLT3 protein expression level of EoL-1 cells treated with DMSO (VC), Dox at concentration of 6 ng/mL, Dox–Cur condition 1 (3 ng/mL Dox + 3 µg/mL Cur), Dox–Cur condition 2 (0.7 ng/mL Dox + 4 µg/mL Cur), Dox–Cur condition 3 (0.5 ng/mL Dox + 4.5 µg/mL Cur), and Dox–Cur condition 4 (0.4 ng/mL Dox + 5.5 µg/mL Cur) for 48 h. Protein bands (B) and percentage (D) of FLT3 protein expression level of EoL-1 cells treated with DMSO (VC), Cur (3 µg/mL), Cur (4 µg/mL), Cur (4.5 µg/mL), and Cur (5.5 µg/mL) for 48 h. The data are shown as Mean ± SD from 3 independent experiments. The significance of mean differences was assessed using one-way ANOVA. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to VC. #$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$ compared to single Dox treatment. Full-length blots/gels are presented in Supplementary Figure S8.

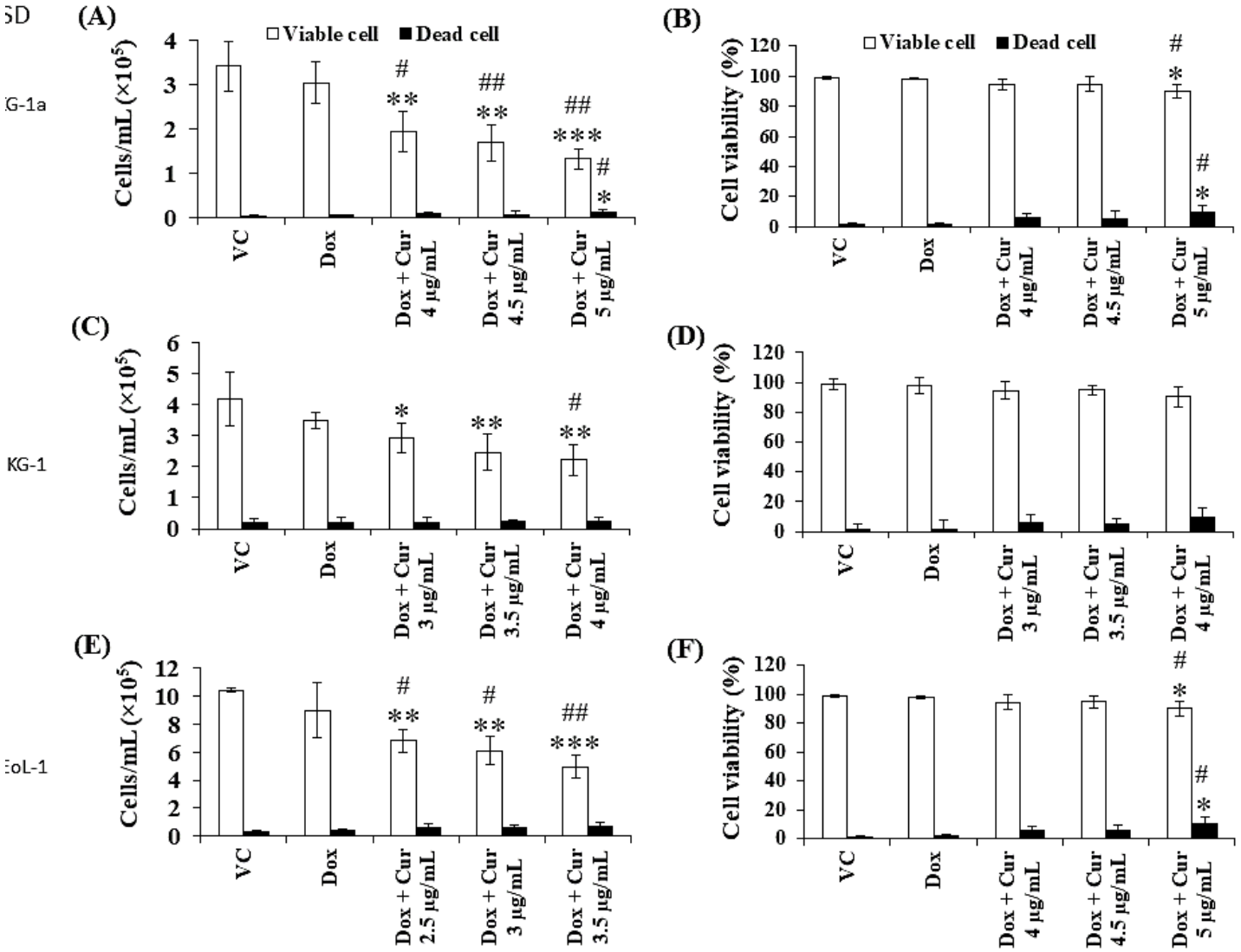

Figure 5

Total cell number and cell viability of KG-1a, KG-1, and EoL-1 cells after treatment with Dox and combination of fixed concentration of Dox and various non-toxic concentration of Cur for 48 h. Total cell number (A) and cell viability (B) of KG-1a cells were treated with DMSO (VC), Dox (15 ng/mL), Dox + Cur (4 µg/mL), Dox + Cur (4.5 µg/mL), and Dox + Cur (5 µg/mL) for 48 h. Total cell number (C) and cell viability (D) of KG-1 cells were treated with DMSO (VC), Dox (22 ng/mL), Dox + Cur (3 µg/mL), Dox + Cur (3.5 µg/mL), and Dox + Cur (4 µg/mL) for 48 h. Total cell number (E) and cell viability (F) of EoL-1 cells were treated with DMSO (VC), Dox (2.8 ng/mL), Dox + Cur (2.5 µg/mL), Dox + Cur (3 µg/mL), and Dox + Cur (3.5 µg/mL) for 48 h. The data are shown as mean ± SD from 3 independent experiments. The significance of mean differences was assessed using one-way ANOVA. *p < 0.05, **p < 0.01, and ***p < 0.001 compared to VC. #p < 0.05, ##p < 0.01, and ###p < 0.001 compared to single Dox treatment.

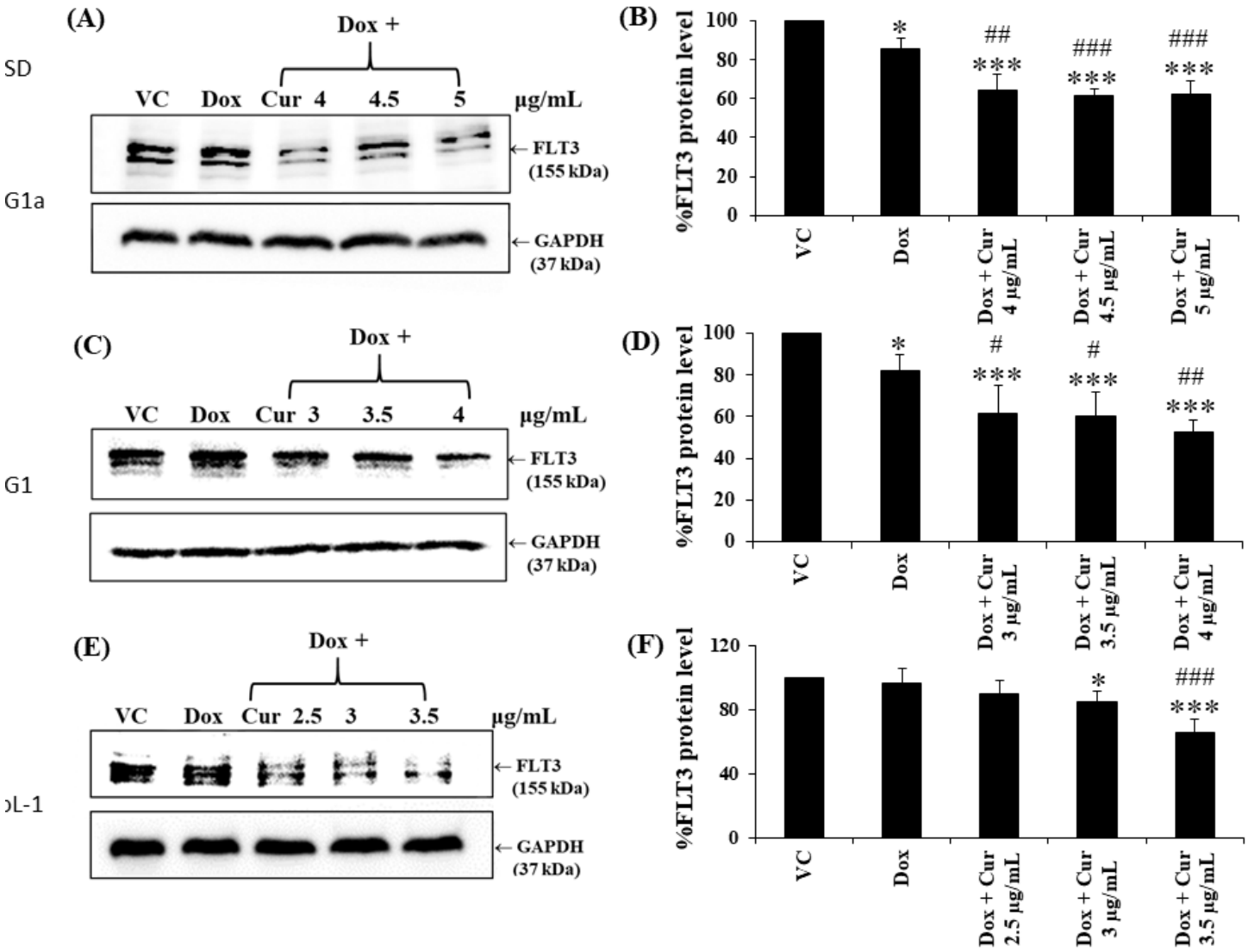

Figure 6

FLT3 protein expression of KG-1a, KG-1, and EoL-1 cells following treatment with Dox and combination of fixed concentration of Dox and various non-toxic concentrations of Cur for 48 h. Protein bands (A) and percentage (B) of KG-1a cells treated with DMSO (VC), Dox (15 ng/mL), Dox + Cur (4 µg/mL), Dox + Cur (4.5 µg/mL), and Dox + Cur (5 µg/mL), for 48 h. Protein band (C) and percentage (D) of KG-1 cells treated with DMSO (VC), Dox (22 ng/mL), Dox + Cur (3 µg/mL), Dox + Cur (3.5 µg/mL), and Dox + Cur (4 µg/mL) for 48 h. Protein band (E) and percentage (F) of EoL-1 cells were treated with DMSO (VC), Dox (2.8 ng/mL), Dox + Cur (2.5 µg/mL), Dox + Cur (3 µg/mL), and Dox + Cur (3.5 µg/mL) for 48 h. The data are shown as mean ± SD from 3 independent experiments. The significance of mean differences was assessed using one-way ANOVA. *p < 0.05, **p < 0.01, and ***p < 0.001 compared to VC. #p < 0.05, ##p < 0.01, and ###p < 0.001 compared with single Dox treatment. Full-length blots/gels are presented in Supplementary Figure S9.

Supplementary Files

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