

ORIGINAL ARTICLE: RESEARCH

Roles of ceramide synthase and ceramide clearence genes in nilotinib-induced cell death in chronic myeloidleukemia cells

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Abstract

In this study, we aimed to increase the sensitivity of human K562 and Meg-01 chronic myeloid leukemia (CML) cells to nilotinib by targeting bioactive sphingolipids, in addition to investigating the roles of ceramide metabolizing genes in nilotinib induced apoptosis. Cytotoxic effects of nilotinib, C8:ceramide, glucosyle ceramide synthase (GCS) and sphingosine kinase-1 (SK-1) inhibitors were determined by XTT cell proliferation assay and synergism between the agents was determined by isobologram analysis. Also, quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) results demonstrated that expression levels of longevity assurance (LASS) genes in response to nilotinib were correlated with sensitivity to nilotinib. For the first time, The results of this study showed for the first time that nilotinib induces apoptosis through upregulating ceramide synthase genes and downregulating SK-1 in CML cells in addition to inhibition of BCR/ABL. On the other hand, manipulating bioactive sphingolipids toward generation/accumulation of ceramides increased the apoptotic effects of nilotinib in CML cells.

Keywords: Chronic myeloid leukemia, nilotinib, ceramide, bioactive sphingolipids, apoptosis

Introduction

Chronic myeloid leukemia (CML) is a disease of hematopoietic stem cells, arising from a reciprocal translocation between the long arms of chromosomes 9 and 22, known as the Philadelphia chromosome [1]. This translocation causes a juxtaposition of ABL and BCR genes, resulting in a BCR-ABL fusion gene that encodes for BCR-ABL oncoprotein which has constitutive tyrosine kinase activity and cytoplasmic relocalization [2]. Therefore, BCR-ABL can activate numerous signal transduction pathways that promote cell proliferation and survival [3–5], including the RAS-ERK cascade, JAK-STAT, phosphatidylinositol 3-kinase (PI3K), and c-Myc pathways [6]. CML starts with a relatively slowly progressing chronic phase, characterized by well-differentiated leukemic cells in the blood. If not treated, the disease can eventually progress into the blastic phase, often

via the accelerated phase [7]. Imatinib was the first tyrosine kinase inhibitor used to specifically recognize the adenosine triphosphate (ATP) binding site of the BCR-ABL protein. However, its continuous administration was associated with the development of resistance, especially in the advanced phase or blast crisis [8]. Different mechanisms were suggested for imatinib resistance [9,10] such as mutations in the ATP binding site of BCR-ABL [11], decreased availability of the drug either by binding to plasma proteins or by overexpression of transporter proteins such as P-glycoprotein on the plasma membrane [12], or overexpression of BCR-ABL. Furthermore, also BCR-ABL independent resistance mechanisms were suggested, such as aberrations in ceramide generating and clearance genes [9]. Nilotinib, an analog of imatinib, has higher selectivity to BCR-ABL as compared to imatinib. Nilotinib disrupts the ATP binding pocket of BCR-ABL tyrosine kinase

and inhibits enzymatic activity by binding to the inactive conformation of the protein and by blocking its interaction with target proteins [13]. Nilotinib can be used both alone [14] and in combination with other agents for the treatment of CML. Nilotinib was designed so that 32/33 mutations in the ATP binding site of the BCR-ABL protein do not decrease its binding efficiency [15].

Sphingolipids are a family of membrane lipids responsible for regulation of the fluidity and subdomain structure of the lipid bilayers [16]. Their bioactive form, ceramide, which is a pro-apoptotic lipid [17], is involved in the regulation of various aspects of cancer pathogenesis and therapy, including apoptosis, cell proliferation, cell migration, senescence, and inflammation [18–21]. Ceramides are generated *de novo* by the LASS (longevity assurance) gene family or by the hydrolysis of sphingomyelin in various cellular compartments. They mediate antiproliferative pathways and also inhibit pro-survival mechanisms [18], mainly by regulating specific protein targets involving AKT, phospholipase D, protein kinase C (PKC), and mitogen-activated protein kinases (MAPKs) [22]. Increased ceramide levels have been observed in response to several cancer chemotherapeutic agents and radiotherapy [23]. The present study indicated that manipulating bioactive sphingolipids toward the generation/accumulation of ceramides increased the apoptotic effects of nilotinib in human K562 and Meg-01 chronic myeloid leukemia cells. Moreover, expression levels of LASS genes in response to nilotinib were correlated with sensitivity to nilotinib. The results of this study may open the way to more effective treatment of CML.

Methods

Reagents

Nilotinib was a gift from Novartis (USA). A 10 mM stock solution of nilotinib was prepared with dimethylsulfoxide (DMSO) and stored at -20°C . C8:ceramide, the glucosylceramide synthase (GCS) inhibitor *N*-(2-hydroxy-1-(4-morpholinylmethyl)-2-phenylethyl)-decaneamide, hydrochloride (PDMP), and sphingosine kinase-1 (SK-1) inhibitor were obtained from Cayman Chemicals (USA) and dissolved in DMSO.

Cell lines and culture conditions

K562 and Meg-01 human CML cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Germany). They were cultured in RPMI 1640 growth medium containing 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen, USA) at 37°C in 5% CO_2 .

Assessment of cellular growth by XTT cell proliferation assay

The IC_{50} (drug concentration that inhibits cell growth by 50%) values of nilotinib and ceramide, and IC_{10} (drug concentration that inhibits cell growth by 10%) values of SK-1 inhibitor and PDMP, were determined by XTT (sodium 3,3'-[1[(phenylamino)carbonyl]-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) cell proliferation assay as described previously [24]. Briefly, 96-well plates were seeded with 2×10^4 cells/well containing $200\mu\text{L}$ of growth medium in the absence or presence of increasing concentrations of the chemicals. The cells were incubated at 37°C in 5% CO_2 . After 72 h, cell suspensions were treated with $50\mu\text{L}$ of XTT reagent for 4 h. Then, the absorbances of the samples were measured by enzyme linked immunosorbent assay (ELISA) reader (Multiskan Spectrum; Thermo Electron Corporation, Vantaa, Finland) at a wavelength of 490 nm. In combination analyses, we combined increasing concentrations of nilotinib with those corresponding to the IC_{10} values of PDMP and SK-1 inhibitor, since we have shown previously that the IC_{10} values of these chemicals are enough to inhibit enzyme activity and provide accumulation of ceramides in the cells. On the other hand, since C8:ceramide by itself triggers induction of the generation of apoptotic ceramide, it can be accepted to act as a pro-drug. Therefore, we combined a concentration corresponding to the IC_{50} value of C8:ceramide with increasing concentrations of nilotinib.

Isobologram analysis for median dose effect

The CalcuSyn for Windows computer program (Biosoft, Cambridge, UK) was used for isobologram analysis [25] of dasatinib with C8:ceramide, PDMP, and SK-1 inhibitor should be replaced with analysis [25] of nilotinib with C8:ceramide, PDMP, and SK-1 inhibitor. In this analysis, experimental data points, represented by dots located below, on, or above the line, indicated synergism, additivity, and antagonism, respectively. The combination index (CI) is a measure of the combined effects of two drugs, using a median effect method. A CI value < 1 indicates a synergistic effect (0.1–0.5 strong synergism; < 0.1 very strong synergism); a CI value of 1 indicates an additive effect; and a CI value > 1 indicates an antagonistic effect (3.3–10 strong antagonism, > 10 very strong antagonism).

Measurement of caspase-3 enzyme activity

Caspase-3 enzyme activity of the cells was assessed using the caspase-3 colorimetric assay kit (R&D

Systems, MN, USA) according to the manufacturer's instructions. First, cells that had been induced to apoptosis with chemical treatment were collected by centrifugation at 1000 rpm for 10 min. Then, the collected cells were lysed by the addition of 100 μ L of cold lysis buffer (1 \times). After incubation of the cells on ice for 10 min, they were centrifuged at 14 000 rpm for 1 min. Supernatants were taken into new Eppendorf tubes and the reaction mixture was prepared in 96-well plates, adding 20 μ L of assay buffer (5 \times), 25 μ L of sample, 50 μ L of sterilized water, and 5 μ L of caspase-3 colorimetric substrate. After 2 h of incubation at 37°C in a CO₂ incubator, absorbance of the plate was read at a wavelength of 405 nm (Multiskan Spectrum; Thermo Electron Corporation).

Detection of changes in mitochondrial membrane potential

JC-1 mitochondrial membrane potential (MMP) (Cell Technology, CA, USA) was used to measure the loss of MMP in K562 and Meg-01 cells. Initially, the cells that had been induced to apoptosis were collected by centrifugation at 1000 rpm for 10 min. Supernatants were removed and 500 μ L of JC-1 stock solution, prepared as 1% dye, was added to the pellets. After the incubation of cells for 15 min at 37°C in a CO₂ incubator, they were centrifuged at 1000 rpm for 5 min. Then, 2 mL of assay buffer was added to the pellets and they were again centrifuged for 5 min at 1000 rpm. All pellets were resuspended with 500 μ L of assay buffer, and 150 μ L of each mixture was added to a 96-well plate in triplicate. The aggregate red form, which remains within the intact mitochondria, has absorption/emission maxima of 585/590 nm, and the green monomeric form, which is released to the cytoplasm due to loss of the MMP, has absorption/emission maxima of 510/527 nm. The plate was read at these wavelengths using a fluorescence ELISA reader (Multiskan Spectrum, Thermo Electron Corporation).

Total RNA isolation and quantitative reverse transcriptase-polymerase chain reaction

The expression levels of ceramide generating and clearance genes and β -actin, the internal positive control, were quantified by SYBR Green based quantitative polymerase chain reaction (qPCR) technique. In total, 3 \times 10⁶ cells/2 mL/well were treated with increasing concentrations of nilotinib and incubated for 24, 48, and 72 h in 5% CO₂. Total RNA was extracted using a Nucleospin Total RNA isolation kit (Macherey-Nagel, Easton, PA, USA) as described by the manufacturer. The amount and quality of RNA were measured by a NanoDrop

Photospectrometer (NanoDrop 1000; Thermo, USA). Some 5 μ g of total RNA was reverse transcribed into cDNA using reverse transcriptase enzyme (Fermentas, USA). The reaction mixture was incubated on ice for 10 min. After incubation at 42°C for 50 min, the reactions were kept at 95°C for 5 min. The resulting total cDNA was then used to determine expression levels of LASS1, LASS2, LASS4, LASS5, LASS6, SK-1, and GCS by using LightCyclerH FastStart DNA Master SYBR Green I kit (Roche, Basel, Switzerland). Expression levels of β -actin were measured, as the internal positive control. Finally, the data were analyzed with REST 2008 software (Corbett Research, Sydney, Australia). The primer sequences were as follows [24]: LASS5-F (5'-ATCTTCTTCGTGAGGCTG-3'), LASS5-R (5'-ATGTCCCAGAACCAAGGT-3') LASS1-F (5'-CTATACATGGA CACCTGGCGCAA-3'), LASS1-R (5' TCAGAAGC GCTTGTCTTCAC CA-3'); LASS2-F (5'-GCTG GAGATTCACAT-3'), LASS2-R (5'-GAAGACGAT GAAGAT-3'); LASS4-F (5'-TGCTGTCCAGTTTCAACGAG-3'), LASS4-R (5'-GAGGAAGTGTTCCTCCAG CG-3'); LASS6-F (5'-CCTCGAGGGAT GGAT TACAAGGATGACGACGATAAGATGGC AGG GATCTTAGCCTGG-3'), LASS6-R (5'-CGG AA TTCCGTTAATCATCCATGGAGCAGGA-3'); SK-1-F (5'-CCGACGAGGACTTTGTGCTAAT-3'), SK-1-R (5'-GCCTGTCCCCCAAAGCATAA C-3'); GCS-F (5'-ATGACAGAAAAAGTAGG CT-3'), GCS-R (5'-GGACACCCCTGAGTGGAA-3'); and β -actin-F (5'-CAGAGCAAGAGAGGCATCC T-3'), β -actin-R (5'-TTGAAGGTCTCAAACATGA T-3').

Results

Antiproliferative effects of nilotinib, C8:ceramide, PDMP, and SK-1 inhibitor on K562 and Meg-01 human CML cells

We have previously determined the antiproliferative effects of nilotinib (IC₅₀ 42 nM [26]), C8:ceramide (IC₅₀ 60 μ M), PDMP (IC₁₀ 20 μ M), and SK-1 inhibitor (IC₁₀ 7 μ M [27]) on K562 cells. In this study it was shown that increasing concentrations of nilotinib (0.01–100 nM) decreased proliferation of Ph+ Meg-01 cells in a dose-dependent manner. As a result, the IC₅₀ value of nilotinib was determined as 2.2 nM for Meg-01 cells [Figure 1(A)], which shows that nilotinib is much more effective on Meg-01 cells as compared to K562 cells. Increasing intracellular concentrations of ceramides, i.e. C8:ceramide, PDMP, or SK-1 inhibitor, also inhibited cell proliferation in a dose-dependent manner as compared to untreated controls. The IC₅₀ value of C8:ceramide was calculated to be 70 μ M for

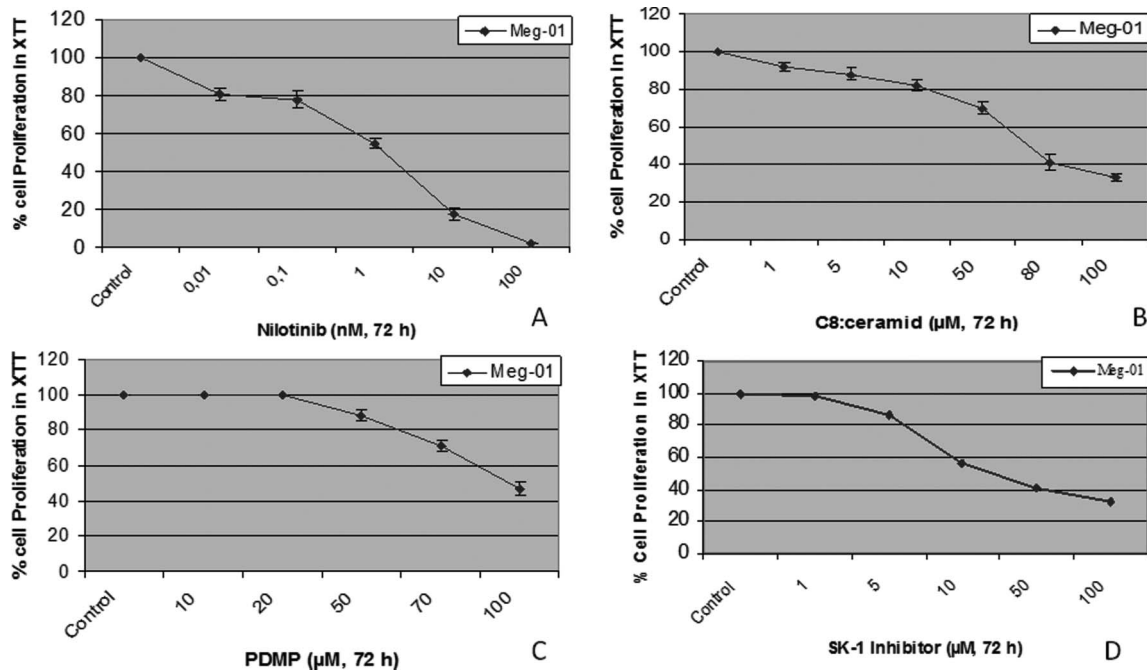


Figure 1. Antiproliferative effects of nilotinib (A), C8:ceramide (B), PDMP (C), and SK-1 inhibitor (D) on Meg-01 cells. The IC_{50} values of nilotinib and C8:ceramide, and the IC_{10} values of PDMP and SK-1 inhibitor, were calculated from cell proliferation plots. Error bars represent standard deviations.

Meg-01 cells [Figure 1(B)]. Since our previous studies demonstrated that the IC_{10} value of PDMP was sufficient to inhibit GCS enzyme activity [24], we calculated the IC_{10} value of PDMP in Meg-01 cells to be $50 \mu\text{M}$ [Figure 1(C)]. Finally, we determined the IC_{10} value of SK-1 inhibitor to be $5 \mu\text{M}$ [Figure 1(D)]. The antiproliferative effects of C8:ceramide, PDMP, and SK-1 inhibitor at similar concentrations have also been confirmed in various types of cancer by independent studies [28–32].

Combination treatment with nilotinib and C8:ceramide, PDMP, or SK-1 inhibitor significantly decreases proliferation of K562 and Meg-01 cells

Combination studies of nilotinib with these chemicals were also carried out to determine whether induction of generation or accumulation of ceramides increased the antiproliferative effects of nilotinib on K562 and Meg-01 cells. Cell proliferation data showed that the inhibition of ceramide clearance by PDMP and SK-1 inhibitor increased the cytotoxic effect of nilotinib as compared to any agent alone in both K562 [Figure 2(A)] and Meg-01 cells [Figure 2(B)]. Interestingly, induction of ceramide generation by the application of C8:ceramide resulted in significant increases in the antiproliferative effects of nilotinib in K562 cells as compared to PDMP and SK-1 inhibitor co-administration [Figure 2(B)]. In addition to these results, the CI values of nilotinib in combination with C8:ceramide, PDMP,

and SK-1 inhibitor were 0.000017, 0.01403, and 0.03471, respectively, in K562 cells [Figure 3(A)]. Combinations of nilotinib with C8:ceramide, PDMP, and SK-1 inhibitor gave CI values of 0.00029, 0.01893, and 0.02374, respectively, in Meg-01 cells [Figure 3(B)]. A CI value < 1 indicates a synergistic effect (0.1–0.5 strong synergism; < 0.1 very strong synergism). These results demonstrated that increasing intracellular concentrations of ceramide via the addition of exogenous ceramide or inhibition of GCS and SK-1 enzymes in nilotinib applied to CML cells resulted in very strong synergism.

Combinations of nilotinib with ceramide metabolism targeting agents increase caspase-3 enzyme activity significantly

While cell proliferation data provide acceptable but approximate data on the effects of these combinations, we conducted the same kinds of experiments in order to investigate the apoptotic effects of nilotinib and increasing concentrations of ceramides via ceramide mimetics/inhibitors. The results demonstrated that the application of 1 and 10 nM nilotinib caused 1.16- and 1.24-fold increases in caspase-3 enzyme activity, while $20 \mu\text{M}$ PDMP, $7 \mu\text{M}$ SK-1 inhibitor, and $60 \mu\text{M}$ C8:ceramide alone increased caspase-3 enzyme activity 1.28-, 1.12-, and 1.36-fold, respectively, as compared to untreated controls. More importantly, combinations

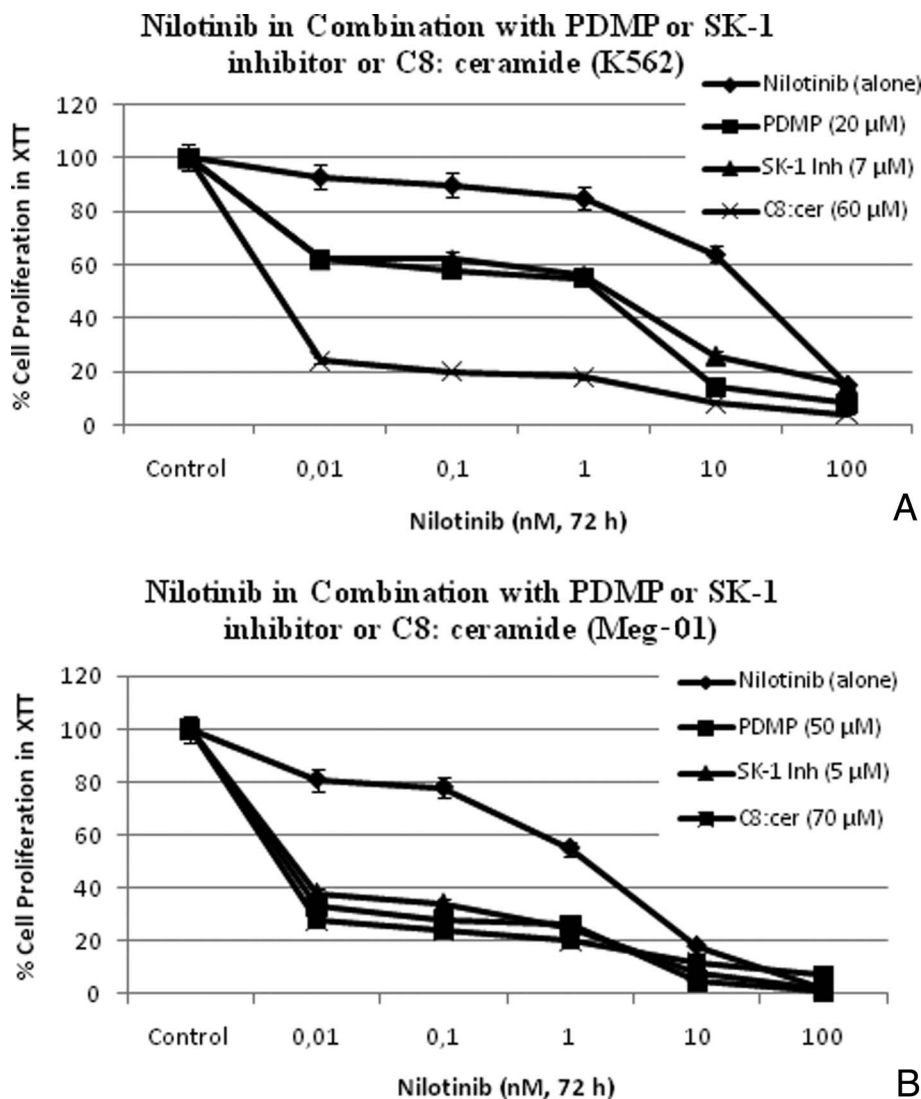


Figure 2. Antiproliferative effects of nilotinib in combination with C8:ceramide, PDMP, and SK-1 inhibitor on K562 (A) and Meg-01 cells (B). Cytotoxicity was determined by XTT cell proliferation assay in a 72 h culture. Error bars represent standard deviations.

of the same concentrations of nilotinib with the same concentrations of PDMP, SK-1 inhibitor, or C8:ceramide increased caspase-3 enzyme activity 2.52- and 2.72-, 1.67- and 1.8-, or 1.8- and 2.51-fold, respectively, compared to untreated controls (Figure 4). When caspase-3 enzyme activity was analyzed for Meg-01 cells we also showed strong synergistic effects, and similarly with K562 cells. There were increases in caspase-3 enzyme activity in response to nilotinib, PDMP, SK-1 inhibitor, and C8:ceramide (Figure 5). Since caspase-3 enzyme activity has a vitally important role in apoptosis, even a slight increase will result in significant cell death. Therefore, increases in the combinations were enough to repress proliferation and induce apoptosis as determined by isobologram analysis of XTT cell proliferation data.

Loss of mitochondrial membrane potential in K562 and Meg-01 cells treated with nilotinib and C8:ceramide, PDMP, or SK-1 inhibitor

In order to confirm the caspase-3 enzyme activity results and examine the roles of mitochondria in nilotinib-induced apoptosis, we also determined the loss of mitochondrial membrane potential. The results of detection of MMP would also give important clues about the synergistic apoptotic effects of nilotinib with ceramide-increasing agents. The results showed that nilotinib, C8:ceramide, PDMP, and SK-1 inhibitor induced a loss of MMP. In agreement with cell proliferation and caspase-3 data, we determined that nilotinib in combination with these agents induced apoptosis significantly through the loss of MMP, as compared to any agent alone, in both K562 (Figure 6) and

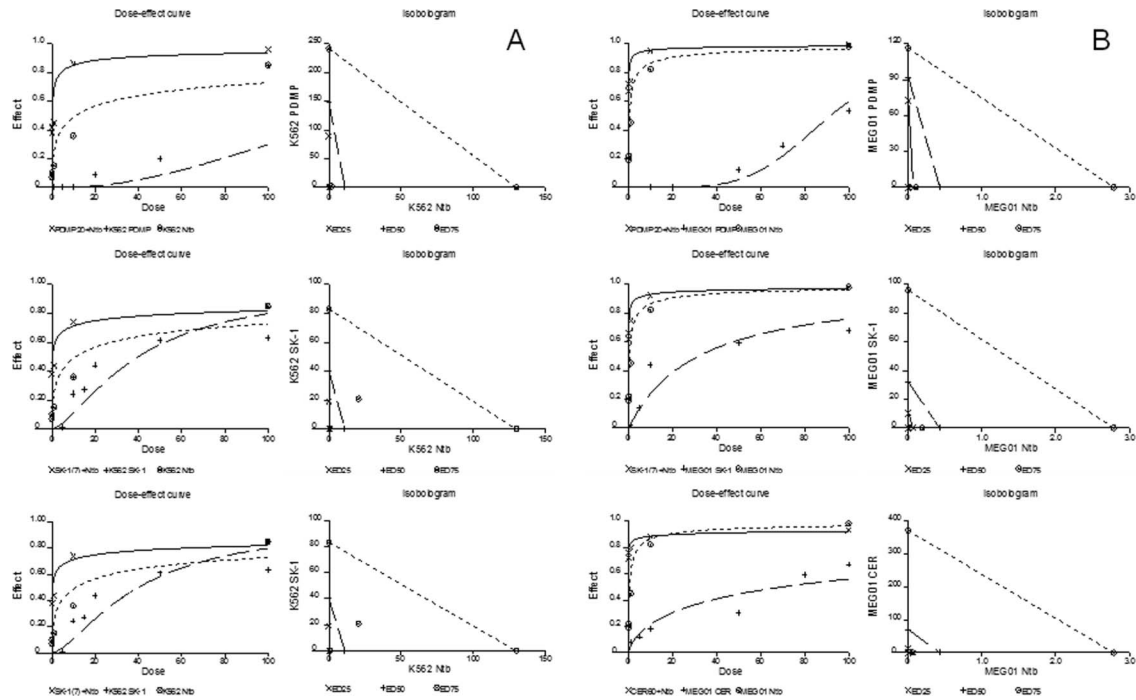


Figure 3. Isobologram analysis of combination of nilotinib with C8:ceramide, PDMP, and SK-1 inhibitor in K562 (A) and Meg-01 (B) cells.

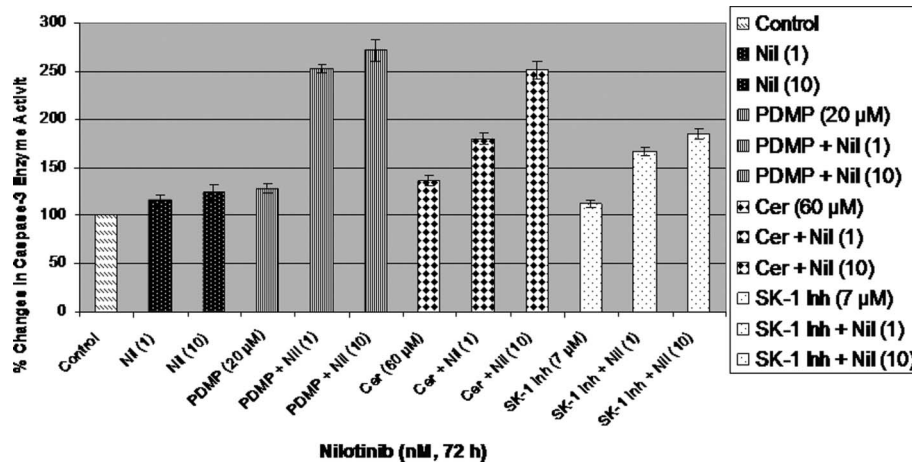


Figure 4. Percent changes in caspase-3 enzyme activity in response to combinations of nilotinib with C8:ceramide, PDMP, and SK-1 inhibitor in K562 cells. Error bars represent standard deviations, and when not apparent they are smaller than graph line thickness.

Meg-01 cells (Figure 7). On the other hand, supporting the previous data, the results of MMP analysis revealed that the combination of nilotinib with C8:ceramide induced apoptosis much more as compared to other combinatorial treatments.

Nilotinib induces de novo ceramide generation and ceramide accumulation as determined by increase in expression levels of LASS genes and decrease in expression levels of SK-1 and GCS genes

We attempted to examine the roles of ceramide metabolizing genes in nilotinib-induced apoptosis.

To this aim, both types of cells were exposed to increasing concentrations of nilotinib (0.1, 1, 10 nM for K562 and 0.01, 0.1, 1 nM for Meg-01), and expression levels of LASS1, LASS2, LASS4, LASS5, LASS6, GCS, SK-1, and β -actin, as internal positive control, were examined by qRT-PCR. The results revealed significant increases in expression levels of ceramide-generating LASS genes in a dose- and time-dependent manner in K562 cells (Figure 8). The most significant increases were observed in LASS1, LASS2, and LASS5 in K562 cells exposed to nilotinib for 24 h [Figure 8(A)], 48 h [Figure 8(B)], and 72 h [Figure 8(C)] and LASS1, LASS2,

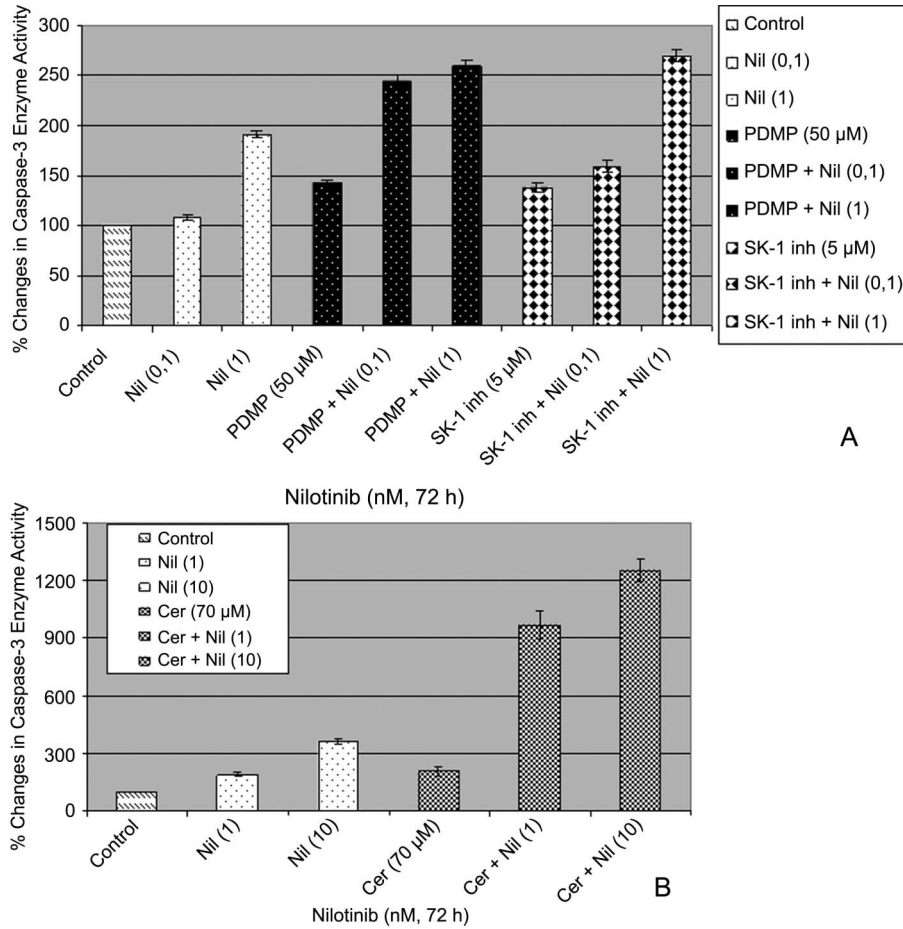


Figure 5. Percent changes in caspase-3 enzyme activity in response to combinations of nilotinib with PDMP and SK-1 inhibitor (A) and C8:ceramide (B) in Meg-01 cells. Error bars represent standard deviations, and when not apparent they are smaller than graph line thickness.

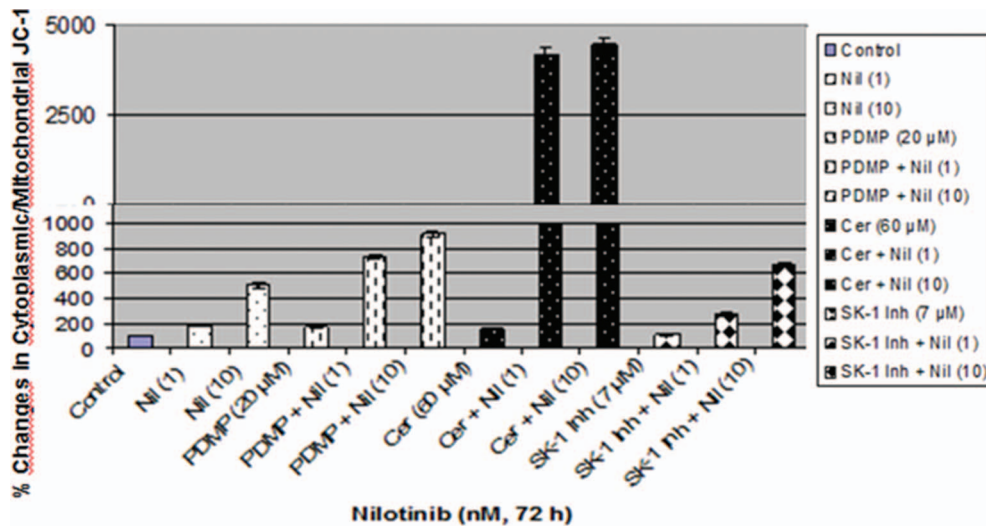


Figure 6. Percent changes in cytoplasmic/mitochondrial JC-1 in K562 cells exposed to combinations of nilotinib with C8:ceramide, PDMP, and SK-1 inhibitor. Error bars represent standard deviations, and when not apparent they are smaller than graph line thickness.

LASS5, and LASS6 in Meg-01 cells (Figure 9). There was also down-regulation of SK-1 and GCS in K562 cells in a time- and dose-dependent manner (Figure 8). There were 68% and 38% decreases in

expression levels of GCS and SK-1 in 10 nM nilotinib-treated K562 cells after 72 h [Figure 8(C)]. While we observed a significant decrease in SK-1 gene expression, there was almost no change in

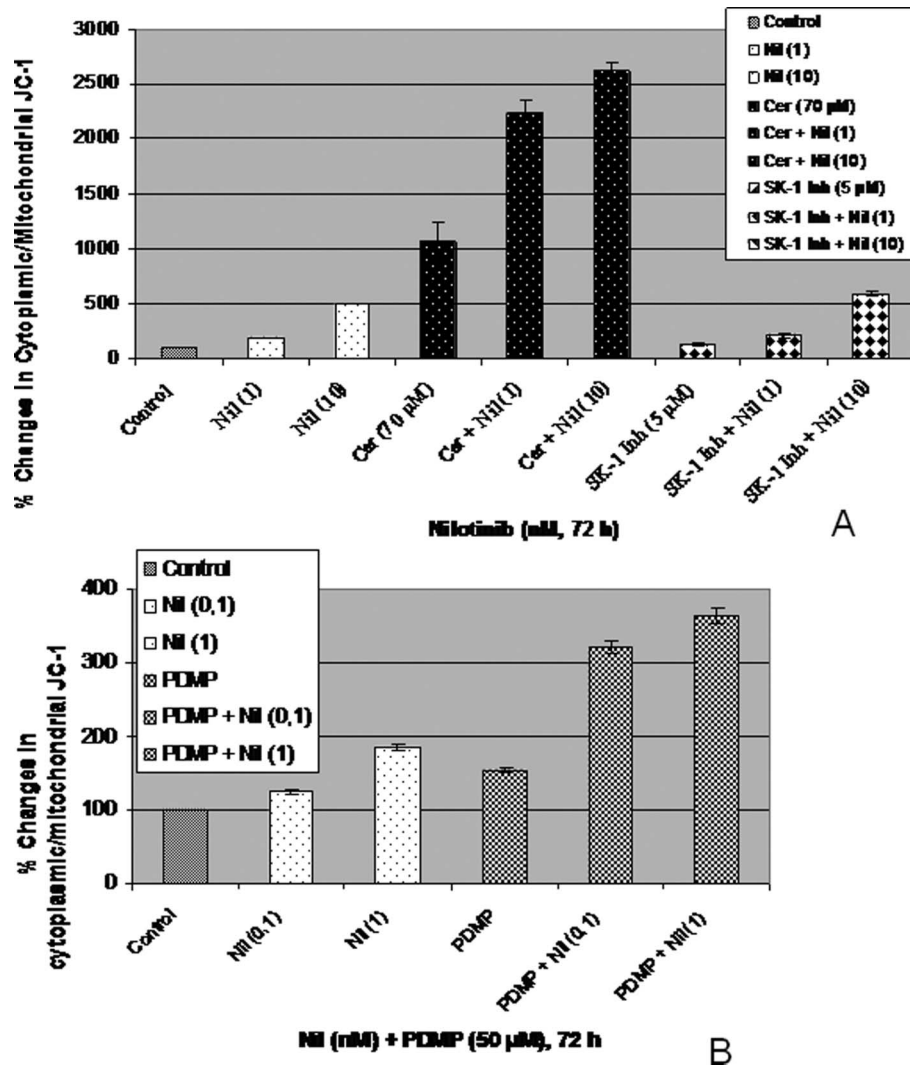


Figure 7. Percent changes in cytoplasmic/mitochondrial JC-1 in Meg-01 cells in response to combinations of nilotinib with SK-1 inhibitor and C8:ceramide (A) and PDMP (B). Error bars represent standard deviations, and when not apparent they are smaller than graph line

the expression pattern of GCS in Meg-01 cells exposed to nilotinib (Figure 9).

Discussion

This study revealed the cytotoxic and apoptotic effects of nilotinib on K562 and Meg-01 cells, especially at lower concentrations. The results also demonstrated that targeting the bioactive sphingolipid metabolism to increase intracellular ceramide concentrations increased the apoptotic effects of nilotinib significantly. Nilotinib was developed for selective inhibition of several tyrosine kinases, such as BCR-ABL, Kit, and PDGF-R (platelet-derived growth factor receptor) expressed in malignant cells [33]. Nilotinib demonstrated significant efficacy among patients with Philadelphia chromosome-positive (Ph+) acute lymphoblastic leukemia and patients with CML in all phases. However, there was

not complete cure of these malignancies [34]. A drug cocktail including another apoptotic molecule such as ceramide could be a good way to obtain better preclinical models in order to reduce side effects and define effective combinations. Although nilotinib is highly specific for Ph+ cells, there is still a possibility of evolving adverse effects [35,36]. This is why providing successful treatment in the clinic without using high-dose nilotinib is an important goal. Combination treatment using ceramides and metabolites at such low concentrations of nilotinib, leading to inhibition of cellular growth and induction of apoptosis, is thus promising for the treatment of CML. The results of this study revealed that the combination of nilotinib and altered ceramide metabolism has already given hopeful results. Manipulating the generation and/or accumulation of the proposed tumor-suppressor lipid, ceramide, could disarm a key mechanism for tumor viability and

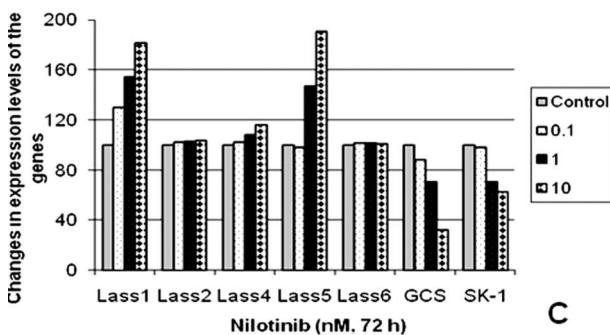
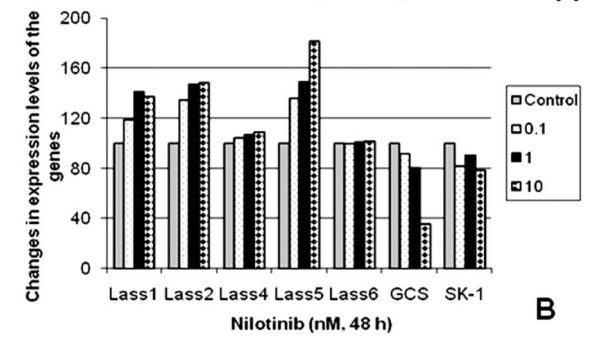
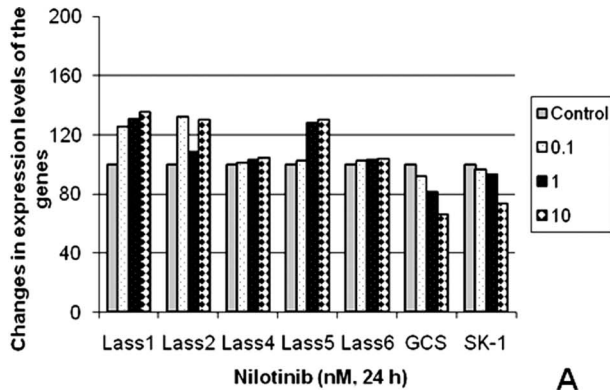


Figure 8. Percent changes in expression levels of ceramide metabolizing genes in response to nilotinib in K562 cells for 24 h (A), 48 h (B), and 72 h (C). Expression level of β -actin was determined as internal positive control.

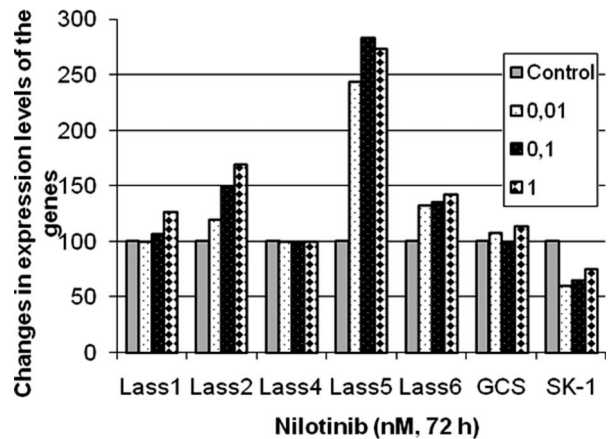


Figure 9. Percent changes in expression levels of ceramide metabolizing genes in response to nilotinib in Meg-01 cells for 72 h. Expression level of β -actin was determined as internal positive control.

growth [18]. Beckham *et al.* have shown that targeting sphingolipid metabolism in different types of cancers has therapeutic potential [37]. A key regulator of ceramide/sphingosine 1-phosphate (S1P) balance is SK-1, an oncogenic enzyme that phosphorylates sphingosine (the catabolite of ceramide) to form S1P. SK-1 not only reduces the levels of apoptotic ceramide but also the generated metabolite, S1P, has significant antiapoptotic potential [38]. Pharmacological or RNA interference (RNAi) inhibition of SK-1 is sufficient to elicit apoptosis even in chemo- or radioresistant cancer cell lines, demonstrating the crucial role of SK-1 in cell survival [39,40]. Thus, SK-1 enzyme inhibition can be a good choice to support the treatment of patients with CML. In our study, nilotinib in combination with SK-1 inhibitor in both cell lines confirmed these assumptions because of significant apoptotic effects. There was an increase of caspase-3 enzyme activity, loss of mitochondrial membrane potential, and also a decrease in cell proliferation in response to inhibition of SK-1. These results suggest that inhibition of SK-1 facilitates treatment of CML in combination with nilotinib therapy. GCS is an enzyme that glucosylates the sphingolipid and transforms apoptotic ceramide into antiapoptotic glucosylceramide, promoting chemoresistance in K562 cells [41]. Liu *et al.* reported that GCS is an important gene for the development of drug resistance in cancer cells [42]. PDMP, a strong GCS inhibitor, overcomes drug resistance in different cell lines including colon, ovary, and breast cancers, and also sensitizes cancer cells to doxorubicin, paclitaxel, and vincristine [43–45]. Although previous studies have shown that several approaches inhibiting GCS reverse drug resistance in cancer cells, there is no study in the literature showing the synergistic effects of nilotinib and PDMP leading to apoptosis. Hence from this perspective, the present results showed for the first time that nilotinib in combination with PDMP inhibited cell proliferation and induced apoptosis significantly. In different types of cancer cells exposed to different stress conditions including chemotherapy and radiotherapy, there was up-regulation of ceramide generating genes and down-regulation of ceramide clearance genes [23,24,46]. In this study, we examined the expression levels of LASS1, LASS2, LASS4, LASS5, LASS6, SK-1, and GCS genes in response to nilotinib, and the results demonstrated that nilotinib increased the expression levels of LASS genes in both cell lines and decreased the expression levels of SK-1 in both cells and GCS in K562 cells. Taken together, these results suggest that targeting bioactive sphingolipids alone induces apoptosis in CML cells, while the combination with nilotinib resulted in significant induction of apoptosis. The results of this study may

open a novel way of increasing the effectiveness of nilotinib in CML treatment in addition to the possibility of its use in other types of cancers.

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