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Simvastatin interacts synergistically with tipifarnib to induce apoptosis in leukemia cells through the disruption of RAS membrane localization and ERK pathway inhibition

Tamer A Ahmed^a, John Hayslip^{b,c}, and Markos Leggas^{a,b}

^aDepartment of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536, USA

^bMarkey Cancer Center, University of Kentucky, Lexington, Kentucky 40536, USA

^cDivision of Hematology and Blood and Marrow Transplantation, University of Kentucky, Lexington, Kentucky 40536, USA

Abstract

Tipifarnib, a farnesyltransferase inhibitor (FTI), was initially designed to disrupt RAS farnesylation and membrane localization necessary for RAS function. However, alternative geranylgeranylation has been postulated as an escape mechanism by which RAS bypasses the effect of FTI treatment. In this study, we demonstrate that simvastatin, an HMG-CoA reductase inhibitor, augments the cytotoxic effect of tipifarnib by blocking the alternative geranylgeranylation of RAS. Notably, this effect was accompanied by disruption of RAS membrane localization and ERK downregulation. In addition, the apoptotic effect of this combination was associated with downregulation of the antiapoptotic Mcl-1 protein and activation of the caspase cascade. These findings demonstrate that combining tipifarnib and simvastatin was successful in targeting RAS/ERK signaling and inducing apoptosis in leukemia cells. Both simvastatin and tipifarnib were used at clinically achievable doses, which make the combination promising for future clinical studies.

Keywords

Tipifarnib; Simvastatin; RAS/ERK; Leukemia

1. Introduction

The RAS/MEK/ERK signaling pathway encompasses several proteins that play key roles in cell proliferation as well as in the prevention of apoptosis of leukemic cells [1]. Aberrant regulation of this pathway is observed in leukemia because of RAS mutations, which lead to

Corresponding author: Markos Leggas, Ph.D., Associate Professor, Department of Pharmaceutical Sciences, University of Kentucky, 789 South Limestone Avenue, Room 323, Lexington, KY 40536-0596, Phone: (859)257-2633, Fax: (859)257-7564, mark.leggas@uky.edu.

CONFLICT OF INTEREST

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its constitutive activation, as well as genetic alteration of upstream signaling molecules of the RAS [2, 3]. Pharmacologic intervention to attenuate this pathway is thus a potential therapeutic strategy for leukemia treatment. However, it has been difficult to identify molecules that directly inhibit the function of RAS, and alternative approaches to prevent or block the membrane localization of RAS have been tried as a way to pharmacologically limit the activation of this pathway [4, 5].

RAS is a small GTP-binding protein that functions as a molecular switch regulating several signaling pathways that play a crucial role in controlling the activity of cell proliferation, differentiation and malignant transformation [6–9]. RAS activation requires a series of posttranslational modifications to allow its association with the inner face of the cell membrane, where it can interact with membrane receptors and activate downstream signaling cascades [4]. The first and most crucial step in RAS posttranslational modification is the covalent attachment of the farnesyl moiety into RAS carboxyl terminal in a process called farnesylation and is catalyzed by the farnesyltransferase (FTase) enzyme [4].

FTase inhibition was proposed as a strategy to impede RAS localization to the membrane and its subsequent activation. Therefore, several farnesyltransferase inhibitors (FTIs) were developed and evaluated in preclinical and clinical settings against a variety of human cancers. The preclinical evaluation of FTIs in cell culture and animal models has shown promising results as potential therapeutic agents and several FTIs progressed into clinical trials [10–12]. However, the efficacy of FTIs as a single agent in patients with solid tumors was limited, but some modest efficacy was observed in hematologic malignances [13, 14]. Resistance to FTIs has been attributed to posttranslational modification of RAS by an alternative lipidation pathway, whereby RAS can be geranylgeranylated by geranylgeranyltransferase I (GGTase I) in the presence of FTIs [15]. This alternative isoprenylation mechanism enables RAS to associate with the cell membrane and retain full biologic activity despite of the blockage of the farnesylation pathway.

Understanding the mechanism by which RAS escapes the effect of FTIs tempted the investigators to change their strategy by targeting both prenylation pathways in order to avoid the cross-geranylgeranylation of RAS and knockdown its activity. Therefore, considerable effort has been made to evaluate the FTIs with geranylgeranyltransferase inhibitors (GGTI) in combination. Although several studies have demonstrated synergistic cytotoxicity and apoptotic activity of FTI/GGTI combinations in different tumor types, significant toxicity was reported in preclinical models, which is mostly related to GGTI, thereby limiting the therapeutic potential of this combination [16, 17]. Recently, GGTI-2418, a novel geranylgeranyltransferase inhibitor, was found to be well tolerated with minimal side-effects in a phase I trial in patients with refractory solid tumors [18].

Simvastatin, an anti-hyperlipidemic drug that inhibits 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase, has been shown in several studies to induce apoptosis in cancer cells through blockade of the geranylgeranylation pathway of small GTPases [19–21]. Unlike GGTIs, statins are known to be well tolerated and have a wide margin of safety. Given the ability of simvastatin to inhibit the alternative pathway of RAS prenylation as well

as its good safety profile, we postulated that simvastatin could overcome tipifarnib resistance and augment its antitumor activity in leukemia cells.

2. Methods

2.1. Chemicals

Simvastatin was purchased from Toronto Research Chemicals Inc. (North York, Canada). Tipifarnib was purchased from Selleck Chemicals LLC (Houston, TX, USA). Absolute ethanol was obtained from Sigma-Aldrich (St Louis, MO, USA). RPMI-1640 medium and penicillin/streptomycin were from Life Technologies (Grand Island, NY, USA), whereas fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA, USA). Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit and NuPage LDS sample buffer were from Invitrogen (Eugene, OR, USA). Antibodies for PARP (#9542), cleaved caspases 3 (#9664), 7 (#9491) and 9 (#9505) and procaspases 3 (#9665), 7 (#9492) and 9 (#9508), Bcl-2 (#2876), Mcl-1 (#4572), Bcl-xL (#2762), Bax (#2772), phospho ERK (#4376), total ERK (#4695), phospho AKT (#9271), total AKT (#9272), RAS (#3339), calnexin (#2433) and GAPDH (#2118) were purchased from Cell Signaling (Danvers, MA, USA). Mevalonate, farnesyl pyrophosphate and geranylgeranyl pyrophosphate were from Sigma-Aldrich (St Louis, MO, USA). Propidium iodide was obtained from (Invitrogen Corporation, Carlsbad, CA, USA). Ribonuclease A from bovine pancreas, resazurin and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St Louis, MO, USA). BCA protein assay was from Thermo Scientific (Rockford, IL, USA) whereas Complete protease inhibitor cocktail was obtained from Roche Diagnostics (Indianapolis, IN, USA).

2.2. Cell culture

All cell lines (KG1 and HL60 acute myelogenous leukemia; K562, chronic myelogenous leukemia; Jurkat, Molt4 and HSB2, acute T cell leukemia) were purchased from ATCC (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium supplemented with FBS, penicillin (100 IU/mL) and streptomycin (100 µg/mL) at 37°C in a humidified incubator with 5% CO₂. FBS was added to medium as follows: 20 % for KG1 and HL60 cells, 10 % for Jurkat, Molt4 and HSB2 cells or 5% for K562 cells.

Cells were suspended in growth medium at 5×10^5 cells/mL for KG1, HL60 and HSB2 and 2.5×10^5 cells/mL for K562, Molt4 and Jurkat and placed in 6- well plates.

2.3. Drug treatment

For cell viability assay, cells were incubated with increasing concentrations of simvastatin (0, 0.4 – 400 µM), tipifarnib (0, 0.01 – 10 µM) or their combination at different concentrations for 72 hr at 37°C. Cells incubated with 10 µL/mL DMSO were used as a control. For all other experiments, cells were treated with simvastatin (1 or 4 µM); tipifarnib (0.1 or 1 µM) or their combinations for 24 or 72 hr. Cells incubated with 10 µL/mL DMSO were used as a control. The tipifarnib and simvastatin concentrations that were used in combination studies were chosen based on clinical evidence suggesting that they could be achieved in the clinic [22, 23].

2.4. Cell viability assay and combination index calculation

A panel of six cell lines of varied leukemic origin including KG1, HL60, K562, Jurkat, Molt4 and HSB2 was used to determine the cytotoxicity of simvastatin and tipifarnib following single drug or combination treatment. Cells were placed in 96 well plates at a density of 50×10^3 cells per well for KG1, HL60 and HSB2 or 25×10^3 cells per well for K562, Jurkat and Molt4 in 100 μ L of the appropriate growth medium. At the end of the incubation period, 10 μ L of resazurin was added to each well and further incubated for 3 hr at 37°C. Cell viability was determined by measuring the fluorescence at 560 nm excitation wavelength and 590 nm emission wavelength using Molecular Devices Spectramax M5 plate reader. Cell viability data were normalized to their corresponding untreated controls and were expressed as a fractional effect (FE), the fraction of unviable cells. Synergism between simvastatin and tipifarnib was assessed using the combination index (CI) method of Chou and Talalay [24, 25].

$$CI = d_1/D_1 + d_2/D_2$$

In this equation, D_1 and D_2 represent the doses of drug 1 and drug 2 alone, required to produce x% effect, and d_1 and d_2 are the doses of drugs 1 and 2 in combination required to produce the same effect. CI value < 1 indicates synergy while values = 1 or > 1 indicate additivity and antagonism, respectively. Experiments were performed in triplicates.

2.5. Apoptosis Assay

Cells were harvested and washed with ice cold phosphate-buffered saline (PBS) buffer, pH 7.2. Cell pellets were resuspended (1×10^6 cells/mL) in annexin binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM CaCl_2 , pH 7.4). To identify dead and apoptotic cells, 1 μ L propidium iodide (PI) and 5 μ L Annexin V-FITC were added to each 100 μ L of cell suspension and samples were incubated for 15 min at room temperature. Samples were diluted to 500 μ L using annexin binding buffer before analysis using FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Cells that were positive for Annexin-V but negative for PI were those in early stage apoptosis while cells positive for both annexin-V and PI were in late stage apoptosis.

2.6. Cell cycle analysis

Cells were harvested and washed twice with ice cold PBS buffer. Cells were then fixed in 3 mL of absolute ethanol overnight at 4°C. Cells were washed twice with ice cold PBS buffer and incubated with propidium iodide (100 μ g/mL) and Ribonuclease A from bovine pancreas (200 μ g/mL) in the dark for 30 min at 37°C. Processed samples were kept at 4°C and protected from light until analysis using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

2.7. Total and fractionated protein isolation

For total lysate preparation, cells were harvested after treatment and washed twice with ice cold PBS. Cell pellets were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1%

Nonidet P-40, 0.5% deoxycholate, 1 mM EDTA, 0.1% SDS) supplemented with Complete protease inhibitor cocktail and incubated for 30 min (4°C) on a rotating shaker. Cell lysates were then centrifuged at 1500 rpm for 2 min to remove any particulates. Protein concentrations of membrane, cytosolic fractions and total cell lysate were measured using the BCA protein assay.

For cytosolic and membrane protein fractions, cells were collected after treatment and washed with ice cold PBS buffer. Cells (1×10^7) were lysed via sonication for 15s in 200 μ L lysis buffer I (1 mM EDTA, 20 mM Tris-HCl (pH 7.4)) supplemented with Complete protease inhibitor cocktail. Lysates were centrifuged at $100,000 \times g$ at 4°C for 1 hr. The supernatant containing the soluble fraction (cytosolic fraction) was collected and the pellet (membrane fraction) was then washed with 1 mL lysis buffer I twice, to remove any remnant of the cytosolic fraction. The membrane pellet was solubilized in 50 μ L lysis buffer II (150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 1% Triton X-100) supplemented with Complete protease inhibitor cocktail and then sonicated for 5s to solubilize any particulate left in the buffer. Protein samples were stored at -20°C until analysis or were processed immediately for immunoblotting.

2.8. Western blot analysis

The expression of total-PARP, cleaved PARP, cleaved caspases 3, 7 and 9 and procaspases 3, 7 and 9, Bcl-2, Mcl-1, Bcl-xL, Bax, phospho ERK, total ERK, phospho AKT, total AKT, RAS, calnexin and GAPDH was evaluated in protein lysates or subcellular fractions, as indicated. Protein samples were boiled at 95°C for 10 min with NuPage LDS sample buffer and 0.1 M DTT. Protein electrophoresis was performed on 10% SDS-polyacrylamide gels (SDS-PAGE) at room temperature and proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) at 4°C. Membranes were blocked for 2 hr at room temperature with tris-buffered saline (TBST) containing 0.05% (v/v) Tween-20 and supplemented with 5% (w/v) nonfat milk or bovine serum albumin. Membranes were incubated with primary antibodies overnight at 4°C at 1:1000 dilution. After washing with TBST, the membranes were probed with HRP- conjugated secondary antibody at 1:2000 dilution (Cell Signaling Technology Inc.) for 1 hr at room temperature. Following washes with TBST, protein bands were visualized by enhanced ECL (Thermo Fisher Scientific, Rockford, IL) using the Kodak Image Station 2000 MM (Eastman Kodak, Rochester, NY).

2.9. Statistical analysis

Data are reported as mean values $\pm$ SD and analyzed statistically with one-way ANOVA followed by Turkey's post-hoc test. P values $\leq$ 0.05 were considered significant.

3. Results

3.1. Simvastatin and tipifarnib combination has a synergistic antiproliferative effect in leukemia cell lines

To evaluate the potential for synergy between simvastatin and tipifarnib we treated KG1, HL60, K562, Jurkat, Molt4 and HSB2 cell lines with either drug alone, to determine the respective IC₅₀ (Supplementary Fig. 1), and then with different concentrations of simvastatin

(1 and 4 μM) and tipifarnib (0.05, 0.1, 0.5 and 1 μM) in combination. Cell viability was measured after 72 hr of treatment using a fluorometric cell proliferation assay as described under methods. Synergism was assessed by calculating CI values, which is based on the mathematical model described by the Chou and Talalay [24, 25]. As shown in Table 1, with the exception of the KG1 cells the combination of simvastatin and tipifarnib was synergistic at all concentrations. Overall, simvastatin at high dose yielded higher fractional effect (FE) in combination with tipifarnib, relative to its lower dose. This effect was more substantial in HL60 cells for both CI and FE values. These results indicate that the combination of simvastatin and tipifarnib had a synergistic cytotoxic effect in various leukemia cell lines.

3.2. The synergistic effects of simvastatin/tipifarnib are mediated by apoptosis

To determine if the combination of simvastatin/tipifarnib was cytotoxic, we investigated whether the reduced cell viability was attributed to apoptosis. Leukemia cells were treated with simvastatin (1 or 4 μM) and tipifarnib (0.1 or 1 μM) concentrations alone or in combination for 72 hr. Subsequently, western blot analysis was performed to analyze the activation of the caspase cascade. As shown in Fig. 1A, proteolytic cleavage of caspase 3 and 7 to their active forms triggered the apoptotic process through the cleavage of other important intracellular substrates such as poly (ADPribose) polymerase (PARP), which is involved in DNA repair. These results were consistent in all leukemia cell lines tested. However, caspase 7 was only activated in Jurkat, Molt4 and HSB2 cells. Moreover, we examined caspase 9 activation (cleavage of caspase 9), which acts upstream of caspase 3 and 7, using western blot analysis. Elevated expression of cleaved caspase 9 was observed in K562, Jurkat, Molt4 and HSB2 cells. HL 60 cells showed no expression of both the full length and cleaved forms of caspase 7 and 9 and PARP. Conversely, KG1 cells were more resistant to simvastatin/tipifarnib with no signs of caspase cascade activation or PARP cleavage.

In fact, several reports suggest the importance of the antiapoptotic and proapoptotic Bcl-2 family proteins in regulating cell survival and apoptosis [26–28]. To further understand the apoptotic effect of simvastatin/tipifarnib, we also examined the expression of Bcl-2 family proteins in leukemia cells treated with simvastatin and tipifarnib alone or in combination for 72hr using western blot analysis. As shown in Fig. 1B, combined treatment of simvastatin and tipifarnib did not substantially alter the expression of either antiapoptotic (e.g., Bcl-2 and Bcl-xL) or proapoptotic (e.g., Bax) proteins. However, simvastatin/tipifarnib resulted in a significant reduction on the expression of the antiapoptotic Mcl-1 protein in all leukemia cells except KG1.

To further confirm the apoptosis inducing effect of the simvastatin/tipifarnib combination, Annexin V-FITC and PI analysis was performed on a subset of leukemia cells. The HL60 and Jurkat cells were treated with simvastatin (1 or 4 μM) and tipifarnib (0.1 or 1 μM) alone or in combination. As shown in Fig. 2A and Fig. 2B, combined exposure to simvastatin and tipifarnib led to a significant increase in early (AnnexinV+/PI-) and late (Annexin V+/PI+) apoptosis in both cell lines, compared to untreated control and single drug treatments.

Together these findings indicate that the synergistic interaction between simvastatin and tipifarnib in human leukemia cells is mediated by apoptosis. Also, KG1 was shown to be more resistant to this combination than other leukemia types.

3.3. Synergistic cytotoxicity of simvastatin/tipifarnib in leukemia cells is not associated with cell cycle arrest

Since the cell proliferation assay cannot distinguish between apoptotic and arrested cells, we examined whether cell cycle arrest is contributing to the decrease in cell viability following simvastatin/tipifarnib exposure. Leukemia cells including KG1, HL60, K562 and HSB2 were treated with simvastatin (4 μ M) and tipifarnib (1 μ M) alone and in combination for 72hr. After treatment, cell cycle distribution was assessed using flow cytometry. The distribution of the cell cycle phases (G1, S and G2/M) showed no significant changes after treatment relative to control in all leukemia cells tested (Fig. 3). These results suggest that the synergetic interaction of simvastatin/tipifarnib is cytotoxic and not cytostatic.

3.4. Co-Treatment of simvastatin and tipifarnib disrupts the localization of RAS in the cell membrane

[6–9][10–12][15][19–21] To evaluate whether coadministration of simvastatin and tipifarnib could disrupt the RAS prenylation process and its membrane association, leukemia cells were treated with simvastatin (4 μ M) and tipifarnib (1 μ M) alone or in combination for 72 hr and cells subjected to a fractionation procedure to isolate the membrane and the cytosolic protein fractions. Western blot analysis was performed to determine RAS location in both fractions. Interestingly, simvastatin/tipifarnib was found to inhibit the membrane association of RAS with its subsequent sequestration into the cytosol, Fig. 4. This effect was not observed in the control cells or those exposed to a single drug treatment. Overall, these findings suggest that simvastatin in the presence of tipifarnib disrupts membrane association of RAS and most likely results in loss of RAS function because of its inability to associate with membrane bound effectors.

3.5. Simvastatin/tipifarnib downregulates the ERK downstream signaling in human leukemia cell lines

It is well known that RAS activation is a crucial step for several cytoprotective and stress related signaling pathways. Therefore, we examined the effect of simvastatin/tipifarnib on the phosphorylation status (activity) of the two main signaling pathways downstream from RAS; ERK and AKT pathways. As shown in Fig. 5, a 24 hr treatment of simvastatin/tipifarnib significantly decreased the phosphorylation of ERK in four of the tested leukemia cell lines including HL60, K562, Molt4 and HSB2. Both KG1 and Jurkat cells showed no expression of the phosphorylated form of ERK. In addition, only Jurkat cells showed high levels of phosphorylated AKT. Interestingly, upon treatment with simvastatin alone or in combination the AKT phosphorylation was reduced. This result suggests that simvastatin/tipifarnib disrupt RAS membrane localization which attenuates ERK signaling..

3.6. Addition of mevalonate and isoprenoids prevents simvastatin/tipifarnib induced apoptosis and reverses the disrupted RAS isoprenylation

To further investigate whether blocking the isoprenylation routes is responsible for the apoptotic effect of simvastatin/tipifarnib, leukemia cells were treated with mevalonate and isoprenoids (FPP and GGPP) in the presence of simvastatin/tipifarnib. Annexin V apoptosis assay and western blot analysis for caspase 3 and Mcl-1 were employed to assess apoptosis. The Annexin V assay revealed that the apoptotic effect of simvastatin/tipifarnib was reversed by the addition of mevalonate, FPP or GGPP in HL60 cells (Fig. 6A and B). Similarly, western blot analysis, in Fig. 6C, indicated that caspase 3 activation (cleaved caspase 3) and Mcl-1 downregulation induced by simvastatin/tipifarnib, in both K562 and HSB2 cells, were reversed by the addition of mevalonate, FPP or GGPP. On the other hand, the effect of this combination on RAS disruption from the membrane was reversed by the addition of mevalonate and GGPP but only partially by FPP (Fig. 6D). These findings indicate that simvastatin/tipifarnib is mediating its apoptotic effect and RAS membrane disruption through the blocking of both the farnesylation and the geranylgeranylation pathways.

4. Discussion

Aberrant activation of the oncogenic RAS signal transduction is commonly observed in hematological malignancies. RAS mutations have been reported in 30% of leukemia, most frequently acute leukemia [2]. FTIs were initially developed to inhibit RAS activation through blocking its farnesylation process [10]. Despite the encouraging preclinical results, FTIs showed limited activity in clinical trials [13, 14]. This is thought to be due to the ability of RAS to get activated through the geranylgeranylation pathway, which acts alternatively to the farnesylation pathway once FTIs are administered [15]. This escape mechanism was only noticed in K-RAS and N-RAS isoforms, which are commonly mutated in solid tumors and leukemia, respectively [15]. Blocking this alternative prenylation pathway is an attractive strategy to evade the resistance to FTIs. Here we report that simvastatin was able to augment the cytotoxicity of tipifarnib in a panel of leukemia cells through blocking the alternative prenylation of RAS by the geranylgeranylation pathway.

In our studies, we employed AML, ALL and CML cells. With the exception of one AML cell line (KG1) the simvastatin/tipifarnib combination was synergistic in all other cell lines. The observed synergy following combination treatment was due to increased apoptosis. The balance between antiapoptotic (e.g. Bcl-2, Mcl-1 and Bcl-xL) and proapoptotic (e.g. Bax and Bad) proteins regulates the release of cytochrome c from the mitochondria into the cytosol, which will lead to the activation of the caspase cascade and the induction of apoptosis. Although our results show that the proapoptotic Bax expression was unchanged by treatment in all cell lines that expressed it, we demonstrate that the induced apoptosis was accompanied by the downregulation of Mcl-1 protein, which is an anti-apoptotic member of the Bcl-2 protein family [29, 30]. In addition, the observed decrease in Mcl-1 expression could be attributed to the loss of ERK signaling, since ERK signaling was previously reported to mediate stabilization of Mcl-1 protein through phosphorylation [31].

Results from both cell viability and apoptotic assays indicate that leukemia cells were differentially affected by the simvastatin/tipifarnib combination. The increased sensitivity to

this combination was more significant in HL60 compared to other leukemia cells tested whereas KG1 cells were more resistant with no signs of apoptosis. Heterogeneity in response was previously reported in a group of AML cell lines toward simvastatin or prenylation inhibitors [32] as well as in primary CD34⁺ AML cells cotreated with simvastatin and tipifarnib [33]. The differential sensitivity of AML cells toward simvastatin was attributed to the differences in interference with prenylation pathways [32]. This was in line with our finding that demonstrates the disruption of RAS isoprenylation in leukemia cells that are responsive to the simvastatin/tipifarnib cotreatment. However, the observed synergy appears to be independent of RAS mutations as both resistant (KG1) and sensitive (HL60, Molt4 and HSB2) cells harbor NRAS mutations [34–36].

In fact, RAS plays a central role in activating several downstream effectors that are known to regulate different cell functions including cell growth, survival and differentiation. Therefore, therapeutic targeting of RAS signaling has become a major endeavor in cancer therapy. Our results suggested that disrupting RAS membrane localization, by simvastatin/tipifarnib cotreatment, significantly decreased ERK phosphorylation in the cell lines tested. This finding is in line with previous work, which demonstrated the disruption of RAS/ERK signaling in multiple myeloma cells treated with FTI/lovastatin [37]. Furthermore, reversal of simvastatin/tipifarnib combination induced apoptosis by mevalonate, FPP and GGPP was notably consistent in the sensitive leukemia cells. This observation confirms that prenylation pathways are the cellular targets of this combination. Concordantly, RAS membrane disruption was also abrogated by the addition of mevalonate and GGPP and partially by FPP. In general, our results indicate that RAS/MEK/ERK pathway might be involved in simvastatin/tipifarnib induced cytotoxicity. That said, RAS as an exclusive target for this combination is still questionable. Several reports suggested that other elusive targets may be involved in mediating the antitumor effect of FTIs [38]. The wide pool of proteins that undergo prenylation makes it difficult to identify a true therapeutic target for simvastatin/tipifarnib. Accordingly, the synergistic interaction between simvastatin and tipifarnib could be attributed to the concurrent deactivation of the farnesylated and the geranylgeranylated proteins, hence other cellular signals may be involved. This might explain the enhanced apoptotic effect of simvastatin/tipifarnib combination observed in Jurkat cells relative to single treatments, despite the lack of pERK expression as well as the limited efficacy of tipifarnib in reducing pAKT levels when added to simvastatin. Nonetheless, the ability of GGPP to rescue the effect of simvastatin/tipifarnib combination suggests a potential role of the alternative geranylgeranylation as a mechanism of resistance to tipifarnib monotherapy.

The fact that resistant AML cell lines require higher doses of simvastatin relative to other leukemia cell lines in order to induce cytotoxicity and to disrupt RAS isoprenylation [32] may indicate the presence of efflux transporters, which could be potentially limiting the intracellular concentrations of the lactone and carboxylate species, respectively. However, neither the lactone nor the carboxylate form of simvastatin was found to be transported by Pgp, BCRP or MRP2 [39, 40], which are known to transport molecules with similar physicochemical properties. This does not preclude the presence of other transporters that may limit the accessibility of the simvastatin carboxylate form to the cancer cell. For example, MRP1 is an efflux transporter that is ubiquitously expressed and primarily transports anionic compounds[41].

The use of simvastatin as anticancer agent was limited by the high doses required to mediate its antitumor activity. However, simvastatin induces apoptosis in combination with tipifarnib at lower concentrations, as low as 1 μ M. It is interesting to note that simvastatin, given at maximum tolerated oral dose (7.5mg/kg, twice daily) to leukemia patients, was found to achieve plasma levels comparable to those used in our study [23]. In addition, recent study has demonstrated that simvastatin was able to inhibit geranylgeranylation pathway in primary AML cells at concentrations similar to those used in our study [42].

In summary, these results suggest that the synergistic cytotoxic effect of simvastatin/tipifarnib combination is, at least in part, due to the disruption of RAS/ERK signaling. Reversal of such effect by the addition of GGPP and FPP indicates that blocking both prenylation pathways is most likely the underlying mechanism of synergy between simvastatin and tipifarnib. These results are significant because the concentrations used in these experiments can be achieved by administration of intermittently dosed high dose simvastatin in the clinical setting. Hence, these results warrant further in vitro study. Ultimately, investigation of alternate formulations or delivery approaches of HMG-CoA reductase inhibitors should be considered to improve their tumor biodistribution, which will enable clinical studies that will prospectively evaluate this promising drug combination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Combined exposure of leukemia cells to simvastatin and tipifarnib induces apoptosis through caspase activation and downregulation of Mcl-1. Leukemia cells were treated for 72 hours with simvastatin and tipifarnib at concentrations indicated, either alone or in combination. At the end of the incubation period, cells were lysed and western blot analysis was performed to monitor the cleavage of caspases and PARP (A) and the expression of BCL-2 family proteins (B). GAPDH was used as a loading control to ensure equivalent loading. SIM, simvastatin. TIP, tipifarnib.

Fig. 2.

Apoptosis induced effect of simvastatin/tipifarnib measured by Annexin V/PI staining.

HL60 and Jurkat cells were treated with simvastatin (4 μM) and tipifarnib (1 μM) alone and in combination for 72hr. Cells were then costained with Annexin and PI with subsequent flow cytometric analysis. A representative dot-plot is shown for each condition (A).

AnnexinV+/PI- stained cells in the bottom right quadrant represent early apoptotic cells whereas late apoptotic or necrotic cells are located in the upper right quadrant with Annexin +/PI+ staining. In panel (B), representative figures of AnnexinV/PI staining of HL60 and Jurkat cells showing the sum of the percentages of early and late apoptotic cells. Results represent means of 3 independent experiments. *, $P > 0.05$, significantly greater than values for cells exposed to simvastatin or tipifarnib alone. SIM, simvastatin. TIP, tipifarnib.

Fig. 3. Simvastatin/tipifarnib does not induce cell cycle arrest in leukemia cells. Leukemia cells were treated for 72 hours with simvastatin (4 μ M) and tipifarnib (1 μ M), alone and in combination, before being stained with PI and analyzed by flow cytometry. Results represent means of 3 independent experiments. SIM, simvastatin. TIP, tipifarnib

Fig. 4. Simvastatin/tipifarnib alters subcellular localization of RAS in human leukemia cells. Leukemia cells were treated with simvastatin (4 μM) and tipifarnib (1 μM), alone and in combination for 72hrs. Cytosolic and membrane fractions were prepared and western blot analysis was performed as described in the method section using the indicated antibodies. Calnexin was used as a membrane marker, whereas GAPDH is a marker of the cytosolic fraction. SIM, simvastatin. TIP, tipifarnib.

Fig. 5.

Co-treatment of simvastatin and tipifarnib blocks ERK phosphorylation in human leukemia cells. Upon treatment with simvastatin (4 μ M) and tipifarnib (1 μ M), alone and in combination for 24hr, leukemia cells were processed for western blot analysis using the indicated antibodies. GAPDH was used as a loading control. SIM, simvastatin. TIP, tipifarnib.

Fig. 6. Depletion of prenyl substrates by simvastatin/tipifarnib is associated with apoptosis induction, caspase activation, Mcl-1 downregulation and RAS membrane disruption. HL60 cells were cotreated with simvastatin (4 μ M) and tipifarnib (1 μ M) in the presence of mevalonate (200 μ M), farnesyl pyrophosphate (10 μ M) and geranylgeranyl pyrophosphate (10 μ M). After 72 hour incubation, cells were harvested and costained with Annexin V/PI with subsequent flow cytometric analysis. A representative dot-plot and bar-figure were shown for each condition (A) and (B), respectively. Alternatively, K562 and HSB2 cells were treated similarly and then processed for western blot analysis to assess the expression of caspase 3 and Mcl-1 (C). GAPDH was used as a loading control. Under the same conditions, K562 and HSB2 cells were processed for western blot analysis to monitor the expression of RAS in both the cytosolic and membrane fractions (D). Calnexin was used as a membrane marker, whereas GAPDH is a marker of the cytosolic fraction. Results represent means of 3 independent experiments. *, $P > 0.05$, significantly lower than values for cells exposed to simvastatin-tipifarnib combination. SIM, simvastatin. TIP, tipifarnib.

Table 1

Combination index (CI) and fractional effect (FE) values (mean ($\pm$ SD)) of simvastatin/tipifarnib cotreatments in human leukemia cell lines.

Cell line Tipifarnib (μ M)/ Simvastatin (μ M)	KG1			HL60			K562			Jurkat			Molt4			HSB2		
	CI	FE		CI	FE		CI	FE		CI	FE		CI	FE		CI	FE	
0.05/1	0.586 ($\pm$ 0.246)	0.271 ($\pm$ 0.053)		0.264 ($\pm$ 0.085)	0.408 ($\pm$ 0.015)		0.267 ($\pm$ 0.159)	0.412 ($\pm$ 0.129)		0.367 ($\pm$ 0.151)	0.287 ($\pm$ 0.125)		0.263 ($\pm$ 0.114)	0.413 ($\pm$ 0.107)		0.413 ($\pm$ 0.313)	0.287 ($\pm$ 0.125)	
0.1/1	1.036 ($\pm$ 0.002)	0.270 ($\pm$ 0.101)		0.401 ($\pm$ 0.030)	0.418 ($\pm$ 0.003)		0.258 ($\pm$ 0.117)	0.474 ($\pm$ 0.085)		0.331 ($\pm$ 0.023)	0.317 ($\pm$ 0.026)		0.331 ($\pm$ 0.123)	0.384 ($\pm$ 0.123)		0.331 ($\pm$ 0.269)	0.317 ($\pm$ 0.026)	
0.5/1	1.414 ($\pm$ 0.254)	0.401 ($\pm$ 0.060)		0.564 ($\pm$ 0.184)	0.536 ($\pm$ 0.031)		0.422 ($\pm$ 0.166)	0.568 ($\pm$ 0.080)		0.561 ($\pm$ 0.064)	0.412 ($\pm$ 0.042)		0.427 ($\pm$ 0.083)	0.536 ($\pm$ 0.085)		0.484 ($\pm$ 0.289)	0.412 ($\pm$ 0.042)	
1/1	0.970 ($\pm$ 0.123)	0.494 ($\pm$ 0.050)		0.613 ($\pm$ 0.181)	0.609 ($\pm$ 0.059)		0.580 ($\pm$ 0.144)	0.596 ($\pm$ 0.047)		0.700 ($\pm$ 0.080)	0.492 ($\pm$ 0.073)		0.391 ($\pm$ 0.134)	0.692 ($\pm$ 0.113)		0.545 ($\pm$ 0.319)	0.492 ($\pm$ 0.073)	
0.05/4	1.051 ($\pm$ 0.298)	0.256 ($\pm$ 0.122)		0.185 ($\pm$ 0.043)	0.745 ($\pm$ 0.045)		0.436 ($\pm$ 0.222)	0.596 ($\pm$ 0.064)		0.606 ($\pm$ 0.268)	0.487 ($\pm$ 0.149)		0.520 ($\pm$ 0.251)	0.503 ($\pm$ 0.085)		0.401 ($\pm$ 0.257)	0.350 ($\pm$ 0.054)	
0.1/4	0.491 ($\pm$ 0.058)	0.395 ($\pm$ 0.065)		0.177 ($\pm$ 0.044)	0.771 ($\pm$ 0.052)		0.427 ($\pm$ 0.223)	0.626 ($\pm$ 0.059)		0.664 ($\pm$ 0.194)	0.578 ($\pm$ 0.149)		0.584 ($\pm$ 0.103)	0.492 ($\pm$ 0.031)		0.505 ($\pm$ 0.217)	0.364 ($\pm$ 0.049)	
0.5/4	0.677 ($\pm$ 0.016)	0.478 ($\pm$ 0.048)		0.190 ($\pm$ 0.037)	0.828 ($\pm$ 0.044)		0.508 ($\pm$ 0.160)	0.673 ($\pm$ 0.032)		0.640 ($\pm$ 0.227)	0.611 ($\pm$ 0.114)		0.519 ($\pm$ 0.182)	0.628 ($\pm$ 0.072)		0.441 ($\pm$ 0.297)	0.494 ($\pm$ 0.098)	
1/4	1.208 ($\pm$ 0.061)	0.517 ($\pm$ 0.006)		0.225 ($\pm$ 0.029)	0.854 ($\pm$ 0.040)		0.612 ($\pm$ 0.250)	0.698 ($\pm$ 0.040)		0.753 ($\pm$ 0.310)	0.646 ($\pm$ 0.091)		0.600 ($\pm$ 0.274)	0.680 ($\pm$ 0.111)		0.544 ($\pm$ 0.306)	0.534 ($\pm$ 0.142)	