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ORIGINAL ARTICLE

Cytotoxic effect of 6-Shogaol in Imatinib sensitive and resistant K562 cells

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Abstract

Chronic Myeloid Leukemia (CML) is a clonal hematopoietic malignancy characterized by the formation of BCR-ABL fusion protein. Imatinib (IMA) is a BCR-ABL tyrosine kinase inhibitor (TKI), which exhibited a high rate of response for newly diagnosed CML patients. Emergence of IMA resistance considered as a major challenge in CML therapy. Recent studies reported the anti-cancer effect of natural extracts such as 6-Shogaol (6-SG) which is extracted from ginger and the mechanisms involved in targeting of cancer cells. In the present study, we aimed to explore the potential anticancer effect of 6-SG on K562S (Imatinib sensitive) and K562R (Imatinib resistant) cells. K562S and K562R cells were incubated with increasing concentrations of 6-SG (5 µM- 50 µM) to determine its cytotoxic and apoptotic effects. Cell viability and apoptosis were investigated with spectrophotometric MTT assay and flow cytometric Annexin V staining, respectively. The mRNA expression levels of apoptotic related genes (BAX and BCL-2) and drug transporter (MDR-1 and MRP-1) genes were evaluated with qRT-PCR. According to our results, 6-SG treatment inhibited cell viability, induced apoptosis in both K562S and K562R cells. Based on our RT-PCR results, 6-SG enhanced pro-apoptotic BAX gene and decreased anti-apoptotic BCL-2 gene expression levels significantly in both treated K562S and K562R cells. Furthermore, 6-SG increased MDR-1 mRNA expression level in K562S and K562R cells in comparison with their control counterparts. Whereas, 6-SG decrease MRP-1 mRNA expression level in K562S cells significantly. It is the first study that reveals the apoptotic effect of 6-SG in CML cell line and IMA resistance. Therefore, 6-SG treatment can be suggested as a promising strategy for CML therapy.

Keywords 6-Shogaol · Imatinib resistance · Chronic myeloid leukemia · Apoptosis

Introduction

CML is a clonal hematopoietic stem cell disorder characterized by the presence of Philadelphia chromosome (Ph) and formation of the BCR-ABL fusion protein. BCR-ABL is a constitutively active tyrosine kinase (TK) and formed as a consequence of reciprocal translocation t (9;22) [1, 2]. IMA is a selective tyrosine kinase inhibitor (TKI) used as highly effective and frontline therapy in CML. Despite the long-term outcomes of IMA therapy, its therapeutic potential is limited due to the emergence of resistance mechanisms [3, 4]. Although IMA induces apoptosis in leukemic cells, it can also trigger resistance in patients, which leads to failure in therapy [5]. Therefore, finding an alternative agent or approach which can effectively overcome the multi-drug resistant cells is necessary in cancer therapy.

Over recent years, application of natural dietary agents and traditional medicine has been extensively accepted as an alternative option for cancer therapy due to their safety and cost effectiveness. Ginger extracts have been reported to have anti-cancer, anti-inflammatory, anti-oxidant effects. 6-Shogaol (6-SG) is a major bioactive constituents of ginger extract (*Zingiber officinale* Roscoe, Zingiberaceae). 6-SG has achieved a great attention among other extracts in terms of its pharmacological properties including anticancer, anti-proliferative, anti-inflammatory and antioxidant properties [6–9]. 6-Shogaol suppresses cell proliferation and triggers

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apoptosis in different human cancer cells such as leukemia [6] colorectal carcinoma [10], hepatocellular carcinoma [11, 12], breast cancer [13], head and neck squamous cell carcinoma [14], prostate cancer [15] and lung cancer cells [16, 17] via multiple mechanisms. For example, 6-SG induced apoptosis in human cancer cells via caspase-dependent cleavage of elF2α [6], suppression of STAT3 and NF-kB Signaling [15], ROS production, caspase activation and GADD 153 expression [10], inducing G(2)/M arrest and aberrant mitotic cell death associated with tubulin aggregation [18]. 6-SG also inhibited MCF-7 cell growth by apoptosis induction and autophagy suppression through targeting notch signaling pathway [19].

Although cytotoxic, anti-proliferative and apoptotic effects of 6-SG have been explored in various cancer cells, its effect on induction of apoptosis in IMA resistant CML cells has not yet been evaluated. In this study, we investigated the apoptotic effect of 6-SG in IMA sensitive K562S and IMA resistant K562R CML cell lines. We examined apoptosis-related and drug transporters genes expression which are involve in IMA resistance in CML. Our data indicated for the first time that 6-SG induced apoptosis in IMA sensitive and resistant CML cell lines through modulation of pro-apoptotic and anti-apoptotic genes.

Materials and methods

Cell culture

K562S (Imatinib sensitive) and K562R (Imatinib resistant) cell lines were grown in RPMI 1640 (Sigma, USA) supplied with 10% fetal bovine serum (FBS) (Sigma, USA) and 100 U/mL of penicillin, 100 lg/mL of streptomycin (Sigma, USA). K562R cells which were resistant to 0.6 µM IMA were kindly gifted by Prof. Carlo Gambacorti-Passerini, University of Milano-Bicocca, Italy. K562R were gradually treated with increasing concentrations of IMA and their resistance was enhanced to 5 µM IMA in our laboratory. K562R cells were cultivated in the presence of the 5 µM IMA [20]. IMA was removed from the media of K562R cells 2 weeks before performing all experiments.

Cell viability assay/cytotoxicity assay

Cytotoxic effects of 6-SG on CML cells was detected by MTT assay. K562S and K562R cells (4 x 10^4cells/well) were cultivated in 96-well plates and incubated with increasing concentrations of 6-SG (5, 10, 15, 25, 50 µM) for 24 and 48 h. The optical density of each samples was measured by spectrophotometric plate reader (Biotek, USA) at 550 and 690 nm. The percentage of untreated cells viability accepted as 100% and percentage of cell viability in each 6-SG treated cells was calculated accordingly. Methanol was used as a solvent for 6-SG and used as a negative control in all groups. The amount of methanol in all 6-SG incubated groups was always kept equal.

Annexin V/7AAD staining

K562S and K562R cell lines (5×10^5 cells/well) were cultivated in 6 well- plates. Cells were treated with 50 µM 6-Shogaol for 48 h. After 48 h cells were collected and washed with PBS two times. Then cells were resuspended in 1X binding buffer (BB) according to manufacturer's protocol (BD Biosciences). 5 µl PE-Annexin V and 5 µl 7AAD was added to 100 µl of cell suspension. After a brief vortex, cells were incubated at room temperature for 15 min. 400 µl 1XBB was added to cells and the cells were analyzed in Accuri C6 flow cytometry.

Real-time quantitative RT-PCR

K562S and K562R cell lines were cultured in 6-well plates at a seeding density of 5×10^5(cells/well) and treated with 50 µM 6-SG for 48 h. Then, total RNA was isolated from cells by Trizol (Invitrogen, USA). Complementary DNA (cDNA) synthesis was carried out using reverse transcriptase (Transcriptor High Fidelity cDNA Synthesis Kit; Roche).

To amplify and detect apoptosis-related (BAX and BCL-2) and drug transporter (MDR-1 and MRP-1) genes qRT-PCR was used. qRT-PCR experiments were performed using SYBR Green PCR Master Mix (Roche) on LC480 instrument. The expression of HPRT mRNA as an endogenous control was used the normalization of the expression level of mRNAs. Primer sequences were listed in Table 1.

Table 1 Primer sequences for Real-time qRT-PCR analysis

Gene	Forward (5′- 3′)	Reverse (5′- 3′)
BCL-2	CGCCCTGTGGATGACTGAGT	GGGCCGTACAGTTCCACAA
BAX	GACGGCAACTTCAACTGGG	AGGAGTCTCACCCAACCAC
MDR-1	AAGGCATTTACTTCAAACTTGTCA	GGATTCATCAGCTGCATTTTC
MRP-1	AGTGCTTTCAGAACACGGTC	TTTCCCAGAAAGAGTAGAAGAGGT
HPRT	TGACACTGGCAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT

Statistical analysis

Statistical software Graph Pad Prism was used for the assessment of the differences between all treated and control group. The one sample t test was used to compare means of two groups and ANOVA was used for comparing means of multiple samples. All of the experimental data were reported as mean ± SD (standard deviation). $P < 0.05$ and $P < 0.001$ were considered as statistically significant.

Results

Cytotoxic effect of 6-SG on CML cells

6-SG was evaluated for its in vitro cytotoxic effect in CML cell lines (K562S and K562R), using MTT assay. K562 cells were incubated with different concentrations (5-50 µM) of 6-SG for 24 and 48 h. Viability of cells incubated without 6-SG was considered as 100%. Cell viability test results were reported in Fig. 1A, B indicating significant cytotoxic activity against K562S ve K562R cell lines. Our results demonstrated that 6-SG incubation inhibited viability of K562S, K562R cells between 5 µM and 50 µM concentrations in a dose dependent manner and significantly ($p < 0.05$, p < 0,001) for 24 and 48 h. 50 µM 6 -SG treatment for 48 h was considered as the most effective dose in both cell lines. Cell viability of K56S and K562R cells treated with 50 µM 6-SG for 48 h were 21.55% ± 2.46 µM and 24.46% ±0.72, respectively.

Effect of 6-SG in induction of apoptosis in CML cells

In order to investigate the type of cell death induced by 6-SG treatment, K562S, K562R cells were incubated with 6-SG (50 µM) for 48 h. Based on the flow cytometry results, the number of viable (PE−/7-AAD−) K562S and K562R cells were decreased significantly after 6-SG treatment ($p < 0.001$) (Fig. 2). The number of early apoptotic (PE+/7-AAD−) in 6-SG treated K562S cells increased significantly ($p < 0.001$) (Fig. 2a, b). Moreover, the number of late apoptotic/dead (PE+/7-AAD+) K562S and K562R cells were increased significantly after 6-SG treatment (p < 0.001) compared to the untreated control (Fig. 2A, C).

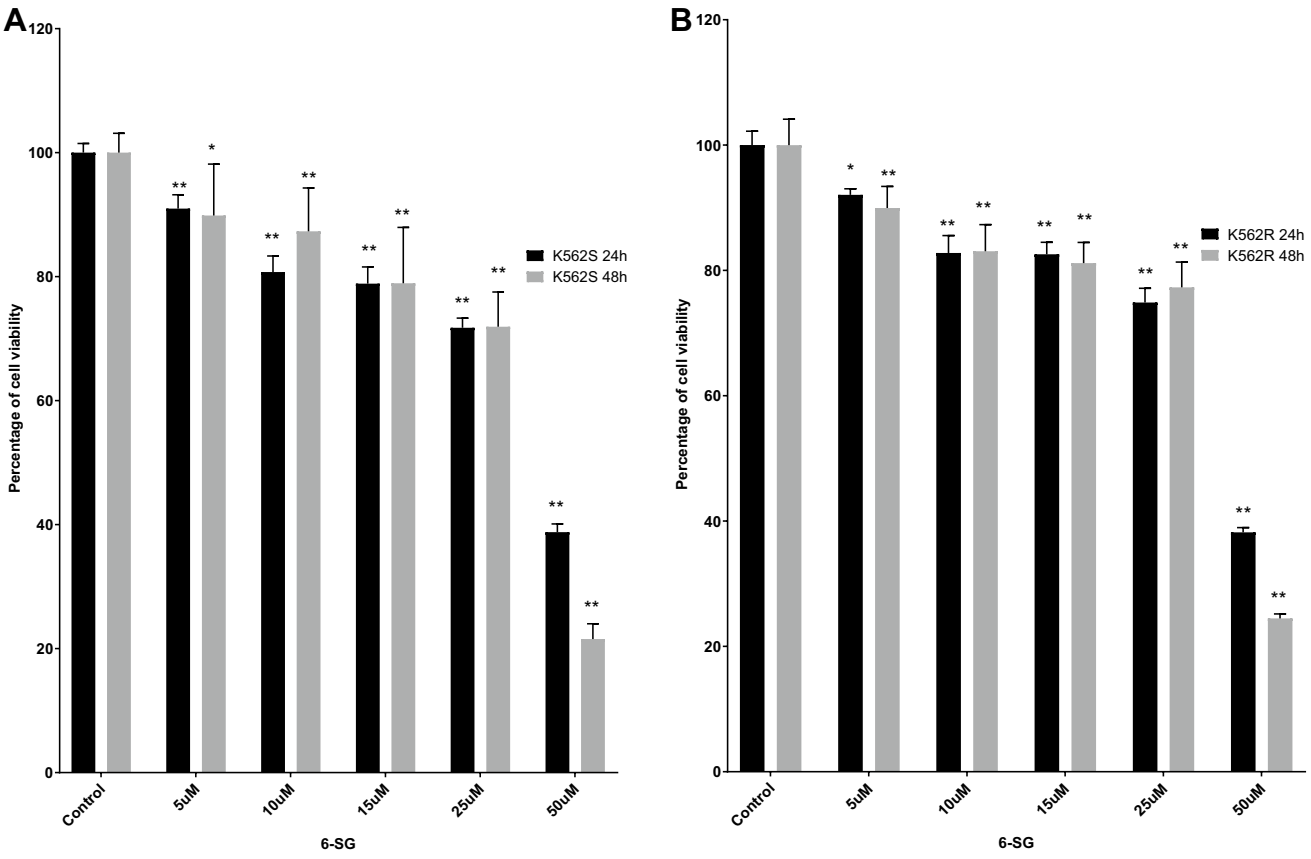
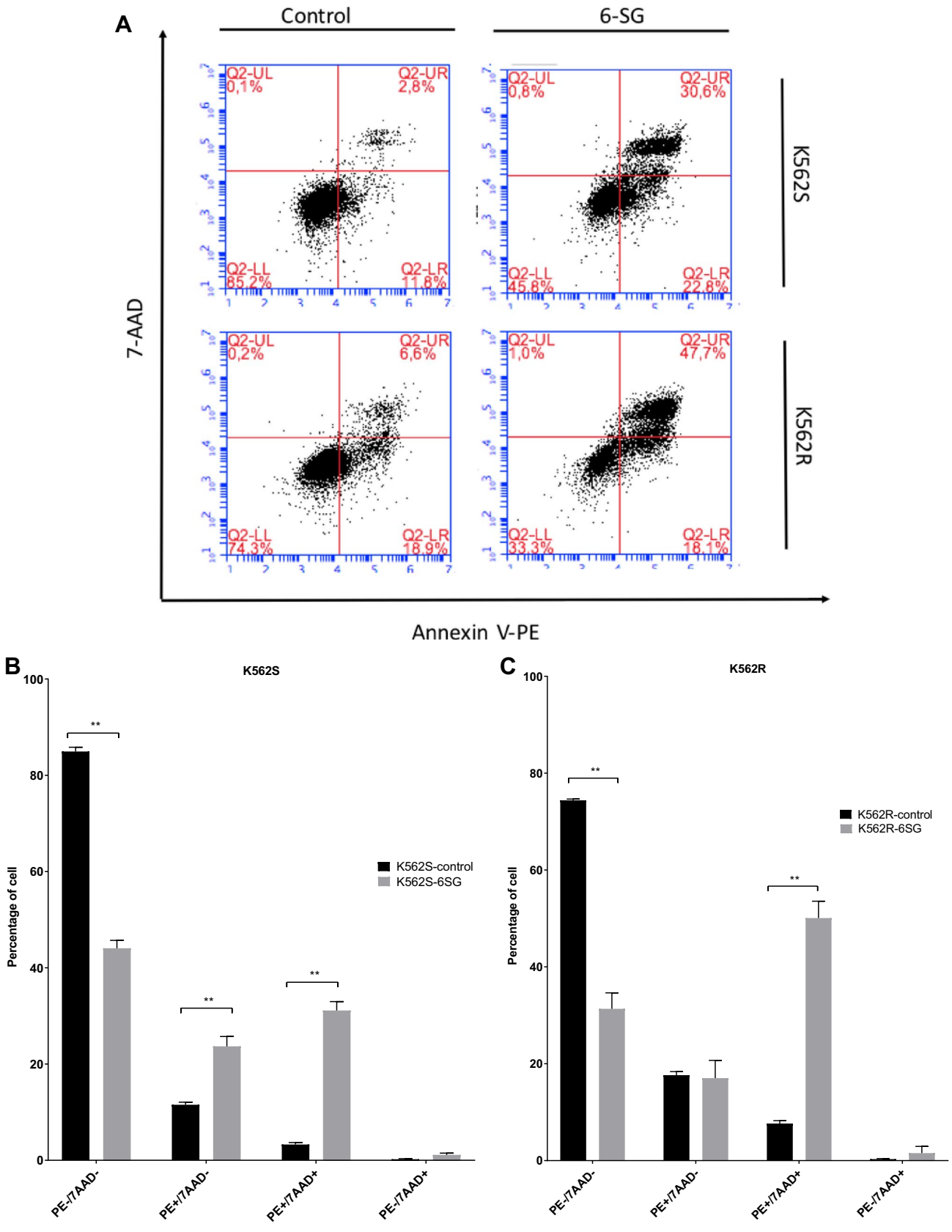


Fig. 1 Effect of 6-SG (5-50 µM) on cell viability of K562S, K562R CML cells. **A** Treatment of K562S; **B** K562 R cells with 6-SG decreased cell viability for 24 and 48 h. (*$p < 0.05$ and ** $p < 0.001$ shows significant differences from the control group)


A
Control
6-SG
K562S
K562R
7-AAD
Annexin V-PE
Q2-UL 0,1%
Q2-UR 2,8%
Q2-LL 85,2%
Q2-LR 11,8%
Q2-UL 0,8%
Q2-UR 30,6%
Q2-LL 45,8%
Q2-LR 22,8%
Q2-UL 0,2%
Q2-UR 6,6%
Q2-LL 74,3%
Q2-LR 18,9%
Q2-UL 1,0%
Q2-UR 47,7%
Q2-LL 33,3%
Q2-LR 18,1%
B
K562S
Percentage of cell
K562S-control
K562S-6SG
PE-/7AAD-
PE+/7AAD-
PE+/7AAD+
PE-/7AAD+
C
K562R
Percentage of cell
K562R-control
K562R-6SG
PE-/7AAD-
PE+/7AAD-
PE+/7AAD+
PE-/7AAD+

◂**Fig. 2** Flow cytometric analysis of Annexin V-PE/7-AAD-stained K562S, K562R CML cells. **A** Flow cytometry results are exhibited as dot plots; **B** Bar graph representation of flow cytometry in K562S; **C** Bar graph representation of flow cytometry in K562R cells. (*$p < 0.05$ and **$p < 0.001$ shows significant differences from the control group)

Effect of 6-SG on mRNA expression of apoptotic and drug transporter genes in CML cells

The effect of 6-SG treatment on the expression levels of apoptotic related genes (BAX and BCL-2) and drug transporters (MDR-1 and MRP-1) were evaluated by qRT- PCR. 6-SG was able to significantly increase the expression of BAX and decrease the BCL-2 mRNA in K562S and K562R cells as compared to the untreated control cells after 48 h ($p < 0.05$) ($p < 0.001$) (Fig. 3A).

Although 6-SG treatment enhanced MDR-1 gene expression levels significantly in K562S and K562R cells ($p < 0.05$) ($p < 0.001$), it decreased MRP-1 gene expression significantly in K562S cells ($p < 0.001$) (Fig. 3B).

Discussion

In the recent decade, introduction of IMA for the therapy of CML increases the efficiency of treatment and near-normal life expectancy in CML patients. Emergence of resistance to IMA therapy leads to relapse and failure [21] and becoming a challenge for CML treatment. Different mechanisms are involved in IMA resistance including apoptosis pathway, autophagy, DNA repair and drug efflux transporters [22, 23]. Hence, new strategies have been developed to overcome TKI resistance. Because of anticancer effect of natural extracts, they have drawn attention and investigated in many studies. 6-SG, pungent component of Ginger extract, is known to exhibit anti-inflammation, anticancer and antioxidant activity [24]. 6-SG induces apoptosis and inhibits the growth of various human cancer cells [24, 25].

Data from MTT assay demonstrated that 6-SG inhibited the growth of K562S and K562R cells in a dose and time dependent manner. 50 µM was the most effective concentration at 24 and 48 h. In order to analyze apoptosis pathway, cells were treated with 50 µM 6-SG for 48 h.

6-Shogaol exhibits inhibitory effect on cell proliferation and induces apoptosis in different human cancer cells via different mechanisms. 6-SG induced apoptosis through eIF2α dephosphorylation and caspase-dependent cleavage of eIF2α in human leukemia cells [6]. Moreover, 6-SG induced apoptosis via STAT3 and NF-kB Signaling suppression in prostate cancer cells [15].

According to our annexin V staining flow cytometry results, 6-SG dose dependently induced apoptosis in K562S and K562R cells. In order to better understand the apoptotic pathways, we also evaluated the mRNA expression levels of BAX and BCL-2 genes expression levels in 6-SG treated cells.

Since apoptosis induction is related to the alterations in the expression pattern of pro-apoptotic and anti-apoptotic genes, mRNA expression levels of BAX and BCL-2 genes were evaluated. Overexpression of BAX and downregulation of BCL-2 are associated with induction of apoptosis [26]. 6-SG, mainly induced mitochondrial pathway of apoptosis, therefore, Bcl-2 family might play crucial role in regulating this pathway. In the study of Pan et al., 6-SG persuaded apoptosis through ROS production, caspase activation and GADD 153 expression. Furthermore, 6-SG increased the protein expression levels of pro-apoptotic genes Bax, Fas and FasL and decreased the anti-apoptotic proteins Bcl-2 and Bcl-xl expression in treated COLO205 cells [10]. Saha et al. reported that 6-SG increased mRNA expression of BAX and decreased expression of BCL2 in prostate cancer cells [15]. 6-SG exhibited apoptotic effects via modulation of STAT3 and MAPKs signaling pathways and suppression of protein expression of STAT3-regulated genes (Bcl-2, Bcl-xl and Survivin) in MDA-MB-231 tumor cells [27]. 6-SG treatment (20 µg) induced apoptosis in human epidermal keratinocytes (HaCaT cells) by enhancing Bax and reducing Bcl-2 expressions when compare to the control [28]. In the present study, the mRNA expression level of BAX gene was significantly augmented in the 6-SG treated K562S and K562 R cells, whereas, 6-SG decrease BCL-2 gene expression level significantly.

Overexpression of drug efflux transporters (MDR-1and MRP-1) are involved in resistance to IMA [29]. Rahimi Babasheikhali et al. reported that ginger extract elevated ABCA2 expression level in the resistant ALL cell lines [30]. It was suggested that increased expression levels of ABCA2 or ABCA3 genes may be considered as a defending mechanism, in order to protecting cells from the ginger as a recognized toxic material [30]. In our previous study, we showed that K562R cells has a high expression level of MDR-1 (ABCB1) compared to K562S cells [20]. For this reason, we evaluated the effect of 6-SG on drug efflux transporters (MDR-1and MRP-1) expression levels. Based on our results, 6-SG did not impair the high expression level of MDR-1 gene in imatinib resistant K562R cells. Whereas, 6-SG treatment reduced MRP-1 expression level in K562S cells. High expression level of MDR-1 is suggested as a defending response of K562 cells against toxic effects of 6-SG. Considering all these observations, we could suggest that 6-SG may overcome IMA resistance and induce apoptosis through increasing pro-apoptotic and decreasing anti-apoptotic genes and other molecular pathways involved in its anticancer potential.

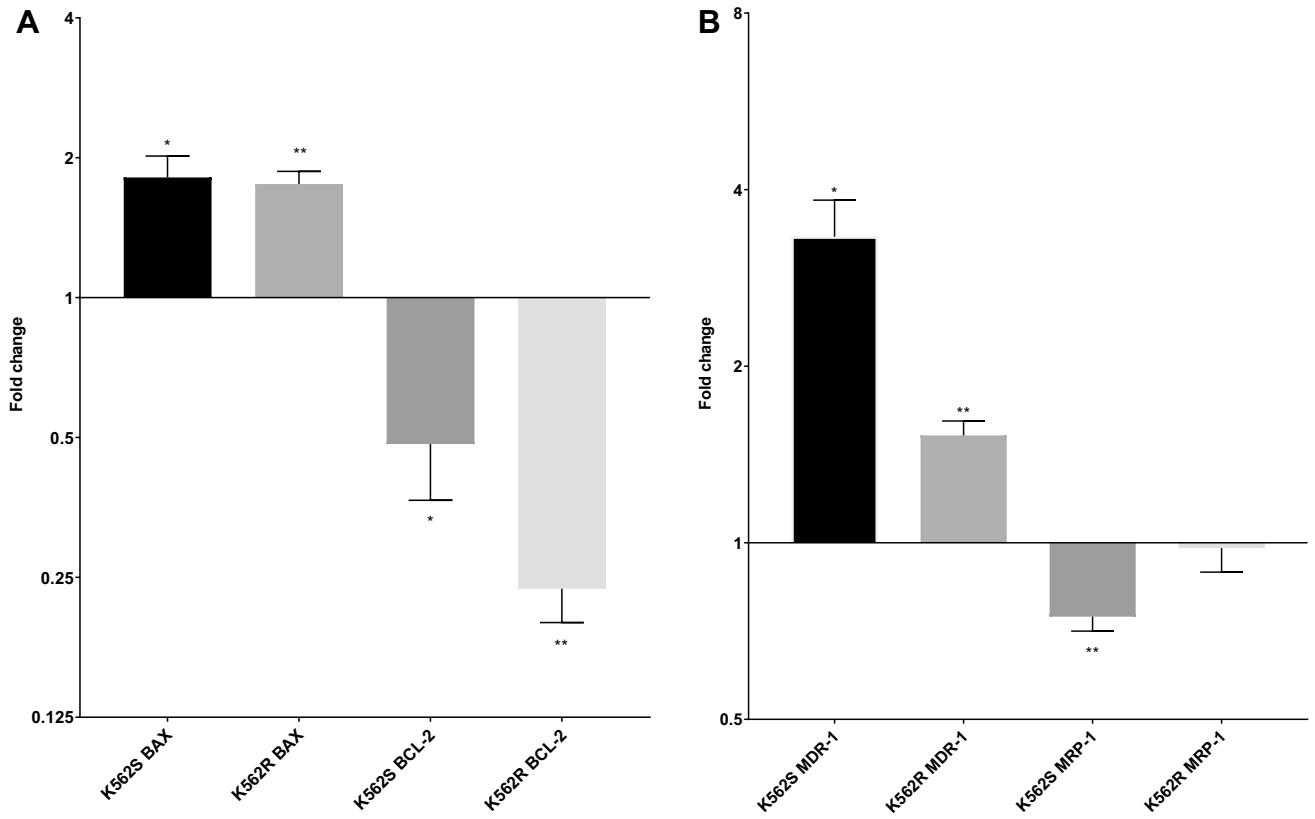


Fig. 3 mRNA analysis of K562S, K562R cells upon treatment with 6-SG for 48 h by Real-time PCR assay. Effects of 6-SG treatment **A** BAX and BCL-2 genes expressions; **B** efflux drug transporters MDR-1 and MRP-1 genes expression in K562S, K562R cancer cells. (*$p < 0.05$ and **$p < 0.001$ shows significant differences from the control group)

Conclusion

In conclusion, this study proved that 6-SG is a potent cytotoxic and anticancer agent that is active against IMA sensitive and resistant K562 cell lines and leads to induction of apoptosis. 6-SG could provide novel strategies in CML treatment via synthesizing potent drugs that selectively target genes. These compounds could also enhance potency of conventional therapies and suppress CML recurrence after achieving a successful therapy.

Authors' contributions Conception and design: AS,TO,YH. Development of methodology: AS, TO. Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): TO, YH, HP, MV, GY, GCY, CA. Writing, review, and/or revision of the manuscript: TO, YH. Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): AS, TO, YH. Study supervision: AS.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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

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