Nanomolar concentration of NSC606985, a camptothecin analog, induces leukemic-cell apoptosis through protein kinase Cδ–dependent mechanisms

Man-Gen Song, Shen-Meng Gao, Ke-Ming Du, Min Xu, Yun Yu, Yu-Hong Zhou, Qiong Wang, Zhu Chen, Yuan-Shan Zhu, and Guo-Qiang Chen

As a promising new class of anticancer drugs, camptothecins have advanced to the forefront of several areas of therapeutic and developmental chemotherapy. In the present study, we report that NSC606985, a rarely studied camptothecin analog, induces apoptosis in acute myeloid leukemia (AML) cells NB4 and U937 and inhibits the proliferation without cell death in breakpoint cluster region–Abelson murine leukemia (bcr-abl) kinase-carrying leukemic K562 cells. For apoptosis induction or growth arrest, nanomolar concentrations of NSC606985 are sufficient. At such low concentrations, this agent also significantly inhibits the clonogenic activity of hematopoietic progenitors from patients with AML. For apoptosis induction, NSC606985 rapidly induces the proteolytic activation of protein kinase Cδ (PKCδ) with loss of mitochondrial transmembrane potential (ΔΨm) and caspase-3 activation. Cotreatment with rottlerin, a PKCδ-specific inhibitor, completely blocks NSC606985-induced mitochondrial ΔΨm loss and caspase-3 activation, while the inhibition of caspase-3 by z-DEVD-fluoromethyl ketone (Z-DEVD-fmk) only partially attenuates PKCδ activation and apoptosis.

Introduction

Acute myeloid leukemia (AML), a heterogenous group of hematopoietic malignancies, is the most common variant of acute leukemia occurring in adults. As documented, approximately 11,000 Americans were diagnosed with AML in 2003, and about 75% would ultimately die of this disease. Significant advances in understanding biologic, molecular, and cytogenetic aspects of this malignancy have been achieved in the past 20 years. Meanwhile, substantial progress is also made in the treatment of patients with AML. Especially, the application of all-trans retinoic acid, its combination with anthracycline-based chemotherapy, and with the addition of arsenic trioxide make patients with acute promyelocytic leukemia (APL), a unique subtype of AML with specific cytogenetic abnormalities, are sufficient. At such low concentrations, this agent also significantly inhibits the clonogenic activity of hematopoietic progenitors from patients with AML. For apoptosis induction, NSC606985 rapidly induces the proteolytic activation of protein kinase Cδ (PKCδ) with loss of mitochondrial transmembrane potential (ΔΨm) and caspase-3 activation. Cotreatment with rottlerin, a PKCδ-specific inhibitor, completely blocks NSC606985-induced mitochondrial ΔΨm loss and caspase-3 activation, while the inhibition of caspase-3 by z-DEVD-fluoromethyl ketone (Z-DEVD-fmk) only partially attenuates PKCδ activation and apoptosis.

These data indicate that NSC606985-induced PKCδ activation is an early event upstream to mitochondrial ΔΨm loss and caspase-3 activation, while activated caspase-3 has an amplifying effect on PKCδ proteolysis. In addition, NSC606985-induced apoptosis by PKCδ also involves caspase-3–independent mechanisms. Taken together, our results suggest that NSC606985 is a potential agent for the treatment of AML. (Blood. 2005;105:3714-3721)

From the Department of Pathophysiology, Shanghai Institute of Hematology, Rui-Jin Hospital, Shanghai Second Medical University, Shanghai, China; the Health Science Center, Shanghai Institutes for Biological Sciences and Graduate School, Chinese Academy of Sciences, Shanghai, China; the Department of Hematology, Zhong-Shan Hospital, Fu-Dan University, Shanghai, China; and the Department of Medicine/Endocrinology, Weill Medical College of Cornell University, New York, NY.


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M-G.S., S-M.G, and K-M.D. contributed equally to this work.

Reprints: Guo-Qiang Chen, No. 280, Chong-Qing South Rd, Shanghai 200025, China; e-mail: chengq@shsmu.edu.cn.

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and irinotecan for the treatment of colorectal carcinoma refractory to 5-fluorouracil or as initial therapy in combination with 5-fluorouracil for the treatment of metastatic colorectal cancer.12

The clinical potentials of camptothecin analogs in the treatment of AML were also investigated. In 1996, Rowinsky et al14 reported that topotecan administered as a single agent had a significant antileukemic activity in patients with AML. Recently, topotecan was investigated as a salvage and front-line therapy for AML in combination with etoposide, cytarabine, or cyclophosphamide, and some efficacious results were obtained.15-17 Further development of novel topo-I inhibitors was spurred by the spectrum of potencies of this class of drugs in terms of enzyme inhibition, antiproliferative activity, toxicities, and pharmaceutical properties. For instance, DX-8951f, a water-soluble hexacyclic camptothecin derivative with more potent topo-I-inhibiting action, has been shown to possess better antileukemic activity in severe combined immunodeficient (SCID) mice with AML.18 Potent preclinical antileukemic activity is also observed for OSI-211, a low-clearance, unilamellar liposomal formulation of a water-soluble camptothecin analog lurtotecan.19

In the present study, we report that very low concentrations (nanomolar, nM) of NSC606985, a rarely studied water-soluble camptothecin ester derivative (Figure 1A),20 can induce leukemic cell apoptosis by proteolytic activation of protein kinase Cζ (PKζ). By using this system, the relationship among PKζ, mitochondrial transmembrane potentials (ΔΨm), and caspase-3 activation in apoptosis regulation is also evaluated.

Patients, materials, and methods

Cell culture and treatment

Leukemic cells, including APL cell line NB4 (kindly provided by Dr Michael Lannotte,21 INSERM u-496, Centre G. Hayem, Hospital Saint-Louis, Paris, France), acute myelomonocytic leukemia U937 cell line, and chronic myeloid leukemia K562 cell line (Cell Bank of Shanghai Institutes for Biological Sciences, Shanghai, China), were cultured in RPMI-1640 medium (Sigma, St Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (FCS; HyClone, Logan, UT) in 5% CO2 95% air humidified atmosphere at 37°C. For experiments, cells were seeded at 2 to 5 × 104 cells/mL and incubated with the indicated concentrations of NSC606985 with or without the PKζ-specific inhibitor rottlerin and caspase-3 inhibitor z-DEVD-fluoromethyl ketone (Z-DEVD-fmk). Etoposide was also used for apoptosis induction in K562 cells, NSC606985 (kindly provided by National Cancer Institute Anticancer Drug Screen standard agent database, Bethesda, MD) was dissolved in double-distilled water as a 25 μM stock solution. Rottlerin (BIOMOL, Plymouth, PA) was prepared in ethanol as 1 mM stock solution and was kept at −80°C. Z-DEVD-fmk (BD Biosciences, San Diego, CA) was dissolved in dimethyl sulfoxide (DMSO) before use. Etoposide (BIOMOL) was also dissolved in DMSO as 20 mM stock solution and kept at −20°C. Cell viability was determined by the trypan-blue exclusion assay, and growth inhibition rate was calculated according to viable cell numbers of treated cells against numbers of untreated cells. For morphologic observation, cells were collected onto slides by cytospin (Shandon, Runcorn, United Kingdom), stained with Wright staining, and examined under light microscope.

DNA gel electrophoresis

Appropriate 106 cells were harvested, and pellets were suspended in lysis buffer (0.1 M NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM EDTA (ethylenediaminetetraacetic acid), 0.5% sodium dodecyl sulfate [SDS], 500 μg/mL protease K). After a 30-minute incubation on ice, samples were centrifuged at 14 000g for 30 minutes, and cellular DNA was extracted. The samples were electrophoresed in 2% agarose gel at 100 V in 40 mM Tris-acetate buffer (pH 7.4) and visualized by ethidium bromide staining.

Nuclear and cytoplasmic fractionation

Cells (about 1 × 107) were incubated in 400 μL lysis buffer (10 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT (dithiothreitol), pH 7.9) with the
supplement of 0.2% Nonidet P-40 (NP-40; [octylphenoxy]polyethoxethanol) and protease inhibitor cocktail (Sigma) for 1 minute on ice. The lysates were microcentrifuged for 1 minute at 2300g. The supernatants were collected for cytoplasmic protein extracts. Then, the pellets were washed with lysis buffer without NP-40 and resuspended in 150 µL extraction buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, and 25% glycerol) and incubated for 20 minutes on ice. The samples were centrifuged at 12,000g for 10 minutes, and the supernatant was collected as nuclear protein extracts.

Western blots

Cells were washed with PBS and lysed with lysis buffer (62.5 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol). Cell lysates were centrifuged at 20,000g for 10 minutes at 4°C, and proteins in the supernatants were quantified. Protein extracts were equally loaded to an 8% Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol). Cell lysates were centrifuged at 20,000g for 1 minute at 2300g. The pellets were washed with lysis buffer without NP-40 and resuspended in 150 µL extraction buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA, and 25% glycerol) and incubated for 20 minutes on ice. The samples were centrifuged at 12,000g for 10 minutes, and the supernatant was collected as nuclear protein extracts.

Statistical analysis

The significance of the difference between groups was determined by the Student t test.

Results

**NSC606985 at nanomolar concentration induces apoptosis in acute promyelocytic leukemia cell line**

In APL cell line NB4 cells, NSC606985 inhibited cell growth in a time- and concentration-dependent manner (Figure 1B, left), which paralleled to the reduced cell viability (Figure 1B, right). This inhibitory effect was observed at nanomolar concentrations. At 48 hours of treatment, 6.25 nM NSC606985 produced 48.2% ± 2.8% cell death, and 50 nM and 100 nM NSC606985 almost caused all cells to die (Figure 1B, right). Further evidence supported that NSC606985 induced cell death by way of apoptosis. NB4 cells treated with NSC606985 presented profound morphologic changes that were characteristics of apoptosis, such as chromatin condensation and nuclear fragmentation with intact cell membrane (Figure 1C). Analysis of nuclear DNA distribution on flow cytometry showed that NSC606985 induced a time-dependent increase in hypoploid cells (also called sub-G1 cells) as a result of the degradation and subsequent leakage of nuclear DNA from cells, an important indication of cells becoming apoptotic (Figure 1D).6 In agreement with this, treatment with NSC606985 in NB4 cells also induced apoptosis-specific DNA-laddering fragmentation on the agarose gel electrophoresis (Figure 1E). Furthermore, annexin V/PI double staining–based flow cytometry analysis, the most sensitive and the most specific test for determining apoptotic cells in suspension culture,24 showed that at early phase after 25 nM NSC606985 treatment, annexin-V+/PI− cells were present (Figure 1F). Subsequently, cells underwent secondary necrosis, as evidenced by increased annexin-V+/PI+ cells (Figure 1F, 48 hours) and necrotic cell–like morphology (Figure 1C).

**NSC606985 produces differential effects in different leukemic cells**

The effect of NSC606985 on cell apoptosis was further examined in 2 other myeloid leukemic cells, U937, a cell line from acute myelomonocytic leukemia, and K562, a cell line from t(9,22)–carrying chronic myeloid leukemia. Like that in NB4 cells, NSC606985 inhibited the cell growth in both U937 and K562 cells in a time- and concentration-dependent manner (Figure 2A-B). Although 6.25 nM NSC606985 was sufficient for potent proliferation-inhibitory effect in K562 cells (Figure 2D), the compound failed to induce apoptosis in the cell line, even at a high concentration (200 nM), as determined by cell morphologic criteria (Figure 2C), DNA fragmentation (Figure 2D), annexin-V/PI assay, and cellular DNA content distribution assay (data not shown). As described by previous reports,25,26 etoposide at 100 µM could induce apoptosis in K562 cells (Figure 2C-D), indicating that the resistance of K562 cells to NSC606985-induced apoptosis was not due to clonal selection.

In U937 cells, nanomolar concentration of NSC606985 also effectively induced apoptosis (Figure 2C-E), although the sensitivity of the cells to NSC606985-induced apoptosis was lower than that of NB4 cells. Under the treatment with NSC606985 at 50-nM concentration for 24 hours, only 21.63% ± 1.64% of U937 cells underwent apoptosis compared with 76.09% ± 5.40% of NB4 cells (P < .001), as assessed by annexin-V analysis. Because of the different sensitivity to NSC606985, NB4 cells and U937 cells were treated respectively with 25 nM and 50 nM NSC606985 for all following experiments, aiming to elucidate the mechanisms of the agent-induced apoptosis.

**NSC606985 at apoptosis-inducing concentrations induces proteolytic activation of PKCδ with mitochondrial transmembrane potential collapse and caspase-3 activation**

To elucidate the mechanisms of NSC606985-induced apoptosis, we first determined the effects of NSC606985 on mitochondrial ΔΨm by double staining of PI and Rh123, a lipophilic cation that is taken up by mitochondria in proportion to the ΔΨm.27 As depicted in Figure 3A, untreated living cells were strongly stained by Rh123 with negative PI. When NB4 cells were treated with 25 nM NSC606985 for 12 hours (Figure 3A, top) or U937 cells treated with 50 nM NSC606985 for 36 hours (Figure 3A, bottom), PI-negative but weaker Rh123-stained cells began to appear and subsequently increased. Following mitochondrial ΔΨm collapse,
with 100 K562 cells were incubated with the indicated concentrations (nM) of NSC606985 or etoposide for 24 and 48 hours. Growth-inhibiting rate (top) and cell viability (bottom) were determined. The values are the mean ± SD of a triplicate experiment. (C) U937 (top row) and K562 cells (bottom row) were treated with or without 50 nM and 100 nM NSC606985, respectively, for 48 hours. Cytomorphology was examined with Wright staining. Images were viewed using an Olympus BX60 microscope equipped with a 100 ×/1.3 objective lens, and were acquired through a SPOT RT camera and SPOT software. For K562 cells, the treatment with etoposide at 100 nM was used as positive control. (D) K562 cells were incubated with the indicated concentrations (nM) of NSC606985 or with 100 μM etoposide for 48 hours. DNA samples from these treated cells were electrophoresed in a 2% agarose gel. (E) PB4 and U937 cells were treated with NSC606985 at the indicated concentrations for 48 hours, and DNA samples from the cells were electrophoresed in a 2% agarose gel. All experiments were repeated 4 times with similar results.

NSC606985 at apoptosis-inducing concentrations also induced a rapid proteolytic cleavage of PKCδ into a 41-kDa catalytic fragment that persistently activates the kinase,28-30 in NB4 cells and U937 cells. This proteolytic effect was clearly observed at 12 and 24 hours after the treatment with NSC606985 in NB4 cells and U937 cells (Figure 3C). This proteolytic effect is also observable in K562 cells (Figure 3C). The similar results could also be seen in etoposide-treated K562 cells (Figure 3C). Moreover, the cleavage of PKCδ was observed in both cytoplasm and nuclei of NB4 and U937 cells (Figure 4B). PKCδ was present in both cytoplasm and the nuclei of untreated U937 cells, while it was undetectable in the cytoplasm of untreated NB4 cells. It appears that the treatment with NSC606985 increased the level of cytoplasmic PKCδ in NB4 cells (Figure 4B).

NSC606985-induced mitochondrial transmembrane potential loss, caspase-3 activation, and apoptosis are blocked by a PKCδ-specific inhibitor

To determine the role of proteolytic activation of PKCδ in NSC606985-induced apoptosis, we treated NB4 and U937 cells with NSC606985 in the presence or absence of rottlerin (1 μM), a specific PKCδ inhibitor.31 As showed in Figure 5A, rottlerin significantly inhibited NSC606985-induced proteolytic cleavage of PKCδ.
PKCβ. In agreement with this effect, the inhibitor also antagonized NSC606985-induced caspase-3 activation, PARP degradation (Figure 5B) and mitochondrial ΔΨm collapse (Figure 5C). Most importantly, rotterlin completely blocked NSC606985-induced apoptosis in both NB4 cells (Figure 5D) and U937 cells (data not shown). These results strongly indicated that NSC606985-induced PKCβ activation was an early event upstream to the mitochondrial ΔΨm collapse and caspase-3 activation and was essential for NSC606985-induced apoptosis in NB4 and U937 cells.

NSC606985-induced PKCβ activation and apoptosis, but not mitochondrial transmembrane potential collapse, are partially antagonized by a caspase-3 inhibitor

To understand the role of caspase-3 and possible relationship between caspase-3 activation and PKCβ cleavage in NSC606985-induced apoptosis, NB4 cells were treated with NSC606985 in the presence or absence of the cell-permeable caspase-3 inhibitor Z-DEVD-fmk. As showed in Figure 6, Z-DEVD-fmk completely blocked NSC606985-induced proteolytic cleavage of PKCβ (Figure 6B) and apoptosis (Figure 6C). Treatment of NB4 cells with 25 nM NSC606985 alone for 12 hours greatly induced cell apoptosis, which was significantly attenuated by the addition of Z-DEVD-fmk (40 μM) 1 hour before NSC606985 treatment, as assessed by the percentage of annexin-V+/PI- cells (P < .01, compared with NSC606985 treatment alone; Figure 6C). However, the addition of Z-DEVD-fmk did not significantly affect NSC606985-induced mitochondrial ΔΨm collapse (P > .05, compared with NSC606985 treatment alone; Figure 6D).

NSC606985 inhibits the formation of CFU-GM and BFU-E of AML patients

Finally, we determined the effects of NSC606985 on the clonogenic activity of fresh BM cells from 4 patients with AML using CFU-GM and BFU-E assays. As shown in Figure 7A-B, treatment with 25 nM and 50 nM NSC606985 significantly inhibited CFU-GM formation and resulted in a 100% inhibition in case 3 and case 4. Except for case 2 and case 4 patients whose BM cells did not form BFU-Es, NSC606985 significantly inhibited BFU-E formation and resulted in a 100% inhibition in case 3 and case 4.
not form BFU-E in untreated condition, the BFU-E formation in two other patients was also significantly inhibited by NSC606985.

**Discussion**

Here, we reported that a camptothecin analog NSC606985 produced a time- and dose-dependent induction of cell apoptosis in leukemia NB4 and U937 cells, as demonstrated by cell morphologic features, sub-G1 cell, DNA-laddering fragmentation, and annexin V+PI− cell analysis. In K562 cells, NSC606985 possessed more potent inhibitory activity on cell proliferation but failed to induce cell apoptosis. Both apoptosis-inducing and proliferation-inhibiting effects were observed at nanomolar concentrations of the agent. Furthermore, apoptosis-inducing or proliferation-inhibiting concentrations of NSC606985 significantly inhibited the clonogenic activity of fresh hematopoietic progenitors from patients with AML. These results indicated that NSC606985 might be a potential therapeutic remedy for the treatment of leukemia.

It has been well known that a complex cellular signaling network contributes to the regulation of apoptosis. In most cases, a central player in the execution of apoptosis is aspartic acid–directed cysteine proteases called caspses, which are activated by the cell surface death receptor pathway and the mitochondria-initiated pathway, the latter inducing the release of proteins such as cytochrome c from the intermembrane space of ΔΨm-collapsed mitochondria. Here, we showed that NSC606985-induced apoptosis paralleled to the mitochondrial ΔΨm loss and caspase-3 activation, but caspase-3–specific inhibitor only partially attenuated NSC606985-induced apoptosis, indicating that caspase-3 pathway partially contributed to leukemic cell apoptosis induced by NSC606985. PKCδ, a ubiquitously expressed member of the novel PKC family, is activated by translocation, tyrosine phosphorylation, or proteolytic cleavage into 41-kDa catalytically active fragment. The isoenzyme enigmatically presents the multifunctional properties and is implicated in the regulation of a variety of cellular processes, including secretion, cell cycle progression, apoptosis, differentiation, and tumor development. In 1994, Trubiani et al reported for the first time that treatment with dexamethasone in thymocytes resulted in a redistribution of PKC to the nucleus and cell apoptosis, indicating a linkage between PKC activation and cell apoptosis. From then on, increasing data supported a direct link between PKCδ activation and cell apoptosis induced by diverse agents such as etoposide, ara-C, taxol, and many others. However, conflicting results have been reported, and the functional significance of PKCδ in cell apoptosis has not been clearly defined, as reviewed by Jackson and Foster. In the present study, we show that, like that seen in etoposide-treated K562 cells, NSC606985 rapidly induced proteolytic activation of PKCδ and subsequently cell apoptosis in NB4 and U937 cells but not in K562 cells. The PKCδ–specific inhibitor rottlerin completely attenuated NSC606985-induced apoptosis, suggesting that PKCδ played a critical role in NSC606985-induced apoptosis, and the NSC606985-induced PKCδ activation was directly linked to NSC606985-induced cell apoptosis (Figure 7C).

It was proposed that PKCδ proteolytic activation was completely dependent on caspase-3, based on the observations that inhibition of caspase-3 activity blocked PKCδ cleavage and apoptosis induced by ultraviolet radiation, oxidative stress, DNA damage, aging neutrophil as well as other inducers. Here, we demonstrated that the blockage of PKCδ activity by rottlerin completely blocked NSC606985-induced mitochondrial ΔΨm loss and caspase-3 activation, indicating that PKCδ activation was an event upstream to mitochondrial ΔΨm loss and caspase-3 activation. This result was consistent with reports that PKCδ directly interacts with mitochondria and alters mitochondrial function for apoptosis induction, presumably mediated through amplifying ceramide formation and the ceramide-mediated mitochondrial amplification loop. Furthermore, we showed that inhibition of caspase-3 activity by Z-DEVD-fmk only partially attenuated PKCδ cleavage and apoptosis without significant alteration in NSC606985-induced mitochondrial ΔΨm loss, supporting the amplifying effect of activated caspase-3 on PKCδ cleavage. More important, these results also proposed that in addition to caspase-3, PKCδ-mediated apoptosis also involved caspase-3–independent mechanisms that remain to be addressed (Figure 7C). It is possibly associated with some potential downstream carriers of PKCδ action in the induction of apoptosis such as DNA-dependent protein kinase, p38 mitogen-activated protein kinase, Rad9, p73 (a structural and functional homolog of the p53 tumor suppressor).

It has previously been reported that subcellular localization of PKCδ affects its apoptosis-inducing effect. DeVries et al showed that the import of PKCδ into the nucleus was required for the initiation of etoposide-induced apoptosis, while the attachment of PKCδ to membrane and translocation to the Golgi complex was required for the ultraviolet- and ceramide-induced apoptosis, respectively. Here, we showed that NSC606985-induced PKCδ cleavage simultaneously occurred in the cytoplasm and nuclei of both NB4 and U937 cells, although PKCδ was differentially distributed within these cells. Unlike untreated U937 cells in which PKCδ was present in the cytoplasm and nuclei, NB4 cells had undetectable level of cytoplasmic PKCδ. Treatment with NSC606985 increases the levels of cytoplasmic PKCδ in NB4 cells. Whether differential subcellular distribution of PKCδ is related to the cell sensitivity to NSC606985-induced apoptosis and whether the NSC606985-induced increase in cytoplasmic PKCδ is translocated from other subcellular compartments remain to be determined. It should be pointed out that a higher level of an anti-PKCδ antibody cross-reactive fragment (about 45 kDa), which existed mainly in the cytoplasm and also down-regulated by NSC606985 treatment, could be clearly seen in NB4 cells, while such a protein was weaker in U937 cells (Figure 4). It remains to be clarified whether the fragment may make NB4 cells more susceptible to NSC606985.

Finally, the cause leading to the difference of effects of NSC606985 on NB4 and U937 cells and on K562 cells remains to be investigated. It has been proposed that the strong resistance of K562 cells to apoptosis induction is related to bcr-abl tyrosine kinase, generated by specific chromosome translocation (9;22). Thus, we speculated that the fusion tyrosine kinase also contribute to the failure of K562 cells to NSC606985-induced apoptosis. However, K562 cells were still extremely resistant to NSC606985-induced apoptosis in the presence of STI571 (data not shown), a specific tyrosine kinase inhibitor, proposing that the resistance of K562 cells to NSC606985-induced apoptosis was independent of the constant tyrosine-kinase activity in these cells, which is consistent with the recent finding by Bueno-da-Silva et al.
of hematopoietic progenitor cells from leukemia patients. The NSC606985-induced apoptosis is mainly mediated through the proteolytic activation of PKCδ, which leads to caspase-3 activation and mitochondrial ΔΨm loss. These results strongly suggest that NSC606985 is a potential new agent for the treatment of leukemia and deserves further preclinical and clinical studies.

References

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