Naringenin Enhances Anti-Proliferation Effect of FMSP On K562 Human Chronic Myelogenous Leukemia Cells Via Targeting Calmodulin Signaling Pathway

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Abstract

Background: FMSP is a synthesized ferrocene derivative which possesses strong anti-proliferative and apoptosis inducing characteristics on tumor cells. Naringenin as a polyphenolic flavonoid is also able to reduce cell viability in tumors.

Methods: Cell viability and proliferation of cancer cells after treatment with these agents were determined with MTT assay. To predict the possible interaction between calmodulin (CaM) and FMSP and naringenin, docking studies were performed. By using fluorescence emission spectra, effects of FMSP and naringenin on CaM structure and activity were studied. CaM-dependent activation of phosphodiesterase 1 (PDE1) by FMSP in relation to naringenin and their combination were compared. Effects of these compounds on PDE1 inhibition, cAMP accumulation, and cAMP-dependent protein kinase A (PKA) activation were assayed.

Results: Our results showed that combination of FMSP and naringenin had more inhibitory effects on CaM structure than FMSP and naringenin alone. Results of docking analyses also confirmed efficient interaction of the two compounds with hydrophobic pocket of calmodulin active site. Kinetic analyses of these agents interaction with CaM showed FMSP and naringenin both competitively inhibited PDE1 activation without changing Vmax parameter. FMSP and naringenin synergistically increased Km values in a higher level compared to FMSP or naringenin alone. Combination of these two agents also had more cytotoxic effects on K562 cells than FMSP alone.

Conclusions: It was shown that mechanism of K562 cell proliferation inhibition by these compounds is based on CaM and consequent PDE inhibition followed by intracellular cAMP level elevation and increased PKA activity in a dose-dependent manner.

Introduction

Chronic myelogenous leukemia (CML) is a neoplastic hematopoietic disease that more commonly occurs in men (50–60 years-old) and is presented by an uncontrollable proliferation of mature granulocytes [1]. CML has been estimated to cause 8990 new cases and 1140 deaths in the United States during 2019 [2]. Different treatment options are available for CML patients but several adverse side effects may occur due to this therapeutics [3]. Therefore, finding novel and effective therapeutic agents with few or no potential adverse effects is necessary. Discovery of various molecular pathways involved in the cancer has led to the development novel molecular targeted treatments that suppress cancer growth [4]. These targeted therapies mainly act by blocking specific molecules required for cell proliferation and tumorigenesis. Specific targeting of key molecules in cancer cells renders these new therapies a higher efficacy and fewer adverse effects in comparison to other conventional therapies [5]. Currently, tyrosine kinase inhibitors (TKIs) have reported as the successful and efficient molecular targeted therapeutics for the treatment of patients with CML, however, some patients show resistance to TKI therapy and need alternative therapeutic approaches to tackle the disease [6].

Cyclic adenosine monophosphate (cAMP) is an intracellular messenger molecule that is able to stimulate cell proliferation in various cell types while inhibit this process in some others. This molecule exerts its effects by the activation of cAMP-dependent protein kinase A (PKA) [7]. Study of cancer cells also has shown that an elevated level of intracellular cAMP plays a pro-apoptotic role within these cells [8]. The intracellular concentration of cAMP is mainly under the control of two enzymes including adenylyl cyclases and cyclic nucleotide phosphodiesterases (PDEs), which are implicated in the synthesis and degradation cAMP, respectively [9]. PDE1, as one of the isoforms of this enzyme, has a binding site for Ca^{2+} /calmodulin (CaM) in its structure by which PDE1 is activated to degrade cAMP in a CaM-dependent manner [10]. It has been revealed that inhibition of PDE1 could cause apoptosis induction in cancer cells and hence this enzyme could be of great importance as a therapeutic strategy to trigger apoptosis of cancer cells [11]. Furthermore, calmodulin itself has been proven to stimulate apoptosis and cell cycle blockage both *in-vitro* and *in-vivo* [12].

Previous studies have indicated that plant-derived phytochemicals have significant ant- cancer activities both alone or in combination with synthetic compounds [13, 14]. Results of our previous investigation showed that 1-ferrocenyl-3-(4-methylsulfonylphenyl)propen-1-one (FMSP, Fig. 1), which was synthesized by our team, as a ferrocene derivative robustly decreased viability of tumor cells *in-vitro* [15]. Naringenin, as a natural polyphenolic flavonoid, has anti-proliferative and apoptosis-inducing properties in many cancer cell types [16–18]. In this study, the effects of FMSP and naringenin on CaM, PDE1, and the cAMP/PKA signaling pathway were investigated in order to reveal their mechanism of action in cell growth inhibition.

Material And Methods

Preparation of FMSP

According to our previously reported protocol, acetyl ferrocene reacted with 4-(methylsulfonyl)benzaldehyde in sodium hydroxide and ethanol to yield 1-ferrocenyl-3-(4-methylsulfonylphenyl)propen-1-one (FMSP) [19]. The synthesized compound was well characterized by elemental analysis, multinuclear (^1H) NMR, and spectroscopic methods such as IR and Mass.

Molecular modeling and docking studies

Docking studies were performed using AutoDock software version 4.0 to search for favorable binding configurations between the small flexible ligands and the rigid protein to predict their interactions. These studies were performed based on the high-resolution crystal structure of calmodulin receptor (PDB code: 1QIW) retrieved from RCSB Protein Data Bank. The co-crystallized ligand (DPD) and water molecules were removed from the protein, Kollman charges were added, nonpolar hydrogens were merged, and AutoDock 4 atom type was assigned to achieve the PDBQT format of the protein. The ligand structures (Naringenin and FMSP) were minimized using HyperChem8.0 (MM⁺ method) and then converted to PDBQT file format with AutoDock tools. A docking grid box was built with (30×30×30) surround the co-

crystallized ligand. The Lamarckian genetic search algorithm was employed, and the docking run was set to 100. Protein residues with atoms greater than 6.0 Å from the docking box were removed for efficiency. The quality of the docked structures was evaluated by measuring the intermolecular energy of the ligand–enzyme assembly.

Determination of kinetic parameters

We used ΔG (H₂O) as an appropriate parameter to calculate macromolecular stability of CaM protein in the presence of FMSP and naringenin. The free energy difference (ΔG) between the native and denatured forms of CaM was calculated by below formula [20]:

$$\Delta G = -RT \ln [F_d / (1-F_d)]$$

$$F_d = (Y_n - Y_{obs}) / (Y_n - Y_d) \quad (1)$$

$$\ln [(Y_n - Y_{obs}) / (Y_{obs} - Y_d)] = -RT \ln K$$

In this formula, R stands for gas constant, T represents the absolute temperature, Y_{obs} indicates fluorescence emission intensity of CaM at different concentrations of FMSP and naringenin, and Y_n and Y_d stand for values of native and denatured states, respectively. The changes in ΔG values were plotted against FMSP and naringenin concentrations, which showed a linear relation based on the following equation [20]:

$$\Delta G = \Delta G (H_2O) - m [D] \quad (2)$$

In the above mentioned formula, $\Delta G (H_2O)$ stands for ΔG at zero concentration of ligand, m is the constant for the dependence of ΔG on ligand concentration, and [D] is the ligand concentration.

Lineweaver-burk plots were created by kinetic analysis of CaM-dependent PDE inhibition in the presence of FMSP and naringenin. Thereafter, in the presence and absence of these two inhibitors, K_m values calculated from these plots. According to the plots, maximum velocity (V_{max}) of the activated PDE1 in the presence and absence of inhibitors is the point at which the lines crossed the y-axis.

Cell culture

The K562 human erythroleukemic cell line was provided by Pasteur Institute, Tehran, Iran. Subsequently, the cancer cells were cultured in RPMI 1640 culture medium (Thermo Fisher Scientific, USA), which were supplemented with 10% FBS (Gibco, Germany), 100 unit/ml penicillin/100 mg/ml streptomycin (Thermo Fisher Scientific, USA), and 1% L-glutamine (Gibco, Germany). The cultured cells were incubated in 5% CO₂ at 37°C conditions.

Cell viability analysis

Cell viability estimation was carried out using 3-[4, 5- methylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide (MTT) assay. In brief, the cultured cells were trypsinized and collected to be seeded in 96-well tissue

culture plates (5000 cells/well) overnight. Then, the cells were treated with various concentrations of FMSP (0–5 μ M) and naringenin (50 μ M) for 48h. Subsequently, a solution containing 10% MTT was prepared using non-complete culture medium and was added to the well for 4 h. After 4-h incubation, MTT-contained medium was replaced by 100 μ L Dimethyl sulfoxide (DMSO) (Sigma-Aldrich Company, USA) to solve the produced MTT crystals. Finally, the absorbance of the solution was read by using an ELISA reader at 570 nm and the cell viabilities were determined as the percentage of untreated control cells.

Quantitative determination of cAMP concentration

The concentration of cAMP molecules within the cells was measured by an enzyme immunoassay kit (Stratagene, La Jolla, CA). Briefly, after treating the K562 cells with the mentioned concentrations of FMSP and naringenin in treatment group and culture medium in controls, about 2×10^5 cells were collected and centrifuged at 1200 rpm and 4°C for 5 min. Cell pellets resuspended by the lysis buffer provided in the kit. According to the kit protocols, cAMP concentration was instantly measured in lysed cells. This method was principally based on the competition between unlabeled cAMP and stable quantity of peroxidase-labeled cAMP for the limited binding sites on an antibody against cAMP molecule.

Measurement of PKA activity

In this present study, a colorimetric assay kit (BioVision, USA) was used for determination of PKA enzyme activity. Briefly, the treated K562 cells were washed with Phosphate-buffered saline (PBS) buffer (pH = 7.4) twice, and the resuspended by lysis buffer (50mMTris–HCl, 2.5mMEDTA, 1mMMgCl₂, 10mMNaF, 10%glycerol, pH 7.2) and sonicated followed by centrifugation at 1200 rpm and 4°C for 5 min. Finally, the activity of PKA enzyme was measured according to the instructions that were provided by the manufacturer.

Effects of PKA blocking on cell growth inhibition

Rp-cAMP (Adenosine 3,5-cyclic monophosphorothioate triethyammonium salt hydrate) was used as a specific inhibitor of PKA (Biolog Life Science Institute, Bremen, Germany). This molecular inhibitor was then diluted by using RPMI culture medium to prepare 100 μ M Rp-cAMP. Briefly, the cultured cells were first incubated with Rp-cAMP (100 μ M) for 20 min and then treated with FMSP (0–5 μ M) and its combination with naringenin (50 μ M).

Statistical analysis

To assess the normality of data and homogeneity of variances, by K–S and Levene's statistical tests were used, respectively. Then, one-way analysis of variance (ANOVA) or Mann–Whitney nonparametric tests were applied to analyze the results of the study. The statistical significance of the obtained results was considered at $p < 0.05$ level between the treated and control groups. Data were analyzed by using SPSS software version 22, and results were indicated as the mean $\pm$ standard error (mean $\pm$ SE).

Results

Molecular modeling and docking studies

The calmodulin receptor pocket was found to be composed of two subunits (A and B) occupied by the co-crystallized inhibitor (DPD). The docking analysis was performed on subunit B of calmodulin.

As shown in Figs. 2A, B, and C, docking results showed that naringenin and FMSP structures exactly fit into the same hydrophobic pocket of calmodulin active site containing Phe92, Met124, Met144, and Met145 as is occupied by the co-crystallized ligand. As specified in the figures, in the docking of naringenin, OH groups form hydrogen binds to Glu11 side chain carboxylic acid and Ile100 oxygen atom. The FMSP is perfectly superimposed on the naringenin structure. This molecule participates in hydrophobic interaction with hydrophobic residues of Phe12, Phe92, Met144 and Met145 and also π - π interactions with aromatic rings of Phe12 and Phe92.

Interaction of FMSP and naringenin with calmodulin

Alteration of fluorescence emission spectra of CaM illustrated the interaction of FMSP and naringenin ligands with CaM as well as drug-induced conformational change in this protein. These data represents inhibitory effects of these two compounds on CaM (Fig. 3A).

Effect of FMSP and naringenin on the activity of PDE-1

The activity of PDE-1 enzyme was measured by increasing concentrations of FMSP, naringenin, and their combination, respectively. This method is based on the hydrolysis of cAMP to AMP, which was quantified by using spectrophotometric technique. Finally, the activity of PDE-1 was calculated and plotted versus different concentrations of FMSP and naringenin (Fig. 3B).

Kinetic analysis of PDE1 activity and inhibition of PDE1 by FMSP and naringenin

Kinetic analysis of PDE-1 enzyme was calculated in the presence and absence of FMSP and naringenin. In the absence of both compounds, the K_m value of PDE1 was $0.05 \pm 0.001 \mu\text{M}$; but adding FMSP and naringenin alone and their combination together increased the K_m values to 1, 0.1 and 3 μM , respectively. These data indicated that the presence of FMSP and naringenin increases the K_m values in a concentration-dependent manner. Moreover, FMSP and naringenin increased K_m values with no apparent impact on V_{max} values. The efficacy order of the combination of FMSP and naringenin, FMSP, and naringenin in increasing K_m values is as follow: FMSP + Naringenin > FMSP > Naringenin. According to the lineweaver-burk plots, either FMSP or naringenin or in combination together competitively inhibit PDE1 enzyme (Fig. 4A). To show the effectiveness of an inhibitor K_i constant is used. This parameter indicates the affinity of the inhibitor to a specific enzyme. We calculated the K_i values of our enzyme-inhibitor reactions by Dickson plot (Figs. 4B,C, and D). The determined K_i constants for FMSP and naringenin and their combination were 5, 10, and 0.5 respectively.

The effect of FMSP and naringenin on calmodulin stability

The stability of CaM protein was estimated in the presence of FMSP, Naringenin and the combination of these two agents separately. The CaM free energy changes (ΔG) for complexes of CaM with FMSP and naringenin alone and their combination are shown in Fig. 5. According to the linear plots, the estimated ΔG (H2O) in the presence of FMSP, naringenin, and the combination of FMSP and naringenin was 1.8, 2.4, and 0.2 kcal mol⁻¹, while this value was 7.1 kcal mol⁻¹ in absence of these two compounds (Table 1).

Table 1
Calmodulin stability parameters in the presence of Naringenin and FMSP

Ligand	Concentration(μ M)	ΔG (H2O) (kCal/mol)	m(kCal/mol/ μ M)	D1/2 (μ M)	R2
Calmodulin	5	7.1	0.101	69.7	0.99
Naringenin	50	2.4	0.042	55.3	0.99
FMSP	5	1.8	0.035	51.4	0.99
FMSP + Naringenin	50 + 5	0.2	0.0151	13.17	0.99

K562 cell viability assay following treatment with FMSP and naringenin

To examine the effect of FMSP alone and its combination with naringenin on the proliferation of K562 cells, different doses of FMSP (0–5 μ M) with a single dose of naringenin (50 μ M) were studied by using MTT assay. Results showed that FMSP alone significantly ($p < 0.05$) reduced the proliferation of these cancer cells in doses more than 2 μ M. Moreover, the combination of FMSP with naringenin with the mentioned doses also meaningfully ($p < 0.05$) diminished the viability of K562 cells in comparison to untreated control cells. Interestingly, the combination of FMSP and naringenin was more effective than FMSP alone in hindering proliferation of human K562 cells (Fig. 6A).

The effect of FMSP alone and its combination with naringenin on intracellular levels of cAMP and PKA activity in K562 cells

Enzyme immunoassay method was used to evaluate the effects of FMSP (0–5 μ M) alone and its combination with naringenin (50 μ M) on cAMP concentration and PKA activity within K562 cell line. Treatment of K562 cells with FMSP and naringenin considerably augmented the levels of intracellular cAMP in these cancer cells (Fig. 6B and C). Furthermore, the exposure of K562 cells to both FMSP alone and its combination with naringenin showed a significant increase in PKA activity compared with control group (Fig. 6D). Interestingly, the activity PKA and the levels of cAMP in combination treatment group were non-significantly higher than the FMSP-treated group.

Activation of PKA by cAMP is required for cell growth inhibition by FMSP alone and its combination with naringenin

To evaluate whether the inhibitory effects of FMSP alone and its combination with naringenin on the viability of K562 cells is mediated by PDE inhibition and the consequent elevation of cAMP and PKA activation, the cancer cells were pretreated with Rp-cAMP as a specific inhibitor of PKA for 20min. Then, the cells were treated with FMSP (0–5 μ M) alone and its combination with naringenin (50 μ M) and MTT assay was used to measure the viability of the cells. As indicated in the Fig. 6B, pretreatment of K562 cell line with the mentioned doses of the inhibitor and treatment of them with two drugs significantly decreased the viability of these cancer cells in comparison to controls. This suggests that the cAMP-dependent activation of PKA is necessary to inhibit the cell proliferation due to FMSP and naringenin treatments.

Discussion

The CaM protein has been reported to regulate different cellular processes including proliferation, apoptosis, autophagy, migration, metastasis, metabolic homeostasis, and expression of various genes [21]. As mentioned before, CaM binds PDE1 to activate this enzyme to degrade intracellular cAMP molecule [10]. PDE1 is involved in the regulation of proliferation and apoptosis in cancer cells [22]. Consequently, emerging importance of CaM antagonists in the treatment of cancer patients has been shown many years ago [23]. Additionally, PDE inhibitors have been developed for the treatment of different diseases [24]. Furthermore, it has been revealed that PDE1 inhibitors are able to kill tumor cells selectively without affecting normal cells [25]. Based on our previous study, FMSP hindered the expression of stemness markers and decreased viability of breast cancer cells by reducing proliferation and inducing apoptosis in these cells [15]. Naringenin has been reported to exert anti-cancer effects on different cells via proliferation inhibition and apoptosis induction [26, 27].

In this study, the first step was carried out to examine the effects of FMSP and naringenin on CaM structure. Our results showed that the combination of FMSP and naringenin efficiently induced conformational alterations on CaM protein and increased its fluorescence emission. As shown in Fig. 3A, inhibitory activity of these two compounds and their combination against CaM is as follows: FMSP + Naringenin > FMSP > Naringenin. The second step of this study was done to assess the impacts of CaM activation on PDE1 activity in the presence of FMSP and naringenin, and FMSP + naringenin compared with untreated control. It was revealed that the combination of FMSP and naringenin more significantly inhibited PDE1 activity than FMSP and naringenin alone. As indicated in Fig. 4A, the K_i constant for FMSP + naringenin was considerably higher than those of FMSP and naringenin alone. Combination of the two compounds synergistically incremented K_m value of PDE1 without any changes in V_{max} parameter. These results indicate that these agents competitively inhibit PDE activity.

The data of ΔG (H₂O) values presented in Fig. 3 are consistent with findings that were mentioned above. The decrease in CaM stability could be attributed to the reduction of ΔG (H₂O) values due to its interaction with the mentioned FMSP and naringenin. Combination of FMSP and naringenin more remarkably decreased ΔG (H₂O) values than FMSP and naringenin alone, indicating that the combined drugs more efficiently decreased CaM stability when compared with single compounds.

In the third step, the effects of FMSP and naringenin on the viability of K562 cell line were evaluated *in-vitro*. The results showed that the combination of FMSP and naringenin more significantly declined the proliferation of K562 cells in comparison to FMSP and naringenin alone (Fig. 6A). We also unraveled that the combination of FMSP and naringenin had more significant effect on the induction of PKA activity and elevation of cAMP levels compared with FMSP alone (Fig. 6B and C).

To evaluate the dependence of the inhibition of cell growth by FMSP and naringenin on PKA activation, effects of these agents on PKA activity were examined. It was revealed that either the combination of FMSP and naringenin or FMSP alone enhanced the activity of PKA (Fig. 6D). In the last step, to understand whether PKA has a role in growth-inhibitory activity of these compounds, K562 cells were pre-incubated with PKA inhibitor (Rp-cAMP) and then treated with FMSP + naringenin and FMSP alone. Our findings revealed that in the presence of Rp-cAMP, inhibitory effects of FMSP + naringenin and FMSP alone on K562 cells was mediated by the accumulation of cAMP and activation of PKA.

Conclusion

Taken together, this study indicated that FMSP is a more potent antagonist of CaM than naringenin. Naringenin could enhance the antagonizing effects of FMSP on CaM protein. By antagonizing CaM, these compounds inhibit PDE1 and consequently increase cAMP levels and PKA activity. Combination of FMSP and naringenin and FMSP alone, reduce K562 cells proliferation in a dose-dependent manner possibly via cAMP/PKA pathway by CaM and PDE1 inhibition. Since both FMSP and naringenin are able to hamper viability of cancer cells, they can be promising anti-proliferative and also pro-apoptotic agents and this necessitate the requirement to investigate their potential anticancer affects in the future researches.

Declarations

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Conflicts of interest: There is no conflict of ontereest to declare.

Availability of data and material: Our data is available upon any request from the corresponding author.

Author Contributions: Shokoofe Noori and Afshin Zarghi designed the experiment and research methodology. Mahsa Azami Movahed and Shabnam Farzaneh performed the experiments, while Sadegh Rajabi and Mohammad Reza Ashrafi were involved in the data analysis and manuscript write-up.

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Figures

Figure 1

Structure of FMSP

Figure 2

Results of docking analysis of FMSP (A), naringenin (B) and superimposition of FMSP on naringenin (C) in calmodulin active site

Figure 3

(A) Calmodulin (CaM) fluorescence spectra at 0.043 mg/ml concentration in 40 mM Tris-HCl buffer, pH 7.4 at 25 °C. Elevation of calmodulin concentration in the free form and in the presence of 10 μM FMSP, naringenin and their combination caused an increase in fluorescence emission of CaM. (B) Calmodulin-dependent activity of PDE1 against different concentrations of FMSP, naringenin, and their combination

Figure 4

(A) Lineweaver-burk plot for the kinetic analysis of PDE1 activity and the mode of inhibition of the enzyme by FMSP and naringenin. Inhibition was performed with 5 μM FMSP and 50 μM naringenin and their combination in different concentrations of cAMP (calmodulin concentration in all the reactions was constant, 100 μM). (B), (C) and (D) Dixon plots: Inhibition was performed with varying concentrations of both compounds and their combination

Figure 5

The changes in the free energy (ΔG) of calmodulin against different concentrations of FMSP, naringenin and their combination

Figure 6

(A) Cytotoxicity impacts of FMSP and its combination with naringenin on K562 cell line. Effects of increasing concentrations of FMSP and its combination with naringenin on the viability of K562 cells were achieved by using MTT assay. Bars indicate the mean $\pm$ SEM of the treatments that were carried out in triplicate. P-values less than 0.05 were indicative of significant differences compared with control group. (B) Intracellular levels of cAMP in K562 cells which were treated with different concentrations of FMSP and its combination with naringenin. (C) Determination of PKA activity in K562 cells by colorimetric assay kit. (D) Effect of the pretreatment of K562 cells with Rp-cAMP, as a specific inhibitor of PKA, in the presence of FMSP alone and its combination with naringenin