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ORIGINAL ARTICLE

SNS-314, a pan-Aurora kinase inhibitor, shows potent anti-tumor activity and dosing flexibility in vivo

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Abstract
Purpose The Aurora family of serine/threonine kinases (Aurora-A, Aurora-B, and Aurora-C) plays a key role in cells orderly progression through mitosis. Elevated expression levels of Aurora kinases have been detected in a high percentage of melanoma, colon, breast, ovarian, gastric, and pancreatic tumors. We characterized the biological and pharmacological properties of SNS-314, an ATP-competitive, selective, and potent inhibitor of Aurora kinases.
Methods We studied the biochemical potency and selectivity of SNS-314 to inhibit Aurora kinases A, B, and C. The inhibition of cellular proliferation induced by SNS-314 was evaluated in a broad range of tumor cell lines and correlated to inhibition of histone H3 phosphorylation, inhibition of cell-cycle progression, increase in nuclear content and cell size, loss of viability, and induction of apoptosis. The dose and administration schedule of SNS-314 was optimized for in vivo efficacy in mouse xenograft models of human cancer.
Results In the HCT116 human colon cancer xenograft model, administration of 50 and 100 mg/kg SNS-314 led to dose-dependent inhibition of histone H3 phosphorylation for at least 10 h, indicating effective Aurora-B inhibition in vivo. HCT116 tumors from animals treated with SNS-314 showed potent and sustained responses including reduction of phosphorylated histone H3 levels, increased caspase-3 and appearance of increased nuclear size. The compound showed significant tumor growth inhibition in a dose-dependent manner under a variety of dosing schedules including weekly, bi-weekly, and 5 days on/9 days off.
Conclusions SNS-314 is a potent small-molecule inhibitor of Aurora kinases developed as a novel anti-cancer therapeutic agent for the treatment of diverse human malignancies.

Keywords Aurora kinase · Experimental therapeutics · Small-molecule kinase inhibitors · Anti-tumor activity · Xenografts

Author names appear in alphabetical order.

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Introduction

SNS-314 is a potent and selective inhibitor of Aurora kinases A, B, and C. Aurora kinases represent a potentially

important therapeutic class of cell-cycle regulatory kinases that control progression through mitosis [1].

Aurora-A localizes to the centrosomes and functions in centrosome regulation and mitotic spindle formation. Aurora-B is characterized as a subunit of the chromosomal passenger protein complex that functions to insure chromosomal segregation and cytokinesis [2–4]. Aurora-C was also recently identified as a chromosomal passenger protein with similar localization during mitosis. Although the role of Aurora-C is less well established, the enzyme is highly expressed in the testes where it appears to play a central role in spermatogenesis [5–7]. Elevated Aurora-A expression has been detected in a high percentage of colon, breast, ovarian, gastric, and pancreatic tumors [8–11]. Aurora-B and Aurora-C are also expressed at high levels in both primary tumors and cell lines [12–18].

Given the central role of all three Aurora kinases in mitotic regulation and the association between their overexpression and tumorigenesis, they are being evaluated as potential targets in cancer therapy. Other inhibitors of these enzymes currently under clinical investigation include AZD1152, PHA-739358, MLN8054, R763, AT9283, and CYC116 [1, 19–22].

Here, we report the characterization of SNS-314, a selective small-molecule inhibitor of Aurora kinases that inhibits proliferation of a wide panel of human cancer cell lines. SNS-314 shows potent antiproliferative activity in HCT116 cells and inhibits soft agar colony formation following only 16-h exposures with the compound. The compound has anti-tumor activity in mouse xenograft models of human colon (HCT116), breast (MDA-MB-231), prostate (PC-3), lung (H1299 and Calu-6), ovarian (A2780) carcinomas and melanoma (A375). Its mechanism of action is consistent with an Aurora kinase inhibitor both in vitro and in vivo as monitored by inhibition of histone H3 serine 10 phosphorylation. SNS-314 represents a new class of highly potent Aurora kinase inhibitors and is being evaluated in a phase-1 dose escalation study designed to assess safety and tolerability in patients with advanced solid malignancies.

Materials and methods

Preparation of Aurora expression plasmids and protein expression

Preparation of the Aurora expression plasmids and protein purification protocols is described in Supplemental data.

Aurora biochemical assays

A homogeneous time-resolved fluorescence (HTRF)-based biochemical IC_{50} assay from Cisbio (Bedford, MA) was used to test for the kinase activity of the three isoforms of Aurora (A, B, and C) in the presence of the compound. A biotin-conjugated histone H3 peptide (Upstate Biotechnology, Dundee, UK) was used as substrate. Aurora-A kinase (7.5 nM) was assayed in 10 mM Tris–HCl pH 7.2, 10 mM $MgCl_2$, 0.1% BSA, 0.05% Tween® 20, 1 mM DTT, 120 nM biotinylated peptide ARTKQTARKSTGGKAPRKQLA-GGK-biotin, 6 μM ATP (2× the K_m for the enzyme) for 1 h at 25°C. The reaction was stopped with 200 mM EDTA. Aurora-B and Aurora-C were assayed at 5 nM enzyme concentration, 120 nM biotinylated peptide, and 300 μM ATP (2× the K_m for the enzymes) for 1 h at 25°C. To detect phosphorylation of histone H3, 0.2 ng/ml europium-conjugated anti-phospho-histone H3 antibody and 8 nM streptavidin-XL665 conjugate were incubated for 45 min at 25°C. The product was detected on an Analyst® AD (Molecular Devices, Sunnyvale, CA). The europium-conjugated antibody was excited at 330–370 nm. Cryptate and fluorescence resonance energy transfer (FRET) emissions were detected at 620 and 665 nm, respectively. GraphPad Prism (GraphPad Software, Inc., San Diego, CA) was used to generate IC_{50} curves and values.

Cell lines

The human colorectal (HCT116, HT29), lung (Calu-6, H1299), prostate (PC-3), ovarian (A2780), breast (MDA-MB-231), cervical (HeLa), pancreatic (MIA PaCa-2), and melanoma (A375) cell lines used in this study were purchased from ATCC (Manassas, VA) and maintained in culture at 37°C with 5% CO_2 according to ATCC guidelines.

Cell-based assays

Phospho-histone H3 (serine 10)

Analysis of phospho-histone H3 (pHH3) levels was performed on HCT116 cells using high-content screening methodology. Briefly, cells were plated at various seeding densities in growth medium on 96-well poly-L-lysine plates. After overnight growth at 37°C, SNS-314 was added to each well for a 1-h treatment. Cells were incubated in 100 μl/well 4% formaldehyde for 15 min at room temperature. They were then permeabilized with 10% Triton® X-100 in phosphate-buffered saline (PBS) and blocked with 10% fetal bovine serum. Cells were incubated in 50 μl/well of pHH3 antibody (Cell Signaling Technology, Danvers, MA) for 1 h at 37°C, washed, and incubated for 35 min at room temperature in 50 μl/well of staining solution (1:100 secondary antibody/1:5,000 Hoechst 33342). Hoechst 33342 (Invitrogen, Carlsbad, CA) was

used to visualize the nuclei of the cells. Images were captured and pHH3 staining was analyzed using the Target Activation application and ArrayScan VTI^{TM} instrument (Cellomics, Inc., Pittsburgh, PA). Data points taken from the parameter Mean_AveIntenCh2 were graphed in GraphPad Prism and fitted into an IC_{50} equation.

Fluorescence-activated cell sorter (FACS) analysis

HCT116 cells were treated for 16 h with different concentrations of SNS-314. They were then fixed in 80% methanol for 5 min, and washed with 0.1% BSA in PBS. Washed cells were resuspended in 10 μg/ml propidium iodide (Sigma, St. Louis, MO), containing 100 μg/ml RNAse, and 0.1% Triton-X, and incubated at 37°C for 1 h. Cell populations were analyzed using established FACS methodologies on a FACScan instrument (Becton-Dickinson, Franklin Lakes, NJ).

Protein preparation and immunoblots

Whole-cell and tumor lysates were prepared in a buffer composed of 20 mM Tris, 13 mM NaCl, 2.5% glycerol, 1% NP-40, 0.1% SDS, and 2 mM EDTA, supplemented with 50 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethanesulfonyl fluoride, phosphatase inhibitor cocktails I and II (Sigma, St. Louis, MO), and a protease inhibitor cocktail (Sigma, St. Louis, MO). Proteins were separated on 15-well 10% Bis–Tris gels run in the MES-SDS running buffer (Invitrogen, Carlsbad, CA). They were then transferred to the nitrocellulose filter paper (Invitrogen, Carlsbad, CA). Total histone H3 and pHH3 (Cell Signaling Technology, Danvers, MA) were detected following an overnight incubation at 4°C in accordance with the manufacturer's instructions. Secondary antibody was detected using the ECL Plus Western Detection Kit (Amersham, Piscataway, NJ) and the proteins were visualized using the BioMax light film (Kodak, Rochester, NY).

Fluorescence imaging

Slides were examined on a LEICA (Solms, Germany) DMIRE2 fluorescence microscope with a 63× oil immersion objective. Images were captured with a LEICA DFC300FX CCD camera and analyzed with Image-Pro software.

Proliferation assays

After the 96-h incubation with various concentrations of SNS-314, cells were incubated with BrdU for 2 h at 37°C. BrdU incorporation was detected as suggested by the manufacturer (BrdU chemiluminescence; Roche, Indianapolis, IN). Chemiluminescence was quantified on the SpectraMax Gemini XS plate reader. Cell proliferation was then determined by normalizing the experimental values (SNS-314 treatment) to control values (DMSO treatment) and reporting the resulting values as percent activity.

Soft agar colony formation assay

HCT116 cells were embedded in 0.35% agar (in fresh medium), at a density of 400 cells/well, and were covered with a layer of 0.35% agar. After overnight growth at 37°C, SNS-314 dilutions were added to wells, and cells were incubated for 24 h at 37°C. The compound was then washed out and plates were incubated for 11 days at 37°C to allow for colony formation. Cell DNA was then labeled with Hoechst 33342. Colonies were imaged and scored with the Morphology Explorer application and ArrayScanV (Cellomics, Inc., Pittsburgh, PA). The number of colonies formed was determined for cells treated with various compound concentrations and expressed relative to the number of colonies scored in control wells.

In vivo efficacy studies

All animal experiments were in accordance with protocols approved by the Sunesis Pharmaceuticals, Inc., Institutional Animal Care and Use Committee and in accordance with "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) and with local, state, and federal regulations. Female mice (*nu/nu*) were allowed to acclimate for 3 days following shipping-related stress and their health was assessed daily by observation. Reverse osmosis-purified water and irradiated food (PicoLab Rodent Diet 20, #5053; Dean's Animal Feeds, San Carlos, CA) were provided ad libitum, and the animals were kept on a 12-h light and dark cycle. Tumor cells were subcutaneously implanted in the right hind flank using 200 μl of a 2.5×10^7 cell/ml suspension (1:1 DPBS with cells, Matrigel). When tumors reached an average volume of 200 mm^3, mice were weighed, randomized by tumor volume ($l \times w \times h \times 0.52$) before initial treatment, assigned to the various study groups, and treated with vehicle or SNS-314. For intraperitoneal (i.p.) administration, SNS-314 was formulated in 20% Captisol® (sulfobutyl ether β-cyclodextrin). Animals were weighed, monitored for signs or symptoms of toxic effects, and measured for tumor volumes twice weekly until an end point was met. The end point for each animal in the study was a measured tumor volume greater than 1,200 mm^3 or 10% of body weight, a greater than 20% body weight loss for two consecutive measurements, clinical observations from which the animal would be considered moribund (emaciation, dehydration, lack of normal movement, etc.), or severe behavior abnormalities.

Tumor growth inhibition (TGI) was determined by examining the tumor volume graph and calculating the percent of inhibition from the vehicle control group on the last day the control contained at least 75% of the animals. TGI was then calculated with the following formula:

$$\mathrm{TGI} = \frac{(\text{control TV}_x - \text{control TV}_\mathrm{i}) - (\text{treatment TV}_x - \text{treatment TV}_\mathrm{i})}{(\text{control TV}_x - \text{control TV}_\mathrm{i})} \times 100$$

where TV_x is the average tumor volume on day x and TV_i is the initial average tumor volume. ANOVA was performed to calculate statistical significance, defined as $p < 0.05$.

Immunohistochemistry

HCT116 tumors were excised on days 4, 11, 18, and 25 from mice treated i.p. with 170 mg/kg SNS-314 on a bi-weekly × 3 schedule, placed in the Streck tissue fixative (Streck, Omaha, NE), paraffin-embedded, sectioned, and the sections transferred to slides. Tumor sections were stained on separate slides with hematoxylin and eosin (H&E), pHH3 (Ser10) antibody (Cell Signaling Technology, Danvers, MA), and caspase-3 antibody. The stained samples were processed and analyzed for number of immunohistochemically positive cells per ten high-power fields in each tumor sample as well as assessed for overall morphological changes.

Results

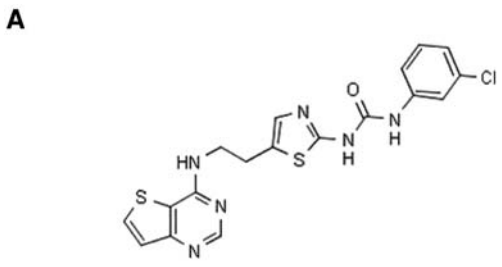
SNS-314 is a novel, potent, and selective inhibitor of Aurora kinases A, B, and C

SNS-314 (Fig. 1a) was identified as a potent and selective small-molecule antagonist of Aurora kinase activity by a FRET-based biochemical IC_{50} assay (Cisbio, Bedford, MA). This assay tests for the kinase activity of the three isoforms of Aurora (isoforms A, B, and C) in the presence of small-molecule inhibitors. In addition to identifying selective Aurora inhibitors, the FRET assay was also used to determine the ATP competitiveness of SNS-314 against Aurora-B. Average IC_{50} data for Auroras A (9 nM), B (31 nM) and C (3 nM) are summarized in Fig. 1b. SNS-314 demonstrated remarkable specificity and was found to inhibit only 24 of 219 kinases by greater than 65% when screened at 1 μM. Full IC_{50} determinations were made for those kinases inhibited by 65% or greater. Of these, only 7 (Fig. 1c) have a calculated IC_{50} value that is within 100-fold of the SNS-314 biochemical IC_{50} (IC_{50}, 5–100 nM) and a further 14 (EphA7, MnK2, MuSK, CHK2, Flt3, cKit, Tie2, KDR, Rse, MSSK1, LOK, Flt1, Ron, and PDGFRα) have an IC_{50} between 100 and 400 nM. Overall, these results confirm that SNS-314 is a reversible, low nanomolar, specific inhibitor of the Aurora kinases.

SNS-314 blocks proliferation in a broad panel of tumor cell lines

The effects of SNS-314 on cellular proliferation were assessed by measuring BrdU incorporation following drug treatment in a panel of human cancer cell lines representing colon, lung, prostate, ovarian, breast, cervical, pancreatic, and melanoma malignancies. Cells were exposed to SNS-314 for 96 h and drug-induced inhibition of cell proliferation activity was evaluated by chemiluminescence detection of BrdU incorporated in DNA. As indicated in Table 1, proliferation of all ten cell lines examined was potently inhibited by SNS-314 with IC_{50} values ranging from 1.8 nM in A2780 ovarian cancer cells to 24 nM in HT29 colon cancer cells. SNS-314 was active in all ten cell lines independently of their relative Aurora-A or Aurora-B protein levels, as detected with Western blots (data not shown). Two cell lines (HCT116 and A2780) showed higher levels of Aurora-B than Aurora-A, whereas five cell lines (PC-3, HeLa, MDA-MB-231, H-1299, and HT29) were characterized by higher levels of Aurora-A than Aurora-B.

The ability of SNS-314 to inhibit clonogenic survival of HCT116 colon cancer cells was assessed by a colony formation assay (Fig. 2a). Cells were treated with SNS-314 (range 4–63 nM) for 8, 16, or 24 h; at the end of treatment, SNS-314 was washed out and cells were grown in drug-free medium for 11 days to allow for the formation of colonies. Figure 2a shows that 8-h exposure to SNS-314 was sufficient to decrease significantly the clonogenic survival of HCT116 cells in a dose-dependent fashion. To evaluate the role played by a possible altered doubling time of cells grown in an anchorage-independent environment, we performed a soft agar colony formation assay using HCT116 cells after treatment with SNS-314. Cells were exposed to SNS-314 (range 4–250 nM) for 24 h, drug was washed out and cells were embedded in soft agar and drug-free medium and incubated at 37°C for 11 days to allow for colony formation. Figure 2b shows that the clonogenicity of HCT116 was completely abrogated after a 24-h exposure to SNS-314



B

Aurora	A	B	C
IC_{50} (nM)	9.0	31	3.4
SD	2.0	7.0	n/a
n	9	10	1

C

Target	IC_{50}* (nM)
Aurora A	1
TrkB	5
TrkA	12
Flt4	14
Fms	15
DDR2	82
Axl	84
c-Raf	100

* Radiometric kinase assay

Fig. 1 SNS-314 is a novel, potent, and selective inhibitor of Aurora kinases A, B, and C. **a** Chemical structure of SNS-314. **b** SNS-314 is a pan-Aurora kinase inhibitor. Enzyme inhibition values for SNS-314 against recombinant Aurora kinases A, B, and C. **c** SNS-314 is a selective inhibitor of Aurora kinases. Enzyme inhibition data for all kinases out of 219 tested that showed an IC50 less than or equal to 100 nM

Table 1 SNS-314 effects on cellular proliferation

Cell line	Tumor type	IC_{50} (nM)
HCT116	Colon	6.4
HT29	Colon	24
Calu-6	Lung	13
H1299	Lung	4
PC-3	Prostate	4.4
A2780	Ovarian	1.8
MDA-MB-231	Breast	8.1
HeLa	Cervical	9.3
MIA PaCa	Pancreatic	9.1
A375	Melanoma	5.9

confirming that drug-induced cell-survival inhibition is a dose- and time-dependent effect sustained after the removal of the drug from the growth medium.

SNS-314 demonstrates a mechanism of action that is consistent with Aurora kinase inhibition

To assess the mechanism of action of SNS-314 in human cancer cells, we measured phosphorylation of histone H3, a substrate of Aurora-B, at Ser10. Quantification of histone H3 phosphorylation was assessed by high-content screening analysis of the binding of a fluorescent anti-pHH3 antibody to nuclei of fixed cells after a 1-h exposure to a SNS-314 dose titration. Histone H3 phosphorylation was potently inhibited by SNS-314 in all 6 cell lines tested with IC_{50} values ranging from 9 nM in HCT116 cells (Fig. 3a) to 60 nM in Calu-6 cells.

Both the expression and activity levels of Aurora-A and Aurora-B remained minimal throughout the G1 and S phases of the cell cycle, increased throughout G2, and reached their peak during mitosis. Thus, we designed

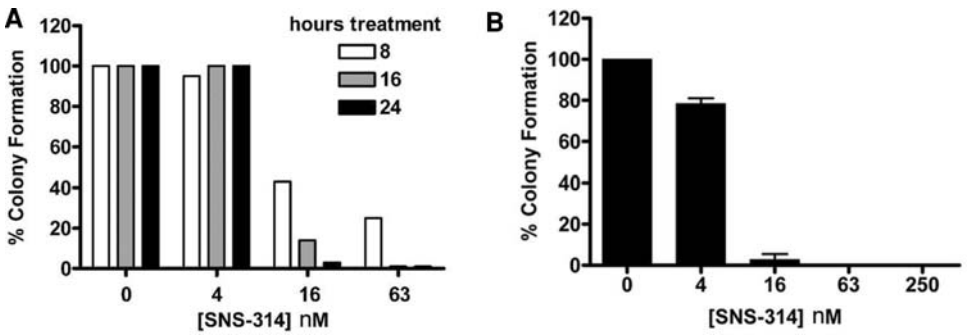


Fig. 2 SNS-314 cell effects are irreversible. **a** Inhibition of colony formation following pulse treatment with various SNS-314 concentrations. HCT116 cells grown in 12-well plates were treated for 8, 16, or 24 h with SNS-314 and drug was washed out. Following the washout, cells were incubated in drug-free medium and colony formation was counted 11 days later using ArrayScan and Cellomics Morphology Explorer software. **b** Inhibition of colony formation in soft agar following pulse treatment with various SNS-314 concentrations. HCT116 cells were treated for 24 h with various SNS-314 concentrations. Following the washout, cells were incubated in drug-free medium and soft agar and colony formation was counted 11 days later with ArrayScan and Cellomics Morphology Explorer software

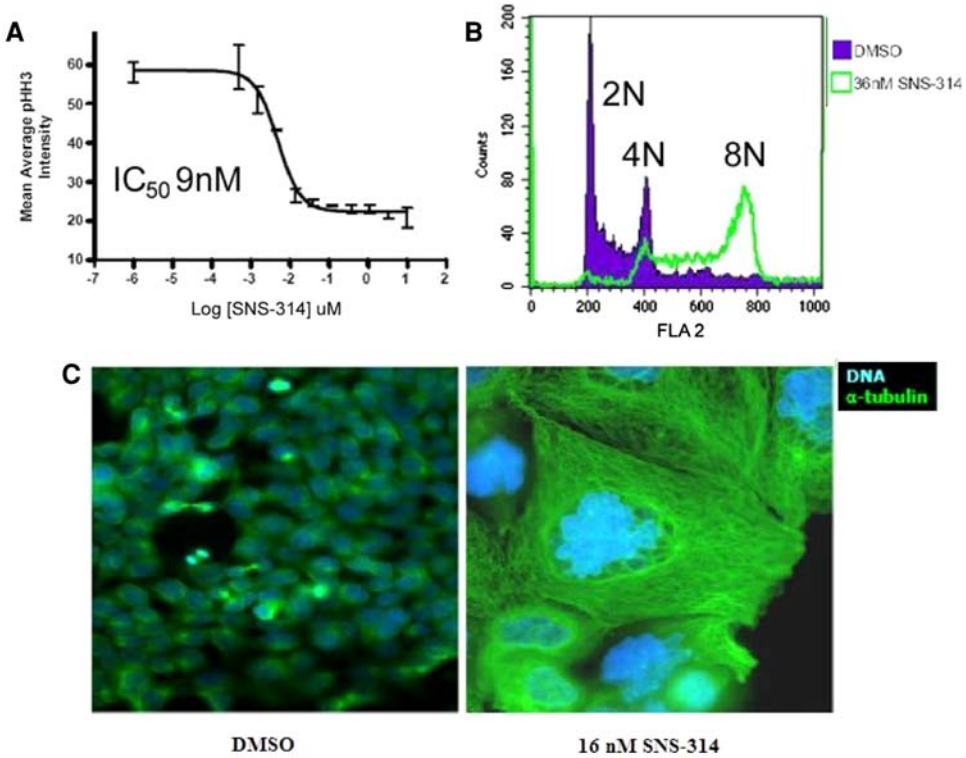


Fig. 3 SNS-314 demonstrates a mechanism of action that is consistent with Aurora kinase inhibition. **a** SNS-314 inhibits phosphorylation of Ser10 of histone H3, a known substrate of Aurora-B. **b** SNS-314 disrupts mitosis as assessed by flow cytometry and immunofluorescence. HCT116 cells treated for 16 h with 36 nM SNS-314 showed a dramatic increase in cells with 4N and 8N DNA content. **c** Treatment of HCT116 cells with 16 nM SNS-314 for 72 h resulted in large nuclei consistent with multinucleated cells and inhibition of cytokinesis

experiments to specifically examine how exposure of cells to SNS-314 affects the cell cycle.

To determine cell-cycle effects of SNS-314, HCT116 cells were grown in 12-well tissue culture plates (100,000 cells/well) for 24 h at 37°C. As shown in Fig. 3b, exposure to SNS-314 for 16 h showed distinct cell-cycle defects, resulting in cell populations with >4N DNA content.

Treatment with SNS-314 caused cells to undergo successive rounds of endoreduplication, as measured by FACS. We examined this phenotype further by visualizing whether there was a coincident increase in cell size. A continual increase in both the DNA content and cell size would be consistent with successive rounds of DNA replication without a corresponding cytokinetic event. This bypass of chromosome segregation and cytokinesis is a hallmark of Aurora-B inhibition. To characterize the effect of SNS-314 on cell size, HCT116 cells were incubated with SNS-314 for extended periods of time and subjected to fluorescence microscopy. Specifically, cells were treated with 16 nM SNS-314 for 72 h. Extended SNS-314 treatment caused substantial increase in cell size, as noted by visual inspection (see Fig. 3c). Cell populations treated for 72 h with SNS-314 contained many large cells that were largely absent in untreated cell samples. The large, treated cells also have an increased nuclear size.

SNS-314 demonstrates anti-tumor activity in vivo

We evaluated the breadth of the anti-tumor effects of SNS-314 in human tumor xenograft mouse models representing a range of human malignancies. Initial in vivo studies to establish the active doses and schedules for SNS-314 were performed in the HCT116 xenograft model of human colon carcinoma. To explore the effects of intensive exposure versus intermittent exposure, dosing schedules ranging from multiple times per day to weekly administration were employed. SNS-314 had significant anti-tumor activity using several dosing schedules.

Intraperitoneal administration of SNS-314 once daily for 5 consecutive days with a 9-day treatment-free period followed by another 5-day cycle was the most active daily dosing schedule, while the most active intermittent dosing schedules were once weekly for 3 consecutive weeks and twice weekly for 3 consecutive weeks (Fig. 4). Therefore, these schedules were used to further investigate the optimal dose activity relationship in six additional tumor models.

SNS-314 displayed significant anti-tumor activity in mouse xenograft models of human breast (MDA-MB-231), prostate (PC-3), lung (H1299 and Calu-6), ovarian (A2780) carcinomas and melanoma (A375) (Table 2). Among the three dose schedules explored, the bi-weekly × 3 schedule was the most consistently efficacious; it provided efficacy with acceptable tolerability (body weight losses within 20% of initial value), achieving between 54 and 91% TGI in all six models tested when SNS-314 was administered at 170 mg/kg. This same dose (170 mg/kg) had caused only moderate or no significant growth inhibition in all six models when administered on a weekly × 3 schedule. The daily treatment schedule (qd ×5, 9d off, ×2) resulted in

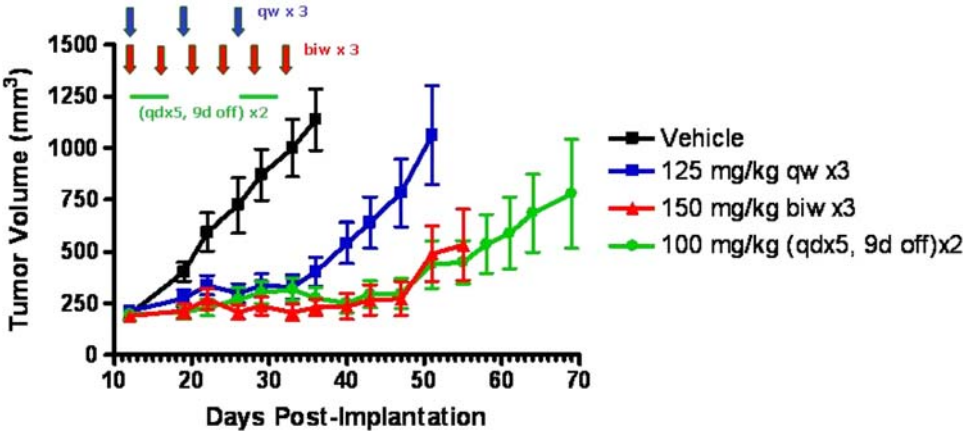


Fig. 4 SNS-314 demonstrates significant and prolonged anti-tumor activity using flexible dosing schedules in HCT116 colon cancer xenografts. SNS-314 was tested in HCT116 tumors on a once weekly (qw), twice weekly (biw), and daily for 5 days with a 9-day interval without drug administration (qd ×5, 9 days off) treatment schedules. Each treatment group included 10 mice

significant TGI at a dose of 102 mg/kg only in two of the models tested: A2780 and Calu-6. In other models (MDA-MB-231, PC-3, H1299, and A375), this dose and schedule was above the MTD while lower doses achieved significant anti-tumor activity in A375, Calu-6 (51 mg/kg), and PC-3 models (85 mg/kg). Partial (tumors whose measured volume is less than 1/2 the measured volume on the first day of dosing for 3 consecutive measurements) or complete (tumors whose volume is less than 63 mm^3 for 3 consecutive measurements) tumor regressions were observed in mice after the well-tolerated bi-weekly treatment schedule in PC-3 and MDA-MB-231 models.

Pharmacodynamic analysis of SNS-314 effects

To correlate the potent xenograft activity of SNS-314 and the post-treatment levels of proteins recognized to be modulated after inhibition of Aurora kinases, we performed in vivo target modulation studies in HCT116 colon carcinoma xenografts. Histone H3 phosphorylation, caspase-3 expression, and changes in the cell ploidy in HCT116 tumors were examined with Western blots and by immunohistochemistry. We also correlated these parameters with plasma and tumor drug concentrations as assessed in the same mouse model. Figure 5a shows that administration to HCT116 tumor-bearing mice of single i.p. doses of 50 or 100 mg/kg SNS-314 led to complete inhibition of pHH3 as early as 3 h post-dose. Inhibition was diminished 10 h after a 50 mg/kg dose, but sustained for at least 10 h after a 100 mg/kg dose. Separate experiments (Fig. 5b) evaluated the inhibition of pHH3 in HCT116 tumor-bearing mice after a single i.p. dose of 170 mg/kg SNS-314 (MTD when administered with a bi-weekly × 3 schedule). The significant inhibition of pHH3 at 6 and 9 h post-dose was still sustained 24 h after treatment in 2 of the 3 HCT116 tumor lysates, each derived from an independent mouse. The suppression of pHH3 correlated with the SNS-314 tumor levels (Fig. 5c). After a single i.p. dose of 170 mg/kg in HCT116 tumor-bearing mice,

Table 2 Tumor growth inhibition after repeat doses of SNS-314 in human xenograft models

Schedule	SNS-314 (mg/kg)	A2780 (ovarian)	A375 (melanoma)	H1299 (lung)	MDA-MB-231 (breast)	PC-3 (prostate)	Calu-6 (lung)
qw × 3	85	0	35.6	0	16.4	56.2*	0
	170	57.3*	34.6	17.9	48.6	56.1*	34.8
biw × 3	42.5	0	1.2	0	20.6	39.5	23.5
	85	9.8	20.6	17.7	19.2	63.6*	28.7
	170	53.8*	65.4*	69.1*	73.8*	67.5*	91.4*
qd ×5, 9d off ×2	25.5	19.1	34.3	28.2	9.7	39.5	33.1
	51	0	64	20.4	46.8	44.9	64.8
	85	nt	nt	nt	nt	78.6*	nt
	102	59.9*	nd	nd	nd	nt	99.5*

Values represent % TGI calculated as indicated in "Materials and methods"

nt not tested, *nd* not determined

* $p < 0.05$; using log rank test

SNS-314 distributed to the tumor tissue and was retained there preferentially over plasma, leading to a 7.5-h half-life in tumors compared to a 4.7-h half-life in plasma.

In addition to inhibition of histone H3 phosphorylation, the Aurora inhibition phenotype is characterized by appearance of large multiploid cells and apoptosis. To evaluate whether these cellular effects were induced by SNS-314 in vivo and correlated with TGI in xenografts, we performed an immunohistochemical analysis of sections from HCT116 tumors from mice treated with 170 mg/kg SNS-314 bi-weekly × 3. Figure 6a shows representative pictures of HCT116 tumors taken on days 4, 11, 18, and 25 at 6 h after the vehicle or 170 mg/kg SNS-314 i.p. dose. Visual counting of tumor cells stained positive for caspase-3 (Fig. 6b) demonstrated that pro-apoptotic effects of SNS-314 were evident as early as day 4 of the experiment when compared to control cells from vehicle-treated mice. Furthermore, the morphology of tumor cells, as evident in H&E-stained sections of HCT116 xenografts, changed with the appearance of increasingly large multinucleated cells at each time point. The SNS-314-induced effects on apoptosis and ploidy were sustained for up to 1 week (day 25) after completion of the 3-week dosing phase. Overall, the difference between plasma and tumor pharmacokinetics and the persistently high drug levels in tumor relative to plasma may partially account for the prolonged SNS-314-induced target modulation in xenograft tumors and contribute to the superior anti-tumor activity of SNS-314.

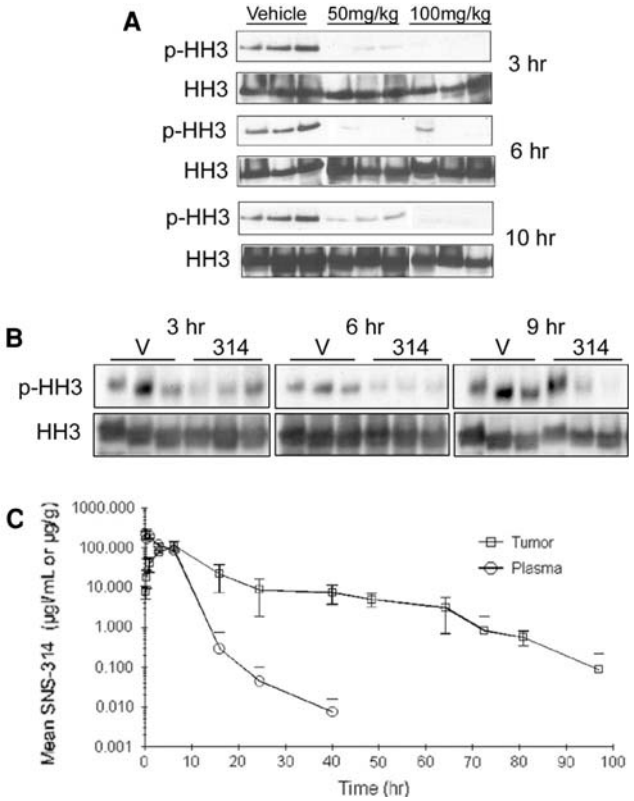


Fig. 5 SNS-314 induces a potent and sustained inhibition of histone H3 phosphorylation in tumors. **a** Western blot analysis of phospho-histone H3 (pHH3 Ser10) and total histone H3 in HCT116 tumor lysates from mice treated i.p. with vehicle or with 50 and 100 mg/kg SNS-314. **b** pHH3 Ser10 and total histone H3 in HCT116 tumor lysates from mice treated i.p. with vehicle or 170 mg/kg SNS-314. Animals were sacrificed at the indicated times after a single drug injection. **c** SNS-314 plasma and tumor concentrations in HCT116 tumor-bearing mice after a single 170 mg/kg i.p. injection

Discussion

SNS-314 was derived from a 2-aminoethyl phenyl benzamide identified by screening and is a potent inhibitor of Auroras A, B, and C [23]. It is highly selective for the three members of the Aurora kinase family with IC_{50} values in the low nanomolar range and shows significant cross-reactivity (>65% inhibition at 1 μM) with only 24 serine/threonine and tyrosine kinases out of a panel of 219 kinases. Seven out of the 24 kinases (Axl, c-RAF, DDR2, Flt4, Fms, TrkA, and TrkB) have SNS-314 IC_{50} values between 5 and 100 nM, while 14 others have IC_{50} values between 104 and 400 nM. Three remaining kinases had SNS-314 IC_{50} values ≥1,000 nM. Several of the kinases most potently inhibited by SNS-314 have been shown to be involved in cancer cell-cycle control and proliferation [24–29], and therefore may contribute to the anti-tumor activity of SNS-314. The potent activity displayed by SNS-314 against TrkA and TrkB kinases may also explain the strong antiproliferative activity of this compound in the PC-3 cells derived from prostate carcinoma, a malignancy characterized by an increased expression of these neurotrophin receptors [30].

The cell activity profile of SNS-314 clearly supports a dominant mechanism of action of an Aurora kinase inhibitor. A series of assays in 12 cancer cell lines of diverse tissue origin showed that SNS-314 consistently decreased phosphorylation of histone H3 on Ser10 and induced a buildup of polyploid cells with 4N or greater DNA content. The average IC_{50} value for inhibition of phosphorylation of histone H3 was 58.7 nM, whereas for induction of polyploidy, the median effective concentration (EC_{50}) was 49.1 nM. The drug-induced failure of cell division led to a profound inhibition of proliferation by SNS-314 in 10 of the 12 human cancer cell lines tested, and this effect was evident independently of the relative Aurora-A or Aurora-B protein expression in each of them.

SNS-314 effects on cancer cell survival are irreversible as indicated by the results of the clonogenic survival assays performed in HCT116 colon carcinoma cells after short exposures to SNS-314. Exposure of HCT116 cells to SNS-314 concentrations in the low nanomolar range for only 8 h is sufficient to decrease significantly cell clonogenic

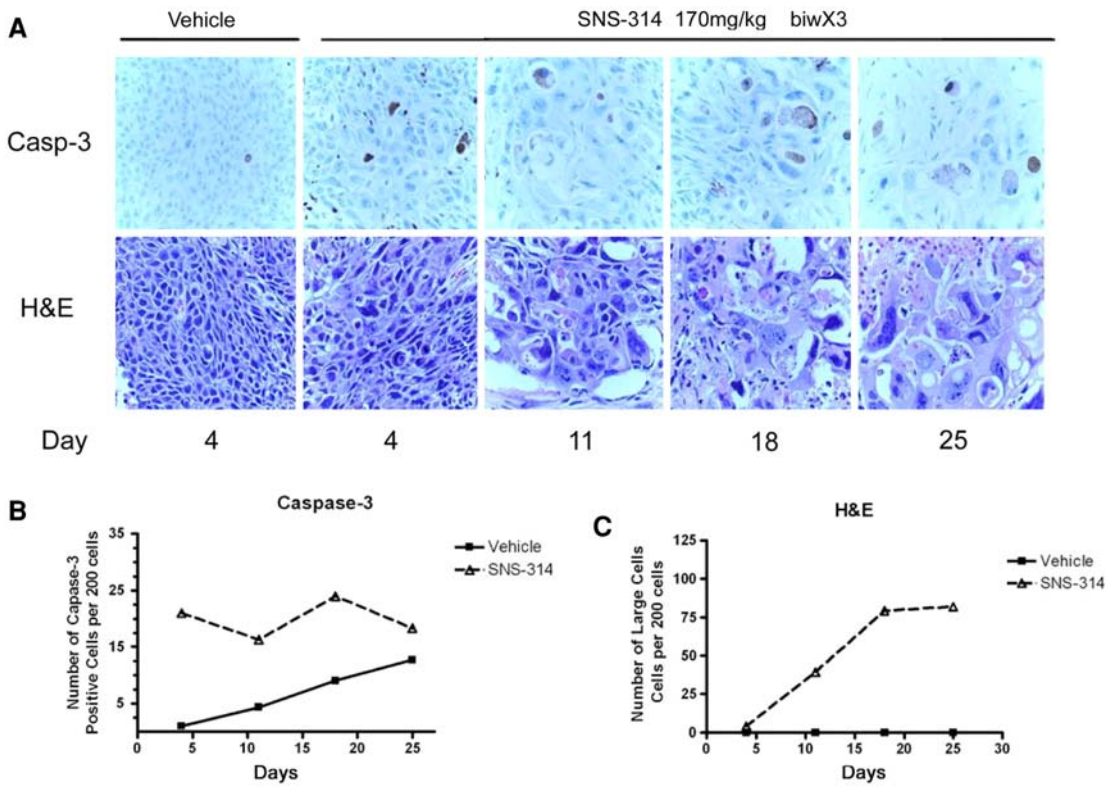


Fig. 6 SNS-314 treatment induces caspase-3 and multinucleated cells in HCT116 tumors. Significant increases in numbers of caspase-3-positive cells are observed in SNS-314-treated tumors indicating induction of apoptosis. Large multinucleated cells persist for at least 25 days after treatment consistent with an Aurora-B-mediated mechanism of action. Representative anti-caspase-3 and H&E sections were prepared from tumors after completion of treatment with 170 m/kg SNS-314 on a bi-weekly schedule for 3 consecutive weeks. **a** SNS-314 was administered i.p. on days 1, 4, 8, 11, 15, 18 with tumors being excised 6 h post-dose on days 4, 11, 18, and 25 (1 week after last dose) of the study. All images were taken at ×40 magnification. **b** Number of cleaved caspase-3-positive cells per 200 cells. Each point represents the average of three scores derived from different mice. **c** Number of cells presenting multinucleated morphology in 200 total cells. Each point represents the average of three different measurements

survival, which is completely abrogated after 16–24-h treatment.

The potent mechanism-based activity of SNS-314 in multiple human cancer cell lines was also translated into a broad anti-tumor in vivo activity in each of the colon, breast, lung, melanoma, and ovarian xenografts investigated to date. HCT116 xenografts were particularly sensitive to i.p. administration of SNS-314 at well-tolerated treatment schedules; these colon carcinoma cells were found to have higher Aurora-B than Aurora-A protein levels, one of the highest percentages of mitotic (MPM-2 positive) cells (Zimmerman et al., unpublished data), and a significant TGI even at intermittent (bi-weekly and weekly) treatment schedules. Other pan-Aurora [20, 21, 31, 32] or Aurora-A-specific [22] inhibitors have been shown to be efficacious in HCT116 xenograft model, but their treatment schedules have always been more intense than the SNS-314 weekly or bi-weekly schedules that we have characterized as having a potent anti-tumor activity. The anti-tumor activity displayed by SNS-314 may be explained also by its specific pharmacokinetic and pharmacodynamic characteristics. After a single i.p. dose of 170 mg/kg SNS-314 in tumor-bearing mice, SNS-314 distributed to tumor tissue and was retained preferentially over plasma, leading to a 7.5-h half-life in tumors and a 4.7-h half-life in plasma. Consistent with the mechanism of action demonstrated by SNS-314 in cells, analysis of tumor tissues from xenografts after drug treatment indicated a profound and sustained inhibition of pHH3, a well-known substrate of Aurora-B [33]. Typically, tumor tissues were also characterized after treatment with SNS-314 by the time-dependent appearance of cell endoreduplication and induction of apoptosis as indicated by polyploidy and caspase induction. These effects were still evident 1 week after completion of drug administration and may explain the prolonged TGI observed after treatment with SNS-314. The same pattern of drug-induced target modulation was observed in different xenografts including HCT116, Calu-6, and A2780. Modulation of pHH3 after treatment with tolerated and efficacious doses of SNS-314 was observed in mouse

epidermis and bone marrow samples [34] suggesting that, as demonstrated also by other Aurora inhibitors [35], the inhibition of pHH3 can be a sensitive and dynamic marker of Aurora inhibition in surrogate tissues.

In conclusion, we show that SNS-314 is a new pan-Aurora kinase inhibitor combining potency, selectivity, and robust in vivo activity. The flexible intermittent dosing and the different schedule options support the clinical evaluation of SNS-314 for the treatment of diverse human malignancies. A dose escalation phase-1 clinical trial is ongoing in patients with solid tumors to assess the safety and pharmacokinetic/pharmacodynamic properties of SNS-314 as well as to establish the MTD that may be used in future single-agent clinical studies of SNS-314.

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