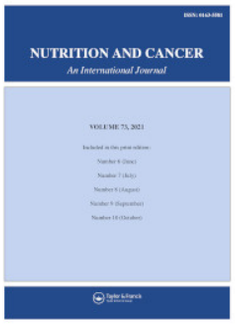

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Involvement of Sphingolipid Metabolism Enzymes in Resveratrol-Mediated Cytotoxicity in Philadelphia-Positive Acute Lymphoblastic Leukemia

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ABSTRACT

Targeting the key enzymes of sphingolipid metabolism including serine palmitoyltransferase (SPT), sphingosine kinase (SK) and glucosylceramide synthase (GCS) has a therapeutic importance. However, sphingolipid metabolism-mediated anti-leukemic actions of resveratrol in Philadelphia-positive acute lymphoblastic leukemia (Ph + ALL) remain unknown. Therefore, we explored potential mechanisms behind resveratrol-mediated cytotoxicity in SD1 and SUP-B15 Ph + ALL cells in the context of sphingolipid metabolism and apoptosis induction. The anti-proliferative and apoptotic effects of resveratrol alone and in combination with SPT inhibitor (myriocin), SK inhibitor (SKI II), GCS inhibitor (PDMP) were determined by MTT cell proliferation assay and flow cytometry, respectively. The effects of resveratrol on PARP cleavage, SPT, SK and GCS protein levels were investigated by Western blot. Resveratrol inhibited proliferation and triggered apoptosis via PARP activation and externalization of phosphatidylserine (PS). Resveratrol increased the expression of SPT whereas it downregulated SK and GCS. Resveratrol's combinations with SKI II and PDMP intensified its anti-leukemic activity by increasing the relocalization of PS while its combination with myriocin suppressed apoptosis. Therefore, resveratrol inhibited cell proliferation and induced apoptosis through modulating SK, GCS and SPT expression, which may be considered as novel biomarkers of resveratrol-induced cytotoxicity in Ph + ALL.

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Introduction

Acute lymphoblastic leukemia (ALL) is the most common childhood leukemia, which accounts for almost 30% of all pediatric leukemia cases. Although 80% of ALL occurs in children, it is also the second most common acute leukemia in adults with more severe outcomes (1). ALL is characterized by abnormal proliferation and accumulation of either malignant and immature B-cell (B-ALL) or T-cell (T-ALL) lymphoid progenitor cells in the bone marrow and blood (2). ALL cells carry various genetic abnormalities including mutations and chromosomal translocations in the genes required for lymphoid development and survival (1–3). Identification of such aberrations has been contributed to our understanding of ALL pathogenesis and used to develop new targeted therapeutics including small molecule inhibitors, monoclonal antibodies and cellular therapies which might be effective in addition to conventional multi-agent intense chemotherapy (3).

Philadelphia chromosome-positive (Ph+) ALL originates from a reciprocal translocation between chromosome 9 and 22, leading to constitutively active BCR-ABL fusion protein with a tyrosine kinase activity. This molecular rearrangement results in overactivated downstream signaling pathways such as PI3K, MAPK, JAK-STAT and RAS, resulting in abnormal proliferation and inhibition of apoptosis (2, 4). The presence of Ph + chromosome increases with age (almost 25% of all ALL cases in adults) and et al., with treatment obstacles. Even though the introduction of tyrosine kinase inhibitors (TKIs) alone and in combination with chemotherapy has dramatically changed the therapeutic outcomes, the development of drug resistance, relapse and fetal cardiovascular and neural toxicities is considered as significant therapeutic problems to overcome (5). Therefore,

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investigating potential nutraceuticals with novel cellular targets for integrated therapies has been paid increasing attention.

Sphingolipids represent a major class of lipids in eucaryotes with significant cellular functions including cell proliferation, cell death, cell differentiation, cell migration and inflammation besides being structural components of the cellular membranes (6). They are synthesized in the endoplasmic reticulum (ER) through multistep complex reactions catalyzed by several different enzymes (6). Bioactive sphingolipids including ceramide (Cer), sphingosine-1-phosphate (S1P) and glucosyl ceramide (GC) regulate multi-stage carcinogenesis and act as important indicators of therapeutic responses to anticancer agents (6, 7). Cer is mainly produced through *de novo* synthesis pathway and serine palmitoyltransferase (SPT) is a rate-limiting enzyme catalyzing the first step of *de novo* pathway at which serine is condensed with palmitoyl-CoA. Cer is a central molecule of sphingolipid metabolism and known to mediate cell death or senescence, hence defined as an apoptotic lipid (6). The intracellular amount of Cer increases in response to chemotherapy, oxidative stress and several agents including resveratrol (8, 9). However, the conversion of Cer into S1P by sphingosine kinases (SK-1 and SK-2) or GC by glucosylceramide synthase (GCS) induces cell proliferation and growth (9, 10). Therefore, S1P and GC are anti-proliferative and anti-apoptotic lipids. Therapeutic potential of targeting sphingolipid metabolism in various cancers including chronic myeloid leukemia (CML), colorectal cancer and glioblastoma has been well documented (11–13). Colorectal cancer patient samples showed upregulation of SK-1 and SK-2, which caused increased metastatic potential (12). Inhibition of SK/S1P pathway in colorectal cancer cells led to down-regulation of adhesion and metastasis-related proteins (12). Shifting of Cer/S1P balance toward S1P in glioblastoma cells was found to increase angiogenic potential (13). The abundance of these sphingolipids is highly regulated by the enzymes, thus, alterations in their expression or activity play significant roles in the determination of cancer cell death or survival.

Resveratrol, a plant-derived polyphenol, is commonly found in grapes, peanuts and berries with chemotherapeutic and chemopreventive potentials (14). Resveratrol targets several signaling pathways involved in initiation, promotion and progression of cancer (14, 15). The mechanisms of resveratrol's anti-carcinogenic potential vary based on the cancer type. It inhibited PI3K/AKT pathway to regulate cell growth, differentiation and proliferation in liver cancer (16). Resveratrol-induced apoptosis in multiple myeloma by inhibiting NF-κB and STAT3 (17). Resveratrol-induced apoptosis in chronic lymphocytic leukemia cells through activation of caspase-3 and cell cycle arrest at G0/G1 (18). It is also known from very limited studies that its chemopreventive or chemotherapeutic effects occur through modulation of sphingolipid metabolism in various cancer types (8, 19, 20). In human gastric cancer cell lines, resveratrol-mediated cytotoxicity was due to the accumulation of Cer and its activity was enhanced via inhibiting SK (21). Resveratrol treatment resulted in Cer accumulation through increased activation of SPT, which caused ER stress and ER-associated caspase activation in nasopharyngeal carcinoma cells (22). However, the roles of bioactive sphingolipids in resveratrol-mediated cytotoxicity in Ph + ALL have not been investigated.

In this study, we aimed to explore the potential mechanisms behind resveratol-mediated cytotoxicity in Ph + ALL in relation to sphingolipid metabolism. We also suggested that targeting sphingolipid metabolism by pharmacological inhibitors could enhance the growth inhibitory effects of resveratrol on Ph + ALL cells.

Materials and Methods

Chemicals

Resveratrol and MTT were purchased from Sigma-Aldrich (USA). Myriocin, SKI II and PDMP were obtained from Cayman Chemicals (Ann Arbor, MI, USA). 10 mM stock solutions were prepared in dimethyl sulfoxide (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

Human SD1 and SUP-B15 Ph + ALL cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). The cells were cultured in RPMI 1640 growth medium (with l-glutamine), supplemented with 10–20% FBS and 1% penicillin-streptomycin at 37 °C in a 5% CO_2 incubator.

MTT Cell Proliferation Assay

Time- and concentration-dependent anti-proliferative effects of resveratrol (10–100 μM), myriocin (1–100 nM), SKI II (0.25–20 μM) and PDMP

(0.25–40 μM) on SD1 and SUP-B15 cells were determined by the MTT cell proliferation assay (23). 1×10^4 SD1 and 2×10^4 SUP-B15 cells were seeded into the wells of a 96 well plate in 100 μl growth medium and incubated for 48 and 72 h. Then, 20 μl MTT solution (5 mg/ml, Sigma-Aldrich) was added to each well to form formazan crystals, which were dissolved in 100 μl DMSO. Absorbance values were measured at 570 nm by a spectrophotometer (Varioskan™ LUX multimode microplate reader, Thermo Fisher Scientific Inc., USA). Based on the cell proliferation graphs, IC_{50} values (concentrations inhibiting cell proliferation by 50%) for resveratrol, myriocin, SKI II and PDMP were calculated.

Design of Combination Analysis

Increasing concentrations of resveratrol were combined with constant concentrations of myriocin, SKI II and PDMP for both cell lines to determine whether there is a cytotoxic synergistic effect or not by MTT assay for 48 h. Concentrations lower than IC_{50} values of SKI II and PDMP were used in combination studies, which were previously shown to be appropriate for the inhibition of these enzymes, hence inducing Cer accumulation (11). SD1 cells were treated with 10, 20 and 40 μM resveratrol combined with 2.5 μM SKI II, 10 μM PDMP and 100 nM myriocin. Similarly, SUP-B15 cells were treated with 5 and 10 μM resveratrol in combination with 1.0 μM SKI II, 1 μM PDMP and 100 nM myriocin. These concentrations alone and in combinations were also used for annexin V FITC/PI double staining.

Annexin V FITC/PI Double Staining by Flow Cytometry

Annexin V-FITC apoptosis detection kit (BioVision, USA) was used to examine the apoptotic effects of resveratrol alone and in combination with SKI II, PDMP and myriocin based on the instructions in the kit. Briefly, 1×10^6/2 ml cells seeded into each well of a 6-well plate were exposed to selected concentrations of each agent alone or in combinations for 48 h. Then, the cells were collected and washed twice with cold PBS. 2 μl of FITC Annexin V and 2 μl of propidium iodide (PI) were added to the cells and incubated for 15 min. The percentage of early and late apoptotic cells was determined by using BD FACSCalibur flow cytometer (BD Biosciences, USA). The histograms were obtained and analyzed using BD FACSDiva™ (BD Biosciences, USA) (23).

Protein Extraction and Western Blot

4×10^6 cells were treated with increasing concentrations of resveratrol for 48 h to detect the changes in the expression of PARP, SPT, SK and GCS by Western blot. Cells were lysed in RIPA buffer (Sigma-Aldrich, USA). The supernatants were collected to measure protein concentrations using RC DC™ 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), SK (1:3000, Cell Signaling, USA), β-Actin (1:3000, Cell Signaling, USA), SPT (1:1000, Novus Biologicals, USA) and GCS (1:1000, Novus Biologicals, USA) overnight at +4 °C and conjugated with appropriate secondary antibodies (1:10000, Jackson Immuno Research, USA). The membranes were visualized using Pierce™ ECL Western Blotting Substrate kit (Thermo Scientific™, USA). Densitometric analysis of immunoreactive bands was carried out using imaging software (Bio-Rad, ChemiDoc, Image Lab™ 3.0). The relative protein levels were quantified relative to an untreated control.

Statistical Analysis

The experiments were performed as two or three independent setups and the results were given as mean ± standard error (SE). Statistical analysis was performed using GraphPad Prism 6.0. One-way ANOVA was used for the analysis of MTT data and two-way ANOVA was used for annexin V-FITC/PI analysis. $P < 0.05$ was considered as statistically significant.

Results

Resveratrol and Sphingolipid Metabolism Inhibitors Suppress Ph + ALL Cell Proliferation

We treated SD1 and SUP-B15 cells with increasing concentrations of resveratrol, SKI II, PDMP and myriocin for 48–72 h to study anti-proliferative effects using MTT assay. As indicated in Figure 1, resveratrol reduced cell viability in a time- and dose-dependent manner. Time-dependent IC_{50} concentrations for SD1 cells were calculated as 43 and 37 μM (Figure 1A). Similarly, IC_{50} concentrations for SUP-B15 cells were 37 and 20 μM (Figure 1B). These results indicated that resveratrol may potently inhibit Ph + ALL cell proliferation. Inhibitors targeting SK and GCS also inhibited cell viability in a time- and

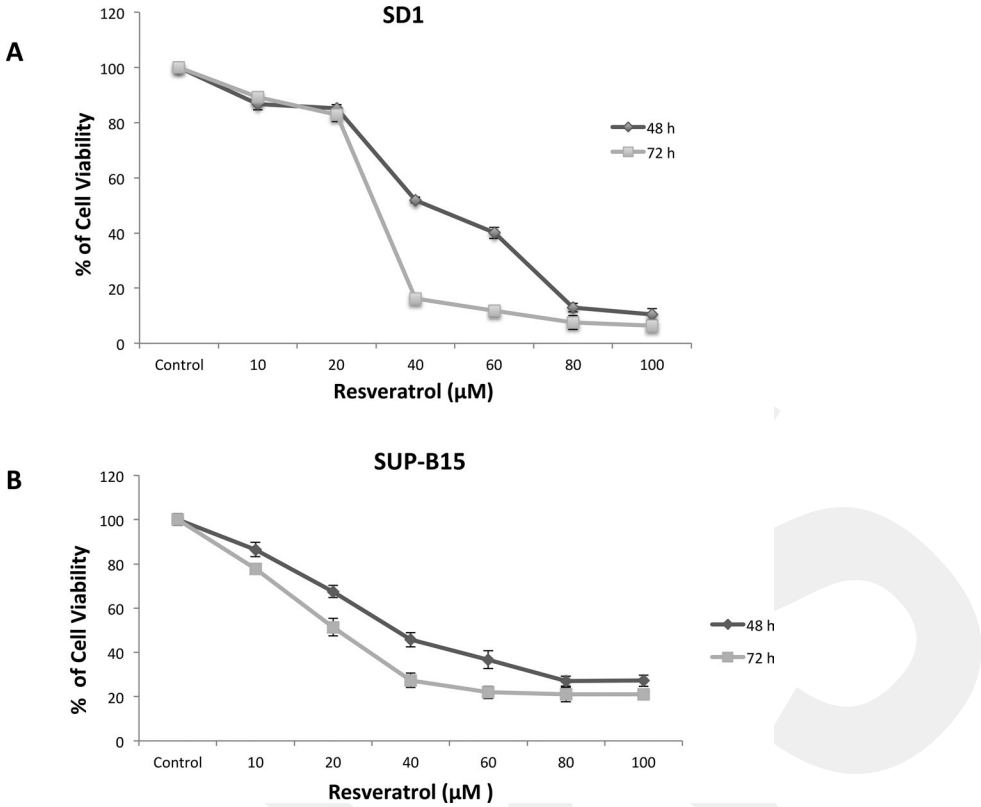

Figure 1. Cytotoxic effects of resveratrol on SD1 (A) and SUP-B15 (B) cells in a time- and concentration-dependent manner. The results derived from the means of three independent experiments are represented as mean ± SE and $P < 0.05$ was considered as significant.

concentration-dependent manner (Figure 2). Time-dependent IC_{50} concentrations were calculated as 6.6 and 4.5 μM for SKI II. IC_{50} values were 36 and 25 μM for PDMP in SD1 cells (Figure 2A). Similarly, IC_{50} concentrations were 4.5 and 2.3 μM for SKI II and 32 and 18 μM for PDMP in SUP-B15 cells (Figure 2B). On the other hand, myriocin did not affect the cell viability significantly for both cells (Figure 2A and B).

Resveratrol Modulates Sphingolipid Metabolism Enzymes

To understand whether the critical enzymes of sphingolipid metabolism could contribute to resveratrol's anti-leukemic activity, alterations in the protein levels of SK (SK-1 and SK-2 isoforms), GCS and SPT in response to resveratrol in SD1 (20 and 40 μM) and SUP-B15 (5 and 10 μM) cells were investigated. It was shown that resveratrol did not cause a significant change in SK-1 expression, but caused 0.32- and 0.54-fold decreases in SK-2 in SD1 cells (Figure 3A, $P < 0.05$). 20 and 40 μM resveratrol significantly reduced GCS expression (0.7- and 0.8-fold, respectively, $P < 0.05$) (Figure 3A) while caused 2.2- and 2.3-fold increases in SPT expression, respectively (Figure 3A, $P < 0.05$). In SUP-B15 cells, 5 and 10 μM resveratrol caused decreases in both SK-1 (0.6- and 0.75-fold, $P < 0.05$) and SK-2 (0.65- and 0.69-fold, $P < 0.05$) expression, respectively (Figure 3B). 10 μM resveratrol reduced GCS expression by 0.32-fold (Figure 3B, $P < 0.05$) and caused a 0.38-fold increase in SPT expression (Figure 3B, $P < 0.05$). These results showed that resveratrol could regulate SK and GCS to increase apoptotic Cer by decreasing its conversion into anti-apoptotic S1P and GC, hence triggering anti-proliferative effect. On the other hand, resveratrol could modulate *de novo* Cer production by upregulating SPT enzyme to induce Cer accumulation, which could lead to inhibition of cell proliferation.

Enhanced Anti-Proliferative Activity of Resveratrol Combined with Sphingolipid Metabolism Inhibitors in Ph + ALL Cells

To further figure out whether inhibiting SK-1 and GCS enzymes could increase the cytotoxic effect of resveratrol or not, SD1 cells were treated with 10, 20, 40 μM resveratrol in combination with 2.5 μM SKI II,

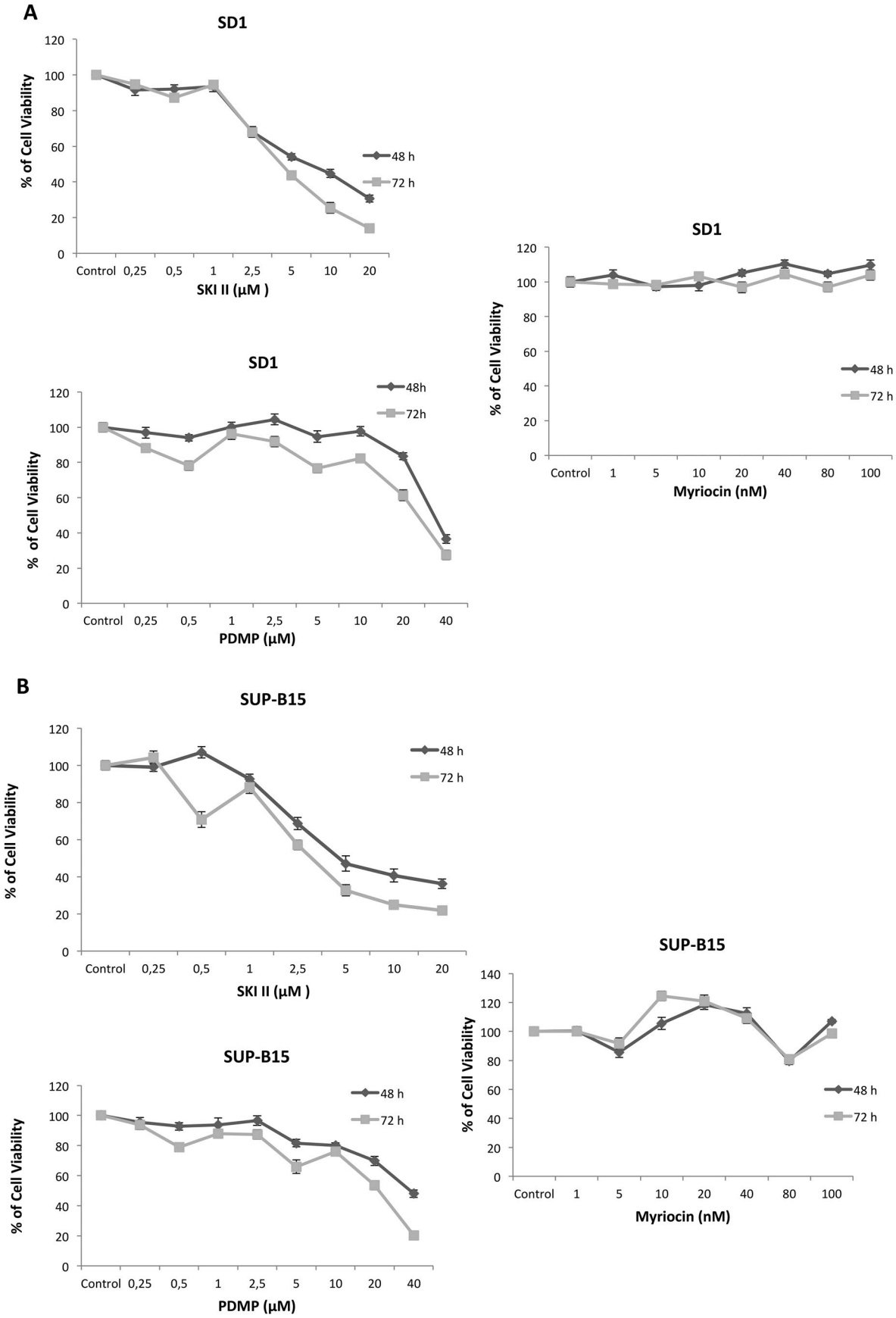

Figure 2. Anti-proliferative effects of SKI II, PDMP and myriocin on SD1 (A) and SUP-B15 (B) cells in a time- and concentration-dependent manner. The results derived from the means of three independent experiments are represented as mean ± SE and $P < 0.05$ was considered as significant.

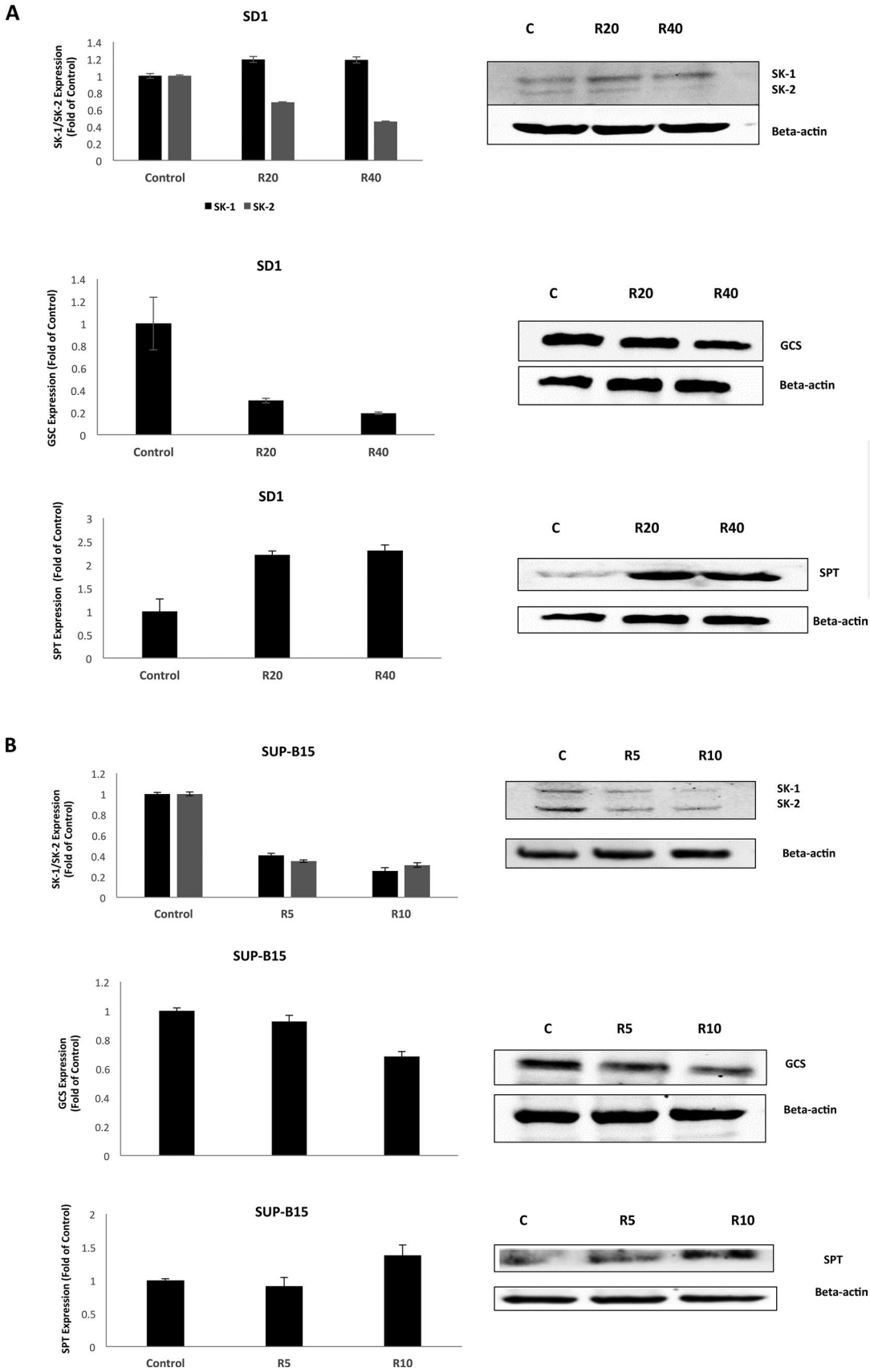

Figure 3. Changes in protein expression of SK-1/SK-2, GCS and SPT in SD1 (A) and SUP-B15 (B) cells treated with resveratrol for 48 h. β-Actin was used as loading control. Experimental sets were repeated twice and representative blots were used for each experiment. The results from two different experimental sets were given as mean ± SE and $P < 0.05$ was considered as significant. The proteins of each group were normalized to their β-actins, and the graphs were plotted by assuming control value as 1.

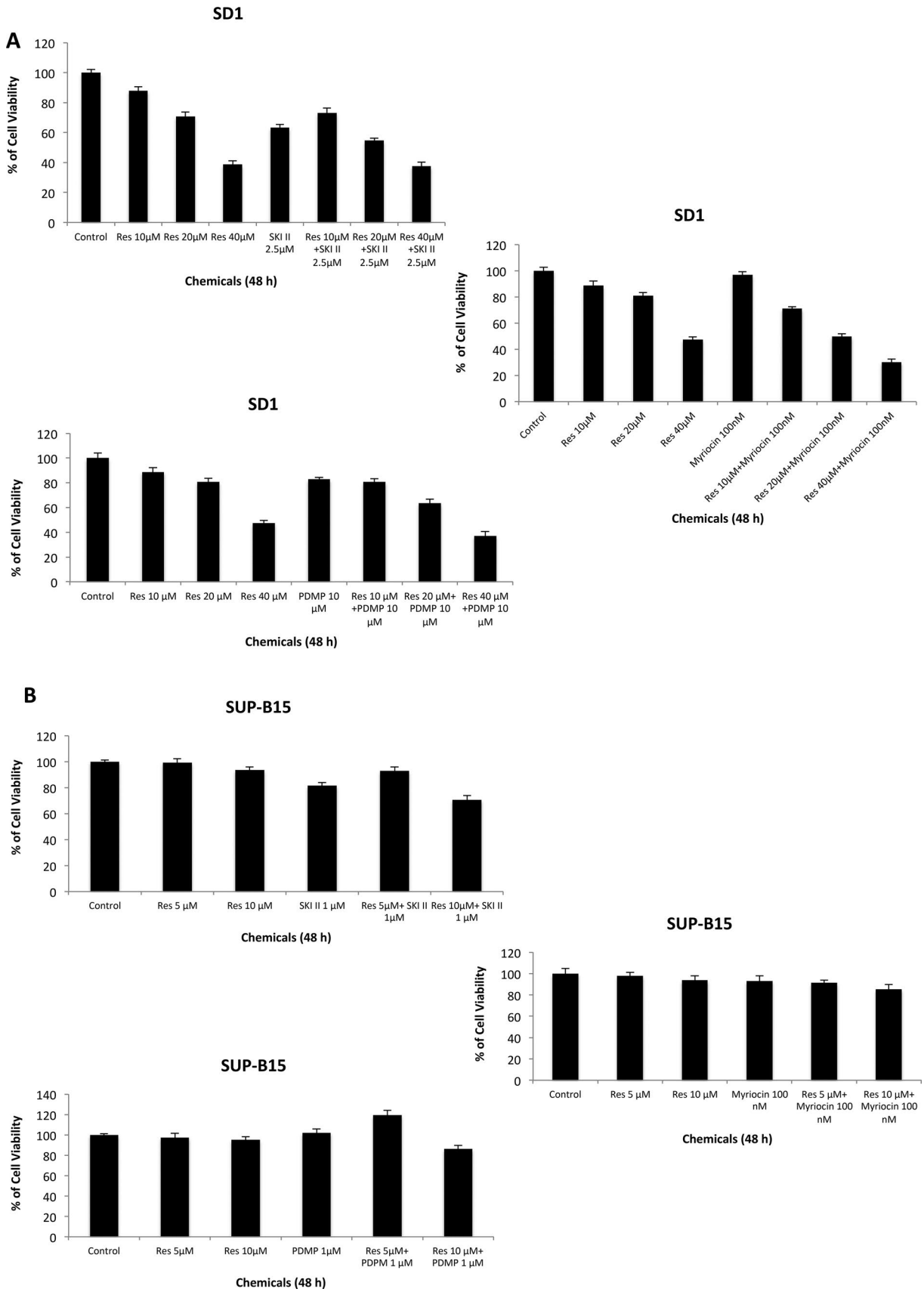

Figure 4. Synergistic cytotoxic effects of resveratrol in combination with SKI II, PDMP and myriocin on SD1 (A) and SUP-B15 (B) cells. The results derived from the means of three independent experiments are represented as mean ± SE and $P < 0.05$ was considered as significant.

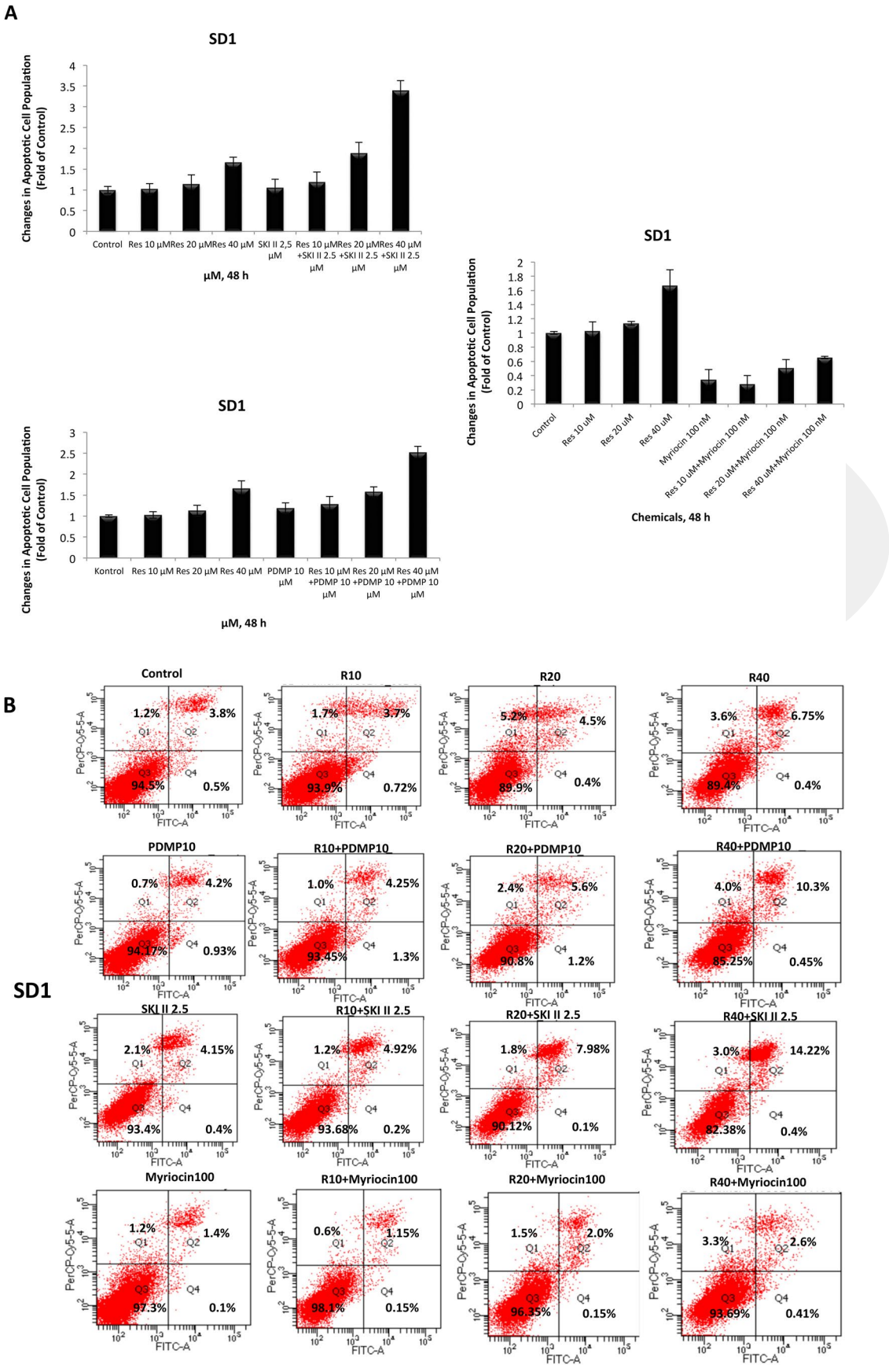

Figure 5. Apoptotic effects of resveratrol and its combinations with SKI II, PDMP and myriocin on SD1 (A, B) cells. The results derived from the means of three independent experiments are represented as mean ± SE and $P < 0.05$ was considered as significant. The percentage of Annexin V-positive/PI-negative early apoptotic population (Q4) and Annexin V-positive/PI-positive late apoptotic population (Q2) was calculated and normalized to untreated control.

10 μM PDMP and 100 nM myriocin to determine the synergistic anti-proliferative effects (Figure 4A). The combinations of resveratrol with 2.5 μM SKI II decreased cell viability to 73%, 54% and 37% as compared to control, respectively ($P < 0.05$). Similarly, increasing concentrations of resveratrol combined with 10 μM PDMP showed that cell proliferation reduced in all combinations (cell viability decreased to 80%, 63% and 37% for combinations, respectively, $P < 0.05$). On the other hand, increasing concentrations of resveratrol with 100 nM myriocin decreased cell viability to 70%, 49% and 30%, respectively (Figure 4A, $P < 0.05$). Similarly, SUP-B15 cells were treated with 5 and 10 μM resveratrol in combination with 1.0 μM SKI II, 1 μM PDMP and 100 nM myriocin (Figure 4B). The combination of increasing concentrations of resveratrol with 1.0 μM SKI II decreased cell growth to 92% and 70%, respectively, as compared to control. Therefore, combination of 10 μM resveratrol with 1.0 μM SKI II was the effective combination on cell proliferation ($P < 0.05$). Similarly, cell proliferation reduced effectively only at 10 μM resveratrol combined with 1 μM PDMP ($P < 0.05$). On the other hand, increasing concentrations of resveratrol with 100 nM myriocin did not affect the cell proliferation (Figure 4B).

Resveratrol's Apoptotic Activity is Enhanced through Inhibiting Sphingolipid Metabolism Enzymes

The relocalization of phosphatidylserine (PS) is one of the hallmarks of apoptosis. Thus, the apoptotic effects of resveratrol and its combinations with GCS, SK and SPT inhibitors were checked by Annexin V/PI staining to figure out the contribution of each enzyme to resveratrol's anti-proliferative effect. Apoptotic SD1 cell population increased by 1.02-, 1.35- and 1.66-fold in response to 10, 20 and 40 μM resveratrol, respectively, as compared to untreated control (Figure 5A and B). The combination of increasing concentrations of resveratrol with 2.5 μM SKI II caused increases in apoptotic cell population as 1.19-, 1.88- and 3.4-fold (Figure 5A and B). Its concentrations with 10 μM PDMP increased apoptotic cell population as 1.3-, 1.6- and 2.5-fold, respectively, when compared to control (Figure 5A and B). The combination of increasing concentrations of resveratrol with 100 nM myriocin resulted in suppression of apoptosis (Figure 5A and B).

SUP-B15 cells were treated with 5 and 10 μM resveratrol and apoptotic cell population increased as 1.14- and almost 2.0-fold as compared to control, respectively (Figure 6A and B). 10 μM resveratrol combined with 1.0 μM SKI II increased apoptotic cell population as 5.6- and 2.9-fold compared to control and 10 μM resveratrol, respectively (Figure 6A and B). Likewise, 10 μM resveratrol with 1.0 μM PDMP increased apoptotic cell population as 2.5-fold compared to control and 1.3-fold compared to 10 μM resveratrol (Figure 6A and B). Combination of 5 and 10 μM resveratrol with 100 nM myriocin resulted in decreases in apoptotic cell population compared to control and resveratrol alone (Figure 6A and B). These results showed that resveratrol-induced apoptosis synergistically in the presence of SK and GCS inhibitors. Inhibiting SK and GCS which are responsible for the conversion of apoptotic Cer to anti-apoptotic S1P and GS, respectively, would lead to accumulation of intracellular apoptotic Cer. Moreover, inhibiting SPT by myriocin, regulatory enzyme involved in *de novo* Cer synthesis, would cause a decrease in the amount of apoptotic Cer, resulting in the suppression of apoptosis in Ph + ALL cells treated with resveratrol.

We also checked PARP cleavage, which is a final substrate of intrinsic pathway of apoptosis, in the cells treated with increasing concentrations of resveratrol for 48 h. Consistent with the annexin V/PI staining results, resveratrol treatment resulted in PARP cleavage in both SD1 and SUP-B15 cells as compared to control ($P < 0.05$) (Figure 7A and B).

Discussion

Anti-carcinogenic properties of resveratrol have been well documented for many cancer types and it is called a pleiotropic flavonoid due to its ability to affect multiple intracellular targets (24). The key enzymes or products of sphingolipid metabolism are considered as novel resveratrol's targets for a variety of solid and hematological tumors in addition to its well-defined intracellular targets (25–27).

In this study, we aimed to investigate the therapeutic potential of resveratrol in human Ph + ALL cells and to determine whether resveratrol could modulate the enzymes of sphingolipid metabolism for its cytotoxic actions for the first time. We determined the inhibitory effect of resveratrol on the proliferation of Ph + SD1 and SUP-B15 ALL cells by MTT assay, annexin V/PI staining and PARP activation. Our results showed a time- and dose-dependent decreases in cell proliferation in response to resveratrol (Figure 1) and resveratrol-induced apoptosis through PARP cleavage (Figure 7), which supported that resveratrol treatment led to increases in apoptotic cell population

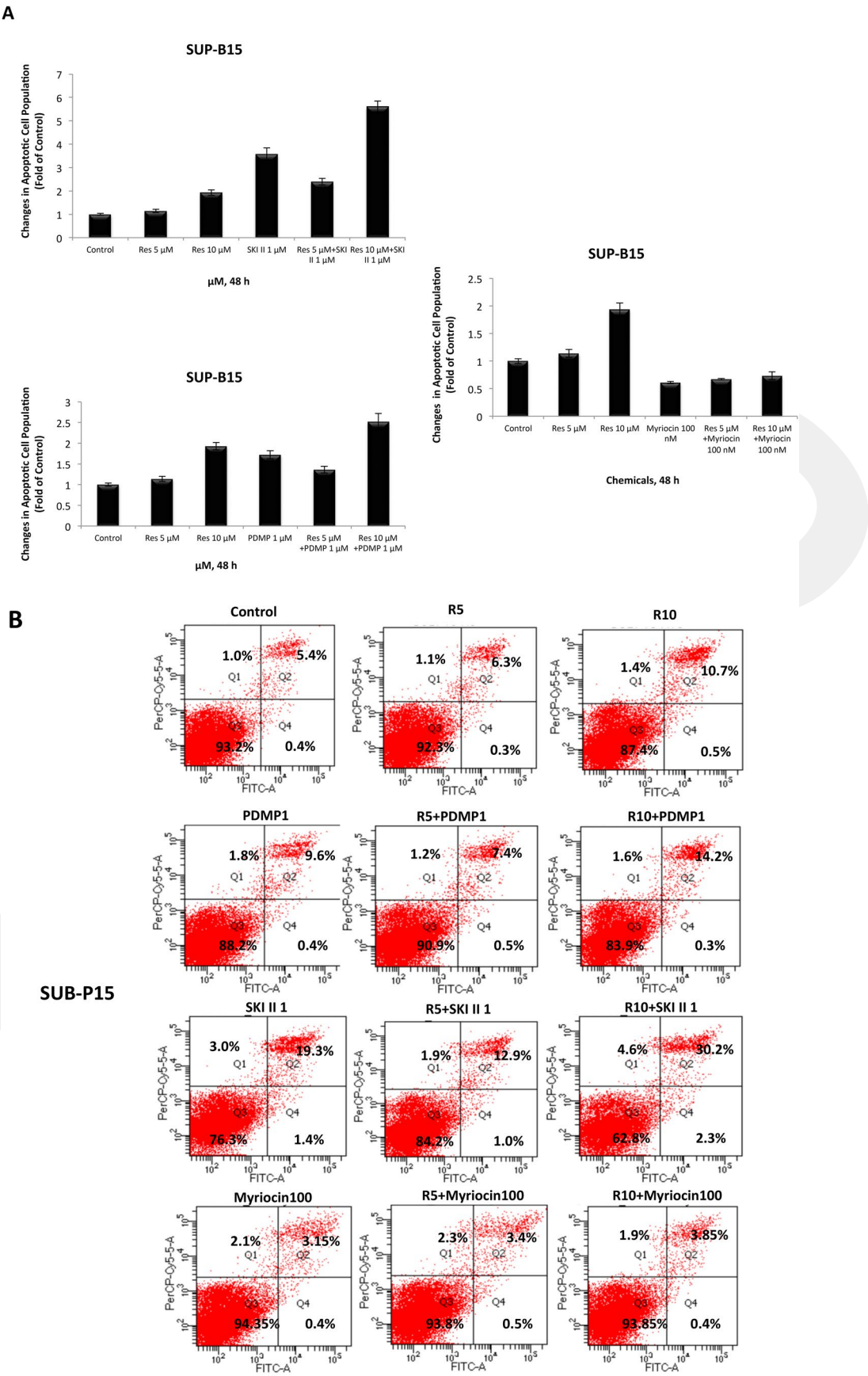

Figure 6. Apoptotic effects of resveratrol and its combinations with SKI II, PDMP and Myriocin on SUP-B15 (A, B) cells. The results derived from the means of three independent experiments are represented as mean ± SE and $P < 0.05$ was considered as significant. The percentage of Annexin V-positive/PI-negative early apoptotic population (Q4) and Annexin V-positive/PI-positive late apoptotic population (Q2) was calculated and normalized to untreated control.

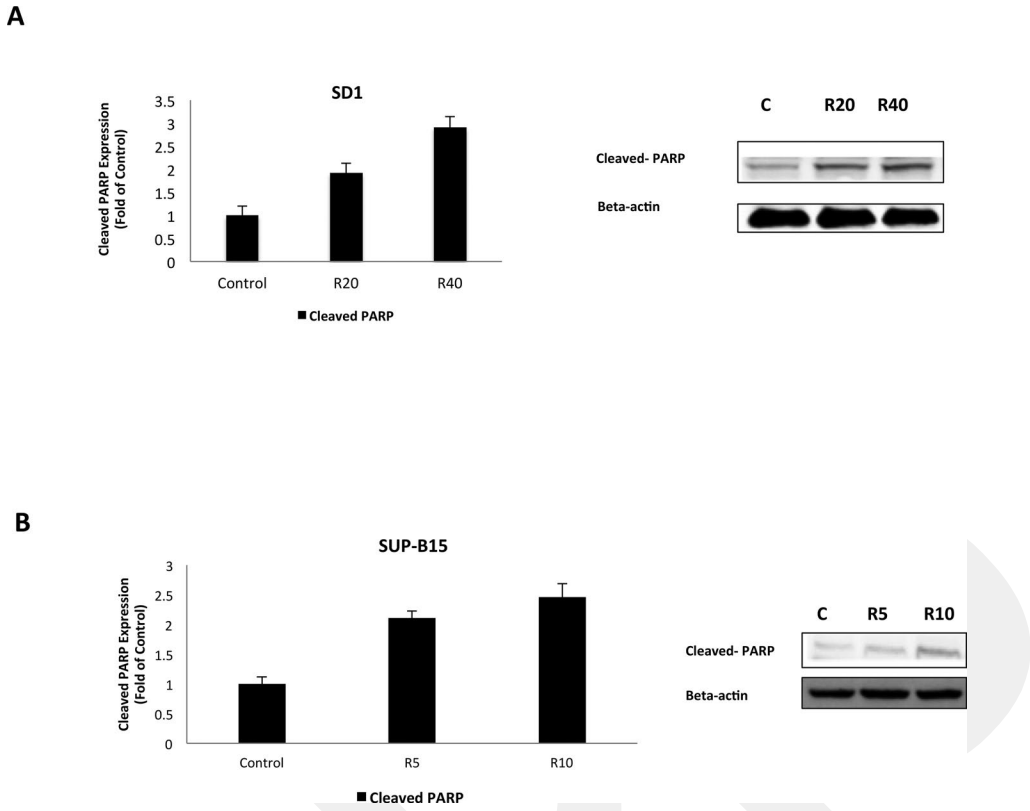

Figure 7. Changes in active PARP expression in SD1 (A) and SUP-B15 (B) cells treated with resveratrol for 48 h. β-actin was used as a loading control. Experimental sets were repeated twice and representative blots were used for each experiment. The results from two different experimental sets were given as mean ± SE and $P < 0.05$ was considered as significant. The truncated PARP of each group was normalized to their β-actins, and the graphs were plotted by assuming control expression as 1.

in both cell lines by PS externalization (Figures 5 and 6). As shown in many cancer types, resveratrol's anti-proliferative action is due to induction of apoptosis via intrinsic pathway. Resveratrol increased PARP cleavage in colorectal cancer cells (28). In HCT116, CO115 and SW48 colorectal cells, a positive regulator of p53 known as SET7/9 was upregulated upon resveratrol treatment, which further triggered PARP-dependent apoptosis. Downregulation of SET7/9 by shRNA attenuated resveratrol-driven apoptosis by restoring PARP activation (28). Resveratrol also inhibited cell proliferation in a time-dependent manner and induced apoptosis in MOLT-4 and HL-60 leukemia cells by inducing PARP cleavage and PS externalization (29). In K562 CML cells, resveratrol induced cell death in a dose-dependent manner via PARP activation and PS externalization (30). Treatment of metastatic breast cancer cells with resveratrol for 48 h inhibited cell proliferation and induced PARP-dependent apoptosis (26) in accordance with the results presented.

The critical role of sphingolipids in the determination of the cell fate is called "sphingolipid rheostat" in cancer development and therapy. The balance between apoptotic Cer and anti-apoptotic S1P or GC contributes to cell survival or death. It has been well documented that Cer accumulation in cancer cells causes cell death while S1P or GC supports cell survival (31). The intracellular amount of apoptotic Cer increases in response to chemotherapeutics or other chemical agents including natural products (19). Therefore, we also checked whether resveratrol could exert its anti-leukemic effects by altering the expression of the rate-limiting enzymes, SK, GCS and SPT, to modulate cellular sphingolipid balance. We found that resveratrol decreased SK and GCS expression responsible for synthesis of anti-apoptotic S1P and GC, respectively, while upregulated SPT regulating the first step of *de novo* Cer synthesis (Figure 3). To further clarify whether the inhibition of SK, GCS and SPT could enhance resveratrol's activity or not, the cytotoxic and apoptotic effects of resveratrol combined with SKI II, PDMP and myriocin were investigated by MTT assay (Figure 4) and annexin V/PI staining (Figures 5 and 6). Resveratrol combined with SKI II and GCS increased apoptotic cell population while its combination with myriocin resulted in suppression of apoptosis as compared to control or resveratrol (Figures 5 and 6). Therefore, it could be concluded

that the anti-leukemic activity of resveratrol might be related to the modulation of the enzymes involved in different steps of sphingolipid metabolism in addition to its direct effect on the expression of SK, GCS and SPT. Furthermore, its efficacy could be enhanced upon combination with inhibitors that could change Cer/S1P-GC balance to determine the cell fate. There are some studies clarifying the roles of resveratrol in the regulation of sphingolipid metabolism in solid cancers. In B16 melanoma cells, 48 h resveratrol treatment suppressed cell viability. Resveratrol caused Cer accumulation and autophagic cell death via inhibition of Akt/mTOR pathway. Inhibition of ceramide synthase by fumonisin reversed resveratrol-mediated autophagy (25). It was shown in metastatic breast cancer that SPT was activated after 48 h resveratrol treatment and its inhibition with myriocin counteracted resveratrol's effects similar to our results (26). The expression and activity of SK-1 was inhibited by resveratrol and resveratrol dimers in 48-h treated MCF-7 breast cancer cells, resulting in cancer cell death by inducing PARP activation (32). In OVCAR-3 ovarian cancer cells, 48 h resveratrol and Cer treatment induced nuclear accumulation of COX-2 via activating ERK1/2 pathway, leading to apoptotic cell death (33). It was also shown that resveratrol and Cer-induced apoptosis was p53-dependent in human ovarian cancer cells. Only a few studies have investigated the possible roles of sphingolipid metabolism in resveratrol-triggered cytotoxicity in hematological malignancies. Resveratrol decreased S1P and increased Cer levels in CML cells by increasing the expression of acid ceramidase (ASMase), which led to inhibition of cell proliferation. This study also suggested that resveratrol upregulated ATF3, EGR1, EGR3 signaling proteins and increased FOXO3 and c-Jun phosphorylation (20). Tian and Yu showed that resveratrol did not change total SK-1 expression, however, it caused its translocation from plasma membrane to cytoplasm and induced apoptosis in K562 CML cells (34). HL-60 cells underwent apoptosis after 72 h treatments with resveratrol plus SK-1 and GCS inhibitors and it downregulated Cer synthesis genes and upregulated Cer metabolizing SK-1 and GCS genes (35). Resveratrol inhibited the proliferation of K562 CML cells after 72 h treatment via altering the mRNA expression of SK-1 and ceramide synthase genes (27). In our study, we also determined the role of *de novo* ceramide synthesis in resveratrol-triggered growth suppression for the first time in leukemia through direct targeting of SPT by resveratrol. Moreover, SK-2 was found as resveratrol's target for both cell lines (Figure 3) although SK-1 is the most studied SK isoform. The involvement of SK-2 in BCR-ABL-independent pathogenesis of ALL models was shown in a recent study (36), also supporting our hypothesis of SK-2 being a novel target of resveratrol in ALL.

Conclusion

The current study showed that resveratrol-induced cytotoxicity might be mediated through its multi-targeted modulatory effect on sphingolipid pathway, since it could regulate Cer-producing and -catabolizing enzymes, which then affected cell proliferation and apoptosis. SK (especially SK-2), GCS and SPT could be considered as novel targets of resveratrol in Ph + ALL. It is also clear that more detailed mechanistic studies such as investigating signal transduction pathways involved in Ph + ALL proliferation and survival should be carried out to clarify the relationship between resveratrol and sphingolipid metabolism in Ph + ALL. Overall, the results would support the potential of resveratrol to be considered as a promising integrative agent for the chemoprevention of Ph + ALL after further detailed in vitro and in vivo studies.

Disclosure statement

The authors report no conflict of interest.

Authors' Contribution

Concept: A.A., Design: A.A. and O.O., Data collection or processing: A.A. and O.O., Analysis or interpretation: A.A. and O.O., Literature search: A.A. and O.O., Writing: A.A., Principal author and supervision: A.A.

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