Synergistic Effects of Methotrexate and Suberoylanilide Hydroxamic Acid in Triggering Apoptosis of Chronic Myeloid Leukemia Cells

Ergul M. ALTUNDAG[1,2], Ayse M. YILMAZ[1,2], Ceyda COREK[1,2], A. Suha YALCIN[1,2], Yavuz TAGA[1,2], Semra KOCTURK[2,3]

[1] Marmara University Faculty of Medicine, Department of Biochemistry, Istanbul
[2] Marmara University Genetic and Metabolic Diseases Research Center, Istanbul
[3] Dokuz Eylül University Faculty of Medicine, Department of Biochemistry, Izmir, TURKEY

ABSTRACT

In this study, we have investigated the effects of suberoylanilide hydroxamic acid (SAHA) against chronic myeloid leukemia (CML) cells in combination studies with methotrexate (MTX), which is a dihydrofolate reductase inhibitor used in combination therapy with other agents or alone. Combination of synergistic ratios of MTX and SAHA led to apoptotic cell death of CML cells via PARP cleavage, cytochrome c release and ROS increase in vitro. We suggest that combination of MTX and SAHA may minimize the toxicity and side effects of SAHA treatment, thus providing lower amounts of each drug in CML treatment.

Keywords: Chronic myeloid leukemia, Methotrexate, Suberoylanilide hydroxamic acid, Apoptosis

ÖZET

Kronik Myeloid Lösemi Hücrelerinin Metotreksat ve Suberoylanilide Hidroksamikasitin Sinerjetik Etkisi ile Apoptoza Sürüklenmesi

MTX, tek başına veya diğer ajanlarla kombine tedavide kullanılan bir dihidrofolat redüktaz inhibitörüdür. Bu çalışmada kronik myeloid lösemi (K562) hücrelerine karşı metotreksat (MTX) ile birlikte kullanılan suberoylanilide hidroksamik asit (SAHA)'nın etkilerini araştırdık. MTX ve SAHA'nın sinerjetik oranları, K562 hücrelerinde in vitro kesilmiş PARP'a , sitokrom c salınımına ve ROS artışı ile apoptotik hücre ölümüne neden oldu. K562 tedavisinde MTX ve SAHA'nın kombinasyonunun her bir ilacın daha düşük dozlarının kullanılmasını sağlayacağını ve böylece SAHA'nın yan etkilerinin ve toksisitesinin azaltılabileceğini düşünmekteyiz.

Anahtar Kelimeler: Kronik myeloid lösemi, Metotreksat, Suberoylanilide hidroksamik asit, Apoptozis

INTRODUCTION

Chronic myeloid leukemia (CML) was the first neoplastic disease for which knowledge of the genotype led to a design therapy and challenged common ideas about cancer treatment.[1] The success of molecular-targeted therapy in CML brought similar approaches for other cancers, but such success has yet to be replicated. In CML cells, Bcr-Abl tyrosine kinase in the cytosol activates several molecular mechanisms known to inhibit apoptosis.[2,3] Additionally, the risk of CML patients developing resistance to the first-line treatment of drugs such as imatinib endeavoured researchers to improve new treatment tools against CML. Inhibitors of histone deacetylases have been the intense focus of developing compounds against epithelial and hematological cancers.[4] In particular, leukemia cells appear to be susceptible to apoptosis under histone deacetylase inhibitor (HDI) treatment.[5,6] While the underlying cellular mechanisms and biological effects elicited by HDIs have yet to be characterized, it is believed that targeting epigenetic deregulation in such a manner has great utility in cancer therapy.

Several HDIs including suberoylanilide hydroxamic acid (SAHA), can block cyclin-dependent kinase activity and inhibit cell-cycle progression ultimately arresting the cell cycle in G1 leading to apoptosis.[7,8] Furthermore SAHA is a known inhibitor of histone deacetylases in human leukemia and cancer cells.[9,10,11] An important finding in predicting the potential utility of HDIs in the clinic is their activity in cell-lines that are resistant to existing chemotherapeutics. For example, Gleevec-resistant Bcr/Abl human chronic myelogenous leukemia (CML) cells are sensitized to Gleevec upon cotreatment with SAHA.[12] Recent studies conducted in CML suggested combinations of drugs for treatment as an effective treatment approach.[13,14]

Combination studies where HDIs have been combined with DNA methyl transferase inhibitors[15,16] suggest that HDIs may synergise with other inhibitors that target DNA regulating processes, altering the pattern of transcriptionally active genes in favor of a drug sensitive profile.[17] These studies have potentially expanded the utility of HDIs in clinical disease and suggest potential widespread applicability in regulating drug resistance.

On the other hand, methotrexate (MTX) has been widely used for the treatment of cancer, especially leukemia.[18] MTX is a dihydrofolate reductase inhibitor first developed for use in combination therapy with other agents or alone, and is widely used as an anticancer drug in various human cancers in particular human leukemia.[19] In humans MTX seems to prevent RNA synthesis rather than DNA synthesis, suggesting inhibition of thymidylate synthesis is the most important mechanism of its cytotoxicity.[20]

HDIs have synergistic or additive antitumor effects with a comprehensive range of antitumor reagents including chemotherapeutic drugs and new targeted therapeutic reagents.[21] Recently MTX was suggested as a HDI but its interaction and combination with SAHA has not been elucidated in CML cells yet. In combined treatment one difficulty is the opposite effects of the choosen drugs. For this reason, we have tried to determine effective dosage and compatibility for the combination of MTX and SAHA in triggering apoptosis of CML cells in vitro.

MATERIALS AND METHODS

Reagents, Cell line and Cell Culture

Methotrexate was purchased from Fluka. Suberoylanilide hydroxamic acid (SAHA), 2’,7’-dichlorodihydrofluorescein diacetate (H2DCF-DA), propidium iodide (PI) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were from Sigma. Annexin V/PI supplied by BD (USA) was formulated according to the manufacturer’s instructions. Antibodies used were: cleaved-PARP antibody (Cell Signalling), cytochrome-c antibody (Santa Cruz Biotech), B-actin (Cell Signalling), and HRP-linked secondary antibody anti-rabbit and anti-mouse IgG (Cell Signalling). Human chronic myelogenous leukemia cells (K562 cells) were obtained from American Type Culture Collection (ATCC, CCL 243). The cells were cultured in RPMI 1640 medium (GIBCO BRL), supplemented with 10% fetal bovine serum (Hyclone Laboratories), 1% L-glutamine and 1 % penicillin-streptomycin in a 5% CO_2 incubator at 37°C.

Cell Viability and IC50 Determination

Cell viability was monitored using the MTT Kit I (Roche Diagnostics, USA). Cells were seeded in 96-well plates at $5x10^3$ cells/well in 100 μl culture media and were exposed to different concentrations of each agent (MTX and SAHA) or their combination for 24 and 48 hours. After treatment, 10 μl of MTT (5 mg/ml in PBS) were added to each well and incubated for 4 hours at 37^0C. The purple-blue MTT formazan precipitate was dissolved in 100 μl of DMSO and the plates were shaken thoroughly on the shaker for 1 min. The absorbance of reporter substrate was measured at 440-650 nm using a microplate reader (Molecular Devices, USA). IC50 (Dm) values were determined as concentrations that reduced cell viability by 50%. Results were expressed as percentage of controls (cells without drug). Fractional effect values were calculated using relative growth values (Fa= [1 - Relative growth] with CalcuSyn software.[22]

Relative growth= $[(OD_{sample}) - (OD_{blank}) / (OD_{control}) - (OD_{blank})]$ x 100 %

Combination Analysis

Chou-Talalay method[22] was used to assess the interaction of drugs. For combination analysis, a single drug dose effect was determined for both Drug A and Drug B. Then, a combination dose-effect (Drug A + Drug B) was determined at a constant combination ratio [(IC50)1 / (IC50)2]. Values of the CI were determined at the IC50 concentration [(fraction affected (Fa)= 0.5)]. Combination index (CI) values were calculated using the CalcuSyn software (Biosoft, UK), where CI= 1 illustrates additive effect, CI< 1 synergistic effect ("synergism") and CI> 1 antagonistic effect ("antagonism").

Apoptosis Assay

After drug treatment for 24 to 48 hours, 1 x 10^6 cells were washed with PBS and ApopNexin FITC apoptosis detection kit (Millipore) was used for analysis. For each assay, ten thousand cells were measured and results were assessed using the CellQuest program (Becton Dickinson FACSCalibur, USA). All experiments were performed in triplicate.

Cell Cycle Analysis

Propidium iodide (PI) staining and flow cytometry were used to determine the stage of the cell cycle. After incubation for 48 hours, cells were harvested and centrifuged. The supernatant was discarded and the pellet was suspended in sterile PBS and 70% cold ethanol. The cells were washed once with PBS, followed by incubation in PBS containing 50 mg/ml PI and 2 mg/ml DNase-free RNase A for 30 min at room temperature in the dark.[23] For each measurement, at least 10,000 events were acquired and fluorescence was measured in the FL2 channel. All experiments were performed in triplicate. Flow cytometric analysis of DNA content was performed and sub-G1 population and G0, G1, G2+M phases were calculated using the Cell Quest software (Becton Dickinson FACSCalibur, USA).

Measurement of ROS Production

Intracellular ROS production was measured using the fluorescent dye, DCFH-DA, which is converted to DCFH by esterases when taken up by the cell. DCFH then reacts with ROS to give a highly fluorescent product (DCF) which can be detected by flow cytometry. Briefly, pelleted cells were suspended in 1 mL of phenol red-free RPMI medium containing 10 μM CM-H2DCF-DA incubated at 37°C for 30 minutes in the dark.[24]

Western Blotting

CML cells were separately cultured with MTX, SAHA or both agents for 48 hours. Cells were harvested and lysed in 200 μl cold lysis buffer (50 mM Tris–HCl, pH 6.8, 15 mM EDTA, 15 mM $MgCl_2$, 50 mM ß-glycerol, 150 μg/ml digitonin containing 1 mM dithiothreitol and 100 mM phenylmethylsulfonyl fluoride). Samples were incubated on ice for 15 minutes and after centrifugation at 14,000 rpm for 10 min the supernatant was collected. Then, sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis was performed according to Laemmli[24] and transferred to nitrocellulose membrane by Turbo-Blot system (Bio-Rad Laboratories, USA). Approximately 40 μg of total proteins were loaded to each well. Protein concen-

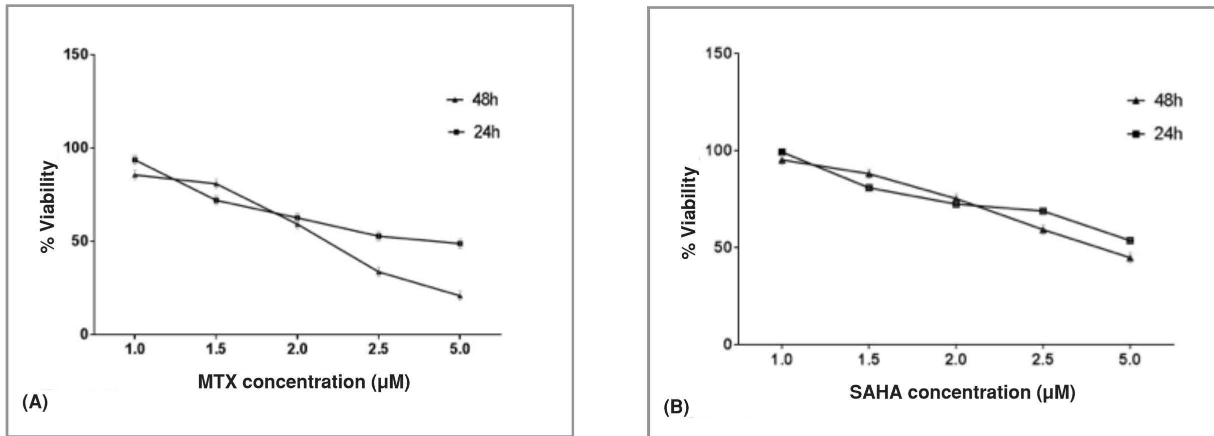


Figure 1. Growth inhibition of K562 cells. Cells were treated with MTX **(A)** and SAHA **(B)** at different concentrations (1-5 µM) for 24 and 48 hours. Percentage viability values are expressed as mean ± S.D of three independent experiments.

tration was determined using BCA assay (Pierce Chemical, USA). Membranes were blocked with 5 % skimmed milk in Tris-buffered saline containing 0.1 % Tween 20, and immunoblotted overnight at 4ºC with the appropriate primary antibody followed by treatment with horseradish peroxidase-linked secondary antibody. Chemiluminescence detection was performed using the West Pico chemiluminescent substrate kit (Thermo Scientific) and the ChemiDoc MP System (Bio-Rad Laboratories, USA).

Determination of Cytochrome C Release From Mitochondria

The release of cytochrome c from mitochondria was determined 48 hours after drug treatment. Briefly, 5 x 10^6 cells were suspended in 50 μl of permeabilization buffer containing 75 mM NaCl, 8 mM Na_2PO_4, 1 mM NaH_2PO_4, pH 7.4, 250 mM sucrose, 1 mM EDTA, 700 μg/ml digitonin. Cells were incubated for 1 min in the same buffer at room temperature after which the pellet was removed by centrifugation for 3 min at 13,000 x g and the supernatant containing cytochrome c protein was obtained.[25]

Statistical Analysis

One-way analysis of variance (ANOVA) was performed to determine the significance of differences between groups, where p values less than 0.05 were considered statistically significant. Analysis of synergism and antagonism was performed using median dose effect analysis with a commercial software (CalcuSyn, Biosoft, UK).

RESULTS

MTX or SAHA application as a single agent for cytotoxicity analysis in K562 cells

To test the effect of MTX and SAHA on CML cells, agents were added to CML cell cultures for 24 and 48 hours. The concentration of MTX ranged from 1 to 5 μM and the concentration of SAHA ranged from 1 to 5 μM. As shown in Figure 1, IC50 (the concentration at which 50% of cell growth is inhibited) value of MTX was calculated as 2.39 μM and that for SAHA was calculated as 3.81 μM. Cytotoxic effects of SAHA and MTX were observed at 48 hours and Fa values were calculated according to relative growth values using the Calcusyn software.

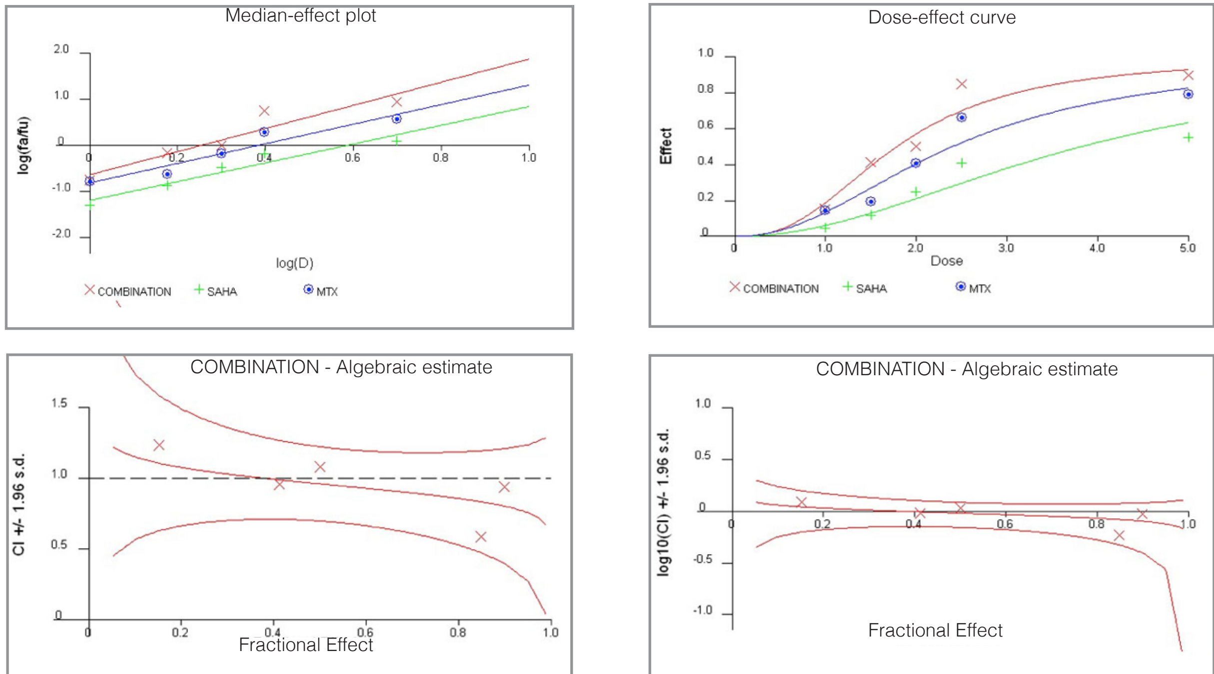


Figure 2. CalcuSyn analysis of SAHA and MTX on growth of K562 cells. Median-effect plot, dose-effect curve and Fa-CI plots are given. Median-effect plot illustrated the value of linear correlation. Dose-effect curve for each drug alone and combined to evaluate synergism or antagonism at different proliferation inhibition levels. Fa-CI plot showed synergistic and addictive effects.

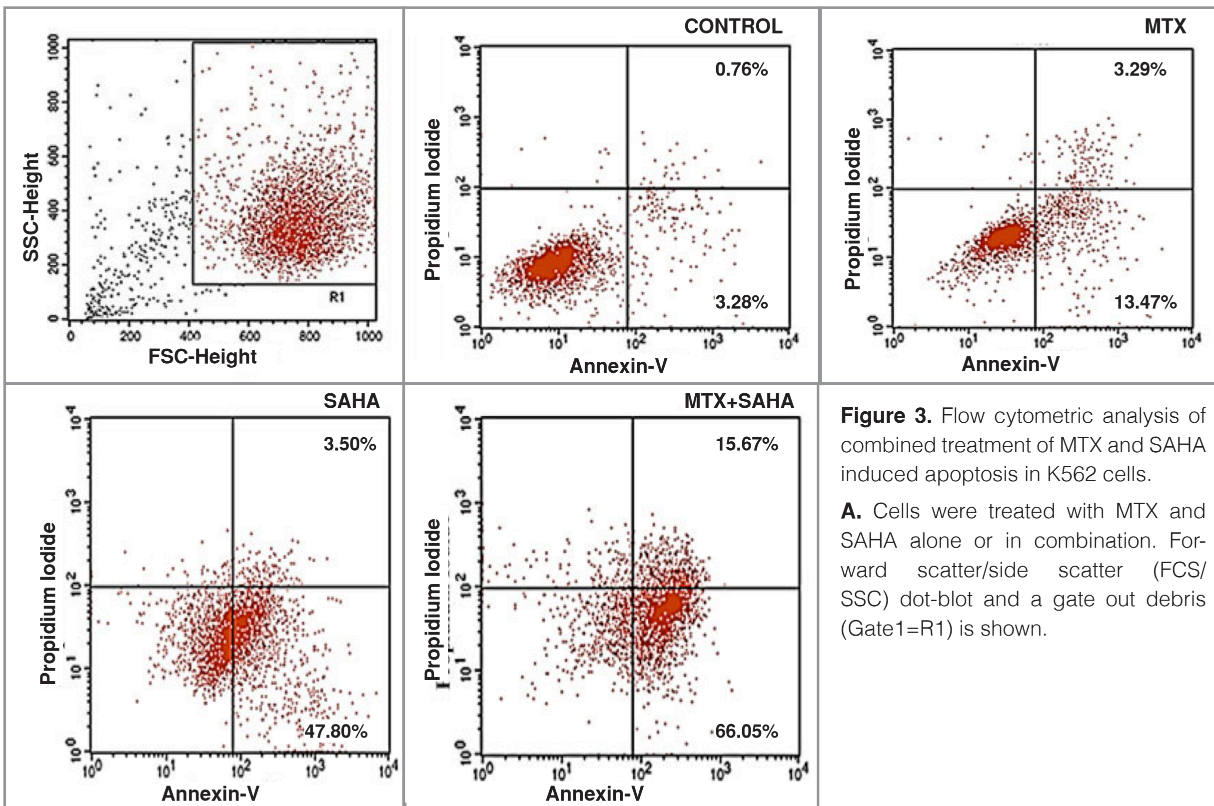


Figure 3. Flow cytometric analysis of combined treatment of MTX and SAHA induced apoptosis in K562 cells.

A. Cells were treated with MTX and SAHA alone or in combination. Forward scatter/side scatter (FCS/SSC) dot-blot and a gate out debris (Gate1=R1) is shown.

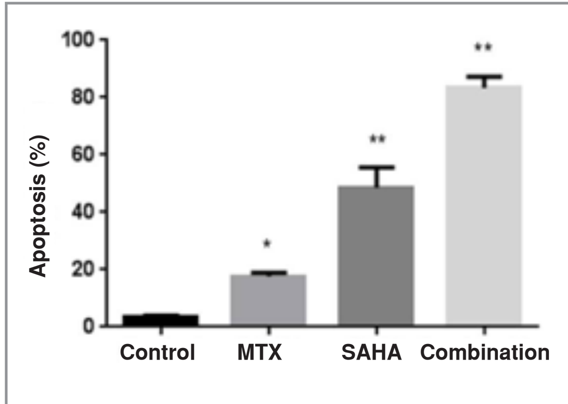


Figure 3. B. Bar graphs represent the means ± S.D of three independent experiments.

* p< 0.05 (Control vs MTX); ** p< 0.0001 (Control vs SAHA and Control vs Combination)

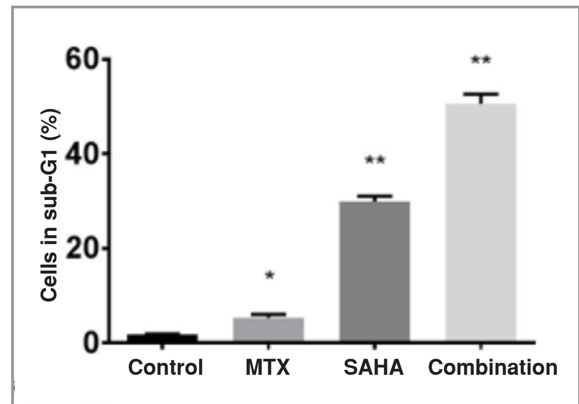
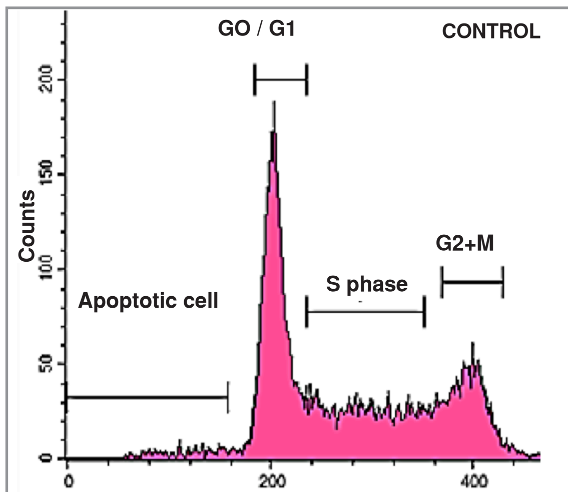
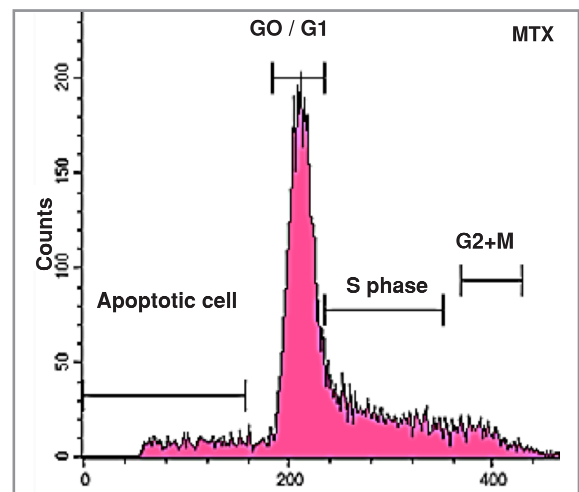
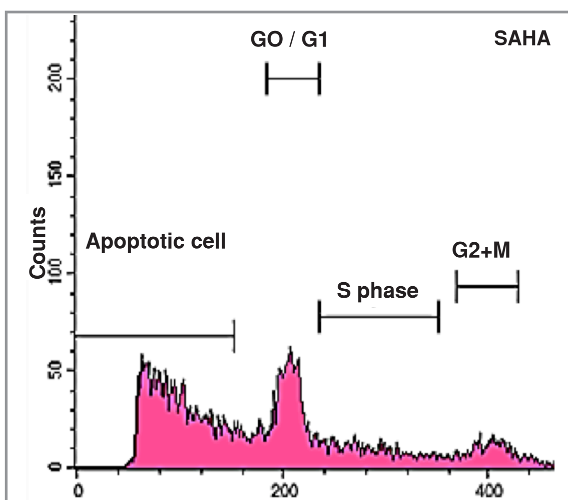


Figure 4. (B) The bar graphs illustrate cell cycle of the sub-G1 (apoptotic cells), Vertical bars represent mean ± S.D of three independent experiments. * p< 0.05 (Control vs MTX), ** p< 0.001 (Control vs SAHA and Control vs Combination).







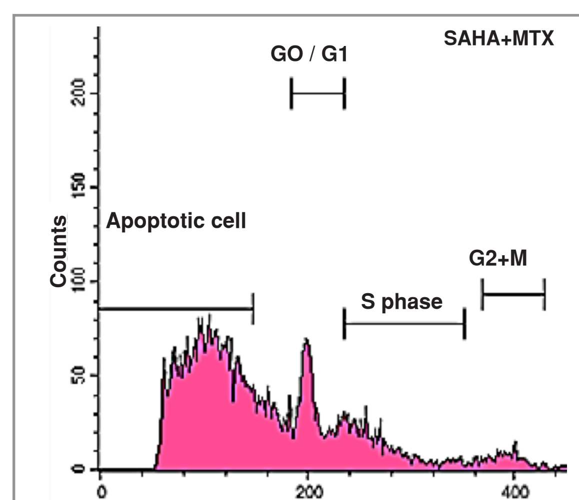


Figure 4. Flow cytometric analysis of combined treatment of MTX and SAHA alterations of cell cycle in K562 cells.
(A) Disruption of the cell cycle was analyzed by flow cytometry showing sub-G1 (apoptotic cells), G0/G1, S, G2+M phases.

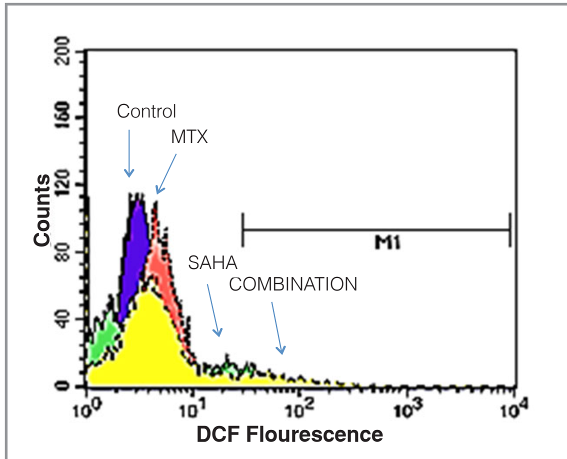


Figure 5. The effect of combination of MTX and SAHA on ROS generation in K562 cells.

(A) K562 cells were treated with 1-5 µM MTX and/or SAHA for 48 hours. DCF fluorescence intensity (FL1) corresponding to the level of ROS generation, was measured by flow cytometry. Representative histogram plot showing M1 marker illustrates the increase in ROS levels.

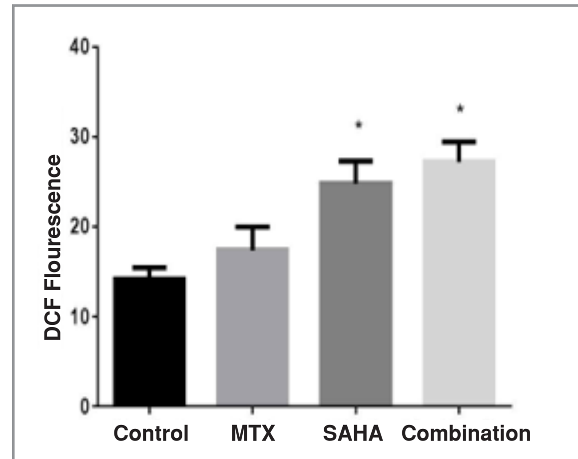


Figure 5. (B) The bar graph shows ROS generation of K562 cells treated with MTX, SAHA and combination in comparison with controls and represent mean ± S.D of three independent experiments. * p<0.001 (Control vs SAHA and Control vs Combination).

Determination of Synergistic Doses of MTX and SAHA in K562 Cells

In our study, median-effect principle was applied to analyze dose-response curves and to quantify synergism or antagonism at varying doses. For this purpose, interactions between MTX and SAHA were first determined using different doses of each drug. For combination analysis ratio of the drugs (IC50-SAHA/IC50-MTX) was found to be 1.5/1. CI value was found to be 0.962 which indicates synergism between the drugs (Figure 2).

Assessment of Apoptotic Effect

Annexin-PI assay was used to examine the effect of MTX and SAHA on cell apoptosis. The percentages of total apoptosis were 17.67, 48.58 and 83.73 for MTX, SAHA and their combination, respectively. The differences between the drugs was significant ($p< 0.05$) and the percentage of apoptosis for combination of the drugs led to a higher amount of apoptosis observed for each drug alone (Figure 3). As loss of DNA is a typical feature of apoptotic cells, DNA fragmentation was quantified by measuring sub-diploid amounts of DNA using PI (Figure 4A). We have observed sub-diploid peaks in all groups and the highest percentage was found in the combination group (Figure 4B). The percentage of sub-diploid DNA was 5.4, 29.9 and 50.6 for MTX, SAHA and their combination, respectively.

Evaluation of ROS Generation

MTX treatment resulted in minimal ROS generation, whereas SAHA and combined treatment resulted in a significant increase ($p< 0.05$) compared to controls (Figure 5). Accordingly, ROS generation may be a marker for the synergistic effect of the two drugs.

Analysis of Apoptotic Protein Expression by Western Blot

Cleaved-PARP and cytochrome c protein levels were significantly increased after cells were exposed to MTX and/or SAHA for 48 hours (Figure 6). According to our results, combination of the two agents resulted in increased cleaved-PARP protein expression. The cytochrome c protein expression in SAHA and combination treatment was higher compared to MTX alone. Thus, we can say that combination of MTX and SAHA was associat-

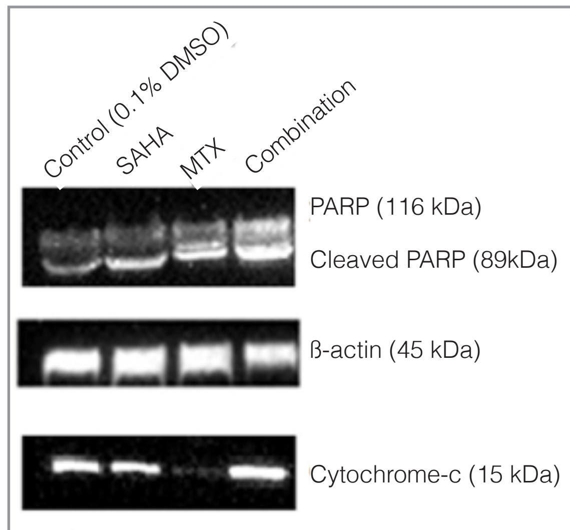


Figure 6. Combination of MTX and SAHA increases cleaved-PARP and cytochrome-c protein expression in K562 cells.

K562 cells were treated by indicated agents (MTX, SAHA, MTX+SAHA) for 48 h, whole cell lysates, cytoplasmic and mitochondrial fractions were prepared. Then, cleaved-PARP and cytochrome c were determined by Western blot analysis.

with single use of HDIs illustrate different side effects.[26] Combination drug therapies can target multiple pathologic processes where lowered drug doses allow minimizing the adverse side effects.[27] Recently it was confirmed that MTX has a similar structure to HDIs and has the ability to inhibit histone deacetylases.[28] Combination of HDIs with MTX has different effects depending on the inhibitor type. The primary problem of combined treatment with some HDIs and MTX is the effects of the drugs which can be opposite.[29,30] For instance, HDIs (e.g. valproate or MS275) increase the resistance of cells to MTX by upregulating thymidylate synthase expression as shown in mouse choroid plexus carcinoma cell lines.[31]

SAHA was the first HDI approved by FDA and has been tested in various clinical trials. The effect of single use of HDI seems to be low in clinical studies and combined treatments of SAHA with other agents have been preferred.[32] Especially, toxicity and side effects such as fatigue, diarrhea, anorexia, and dehydration, as well as myelosuppression, bone loss and thrombocytopenia cause some problems for SAHA treated patients.[33] Therefore combined trials are needed to reduce cytotoxicity and side effects. We have selected MTX and SAHA for combination and assessed the effects of combination to find a synergistic effect of these two agents. The importance of our study is minimizing cytotoxicity with combination of SAHA and MTX which can be used to reduce side effects of the treatments in CML.

Assessment of the degrees of synergism or antagonism of the drug combination was performed by Chou-Talalay method. Combined treatment of MTX and SAHA showed synergistic effects with CI values < 1 in CML cells. We have also found that the two agents have a synergistic effect in CML cells and that IC50 of MTX and SAHA decreased when the two agents were used in combination. It has been shown that MTX and SAHA synergistically increased apoptotic cell death in Bp-ALL and T-ALL cell models[34] and the combination of MTX and valproic acid has a better effect than each drug alone against solid Ehrlich tumors in mice.[35] In our study, high-doses of MTX and SAHA alone could be lowered approximately 2-fold by combining both agents while the rate of apoptosis was increased. Thus, apoptosis of cells was at an increased rate with reduced toxicity.

Both MTX and SAHA can induce cell death and arrest cell growth when used alone as single agents.[9,36,37] Additionally, combining HDIs with other pro-apoptotic agents resulted in synergistic levels compared to single agents.[38,39] HDIs have the capacity to act synergistically with a diverse range of pharmacological and biological agents to kill tumor cells.[40] Several investigators found that increased ROS formation plays a critical role in cell death induced by SAHA and other HDIs. Apoptotic mechanism of SAHA has been explained to initiate cell death via mitochondria-mediated death pathway which is characterized by cytochrome c release and ROS generation, but does not require the activation of key caspases such as caspase-8 or caspase-3.[41,42] We have observed similar findings for SAHA and our results suggest that SAHA-mediated ROS formation plays an important role in CML cells. In our study, use of SAHA increased

ROS generation either alone or in combination with MTX. Our results also revealed that combination of MTX and SAHA increased cleaved-PARP protein expression when compared to MTX or SAHA alone. Pei et al.[43] showed that combined treatment of myeloma cells with bortezomib and HDI triggers PARP degradation and release of cytochrome c. Besides, SAHA-related cytochrome c release was increased in combination thus initiating apoptosis. Leclerc et al.[34] reported that co-treatment of SAHA and MTX synergizes to induce apoptotic cell death in ALL cells. Here, we have shown that combination of MTX and SAHA produces a synergistic effect on apoptosis of CML cells and suggest that combination of these two agents can be effectively used to kill CML cells. In conclusion, in our study synergistic effects of MTX and SAHA in triggering apoptosis have been shown for the first time and we suggest that this combination might be a good candidate in CML treatment. However, this in vitro study should be further confirmed by corresponding in vivo studies.

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Correspondence
Prof. Dr. Semra KOÇTÜRK
Dokuz Eylül Üniversitesi
Tıp Fakültesi
Biyokimya Anabilim Dalı
İnciraltı
İZMİR / TURKEY

Tel: (+90.232) 412 44 07 / 4407
Fax: (+90.232) 464 81 35
e-mail: semra.kocturk@deu.edu.tr