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ORIGINAL PAPER

Resveratrol triggers anti-proliferative and apoptotic effects in FLT3-ITD-positive acute myeloid leukemia cells via inhibiting ceramide catabolism enzymes

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Abstract

Resveratrol possesses well-defined anti-carcinogenic activities. However, how resveratrol exerts its anti-leukemic actions by modulating anti-apoptotic ceramide catabolism enzymes, mainly sphingosine kinase (SK-1) and glucosylceramide synthase (GCS), in FLT3-ITD AML remains unclear. Resveratrol, SKI II (SK inhibitor) and PDMP (GCS inhibitor) were evaluated alone or in combinations for their effect on cell proliferation (MTT assay), apoptosis (annexin V-FITC/PI staining by flow cytometry) and cell cycle progression (PI staining by flow cytometry) in MOLM-13 and MV4-11 cells. The combination indexes (CIs) were calculated based on cell proliferation data using CompuSyn software. Caspase-3 and PARP activation, changes in SK-1 and GCS levels by resveratrol alone or PARP cleavage in co-treatments were determined by western blot. Resveratrol and inhibitors alone inhibited cell proliferation in a dose- and time-dependent manner. Resveratrol downregulated SK-1 and GCS expression in both cell lines. It induced apoptosis by phosphatidylserine (PS) exposure together with caspase-3 and PARP cleavage and arrested the cell cycle slightly at the S phase. Co-administrations intensified resveratrol's effect by inhibiting cell proliferation synergistically (A CI of < 1) or additively (A CI 1.0–1.1) and inducing apoptosis via PS relocalization and PARP cleavage. Resveratrol plus SKI II did not affect cell cycle progression significantly, however, resveratrol plus PDMP blocked cycle progression at G0/G1 and S phases for MOLM-13 cells and MV4-11 cells, respectively. Overall, resveratrol may inhibit FLT3-ITD AML cell proliferation by inhibiting ceramide catabolism and be evaluated as a chemopreventive after detailed analysis of the crosstalk between resveratrol and ceramide catabolism pathway.

Keywords Apoptosis · FLT3-ITD acute myeloid leukemia · Glucosylceramide synthase · Resveratrol · Sphingosine kinase

Introduction

Fms-related tyrosine kinase 3 (FLT3) is a receptor tyrosine kinase commonly expressed on hematopoietic cells with crucial functions in both normal and abnormal hematopoiesis. Normally, FLT3 expression is restricted to immature blood cells until they fully differentiate into functional blood cells. After binding of FLT3 ligand to FLT3, dimerization of the receptor results in activation of signaling pathways like MAPK/ERK and PI3K/AKT [1]. Almost 30% of acute myeloid leukemia (AML) patients possess the activating mutations of FLT3, which are tyrosine kinase domain (TKD) mutations and FLT3-internal tandem duplications (ITDs) in the juxtamembrane domain [2]. The presence of FLT3-ITD mutation is associated with a higher relapse rate, poorer prognosis and shorter overall survival due to continuous activation of downstream MAPK/ERK, PI3K/AKT and STAT5 pathways which are responsible for sustained cell proliferation and resistance to apoptosis [3]. The roles of FLT3-ITD in the molecular development of malignancy have made it a significant target by designing FLT3 inhibitors including first and second generation inhibitors although conventional intense chemotherapy is the first choice in routine clinic treatment [2, 3]. Food and Drug Administration (FDA) approved some inhibitors such as midostaurin

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and gilteritinib for clinical use in FLT3-ITD AML patients either as monotherapy or combinational therapy, however, the development of early onset of acquired resistance and the presence of refractory disease are still problems in effective treatment [4]. Therefore, potential addition of natural products [5, 6] as an integrative therapy component could be contributing to FLT3-ITD AML treatment after their mechanism of action and possible new intracellular targets have been defined.

Sphingolipid family members have impacts on crucial cellular functions including cell proliferation, cell death, migration and multi-drug resistance in addition to their structural roles in cellular membranes [7]. Ceramide (Cer) is a central molecule in sphingolipid metabolism and has been recognized as an important lipid determinant of cell fate. Cer can be generated by de novo and salvage pathways regulated by sequential activities of different enzymes which could be defined as diagnostic and disease progression markers [8]. Accumulation of Cer in the cells in response to stress conditions such as chemotherapeutics, radiation and natural products associates with suppression of cell proliferation and induction of cell death, which defines it a pro-apoptotic lipid [9, 10]. On the other hand, conversion of Cer into sphingosine-1-phosphate (S1P) or glucosyl ceramide (GC) by sphingosine kinase (SK) or glucosylceramide synthase (GCS), respectively is related to increased cell proliferation, resistance to apoptosis and drugs, hence they are anti-apoptotic lipids [11]. Alteration of the balance between pro-apoptotic and pro-survival sphingolipids in the cell is defined as ''sphingolipid rheostat'', which is a critical decision-making strategy for cell fate [12]. In a recent study, SK and its product S1P increased in large granular lymphocyte leukemia patients and targeting of SK by both genetic and pharmacologic approaches resulted in decreased cell proliferation via degradation of anti-apoptotic Mcl-1 [13]. Targeting of acid ceramidase by a specific inhibitor in in vitro and in vivo models of AML resulted in Cer accumulation and decreased S1P levels, which caused apoptosis [10]. Likewise, the overexpression of GCS was associated with poor prognosis in oral cavity cancer [14]. Silencing of GCS in combination with chemotherapy in glioblastoma resulted in decreased proliferation and increased apoptosis [15]. Hence, sphingolipid metabolizing enzymes involved in regulation of "sphingolipid rheostat" could be recognized as therapeutic targets.

Resveratrol, a plant-derived phytoalexin, has been studied extensively in various kinds of cancers ranging from solid tumors to hematological malignancies [16]. It could be called a promising adjuvant in cancer therapies due to its multi-targeted effects on different cellular processes including cell proliferation, apoptosis, invasion, metastasis, EMT progress, stemness and tumor microenvironment [17]. Common pathways targeted by resveratrol include NFκB [18], PI3K/AKT [19], Sonic hedgehog [20] and JAK/STAT pathways [21]. Additionally, sphingolipid signaling/metabolism has been identified a new target of resveratrol in cancers [22]. In hepatocellular carcinoma cells, resveratrol increased the accumulation of Cer, sphinganine and sphingosine together with increased expression of enzymes in de novo Cer synthesis pathway, resulting in growth inhibition [23]. Increased dihydroceramide levels were found to be the reason behind resveratrol-mediated cytotoxicity in gastric cancer cells [24]. Resveratrol is reported to be an effective chemopreventive agent in solid cancers such as hepatocellular carcinoma [23] and limited hematological cancers including chronic myeloid leukemia (CML) and AML through its modulatory activity on sphingolipid metabolism [25, 26]. The molecular signaling mechanisms by which resveratrol exerts its anti-leukemic effects in the context of Cer catabolism in FLT3-ITD AML remain incompletely unclear even-though we showed its effects on de novo pathway of Cer production (unpublished data). In this study, we aimed to investigate whether resveratrol could modulate the Cer catabolizing enzymes, mainly focusing on SK-1 and GCS, for its anti-cancer effects in human FLT3-ITD-positive AML. Hence, the molecular mechanism behind resveratrol's action may be related to SK-1 and GCS that produce anti-apoptotic S1P and GC, respectively.

Materials and methods

Chemicals

Resveratrol and MTT were purchased from Sigma-Aldrich (USA). SKI II and PDMP were obtained from Cayman Chemicals (Ann Arbor, MI, USA). 10 mM stock solutions were prepared in DMSO. The final concentration of DMSO did not exceed more than 0.1% in culture. Penicillin–streptomycin, RPMI 1640, and fetal bovine serum (FBS) were obtained from Invitrogen (Paisley, UK).

Cell lines and culture conditions

Two different FLT3-ITD AML cell lines (MOLM-13 and MV4-11) were obtained from DSMZ (German Collection of Microorganisms and Cell cultures). MOLM-13 and MV4-11 cells were grown in RPMI 1640 (+ L-glutamine, GibcoTM) medium containing 10–20% FBS and 1% penicillin–streptomycin in a 5% CO_2 incubator at 37 °C.

MTT assay

The anti-proliferative effects of resveratrol (5–60 µM), SKI II (1–80 µM) and PDMP (1–100 µM) on MOLM-13 and MV4-11 cells were assessed by MTT cell proliferation assay

as described [27]. Briefly, the cells were seeded into a 96 well plate at a density of 1×10^4 cells/well and incubated for 48–72 h. 20 µl MTT solution (5 mg/ml, Sigma-Aldrich) was added after incubation to observe formazan crystals which were dissolved in 100 µl DMSO. Then, absorbance values were measured at 570 nm and the cell proliferation/viability graphs were obtained. Based on the graphs, IC_{50} values (concentration inhibiting cell viability by 50%) for resveratrol, SKI II and PDMP were calculated based on the cell proliferation/viability plots using GraphPad software (San Diego, CA).

Calculation of combination indexes (CIs)

Increasing concentrations of resveratrol were combined with increasing concentrations of inhibitors at a fixed molar ratio for 48 h. Then, the therapeutic effect of a resveratrol-inhibitor combination was assessed by MTT assay. Combined-effects analyses, based on the method of Chou and Talalay, were performed to establish whether combinations of resveratrol and Cer catabolism inhibitors result in synergism, additivity or antagonism using CompuSyn software (Biosoft, Cambridge, United Kingdom) [28]. A CI of < 1, 1.0–1.1, or > 1.1 is indicative of synergistic, additive/nearly additive, or antagonistic effects, respectively [29].

Annexin V-FITC/PI double staining for flow cytometric apoptosis detection

Cell apoptosis was assessed using Annexin V/FITC Apoptosis Detection Kit (BioVision, Inc). 7.5×105 cells/well were treated with resveratrol, SKI II, PDMP alone and in combination for 48 h in a 6 well plate. After incubation at 37 °C, the cells were collected at 1800 rpm for 10 min, washed with cold 1X PBS and resuspended with 200 µl annexin binding buffer. Then, 2 µl propidium iodide and 2 µl Annexin V/FITC were added. Following incubation at room temperature for 15 min, apoptotic cells were detected using a BD FACSCalibur flow cytometer (BD Biosciences) within 1 h. The results were analyzed using BD FACSDivaTM (BD Biosciences). Early and late apoptotic cells were analyzed [27].

Cell cycle analysis by flow cytometry

The cells were seeded into a 6 well plate at a density of 7.5×10^5 cells/well and treated with resveratrol, SKI II, PDMP alone and in combination for 48 h. Then, the cells were centrifuged at 260×*g* for 10 min and cell pellets were washed twice with 1 ml cold PBS. 3 ml cold ethanol was added to each sample and suspensions were incubated for at least 24 h in − 20 °C. Samples were centrifuged at 260×*g* for 10 min and supernatant was removed. Pellets were homogenized in 5 ml cold PBS and centrifuged. 1 ml PBS-Triton X100 and 100 µl RNase-A (200 µg/ml, Sigma-Aldrich) were added and incubated at 37 °C for 30 min. Then, 100 µl propidium iodide (1 mg/ml, Sigma-Aldrich) were added and incubated at room temperature for 10–15 min. Cell cycle analysis was carried out by BD FACSCalibur flow cytometer (BD Biosciences) and histograms were obtained using BD FACSDiva™ (BD Biosciences) [27].

Western blot

5×10^6 cells were treated with resveratrol, SKI II, PDMP alone and in combinations for 48 h. The expression of PARP, Caspase-3, SK-1 and GCS were checked by western blot. Cells were lysed in RIPA buffer (Sigma-Aldrich, USA). The supernatants were collected to measure protein concentrations using RC DCTM Protein Assay Kit (Bio-Rad, USA). 30 µg/well total protein was separated by 8–15% SDS-PAGE and transferred to PVDF membranes. The membranes were blotted with primary antibodies for PARP (1:3000, Cell Signaling, USA), caspase-3 (1:1000, Cell Signaling, USA), SK (1:3000, Cell Signaling, USA), Beta Actin (1:3000, Cell Signaling, USA) and GCS (1:1000, Novus Biologicals, USA) overnight at + 4 °C and conjugated with appropriate secondary antibodies (1:10,000, Jackson Immuno Research, USA). The membranes were visualized with Pierce ™ ECL Western Blotting Substrate kit (Thermo ScientificTM, USA). Immunoreactive bands and their densitometric analysis were carried out using the imaging software (Bio-Rad, ChemiDoc, Image LabTM 3.0).

Statistical analysis

The data were analyzed with GraphPad software (San Diego, CA) and expressed as the mean ± standard error (SEM) of three independent experiments. Differences among multiple groups were evaluated using one-way ANOVA followed by Dunnett's test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Resveratrol inhibits the proliferation of FLT3-ITD AML cells

The effect of resveratrol on viability of MOLM-13 and MV4-11 cells was checked to identify the appropriate anti-leukemic concentrations of resveratrol using MTT assay. The cells were treated with increasing concentrations of resveratrol (5 to 60 µM). As presented in Fig. 1a and b, resveratrol significantly reduced the viability in a time and concentration-dependent manner compared to the control, with IC_{50} values of 18 µM for MOLM-13 and 28 µM for

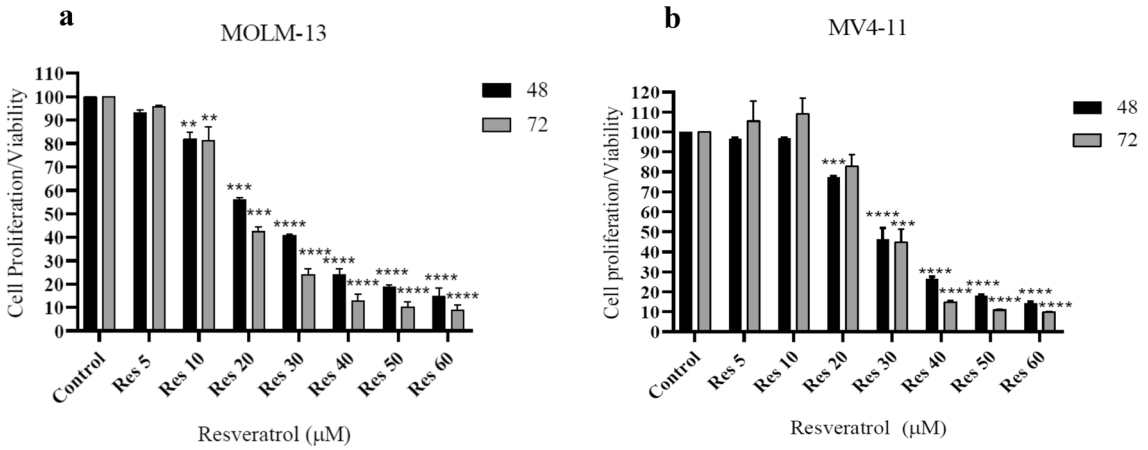


Fig. 1 Time- and concentration-dependent cytotoxicity of resveratrol on MOLM-13 (**a**) and MV4-11 (**b**) cells. Data are presented as the mean ± standard error (SEM) of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ vs. control

MV4-11 cells at 72 h, respectively. IC_{50} values (22 μM and 30 μM, respectively) were consistent with our unpublished findings after 48 h treatment of the cells with 10–30 μM resveratrol.

Resveratrol targets ceramide catabolism via decreasing the expression of SK-1 and GCS

To understand whether resveratrol modulates the enzymes involved in the regulation of sphingolipid rheostat for its anti-leukemic activity, the cells were treated with increasing concentration of resveratrol (10 to 30 μM) for 48 h followed by western blotting to assess the changes in protein expression levels of SK-1 and GCS. Resveratrol treatment resulted in downregulation of SK-1 and GCS significantly in both cell lines. Specifically, there were 0.7-fold and 0.4-fold decreases in SK-1 and 0.5-fold and- fold 0.3-fold decreases in GCS expression for MOLM-13 and MV4-11 cells in response to 30 μM resveratrol, respectively (Fig. 2a and b). Therefore, Cer catabolizing enzymes could be the direct targets of resveratrol in FLT3-ITD AML.

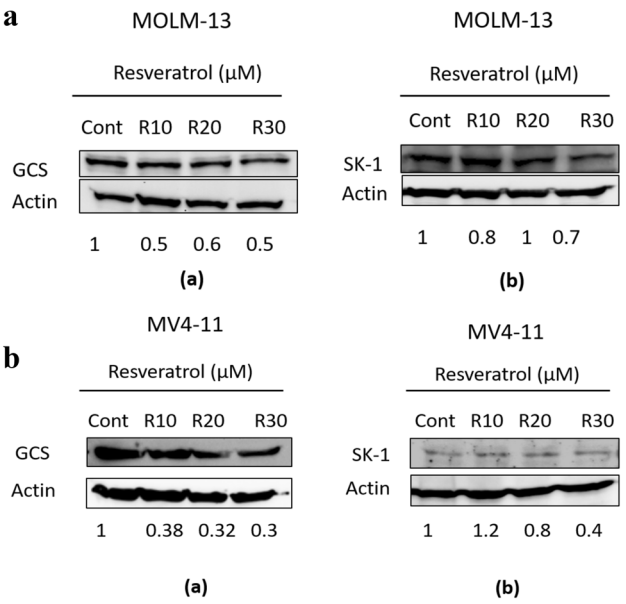


Fig. 2 Concentration-dependent effects of resveratrol on GCS and SK-1 protein levels in MOLM-13 (**a**) and MV4-11 (**b**) cells after 48 h treatment

Inhibition of ceramide catabolizing enzymes potentiates resveratrol-induced anti-proliferative effect

In order to evaluate the combined effects of resveratrol with SKI II and PDMP, cytotoxic effects of SKI II and PDMP alone were firstly determined in a time- and concentration-dependent manner by MTT assay. In both MOLM-13 and MV4-11 cells, SKI II and PDMP treatments decreased cell viability as compared to untreated controls. IC_{50} concentrations of SKI II and PDMP on MOLM-13 cells were 19 μM and 30 μM for 48 h and 12 μM and 27 μM for 72, respectively. IC_{50} on MV4-11 cells were 9.5 μM and 6.6 μM for 48–72 h SKI II treatment and 45 μM and 35 μM for 48–72 h PDMP treatment (Fig. 3a–d). To further understand whether SKI II or PDMP enhances the cytotoxic effect of resveratrol, we evaluated the combinational activity of resveratrol (10–30 μM) with SKI II (5–15 μM) and PDMP (5–15 μM) for MOLM-13 cells and SKI II (5–15 μM) and PDMP (20–60 μM) for MV4-11 cells. The results showed that all combinations decreased cell viability when compared to untreated controls in MOLM-13 and MV4-11 cells (Fig. 4a and b). Combinations of 10 μM resveratrol with 5 μM SKI II and PDMP suppressed cell proliferation significantly in MOLM-13 cells as compared to resveratrol alone. 10 μM

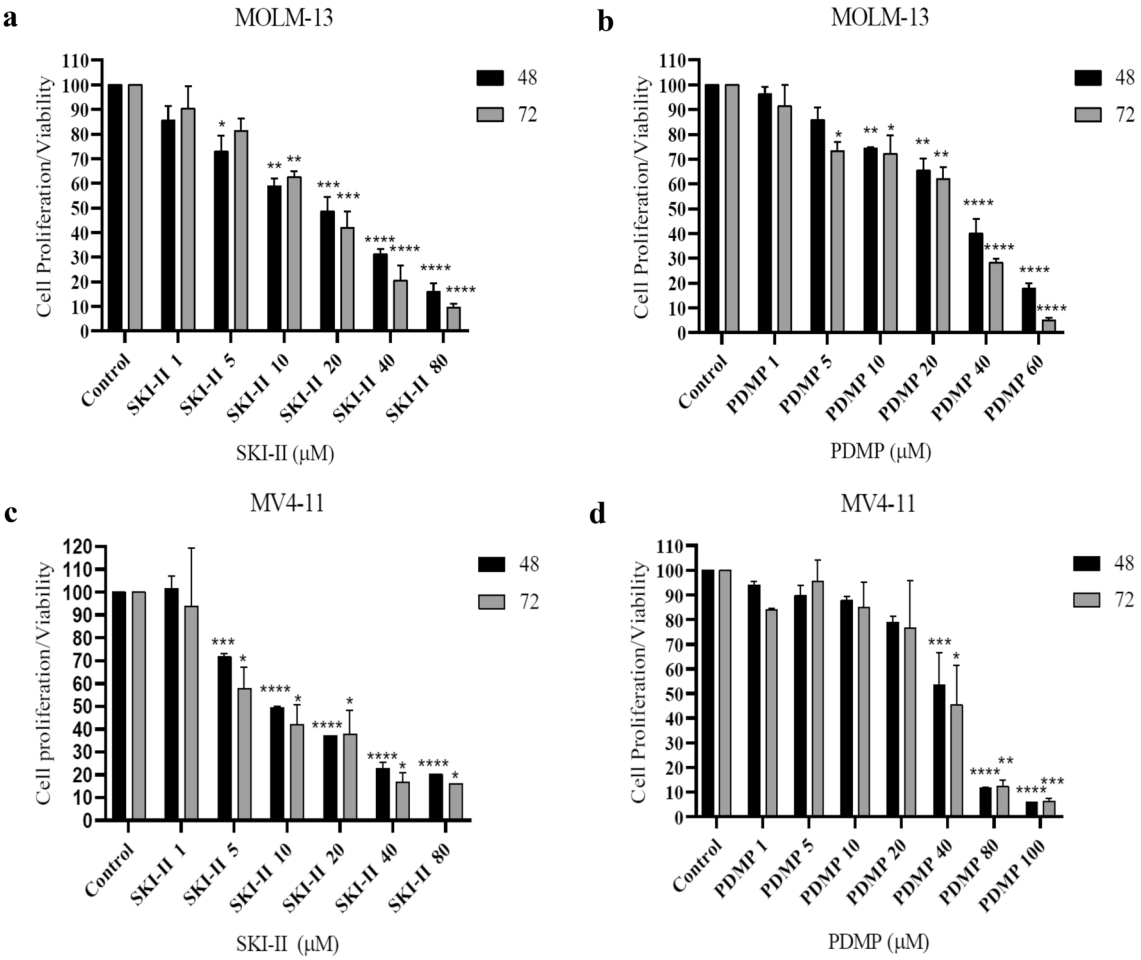


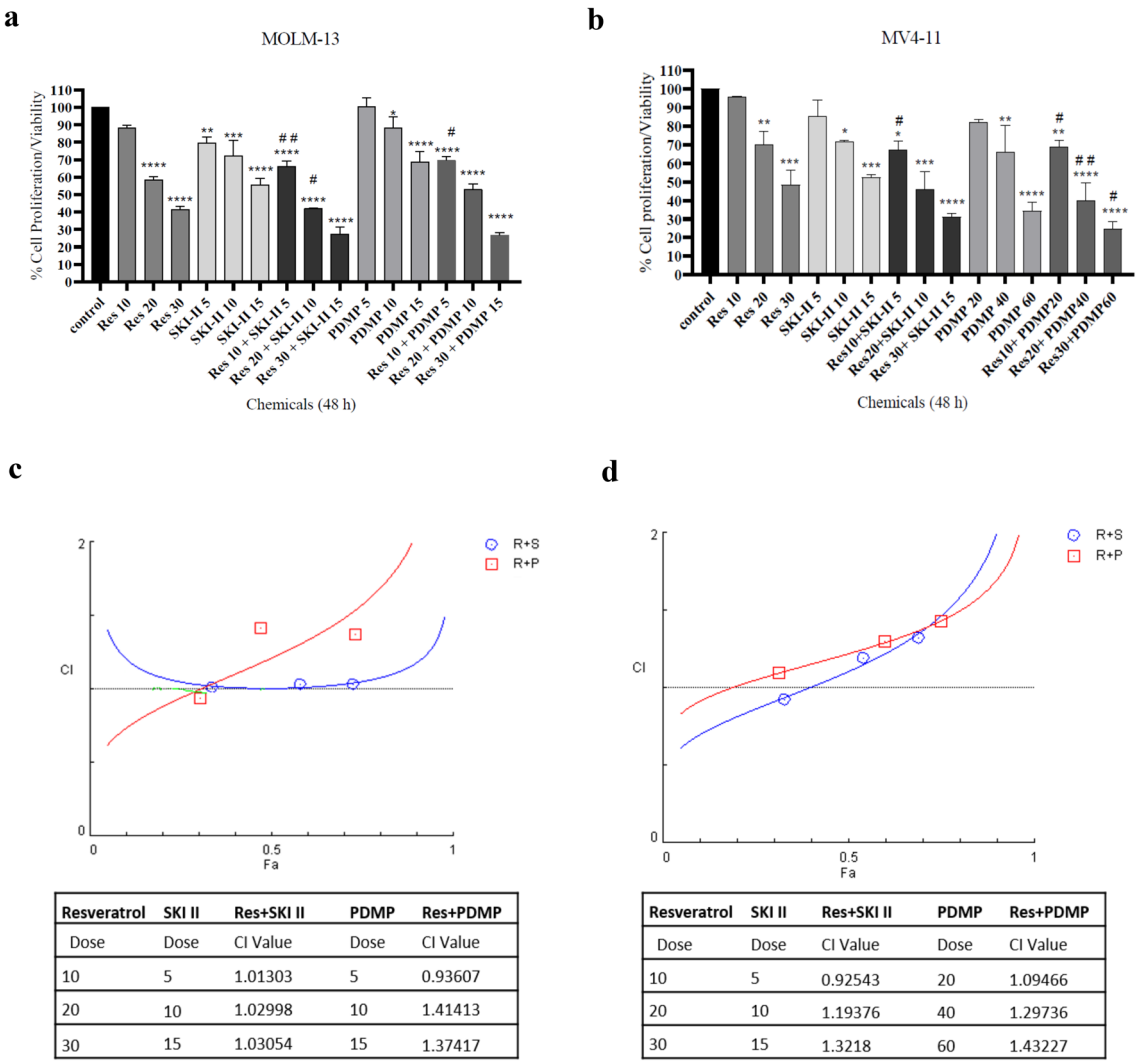
Fig. 3 Time- and concentration-dependent anti-proliferative effects of SKI II and PDMP on MOLM-13 (**a**, **b**) and MV4-11 (**c**, **d**) cells. Data are presented as the mean ± standard error (SEM) of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ and $^{****}P<0.0001$ vs. control

resveratrol together with 5 μM SKI II and all combinations with PDMP displayed suppressive effects on cell viability of MV4-11 cells compared to individual concentrations of resveratrol (Fig. 4a and b). Isobologram analysis of co-administrations revealed that 10 μM resveratrol plus 5 μM PDMP had synergistic effect although additive effects of three combined concentrations of resveratrol and SKI II were shown for MOLM-13 cells. In MV4-11 cells, 10 μM resveratrol plus 20 μM PDMP showed nearly additive effect while 5 μM SKI II in combination with 10 μM resveratrol had synergistic effect (Fig. 4c and d).

Resveratrol in combination with SKI II and PDMP promotes apoptosis in FLT3-ITD AML cell lines

To investigate whether apoptosis is involved in a decrease in cell viability, we measured the cleavage of caspase-3 and PARP, well-recognized markers for apoptosis, after 48 h exposure to resveratrol. We observed dose-dependent increases in the protein expression of cleaved caspase-3 and cleaved PARP in both cells. There were 14- fold and 12.5-fold increases in cleaved caspase-3 and cleaved PARP levels, respectively in MOLM-13 cells at 30 μM resveratrol; likewise, for MV4-11 cells, 14.3-fold and 10.8-fold increases were detected in caspase-3 and PARP cleavages at 30 μM resveratrol (Fig. 5a and b).

Apoptotic effects of resveratrol alone and in combination with SKI II and PDMP were also evaluated by flow cytometry. The percentage of early and late apoptotic cells increased in a dose-dependent manner as compared to the control in MOLM-13 and MV4-11 cells (Fig. 6). The percentages of apoptotic MOLM-13 and MV4-11 cell population were 10.35%, 17.8% and 42% and 15.6%, 40.05% and 63.95% at 10, 20 and 30 μM resveratrol, respectively (Fig. 6a and b) when compared to the controls (7.5% and 11.4% for MOLM-13 and MV4-11 cells, respectively), which were in accordance with caspase-3 and PARP activation (Fig. 5). Flow cytometry results also showed



Resveratrol	SKI II	Res+SKI II	PDMP	Res+PDMP
Dose	Dose	CI Value	Dose	CI Value
10	5	1.01303	5	0.93607
20	10	1.02998	10	1.41413
30	15	1.03054	15	1.37417

Resveratrol	SKI II	Res+SKI II	PDMP	Res+PDMP
Dose	Dose	CI Value	Dose	CI Value
10	5	0.92543	20	1.09466
20	10	1.19376	40	1.29736
30	15	1.3218	60	1.43227

Fig. 4 Anti-proliferative effects of resveratrol combined with SKI II and PDMP on MOLM-13 (**a**) and MV4-11 (**b**) cells. Data are presented as the mean ± standard error of three independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ****$P<0.0001$ vs. control. $^{\#}P<0.05$, $^{\#\#}$P<0.01 vs. resveratrol alone. Combination indexes (CI) were calculated and isobolograms were drawn using CompuSyn software in MOLM-13 (**c**) and MV4-11 (**d**) cells. CI of < 1, 1.0–1.1, or > 1.1 is indicative of synergistic, additive/nearly additive, or antagonistic effects, respectively. *R* resveratrol, *S* SKI II, *P* PDMP

that the combination of resveratrol with SKI II or PDMP significantly promoted the apoptosis of MOLM-13 and MV4-11 cells (Fig. 6). In MOLM-13 cells, 30 µM resveratrol in combination with 15 µM SKI II or PDMP caused significant apoptosis compared to the controls (50.15% and 46.35% vs. 7.5%, respectively) (Fig. 6a). Similarly, 30 µM resveratrol in combination with 15 µM SKI II or 60 µM PDMP induced apoptosis markedly in MV4-11 cells (71.25% and 94.2% vs. 11.4%) (Fig. 6b). Additionally, the combination of resveratrol with SKI II or PDMP significantly increased PARP activation in both MOLM-13 and MV4-11 cells when compared to the control (Fig. 6c).

Effect of resveratrol in combination with SKI II and PDMP on cell cycle progression

Flow cytometry data in Fig. 7a and b showed that there was a slight accumulation of MOLM-13 and MV4-11 cells at S phase (31.3% vs. 26% control for MOLM-13; 29.4% vs. 23.15% control for MV4-11) in response to resveratrol especially at 30 µM (Fig. 7a and b), consistent with what we showed in our unpublished data. Increasing concentrations of SKI II induced slight to moderate accumulation of both MOLM-13 and MV4-11 cell populations in G0/G1 phase compared with the control (70.35%, 73.25% and 77.2% vs.

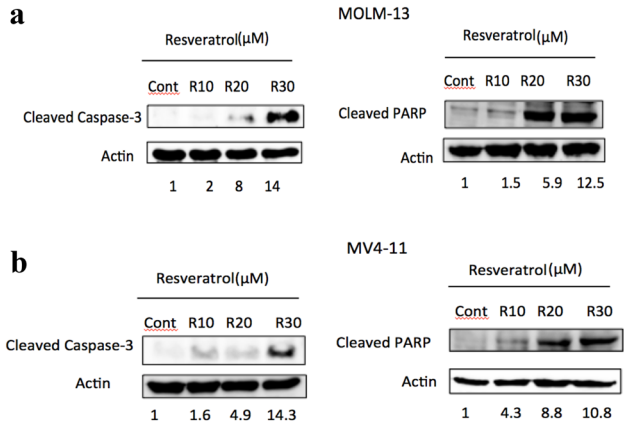


Fig. 5 Effects of resveratrol on cleaved caspase-3 and PARP protein levels in response to increasing concentrations of resveratrol in MOLM-13 (**a**) and MV4-11 (**b**) cells

65.1% control for MOLM-13; 73%, 76.1% and 73.95% vs. 70.25% control for MV4-11). Individual PDMP concentrations showed different cytostatic effects on MOLM-13 and MV4-11 cells, which were a slight G2/M (12.35%, 14.95% and 12.9% vs. 8.9% control) and G0/G1 (71.9%, 77.3% and 86% vs. 70.25% control) arrest, respectively. In resveratrol and SKI II combination group, there were no significant changes in all phases of cell cycle for both cell lines except 20 μM resveratrol with 10 μM SKI II for MOLM-13 cells, resulting in a slight G2/M arrest (14.45% vs. 8.9% control, vs. 11% resveratrol alone) However, resveratrol in combination with PDMP resulted in G0/G1 phase arrest in MOLM-13 cells when compared to the control (65.8%, 67%, 75.9% vs. 65.1% control) or resveratrol alone (61.5%, 63% and 61.55%), however, combination of highest concentrations of resveratrol and PDMP caused an accumulation at S phase for MV4-11 cells (39.45 vs. 23.15 control and vs. 29.4% resveratrol alone).

Discussion

The multi-targeted mechanisms underlying the growth inhibitory actions of resveratrol have been well reported in several cancer types ranging from solid tumors to hematological cancers in which apoptosis induction through either extrinsic or intrinsic pathways was the major mechanism behind resveratrol-mediated cytotoxic effects [30]. In this study, we also confirmed that resveratrol inhibited the proliferation of MOLM-13 and MV4-11 cells in a concentration- and time-dependent manner through induction of intrinsic route of apoptosis (increased levels of cleaved caspase-3 and cleaved PARP) and a slight cell cycle arrest at S phase. Additionally, recent studies speculating how the modulation of sphingolipid metabolism's enzymes or products, especially the alterations in the balance of sphingolipid rheostat by resveratrol, could explain its anti-cancer properties [22]. However, to our knowledge, there were no studies investigating resveratrol's effect on FLT3-ITD-positive AML in the context of Cer catabolism. Therefore, we suggested that resveratrol could manipulate two important Cer catabolizing enzymes, SK-1 and GCS, to contribute to apoptosis induction and cell cycle arrest. It was clear that resveratrol decreased the expression of SK-1 and GCS which are responsible for the conversion of Cer into S1P and GCS, respectively, hence decreasing intracellular Cer accumulation and enhancing cell survival. This could be the main mechanism related to resveratrol's anti-leukemic effects in MOLM-13 and MV4-11 cells. It is very well known that SK-1 and GCS favor cell proliferation and reduce apoptotic response, hence, it is commonly upregulated and considered as therapeutic targets in cancer [31, 32]. In K562 CML cells, resveratrol did not affect the total SK-1 expression, however, it inhibited the translocation of SK-1 from cytoplasm to plasma membrane where it catalyzes the formation of S1P and reduced its enzymatic activity, therefore, suppressing CML proliferation [25]. Resveratrol inhibited prostate cancer cell proliferation by downregulating SK-1 in an ERK-dependent manner in both in vitro and in vivo models [33]. Resveratrol modulated both Cer catabolism and de novo Cer pathway by downregulating SK-1 and GCS and upregulating serine palmitoyltransferase, respectively in philadelphia-positive acute lymphoblastic leukemia for its anti-leukemic activities [34]. The modulatory effect of resveratrol on GCS is less studied as compared to SK-1. In HL-60 and K562 leukemia cells, resveratrol suppressed GCS mRNA expression in addition to SK-1 expression [26, 35]. Therefore, this report is the first one implicating the roles of GCS and SK-1 in resveratrol-induced anti-leukemic effects in FLT3-ITD AML.

We further investigated whether pharmacological inhibition of SK-1 and PDMP may be a direct factor involved in enhanced cytotoxicity caused by co-treatments and found that co-administrations decreased cell viability and induced apoptosis in FLT3-ITD AML cells by mainly increasing PARP cleavage, which could be related to increased levels of anti-proliferative Cer. To lesser extent, cell cycle arrest could be a contributing factor for the improved activity of resveratrol in the presence of SKI II or PDMP, which was specific to each agent in each cell line as explained in Fig. 7a and b. In accordance with the presented data, it was shown in K562 and HL-60 cells that administration of resveratrol together with SKI II or PDMP inhibited cell viability and induced apoptosis by increasing mitochondrial membrane depolarization and caspase-3 enzyme activity [26, 35]. In HT29 colon cancer cells, resveratrol plus dimethylsphingosine (SK inhibitor) markedly increased cytotoxic effects of resveratrol [24]. Similarly, resveratrol's apoptotic activity was enhanced

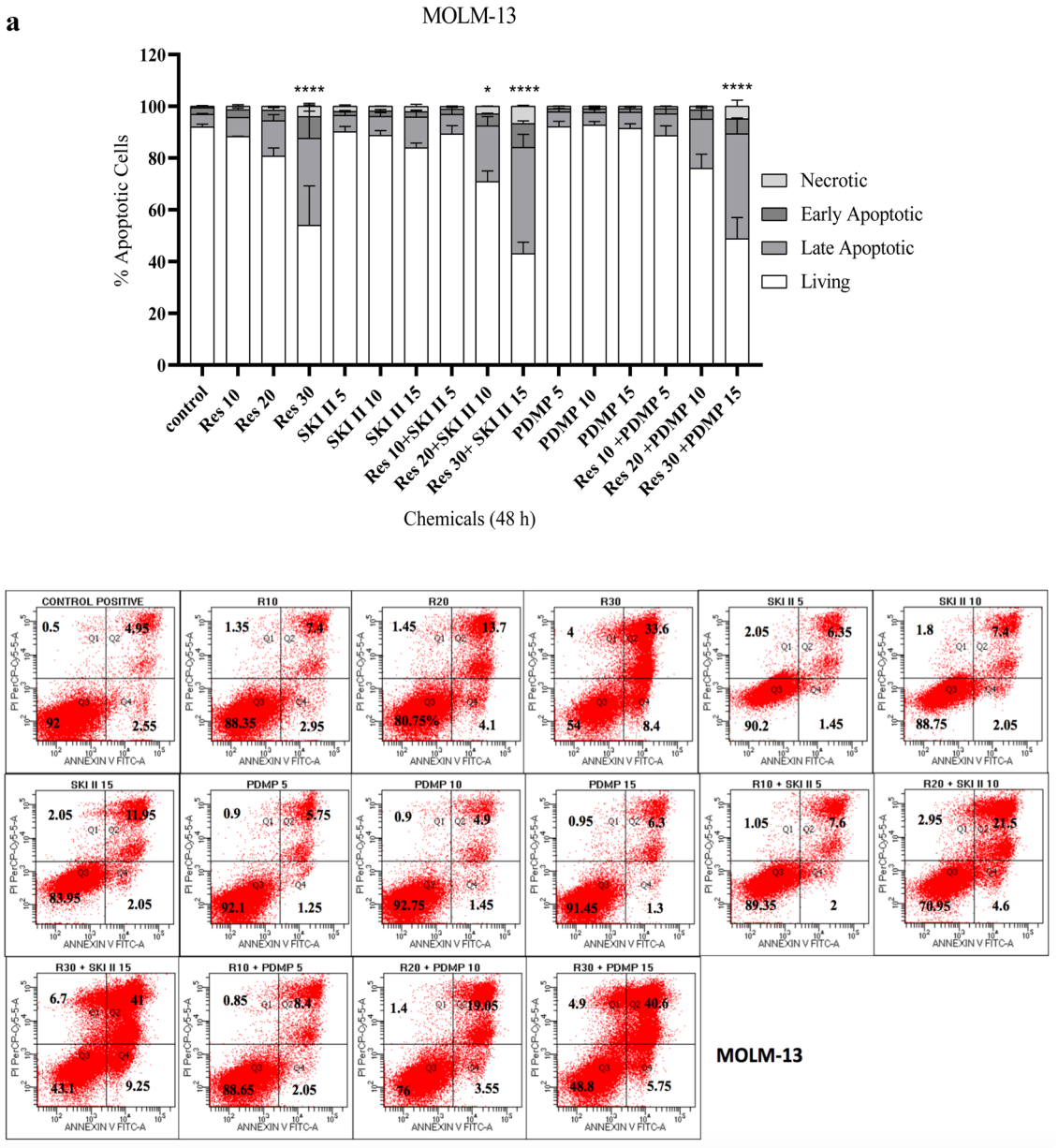
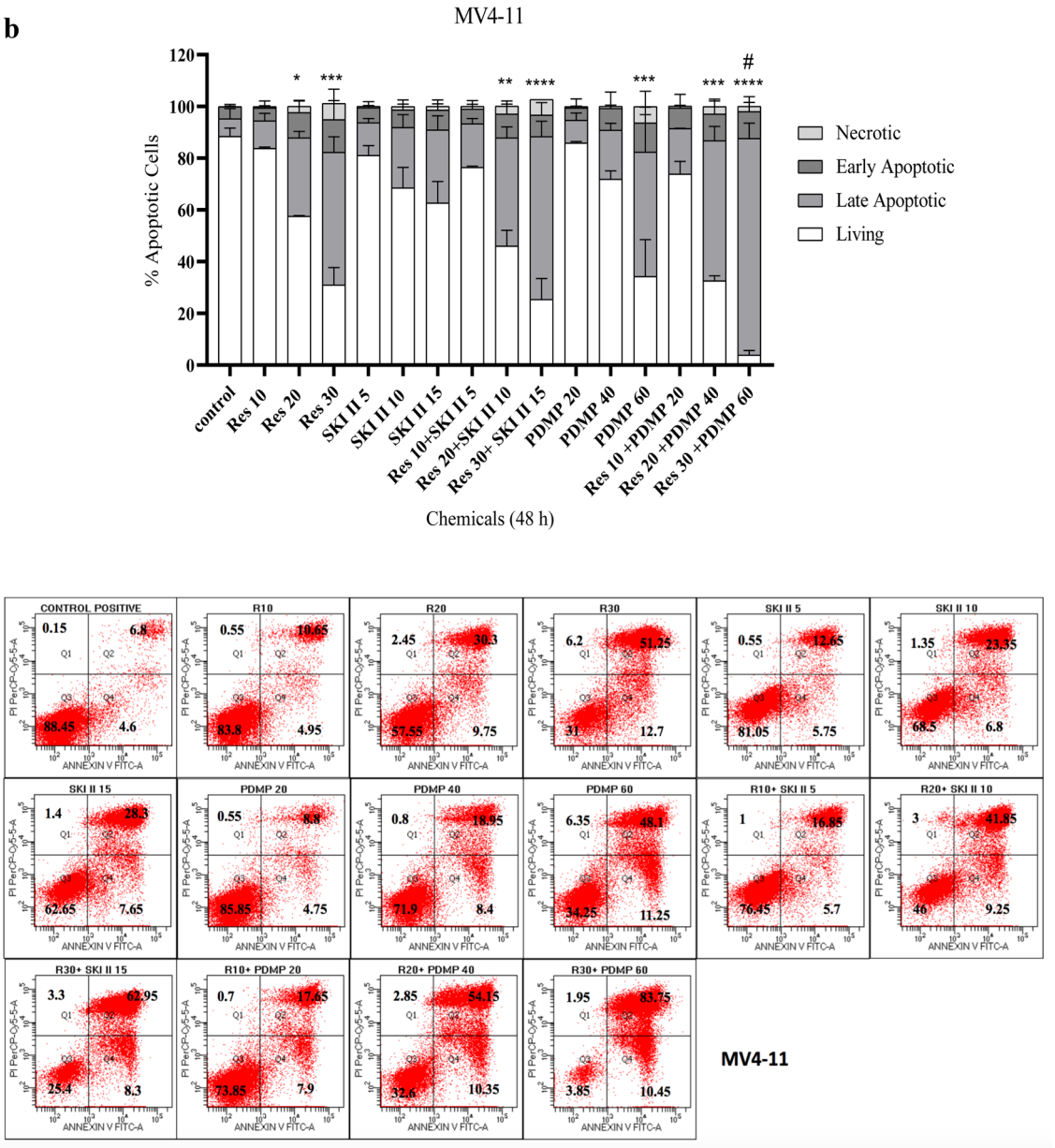


Fig. 6 Resveratrol combined with SKI II and PDMP promotes the apoptotic effects on MOLM-13 (**a**) and MV4-11 cells (**b**). All data are expressed as the mean ± standard error (SEM) of three independent experiments. $^{*}P<0.05$, $^{**}P<0.005$, $^{***}P$:0.001 and $^{****}P<0.0001$ vs. control. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. control. $^{\#}P<0.05$ vs. resveratrol alone. Q2 and Q4 in histograms represent late and early apoptotic cells, respectively. PARP cleavage was checked by western blot in MOLM-13 and MV4-11 cells treated with resveratrol, SKI II or PDMP alone or in combination for 48 h (**c**)



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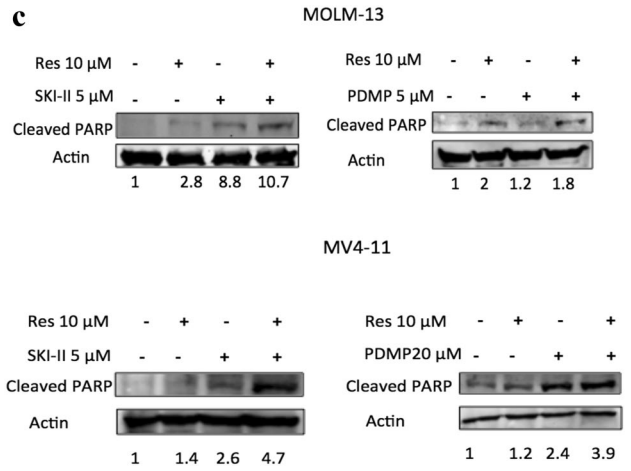

Fig. 6 (continued)

in the presence of SKI II or PDMP in SD1 and SUP-B15 philadelphia-positive acute lymphoblastic leukemia cells [34].

Being of SK-1 as an important therapeutic target and SK-1 inhibiting function of resveratrol has opened the way to design resveratrol analogs with higher SK-1 inhibitory function [36, 37]. Resveratrol and its newly synthesized dimers inhibited SK-1 expression and activity and induced PARP-dependent apoptosis in MCF-7 breast cancer cells [36], which also supported our hypothesis of resveratrol as a potential inhibitor of SK-1.

In conclusion, resveratrol could be considered as a promising nutraceutical to be integrated into FLT3-ITD AML treatment, since it induced apoptosis through intrinsic pathway of apoptosis and inducing S phase arrest. Additionally, resveratrol decreased SK-1 and GCS expression which might be the major mechanism of resveratrol-induced proliferation inhibition and apoptosis based on the interpretation of the

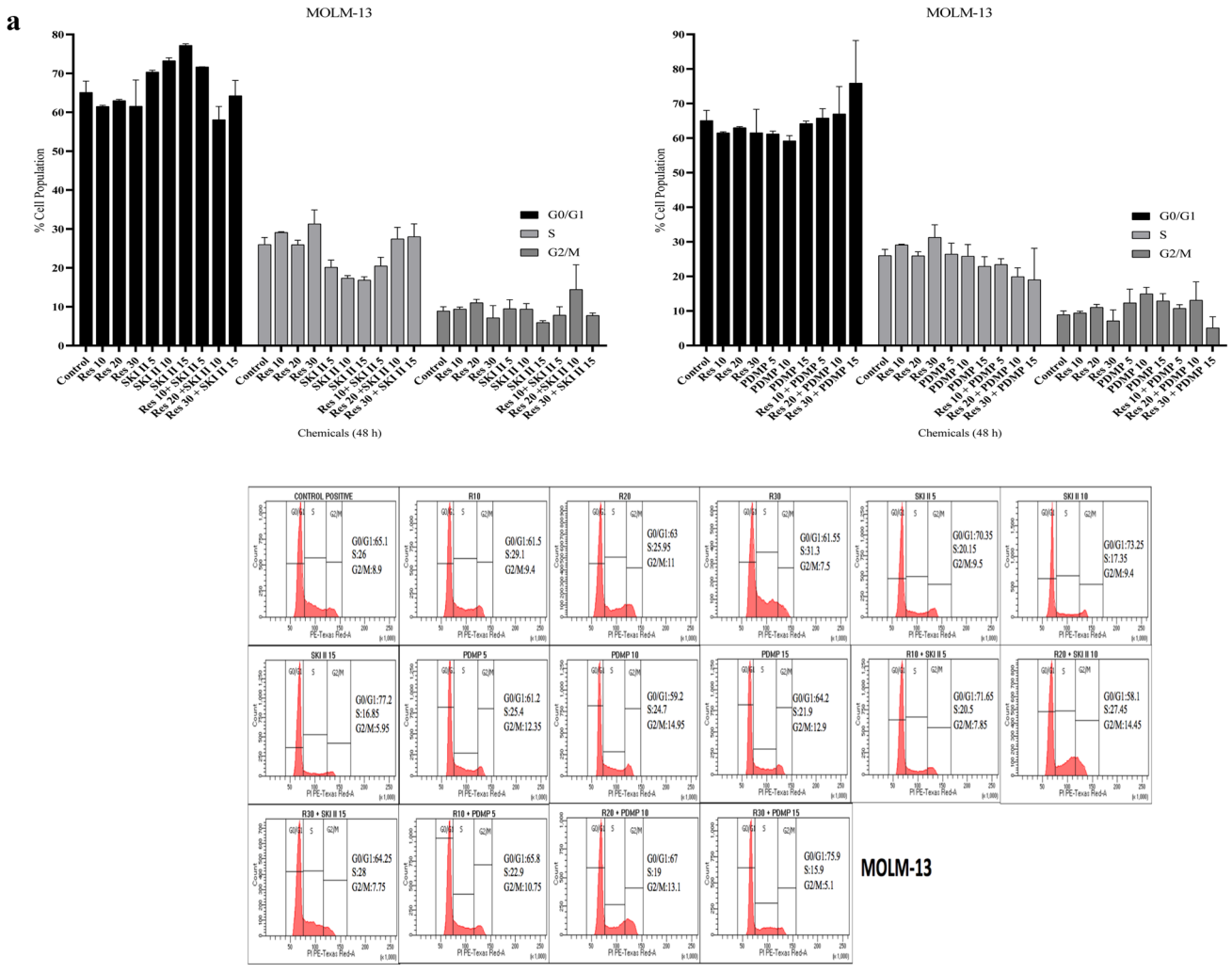

Fig. 7 Effects of resveratrol plus with SKI II or PDMP on cell cycle distribution in MOLM-13 (**a**) and MV4-11 (**b**) cells. Histograms display the percentages of cell populations accumulated in each phase of cell cycle

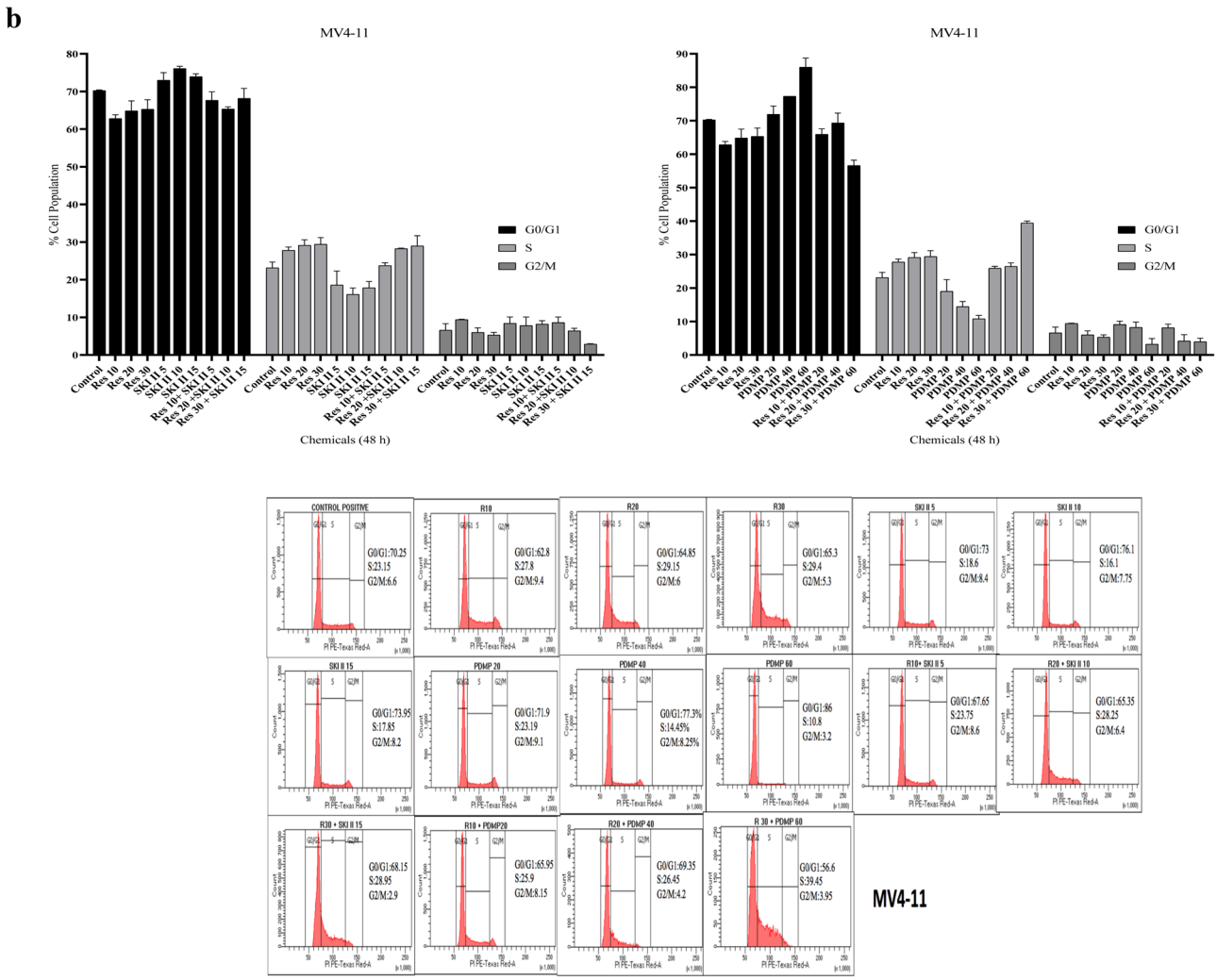

Fig. 7 (continued)

data from combinational treatments. Therefore, we could suggest that SK-1 and GCS represent novel targets of resveratrol in FLT3-ITD AML after identifying the actual relationship between these enzymes and resveratrol.

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Author contributions AA designed and supervised the study; NŞE performed the experimental procedures; NŞE and AA analyzed the data and reviewed the manuscript; and AA wrote the paper. All authors read and approved the final manuscript. This study is derived from the master thesis of NŞE.

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Declarations

Conflict of interest The authors declare that they have no known competing financial interest or personal relationships that could influence their work.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate Not applicable.

Consent for publication Not applicable.

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