Cyclin-Dependent Kinase 7/9 Inhibitor SNS-032 Abrogates FIP1-like-1 Platelet-Derived Growth Factor Receptor α and Bcr-Abl Oncogene Addiction in Malignant Hematologic Cells

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Abstract

**Purpose:** The "gate-keeper" mutations T674I platelet—derived growth factor receptor α (PDGFRα) in hypereosinophilic syndrome (HES) and T315I Bcr-Abl in chronic myeloid leukemia (CML) are resistant to imatinib and the second-generation small-molecule tyrosine kinase inhibitors (TKI). However, to combat acquired resistance to imatinib, an alternative approach is to decrease the expression of the addicted gene to efficiently kill resistant malignant hematologic cells. The purpose of this study was to evaluate the strategy of shutting down the transcription and expression of FIP1-like-1 (FIP1L1)–PDGFRα and Bcr-Abl with SNS-032, an inhibitor of cyclin-dependent kinase 7 (CDK7) and CDK9 in phase I clinical trials.

**Experimental Design:** The effects of SNS-032 on PDGFRα and Bcr-Abl signaling pathways, apoptosis, and cell cycling were analyzed in TKI-resistant cells of HES and CML. The *in vivo* antitumor activity of SNS-032 was assessed with xenografted BaF3-T674I FIP1L1-PDGFRα and KBM5-T315I Bcr-Abl cells in nude mouse models.

**Results:** SNS-032 inhibited the phosphorylation on Ser5 and Ser2 of RNA polymerase II. SNS-032 decreased both the mRNA and protein levels of FIP1L1-PDGFRα and Bcr-Abl and inhibited the proliferation of malignant cells expressing FIP1L1-PDGFRα or Bcr-Abl. It also decreased the phosphorylation of downstream molecules. It induced apoptosis by triggering both the mitochondrial pathway and the death receptor pathway.

**Conclusions:** This CDK7/9 inhibitor potently inhibits FIP1L1-PDGFRα—positive HES cells and Bcr-Abl-positive CML cells regardless of their sensitivity to imatinib. SNS-032 may have potential in treating hematologic malignancy by abrogating oncogene addiction. *Clin Cancer Res; 18(7); 1966–78. ©2012 AACR.

Introduction

In "oncogene addiction", malignant cells depend highly on the continual activation of intracellular signaling by a specific oncogene despite a multitude of additional carcinogenic genetic changes (1–3). Thus, targeted therapy to inhibit "addicted" oncoproteins has shown some success: small-molecule tyrosine kinase inhibitors (TKI) have been used to block Bcr-Abl, KIT, and PDGFRα in chronic myeloid leukemia (CML), gastrointestinal stromal tumors (GIST), and hypereosinophilic syndrome (HES), respectively (2, 4–6).

The importance of platelet-derived growth factor receptor α (PDGFRα) in HES and Bcr-Abl in CML are good examples of oncogene addiction (7, 8), but the malignant cells can adapt to the challenge of TKIs, and acquired resistance to imatinib is an emergent problem (9, 10). Mutation in the kinase domain of PDGFRα or Bcr-Abl is a common mechanism of resistance to imatinib. Particularly, the "gatekeeper" mutations T674I PDGFRα in HES and T315I Bcr-Abl in CML, which are analogous to T670I KIT in GISTs, are good examples of oncogene addiction (7, 8), but the malignant cells can adapt to the challenge of TKIs, and acquired resistance to imatinib is an emergent problem (9, 10). Mutation in the kinase domain of PDGFRα or Bcr-Abl is a common mechanism of resistance to imatinib. Particularly, the "gatekeeper" mutations T674I PDGFRα in HES and T315I Bcr-Abl in CML, which are analogous to T670I KIT in GISTs, are resistant to the second-generation TKIs (e.g., nilotinib, dasatinib, and midostaurin; refs. 2, 7, 11). Recently, the third-generation TKIs (e.g., AP24534, HG-7-85-1, and...
Imatinib-Resistant Cells Are Responsive to SNS-032

Translational Relevance

Resistance to tyrosine kinase inhibitors (TKI) such as imatinib is an emerging problem for molecular targeted therapy. The second-generation TKIs (e.g., nilotinib and dasatinib) can overcome resistance caused by most mutations except the "gate-keeper" mutations [e.g., T674I platelet-derived growth factor receptor α (PDGFRα) in hypereosinophilic syndrome (HES), T315I Bcr-Abl in chronic myelogenous leukemia (CML)]. An alternative approach for many of these TKI-resistant mutants is to decrease the expression of an "addicted" oncogene, which ensures continual activation of intracellular signaling, to kill the malignant cells. Here, we report that SNS-032 [an inhibitor of cyclin-dependent kinase 7 (CDK7) and CDK9] decreased both the mRNA and protein levels of FIP1-like 1 (FIP1L1)–PDGFRα and Bcr-Abl, abrogated their downstream signaling, respectively, and inhibited the growth of HES and CML cells. The CDK inhibitor SNS-032 may have potential for treating FIP1L1-PDGFRα-positive HES or CML regardless of their sensitivity to imatinib by abrogating oncogene addiction.

DCC-2036; refs. 12–15) targeting these "gate-keeper" mutants have been developed, but their efficacy and safety remain to be validated in clinical trials.

An alternative strategy to treat malignancies with oncogene addiction and TKI resistance has shown promise without significant host toxicity: the expression of the oncoprotein PDGFRα and Bcr-Abl suppressed by HSP inhibitors (e.g., 17-AAG, IPI-504, and celastrol; refs. 16–19), transcription inhibitors (e.g., flavopiridol and triptolide; refs. 11, 20, 21), or translation inhibitors (e.g., homoharringtonine; ref. 22). Despite the cell-wide impact of stopping transcription or translation, the relatively fast protein turnover rate of the "addicted" oncoproteins provides a therapeutic window to selectively kill malignant cells by pulse treatments with these inhibitors (22–24).

Cyclin-dependent kinases (CDK) regulate cell-cycle progression, and a pot inhibitor of CDK2, 7, and 9 being investigated for treating hematologic malignancies is SNS-032 (also known as BMS-387032; ref. 25). SNS-032 inhibits CDK7 and 9, thereby blocking the phosphorylation of Ser5 and Ser2 of the C-terminal domain of RNA pol II, thereby inhibiting transcriptional initiation and elongation (26, 27).

Both HES and CML are myeloproliferative diseases (9, 28). HES is characterized by symptomatic persistent eosinophilia (>1,500 eosinophils/μL in the peripheral blood for >6 months) with target organ damage (e.g., heart, lung, and skin; refs. 29, 30), and CML is characterized by expansion of immature granulocyte filled in peripheral blood, bone marrow, and spleen (15, 31). In this study, we aimed to evaluate the antineoplastic activity of SNS-032 in HES and CML cells and examine whether it abrogates oncogene addiction by inhibiting RNA synthesis. SNS-032, at nanomolar concentrations, inhibited the expression of FIP1-like-1 (FIP1L1)–PDGFRα and Bcr-Abl. SNS-032 inhibited cell cycling and growth and induced apoptosis both in wild-type and mutant malignant hematologic cells. We assessed the in vivo effect of SNS-032 in nude mouse xenograft models. Abrogating oncogene addiction by inhibiting RNA synthesis is at least one mechanism of action of SNS-032 in addition to inhibiting cell cycling in the FIP1L1–PDGFRα-positive HES cells and the Bcr-Abl–positive CML cells, regardless of resistance to imatinib.

Materials and Methods

Reagents

SNS-032 synthesized in our laboratory was dissolved in dimethyl sulfoxide (DMSO) for a 20 mmol/L stock solution, which was frozen in aliquots and stored at −20°C. Imatinib was a product of Novartis Pharmaceuticals (22). Caspase-3 inhibitor benzoxycarbonyl-Asp (OMe)-γ-carboxy-Asp (OMe)-Val-Asp (OMe)-fluoromethylketone (z-DEVD-fmk) was obtained from BD Biosciences. MG132 was purchased from EMD Biosciences. Antibodies against PARP, Bcl-2, X-linked inhibitor of apoptosis protein (XIAP), active caspase-3, and cytochrome c (clone 6H2.B4) were from BD Biosciences Pharmingen; rabbit polyclonal antibodies against c-Abl (C-19), apoptosis-inducing factor (AIF), Bax, Bcl-XL, and Mcl-1 (S-19) were from Santa Cruz Biotechnology; anti-Bim was from Stressgen; antibodies against phospho-PDGFRα (Y1018), phospho-c-Abl (Y245), caspase-3, -8, -9, phospho-Erk1/2 (T202/Y204), extracellular signal–regulated kinase (Erk) 1/2, phospho-Akt (Ser473), and Akt were from Cell Signaling Technology; anti-survivin was from Novus Biologicals; rabbit anti-human antibodies against RNA polymerase II (pol II), phospho-Pol II (Ser2), and phospho-Pol II (Ser5) were from BD Biosciences Pharmingen; antibodies against phospho-STAT3 (Y705), anti-STAT3, phospho-STAT5 (Y694/Y699; clone 8-5-2), anti-STAT5, and anti-PDGFRα were from Upstate Technology; cycloheximide and anti-actin were from Sigma-Aldrich; and anti-mouse immunoglobulin G and anti-rabbit immunoglobulin G horseradish peroxidase–conjugated antibodies were from Pierce Biotechnology (20, 22, 32).

Cell culture

The EOL-1 cell line, which carries the FIP1L1-PDGFRα fusion oncogene (33), and BaF3 cells expressing wild-type or T674I FIP1L1–PDGFRα were previously described (8, 20). Cells were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal calf serum (Kibbutz Beit). The KBM5 cells expressing 210-kDa wild-type (KBM5) or T315I Bcr-Abl (KBM5-T315I) were cultured as previously described (32). Cells are incubated at 37°C and in water vapor–saturated air with 5% CO2 at one atmospheric pressure.

Cell viability assay

The MTS assay (CellTiter 96Aqueous One Solution reagent; Promega) was used to evaluate cell viability as...
described previously (22, 32). Briefly, 2 × 10^5 cells per mL in 100 μL were exposed to various concentrations of SNS-032 for 72 hours. Control cells received DMSO for a final concentration the same as the highest concentration of SNS-032 but less than 0.1% v/v. Twenty microliters of the MTS solution per well was added 4 hours before culture termination. The absorbance was read on a 96-well plate reader at wavelength 490 nm.

**Cell-cycle analysis by flow cytometry**

After drug treatment, cells were collected, fixed, and stained with propidium iodide (PI; Sigma-Aldrich) according to standard protocols. Cell-cycle distribution was determined by use of a FACSCalibur flow cytometer equipped with CellQuest Pro software (Becton Dickinson; refs. 22, 32).

**Measurement of apoptosis by flow cytometry**

Apoptosis was measured by use of Annexin V-FITC (fluorescein isothiocyanate; for EOL-1, KBM5) or Annexin V-phycocerythrin (PE; for PDGFRα-positive BaF3 cells) apoptosis detection kits (BD Biosciences Pharmingen) and analyzed with use of a FACSCalibur flow cytometer and CellQuest Pro software as described (22, 32).

**RNA isolation and real-time quantitative RT-PCR**

Five micrograms of total RNA extracted with TRIzol (Invitrogen) was used for reverse transcription with the RNA PCR first-strand SuperScript Kit (Invitrogen). Then, 50 ng of total cDNA was used for real-time quantitative PCR (qRT-PCR) with the SYBR Premix Ex Taq Kit (TaKaRa). Each PCR reaction was carried out in a 20-μL volume on a 96-well optical reaction plate in Roche LightCycler 480. The relative gene expression was analyzed by the comparative C_t method with 18S ribosomal RNA used as an endogenous control after confirming that the efficiencies of the target and the endogenous control amplifications were approximately equal.

The specific primers for real-time PCR were FIP1L1-PDGFRα forward, 5'-GGG CAA ATG AGA ACA CCA GCA CCA-3' and reverse, 5'-CCA GAC CCG ACC AAG CAC TAG T-3'; Bcr-Abl forward, 5'-AAG CCC ACC AAG CCC ACT GTC TAT-3' and reverse, 5'-CTT CGT CTG AGA TAC TGG ATT CCT-3'; Mcl-1 forward, 5'-GAA AGC TGC ATC GAA CCA TT-3'; and reverse, 5'-ACA TTC CTG ATG CCA CCT TC-3'; 18S forward, 5'-AAA CGG CTA CCA CAT CCA AG-3' and reverse, 5'-CCCT CCA GTG CCT CGT TA-3'.

**Western blot analysis**

Western blot analysis, except for AIF and cytochrome c release detection, involved whole-cell lysates prepared in radioimmunoprecipitation assay (RIPA) buffer (1 × PBS, 1% w/v NP-40, 0.5% w/v sodium deoxycholate, 0.1% w/v SDS) supplemented with stock solutions to final concentrations of 10 mmol/L β-glycerophosphate, 1 mmol/L sodium orthovanadate, 10 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 × Roche Complete Protease Inhibitor Cocktail (Roche; ref. 32). The cytosolic fraction was prepared with digitonin extraction buffer [10 mmol/L PIPES (pH 6.8), 0.015% (w/v) digitonin, 300 mmol/L sucrose, 100 mmol/L NaCl, 3 mmol/L MgCl_2, 5 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride] as described previously to evaluate the level of AIF and cytochrome c in the cytosol (34).

**Clonogenicity assay**

BaF3 cells expressing wild-type or T674I FIP1L1-PDGFRα (2 × 10^5/mL) or KBM5 cells expressing wild-type or T315I Bcr-Abl were treated with increasing concentrations of SNS-032 or diluent (DMSO, control) for 24 hours and washed with PBS, then seeded in methylcellulose media (Cat# 03231; Stem Cell Technologies) for mouse cells (BaF3) or Iscove's medium containing 0.3% agar and 20% fetal calf serum (KBM5) in the absence of drug treatment, respectively. After incubation for approximately 7 days at 37°C in a tissue culture chamber, colonies of 50 or more cells were counted under an inverted phase-contrast microscope.

**siRNA transfection**

ON-TARGET plus SMART pool siRNA duplexes against human Mcl-1 and ON-TARGET plus Non-Targeting siRNAs (mock siRNA; cat# D-001810-0X) were synthesized by Dharmacon RNA Tech. Transfection of anti-Mcl-1 or the control siRNA duplexes into EOL-1 cells involved the Cell Line Nucleofector Kit T (Anaxa) and program O-17 according to the manufacturer’s instructions (20).

**Nude mouse xenograft model**

Nude nu/nu BALB/c mice were bred at the animal facility of Sun Yat-Sen University, Guangzhou, China. The mice were housed in barrier facilities with a 12-hour light–dark cycle, with food and water available ad libitum. A mixture of 1 × 10^7 of BaF3-T674I cells with Matrigel (BD Biosciences Pharmingen) or KBM5-T315I cells (3 × 10^7) were inoculated subcutaneously on the flanks of 4- to 6-week-old male nude mice (17). Tumors were measured every other day with use of calipers. Tumor volumes were calculated by the following formula: V = a^2 × b × 0.4, where a is the smallest diameter and b is the diameter perpendicular to a. Four days after subcutaneous inoculation, when tumors were palpable (~100 mm^3), mice were randomized to receive treatment with vehicle (tissue culture medium containing DMSO 0.1% v/v) or SNS-032 (15 mg/kg injected intraperitoneally every 2 days) for about 2 weeks. SNS-032 was dissolved in tissue culture grade DMSO before dilution. The body weight, feeding behavior, and motor activity of each animal were monitored as indicators of general health. The animals were then euthanized, and tumor xenografts were immediately removed, weighed, stored, and fixed. All animal studies were conducted with the approval of the Sun Yat-Sen University Institutional Animal Care and Use Committee.

**Immunohistochemical staining**

Formalin-fixed xenografts were embedded in paraffin and sectioned. Tumor xenograft sections (4 μm) were
Immunostained by use of the MaxVision Kit (Maixin Bio). After staining with the primary antibodies of rabbit anti-human Ki67 or c-Abl, MaxVision reagent (50 μL) was applied to each slide (17). Color was developed with 0.05% diaminobenzidine and 0.03% H2O2 in 50 mmol/L Tris-HCl (pH 7.6), and the slides were counterstained with hematoxylin. A negative control for Ki67 was included for each xenograft specimen by substituting the primary antibody with pre-immune goat serum.

Statistical analysis

All experiments were carried out at least 3 times, and results are expressed as mean ± 95% confidence intervals (CI) unless otherwise stated. Comparisons between 2 groups were analyzed by t test and between more than 2 groups by ANOVA with post hoc comparison by Tukey test. GraphPad Prism 5.0 (GraphPad Software) was used for statistical analysis. P < 0.05 was considered statistically significant.
Results

SNS-032 downregulates mRNA of PDGFRα and Bcr-Abl

Because SNS-032 is a selective inhibitor of CDK7 and 9, consequently disabling RNA pol II and gene transcription (35, 36), we first examined its effect on the expression of the addicted genes FIP1L1-PDGFRα (HES) and Bcr-Abl (CML). Exposing HES cells to increasing concentrations of SNS-032 or a fixed concentration of SNS-032 for various durations led to a concentration- and time-dependent decrease in phosphorylated RNA pol II at Ser2 and Ser5 (Fig. 1A) but not total protein. Similar results were obtained in CML cells (Fig. 1B). These findings are in accordance with the mechanism of action of SNS-032 inhibiting the activity of RNA pol II (27, 35, 37) by competing with ATP-binding pocket to CDK7 and 9 (35, 36). The levels of total and phosphorylated PDGFRα and Bcr-Abl were decreased concentration and time dependently (Fig. 1A and B). qRT-PCR revealed that SNS-032 decreased the mRNA levels of PDGFRα and Bcr-Abl concentration dependently in EOL-1, KBM5, and KBM5-T315I cells (Fig. 1C). Time course experiments showed that PDGFRα mRNA decreased after 3 hours and continued to decrease with 75 nmol/L SNS-032 (Fig. 1D). Therefore, SNS-032 treatment led to downregulated mRNA of PDGFRα and Bcr-Abl.

SNS-032 blocks FIP1L1-PDGFRα and Bcr-Abl downstream signaling

Because of the appreciably decreased levels of phosphorylated and total FIP1L1-PDGFRα and Bcr-Abl after SNS-032 treatment, we further examined their downstream signaling. SNS-032 concentration and time dependently decreased the basal phosphorylation levels of STAT3,
Figure 3. SNS-032 inhibits the growth of wild-type (WT) or mutant cells of HES and CML. A, SNS-032 inhibits the cell viability of HES and CML cells. HES or CML cells were exposed to increasing concentrations of SNS-032 for 72 hours and then underwent MTS assay. Data are mean ± 95% confidence intervals from 3 independent experiments. B, clonogenicity of BaF3 cells expressing WT or T674I FIP1L1-PDGFRα in methylcellulose and KBM5 cells with WT or T315I Bcr-Abl in soft agar was inhibited by SNS-032 in a concentration-dependent manner. Data are mean ± 95% confidence intervals. C, effect of SNS-032 on the distribution of cell-cycle phases in HES and CML cells. The indicated malignant hematologic cells were exposed to SNS-032 for 12 (EOL-1 cells) or 24 hours (BaF3 and KBM5 cells), respectively, and then analyzed by flow cytometry after staining with propidium iodide.
Figure 4. SNS-032 induces apoptosis in HES cells expressing FIP1L1-PDGFRα. A, EOL-1 cells (for 12 hours, top) and BaF3 cells expressing WT or T674I FIP1L1-PDGFRα (for 24 hours) were treated with SNS-032 at increasing concentrations (middle) or with 800 nmol/L SNS-032 for various durations (bottom), then underwent Annexin V staining for flow cytometry of cell death. Data are mean ± 95% confidence intervals from 3 independent experiments. ***, P < 0.01; ****, P < 0.0001, ANOVA with post hoc comparison with control by Tukey test. B, immunoblotting of PARP cleavage and caspase-3 activation in whole-cell lysates of HES cells treated with SNS-032 at different concentrations and for different durations. C, time course measurement of AIF and cytochrome c in cytosolic fraction of EOL-1 (150 nmol/L), BaF3-WT and BaF3-T674I (800 nmol/L) cells treated with SNS-032. D, effect of SNS-032 on apoptosis-related proteins. EOL-1 cells and BaF3 cells were treated with the indicated concentrations for 24 hours (top) or for various durations with a fixed concentration of SNS-032 (150 nmol/L for EOL-1, 800 nmol/L for BaF3, bottom); levels of XIAP, Mcl-1, Bax, Bim, and survivin were examined by immunoblotting in whole-cell extracts.
Erk1/2, and Akt in HES cells (Fig. 2A and B). The decrease in phosphorylated STAT3, Erk1/2, and Akt preceded the decrease in their total protein levels, suggesting that a decrease in the upstream signals from phosphorylated PDGFRα contributed to the decrease in the downstream signals of STAT3, Erk1/2, and Akt. Similarly, we discovered that the levels of phosphorylated downstream targets of Bcr-Abl were downregulated in SNS-032–treated CML cells (Fig. 2C). Thus, SNS-032 abolished the kinase activity of both PDGFRα and Bcr-Abl and activation of their downstream targets.

**SNS-032 inhibits the growth of PDGFRα- or Bcr-Abl–positive cells**

We next evaluated the effect of SNS-032 on viability of HES and CML cells. EOL-1, BaF3-WT, or T674I FIP1L1-PDGFRα cells were incubated with or without various concentrations of SNS-032 for 72 hours, and cell viability was evaluated by trypan blue exclusion. SNS-032 significantly decreased cell viability in a concentration-dependent manner in all cell lines (Fig. 3A). A 50% inhibitory concentration (IC50) was determined for each cell line, and SNS-032 showed an IC50 value of 75 nmol/L for EOL-1 cells, 300 nmol/L for BaF3-WT cells, and 1000 nmol/L for T674I FIP1L1-PDGFRα cells (Fig. 3B). These results indicate that SNS-032 effectively inhibited the growth of PDGFRα- or Bcr-Abl–positive cells.
viability, as measured by MTS assay, was potently decreased by SNS-032 in EOL-1 cells, wild-type- and T674I FIP1L1-PDGFRα–expressing BaF3 cells (IC50 = 135, 508, and 417 nmol/L, respectively; Fig. 3A, left). In KBM5 or KBM5-T315I cells, MTS assay showed that the IC50 values were 231 and 97 nmol/L, respectively (Fig. 3A, right). In independent experiments, we also assessed the effect of SNS-032 on clonogenicity of BaF3-WT or T674I FIP1L1-PDGFRα and KBM5 or KBM5-T315I cells. These cells were exposed to increasing concentrations of SNS-032 for 24 hours and then assayed for colony formation in the absence of drugs with the methods described in Materials and Methods. SNS-032 potently decreased the number of surviving clonogenic BaF3 and KBM5 cells in a dose-dependent manner [IC50 = 617 nmol/L (BaF3-WT), 508 nmol/L (BaF3-T674I), 58 nmol/L (KBM5), and 39 nmol/L (KBM5-T315I); Fig. 3B].

Flow cytometric analysis revealed no significant alteration in cell-cycle distribution in EOL-1, BaF3, and KBM5 cells treated with various concentrations of SNS-032 for 24 hours, except for the appearance of a sub-G1 apoptotic population (Fig. 3C). Therefore, SNS-032 potently inhibited the growth of EOL-1, T674I FIP1L1-PDGFRα–expressing BaF3, and KBM5-T315I cells without significantly affecting the cell cycle.

**SNS-032 induces apoptosis in HES and CML cells**

To confirm the capability of SNS-032 to induce apoptosis, control and SNS-032–treated HES and CML cells were assessed by flow cytometry after staining with Annexin V and PI or 7-AAD. After 12 hours of 150 nmol/L SNS-032 treatment, the population of apoptotic (Annexin V–positive) EOL-1 cells reached about 85% (Fig. 4A, top). SNS-032 induced a substantial apoptosis in BaF3-WT and BaF3-T674I in a concentration- and time-dependent manner (Fig. 4A, middle and bottom).

Similarly, the population of apoptotic cells was about 55% (KBM5) and 61% (KBM5-T315I) after incubating with 250 nmol/L SNS-032 for 24 hours (Supplementary Fig. S1A). As well, SNS-032 induced a concentration- and time-dependent specific cleavage of PARP (Fig. 4B) and caspase-8, -9, and -3 in HES cells. Similar results were also obtained in CML cells (Supplementary Fig. S1B). These data supported the occurrence of apoptosis in these malignant hematologic cells after SNS-032 treatment.

**SNS-032 leads to release of cytochrome c and downregulation of Mcl-1**

Mitochondrial depolarization is an early event during the onset of apoptosis. We therefore tested the release of AIF and cytochrome c from mitochondria into the cytosol. In both HES and CML cells exposed to SNS-032 for various durations, SNS-032 induced a substantial time-dependent release of AIF and cytochrome c into cytosol (Fig. 4C and Supplementary Fig. S1C), which implies that SNS-032 triggered the intrinsic (mitochondrial) pathway of apoptosis.

To elucidate the mechanism of SNS-032–induced apoptosis, we evaluated the expression of apoptosis-related proteins. Western blot analysis in HES cells revealed a substantial decrease in levels of Mcl-1 in a concentration- and time-dependent manner, with no alteration in levels of Bax, Bim, and survivin (Fig. 4D). The Mcl-1 protein levels were downregulated in CML cells as well (Supplementary Fig. S1D). An intriguing feature of SNS-032–induced change in Mcl-1 was the conversion of its precursor (42 kDa) to a 28-kDa proapoptotic fragment associated with the onset of apoptosis in EOL-1 cells (Fig. 4D, left).

**Mcl-1 is a critical regulator in SNS-032–mediated apoptosis in HES cells**

To examine the relative contribution of Mcl-1 in SNS-032–induced apoptosis, EOL-1 cells transfected with empty vector or the Mcl-1 plasmid were exposed to various concentrations of SNS-032 for 12 hours. The cells transfected with empty vector underwent extensive apoptosis with SNS-032 exposure, whereas Mcl-1–transfected cells showed no increase in apoptosis with SNS-032 exposure, as evaluated by specific cleavage of PARP and trypan blue dye exclusion (Fig. 5A, left). On the other hand, siRNA silencing of Mcl-1 significantly potentiated the capability of SNS-032 to induce apoptosis (Fig. 5A, right). Of note, silencing Mcl-1 in the absence of SNS-032 only slightly induced apoptosis. Therefore, Mcl-1 is a critical factor in SNS-032–mediated apoptosis in HES cells.

**SNS-032 leads to Mcl-1 downregulation by inhibiting its transcription and inducing its cleavage by caspase-3 activation**

We next investigated the mechanism of Mcl-1 downregulation in response to SNS-032 treatment by qRT-PCR. The mRNA level of Mcl-1 was decreased concentration and time dependently (Fig. 5B), which suggests that SNS-032 decreases the expression of Mcl-1 at the transcriptional level.
inhibits Mcl-1 at the transcription level. Because the half-life of Mcl-1 protein is relatively short (8), we assessed whether SNS-032 affected the turnover rate of Mcl-1 protein. We incubated EOL-1 cells with 100 nmol/L SNS-032 for 1 hour and then added the translation inhibitor cycloheximide. Western blot analysis revealed a progressive decrease in Mcl-1 protein level with cycloheximide treatment in control cells not treated with SNS-032 (Fig. 5C), and treatment with SNS-032 accelerated the decrease in Mcl-1 level, which suggests that SNS-032 accelerated the turnover of Mcl-1 protein in the absence of de novo protein synthesis.

We next examined whether the ubiquitin/proteasome pathway was involved in the increased turnover of Mcl-1 after SNS-032 treatment. Pharmacologic inhibition of proteasomes by MG132 pretreatment (30 minutes) and continual treatment did not abrogate the SNS-032–induced decrease in Mcl-1 level (Fig. 5D, left), indicating that downregulation of Mcl-1 does not depend on proteasome pathway.

Early reports showed that activation of caspase-3 by chemotherapeutic agents led to cleavage of Mcl-1 at Asp127, thus resulting in 2 fragments, 28-kDa Mcl-1128–350 and 17-kDa Mcl-11–127 (38). In contrast to the intact form of Mcl-1, Mcl-1128–350 has a proapoptotic function during apoptosis. Because of the time-dependent conversion of intact Mcl-1 into the proapoptotic form (Mcl-1128–350, 28 kDa; Fig. 4D, left), we suspected that Mcl-1 might be cleaved by the activated caspase-3. Pretreatment (30 minutes) and continual treatment with the caspase-3 inhibitor z-DEVD-fmk abrogated the conversion of intact Mcl-1 into Mcl-1128–350 induced by SNS-032 (Fig. 5D, right). The results suggested that caspase-3 may be the main effector of the accelerated decrease in Mcl-1 level in the absence of de novo protein synthesis with SNS-032 exposure.

**SNS-032 inhibits the growth of xenografted BaF3-T674I FIP1L1-PDGFRα or KBM5-T315I cells in nude mice**

We assessed the in vivo effect of SNS-032 with a nude mouse xenograft model. Four days after subcutaneous inoculation of BaF3-T674I cells or KBM5-T315I cells in mice, when tumors were palpable (~100 mm³), the mice were randomized to receive treatments with vehicle or SNS-032 for approximately 2 weeks. The growth curve (the estimated tumor size calculated by tumor dimension vs. time) was significantly inhibited by SNS-032 (Fig. 6A). The mean weight of tumors in the treated group was significantly lower than in the control group (P < 0.0001; Fig. 6B). The body weights of the mice were stable, with no significant differences between treated and control mice (data not shown).

Motor activity and feeding behavior were all normal.

The levels of RNA pol II, PDGFRα, Bcr-Abl, and downstream molecules were significantly lower in tumor tissues from mice treated with SNS-032 than in control treatment (Fig. 6C), so SNS-032 may abolish the kinase activity of PDGFRα and Bcr-Abl and activation of its downstream targets in vivo. Immunohistochemical analysis with an anti–Ki-67 antibody (to measure cell proliferation) revealed that SNS-032 decreased the proliferation of xenografted BaF3-T674I and KBM5-T315I cells (Fig. 6D). Immunohistochemical analysis with an anti–c-Abl antibody revealed that the c-Abl immunoreactivity was appreciably decreased after treatment with SNS-032 (Fig. 6D, right). Therefore, SNS-032 inhibited both xenografted BaF3-T674I cells and KBM5-T315I cells in vivo.

**Discussion**

Some cases of HES feature oncogene addiction of FIP1L1-PDGFRα, a chimaera formed by deletion of an 800-kb region on chromosome 4q12 (9). Most patients with CML were generated by Bcr-Abl, a fusion gene formed by a reciprocal chromosomal translocation t(9;22)(q34;q11) (39–41). Both patients with HES with FIP1L1-PDGFRα addiction and patients with CML generally respond to imatinib. However, acquired resistance to multiple TKIs that is mediated by the gatekeeper point mutations (e.g., T674I in FIP1L1-PDGFRα, T315I in Bcr-Abl) presents a challenge for therapy for HES and CML (7, 15).

New generations of TKIs have been designed (i.e., nilotinib, PKC412, dasatinib, sunitinib, and sorafenib). These inhibitors may have activity against the gatekeeper T674I mutant (42–45). For nilotinib, its effect on T674I FIP1L1-PDGFRα mutant is controversial. Nilotinib was found to inhibit BaF3 cells carrying T674I FIP1L1-PDGFRα (IC₅₀ = 376 nmol/L; ref. 46), but another report suggested that nilotinib was not active (42). Whether sunitinib has activity against the T674I mutation is also unknown. In CML treatment, nilotinib and dasatinib can effectively inhibit the activity of most point mutations (e.g., E255K, M351T) but T315I in Bcr-Abl (15, 28, 31).

To search for an alternative therapy for TK-resistant HES and CML, particularly those harboring T674I and T315I (gatekeeper mutations), we evaluated SNS-032, a potent inhibitor of CDK7 and 9. SNS-032 blocked transcriptional phosphorylation of RNA pol II by CDK7 and 9 (27). SNS-032 was originally designed as a selective, specific CDK inhibitor against CDK2, 7, and 9. The enzymatic activity assay showed that the IC₅₀ values against CDK2, 7, and 9 were 38 to 48, 62, and 4 nmol/L, respectively, whereas the IC₅₀ values against other kinases were more than 1,000 nmol/L (36). kinase-binding profiling analysis showed that SNS-032 binds with tyrosine kinases PDGFRα and c-Abl with IC₅₀ value more than 10 nmol/L (44, 47). Taken together, these reports support that SNS-032 does not directly inhibit the kinase activities of PDGFRα and Bcr-Abl. We confirmed the inhibitory effect of SNS-032 on transcription. In both HES and CML cells, SNS-032 significantly inhibited RNA polymerase level, thus decreasing levels of FIP1L1-PDGFRα and Bcr-Abl mRNA and protein regardless of the T674I or T315I mutation status (Fig. 1C). SNS-032 inhibited transcription at the endogenous FIP1L1-PDGFRα promoter in EOL-1 cells and the artificial promoter in the expression plasmid for FIP1L1-PDGFRα in BaF3 cells with equal potency. SNS-032 decreased the PDGFRα and Bcr-Abl protein levels, resulting in loss of kinase activities, as assayed by the phosphorylation of downstream targets.
Our *in vitro* and the mouse model results showed that SNS-032 significantly inhibits the growth of imatinib-resistant BaF3-T674I and KBM5-T315I cells. PDGFRα in the subset of PDGFRα-positive HES and Bcr-Abl in CML are typical models of oncogene addiction, and our previous reports have shown that silencing PDGFRα in both EOL-1 cells and BaF3 cells expressing FIP1L1-PDGFRα after transfection with siRNA duplexes induces apoptosis (20). Similar to triptolide (20), flavopiridol (11), and IPI-504 (18), SNS-032 may impact multiple molecules, but downregulation of PDGFRα and Bcr-Abl is likely the major factor to induce apoptosis in PDGFRα-positive HES cells and CML cells, respectively.

We identified a substantial role of Mcl-1 in SNS-032–induced apoptosis. Mcl-1 is often overexpressed in leukemic cells, and it protects malignant cells against apoptosis induced by chemotherapeutic agents such as flavopiridol, triptolide, and sorafenib (11, 20, 24, 40). Lowering Mcl-1 level by antisense oligonucleotides or siRNA can increase the sensitivity of leukemia cells to chemotherapy (41). Our qRT-PCR data showed that SNS-032 inhibited Mcl-1 at transcription levels. Time chase experiments showed that the turnover of Mcl-1 was only slightly accelerated and not responsible for the substantial decrease in Mcl-1 level with SNS-032 treatment. Analysis of posttranslational protein stability showed that the decrease in Mcl-1 level depended on activated caspase-3 but not the proteasomes. SNS-032 may induce the cleavage of intact Mcl-1 (42 kDa) at Asp127, thereby generating the Mcl-1 128–350 (28 kDa) fragment to potentiate apoptosis (38, 48). Thus, Mcl-1 generated by activated caspase-3 after SNS-032 treatment may provide positive feedback to apoptosis. Downregulation of Mcl-1 by SNS-032 likely contributes to apoptosis of EOL-1 cells. Silencing Mcl-1 by siRNA sensitized EOL-1 cells to SNS-032 (Fig. 5A, right); conversely, EOL-1 cells ectopically expressing Mcl-1 cDNA were resistant to SNS-032–induced apoptosis (Fig. 5A, left). Taken together, our results and those of others support a significant role of Mcl-1 in SNS-032–mediated apoptosis in leukemia cells (27). Together, our findings suggested that Mcl-1 mRNA level decreased by SNS-032 treatment may be the primary reason for the reduction of Mcl-1 protein, and the caspase-3–mediated Mcl-1 cleavage triggered by apoptosis may accelerate the process by offering a positive feedback.

In summary, SNS-032, a potent inhibitor of CDK7 and 9, which leads to inhibition of RNA pol II function, potently inhibits cell proliferation and induces apoptosis in resistant malignant cells expressing PDGFRα or Bcr-Abl. SNS-032 decreases both PDGFRα and Bcr-Abl mRNA levels and subsequent downstream signaling. It downregulates Mcl-1 and triggers the intrinsic apoptosis pathway. Caspase-3–mediated Mcl-1 cleavage may potentiate the SNS-032–induced apoptosis. SNS-032 inhibited the growth of xenografted cells expressing T674I FIP1L1-PDGFRα and the xenografted KBM5-T315I cells in nude mice. Therefore, inhibiting transcription with SNS-032 is a strategy to treat HES or CML, including TKI-resistant patients, that warrants further clinical investigation.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

**Conception and design:** Y. Wu, J. Pan  
**Development of methodology:** X. Shi  
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** Y. Wu, X. Sun, K. Ding, J. Pan  
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** C. Chen, S.-C. J. Yeung, J. Pan  
**Writing, review, and/or revision of the manuscript:** Y. Wu, S.-C. J. Yeung, J. Pan  
**Study supervision:** J. Pan  
**Carried out experiments:** Y. Wu, X. Shi, B. Jin  
**Reviewed data:** C. Chen

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**References**


10. Lierman E, Michaux L, Beultens E, Pierre P, Marynen P, Cools J, et al. FIP1L1-PDGFRalpha DB42, a novel panresistant mutant, emerging from a...
34. Pan J, Xu G, Yeung SC. Cytochrome c release is upstream to activation of caspase-9, caspase-6, and caspase-3 in the enhanced apoptosis of anaplastic thyroid cancer cells induced by manumycin and paclitaxel. J Clin Endocrinol Metab 2001;86:4731–40.
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