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ORIGINAL ARTICLE

SNS-032 is a potent and selective CDK 2, 7 and 9 inhibitor that drives target modulation in patient samples

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Abstract
Purpose SNS-032 (formerly BMS-387032) is a potent, selective inhibitor of cyclin-dependent kinases (CDK) 2, 7 and 9, currently in phase 1 clinical trial for chronic lymphocytic leukemia (CLL) and multiple myeloma (MM). We used the MM cell line RPMI-8226 to evaluate the relationship between duration of SNS-032 exposure, target modulation of CDKs 2, 7 and 9, and induction of apoptosis. We also assessed target modulation in patient peripheral blood mononuclear cells (PBMCs) from phase 1 solid tumor patients treated with SNS-032.
Methods Proliferation and colony forming assays were used to evaluate cytotoxicity, Western blot analyses to evaluate target modulation, FACS analysis to assess cell cycle distribution, RT-PCR to evaluate transcriptional inhibition.
Results SNS-032 blocks the cell cycle via inhibition of CDKs 2 and 7, and transcription via inhibition of CDKs 7 and 9. Treatment of RPMI-8226 MM cells at 300 nM (IC_{90}) for 6 h was sufficient for commitment to apoptosis. This correlated with inhibition of CDKs 2, 7 and 9, as reflected in substrate signaling molecules. SNS-032 activity was unaffected by human serum. Target modulation was observed in PBMC from treated patients.
Conclusions These results demonstrate SNS-032 target modulation of CDKs 2, 7 and 9, and establish 6 h exposure as sufficient to commit RPMI-8226 MM cells to apoptosis. Combined with the demonstration of target modulation in PBMC from phase 1 solid tumor patients treated with SNS-032, these data support the ongoing clinical study of SNS-032 in MM and CLL.

Keywords SNS-032 · Chronic lymphocytic leukemia · Multiple myeloma · Cyclin-dependent kinase · Transcription · Survival factors

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Introduction

Cancer is a disease characterized by deregulated cell proliferation and prolonged cell survival, processes key to the development and maintenance of tumors. The signaling networks driving these processes remain the focus of extensive research aimed at identifying new cancer therapeutics. Cyclin dependent kinases (CDKs) act in concert with their activating cyclin partners and subunit inhibitors to control cellular proliferation and transcription, and are potential targets for anticancer therapy [1–3].

Targeting transcription to treat cancers requires that the drug have suitable pharmacologic properties to enable short-term blockade of the transcriptional machinery, otherwise unacceptable toxicities would be anticipated. Cancers that are dependent on short-lived transcripts and proteins such as survival signaling proteins, cytokines and growth factors might therefore be most sensitive to agents that block transcription, as cells that depend on these proteins are susceptible to an induced "oncogenic shock" [4]. Multiple

lines of evidence suggest that hematologic malignancies are highly dependent on survival signaling proteins, and disrupting the relative balance of pro-survival and pro-apoptotic proteins is sufficient to drive these cells into apoptosis [5–9]. Intermittent dosing of agents that transiently down-regulate survival signaling proteins may selectively cause apoptosis in tumor cells that are maintained by the aberrant expression of these proteins, while sparing normal cells in which the survival/apoptotic balance is correctly regulated. As a potent and specific inhibitor of CDKs 2, 7 and 9, SNS-032 is ideally positioned to test this hypothesis.

Most CDK family members function as direct regulators of specific phases of the cell cycle. Their activation is required for progression through key cell cycle checkpoints, and their in vitro inhibition causes arrest at the G1/S or G2/M boundaries [10]. The CDK2/cyclin E complex regulates entry of cells into S phase through phosphorylation of Rb, which releases the transcription factor E2F from the Rb–E2F complex to promote S phase entry [11]. The subsequent activation of CDK2/cyclin A is then responsible for S phase progression. As the cell progresses into S-phase, the phosphorylation of cyclin E by CDK2 leads to its degradation. In contrast to cyclin E, the phosphorylation of Cdc6 (a key factor in pre-replication complex assembly and the regulation of DNA replication) by CDK2 prevents proteolytic degradation of the protein [12]. The CDK7/cyclin H complex (also known as cyclin activating kinase or CAK) is the master regulator of the cell cycle; full activation of the CDKs that control the cell cycle requires phosphorylation by CDK7 [13, 14].

In addition to its pivotal role in the cell cycle, CDK7 also functions as a transcriptional regulator. As part of the TFIIH complex (CDK7/cyclin H/Mat1), CDK7 phosphorylates the C-terminal domain (CTD) of RNA polymerase II (RNA pol II) at serine 5 (pSer5), thereby activating the polymerase for transcriptional initiation [15–17]. Transcriptional elongation is controlled by another CDK family member, CDK9. CDK9/cyclin T (pTEFb) phosphyorylates RNA pol II at serine 2 (pSer2) in the CTD. Therefore, the phosphorylation of RNA pol II CTD by CDK7 and 9 is critical for transcription and sustained expression of short half-life proteins and transcripts [18, 19].

The molecular events that distinguish the activities of CDKs 2, 7 and 9 can be leveraged as pharmacodynamic biomarkers for SNS-032. CDK2 inhibition can be assessed by evaluating levels of cyclin E, Cdc6 or phosphorylation of Rb. Decreased phosphorylation of pSer5 and pSer2 of RNA pol II CTD would indicate inhibition of CDKs 7 and 9, respectively. Coupling assessment of pharmacodynamic biomarkers with phenotypic outcome—namely inhibition of proliferation, down regulation of transcripts and proteins, and apoptosis—forms a temporal link between the two.

SNS-032 has previously been described as a selective inhibitor of CDK2 with potent anti-tumor activity in animal models [20]. The studies reported here reveal that SNS-032 also inhibits CDK7/cyclinH and CDK9/cyclinT at low nanomolar concentrations in biochemical assays. SNS-032 is currently in a phase 1 clinical study in chronic lymphocytic leukemia (CLL) and multiple myeloma (MM), both B cell malignancies in which signaling through short-lived proteins is key to disease maintenance and progression [21–23]. The dose administration schedule in this phase 1 study comprises a 15 min loading dose followed by a 6 h intravenous infusion. The pharmacodynamic studies reported here are modeled after this dosing regimen. Mechanism-based target modulation of CDKs 2, 7 and 9 resulting in inhibition of cell cycle progression and transcription, followed by apoptosis, is demonstrated in cellular assays. Target modulation in clinical samples was observed, through modulation of pharmacodynamic biomarkers, in peripheral blood mononuclear cells (PBMC) isolated from patients treated with SNS-032 in a previously completed phase 1 trial in solid tumors.

Materials and methods

Drugs

SNS-032 was provided as a 13 mM stock dissolved in 2.1 mM L-tartaric acid (pH 4.0) containing 0.9% sodium chloride. Flavopiridol was provided as a 10 mM stock dissolved in DMSO. Stock solutions were diluted in DMSO.

Compound profiling

Biochemical screening was performed using KinaseProfiler™ (Millipore, Billerica, MA).

Cells and cell culture

HCT116 human colon carcinoma and RPMI-8226 MM cell lines were obtained from American Type Culture Collection, Manassas, VA, USA. RPMI-8226 cells were grown in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS) or 10% human serum (HS) (Mediatech Inc., Manassas, VA, USA). The HCT116 cells were grown in DMEM media supplemented with 10% FBS.

Isolation of human PBMC

Patient-derived PBMC were obtained with informed consent according to IRB procedures from patients with advanced solid malignancies as part of a phase 1 dose-escalation study. Patients received a 1 h i.v. infusion of

SNS-032 daily × 5 every 21 days. Blood samples were obtained pre-infusion (PI), at the completion of infusion (CI) and 4 h post start of infusion. Whole blood was collected into Cell Preparation Tubes™ (BD, Franklin Lakes, NJ, USA) containing sodium heparin and kept at room temperature (for a maximum of 3 h) until processed. Tubes were centrifuged at room temperature for 20 min at 1,700×*g*. The PBMC/plasma layer was poured into a sterile 50 mL polypropylene tube and centrifuged at 4°C for 10 min at 300 ×*g*. The plasma layer was removed and the pelleted PBMC washed once in 10 mL 4°C PBS (Cellgro). PBMC were centrifuged at 4°C for 10 min at 300×*g* and the PBS was removed. PBMC were resuspended in 1 mL 4°C PBS, transferred to a 1.5 mL microcentrifuge tube, and centrifuged at 4°C for 5 min at 3,000×*g*. The PBS was removed and the PBMC pellet snap-frozen in a dry-ice ethanol slurry and stored at −80°C until analysis.

Cell cycle analysis

HCT116 cells were treated with 30 nM, 300 nM or 3 μM SNS-032 for 24 h. Adherent cells were detached with 0.1% trypsin–EDTA solution, combined with floating cells, and centrifuged at 4°C for 10 min at 300×*g*. Cell pellets were washed with 4°C phosphate buffered saline (PBS) and fixed in 70% ethanol. Cells were centrifuged as above and the pellet washed with 4°C PBS containing 1% bovine serum albumin (BSA). Cells were incubated in PBS containing 1% BSA, 10 μg/mL propidium iodide (PI) (Invitrogen, Carlsbad, CA, USA), 100 μg/mL RNase A (Sigma-Aldrich, St. Louis, MO, USA), and 0.1% Triton X-100. Cells were analyzed by FACS (BD, Franklin Lakes, NJ, USA) for total DNA content.

Western blot analysis

Cell pellets were washed once in 4°C PBS and lysed in 1× RIPA buffer (Cell Signaling Technology, Danvers, MA, USA) containing phosphatase (Sigma-Aldrich) and protease inhibitors (Roche Applied Science, Indianapolis, IN, USA). Lysates were normalized for total protein content and 25 μg of protein was mixed with sample loading buffer (Invitrogen, Carlsbad, CA, USA). Samples were elecropheresed on 4–12% or 8–20% Tris-glycine NuPage gels (Invitrogen). Gels were transferred to nitrocellulose membrane and blocked using tris-buffered saline containing 0.1% Tween and 5% nonfat dry milk. After primary and secondary antibody incubations, blots were resolved using the ECL plus western blotting development kit (Amersham Biosciences, Piscataway, NJ, USA). Images were acquired using a flatbed scanner (Epson, Long Beach, CA, USA).

Antibodies

Primary antibodies were RNA polymerase II CTD pSer 2, RNA polymerase II CTD pSer 5, total RNA polymerase II, Cdc6 (Abcam Inc., Cambridge, MA, USA), Mcl-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin E, total PARP (Cell Signaling Technology), *β*-actin (Sigma-Aldrich). Secondary antibodies conjugated with HRP were anti-mouse (Cell Signaling Technology), anti-rabbit (Zymed Laboratories, South San Francisco, CA, USA).

Cellular RNA Pol II CTD Ser2 and Ser5 phosphorylation assay

RPMI-8226 cells were plated in a poly-L-lysine 96-well plate and treated for 16 h with a titration of SNS-032 ranging from 0.17 nM–10 μM or with 0.1% DMSO control. Cells were fixed with 3.7% formaldehyde and permeabilized with 100% ice-cold methanol. After blocking with 5% BSA in PBS, cells were probed with either anti-phospho-RNA Pol II CTD Ser2 or Ser5 antibodies. Cells were washed with PBS and stained for 1 h with anti-rabbit AlexaFluor 488 secondary antibody and with Hoechst 33342 (Invitrogen) nuclear stain. Phosphorylation levels in the cells were measured by immunofluorescence using a Cellomics ArrayScan (Thermo Scientific, Pittsburg, PA, USA) high content screening device.

Colony formation assay

RPMI-8226 cells were treated with SNS-032 or 0.1% DMSO control for up to 24 h. Cells were harvested, washed, re-plated in poly-L-lysine 96-well and grown for an additional 7 days. Colonies were counted using a Cellomics ArrayScan (Thermo Scientific).

Apoptosis assay

Apoptosis was assessed using the annexin V/propidium iodide assay (BD) and the Terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) assay per manufacturer's instructions. Levels of apoptosis were analyzed by FACS.

Real-time reverse transcription-PCR (RT-PCR)

RNA was isolated from treated cells using the RNAqueous 4PCR kit (Ambion Inc., Austin, TX, USA) and cDNA synthesized using the cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). cDNA samples were analyzed by RT-PCR analysis by standard methods with a TaqMan device and gene expression kits (Applied Biosystems). Relative Quantification (RQ) was

calculated as a ratio of transcript levels in compound-treated cells compared to vehicle control. All samples were normalized to 18S rRNA, an RNA polymerase I transcript that is not modulated by inhibition of RNA polymerase II.

Measurement of cell proliferation by MTT

Cells were plated in 96-well plates and treated with SNS-032 or DMSO control for 16 h. Cells were washed and incubated in fresh media for an additional 72 h. MTT reagent was added directly to the media and incubated for 1.5 h. MTT lysis buffer was added and incubated overnight. Samples were analyzed at 595 nM using the SpectraMax plate reader (Molecular Devices, Sunnyvale, CA, USA).

Results and discussion

An understanding of disease pathogenesis at the molecular level allows for the identification of novel targeted therapeutic approaches, and provides the opportunity to assess mechanism-based pharmacodynamic activity from discovery through development. The identification of SNS-032 as a potential therapeutic for MM and CLL links the unique and selective target kinase inhibition profile of the molecule to the pathology of these diseases.

SNS-032 is a potent, selective inhibitor of CDKs 2, 7 and 9

Profiling SNS-032 for inhibitory activity against 200 kinases indicated that SNS-032 is a potent and selective inhibitor of CDKs 2, 7 and 9 (Table 1). The inhibitory profile of SNS-032 was highly selective for potent inhibition of CDKs 2, 7 and 9 (IC_{50} 38-48, 62 and 4 nM, respectively) and modest inhibitory activity against CDKs 4 and 5 (925 and 340 nM, respectively), as well as against GSK3α and β (230 and 660 nM, respectively). SNS-032 showed no activity against 190 additional human kinases (IC_{50} > 1,000 nM). Other CDK inhibitors are currently under evaluation in the clinic, including flavopiridol, seliciclib and AT7519. Based on published data, SNS-032 is both more potent than seliciclib and more selective than flavopiridol [1, 24–28]. Compared to AT7519, SNS-032 is more selective, has similar potency for CDK2 and markedly greater potency for CDK 7 and CDK9 [29]. Based on the relative potency of CDK9 inhibition, SNS-032 is likely a more effective inhibitor of transcription than AT7519.

SNS-032-treated cells show target modulation consistent with CDK2 inhibition

SNS-032-mediated cellular inhibition of CDK2 in RPMI-8226 cells was demonstrated by analysis of cyclin E and cdc6 levels during exposure to SNS-032, and following 6 h exposure, washout and further incubation for 2 h. A dose-dependent stabilization of cyclin E was observed by 4 h (Fig. 1a) that persisted for at least 2 h post-washout (Fig. 1b). Concomitant decrease in Cdc6 was demonstrated by reduction in full-length protein at 4 h (Fig. 1a). As for cyclin E, this effect continued to 2 h post-washout (Fig. 1b).

SNS-032 causes G2 arrest in HCT116 cells

HCT116 colon carcinoma cells were treated with 300 nM (IC_{70}) SNS-032 for 24 h to determine the effect on the cell cycle. HCT116 cells were less sensitive to the cytotoxic effects of SNS-032 than RPMI-8226, allowing analysis of

Table 1 Enzymatic IC_{50} against a panel of serine/threonine and tyrosine kinases, assessed for inhibition by SNS-032, flavopiridol, seliciclib and AT5719

Kinase	SNS-032 IC50 nM	Flavopiridol IC50 nM	Seliciclib IC50 nM	AT7519 IC50 nM
CDK2/Cyclin A	**38**	**100**	710	**44**
CDK2/Cyclin E	**48**	170	**100**	510
CDK7/Cyclin H	**62**	~300	490	2,800
CDK9/Cyclin T	**4**	**6**	600	**<100**
CDK 5/p35	340	~100	160	**18**
CDK1/Cyclin B	480	41 (K_{iapp})[a]	2,700	190
CDK4/Cyclin D	925	65 (K_{iapp})[a]	>10 μM	67
CDK6/Cyclin D	>1,000	~100	>100 μM	660
GSK3α	230			
GSK3β	660			98
190 additional human kinases	>1,000	PKCδ 890 PKCε 480	DYRK1A 3100 ERK2 1170	Reported >1,000 for 17 kinases

Data in bold represent $IC_{50} \leq 100$ nM

[a] Apparent K_i

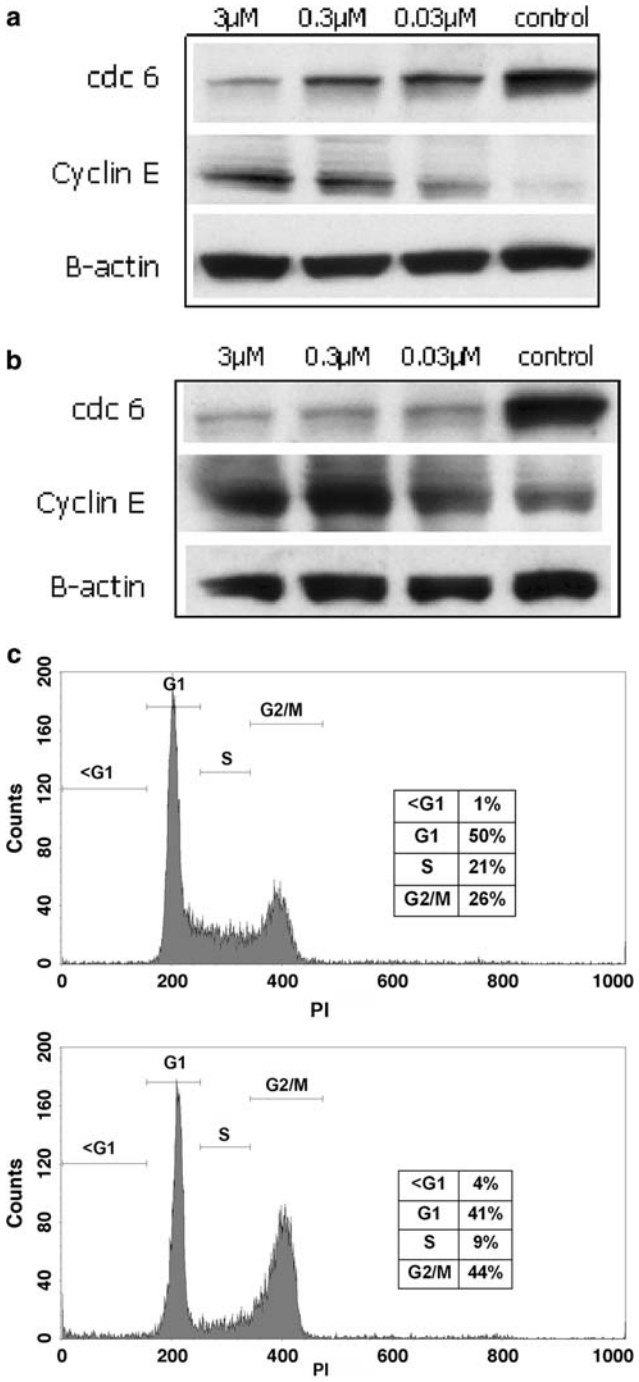

Fig. 1 Modulation of cellular CDK2 activity by SNS-032. **a** Inhibition of CDK2 activity was observed by a dose-dependent stabilization of cyclin E and a decrease in Cdc6 in RPMI-8226 MM cells treated with SNS-032 for 4 h. **b** Effects were sustained following 6 h of treatment, washout of drug and 2 additional hours of incubation. **c** SNS-032 arrested cell cycle progression at G2/M in asynchronous HCT116 cells treated for 24 h with 300 nM SNS-032 compared to 0.1% DMSO control

cell cycle progression not confounded by apoptosis. Accumulation at the G2/M boundary occurred (Fig. 1c), likely by SNS-032 blocking CDK7-induced activation of CDK1.

SNS-032 inhibits phosphorylation of RNA polymerase II CTD and induces down-regulation of short half-life proteins

Phosphorylation of Ser5 and Ser2 of RNA Pol II CTD was measured as a read-out of CDK7 and CDK9 activity, respectively, in RPMI-8226 MM cells treated with SNS-032. Cellular IC_{50} values for SNS-032 of 231 nM and 192 nM were identified for inhibition of CDK7 and CDK9, respectively (Fig. 2a, b).

Transiently inhibiting transcription affects proteins and transcripts with short half-lives, including survival signaling proteins such as Mcl-1 and XIAP, cell cycle related proteins such as D type cyclins and cytokines and growth factors such as VEGF. The effects of SNS-032 on these proteins were studied in RPMI-8226 MM cells. CDK9 inhibition and PARP cleavage were also evaluated, to establish a temporal link between CDK inhibition, down-modulation of survival factors, and apoptosis. As shown in Fig. 2c, CDK9 inhibition was demonstrated within 2 h of treatment by decreased phosphorylation of Ser2 on RNA pol II CTD. Transcripts (evaluated by RT-PCR) encoding VEGF, XIAP and Mcl-1 were most sensitive to SNS-032-mediated transcriptional inhibition (Fig. 2d). Cyclin D1 and D2 transcripts were more stable, although down-regulation was observed (Fig. 2d). Survival signaling proteins Mcl-1 and XIAP were decreased (Fig. 2e), becoming undetectable after 6 h of exposure. Bcl-2 protein remained constant, consistent with the long half-life of the Bcl-2 protein [30, 31]. CDK9 target inhibition correlated temporally with the induction of PARP cleavage, indicative of apoptosis. At 6 h only cleaved PARP, with no intact PARP, was detected (Fig. 2c).

SNS-032 inhibition of proliferation, colony formation and induction of apoptosis correlates with CDK 2, 7 and 9 target modulation

The SNS-032-induced pharmacodynamic changes were correlated with phenotypic effects of inhibition in both proliferation and colony formation assays. A proliferation IC_{50} of 148 nM was established for SNS-032 in RPMI-8226 MM cells (Fig. 3a) using an MTT assay. Essentially complete inhibition of colony formation was observed following 8 h of SNS-032 exposure, while 4 h exposure was partially inhibitory to colony growth (Fig. 3b).

The induction of apoptosis was analyzed by annexin V staining for early stage apoptosis (Fig. 3c) and by TUNEL staining to detect DNA fragmentation for late-stage apoptosis (Fig. 3d). Six hour treatment was studied to support the 6 h pharmacologically-derived infusion regimen [32] employed for SNS-032 in the ongoing phase 1 clinical trial. Apoptosis was observed within 2–4 h of SNS-032 treatment

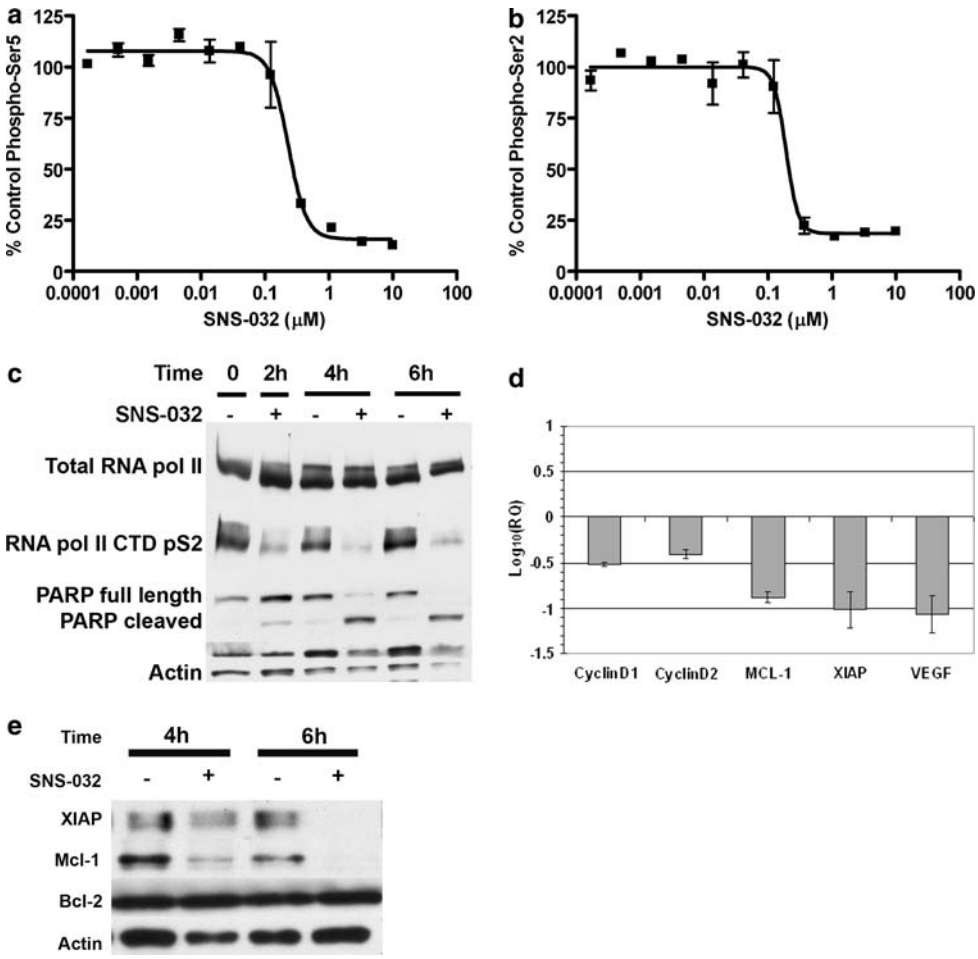

Fig. 2 Modulation of cellular CDK7 and CDK9 activity by SNS-032. Cellular inhibition of CDK7 (**a**) and CDK9 (**b**) was demonstrated in RPMI-8226 MM cells by assessing decreased pSer5 or pSer2 of RNA Pol II-CTD, respectively. Cells were treated with 0.17 nM–10 μM SNS-032 for 16 h and compared to 0.1% DMSO treated control cells. SNS-032 cellular IC_{50} were 231 nM (CDK7) and 192 nM (CDK9). Cellular inhibition of transcriptional CDKs correlated with decreases in short half-life survival proteins and apoptosis. (**c**) RPMI-8226 MM cells treated with 300 nM SNS-032 for up to 6 h showed decreases of pSer2 RNA Pol II-CTD and increase in cleaved PARP. **d** Decreases in transcripts for cyclins D1 and D2, Mcl-1, XIAP and VEGF were quantified by RT-PCR after 300 nM SNS-032 treatment for 6 h. Transcripts were normalized to 18S RNA levels. **e** Decreased survival signalling protein levels were observed for the short-lived XIAP and Mcl-1, but not for the more stable Bcl-2 [30, 31]

and progressed following washout of the drug at 6 h (Fig. 3c, d). Thus, 6 h treatment at 300 nM is sufficient to commit RPMI-8226 MM cells to apoptosis.

SNS-032 activity is unaffected by human serum, in contrast to flavopiridol

SNS-032 has consistent low-moderate plasma protein binding across species (62–69%) [20] while flavopiridol is highly protein bound in human plasma but not in bovine plasma [32, 33]. The impact of plasma protein binding on the cellular activity of flavopiridol and SNS-032 was evaluated in a cell proliferation assay, in the presence of either 10% human or bovine serum. The activity of SNS-032 was similar in either human or bovine serum (Fig. 4a); in contrast, the activity of flavopiridol is reduced by >60% in the presence of 10% human serum (Fig. 4b). In the presence of human serum, SNS-032 was 5-fold more potent than flavopiridol. The high human plasma protein binding of flavopiridol may explain the lack of consistent evidence of mechanism-based target modulation [33, 34].

Pharmacodynamic activity is observed in PBMC from patients treated with SNS-032

Patient PBMC collected in a phase 1 study of SNS-032 in advanced solid tumors were evaluated for evidence of CDK7 and 9 inhibition and down-regulation of Mcl-1 [35]. The PBMC from patients administered lower doses of drug showed evidence of a compensatory signaling response, as represented in Fig. 5a (similar data were obtained at doses of 6 and 12 mg/m^2) [36]. At higher doses, as shown in Fig. 5b–d, dose-dependent modulation of CDK7 and CDK9 (pSer5 and pSer2 RNA pol II CTD) and down-regulation of Mcl-1 were detected. The inhibition of CDK9 appeared to be greater than that of CDK7, correlating with SNS-032 lower biochemical IC_{50} for CDK9. This is consistent with data obtained in PBMC from CLL patients treated in the ongoing phase 1 clinical trial of SNS-032 [37]. Dose-dependent down-modulation of actin was observed on Day 1, suggesting global inhibition of RNA pol II-mediated transcription by SNS-032.

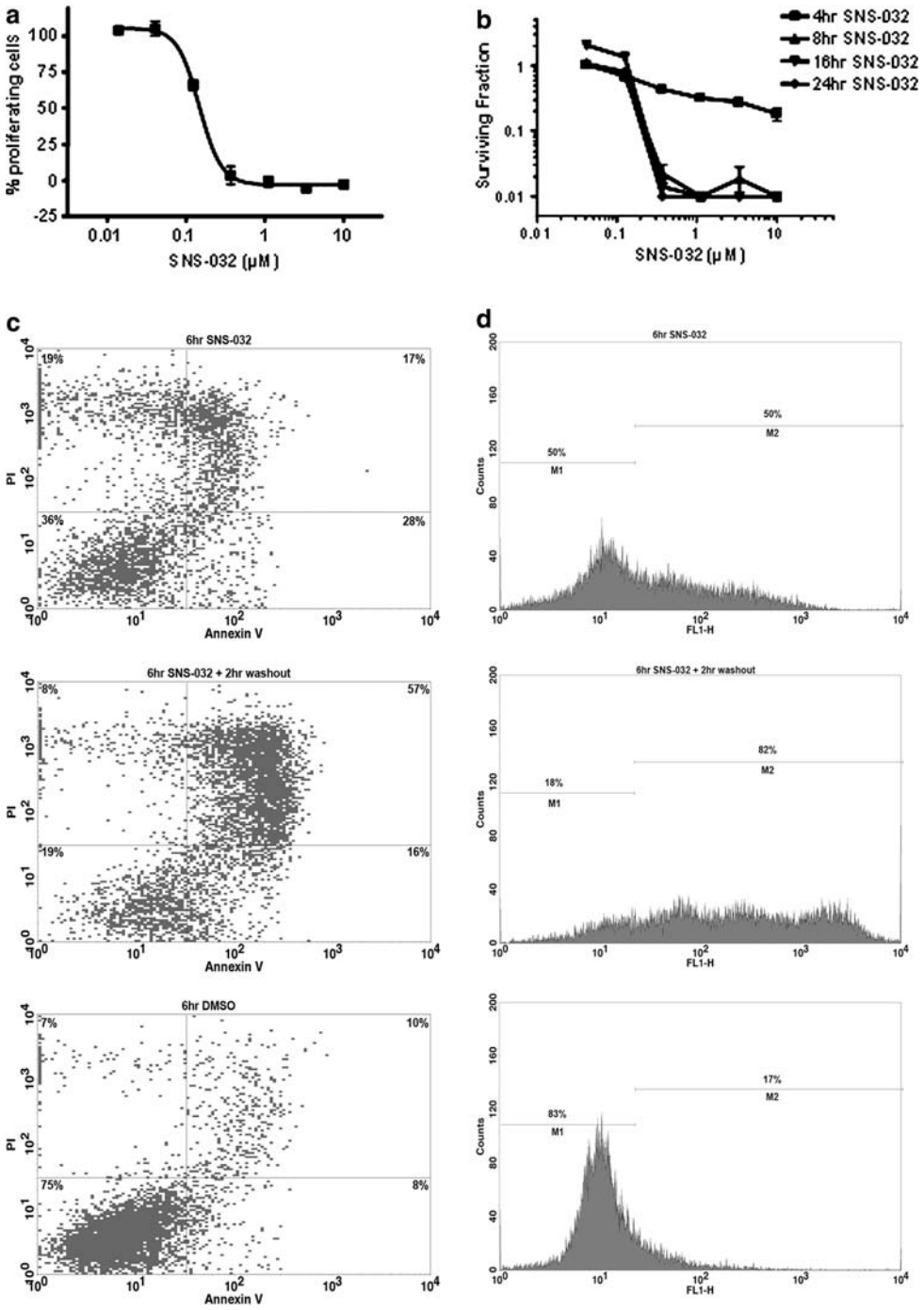

Fig. 3 Phenotypic effects of SNS-032 treatment in RPMI-8226 MM cells. Inhibition of **a** proliferation and **b** colony formation was dose- and time-dependent. For the proliferation assay, cells were treated for 16 h, SNS-032 removed and incubation continued for 72 h before MTT analysis. The proliferation IC_{90} was 275 nM. Colony growth inhibition was evaluated in cells treated with a dose-titration of SNS-032 for 4, 8, 16 or 24 h, followed by washout and growth for 7 days. Maximal inhibition was observed by 8 h SNS-032 exposure. **c** Apoptosis was evaluated in RPMI-8226 MM cells by annexin V binding and combined annexin V bindin/PI staining (*lower and upper right quadrants*, respectively). After 6 h 300 nM SNS-032 treatment, apoptotic cells increased from 18 to 45%. Six hour SNS-032 exposure was sufficient to commit cells to apoptosis, as shown by the increase in apoptotic cells to 73% in cells treated for 6 h and incubated for an additional 2 h without drug. **d** Similar results were observed by TUNEL staining. Apoptotic cells were defined by TUNEL staining indicated by M2. Apoptosis increased from 17% in control cells to 82% in cells treated for 6 h with SNS-032, followed by washout and further incubation for 2 h

Conclusions

The molecular signature of SNS-032 resulted in cellular effects leading to apoptosis. This allowed the identification and investigation of pharmacodynamic markers that demonstrated mechanism-based target modulation and regulation of disease-relevant proteins by SNS-032. Studies of SNS-032 in RPMI-8226 MM cells showed a dose- and time-dependent de-stabilization of Cdc6, stabilization of cyclin E (markers of CDK2 inhibition) and inhibition of pSer5 and pSer2 of RNA Pol II CTD (markers of CDK7 and 9 inhibition, respectively). Down-regulation of transcripts encoding survival signaling proteins, cyclins D1 and D2 and VEGF occurred within 4–6 h of exposure. As a consequence of transcriptional inhibition, down-regulation of the survival signaling proteins Mcl-1 and XIAP occurred within this same time frame, whereas the level of Bcl-2 remained constant, consistent with the stability of Bcl-2 protein [30, 31]. Apoptosis occurred after a relatively short exposure to SNS-032. These data were leveraged in the

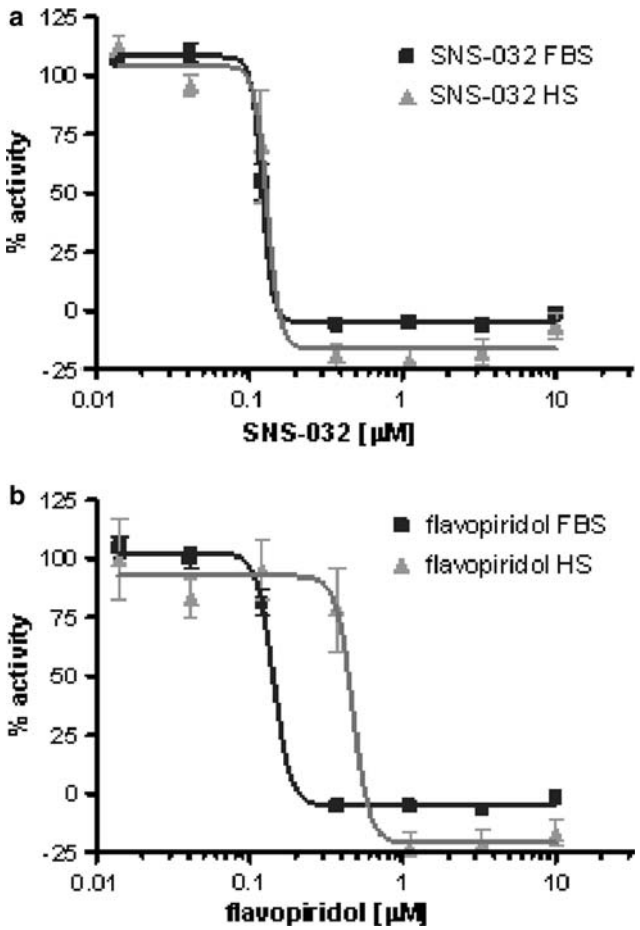

Fig. 4 The effect of human plasma protein binding on SNS-032 and flavopiridol activity. Proliferation assays were performed in RPMI-8226 MM cells incubated in either 10% FBS or 10% HS. **a** SNS-032 activity was unaffected, with cellular IC_{50} of 111 nM and 79 nM in FBS versus HS, respectively. **b** Flavopiridol activity decreased in HS as the cellular IC_{50} increased from 140 to 394 nM

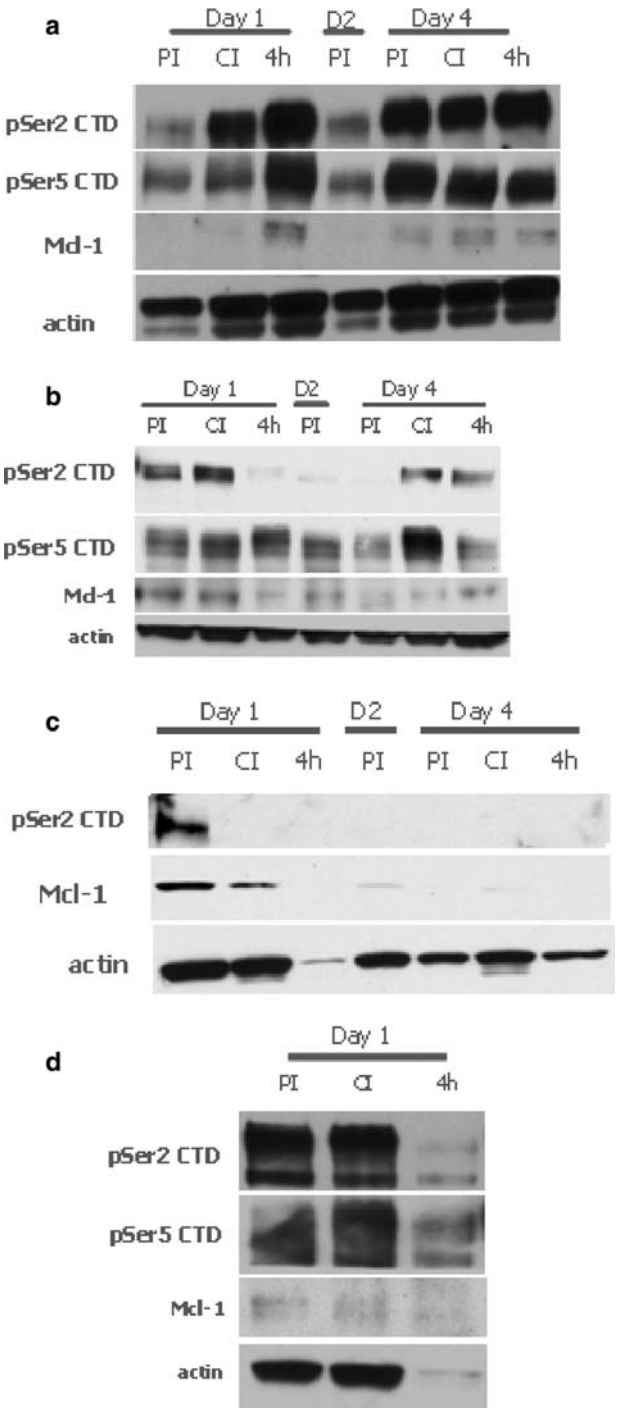

Fig. 5 Dose-dependent modulation of CDK 2, 7, and 9 regulated proteins was observed in PBMC from SNS-032 treated advanced solid tumor patients. PBMC were taken in cycle 1, on day 1 pre-infusion (PI), at completion of infusion (CI) and 4 h post infusion start, on day 2 (PI) and day 4 (as for day 1). **a** Interestingly, increased phosphorylation of RNA Pol II CTD and Mcl-1 was observed at SNS-032 dose of 6 mg/m^2. At doses of 24 mg/m^2(**b**), 48 mg/m^2 (**c**) and 60 mg/m^2(**d**), inhibition of CDK9 was apparent with decreased pSer2 RNA Pol II CTD. CDK7 inhibition, decreased pSer5 RNA Pol II CTD, was observed at 60 mg/m^2. At 48 mg/m^2, Mcl-1 was detected at baseline and decreased with SNS-032 treatment. Decreased actin was seen at both the 48 and 60 mg/m^2 dose levels. Not all targets were evaluable for each patient sample

design of a pharmacologically-derived dosing regimen that sustains biologically active concentrations of SNS-032 (IC_{90} or greater) for 6 h in a Phase 1 clinical trial in MM and CLL.

MM is caused by the monoclonal proliferation of malignant plasma cells and their migration to and expansion within the bone marrow microenvironment. An array of oncogenic processes is implicated—spanning proliferation, survival, migration, stromal and endothelial cell interactions and angiogenesis [22, 38, 39]. The adhesion of the myeloma cell to the bone marrow stromal cells causes the production of cytokines including IL-6 and VEGF. In addition to causing down-regulation of survival signaling proteins, SNS-032 reduced both VEGF expression [40] and transcription of cyclins D1 and 2 [41]. The preferential cytotoxicity of SNS-032 towards CD138+ MM cells verus normal (CD138−) cells has been previously reported [42].

CLL is characterized by an accumulation of clonal B cells that, through interactions within the tumor microenvironment and defects in the apoptotic machinery, have evaded normal programmed cell death [43]. CLL cells are susceptible to the down-regulation of survival factors including Mcl-1 [30]. Flavopiridol has demonstrated clinical activity in CLL [32] and was shown in vitro to down-regulate the pro-survival proteins Mcl-1 and XIAP in CLL cells. It was recently demonstrated that the in vitro potency of SNS-032 for primary CLL cells was 10- to 30-fold greater than flavopiridol, that target modulation and down-regulation of survival signaling molecules correlated with cell killing, and that cell killing was preferential for primary CLL cells versus normal PBMC [34].

The mechanistic and kinetic assessment of SNS-032 was leveraged for: (a) the selection of MM and CLL as clinical indications, (b) the design of a pharmacologically-derived dose regimen that integrates cell-based data with pharmacokinetics, (c) biomarker selection for correlative studies to demonstrate pharmacodynamic activity and characterize possible relationships with pharmacokinetics and clinical response. SNS-032 demonstrated target modulation, anti-proliferative activity and induction of apoptosis. The ongoing clinical study of SNS-032 in CLL and MM tests the hypothesis that the targeted inhibition of CDKs may be effective in the treatment of hematologic malignancies driven by deregulated proliferation and dependence on survival signaling proteins and cytokines.

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