Orlistatand and Rosuvastatin Suppressed Proliferation of K562 Human Myelogenous Leukemia Cell Line through AMPK/Akt/c-Myc Signaling

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Abstract

Background: Chronic myeloid leukemia is a myeloproliferative cancer with worldwide incidence, has become as a clinical concern due to chemoresistance in the patients received chemotherapy. Here, we investigated the effect of Orlistat and Rosuvastatin on K562 human myelogenous leukemia cell line in vitro and attempted to illuminate their possible underlying mechanisms.

Methods: Cells were exposed to Orlistat and Rosuvastatin, the inhibitors of lipogenesis, then survival and apoptosis rate of K562 cells were examined by MTT assay and flow cytometric analysis respectively. The real time-PCR analysis was used to quantify mRNA levels of Bax, Bcl-2, and Hsp-70 genes. Cell cycle analysis was performed using flow cytometry, whereas the subcellular distribution of c-Myc was measured via immunofluorescence imaging technique. Additionally, the protein level of AMPK, p-AMPK Akt-1, and p-Akt-1 were studied by western blotting.

Results: The results showed Orlistat and Rosuvastatin had synergistic anticancer effects on cells and in comparison with the control group, viability and apoptosis rate decreased and increased in treated cells respectively in a dose/time-dependent manner ($P < 0.05$). The mRNA levels of Bax increased while expression of Hsp-70 decreased ($P < 0.05$). K562 cells treated with Orlistat and Rosuvastatin showed a cell cycle arrest in sub-G1 phase and a decreased level of c-Myc positive cells. Upon outlining the mechanism, it was revealed that AMPK/p-AMPK and p-Akt-1/Akt-1 ratio decreased in treated cells ($P < 0.05$).

Conclusions: Data suggest Orlistat and Rosuvastatin could synergically suppress proliferation of K562 cells through AMPK/Akt/c-Myc axis, proposing a theoretical basis for upcoming application in the treatment of chronic myeloid leukemia.

Background

Chronic myeloid leukemia (CML), a malignancy of bone marrow resident stem cells, is the globally leading cause of cancer-related death [1]. CML is characterized by a high proliferation rate of myeloid cells that results in uncontrolled releasing of immature myeloid lineage into blood [1]. Different therapies including chemotherapy, and stem cell transplant have been applied by physicians to eliminate cancerous cells of CML affiliated individual and improve lifespan [2, 3]. Despite improvement in survival rate of CML patients, however, the chemoresistance and mortality remain challenges in CML cancer treatment [2, 4]. Growing evidence indicates lipogenesis process contribute to support tumorigenesis in cancer cells [5, 6]. With respect to increased level of lipogenesis-based enzymes such as fatty acid synthase (FASN) and 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) in tumor cells, they can be potentially targeted to eliminate tumor cells [7, 8]. FASN, a homodimer of two protein subunits, catalyzes fatty acid synthesis, specially produces palmitate from malonyl-CoA and acetyl-CoA [9]. HMGCR is involved in the mevalonate metabolic pathway and mediates synthesis of cholesterol and isoprenoids compounds [10]. Recent trends in cancer treatment have led to a proliferation of studies that

evaluate lipogenesis in cancer cells [11]. In this regard, several laboratories showed that inhibition of FASN and HMGCR may elucidate a mechanism by which participates to block lipogenesis in tumor cells, subsequently induces cell death [12, 13]. For example, treatment of MDA-MB468 breast cancer cells with Cerulenin, a FASN inhibitor, caused apoptosis and delayed proliferation rate of tumor cells [14]. Orlistat, tetrahydrolipstatin, is an anti-obesity drug that inhibit lipase enzymes and absorption of fats from digestive tract [15]. Recently, the anti-cancer properties of Orlistat have been documented [16]. Statins, the cholesterol reducing compounds, such as Rosuvastatin is being used to inhibit cardiovascular disease in patients at high risk and treat abnormal cholesterol and fats through inhibition of HMGCR [17]. It is well established that inhibition of HMGCR by statins disturbed lipogenesis in tumor cells, accordingly, decreased cell survival [18]. Regarding up-regulated lipogenesis in tumor cells, however, there has been little discussion about the effect of anti-obesity compounds including Orlistat and Rosuvastatin on human myeloid cancer cells. The evidence for this relationship is inconclusive, hence, the aim of this study is to shine light on effect of Orlistat and Rosuvastatin on apoptosis rate of K562 cells *in vitro*.

Methods

Cell culture

To perform experiment, K562, human myelogenous leukemia cell line (Pasteur Institute, Iran) were cultured in RPMI1640 (Biowest, France) cell medium containing 10% FBS (Gibco) and 1% penicillin-streptomycin (Gibco). Cells were kept in a humidity incubator at 37 °C. Every 2-3 days the supernatant was replaced by the fresh medium. The cells at 3-6 subcultures were enrolled to downstream experiments. All experiments were repeated in three sets.

Treatment protocol

K562 cells were divided into five groups and treated with 0 μ M, 20 μ M, 40 μ M, 60 μ M, and 80 μ M of Orlistat (Sigma) and Rosuvastatin (Sigma) or with a mixture of Orlistat and Rosuvastatin for 24 h, 48 h, and 72 h. After IC₅₀ analyzing, for downstream measurements, cells were co-cultured with Orlistat and Rosuvastatin over a period of 72 h. Compounds were dissolved in 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (DMSO, Merck) solution.

MTT assay

K562 cells (6×10^3 cell /well) were incubated in 96-well tissue culture plates with Orlistat and Rosuvastatin or with a combination of Orlistat and Rosuvastatin over a period of 24 h, 48h, and 72 h. In short, 10 μ l of 5 mg/mL MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) (MTT, Sigma) solution in PBS (phosphate-buffered saline) was added to each well and incubated for 4 h. Next, 100- μ l dimethylsulfoxide (DMSO) was poured and the plates were again kept for 15 min in the dark. Absorbencies were recorded at 570 nm using a reader system (Bioteck, elx800). After first round cell survival analysis, we used IC₅₀ of Orlistat and Rosuvastatin for subsequent analyses.

Hoechst staining

Hoechst-33258 staining dye solution (Sigma, CAS Number: 23491-45-4) was used to labeling DNA for monitoring apoptosis in fluorescence microscopy. Briefly, 1×10^5 cells were seeded in per well of 24-well plates. After treatment period, cells were fixed by methanol (Merck) and kept in -20 ° C for 15 min. Next, methanol was removed and cells incubated with 1 mg Hoechst at RT for 30 min. Visualization of cells stained with Hoechst was accomplished at 461 nm by fluorescence microscopy (Zeiss, Germany).

Flow cytometric analysis

We used Annexin-V, a marker of apoptosis, to detect percentage of apoptotic K562 cells. In brief, according to Annexin-V kit's (eBioscience) protocol, 2×10^5 cells were incubated with 300 ml of binding buffer at RT for 15 min. Then, Annexin-V (5 ml) was mixed with cell suspension at RT for 15 min in a dark condition. Cells were washed twice with PBS and suspended in the 1ml final volume of binding buffer. Analysis was done by a flowcytometric system (BD FACSCalibur) and FlowJo (version 7.6.1) software.

Real time-PCR analysis

To evaluate effect of Orlistat and Rosuvastatin on expression of Bax, Bcl-2, and Hsp-70, we performed real time-PCR analysis. Total RNA was extracted from K562 cells using a RNA purification kit (Yekta Tajhiz Azma, Iran). The quality of RNAs was calculated by a Nano drop spectrophotometer system (BioTek). Next, cDNA was constructed using 1000 ng of total RNA according to manufacturer's recommendation (Yekta Tajhiz Azma, Iran). An optimal mixture of primers, cDNAs, and Sybergreenmaster mix reagent (Yekta Tajhiz Azma, Iran) was subjected to real time-PCR system (Labgene scientific SA, Swiss). The transcription levels of Bax, Bcl-2, and Hsp-70 genes were measured against GAPDH as housekeeping gene. Sequences of primers designed by Oligo-7 software (ver 7.60) were presented in Table 1.

Table 1: list of primers

Genes	Forward	Reverse	Tm
Bax	GCTTCAGGGTTTCATCCAGG	GCTGCCACTCGGAAAAAGAC	60
Bcl-2	GATGGGATCGTTGCCTTATGC	TTCTGGTGTTTCCCCCTTGG	60
Hsp-70	GCCGAGCATTCTCTGATCCA	AACACTTTCGGCTGTCTCCT	60
GAPDH	CAAGTTCAACGGCACAGTCAAG	ATACTCAGCACCAGCATCACC	59

Western blotting

We did western blotting to measure protein level of AMPK, p-AMPK, Akt, and p-Akt involved in growth of K562 cells. Cells lysates were obtained using RIPA buffer (Merck) supplemented with a protease inhibitor cocktail (Sigma). Briefly, 100 µg of each protein sample was injected onto a 10% sodium dodecyl sulfate–polyacrylamide (SDS) gel and after electrophoresing, proteins were transferred to a 0.2 µm polyvinylidenedifluoride (PVDF, Millipore) membrane. Protein bands on membrane were probed with anti-human AMPK, p-AMPK, Akt, and p-Akt primary antibodies (Santa Cruz) at 4 °C overnight after blocking with 5% skim milk in TBST over 1 h. Bands were visualized by HRP-linked secondary antibody (Sigma) and ECL plus solution (Roche). Density of proteins bands was measured with ImageJ software ver.1.45p (NIH). The protein level of β-actin (Sigma) was used to normalization of target proteins.

Cell cycle analysis

To determine possible effect of drugs on cell cycle, we used propidium iodide (PI) staining (Sigma) with a flow cytometry (BD FACSCalibur). Following centrifuging at 1500 rpm for 5 min, K562 cells were fixed with 70% ethanol at -20 °C overnight. Then, cells were incubated with pre-chilled PI stain solution (20 mg/mL PI, 0.1% Triton X-100, and 20 mg/mL RNase A; Invitrogen) for 15 min in a dark room. Finally, cells were subjected to flow cytometry (BD FACSCalibur) and data analyzed by flow Jo software ver 7.6.1

Immunofluorescence imaging

The intracellular distribution of c-Myc was imaged by immunofluorescence imaging (IF) technique. For this purpose, 1×10^5 cells were plated in each well of 24-well tissue culture plates and exposed to treatment protocol. For fixation, K562 cells were incubated with 4% paraformaldehyde (PFA) solution and permeabilized with 0.1% Triton 100-X solution (Sigma). After 30 min blocking with 1% bovine serum albumin (BSA, Sigma), cells were incubated with the primary anti-human c-Myc antibody (Santa Cruz) overnight at 4 °C. Next, cells were washed twice with PBS and incubated with secondary FITC-conjugated antibody (Sigma) for 40 min at 37 °C in the dark. For nuclear visualization, DAPI (4', 6-diamidino-2-phenylindole) solution (Sigma) was used. Cells were imaged via a fluorescent microscope (Zeiss, Germany) and analyzed with cellSens imaging software.

Statistical analysis

Statistical analyses were performed by SPSS software V25.0 (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± SD. One-way analysis of variance (ANOVA) and Tukey's post hoc test were used to assess the significant differences ($P < 0.05$) between groups. In graphs, the significant values are shown by asterisks as follows * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ***** $P < 0.00001$.

Results

Orlistat and Rosuvastatin decreased K562 cells viability

To investigate the potential cytotoxic effects of Orlistat and Rosuvastatin on K562 cells, we used conventional MTT assay. We found that compared to control group, Orlistat significantly decreased viability of K562 cells in a dose/time-dependent manner ($P_{\text{control vs. O20}} < 0.05$; $P_{\text{control vs. O40, O60, and O80}} < 0.00001$ for 24h, $P_{\text{control vs. O20, O40, O60, and O80}} < 0.00001$ for both 48h and 72h) (Fig 1 a,b and fig 2a). As well, there exist a significant difference between other groups at the same times ($P_{\text{O20 vs. O60 and O80}} < 0.01$ for 24 h, $P_{\text{O20 vs. O80}} < 0.001$; $P_{\text{O40 vs. O80}} < 0.01$ for 48 h, $P_{\text{O20 vs. O60}} < 0.01$; $P_{\text{O20 vs. O80}} < 0.0001$; $P_{\text{O40 vs. O80}} < 0.01$ for 72h), which indicating dose-dependent decrease in the cell survival.

Similarly, data showed that Rosuvastatin reduced the cell viability values of K562 cells dose/time-dependently ($P_{\text{control vs. R20 and R40}} < 0.01$; $P_{\text{control vs. R60 and R80}} < 0.0001$ for 24h, $P_{\text{control vs. R20, R40, R60, and R80}} < 0.00001$ for both 48h and 72h). Furthermore, we found that R60 and R80 profoundly decreased cell viability as compared to other treated cells ($P_{\text{R20 and R40 vs. R80}} < 0.01$; $P_{\text{R20 vs. R60}} < 0.01$; $P_{\text{R20 vs. R80}} < 0.001$; $P_{\text{R40 vs. R60}} < 0.05$ for 24h, $P_{\text{R20 vs. R60 and R80}} < 0.001$; $P_{\text{R40 vs. R60}} < 0.01$; $P_{\text{R20 vs. R80}} < 0.001$ for 72 h) (Fig 1c, d and fig 2b).

We evaluated the combination cytotoxic effect of compounds on K562 cells at the same times. The combination drug was prepared according to the drugs IC₅₀ concentrations (The 50% inhibitory concentration). Result indicated that Orlistat and Rosuvastatin combination more significantly inhibited K562 cells viability in a dose-dependent manner ($P_{\text{control vs. O+R20, O+R40, O+R60, and O+R80}} < 0.00001$). The viability of cells was significantly low in high dose groups rather than 20 O+R and 40 O+R groups ($P_{\text{O+R20 vs. O+R60}} < 0.01$; $P_{\text{O+R20 vs. O+R80}} < 0.001$; $P_{\text{O+R40 vs. O+R80}} < 0.001$ for 24h, $P_{\text{O+R20 vs. O+R60}} < 0.01$; $P_{\text{O+R20 vs. O+R80}} < 0.0001$; $P_{\text{O+R40 vs. O+R80}} < 0.01$; $P_{\text{O+R60 vs. O+R80}} < 0.05$ for 48h, $P_{\text{O+R20 vs. O+R60}} < 0.01$; $P_{\text{O+R20 vs. O+R80}} < 0.001$; $P_{\text{O+R40 vs. O+R60 and O+R80}} < 0.05$ for 72h) (Fig. 1e, f). IC₅₀ concentrations of compounds were evaluated after 72 hours by MTT assay data and Compusyn software. According to Compusyn analysis the IC₅₀ were 52 mM and 57 μ M for Orlistat and Rosuvastatin respectively. As shown in Fig. 2, we found that Orlistat and Rosuvastatin act in a synergic manner and caused a profound decrease in cell viability. In all applied concentrations of combination compounds, orlistat and Rosuvastatin showed synergic effect (Fig 2c).

Orlistat and Rosuvastatin induced apoptosis in K562 cells

The Hoechst staining test was used to detect apoptotic cells. Representative images of Hoechst staining were shown in Fig. 3. The number of apoptotic K562 cells (yellow arrows) increased in treated cells. The Hoechst staining makes the condensed chromatin of apoptotic K562 cells more brightly than chromatin of control cells.

To further insight into apoptosis we monitored the apoptosis rate of K562 cells via Annexin V test. The result indicated that Orlistat and Rosuvastatin significantly increased the percentage of apoptotic cells ($P_{\text{control vs. O and R}} < 0.05$; $P_{\text{control vs. O+R}} < 0.001$) (Fig. 3 b, c). Besides, Orlistat and Rosuvastatin synergically

showed a high level of apoptotic cells ($61.22 \pm 3.57 \%$), when compared with O ($46.21 \pm 4.26 \%$) and R ($44.15 \pm 4.14 \%$) groups ($P < 0.05$).

Orlistat and Rosuvastatin altered expression of Bax, Bcl2, and Hsp-70 genes

To inquiry underlying mechanisms involved in apoptosis, we performed real time-PCR analyze for Bax and Bcl-2 genes involved in apoptosis pathway [19]. Our results showed the Orlistat and Rosuvastatin significantly increased mRNA level of Bax as compared to control group ($P < 0.01$ and $P < 0.05$, respectively). Orlistat and Rosuvastatin synergically increased Bax expression ($P < 0.01$) (Fig. 4). Moreover, the pattern of Bcl-2 expression was decreased in treated groups but did not significantly altered ($P > 0.05$). Data revealed that the Bax/Bcl-2 ratio was significantly increased in treated groups (1 vs. 2.6 ± 0.3 and 2.63 ± 0.29 fold changes; $P_{\text{control vs. O and O+R}} < 0.01$; 1 vs. 2.24 ± 0.25 fold changes; $P_{\text{control vs. O+R}} < 0.001$).

Study on Hsp-70 expression pattern indicated that Orlistat and Rosuvastatin treatment either lonely or in a mixture form decreased mRNA level of Hsp-70 as compared with control cells ($P < 0.0001$) (Fig. 4). Despite increase in mRNA level of Hsp-70 in treated cells, we did not observed significant difference between treated groups ($P > 0.05$).

Orlistat and Rosuvastatin induced a block of cell cycle progress at the sub-G1 phase in K562 cells

In order to examine the role of Orlistat and Rosuvastatin on cell-cycle progression, DNA content was measured in cells treated with Orlistat and Rosuvastatin using PI staining (Fig. 5a). Results show that Orlistat and Rosuvastatin had potential to induce arrest in sub-G1 phase (control: $4.1 \pm 0.95 \%$ vs. O: $38.05 \pm 2.05 \%$; $P < 0.001$, control $4.1 \pm 0.95 \%$ vs. R: $25.15 \pm 3.89 \%$; $P < 0.01$, control: $4.1 \pm 0.95 \%$ vs. O+R: $51.85 \pm 3.32 \%$; $P < 0.001$) (Fig. 5a, b).

Additionally, we observed that there was significant difference between treated groups ($P_{\text{R vs. O}} < 0.05$; $P_{\text{R vs. O+R}} < 0.01$; $P_{\text{O vs. O+R}} < 0.05$). Based on the data, percentage of G0/G1 cells were reduced in treated cells as compared to control cells ($P_{\text{control vs. O}} < 0.05$; $P_{\text{control vs. O+R}} < 0.01$). Similarly, percentage of G2/M cells were low in treated cells ($P_{\text{control vs. O and R}} < 0.01$; $P_{\text{control vs. O+R}} < 0.001$), whereas percentage of S cells not significantly affected ($P > 0.05$) (Fig. 5a, b). Combination form had more effective role than orlistat and rosuvastatin in sub-G1 cell cycle arrest.

The subcellular distribution of c-Myc was reduced in treated K562 cells

Expression of c-Myc was also monitored by IF assay. Data confirmed that Orlistat and Rosuvastatin were capable of inhibiting expression of c-Myc inside cells (Fig. 6a). Compared with control, cells cultured with Orlistat and Rosuvastatin showed a decreased number of c-Myc positive cells.

Orlistat and Rosuvastatin altered protein level of AMPK, p-AMPK (T172), Akt-1, and p-Akt-1 (S473) in K562 cells

We also investigated growth/proliferation dynamic of treated K562 cells via AMPK and Akt pathway using western blotting analysis. AMPK and Akt proteins contribute to modulate stress condition and energy balance inside cells [20, 21]. As shown in Fig. 6b and c, western blotting analysis showed that Orlistat and Rosuvastatin treatment increased AMPK expression ($P_{\text{control vs. O and R}} < 0.05$). In addition, we found a significant decrease in protein level of O+R group as compared with other treated groups ($P_{\text{O+R vs. O and R}} < 0.05$). In comparison to control group, a significant increased levels of p-AMPK protein in treated cells was observed ($P_{\text{control vs. O and O+R}} < 0.001$; $P_{\text{control vs. R}} < 0.01$) (Fig. 6b, c). We found that p-AMPK decreased in R group (1.73 ± 0.18 fold), when compared with both O (2.15 ± 0.06 fold) and O+R group (2.16 ± 0.05) ($P < 0.05$). In addition, we found that AMPK /p-AMPK ratio significantly decreased in treated cells ($P_{\text{control vs. O and O+R}} < 0.001$; $P_{\text{R vs. O+R}} < 0.05$) (Fig. 6c). Then we investigated p-Akt-1/Akt-1 status inside cells. Data indicated that Orlistat and Rosuvastatin suppressed p-Akt-1 in K562 cells ($P_{\text{control vs. O and O+R}} < 0.0001$; $P_{\text{control vs. R}} < 0.05$; $P_{\text{R vs. O and O+R}} < 0.0001$) (Fig. 6b, d). In contrast, protein level of Akt-1 increased in treated cells ($P_{\text{control vs. O, R, and O+R}} < 0.05$). Analysis of p-Akt-1/Akt-1 ratio showed that this ratio diminished in treated cells ($P_{\text{control vs. O and O+R}} < 0.0001$; $P_{\text{control vs. R}} < 0.001$; $P_{\text{R vs. O and O+R}} < 0.0001$) (Fig. 6d). This result suggests that Orlistat and Rosuvastatin hinder proliferation of cells through activating AMPK and inhibiting p-Akt-1 enzymes.

Discussion

At the present study, it was hypothesized that Orlistat and Rosuvastatin, anti-lipogenesis compounds, could suppress K562 cells. Though outstanding advancement has been made through the introduction of chemotherapy and targeted molecular treatment over the last decades, new/supplementary approaches to eliminate tumor cells and increase life span of CML patients are desirable [22, 23]. Related to Orlistat and Rosuvastatin dose intensity, we initially showed that cell viability decreased in a dose and time-dependent manner. Moreover, simultaneously, we found that the combination doses of Orlistat and Rosuvastatin synergically declined viability rate of cells in a dose and time-dependent mode. These data support a concept that these compounds, in a mixture form, more profoundly inhibited K562 growth rather than in a single form at the same time. The findings are consistent with those of Sokolowska and co-workers who investigated effect of Orlistat on human pancreatic cancer cells (PANC-1) and showed that Orlistat decreased cell viability and induced apoptosis [16]. In the similar vein, it was demonstrated that orlistat was capable of inducing inhibition in proliferation rate and promoting mortality [24]. Previously, it was shown that Rosuvastatin had potential to inhibit HMGCR/ SREBP-2 /Ras signaling which, in turn, disrupted cell growth and lipogenesis in cancer cells [25]. Besides this pathway, there is evidence that Rosuvastatin may inhibit endothelial and cancer cells through JNK/NF- κ B axis and P21 pathways respectively [26, 27].

In an attempt to elucidate the possible effect of these compounds on apoptosis rate of cells, Hoechst staining showed an increased level of the nuclear segmentation, subsequently, apoptosis in treated cells profoundly in O+R group cells. Further test carried out with K562 cells, as shown in Fig. 3, we observed markedly apoptosis rate in treated cells. Notably, we revealed that combination of Orlistat and

Rosuvastatin increased percentage of apoptotic cells as well as expression of Bax gene and the Bax/Bcl-2 ratio. To the best of our knowledge, up-regulated levels of Bax gene and Bax/Bcl-2 ratio represent an induction in apoptotic pathway [28]. In our opinion, Orlistat and Rosuvastatin disrupted lipogenesis through targeting FASN the HMG-COA [29] and contribute to induce cell death in particular in the mixture form [24]. We showed that the mRNA levels of Hsp-70 were decreased in treated cells. Confirmed that, Hsp-70, a stress inducible chaperone, involved in tumorigenesis and chemotherapy [30]. Based on our knowledge, there are insufficient reports, however, a study by Schmeer *et al.* revealed that statins suppressed Hsps in retina ischemia/reperfusion *in vivo* [31]. Hsp-70 has been demonstrated that inhibit apoptosis [32]. In this regard, we would imagine that Orlistat and Rosuvastatin may block Hsp-70 which subsequently induced apoptosis in cells [33]. Furthermore, cell cycle analysis indicated Orlistat and Rosuvastatin lonely or synergically caused arrest in Sub-G1 step confirming apoptosis rate in K562 cells. This view is supported by Papaevangelou and colleagues who reported that Orlistat caused arrest in G1 phase and decreased DNA synthesis in cisplatin-resistant ovarian carcinoma model[34]. Similarly, Zeybek *et al.* revealed that Rosuvastatin made arrest in G1 phase in B-CPAP, papillary thyroid cancer cells [35, 36].

We further found that upon increase in k562 cells apoptosis rate in treated groups, c-Myc distribution inside treated cells was decreased. This finding is vital owing to the key roles of c-Myc in tomorigenesis and proliferation [37]. This transcription factor is the most often up-regulated oncogene in human tumors that mediates reprogram and the most vital processes for proliferation, survival, and metabolism [38]. In accordance with the present results, previous study has demonstrated that Statins down-regulated c-Myc and tumor growth through miR-33b molecule; a c-Myc key regulator [39]. These results lends support to Wang and co-workers who reported that Simvastatin decreased c-Myc and induced breast cancer cell death [40]. Notably, these finding may suggest a relationship between cell cycle arrest and decreased level of c-Myc in treated cells [41, 42]. As well, these results support a concept that c-Myc inhibition contribute to induce apoptosis not only by cell cycle arrest but also through distributing bioenergetic balance and metabolic process [43].

We, at that time, explored the possible underling mechanisms that resulted in c-Myc suppression. In this regard, western blotting analysis showed a decreased amount of AMPK/p-AMPK ratio suggesting hyperphosphorylation of AMPK. As known, AMPK, a key regulator of cellular energy homeostasis, is activated in response to stresses that exhaust cellular ATP supplies [44]. This enzyme is a multifunctional enzyme and mediates different signaling pathway including apoptosis, proliferation, and autophagy [44, 45]. It seems possible that these results are due to inhibiting/distributing cellular energy homeostasis through blocking lipognesis in K562 cells that contributes to activate AMPK and inhibit proliferation through c-Myc pathway [46]. On the other hand, phosphorylation of AMPK occurs in theronine 172 byLKB1 which may additionally active P53 in cells [47] and cause apoptosis flow in cells. Furthermore, Patel and colleagues have reported that AMPK was profoundly activated in apoptotic cells indicating involvement in apoptosis pathway [48]. As mentioned above, AMPK has been reported that to involve in different signaling pathway, and further inquiries are essential to uncover any possible cross links between pathways and elucidate the majority of a pathway in suppression of K562 cells. As apparent from Fig 6b and c, combination form of compounds had potentially more effect on AMPK activation

rather than the two compounds alone, thus it can be concluded that Rosuvastatin and Orlistat synergism effect may be exerted by AMPK activation.

We also found incubation of cells with Orlistat and Rosuvastatin increased protein level of active Akt-1, however, p-Akt-1/Akt-1 ratio decreased in treated cells, which is an indicative of Akt-1 suppression inside cells. Interestingly, Orlistat and combination form similarly inhibited Akt-1 activation (Fig 6b, d), therefore, it can be concluded that result from R+O group somewhat may be run by Orlistat. It was recently demonstrated that inhibition of the protein geranylgeranylation by statins blocked Akt activation, which, in turn, induced apoptosis in cancer cells [49]. Akt plays a pivotal function in various cellular processes including apoptosis, proliferation, glucose metabolism, transcription, and migration. Well-known, Akt-1 act as a tumorigenesis factor through regulating c-Myc and inhibits apoptosis flow as well as cell cycle arrest in tumor cells [50]. Regarding the results of our experiment, it is reasonable to suggest that decreased level of p-Akt-1/Akt ratio could increase apoptosis rate and cell cycle arrest in treated cells. Indeed, AMPK and Akt contribute to regulate c-Myc in downstream; that activated AMPK (p-AMPK) inhibits c-Myc, whereas, activated Akt (p-Akt) mediates c-Myc activation [46, 51] which may correlates with our results. Beside this, presumably, mTOR (mammalian target of rapamycin), a serine kinase which causes phosphorylation on S473 of Akt-1, may be negatively affected by Orlistat, Rosuvastatin, and their combination; which could be considered in further studies [52]. In summary, through studying treated K562 cells with Orlistat and Rosuvastatin alone or in combination form affecting cell survival, cell cycle, and proliferation signaling pathway, we shed light into cellular mechanisms of apoptosis and cell proliferation pathway inside K562 cells. We showed that K562 cells were suppressed under Orlistat and Rosuvastatin treatment. It may be assumed that AMPK/AKT/c-Myc axis participate in inhibiting proliferation and inducing apoptosis in K562 cells.

Conclusion

The evidence from this study suggests that Orlistat and Rosuvastatin treatment suppressed the proliferation of K562 cells more effectively in combination form through interfering with growth kinetics. Our findings indicated that Orlistat and Rosuvastatin could modulate AMPK/Akt-1/c-Myc signaling; which resulted in cell cycle arrest, apoptosis, and cell fall. However, further studies are necessary to clarify the exact underlying mechanisms, signaling pathways involved in K562 suppression.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

Authors have no competing interests to disclose

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Author Contributions

Y. P. and B.M. conceptualization with equal edited the manuscript. B.M. collected data and Y. P. performed software and analysis.

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Figures

Figure 1

Viability of K562 cells by MTT assay treated with Orlistat (a, b), Rosuvastatin (c, d), Orlistat + Rosuvastatin (e, f) over a period of 24 h, 48 h, and 72 h. N= 3, One-way ANOVA and Tukey test was used. *P< 0.05, **P< 0.01, ***P< 0.001, ****P< 0.0001, and *****P< 0.00001.

Figure 2

Determination of IC₅₀ value of Orlistat (a), Rosuvastatin (b) and synergic effect of Orlistat and Rosuvastatin (c). Combination index was > 1, indicating that Orlistat and Rosuvastatin affected cells in a synergic manner.

Figure 3

Hoechst staining, blue fluorescent dye, shows apoptotic cells using a fluorescence microscopy (a), yellow arrows illustrate chromatin dense in apoptotic cells. Flow cytometric analysis of apoptosis rate in treated cells (b, c). The percentage of apoptotic cells increased in treated groups. N=3, One-way ANOVA and Tukey test was used. *P< 0.05, **P< 0.01, ***P < 0.001.

Figure 4

Real time-PCR analysis of Bax, Bcl-2, Bax/Bcl-2 ratio, and Hsp-70 genes. N=3, One-way ANOVA and Tukey test was used. *P< 0.05, **P< 0.01, ****P < 0.0001.

Figure 5

Cell cycle analysis by flow cytometry and PI staining (a,b). N= 3, One-way ANOVA and Tukey test was used. *P< 0.05, **P< 0.01, ***P< 0.001.

Figure 6

Representative images of c-Myc + cells after treatment with Orlistat and Rosuvastatin (c). Quantitation of p-AMPK, AMPK, p-Akt, and Akt protein level by western blotting and densitometric quantitation of the bands (a, b, d). N= 3, One-way ANOVA and Tukey test was used. *P< 0.05, **P< 0.01, ***P< 0.001, ****P< 0.0001.