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ORIGINAL ARTICLE / ÖZGÜN MAKALE

AN INVESTIGATION ON THE EFFECTS OF SIRT5 MODULATORS ON SIRT5 AND CYTOCHROME C PROTEIN EXPRESSIONS IN K562 CHRONIC MYELOID LEUKEMIA CELL LINE

K562 KRONİK MİYELOİD LÖSEMİ HÜCRE HATTINDA SIRT5 MODÜLATÖRLERİNİN SIRT5 VE SİTOKROM C PROTEİN EKSPRESYONLARI ÜZERİNE ETKİLERİNİN ARAŞTIRILMASI

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ABSTRACT

Objective: *SIRT5 is a mitochondrial protein that removes acetyl, malonyl and succinyl groups from lysine moieties in target proteins and interacts with cytochrome c and causes its deacetylation. There is no study on the effects of SIRT5 in K562 chronic myeloid leukemia cells. Resveratrol and Suramin are known to play a role in modulating the deacetylase and desuccinylase activities of SIRT5. It has been reported that Resveratrol induces apoptosis of K562 cells but effects of Suramin on the apoptosis of K562 cells are largely unknown. In this study, it was aimed to elucidate the effects of SIRT5 modulators Resveratrol and Suramin on proliferation and apoptosis of K562 cells and on SIRT5 and cytochrome c protein, a known target of SIRT5.*

Material and Method: *K562 chronic myeloid leukemia cells were treated with increasing concentrations of suramin and resveratrol, cell proliferation was determined by MTT assay and BrdU incorporation. Apoptosis was determined with Annexin V staining by Flow cytometry. Western Blot analysis was performed to determine the effect of resveratrol and suramin on SIRT5 and Cytochrome c protein expression levels.*

Result and Discussion: *Our results showed that suramin did not affect SIRT5 and cytochrome c protein expressions significantly and resveratrol decreased SIRT5 and increased cytochrome c expression. Suramin did not cause any changes on the apoptosis of K562 cells. Resveratrol decreased cell proliferation and induced*

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apoptosis of K562 cells in accordance with the literature. The SIRT5-lowering effect of Resveratrol may have mediated its apoptotic effects.

Keywords: *Apoptosis, K562, Resveratrol, SIRT5, Suramin*

ÖZ

Amaç: *SIRT5, hedef proteinlerdeki lizin rezidülerinden, asetil, malonil ve süksinil gruplarını uzaklaştıran ve sitokrom c ile etkileşerek, onun deasetilasyonuna neden olan bir mitokondriyal proteindir. SIRT5'in K562 kronik miyeloid lösemi hücrelerindeki etkilerine ilişkin bir çalışma bulunmamaktadır. Resveratrol ve Suramin'in SIRT5'in deasetilaz ve desüksinilaz aktivitelerini modüle etmede rol oynadığı bilinmektedir. Resveratrol'ün K562 hücrelerinin apoptozunu indüklediği bildirilmiştir. Ancak Suramin'in K562 hücrelerinin apoptozu üzerindeki etkileri büyük ölçüde bilinmemektedir. Bu çalışmada, SIRT5 modülatörleri Resveratrol ve Suramin'in K562 hücrelerinin proliferasyonu ve apoptozu ile SIRT5 ve SIRT5'in bilinen bir hedefi olan sitokrom c proteini üzerindeki etkilerinin aydınlatılması amaçlanmıştır.*

Gereç ve Yöntem: *K562 kronik miyeloid lösemi hücrelerine artan konsantrasyonlarda Suramin ve Resveratrol uygulandı. Hücre proliferasyonu MTT analizi ve BrdU inkoporasyon yöntemi ile belirlendi. Apoptoz, Akım sitometrisi ile Annexin V boyaması ile belirlendi. Resveratrol ve Suramin'in SIRT5 ve Sitokrom c protein ekspresyon seviyeleri üzerindeki etkisini belirlemek için Western Blot analizi yapıldı.*

Sonuç ve Tartışma: *Sonuçlarımız, Suramin'in SIRT5 ve sitokrom c protein ekspresyonlarını önemli ölçüde etkilemediğini ve Resveratrol'ün SIRT5'i azalttığını ve sitokrom c ekspresyonunu artırdığını göstermiştir. Suramin, K562 hücrelerinin apoptozunda herhangi bir değişikliğe neden olmamıştır. Resveratrol, literatüre uygun olarak hücre proliferasyonunu azaltmış ve K562 hücrelerinin apoptozunu indüklemiştir. Resveratrolün, SIRT5 protein ekspresyonunu azaltıcı etkisi ile apoptotik etkilerine aracılık etmiş olabileceği düşünülmektedir.*

Anahtar Kelimeler: *Apoptoz, K562, Resveratrol, SIRT5, Suramin*

INTRODUCTION

Chronic myeloid leukemia (CML) has been characterized by Philadelphia chromosome and Bcr-Abl protein [1]. Although tyrosine kinase inhibitors are used in the treatment of this disease, resistance to these inhibitors limits the treatment of the disease. Identification of new therapeutic agents and molecular pathways are promising strategies for the treatment of chronic myeloid leukemia [2].

Sirtuins (SIRT1-7) which require nicotinamide adenine dinucleotide (NAD(+)) for their activity are protein deacetylase enzymes and their functions are related with molecular pathways involved in tumorogenesis [3]. Although Sirtuin 5 (SIRT5) is mainly located in the mitochondrial matrix, it is also present in the cytosol, peroxisomes and nucleus. SIRT5 has vital roles in the regulation of mitochondrial proteins, mitochondrial respiration, nitrogen metabolism, glycolysis, pentose phosphate pathway and apoptosis [4-6]. However, increased SIRT5 mRNA expression has been shown in many cancer types including mammary tumors, gastric, colorectal and prostate cancer [7-9] and SIRT5 may exhibit tumor promoter functions and leads cisplatin resistance of ovarian cancer cells [10]. Several studies have reported decreased SIRT5 expression in glioblastoma tissues and hepatocellular carcinoma [3, 11]. However, there isn't any study in the literature that determines the expression of SIRT5 protein in chronic myeloid leukemia cells.

It has been shown that Resveratrol induces apoptosis of K562 chronic myeloid leukemia cell line [12-16] and one study reports the effects of Suramin on K562 cell proliferation [17]. There is no study in the literature investigating the effects of Resveratrol and Suramin on the protein expression levels of SIRT5 in K562 chronic myeloid leukemia cells. In this study, we explored the effects of SIRT5

modulators (Resveratrol and Suramin) on the cell viability and apoptosis of K562 chronic myeloid leukemia cells and also on the protein expression levels of SIRT5 and cytochrome c.

MATERIAL AND METHOD

Cell Culture

K562 chronic myeloid cell line was cultured in RPMI 1640 medium (Sigma) supplemented with 1% L-glutamine (Capricorn Scientific), 1% penicilin-streptomycin (Capricorn Scientific), 10% heat inactivated fetal bovine serum (Capricorn Scientific) at 37°C incubator which has %5 CO_2 and %95 O_2.

Cell Viability

In order to detect the effects of Suramin (Calbiochem) or Resveratrol (Sigma) on the proliferation of K562 cells, firstly cells (20.000 cells/well) were seeded to 96 well plates and treated with Suramin (0.1-100 μM) and Resveratrol (0.1-100 μM) for 72h. All wells (Suramin and Resveratrol treated and untreated) contained equal amounts of DMSO. Imatinib mesylate (IMA) was used as a positive control. Following incubation, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution was added to the wells then incubated for 4h. To dissolve formazan crystals, Sodium Dodecyl Sulfate-Hydrochloric acid (SDS-HCl) (0.01M) was used. Absorbance at 550 nm was measured using Microplate Reader (Molecular Devices-Spectra Max spectrophotometer, Sunnyvale, CA, USA). Cells which weren't treated with any substances were used as negative controls and viability of control cells was accepted as %100.

5-Bromo-2'-deoxyuridine (BrdU) Incorporation

Proliferation of K562 cells was determined by 5-bromo-2'-deoxyuridine (BrdU) incorporation assay according to BrdU Elisa kit (Cell Signalling) protocol. Birefly, cells (20.000 cells/well) were seeded to 96-well plates and SIRT5 modulators (0.1-100μM) were applied. IMA was used as a positive control. After 72h incubation, BrdU solution was added. Following 24 h incubation, cells were centrifuged (300g, 10 min) and supernatant was discarded. Fixing/denaturating solution 1X detection antibody solution was added. After washing steps, 1X HRP seconder antibody solution was added and after HRP substrate (TMB) substrate and the stop solution addition absorbance (at 450nm) was measured.

Western Blot Analysis

Western Blot analysis was performed to determine the effect of SIRT5 modulators on SIRT5 and Cytochrome c protein expression levels. K562 chronic myeloid leukemia cells were seeded to 6 well plates (500.000 cells/well) and incubated with Suramin (100μM) and Resveratrol (100μM) for 72 h. Total protein isolation was performed according to the manufacturers' recommendations (Active Motif). Protein concentrations of cell lysates were determined with Bradford Method (Thermo). Anti-beta actin primary antibody was used as housekeeping loading control. Proteins were separated by SDS

poliacrylamide gel electrophoresis and were transferred to nitrosellulose membrane and incubated with anti-SIRT5 (Cell Signaling Technology) and anti-Cytc (Cell Signalling Technology) primary antibodies. Proper secondary antibodies (Cell Signaling Technology) were used and protein bands were visualized with chemiluminesans imager (Licor CLX). Protein bands were analysed by Image J program.

Apoptotic Analysis

To determine the apoptotic effect of Suramin and Resveratrol, K562 cells were harvested in 6 well plates, and exposed to Suramin (100μM) and Resveratrol (100μM) for 72h. Cells were stained with Annexin V-7AAD and apoptosis was assessed with Flow cytometry. Cells treated with IMA were used as positive control.

Statistical Analysis

Statistical Analysis was performed by GraphPad Prism 7 software. Data were expressed as mean ± standart deviation. Differences between groups were determined by One Way Anova variance analysis and Tukey post hoc analysis was used. One Way Anova variance analysis was chosen to determine whether there are any statistically significant differences between the means of three or more independent (unrelated) groups. Tukey test was used since it is the most commonly used and powerful post-hoc test. $p<0.05$ is accepted as statistically significant.

RESULT AND DISCUSSION

Determination of the effects of SIRT 5 modulators on the proliferation of K562 cells by MTT Assay

To analyse the effects of SIRT5 modulators (Suramin and Resveratrol) on the viability of K562 cell line, cells were incubated with these modulators for 72h and MTT and BrdU Incorporation assays were performed. According to our results, Resveratrol (40-100μM) decreased the viability of cells significantly ($p<0.001$), whereas Suramin (0.1-100μM) did not exhibit any significant effect on the viability of K562 cells (Figure 1a and figure1b).

Determination of the effects of SIRT 5 modulators on the proliferation of K562 cells by BrdU incorporation

While there was no significant difference between the cells treated with Suramin (0.1-100μM) and the control group, a significant difference was observed between the control group and the cells treated between 40-100 μM Resveratrol ($p <0.05$). IMA (0.5μM) was used as a positive control (Figure 2a and 2b).

Determination of SIRT5 and Cytochrome c protein expression by Western Blot Analysis

Western blot analysis was performed to determine the effect of SIRT5 modulators on the expression of SIRT5 and Cytochrome c protein. According to our results, Resveratrol (100µM) decreased SIRT5 protein significantly ($p<0.05$), but Suramin did not show any significant effect on SIRT5 protein expression (Figure 3a and 3b). Moreover, Resveratrol significantly increased Cytochrome c protein expression ($p <0.05$), but Suramin did not show any significant effect on Cytochrome c protein expression (Figure 3c and 3d).

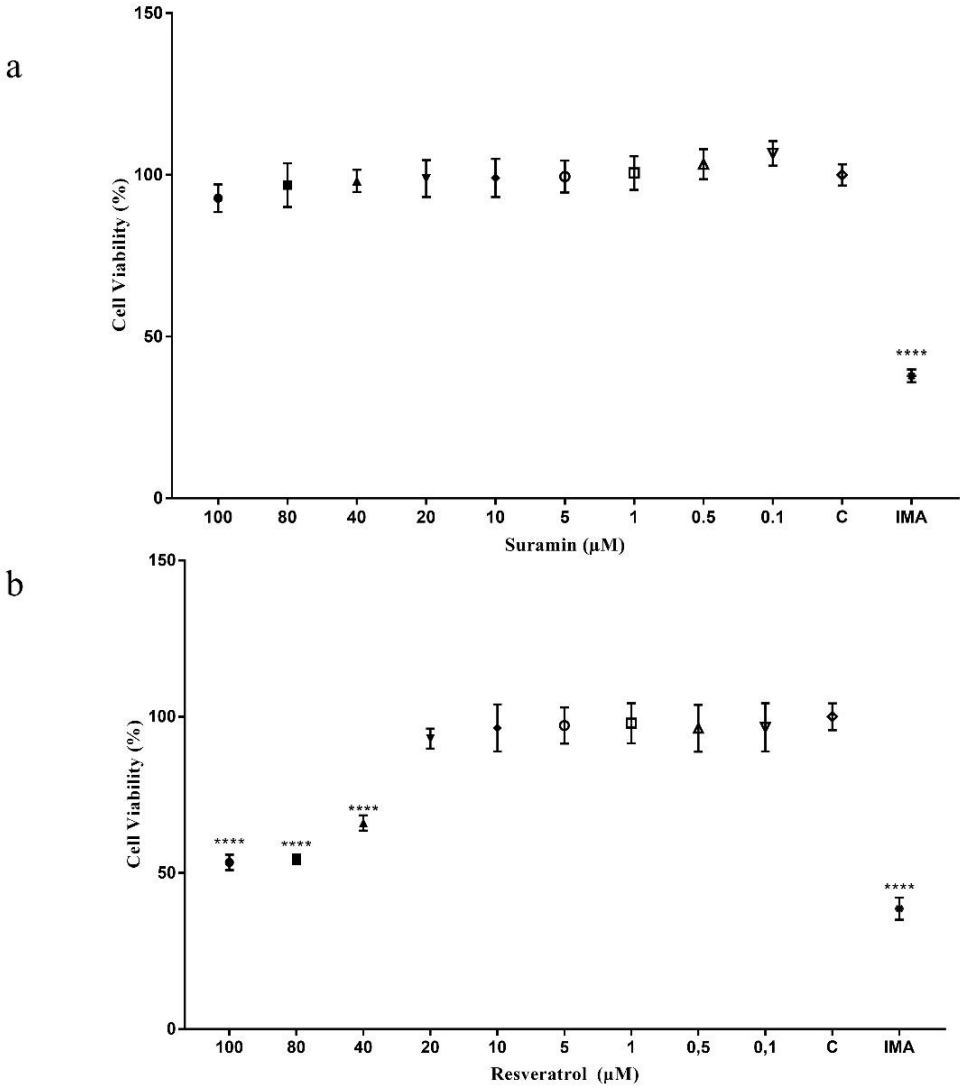

Figure 1. Effect of Suramin (a) and Resveratrol (b) on the viability of K562 cells by MTT Assay. Cells without Suramin or Resveratrol were used as a control group which is denoted by C (Control) and contains equal amounts of DMSO with the cells treated with different concentrations of Suramin or Resveratrol. IMA was used as a positive control. While Suramin did not affect cell viability significantly between 0.1-100µM concentration range, Resveratrol decreased cell viability between 40-100 µM concentration range significantly ($p<0.0001$). **** $p<0.0001$ compared to control.

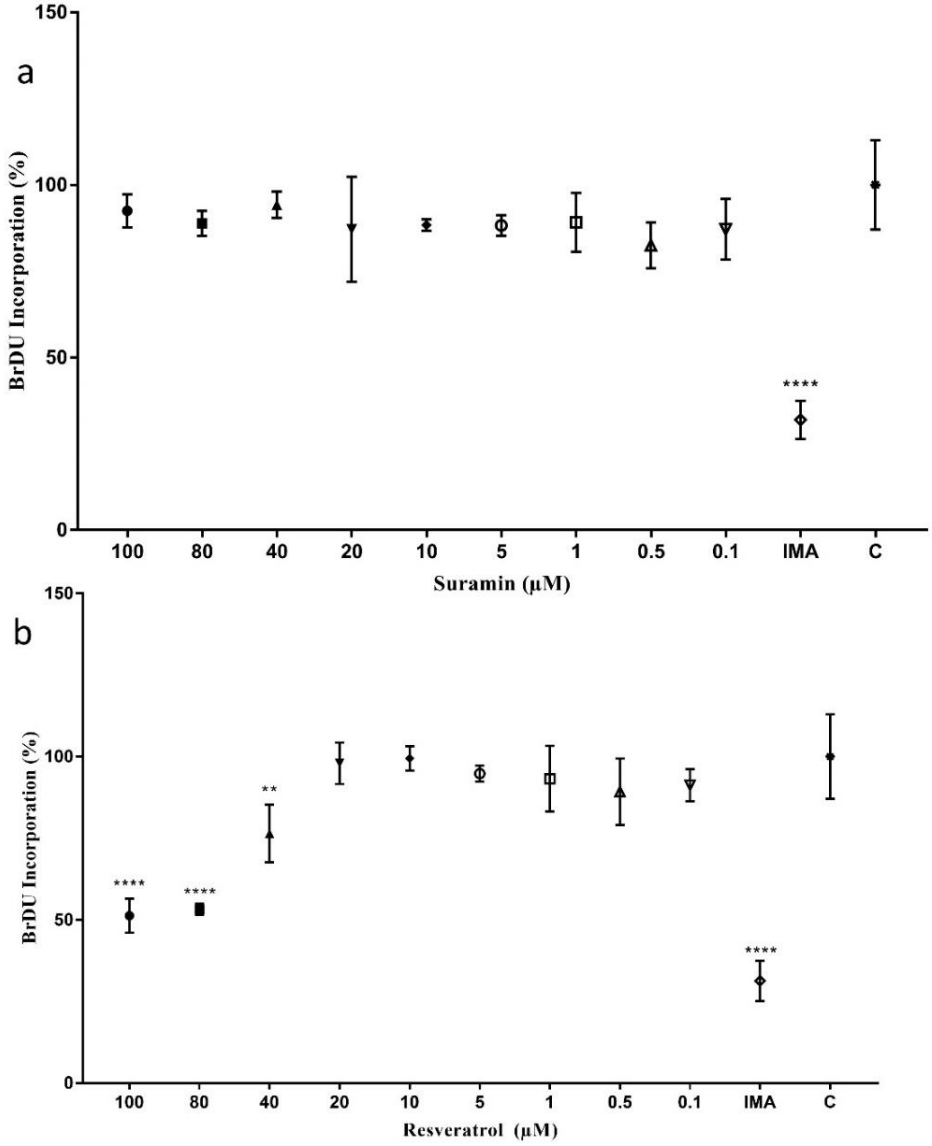

Figure 2. Effect of Suramin (A) and Resveratrol (B) on the viability of K562 cells by BrdU Assay. Cells without suramin or Resveratrol were used as control group which is denoted by C (Control) and contains equal amounts of DMSO with the cells treated with diffrent concentrations of suramin or Resveratrol. IMA was used as a positive control. While suramin did not affect cell viability significantly between 0.1-100µM concentration range, Resveratrol decreased cell viability between 40-100 µM concentration range significantly ($p<0.05$). *** $p<0.001$ and ****$p<0.0001$ compared to control.

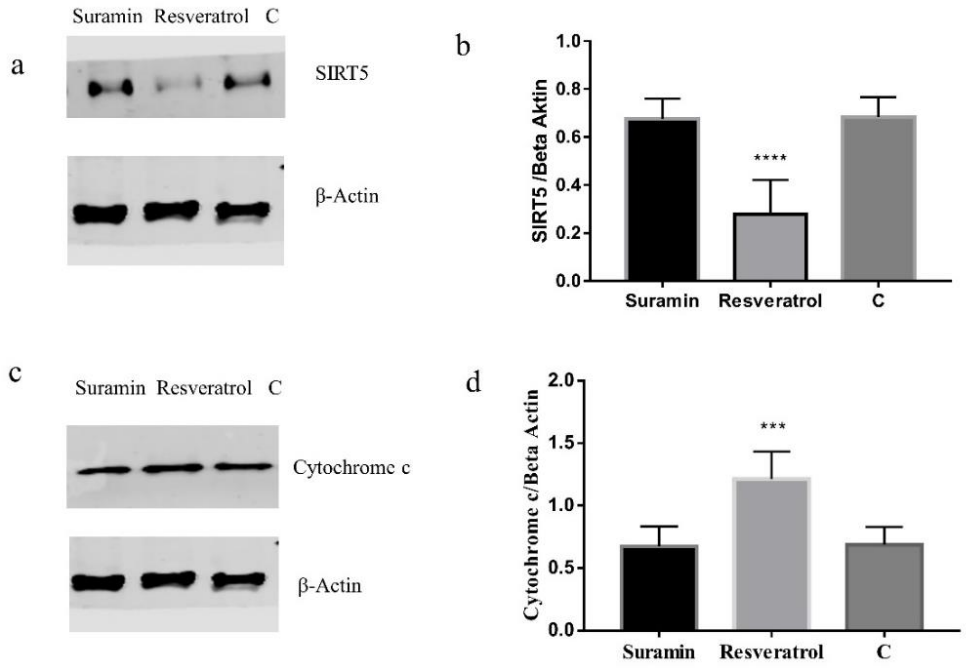

Figure 3. Effect of Suramin (100µM) and Resveratrol (100µM) on SIRT5 (a and b) and Cytochrome C (c and d) protein expression in K562 cell line. Resveratrol decreased SIRT5 and increased Cytochrome c protein expression significantly ($p<0.05$). Suramin did not effect significantly both SIRT5 and Cytochrome c protein expressions. *** $p<0.001$ and ****$p<0.0001$ compared to control.

Effects of Suramin and Resveratrol on Apoptosis of K562 cells

As a result of our analysis, it was determined that Resveratrol induces apoptosis of K562 cells ($p<0.05$) and Suramin did not exhibit any significant apoptosis inducing effect in K562 cells (Figure 4a and 4b).

In this study, we investigated the effects of Suramin and Resveratrol on the protein expressions of SIRT5 and its target Cytochrome c in K562 chronic myeloid leukemia cell line. We aimed to determine the correlations between the alterations of the SIRT5-cytochrome c axis and K562 cell apoptosis. Sirtuins which are NAD+ dependent protein deacetylases and mono-ADP-ribosyltransferases play important roles in cell proliferation, apoptosis, aging, and caloric restriction [18]. Association between sirtuins and cancer was revealed in recent studies [19, 20].

Cytochrome c is the target of SIRT5 protein and it is released from mitochondria to cytoplasm and plays an important role in apoptosis [21]. Guan et al reported that SIRT1/SIRT5 can cause deacetylation of tumor suppressor promyelocytic leukemia protein (PML) [22] and Zhang et al. showed deacetylation of Cytochrome c by SIRT5 and co-localization of SIRT5 and Cytochrome c [23].

Resveratrol is a natural phenol found in a variety of foods including grapes and berries. It was reported that it regulates many molecular targets and exhibits antitumor effects in many types of cancer [13, 24]. Resveratrol induces apoptosis of K562 cells via Bax and cytochrome c release [25] and its therapeutic effects has been shown in IMA resistant and IMA sensitive K562 cells [12]. In this study,

the role of Resveratrol on K562 cell apoptosis is evaluated together with its effect on SIRT5 protein expression. We found that Resveratrol decreases the viability of K562 cells and increases apoptosis significantly. It has been shown that in endothelial cells, there is a relationship between the anti-inflammatory effects of Resveratrol and SIRT1 and SIRT5 proteins [26]. Although it has been shown that Resveratrol activates deacetylase activity of SIRT5, Resveratol did not impact SIRT5 protein expressions in H295R cells [27, 28]. This is the first study investigating the effects of Resveratrol on SIRT5 protein expression in K562 cells. In our study, we found that Resveratrol decreases SIRT5 protein expression and increases cytochrome c expression significantly.

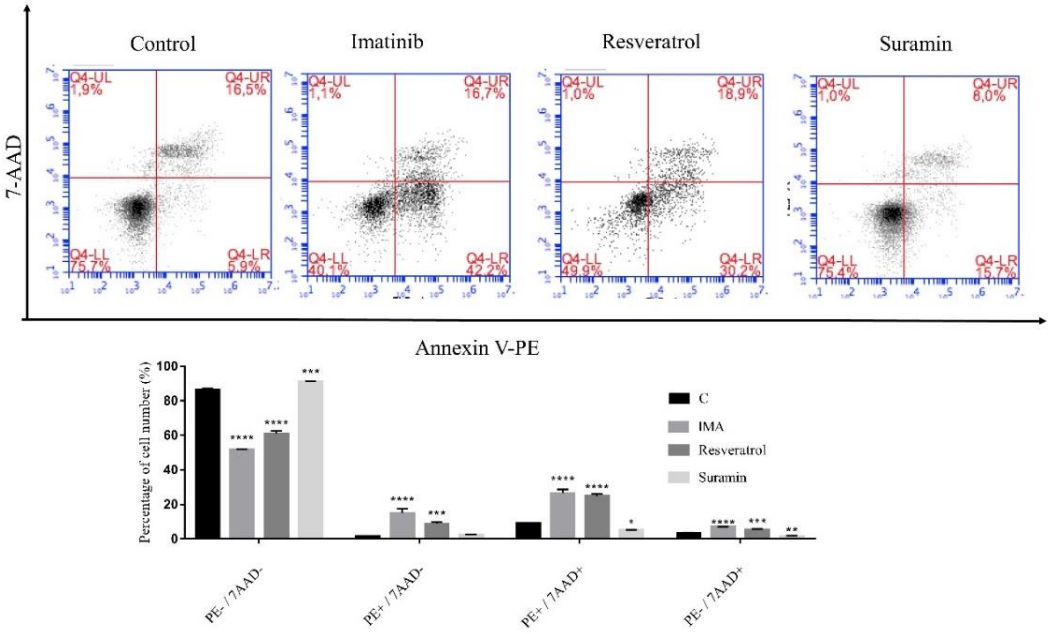

Figure 4. Effect of Suramin and Resveratrol on the apoptosis of K562 cell line. IMA was used as positive control. Cells without Suramin or Resveratrol were used as negative controls (C). (a) According to flow cytometry results, Resveratrol (100µM) increased apoptosis of K562 cells significantly ($p<0.05$) and Suramin (100µM) did not show any effect on apoptosis significantly. (b) Flow cytometry results were represented as bar graph. Viable cells are Annexin V-PE -/7AAD -, early apoptotic cells are Annexin V-PE +/7AAD-), late apoptotic cells are Annexin V-PE +/7AAD +. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ compared to control

Suramin, polysulfonated naphthylurea, has an antiproliferative and antiviral activity [29]. Suramin has also been considered as a pharmacological inhibitor of SIRT5 deacetylase activity [30, 31]. Suramin has broad range functions from antiparasitic/antiviral to anticancer [32]. In studies on the role of Suramin in cancer, it was shown that Suramin leads to growth inhibition of human stomach cancer cell lines (SNU-5 and SNU-16) [33] and inhibition of the proliferation of pancreatic cancer cells [34]. While Suramin has marked inhibitory effect on cell proliferation of KMS-11, 12, KMM-1, KMS5 myeloma cells and Raji, KMS-9 and HL-60 hematopoietic cell lines, it hasn't any singnificant

antiproliferative effect on RPMI 8226 and K562 cell lines [17]. There is no study in the literature investigating the effects of Suramin on apoptosis in K562 cells. In our study, we found that Suramin did not affect the proliferation and apoptosis of K562 cells as well as it did not affect SIRT5 and cytochrome c protein expression significantly. According to Schuetz at al., Suramin inhibited NAD (+) dependent decetylation activity of SIRT5 via binding to SIRT5 [31]. On the other hand, in our study another SIRT5 modulator Resveratrol was found to increase cytochrome c protein expression significantly in accordance with the literature [35]. This study reveals that two different SIRT5 modulators may have different effects on the protein expression of SIRT5 and apoptosis.

In conclusion, SIRT5 modulators exhibited different effects on the proliferation and apoptosis of K562 cells. Suramin did not show any significant antiproliferative and apoptosis inducing effect on K562 cells. On the other hand, Resveratrol was found to reduce cell proliferation and induce apoptosis significantly. Suramin had no significant effect on SIRT5 and Cytochrome c protein expressions, whereas Resveratrol decreased SIRT5 protein expression and increased cytochrome c protein expression significantly. This study will contribute to future studies on the role of SIRT5 protein in the treatment of chronic myeloid leukemia.

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AUTHOR CONTRIBUTIONS

Concept: *A.K., T.O., A.Z.K*; Design: *A.K., A.Z.K., T.O.*; Control: *A.K., A.Z.K., T.O., Y.H.*; Sources: *A.K., T.O., A.Z.K., Y.H., A.S.;* Materials: A.K., *T.O., A.Z.K., Y.H., A.S.*; Data Collection and/or processing: *A.K., T.O., A.Z.K., Y.H., A.S.*; Analysis and/or interpretation: *A.K., T.O., A.Z.K., Y.H., A.S.*; Literature review: *A.K., T.O., A.Z.K., Y.H., A.S.*; Manuscript writing: *A.K., T.O., A.Z.K., Y.H., A.S.*; Critical review: *A.K., T.O., A.Z.K., Y.H., A.S.*; Other: *A.K., T.O., A.Z.K., Y.H.*

CONFLICT OF INTEREST

Authors confirm no declaration of interest.

ETHICS COMMITTEE APPROVAL

The authors declare that the ethics committee approval is not required for this study.

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