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ORIGINAL PAPER

Tomatidine, a Steroidal Alkaloid, Synergizes with Cisplatin to Inhibit Cell Viability and Induce Cell Death Selectively on FLT3-ITD+ Acute Myeloid Leukemia Cells

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Abstract

Background Acute Myeloid Leukemia (AML) is a hematological cancer that frequently presents with a range of side effects and drug resistance during anticancer drug treatment. The current study aims to achieve increased efficacy by combining lower doses of cisplatin with increasing concentrations of tomatidine in AML cells to increase efficacy.

Methods Anti-proliferative effects of single and combination of cisplatin and tomatidine were assessed via MTT cell viability assay. The Annexin V/Propidium Iodide Double Staining method was used to measure the apoptotic effects of combined tomatidine and cisplatin treatment. Then, Western Blot analysis was performed to measure Poly (ADP-ribose) polymerase (PARP) and Caspase-3 protein expression levels.

Results Cisplatin treatment with lower concentrations displayed high cytotoxic effects on AML cells, compared with tomatidine. The combination of the Inhibitory Concentration (IC) 20 value of cisplatin and increasing doses of tomatidine exhibited a significant decrease in cell viability relative to single treatments. The combination index analysis revealed a mild synergistic effect of cisplatin IC20 and varying tomatidine doses. The apoptosis induced when cisplatin was combined with 500 µM tomatidine by almost 20%, while the percentage of apoptosis in combination with 1 mM tomatidine was measured by 50% for both cell lines. The upregulation of proapoptotic cleaved-PARP (3.2 and 1.08-fold for THP-1 and MOLM-13, respectively) and downregulation in Caspase-3 (0.23 and 0.13-fold for THP-1 and MOLM-13, respectively) was detected.

Conclusions Together, the study indicated that when tomatidine combined with cisplatin on AML cell lines, a combinatorial anti-proliferative and apoptotic effect is observed. The combination of cisplatin with tomatidine may be a promising approach.

Keywords Cancer · Tomatidine · Cisplatin · Apoptosis · Acute myeloid leukemia · Combination therapy

Introduction

Acute Myeloid Leukemia (AML) is a hematopoietic stem cell differentiation disorder in myeloid tissue [1, 2]. AML malignancy arises from hematopoietic stem and precursor cells (HSPCs) [1–3]. It is characterized as genetically heterogeneous since multiple mutation-related genetic alterations occur during the differentiation of myeloid cells [4, 5]. Mutations of the FLT3 gene are one of the standard diagnosis markers in AML as almost 30% of all AML cases occur due to the FMS-like tyrosine kinase 3 (FLT3) receptor mutation. The mutated gene affects the FMS-like tyrosine kinase 3 (FLT3) receptor. In a prominent aspect, the mutations could be either point mutations/deletion in the tyrosine kinase domain (FLT3-TKD) or internal tandem duplication mutations at the juxtamembrane domain (FLT3-ITD) of the receptor. These two major mutations keep the FLT3 receptor always active in a ligand-independent manner. Since the FMS-like tyrosine kinase 3 (FLT3) receptor is responsible for a hematopoietic cell's proliferation, differentiation and survival, these mutations result in abnormalities in hematopoietic cells [6, 7]. In genetically-based

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recurrent abnormalities, FLT3 (ITD version – around 20–25%) and NPM1 mutations are prevalent mutations seen in AML diagnosis [6].

This highly malignant cancer type has various treatment options, such as stem cell transplantation, radiotherapy, specific biological treatment techniques, and chemotherapy [3]. Cisplatin, Cisplatinum, or cis-diamminedichloroplatinum (II) is a platinum-containing chemotherapeutic drug preferred for AML treatment [8, 9]. The action mechanism of cisplatin involves a crosslink between purine bases in DNA. The DNA repair mechanism recognizes crosslinked purine bases as DNA damage, and the apoptosis mechanism is induced [9]. However, cisplatin is a highly toxic anticancer drug [10]. Its side effects and drug resistance potency are accepted as the significant harmful impacts of this FDA-approved anticancer drug. Combination therapies with other anticancer drugs and natural products have been used to treat AML to overcome side effects and drug resistance [8, 9]. So far, it is discovered that combining cisplatin with natural anticancer agents increases the chemosensitivity of carcinogenic cells against cisplatin as well as being a great alternative to combinatorial chemotherapy.

The current study covers the combinational effect of the FDA-approved anticancer drug cisplatin and the natural product, tomatidine. Tomatidine and its glycoside α-tomatine are plant-based steroidal alkaloids that are generally isolated from *S. lycopersicon L.* (tomato) and *S. aculeastrum* (Goat bitter apple/soda apple) because of their high abundance [11]. Tomatine is the actual chemical material found in Goat bitter apple/soda apple. The actual conversion of tomatidine from α-tomatine occurs in living organisms' intestines [11–13]. It is classified as anti-metastatic, anti-inflammatory, and anti-proliferative in terms of cell signaling mechanism [12]. Besides the effectiveness on muscle atrophy and weakness, the anticancer effect of tomatidine and its derivatives (i.e. glycosides) has been proven on various cancer types [14]. Tomatidine is known for its low cytotoxic effect when compared to tomatine [11]. Tomatidine does not show enormous declines in cell viability up to a concentration of 100 µM treatment [11, 15]. Although there are biological evaluations of tomatidine on various types of cancers, the actual mechanism is not well understood in AML.

The purpose of the current study is to reveal the combinational effect of tomatidine and cisplatin and achieve increased efficacy by using low cisplatin doses together with tomatidine. This study also aims to compare the FLT3-ITD-positive and FLT3 wild-type AML cell lines in the course of the study by using two different cell lines, MOLM-13 and THP-1, which are FLT3-ITD-positive and FLT3 wild-type, respectively. It is believed that using such a combination is beneficial to use decreased cisplatin concentration for therapy.

Materials and Methods

Chemicals

Tomatidine-HCl, cisplatin and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) were purchased from Sigma. The stock solution concentration of tomatidine-HCl was calculated as 27.65 µM, prepared in DMSO, and stored at −20 °C. Cisplatin was prepared in NaCl as 3.3 mM and stored at +4 °C. RPMI-1640, Fetal Bovine Serum (FBS), Penicillin/streptomycin and trypsin were purchased from Euroclone.

Cell Culture and Maintenance

MOLM-13 was obtained from the German Collection of Microorganisms and Cell Cultures (DMSZ). THP-1 cell line was kindly provided by Dr. Aysun Adan, Abdullah Gul University. PCS 201-012 human fibroblast cell line was kindly donated. The cells were grown in RPMI 1640 medium that contained 100 U/mL of penicillin and streptomycin and 10% FBS as supplements and were maintained in a 5% CO_2 environment at 37 °C. The cells were seeded at a density of 1×10^6 cells/ml after they reached confluency, and they were split at ratios of 1:2 every two days. Thereafter, the cells were gathered and centrifuged at 700 rpm for 5 min. After discarding the supernatant, fresh media were used to reconstitute the pellet.

Cell Viability Assay

MTT assay was used to measure the anti-proliferative effects of cisplatin and tomatidine on MOLM-13, THP-1 and fibroblast cell lines. 1×10^4 cells/well were seeded into each well of 96-well plates containing 200 µl of growth medium. The single and combination treatments of cisplatin, tomatidine, and their combinations were performed for 24, 48, and 72 h. The IC (drug concentration that inhibits cell growth) of cisplatin and tomatidine was determined by the viability assay. After incubation, 10 µL MTT dye (Sigma) was added to cells and incubated for 2–4 h in order to allow for formation of formazan crystals. Afterward, the plates were centrifuged at 1800 rpm for 10 min, then formazan crystals were dissolved in 100 µL DMSO. Formazan crystal intensity was measured at 570 nm by Varioskan™ LUX multimode microplate reader (Thermo Scientific™). GraphPad Prism Version 8.0.2 was used to determine the IC10, IC20, IC30 values of cisplatin.

Combination Index (CI) Analysis – Isobologram Test

The synergistic and/or antagonistic effects of cisplatin and tomatidine co-treatments were analysed via a Windows

Software Program, CalcuSyn (CalcuSyn software, Biosoft, Cambridge, UK). Experimental data points represented placed below, on, or above the line symbolize synergism, additivity, and antagonism, respectively. The effects of the drug combination were evaluated using the combination index (CI) based on Chou-Talalay's multidrug effect equation [16]. A CI value < 1 indicates a synergistic effect (0.1–0.5 strong synergism; < 0.1 very strong synergism); a CI value of 1 indicates an additive effect; and a CI value > 1 an antagonistic effect (3.3–10 strong antagonism; > 10 very strong antagonism).

Apoptosis Assay

The apoptotic cell death was assessed by using a dual staining via Annexin V-FITC apoptosis detection kit (Biolegend). 1×10^6 cells/well were treated with IC20 (inhibitory concentration of 20% of the cells) value of cisplatin (2.6 µM for THP-1 and 0.9 µM for MOLM-13) in combination with 500 µM and 1 mM tomatidine for 48 h in 6-well plate. After incubation, cells were collected at 1700 rpm for 5 min at 4 °C, washed with cold PBS (twice) and resuspended with 200 µL 1X Annexin binding buffer. Then, 2 µL Annexin V-FITC and 4 µL Propidium Iodide (PI) (Biolegend) were added to each obtained cell suspension. Following incubation at room temperature for 15 min, apoptotic cells were detected using a BD FACSAria III Cell Analyzer [17].

Protein Extraction and Western Blot

5×10^6 cells/well were treated tomatidine 500 µM alone, and in combination with the IC20 of cisplatin for 48 h to detect the expression levels of PARP and caspase-3 by western blot. Following the incubation, the cells were lysed in RIPA buffer (50 mM Tris-HCl pH:8.0, 1% NP-40, 150 mM NaCl, 0.1% SDS, 0.5% Sodium deoxycholate, protease inhibitor). The protein quantification was conducted via BioRad DC™ Protein Quantification Kit (Bio-Rad, USA). 20 µg/well total proteins were separated by 8–15% SDS-PAGE and transferred to the PVDF membrane. The membrane was blotted with primary antibodies for PARP (1:3,000, Cell Signaling), caspase-3 (1:1,000, Santa Cruz), and GAPDH (1:2,000, Santa Cruz) overnight at 4 °C and then conjugated with appropriate secondary antibodies (peroxidase affiniPure goat anti-mouse IgG (1:10,000)). The membranes were visualized with Pierce ™ ECL Western Blotting Substrate kit (Thermo Scientific™, USA). Proteins were detected via chromogenic substrate (ECL). The intensity of immunoreactive bands and their densitometric analysis were carried out using imaging software (Bio-Rad, ChemiDoc, Image Lab™ 3.0).

Statistical Analysis

Each independent experiment was repeated three independent times ($n = 3$) and illustrated as the mean ± standard deviation (SD). One-way ANOVA and Two-way ANOVA were used to analyze the obtained data, followed by Tukey's Multiple Comparison Test for statistical significance. The significance of the data was interpreted by considering a $p < 0.0001$.

Results

Cytotoxic Effect of Tomatidine and Cisplatin on Acute Myeloid Leukemia Cell Lines

In order to determine the antiproliferative effects of the compounds, the cells were treated with increasing concentrations of tomatidine (50 to 500 µM) and cisplatin (0.5 to 20 µM) for three different time points. The cell viability reduction was detected for both THP-1 and MOLM-13 cells in a time- and dose-dependent manner compared to the untreated control (Fig. 1). However, the sensitivity of cells against the same drug treatment varied. While single tomatidine treatment at 500 µM concentration reduced the cell viability of THP-1 cells as 42.5%, 74%, and 90.6% (Fig. 1a), the recorded reductions for MOLM-13 cells were 73.7%, 90.1%, 91.5% at 24, 48, and 72 h experiment sets respectively (Fig. 1b). Following of single tomatidine treatment, similar sensitivity differences were recorded in single cisplatin treatments as well. As is shown in Fig. 1c, d, cisplatin demonstrated a time- and dose-dependent inhibition of cell viability. While 1 µM cisplatin concentration resulted in a significant decrease for 24 h and 48 h in cell viability, the same drug administration of cisplatin did not significantly affect the cell viability of THP-1 cells. Surprisingly, the effect of 5 µM cisplatin administration on MOLM-13 cells was almost equivalent to 20 µM cisplatin treatment of THP-1 cells.

In addition to the dose determination process, the effect of the tomatidine on healthy fibroblast cells was evaluated to assess the cytotoxicity. We aimed to test the concentration scale that we used on AML cells as well as the very high concentration of tomatidine to check its safety. The fibroblast cell was treated with tomatidine from 50 µM to extremely high 5 mM concentration for 48 h (Fig. 1e). The fibroblast viability was not significantly affected with the tomatidine at doses of 50 µM and 100 µM which we used on AML cells. Tomatidine showed a 25.1% reduction at 200 µM concentration, while 500 µM and 5 mM concentrations resulted in a 43.5% and 79.5% decrease in cell viability, respectively,

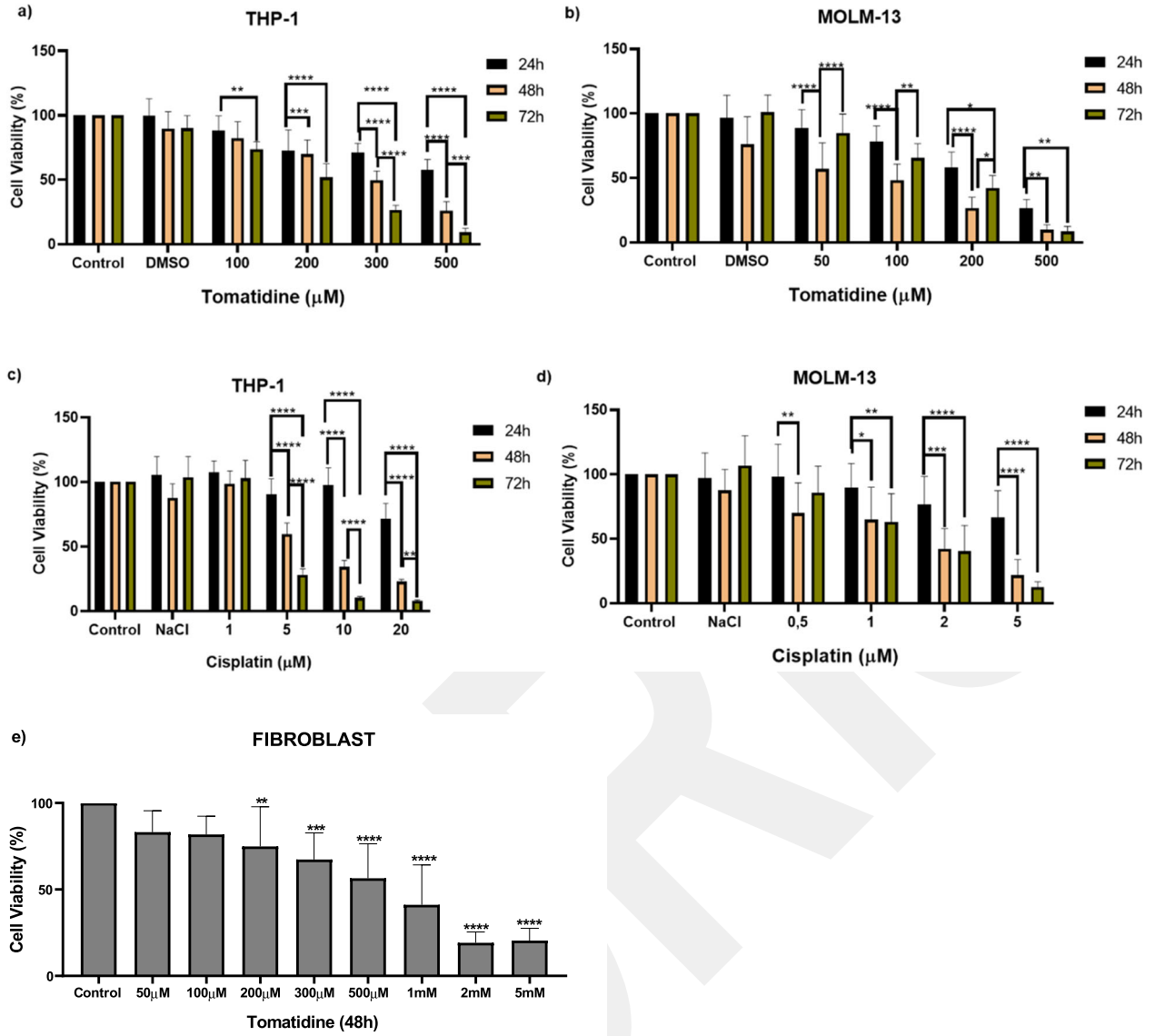

Fig. 1 The cell viability effect concentrations of tomatidine and cisplatin on THP-1 (**a**, **c**), MOLM-13 (**b**, **d**) for 24 h, 48 h, and 72 h. The effect of tomatidine on fibroblast cells are assessed for 48 h (**e**). Experiments are shown as means ± SD of three independent experimental setups in triplicates ($n = 3$) *(*$P < 0.05$, **$P < 0.005$, ***$P < 0.001$, ****$P < 0.0001$)*

compared to DMSO-treated control cells. In conclusion, 500 μM and 1 mM tomatidine were chosen for further experiments that were considered as subtoxic concentrations on fibroblasts.

Combined Cytotoxicity Effects of Tomatidine and Cisplatin Combination along with Their Synergistic Effect on AML Cell Lines

To understand whether the combination of cisplatin and tomatidine has a synergistic combinatorial inhibitory effect on cell viability, increasing concentrations of tomatidine (from 100 to 500 μM) was combined with constant Inhibition Concentration (IC) 10 (1.4 μM; 0.6 μM), 20 (2.6 μM; 0.9 μM), and 30 (3.7 μM; 1.3 μM) values of cisplatin for 48 h on both cell lines (THP-1; MOLM-13) (Fig. 2a, b). The most effective results were obtained from the combinatorial administration of IC20 of cisplatin with increasing concentrations of tomatidine on AML cells in cell proliferation when compared to both untreated controls and single drug treatments (Fig. 2a, b). Then we proceed by combining IC20 value of cisplatin and increasing tomatidine doses from 50 to 500 μM on THP-1 and MOLM-13 cells (Fig. 2c–f). In THP-1, the combination of 500 μM with IC20 of cisplatin demonstrated a significant decrease in cell viability when compared to single cisplatin and low-dose tomatidine combinations (Fig. 2c, d). Besides, for MOLM-13, the

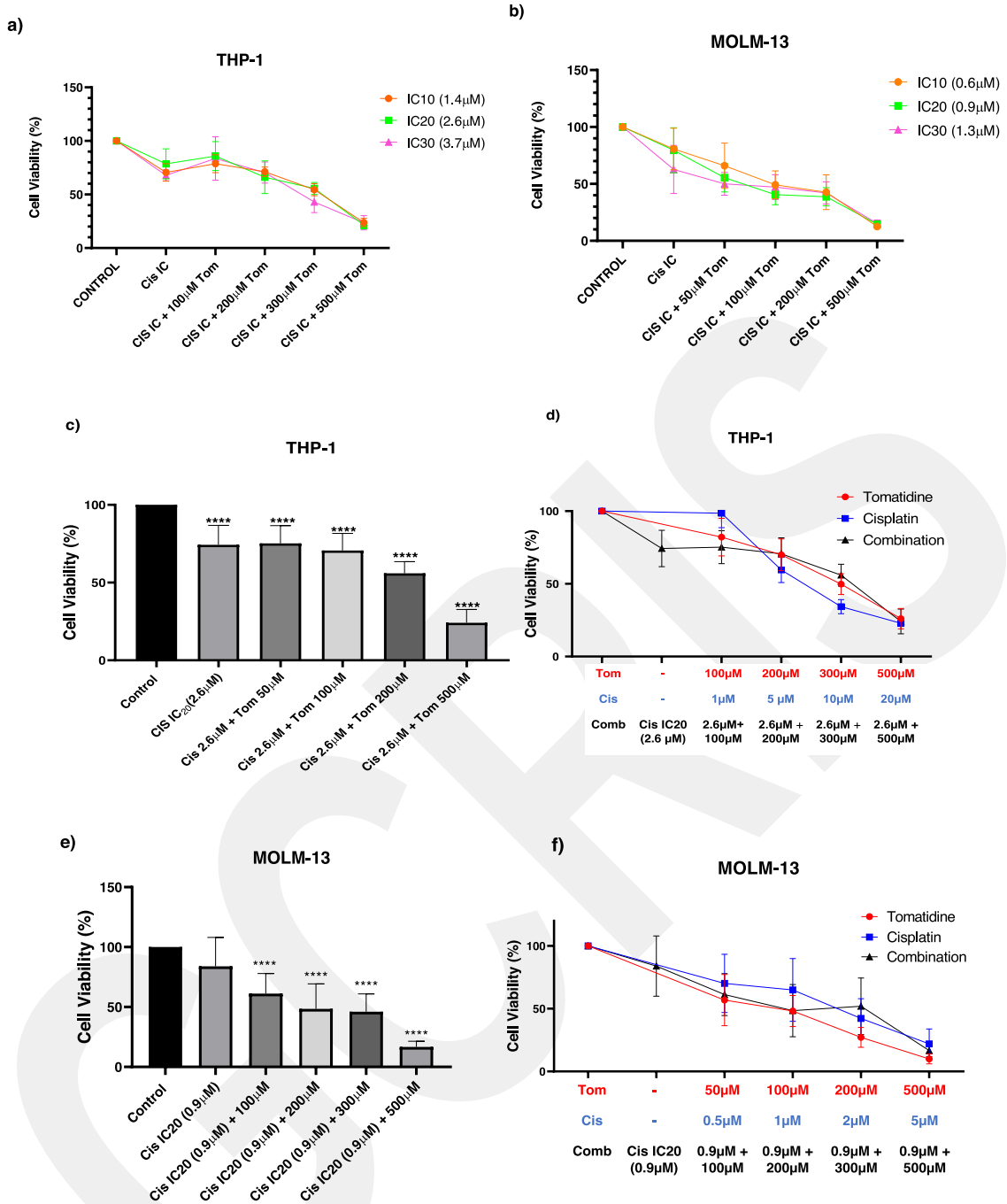

Fig. 2 Combined cytotoxic effects of cisplatin and tomatidine on THP-1 and MOLM-13 cells for 48 h of drug treatment. The overall combinatorial effects of IC10 (1.4 μM–0.6 μM), IC20 (2.6 μM–0.9 μM) and IC30 (3.7 μM–1.3 μM) for THP-1 (**a**) and MOLM-13 (**b**) cells. The combined cytotoxic effect of IC20 cisplatin with increasing concentrations of tomatidine on THP-1 (**c**, **d**) and MOLM-13 (**e**, **f**) cells. In Fig. 2d, f, the comparison of single cisplatin, tomatidine, and combinational treatments are demonstrated with circle, square lines, and triangle lines, respectively. Experiments are shown as means ± SD of three independent experimental setups in triplicates (n = 3) *(***P < 0.001, ****P < 0.0001)*

combination of 0.9 μM cisplatin with 500 μM tomatidine showed a better decrease in cell viability when compared to THP-1 cells (Fig. 2e, f).

To further elucidate the effect of the combination of cisplatin and tomatidine, the isobologram analysis was applied to investigate the synergistic effect of cisplatin and tomatidine combination for both cell lines via computational study. The CI (Combination Index) values for IC20 of cisplatin in combination with tomatidine were listed in Table 1 for THP-1 and MOLM-13 cells.

In THP-1 cells, the combination of 500 µM tomatidine and 2.6 µM cisplatin indicated a mild synergistic effect detected with computational studies (Fig. 3, Table 1). It is also noteworthy to add that while single cisplatin IC20 value caused a 25.7% reduction in cell viability of THP-1 cells, this decrease was detected as 75.9% due to the combination of cisplatin IC20 (2.6 µM) with 500 µM tomatidine under in vitro conditions (Fig. 2c). When this combination effect was compared with single tomatidine treatments in Fig. 2d, the cell viability declined 74% after 500 µM tomatidine treatment. The results of the computational study revealed that the administered combination of 0.9 µM cisplatin and 50 µM tomatidine was the only detected administration as the synergistic combinatorial effect in the case of MOLM-13. The combination of the increasing tomatidine doses showed an additive or nearly additive effect (Fig. 3). In the case of in vitro MOLM-13, the combination of 0.9 µM (IC20 value) cisplatin with 500 µM tomatidine 83.4% inhibition in cell viability was detected. It was a 16.01% (Fig. 2e) and 90.1% (Fig. 2f) reduction in cell viability due to the treatment of single IC20 cisplatin and a single 500 µM tomatidine, respectively.

To sum up, the concentration of tomatidine in the synergy case varied for THP-1 (500 µM concentration was synergistic) and MOLM-13 (50 µM concentration was synergistic), assumably due to their different genetic backgrounds. Unlike computational study results, 500 µM tomatidine with IC20 cisplatin concentrations introduced significant reductions in cell viability compared to single IC20 cisplatin and single 500 µM tomatidine treatments.

Table 1 Combination Index (CI) values of MOLM-13 and THP-1 cells treated by constant cisplatin with increasing tomatidine concentrations

	Cisplatin only	Tomatidine only	Cisplatin:tomatidine combination
MOLM-13	**Dose**	**Dose**	**CI value**
	0.9 µM	50 µM	0.89316
	0.9 µM	100 µM	1.02366
	0.9 µM	200 µM	1.89585
	0.9 µM	500 µM	1.42862
THP-1	2.6 µM	100 µM	1.26107
	2.6 µM	200 µM	1.24499
	2.6 µM	300 µM	1.42253
	2.6 µM	500 µM	0.93319

CI < 1 synergistic, CI = 1.0–1.1 additive, CI > 1.1 antagonistic effect

Co-administration of Cisplatin and Tomatidine Exerted Apoptotic Effects on AML Cells

The apoptotic effect of previously determined cisplatin IC20 values with increasing doses of tomatidine was tested to understand the mechanism behind the detected cell death after cytotoxicity assay. The experimental design contained the 0.9 µM (for MOLM-13) and 2.6 µM (for THP-1) cisplatin doses with 500 µM and 1 mM tomatidine drug concentrations. The comparative analyses of the results were conducted according to the Total Apoptosis and Living Cells (untreated controls).

After single 500 µM and 1 mM tomatidine treatments, there was a significant increase in apoptotic bodies in both cell lines (please check Fig. 4). Unlike the single 500 µM tomatidine treatment, no significant increase in apoptotic bodies was detected due to cisplatin's single drug treatment for both MOLM-13 and THP-1. The single 500 µM effect on total apoptotic bodies was 16% and 12% for MOLM-13 and THP-1 cells, respectively. Compared to the untreated control, the single 1 mM tomatidine treatment resulted in a 10.1-fold and a 3.4-fold increase in total apoptotic bodies for MOLM-13 and THP-1, respectively. The notable difference in the effect of 500 µM and 1 mM single tomatidine treatments was 3.5-fold in the total apoptotic body population.

The results obtained from the combination treatments were promising. The administration of 0.9 µM cisplatin with 500 µM tomatidine induced a 15.7% increase in the apoptotic MOLM-13 cell population. In comparison, this induction was 47.8% after the combinatorial treatment of 0.9 µM cisplatin with 1 mM. Likewise, induction of apoptosis for the THP-1 cell population was detected as

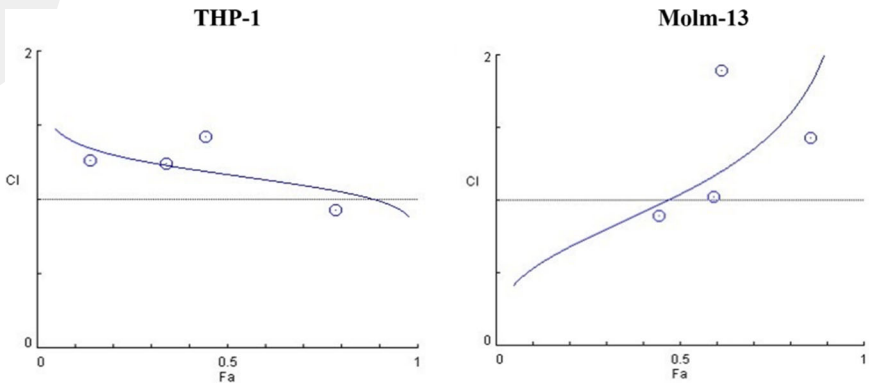

Fig. 3 Synergistic effect of THP-1 and MOLM-13 for co-administration of cisplatin IC20 value with increasing tomatidine concentrations, calculated via CompuSyn. Fa, Fractional Inhibition of Bioluminescence

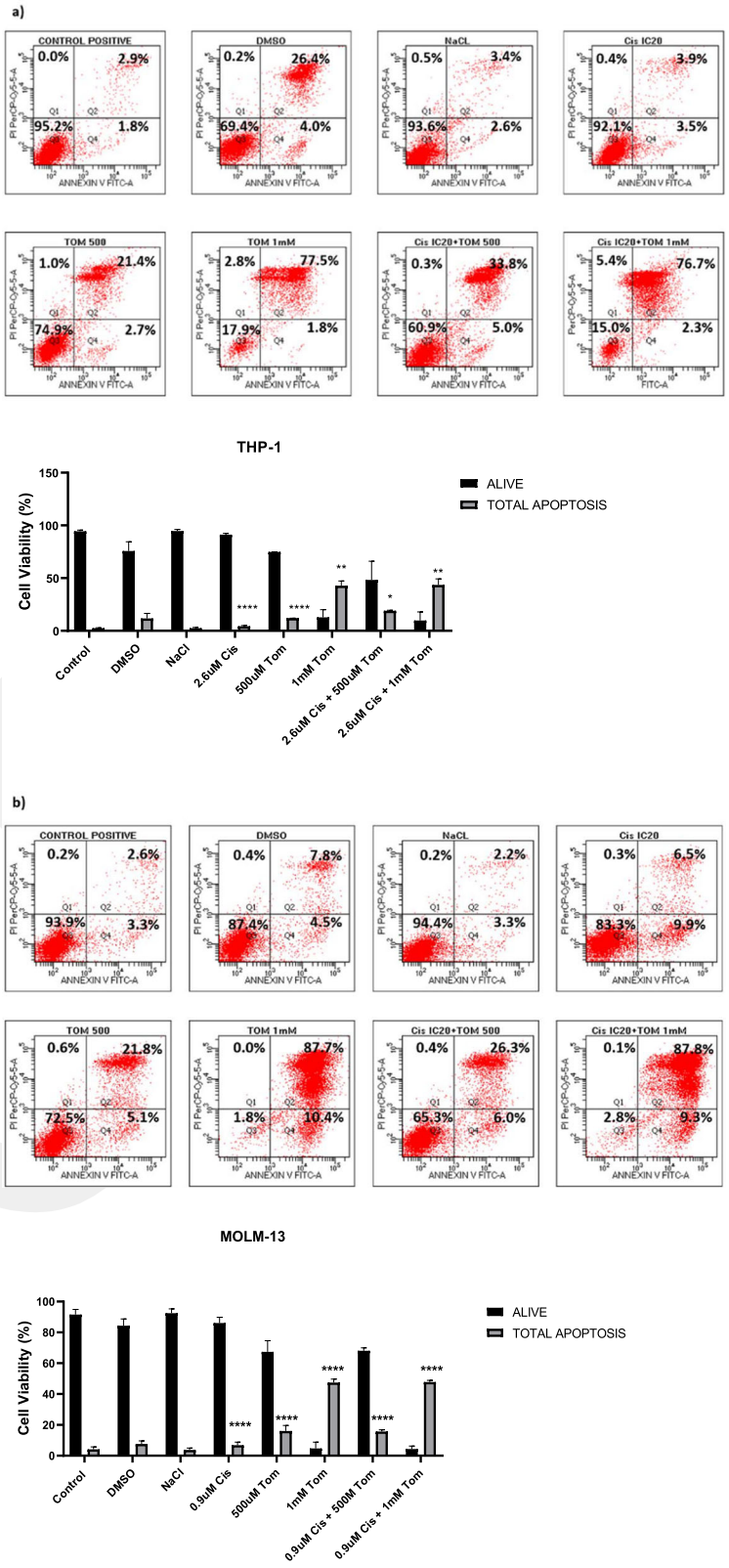

Fig. 4 Induction of apoptosis in THP-1 (**a**) and MOLM-13 (**b**) cells in response to cisplatin and tomatidine combination for 48 h. The representative experiment was shown on the left panels and the percentages represent one of the repeats of flow cytometry results on histograms. The average percentage of the Q1 quadrant represents the necrotic cells (Annexin V-FITC$^-$/ PI$^+$), the Q2 quadrant indicates late apoptosis (Annexin V-FITC$^+$/ PI$^+$), the Q3 quadrant represents the number of living cells (Annexin V FITC$^-$/PI$^-$) and the Q4 quadrant indicates early apoptosis (Annexin V-FITC$^+$/PI$^-$) as expressed in graphs. The percentage of the Q2 + Q4 quadrants represents the amount of total apoptosis. Graphical results were interpreted according to the average of two biological replicates and bar chart results were taken as a basis for analysis

19.1% and 43.6% after the combinatorial drug treatment of 2.6 µM cisplatin with 500 µM and 1 mM tomatidine administration to the cells, respectively. While the cells treated with single tomatidine accumulated in the Q2 quadrant (early apoptosis), when treated with cisplatin, the tendency of apoptotic bodies was detected mainly in

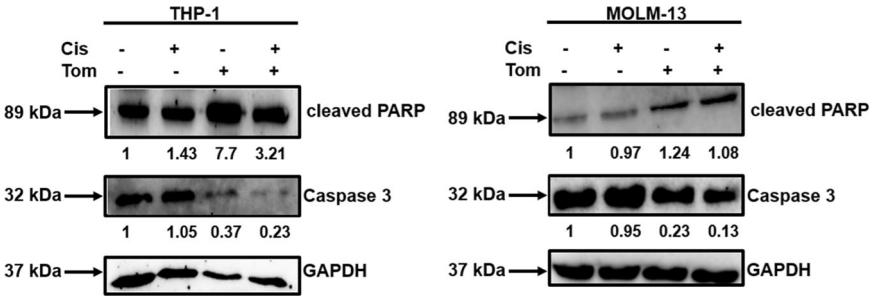

Fig. 5 Western blot analysis of single or combination treatment of cisplatin and tomatidine on THP-1 (the left panel) and MOLM-13 (the right panel) cells for 48 h. The expression levels of cleaved PARP protein, Caspase 3 protein, and housekeeping protein GAPDH protein levels were detected, and amounts were noted under each band. The overall experiment was done as 2 biological replicates. Results were normalized according to the control groups, and the protein levels of control groups were indicated as 1.

the Q4 quadrant (late apoptosis). Combining cisplatin with tomatidine disrupted this accumulation trend by increasing the number of apoptotic bodies in the Q4 quadrants.

Collectively, these data indicated that the tomatidine treatment decreased leukemia cell proliferation via induction of apoptosis relative to untreated cells. Even if the changes are minor, the results are still promising. Regarding the limitations of this combination, it could be argued that can be more effective with the combination of tomatidine and with high doses of cisplatin.

Effects of Cisplatin and Tomatidine Combination on Apoptotic Proteins

The next step was to validate further the apoptotic mechanisms that have been induced so far. The drug dosage was set using cisplatin IC20 with 500 µM tomatidine combination based on the cytotoxicity and apoptosis results. The expression levels of total Caspase-3 and cleaved PARP proteins (apoptotic cell death markers) were measured in this part of the study. While interpreting the results and measuring the protein levels, the untreated control groups were taken as reference points.

While sole tomatidine and cisplatin administrations led to an approximate 8.0- and 1.4-fold elevation for cleaved PARP protein levels, the protein band of combinatorial treatment was detected as 3.2 in THP-1 cells. Interestingly, the decrease in total caspase-3 levels was significant for sole and combination tomatidine cases compared to single cisplatin treatments (left panel of Fig. 5).

MOLM-13 cells showed a mild increase in cleaved PARP in response to the combinations of the compounds. The increase of cleaved PARP protein levels had a minor difference with single tomatidine administration. While the singular tomatidine dose resulted in a 1.24-fold increase in the protein expression of cleaved PARP, this increase was detected as 1.08-fold in the combination treatment group. Oppositely, total caspase-3 levels were detected as 0.13-fold in response to the combination treatment. In this case, the protein expression level after a single cisplatin treatment was 0.97-fold compared to the untreated control (right panel of Fig. 5).

Discussion

AML is a disease characterized by the accumulation of immature myeloid cells called blasts in the peripheral blood, bone marrow, spleen, and liver, eventually leading to hematopoietic malignancy [18, 19]. Chromosomal abnormalities, genetic disorders and abnormal signaling pathways may play a role in the pathogenesis of AML, converting AML into a highly complex disease [20, 21]. Therefore, traditional treatment methods are not very successful in combating AML and this shows that there is always a need for new treatments against AML. In this study, two AML cell lines were used with different FLT3 surface expressions; THP-1 (FLT3 wild type) and MOLM-13 (heterozygous FLT3-ITD) [22]. The aim of the study is to compare the sensitivity of different FLT3-expressing cell lines.

Cisplatin is a commonly used chemotherapeutic drug that is used against solid tumors such as bladder, head and neck, lung, and ovarian cancers and hematological malignancies such as leukemias and lymphomas [9]. However, with the occurrence of drug resistance and the presence of unwanted side effects, it is beneficial to apply combination treatments in order to lower the cisplatin dose to reduce the side effects. Steroidal alkaloids are among a group of compounds that could have biological activities such as anti-cancer, anti-microbial, and anti-inflammatory [23]. Based on their anti-cancer potential, many steroidal alkaloids are tested in clinical trials [24]. The combination of cisplatin with natural products has been investigated in the treatment of various malignancies including leukemia.

The advantage of combining cisplatin with a natural product, curcumin for instance, could reduce the side effects of cisplatin because of using lower concentrations of cisplatin in a combination strategy [25]. Zou et al [26] showed that curcumin sensitizes breast cancer cells to cisplatin. The combination treatment of AML cells with cisplatin combined with curcumin, induced apoptosis and demonstrated a better antiproliferative effect than single treatment on head and neck squamous cell carcinoma [27], hepatic cancer [28] and myeloid leukemia cells [29]. Moreover, the combination of tomatidine and curcumin showed anticancer effect on prostate cancer cells [30]. Another study shows that cisplatin combination with resveratrol induced apoptosis [31]. There are similar studies of cisplatin combination with natural products, such as quercetin, inducing cell death [32]. The practice of dietary agents for treating diseases is well known since ancient times. Recently, some studies have been focused on steroid-like compounds present in a number of dietary agents. Tomatidine demonstrates anticancer activities against many types of cancers [33–35]. Hsieh et al [11] showed that tomatidine represses invasion and metastasis of osteoclastoma cells in vitro through c-Raf–MEK–ERK pathway. Another study demonstrated that tomatidine inhibited the invasion of lung cancer cells via inactivation of matrix metalloprotease (MMP) expression. In addition to the repression of MMP expression, tomatidine was found to decrease the nuclear level of nuclear factor kappa B (NF-KB) and the study discusses the antimetastatic potential of tomatidine in cancer therapy [36]. It was also previously demonstrated that tomatidine could act as an anti-inflammatory molecule in inflammatory diseases and cancer [37]. Tomatidine was also shown to sensitize the chemoresistant human adenocarcinoma cells to adriamycin and vinblastine and thus the compound serve as a chemosensitizer in combination therapy [38]. The result of another study supported the idea of desensitizing effect of tomatidine by combining tomatidine with gemcitabine on the Pancreatic Ductal Adenocarcinoma (PDAC) in 3D in vivo studies [39]. While cisplatin and tomatidine both demonstrated anticancer effects on various types of cancers, their combination has not been investigated in the context of AML. Hence, in the present study, it was investigated whether cisplatin combination with tomatidine has a synergistic anti-proliferative effect on AML cells. To the best of our knowledge, this is the first report indicating a strong combined effect of low concentrations of tomatidine and cisplatin on AML cells. In the current study, we combined tomatidine, a tomato glycoalkaloid, with cisplatin and investigated the anticancer effects on AML cells. Our motivations are firstly, to sensitize AML cells to cisplatin by using tomatidine, secondly to suggest a treatment strategy to decrease the side effects of cisplatin by using lower doses as a combination and thus suggesting that tomatidine might be considered as an adjunct therapy for AML. Firstly, our study demonstrated that single administration of tomatidine and cisplatin reduced the cell proliferation of both AML cell lines. After 48 h of 500 µM tomatidine treatment, 74% and 90.1% cell proliferation inhibition were achieved for MOLM-13 and THP-1 cells, respectively. These results showed that single tomatidine treatment was more potent on FLT3-ITD MOLM-13 cells than FLT-3 wild-type THP-1 cells. Similarly, at 1 µM cisplatin concentration, MOLM-13 cells were more sensitive and susceptible than THP-1 cells to only cisplatin treatment. Similar considerable differences in sensitivity of FLT3-wild type and -ITD mutant cells against small molecules, such as midostaurin, idasanutlin and cobimetinib, were discussed in the literature [40, 41]. In these studies, FLT3-ITD mutant cells were more sensitive than FLT3-wild-type cells and more susceptible to FLT3 inhibition. These results may be reflected by the FLT3 mutant or wild-type expression status and thus different pathways activated in the cells. As a next step in this study, the IC20 values of cisplatin and increasing concentrations of tomatidine combined to investigate a synergy for these drugs. Similar to the cytotoxicity results, MOLM-13 cells were more sensitive because when IC20 of cisplatin (0.9 µM) and 50 µM tomatidine were combined, a strong synergistic effect was observed (Fig. 2b). However, for THP-1 cells, synergy was obtained when IC20 of cisplatin (2.6 µM) and only when 500 µM tomatidine were combined (Fig. 2a). These results revealed that FLT3-ITD mutation-bearing MOLM-13 cells were more susceptible to the combination treatment. Our results showed that 50 µM tomatidine for MOLM-13 and 500 µM tomatidine for THP-1 cells with IC20 of cisplatin was associated with strong synergistic effects. This is in line with the study in which two small molecule inhibitors of FLT3 (lestaurtinib and PKC412) were combined with the chemotherapeutic agent cytarabine (AraC), a significant synergy was demonstrated in lestaurtinib/AraC combination in FLT3-ITD mutant but not in wild type cases [42].

As emphasized earlier, the motivation of this study is to combine these compounds to reduce the side effects of cisplatin by using low doses of cisplatin. We assume that using low doses of cisplatin in combination with tomatidine would decrease the toxicity of the compound. As mentioned before, one of the questions of this study was to investigate whether low cisplatin combined with tomatidine would have similar or better effects than high single cisplatin concentrations. This would be especially important to avoid the possible side effects of high doses of cisplatin. The results of the current study revealed that combining low doses of cisplatin with tomatidine shows a better cytotoxic effect with higher doses of cisplatin. For MOLM-13, 78% of cell inhibition was observed when 5 µM cisplatin was administered (Fig. 1d) while when 0.9 µM cisplatin was

combined with tomatidine, an 83.4% reduction in cell viability (Fig. 2b) after 48 h. The findings of our study add to the literature that FLT3-ITD mutant and wild-type AML cells have considerable differences in cytotoxicity and combination therapy.

Apoptosis is considered to be the main fundamental mechanism for the cytotoxic effects of most anti-cancer agents. Therefore, we next checked apoptosis induction and apoptotic protein expression levels in MOLM-13 and THP-1 cells, treated with cisplatin together with tomatidine. Tomatidine induced apoptosis in both MOLM-13 and THP-1 cells whilst cisplatin was less effective in the induction of apoptosis. On the other hand, the co-administration of drugs increased the total apoptotic cell population in only MOLM-13 cells as compared to control or tomatidine and cisplatin alone. Cisplatin binds to DNA and induces DNA damage culminating in mitochondria-mediated apoptosis. Contrary to the findings of the high apoptotic effect of the cisplatin in literature, our results did not reveal a significant apoptotic effect when single cisplatin was administered. When these results were compared to previous reports, a significant apoptosis induction effect was observed when high cisplatin doses were administered in melanoma, colon, and ovarian cancer cells. The mean cisplatin concentration shown to be used to induce apoptosis in vitro tumor cell lines was 52 µM and the median concentration was 20 µM [43–45]. On the contrary, the IC20 values of cisplatin for MOLM-13 and THP-1 were 0.9 µM and 2.6 µM, respectively. We argue that this could be the reason behind the low apoptotic cell death when single cisplatin was used (Fig. 4a, b). However, when we combined cisplatin and tomatidine, significant apoptotic cell death was achieved. Indeed, this was the motivation of our study in which the combination of low-dose cisplatin with tomatidine results in a higher degree of apoptosis.

When comparing our results to those of previous studies, it was demonstrated that treatment of the tomatidine cell showed the apoptosis rate of cells increased significantly compared with that of the control group in ovarian cell and mouse C2C12 myoblast. In addition, it was reported that *C. elegans* exposed to 50 µM tomatidine died at relatively early ages compared to control *C. elegans* which are not exposed to tomatidine [46–48]. Similarly, we presented an apoptotic effect in the AML cell lines. In order to better understand the apoptosis effect combination of cisplatin and tomatidine, different doses of cisplatin can be studied in more detail.

To further explore the underlying mechanism of tomatine and cisplatin combination-induced apoptosis, western blotting was utilized to detect the expression of apoptosis-related proteins. The results showed that alone and combination treatment remarkably differed c-PARP and total caspase expression in both cell lines compared to the control (Fig. 5). Henkels et al., and Zhang et al., demonstrated a decrease in PARP expression by cisplatin dose-dependent treatment in ovarian cell lines and non-small cell lung cancer [49, 50]. Another study by Henkels et al demonstrated a model in which cisplatin-induced programmed cell death in the cisplatin-sensitive and resistant ovarian cell lines proceeds via caspase-3-independent and -dependent pathways [51]. Cisplatin treatment induced the activation/cleavage of caspase-3, -6, -7, -8, -9 in all ovarian cell lines [52]. Our data are the first to show the role of baseline expression of apoptosis protein and how its expression is altered by cisplatin and tomatidine combination in FLT3-ITD+ AML.

Conclusions

In conclusion, the present study demonstrates that tomatidine had a significant inhibitory effect on THP-1 and MOLM-13 AML cell lines. It was demonstrated for the first time, to the best of our knowledge, that tomatidine induces apoptosis in combination with cisplatin in AML cells. The present study also indicates that there is a selective synergistic anti-proliferative effect of the cisplatin and tomatidine combination at low doses on FLT3-ITD+ cells. The AML cells that were used in this study, with different FLT3 expression, were shown to have distinguishable responses in the way that FLT3-ITD expressing cells were more sensitive to the combination treatment. Together with the accumulating knowledge that natural products have a protective role from cisplatin cytotoxicity, it is important to note that combination therapies would be effective against cisplatin resistance [53]. The findings of the study could indicate that tomatidine could be an adjuvant compound for cisplatin-treated AML. Further research should be conducted to demonstrate this combination's effect in different cancer models including in vivo cancer models.

Data availability

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by H.B.A. and M.Y. The first draft of the manuscript was written by H.B.A., M.Y. and E.B.G.A. and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Abbreviations

AML	Acute myeloid leukemia
FLT3	FMS-like tyrosine kinase 3
ITD	Internal tandem duplication
FACS	Fluorescence-activated cell sorting
PARP	Poly (ADP-ribose) polymerase

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