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Research Article

Cytotoxic Effects of Resveratrol and Its Combinations with Ceramide Metabolism Inhibitors on FLT3 Positive Acute Myeloid Leukemia

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Abstract

Sphingolipids determine the cell fate by regulating cell proliferation and growth. Ceramide, growth inhibitory lipid, might be produced through *de novo* pathway or salvage pathway, which is converted to proliferation inducers sphingosine-1-phosphate (S1P) and glucosyl ceramide (GC) by sphingosine kinase (SK) and glucosyl ceramide synthase (GCS), respectively. It is aimed to investigate therapeutic potential of resveratrol on FLT3 overexpressing acute myeloid leukemia (AML) cells by pharmacological targeting of ceramide metabolism. The cytotoxic effects of resveratrol, SK inhibitor (SKI II), GCS inhibitor (PDMP) and the combinations of resveratrol with SK-1 inhibitor and GCS inhibitor on THP-1 and OCI-AML3 FLT3 overexpressing AML cells were investigated by MTT cell viability assay in a time- and concentration-dependent manner. Apoptotic effect of resveratrol was analyzed by annexin V/PI double staining using flow cytometry. Resveratrol decreased cell viability and induced apoptosis in both cell lines ($p<0.05$ considered significant). There were synergistic cytotoxic effects of resveratrol with co-administration of SK-1 inhibitor and GCS inhibitor at 48 h ($p<0.05$ considered significant). This preliminary data showed for the first time that resveratrol might inhibit the viability of FLT3 overexpressing AML cells through targeting ceramide metabolism and inducing apoptosis, which needs to be further clarified mechanistically.

Keywords: FLT3 acute myeloid leukemia, resveratrol, sphingosine kinase, glucosyl ceramide synthase

Resveratrol ve Seramid Metabolizması Enzim İnhibitörleri İle Kombinasyonlarının FLT3 Pozitif Akut Miyeloid Lösemideki Sitotoksik Etkileri

Öz

Sfingolipidler hücre büyümesi ve çoğalmasını kontrol ederek hücrenin yaşam veya ölüm arasındaki kararını belirlemektedir. Büyümeyi baskılayıcı etkisi olan *de novo* yol izi veya salvage yol izi ile sentezlenen seramid sfingozin kinaz (SK) ve glukosil seramid sentaz (GSS) enzimleri tarafından sırasıyla hücre çoğalmasını destekleyici sfingozin-1-fosfat (S1F) ve glukozil seramide (GS) dönüştürülmektedir. Bu çalışmada, resveratrolün FLT3'yi aşırı ifade eden THP-1 ve OCI-AML3 hücreleri üzerindeki terapötik potansiyeli seramid metabolizmasının farmakolojik olarak hedeflenmesi ile araştırılmıştır. Resveratrol, SK inhibitörü (SKI II), GSS inhibitörü (PDMP) ve resveratrolün inhibitörler ile kombinasyonlarının THP-1 ve OCI-AML3 hücreleri üzerindeki sitotoksik etkileri konsantrasyona ve zamana bağlı olarak MTT hücre canlılık testi ile saptanmıştır. Resveratrolün apoptotik etkisi aneksin-V/PI ikili boyaması yapılarak akım sitometresi ile belirlenmiştir. Resveratrol her iki hücrede hücre canlılığını azaltmış ve apoptozu indüklemiştir ($p<0.05$ anlamlı olarak değerlendirilmiştir). Resveratolün SK ve GSS inhibitörleri ile kombinasyonlarının 48 saatlik muamele sonucu sinerjistik sitotoksik etki gösterdiği belirlenmiştir ($p<0.05$). Elde edilen bu sonuçlar, resveratrolün FLT3'yi aşırı ifade eden AML'de seramid metabolizmasını hedefleyerek etki gösterebileceğini literatürde ilk defa göstermiştir ve çalışma mekanistik olarak araştırılabilecektir.

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Anahtar Kelimeler: FLT3 akut miyeloid lösemi, resveratrol, sfingozin kinaz, glukosil seramid sentaz

1. Introduction

Acute myeloid leukemia (AML) is a highly aggressive disease characterized by abnormal proliferation and accumulation of myeloblasts with a reduced differentiation and maturation capacity in the bone marrow and blood stream (Grove and Vassiliou, 2014). Molecular detection of some genetic mutations determines the prognostic AML subgroups including The Fms-like tyrosine kinase 3 (FLT3) overexpression or FLT3 mutations such as FLT3 tyrosine kinase domain mutations or FLT3-internal tandem duplications (Martelli et al., 2013; Estey, 2018). FLT3 gene encodes a type III receptor tyrosine kinase and has a key role in the development of the hematopoietic and immune system (Testa and Pelosi, 2013). FLT3 is normally expressed on hematopoietic stem cells and early hematopoietic progenitor cells until they fully differentiate into their functional forms (Daver et al., 2019). Binding of FLT3 ligand (FL) to FLT3 induces receptor dimerization and autophosphorylation, which results in the activation of downstram RAS, PI3K/AKT and STAT5 signaling pathways leading to cell proliferation and supression of apoptosis (Lyman, 1995; Daver et al., 2019). Then, FLT3 expression becomes very weak or completely absent in functional blood cells. Therefore, overexpression of wild type (WT) FLT3 receptor is a clinical obstacle for a high percentage of AML cases (%70-100), which contributes to disease pathogenesis and aggressiveness and associates with poor prognosis (Ozeki et al., 2004). The standart treatment of AML patients consists of cytotoxic chemotherapy. However, high-risk patients with overexpressed WT FLT3 are usually not cured with conventional chemotherapy (Robak and Wierzbowska, 2009). Although FLT3 inhibitors including midostaurin, sorafenib and gilterinitib have been changed the course of treatment strategies after discovering the importance of FLT3 in disease formation, the development of drug resistance and toxic side effects are still significant problems to overcome (Konig and Lewis, 2015; Estey, 2018). Therefore, investigating compounds that can inhibit the proliferation of FLT3 positive AML and identification of new signaling pathways such as sphingolipid signaling to target could be considered as promising strategies.

Bioactive sphingolipids are a lipid family with important members including ceramide (Cer), sphingosine-1-phosphate (S1P) and glucosyl ceramide (GC), which have significant roles in the regulation of cell division, growth, metastasis and apoptosis (Truman et al., 2014). The functions regulated by sphingolipids are directly related to initiation and progression of cancer and response to anticancer therapies (Ogretmen and Hannun, 2004; Kroll et al., 2020). Cer produced through *de novo* synthesis pathway or salvage pathway is a central molecule in sphingolipid metabolism playing significant roles in the supression of cell proliferation and growth (Morad and Cobat, 2012). On the other hand, the conversion of Cer into S1P by sphingosine kinase (SK) or/and into GC by glucosyl ceramide synthase (GCS) induces the proliferation of cancer cells (Giussani et al., 2014). Since Cer metabolism determines the cell fate which is called "sphingolipid rheostat" (Adan-Gokbulut et al., 2013), the enzymes regulating various steps of sphingolipid metabolism have been considered as potential biomarkers and chemotherapeutic targets in various malignancies (Kroll et al., 2020). Therefore,

it is also important to elucidate their roles in FLT3 positive AML pathogenesis and therapy.

Resveratrol (3,4',5-trihydroxy-trans-stilbene) is a natural phytoalexin which is mainly found in grapes, peanuts and blueberries (Kundu and Surh, 2008). Several studies have shown that resveratrol has anti-carcinogenic potential and plays key roles to suppress the proliferation of leukemia and solid cancer cells by targeting multiple and different cellular signaling pathways and molecules (Shukla and Singh, 2011; Rauf et al., 2018). However, there is no study investigating its therapeutic potential and mechanisms of action in WT FLT3 overexpressing AML.

In this project, it is aimed to investigate the therapeutic potential of resveratrol in FLT3 overexpressing AML through modulation of Cer metabolism. Furthermore, potential novel combination therapy is investigated by administrating resveratrol with SK inhibitor and GCS inhibitor.

2. Material and Methods

2.1. Chemicals

Resveratrol and MTT were obtained from Sigma-Aldrich (USA). SKI II and PDMP were purchased from Cayman Chemicals (Ann Arbor, MI, USA). 10 mM stock solutions of these agents were prepared in DMSO and stored at −20 °C. The final concentration of DMSO did not exceed more than 0.1% in culture. Penicillin-streptomycin, RPMI 1640, and fetal bovine serum were obtained from Invitrogen (Paisley, UK).

2.2. Cell lines and culture conditions

Human THP-1 and OCI-AML3 WT FLT3 AML cells were obtained from German Collection of Microorganisms and Cell cultures (Germany, DSMZ). THP-1 cells were cultured in RPMI-1640 growth medium (with L-glutamine) including 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37ºC in a 5% CO_2 incubator. OCI-AML3 cells were cultured in alpha-MEM growth medium supplemented with 20% FBS and 1% penicillin-streptomycin at 37ºC in a 5% CO_2.

2.3. MTT cell viability/proliferation assay

Time and concentration-dependent cytotoxic effects of resveratrol (5-60 μM), SKI II (1-80 μM) and PDMP (1-80 μM) on THP-1 and OCI-AML3 cells were determined by MTT cell proliferation assay (Adan and Baran, 2015). In short, $1x10^4$ THP-1 and OCI-AML3 cells were seeded into each well of 96 well plates in 100 μl growth medium and incubated for 48 and 72h. 20 μl MTT solution (5mg/mL, Sigma Aldrich) was added to each well after 48-72 h and incubated for another 3h followed by dissolving formazan crystals in 100 μl DMSO. Then, absorbance values were measured at 570 nm by a spectrophotometer to draw the cell proliferation/viability graphs. 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.

2.4. Synergistic cytotoxic effects of combinations

Increasing concentrations of resveratrol were combined with constant concentrations of SKI II and PDMP for both cell lines in order

to determine whether there is cytotoxic synergisim or not. MTT assay was performed as described after 48h incubation. Concentration values lower than IC_{50} for SK I II and PDMP were used in combination studies, which were previously found to be appropriate to inhibit these enzymes for Cer accumulation (Baran et al., 2007). THP-1 cells were treated with 5- and 10 μM resveratrol combined with 5.0 μM SKI II and PDMP. Similarly, OCI-AML3 cells were treated with increasing concentrations of resveratrol (5- and 10 μM) in combination with 1.0 μM SKI II and 5 μM PDMP

2.5. Annexin-V/PI double staining

THP-1 and OCI-AML3 cells were treated with increasing concentrations of resveratrol for 48 h. THP-1 cells were treated with 5-, 10- and 20 μM resveratrol. Similarly, OCI-AML3 cells were treated with same increasing concentrations of resveratrol. Annexin V-FITC apoptosis detection kit (BioVision, USA) was used to examine apoptotic effects of resveratrol based on the instructions in the kit. Briefly, $1x10^6$/2ml cells seeded into each well of 6-well plate were treated with increasing concentration of resveratrol for 48 h. Then, cells were collected, washed twice with cold PBS. 2 μl of FITC Annexin V and 2 μl of PI were added to the cells suspended in 200 μl binding buffer and incubated for 15 minutes in the dark at room temperature. The percentage of early and late apoptotic cells were determined by flow cytometry (Adan and Baran, 2015)

2.6. Statistical analysis

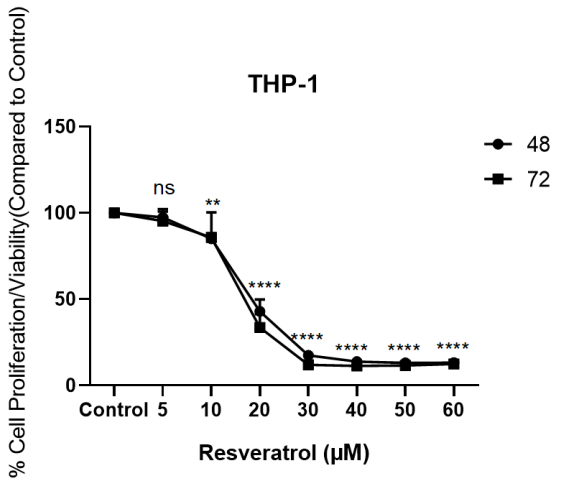
The experiments were performed in three independent setups and the results are given as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 6.0 program. Two-way ANOVA (one way analysis of variance) was used for the analysis of MTT results and annexin V-FITC /PI analysis. $p<0.05$ was considered as statistically significant.

3. Research Findings

3.1. Resveratrol suppressed cell proliferation in FLT3 positive AML cells

In order to determine the effect of resveratrol on the viability of FLT3 overexpressing THP-1 and OCI-AML3 cells, the cells were treated with increasing concentrations of resveratrol (5 to 60 μM) for 48 and 72 h. In both cell lines, resveratrol decreased cell viability in a time- and concentration dependent manner (Figure 1). The concentration inhibiting cell proliferation by 50% (IC_{50}) were calculated as 18- and 16 μM after 48 and 72 h treatment for THP-1 cells (Figure 1A). The proliferation of OCI-AML3 cells was inhibited by 50% at 18- and 15 μM for 48 h and 72 h treatment, respectively (Figure 1B).

A.



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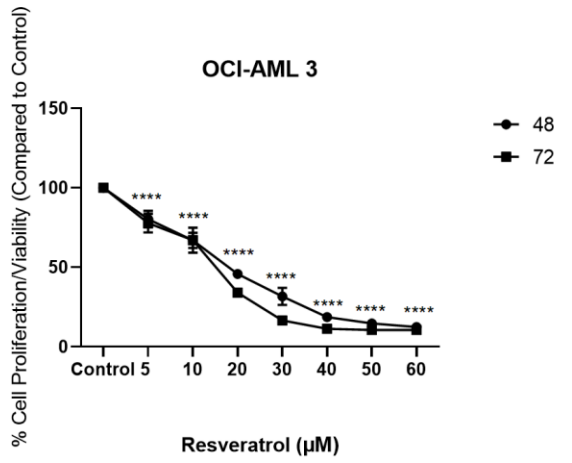
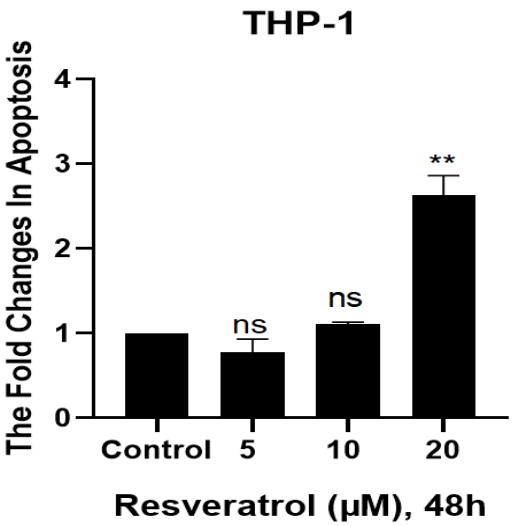


Figure 1. Effect of resveratrol on proliferation/viability of THP-1 (A) and OCI-AML3 (B) cells in a time- and concentration dependent manner. The results derived from the means of three independent experiments are represented as mean± SD and p <0.05 was considered as significant (**p<0.005, ****p<0.0001 statistically significant differences compared to control)

3.2. Resveratrol induced apoptosis through externalization of phosphatidylserine

To identify the mechanism for the antiproliferative effects of resveratrol on THP-1 and OCI-AML3 cells, annexin V-/PI double staining was performed to detect the relocalization of phosphatidylserine (PS) from inner membrane to outer membrane, which is a hallmark of apoptosis. As shown in Figure 2A, THP-1 cells treated with increasing concentrations of resveratrol (5-10 and 20 μM) for 48 h, compared with untreated control cells, 20 μM resveratrol resulted in 2.6- fold increase in apoptotic cells. Similarly, OCI-AML3 cells underwent apoptosis after treatment with 10- and 20 μM resveratrol (Figure 2B). There were 2.3- and 4.4 fold increases in the percentage of apoptotic cells. Based on the results, OCI-AML3 cells were more sensitive to resveratrol at 10- and 20 μM.

A.



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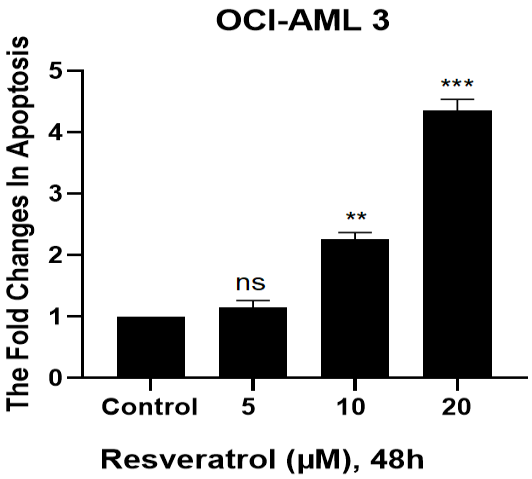
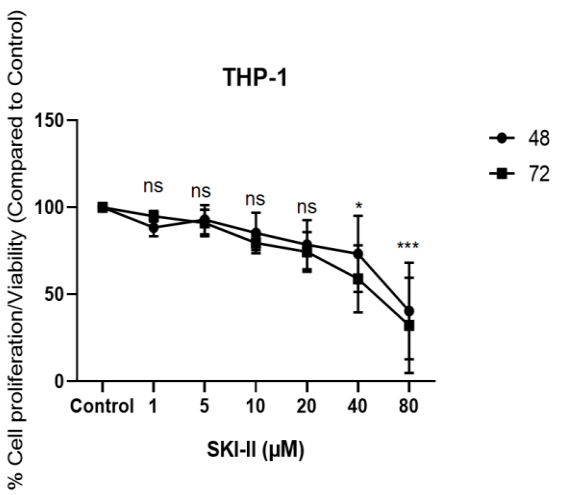


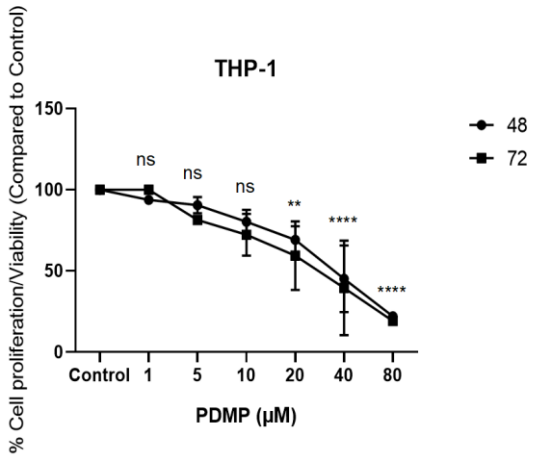
Figure 2. Concentration-dependent changes of PS externalization in resveratrol-treated THP-1 cells (A) and OCI-AML3 cells (B). The results derived from the means of three independent experiments are represented as mean± SD and p <0.05 was considered as significant (**p<0.005, ***p< 0.0001 statistically significant differences compared to control)

3.3. Cytotoxic effects of ceramide metabolizing enzyme inhibitors on THP-1 and OCI-AML3 cells

To figure whether resveratrols' suppressive effects on cell viability could be further supported by targeting sphingolipid metabolism enzymes out, we firstly determined antiproliferative effects of SK inhibitor (SKI II) and GCS inhibitor (PDMP) on THP-1 and OCI-AML3 cells by MTT cell proliferation assay. The cells were treated with increasing concentrations of SKI II and PDMP (1-80 μM) for 48 and 72 hs. Based on the cell proliferation graphs, SKI II and PDMP had cytotoxic effects on THP-1 cells in a time- and concentration dependent manner, especially at higher concentrations (Figure 3A). In contrast, OCI-AML3 cells' growth was inhibited in almost all concentrations in a time-dependent manner (Figure 3B). Time-dependent IC_{50} concentrations for THP-1 cells were calculated as 77- and 65 μM for SKI II and 57- and 53 μM for PDMP (Figure 3A). Similarly, IC_{50} concentrations for OCI-AML3 cells were calculated as 10- and 5 μM (SKI II) and 53- and 17 μM (PDMP) in a time dependent manner (Figure 3B).

A.





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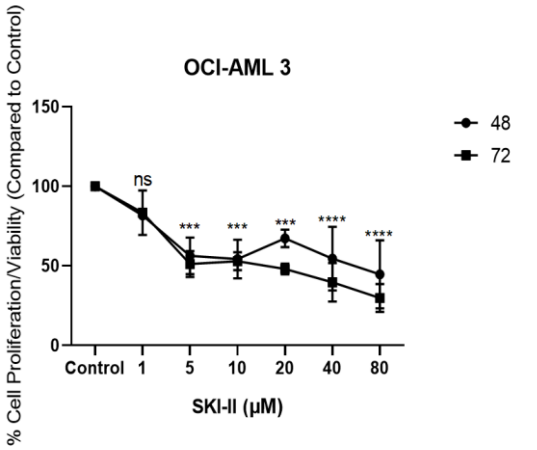


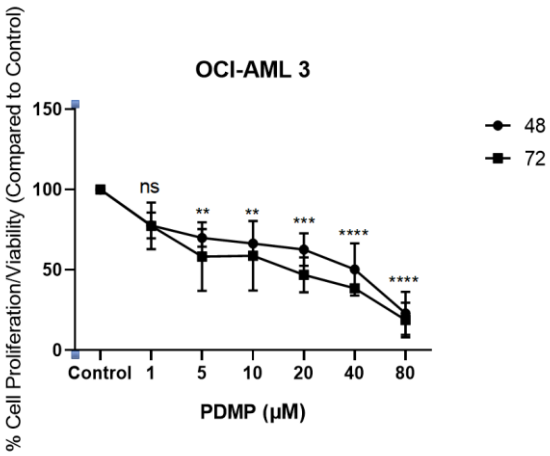


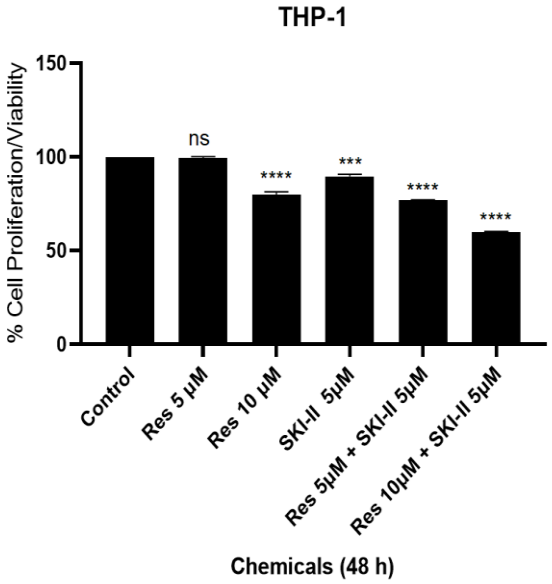
Figure 3. Effects of ceramide metabolizing enzyme inhibitors (SKI-II and PDMP) on proliferation/viability of THP-1 (A) and OCI-AML3 (B) cells in a time- and concentration dependent manner. The results derived from

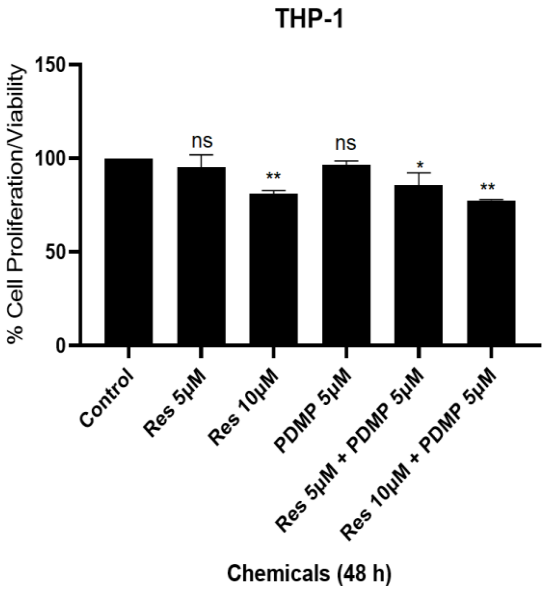
the means of three independent experiments are represented as mean± SD and p <0.05 was considered as significant (*p:0.0105, **p:0.0015, ***p:0.0004 ****p<0.0001 statistically significant differences compared to control)

3.4. Synergistic antiproliferative effects of resveratrol by targeting SK and GCS

Combination studies were also performed to figure out whether Cer, which is known to be apoptotic molecule and expected to be increased in the cells after inhibiting SK and GCS enzymes, could increase the cytotoxic effects or not. Therefore, THP-1 cells were treated with increasing 5- and 10 μM resveratrol in combination with 5 μM SKI II and 5 μM PDMP to determine the synergistic antiproliferative effects. The combination of increasing concentrations of resveratrol with 5 μM SKI II decreased cell growth as compared to control. Similarly, increasing concentrations of resveratrol combined with 5 μM PDMP showed that cell proliferation decreased in all combinations (Figure 4A). On the other hand, in OCI-AML3 cells, only 10 μM resveratrol together with 1 μM SKI-II and 5 μM PDMP reduced the number of viable cells more strongly than treatment with single agents (Figure 4B).

A.





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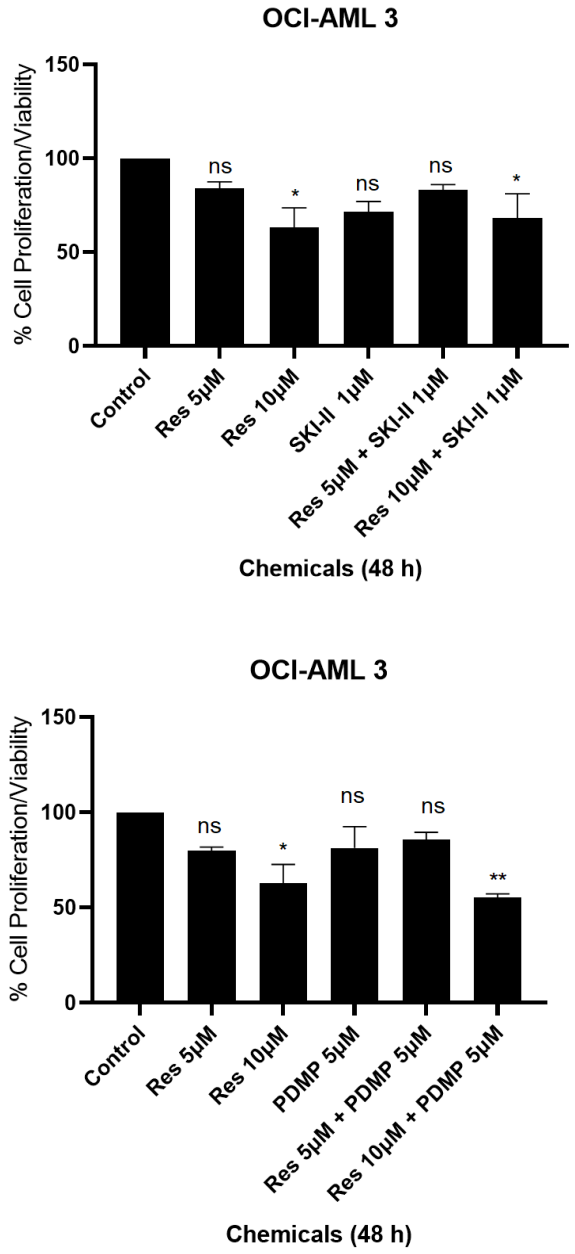

Figure 4. Synergistic effects of resveratrol in combination with ceramide metabolizing enzyme inhibitors (SKI-II and PDMP) on the proliferation/viability of THP-1 (A) and OCI-AML3 (B) cells. The results derived from the means of three independent experiments are represented as mean± SD and p <0.05 was considered as significant (*p<0.05, **p<0.001, ***p<0.0005 ****p<0.0001 statistically significant differences compared to control)

4. Discussion and Conclusion

The potential therapeutic effects of resveratrol have been shown in various cancer types including colon, breast, lung, skin, bone, cervical, ovarium and blood cancers which are associated with targeting of different multiple signaling molecules in the cells to modulate carcinogenesis (Athar et al., 2009; Rauf et al., 2018). For instance, resveratrol inhibits invasion and metastasis of *in vitro* and *in vivo* models of colon cancer by supressing Akt signaling and N-cadherin and enhancing E-cadherin expression (Yuan et al., 2019). Although there are accumulation of resveratrol related studies in solid cancers, it is also effective in hematological malignancies as a chemopreventive agent. In a recent study, resveratrol has been shown to regulate autophagy and trigger apoptosis via caspase-3 dependent apoptosis (Siedlecka-Kroplewska K, et al., 2019). Resveratrol targets miR-196b and miR-1290 to induce antileukemic effect in acute lymphoblastic leukemia by increasing the expression of insulin-like growth factor binding protein 3 (Zhou W, et al., 2017). In addition, the role of sphingolipid metabolism in resveratrol-induced anticancer effects has been defined recently as a new target in solid tumors like melanoma and breast cancer and in some hematological malignancies including chronic myeloid leukemia (CML) (Dei Cas and Ghidoni , 2018; Wang et al., 2014; Scarlatti et al., 2003; Kartal et al., 2011). In melanoma cells, resveratrol induced Cer accumulation which functions as protective autophagy (Wang et al., 2014) by supressing AKT/mTOR pathway. Resveratrol treatment resulted in endoplasmic reticulum stress mediated apoptosis in nasopharyngeal cancer cells (Chow et al., 2014). In hepatocellular carcinoma cells, resveratrol increased Cer

concentration and the expression of enzymes involved in *de novo* Cer synthesis pathway (Charytoniuk et al., 2019).

In this study, we investigated the therapeutic potential of resveratrol in human FLT3 overexpressing AML cells for the first time and identified the role of sphingolipid metabolism in resveratrol-triggered inhibition of cell proliferation. We determined the therapeutic effect of resveratrol on FLT3 positive THP-1 and OCI-AML3 cells by checking its effects on cell viability/proliferation and apoptosis with annexin-V/PI staining. The results indicated a time and dose-dependent decreases in cell proliferation in response to resveratrol (Figure 1A and Figure 1B) and resveratrol induced apoptosis by causing increases in apoptotic cell population in both cell lines by PS externalization (Figure 2A and 2B). As shown in many cancer types, one of the common mechanisms of resveratrol's action is to induce apoptosis through PS relocalization shown in human melanoma cells (Zhao et al., 2018), 232B4 chronic lymphocytic leukemia (Adan-Gokbulut et al., 2013) and ovarian cancer cells (Baribeau et al., 2014). Therefore, our results are in accordance with the literatüre and resveratrol might have therapeutic effect on FLT3 positive AML cells.

Sphingolipids determines the cell fate, which is called ''sphingolipid rheostat''. The alteration of the balance towards apoptotic Cer or antiapoptotic S1P or GC dictates cell survival or death. Ceramide accumulation in cancer cells cause apoptosis or growth arrest while S1P or GC support cell survival (Newton et al., 2015; Kroll et al., 2020). For instance, SK-1 is identified as a novel biomarker in hepatocellular carcinoma tissues as compared to normal tissues (Cai et al., 2017).

Therefore, to further check whether inhibition of SK and GCS could enhance resveratrol's antileukemic activity or not, the antiproliferative effects of resveratrol combined with SKI II and PDMP were investigated by MTT cell proliferation assay after determining the IC_{50} values of each inhibitor on THP-1 and OCI-AML3 cells (Figure 3A and 3B). Resveratrol combined with SKI II and GCS decreased cell viability as compared to untreated cells (Figure 4A and 4B). Therefore, it can be speculated that inhibition of cell proliferation by resveratrol could be associated with modulation of different steps of sphingolipid metabolism. Furthermore, its efficacy could be enhanced upon combination with inhibitors. Although there are very limited studies investigating the relationship between resveratrol and sphingolipid metabolism in hematological cancers, our results are in accordance with them. For instance, resveratrol decreased S1P and increased Cer levels in K562 CML cells to inhibit cell proliferation by increasing the expression of acid ceramidase (ASMase) (Mizutani et al., 2016). The proliferation of HL60 cells inhibited after resveratrol treatment combined with SK-1 and GCS inhibitors (Cakır et al., 2011). In another study, K562 CML cells' proliferation inhibited by resveratrol alone, which is further enhanced by combining with SK and GCS inhibitors (Kartal et al., 2011). In this study, caspase-3 dependent apoptosis induced by resveratrol alone was further enhanced by inhibition of SK and GCS. Additionally, resveratrol increased the expression of Cer generation genes while decreased the expression of SK and GCS genes. Tian and Yu (2015) showed that although resveratrol did not change total SK-1 expression, it caused its translocation from

plasma membrane to cytoplasm and induced apoptosis in K562 CML cells by altering sphingolipid rheostat towards Cer accumulation.

In conclusion, our results represented a potential resveratrol mediated antileukemic mechanism in human FLT3 positive AML cells. Inhibition of cell viability in resveratrol-treated WT FLT3 AML cells might be related to indcution of apoptosis and additive effect of inhibition of sphingolipid metabolism. Taken together, the results support the potential of resveratrol to be considered as a promising chemopreventive agent for the treatment of FLT3 positive AML after further mechanistic analysis and *in vivo* studies.

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6. Authors' Contributions

Concept: A.A., Desing: A.A., Data Collection or Processing A.A., N.Ş.E, Analysis or Interpretation: A.A., N.Ş.E, Literature Search: A.A., N.Ş.E, Writing: A.A., Final Approval of Manuscript: A.A., N.Ş.E, Supervisor and Principal Author, A.A.

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