



SNS-032 Is a Potent and Selective Inhibitor of CDK2, 7 and 9 and Induces Cell Death by Inhibiting Cell Cycle Progression and the Expression of Antiapoptotic Proteins

SUNESIS

Samer Nuwayhid, David Stockett, Jenny Hyde, Alex Aleshin, Duncan H. Walker, Michelle R. Arkin

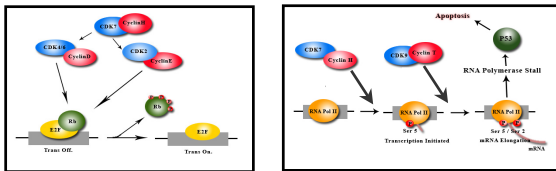
Sunesis Pharmaceuticals Inc, S San Francisco, CA

ABSTRACT

The cell cycle-regulated cyclin-dependent kinases (CDKs), CDK1, 2, and 4 have been extensively studied as potential therapeutic targets in cancer. Recent research has additionally underscored the potential role of several constitutively active CDKs including CDK7 and 9 as cancer targets. Phosphorylation of the c-terminal domain (CTD) of RNA Polymerase II by CDK7 and 9 are critical steps in transcriptional regulation. Inhibition of these kinases is predicted to have the greatest effect on the expression of proteins with short t_{1/2} and short-lived mRNA, including proteins involved in apoptotic regulation. CDK7 also activates cell-cycle CDKs 1, 2, 4 and 6. SNS-032 (formerly BMS-387032) has previously been described as a selective inhibitor of CDK2 with potent antitumor activity in animal models. Here we show that in addition to inhibition of CDK2, SNS-032 also inhibits CDK7/cyclinH and CDK9/cyclinT at low nanomolar concentrations in biochemical assays. The compound is highly selective for CDK inhibition; in a panel of 208 kinases, only four non-CDK proteins were inhibited by >50% at 1 μM SNS-032. The cellular pharmacology of SNS-032 mirrors the biochemical data. Cells treated with SNS-032 show a rapid cell cycle arrest and onset of cell death that corresponds with inhibition of multiple substrates of CDK2, 7, and 9. For instance, inhibition of Rb phosphorylation, accumulation of cyclin E protein and cell-cycle arrest at G1 and G2 are observed in multiple cell lines in a time and dose-dependent manner, consistent with inhibition of CDK2 and CDK7. Furthermore, SNS-032 inhibits CDK9-mediated phosphorylation of Ser2 in the CTD with an IC₅₀ = 200 nM. Corresponding with inhibition of RNA polymerase II, the short half-life, anti-apoptotic protein Mcl-1 is rapidly depleted from cells, coincident with the phosphorylation of p53. Expression of Mcl-1 is a candidate predictor of aggressive disease and resistance to chemotherapy in CLL, and is essential for survival of B-cell lymphoma and multiple myelomas, supporting the use of SNS-032 as a treatment for these diseases. SNS-032, a selective inhibitor of multiple CDKs involved in apoptosis and cell cycle regulation, has potential for antitumor activity in both solid and hematological cancers. SNS-032 is currently in phase 1 clinical studies.

BACKGROUND

SNS-032, was designed as a selective CDK2 inhibitor. Here, we show that in addition to CDK2, CDK 7 and 9 inhibitory activities also contribute to the biological activity of the molecule. The CDK2/cyclin E complex regulates entry of cells into S phase by phosphorylating Rb, a negative regulator of the transcription factor E2F. CDK2 phosphorylates a number of additional substrates, including cyclin E, signaling its degradation. Inhibiting CDK2 should therefore arrest cells in G1 and stabilize cyclin E. The cell-cycle CDKs (CDK1, 2 4 and 6) are activated by phosphorylation by CDK7/cyclin H (also called CAK). Inhibition of CDK7 would therefore also result in cell-cycle arrest at multiple points in the cell cycle due to failure to activate the cell cycle CDKs. CDK 7 and 9 activate transcription by phosphorylating the CTD of RNA pol II. Inhibition of CTD phosphorylation has been shown to inhibit transcription and reduce expression of short lived proteins, including those involved in apoptosis regulation. Stalling of RNA polymerase has also been shown to activate p53, leading to apoptosis. Thus, the CDK7 and 9 inhibitory activities of SNS-032 are expected to cause cytotoxicity via induction of apoptosis.



METHODS

Cell lines and Cell culture: HCT116 and RPMI8226 cell lines were obtained from ATCC. All cell lines were cultured in RPMI1640 (Cellgro) media supplemented with 10% FBS.
Westerns: Cell lysates (6-10 μg protein) were loaded onto 4-12% NuPage Bis-Tris gel and then transferred to nitrocellulose or PVDF membrane (western dependent) and probed using ¹²⁵I (RNA pol II ser 2, abcam #ab5095; RNA pol II ser 5, abcam #ab5131; total RNA pol II, Covance #MMS126R; Mcl-1, Cell Signaling #4572; p-53, Cell Signaling #9284, p-cdk2(t160), Cell Signaling #2561; cyclin E, Upstate #05363; β-actin, sigma #A2228) and 2° (HRP-goat anti-rabbit IgG, Zymed #626120; HRP-anti-mouse, Cell Signaling #7076)
Array Scan: HCT116 cells were treated for 16 hours with serial dilutions of SNS-032 and fixed and permeabilized with 100% MeOH. The cells were then stained with either anti-RNA polymerase II serine2 (Abcam #ab5095) or anti-RNA polymerase II serine5 (Abcam #ab5131) antibodies in combination with AlexaFluor 488 anti-rabbit IgG secondary antibody (Invitrogen #A11008). The cell nuclei were stained using Hoechst 33342 (Invitrogen #3570). Fluorescence levels in the cells were then analyzed by HCS using a Cellomics ArrayScan instrument.
MTT: Cells were plated at 4000 per well in a 96 well plate, incubated for 24 hours and then treated with compound. After treating cells for 72 hours, cells were incubated with 5% MTT for 1 hour and lysed. MTT was read at 595nm. Fraction of alive cells = [absorbance of sample well-avg(no cell control)] / [Avg(absorbance of DMSO only control)-avg(no cell control)].

SNS-032 Is Selective for Cdk2 and Displays Broad Cytotoxic Activity

Table 1. SNS-032 is a selective inhibitor of a subset of CDKs

Kinase	Ki (nM)	Kinase	Ki (nM)
cdk9/cyclin T	5	cdk5/p35	340
cdk2/cyclin E	20	cdk4/cyclin D	940
cdk7/cyclin H	60	gsk3α	230
cdk2/cyclin A	70	gsk3β	660
cdk1/cyclin B	280	cdk6/cyclin D	>1000
		198 other kinases	>1000

Table 2. Cytotoxicity of SNS-032.

Cell line	IC ₅₀ (nM)
A2780	39
HCT116	70
A549	43
WM2664	45

SNS-032 Inhibits Cell Cycle Progression

Cell Cycle Arrest by SNS-032 is consistent with CDK2 and 7 inhibition

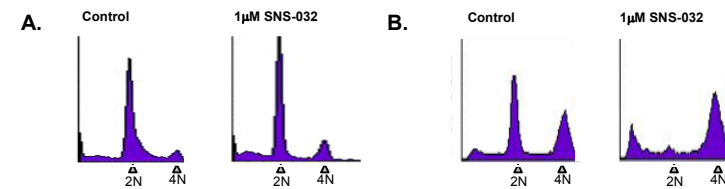


Figure 1. FACS analysis. Asynchronous HCT116 cells(A) or HCT116 cells synchronized with 5mM hydroxyurea (B) were treated with 1μM SNS-032 and incubated for 16 hours at 37°. Following treatment cells were fixed with 70% ethanol, stained with PI and analyzed for total DNA content.

Asynchronous HCT116 cells show G1 arrest and loss of S phase after treatment with SNS-032, consistent with CDK2 inhibition. Cells synchronized in S-phase show G2 arrest and apoptosis with 1μM SNS-032. This effect is consistent with inhibition of CDK7 resulting in failure to activate CDK1.

Treatment with SNS-032 decreases pCDK2 and stabilizes cyclin E

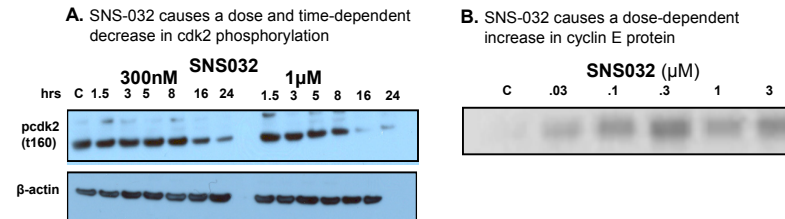


Figure 2. HCT116 cells were treated with 300 nM and 1 μM SNS-032 as indicated (A) or treated with increasing concentrations of SNS-032 for 8 hours (B). SNS-032 treatment of HCT-116 cells results in both a decrease in pCDK2 at Thr160 and an increase in cyclin E levels. These results are consistent with the inhibition of CDK2 through direct effects on CDK2 and indirectly through inhibition of CDK7

SNS-032 Inhibits CDK9 and Cdk7-mediated Phosphorylation of RNA Polymerase II CTD

SNS-032 induces a dose-dependent decrease in phosphorylation of RNA Pol II CTD at both Ser 2 and Ser 5

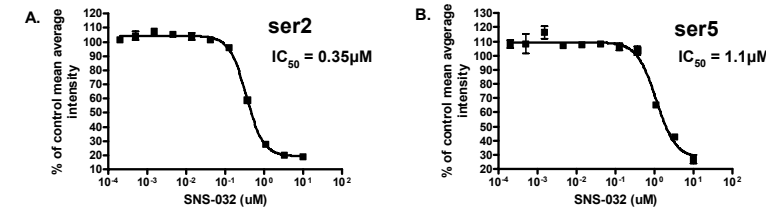


Figure 3. Panel A shows levels of p-ser2 in the CTD in HCT116 cells following 16 hours of treatment with serial dilutions of SNS-032. Panel B shows levels of p-ser5 in the CTD in HCT116 cells following 16 hours of treatment with serial dilutions of SNS-032. Phosphorylation levels in both figures are represented as a % phosphorylation relative to untreated cells.

SNS-032 induces a time-dependent decrease in RNA Pol II on both Ser 2 and Ser 5

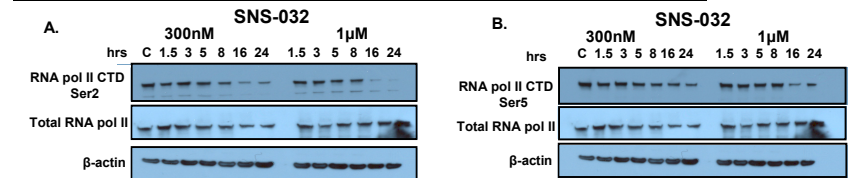


Figure 4. A timecourse of phosphorylation of RNA Pol II CTD ser 2 (panel A) and ser 5 (panel B) was determined by western blotting. Consistent with the biochemical data, SNS-032 more potently inhibits ser2 phosphorylation (substrate for CDK9).

SNS-032 Treatment Leads to Downregulation of Mcl-1 in RPMI8226 Cells

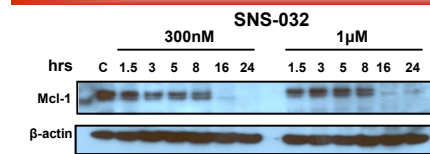


Figure 5. Western blots. RPMI8226 myeloma cells, which overexpress MCL-1, were treated with 300nM and 1μM SNS-032 for various time points as indicated. SNS-032 was able to induce both a time and dose-dependent decrease in MCL-1, consistent with inhibition of CDK9 and CDK7-dependent transcription of this short half-life protein

SUMMARY AND CONCLUSION

- SNS-032 is a selective CDK inhibitor, preferentially targeting CDK2, CDK7 and CDK9 in vitro.
- In cell models, SNS-032 shows dual activity, targeting both cell cycle progression and apoptosis pathway proteins.
 - SNS-032 Inhibited CDK9 and 7-mediated phosphorylation of ser 2 and ser 5 of the CTD of RNA pol II and in turn downregulates the antiapoptotic protein Mcl-1.
 - SNS-032 induced a cell cycle arrest, and increased cyclin E levels are consistent with inhibition of cell cycle CDKs
- Mcl-1 is a key survival factor in many B-cell malignancies. SNS-032 is being pursued as treatment for these diseases.