Chemoprevention of Mouse Intestinal Tumorigenesis by the Cyclin-Dependent Kinase Inhibitor SNS-032

Amelie Boquoi, Tina Chen and Greg H. Enders

Abstract

Despite advances in screening and treatment, colorectal cancer remains the second leading cause of cancer-related death in the United States. Cyclin-dependent kinases (Cdk) are deregulated in colorectal cancer by silencing of the Cdk inhibitor p16\textsuperscript{Ink4a} and other mechanisms. We tested whether the small molecule Cdk inhibitor SNS-032 (formerly BMS-387032), which targets Cdk2, Cdk7, and Cdk9, can prevent intestinal tumorigenesis in mouse models. We generated mice with high intestinal tumor loads by combining the multiple intestinal neoplasia (Min) mutation with Ink4a/Arf mutations and inducing colitis with dextran sulfate sodium. p16-null Min mice \((n = 17)\) began dextran sulfate sodium treatment at week 5 and i.p. injection of carrier or SNS-032 at week 6. Mice were sacrificed at week 12. SNS-032 was well tolerated and reduced colon tumor burden to 36% of that in carrier-treated mice \((P < 0.001)\). We then extended the study to Ink4/Arf-null Min mice \((n = 14)\) and increased the drug dose frequency. SNS-032 treatment reduced the intestinal tumor number to 25% and intestinal tumor burden to 16% of carrier-treated mice \((P < 0.0001)\). DNA synthesis in non-neoplastic and tumor epithelial cells, detected by bromodeoxyuridine incorporation, was modestly reduced by acute SNS-032 treatment. The mitotic index, detected by histone H3 phosphorylation, was distinctly decreased \((P < 0.03)\), and apoptosis, detected by caspase 3 activation, was increased \((P < 0.005)\). These results show the chemoprevention of intestinal tumorigenesis by SNS-032. Our findings support further study of Cdk inhibitors for chemoprevention and therapy of colon cancer.

Despite advances in screening and treatment, colorectal cancer remains the second leading cause of cancer-related deaths in the United States. Patients with inherited predispositions, chronic ulcerative colitis, or a personal history of colon tumors are at particular high risk. Chemoprevention constitutes an appealing alternative method to combat the disease in such patients. Most chemoprevention has targeted inflammatory mediators (1–3). Among other molecular pathways that are deregulated in colorectal cancer are the cyclin-dependent kinases (Cdk), enzymes that promote cell cycle progression and transcription of genes involved in cell replication and survival. The Cdk inhibitor p16\textsuperscript{Ink4a} is frequently inactivated in sporadic and ulcerative colitis–associated neoplasia (4–7). Several small molecule Cdk inhibitors have been developed for cancer therapy and are undergoing clinical investigation (8, 9).

Although Cdk inhibitors have been studied for activity against human colorectal cancer cell lines in mouse xenograft studies (10), to our knowledge, no studies have tested their efficacy in treating intestinal tumors arising \textit{in situ}. Xenograft studies have the advantage of assessing drug efficacy against human colorectal cancer cells but carry the drawbacks of using immunocompromised mice and tumor growth in an artificial setting, typically a pocket of subcutaneous tissue formed by needle injection. We focused our studies on the potential of a newer inhibitor, SNS-032 (11, 12), to suppress intestinal tumorigenesis in a preclinical model. Thus, these studies have the advantages of using immunocompetent hosts, avoiding idiosyncrasies of established cell lines, examining tumor growth in native contexts, and allowing drug access via the native vasculature. Furthermore, specific premalignant states and genotypes can be assessed that mimic those found in human populations. Thus, studies of drug effect on tumorigenesis \textit{in situ} can have valuable implications for both therapy and chemoprevention.

Materials and Methods

Animals

Multiple intestinal neoplasia (Min) mice in a C57/B16 background were purchased from The Jackson Laboratory. p16-null mice (13), initially in a mixed 129Sv/FVB/C57B16 genetic background (at least 50% C57B1/6), were repeatedly backcrossed with C57/B16.
mice over at least 10 generations. Ink4a/Arf-null mice in a C57/B16 background were obtained from the National Cancer Institute Mouse Models of Human Cancer Consortium (strain no. 01XB2). Genotyping was done via PCR using tail-DNA.

**Treatments**

Colitis was induced in 17 p16-null Min mice by providing mice with drinking water containing 4% dextran sulfate sodium (DSS); molecular weight range, 36,000-50,000; MPBio) at 5 wk of age. DSS was administered in two cycles, with each cycle consisting of 3 d of DSS and 11 d of untreated water. SNS-032 (kindly provided by Sunesis Pharmaceuticals, Inc., San Francisco, CA) was administered by i.p. injection twice a week at 30 mg/kg in 2.1 mmol/L tartaric acid/0.9% sodium chloride (pH 4.2) during weeks without DSS. Mice were sacrificed 5 to 6 h after the last SNS-032 injection. Bromodeoxyuridine (BrdUrd; 100 μL of a 10 mg/mL solution; Sigma-Aldrich) was injected i.p. 4 h before euthanasia. Fourteen Ink4a/Arf-null Min mice were treated the same way except that DSS dose was reduced to 3% for 11 mice and SNS-032 dosing was increased to thrice a week in 6, 8, 9, and 10. Mice were euthanized on week 12. By this time point, Min tumors are readily recognized by examination of dissected intestines under a dissecting microscope but there is little disease-related morbidity or mortality (15, 20). Colon tumor number and maximum diameter were scored by an observer blinded to the treatment groups and used to calculate tumor burden (total tumor area).

**Histopathologic analysis**

Intestines from mice euthanized by carbon dioxide inhalation were resected, opened longitudinally under a dissecting microscope (Motic with Motic Images Plus 2.0.2 software, Ted Pella, Inc.) and cleared of contents with a Kimwipe (Kimberly Clarke). An observer blinded to the treatment groups counted tumors and measured greatest tumor diameter, using an eyepiece reticle. Between three and six tumors were harvested per mouse. Sections were fixed in formalin, embedded in paraffin, sectioned, stained with hematoxylin, and subjected to immunohistochemistry. Standard procedures were used for antigen retrieval and tissue staining, as previously described (11). Primary antibodies used were directed against BrdUrd (1:100; Becton Dickinson #555627), cleaved/activated caspase 3 (aCasp3; Asp175, 1:200; Cell Signaling #9661), and phosphorylated histone H3 (PH3, 1:100; Cell Signaling #9701). Secondary antibodies were biotinylated anti-mouse IgG (H+L, 1:200; Vector Labs #BA-2001) and biotinylated anti-rabbit IgG (H+L, 1:200; Vector Labs #BA-1000).

Tissue pieces were embedded with the lumen perpendicular to the bottom of the block, and only well-oriented tissue (with intact crypts reaching the lumen) were scored. aCasp3 and PH3 staining in epithelial cells was quantitated by counting positively stained cells per ×40 microscope field (Nikon Eclipse E800 Microscope with Nikon ACT-1C software). BrdUrd staining was quantitated by counting positive cells per total epithelial cell count in a ×40 field and calculating the percentage of positive cells.

**Statistical analysis**

Total numbers of tumors or tumor burden were compared using a likelihood ratio test, in which the numbers were assumed to be Poisson distributed, or a Wilcoxon two-sample test. A random effects model was used to compare tumor sizes in drug-treated or carrier-treated animals. The Wilcoxon test was also used for comparisons of BrdUrd, PH3, or aCasp3 staining.

**Results**

**The model**

The Min (multiple intestinal neoplasia) line represents the best-studied mouse model of intestinal tumorigenesis (16). These mice are heterozygous for an inactivating mutation in the adenomatous polyposis coli tumor suppressor protein, the most common mutation in human colon tumors. Adenomas form throughout the small intestine (jejunum and ileum) and large intestine (colon). The functional similarity of this phenotype to the human genetic disease adenomatous polyposis coli (or familial adenomatous polyposis) is underscored by recent evidence that microadenomas form throughout the small intestine in the human disease (17). We combined the Min mutation with lesions in the Ink4a/Arf pathway, for their ability to augment colon tumorigenesis and mimic Ink4a/Arf inactivation in human disease (5, 15, 18, 19). In addition, we entertained the possibility that tumorigenesis driven in part by Cdk deregulation might be particularly susceptible to Cdk-targeted therapy. We initiated colitis in these mice by administration of DSS. This treatment is known to augment Min tumorigenesis (20). Moreover, human ulcerative colitis is strongly associated with p16 inactivation (6, 7) and colon tumorigenesis (21–23). Therefore, although no mouse model is without caveats, this combination of predisposing factors mirrors high-risk states for colon neoplasia in humans.

**Reduced tumor burden in p16-null Min mice treated with SNS-032**

Carrier or SNS-032 was administered to p16-null (24) Min mice by i.p. injection at doses shown to be effective in other experimental settings (11). DSS was administered on weeks 5 and 7 (Fig. 1). Drug or carrier was administered on weeks 6, 8, 9, and 10. Mice were euthanized on week 12. By this time point, Min tumors are readily recognized by examination of dissected intestines under a dissecting microscope but there is little disease-related morbidity or mortality (15, 20). Colon tumor number and maximum diameter were scored by an observer blinded to the treatment groups and used to calculate tumor burden (total tumor area).

SNS-032 was well tolerated under these conditions, without obvious side effects. Drug-treated animals showed 43% of the tumors and 36% of the tumor burden of carrier-treated mice (Fig. 2; P < 0.003 and 0.001, respectively). Although death was not a designed end point in this study, two carrier-treated
mice died during DSS treatment and one during the month before scheduled sacrifice, accounting for the lower number of carrier-treated mice scored. These results provide evidence for chemoprevention of colon tumorigenesis by SNS-032 in a model of colon tumorigenesis in situ.

**Reduced tumor burden in Ink4a/Arf-null Min mice treated with SNS-032**

We then extended our studies to Ink4a/Arf-null mice, which are defective in both p16 and Arf (13). Simultaneous silencing of both overlapping genes at the Ink4a/Arf locus is seen in a substantial fraction of human colon tumors (25). Moreover, Ink4a/Arf-null Min mice show a modestly more aggressive tumor phenotype than the corresponding p16-null mice (15, 18). Early in the course of the experiments, we found that the Ink4a/Arf-null Min mice did not tolerate the DSS regimen used in p16-null mice, with several (both carrier and drug-treated animals) demonstrating major weight loss or death. We therefore reduced the concentration of DSS in the drinking water from 4% to 3%. No animal experienced major weight loss or overt illness on the new regimen. Because there were no significant side effects of SNS-032 when administered twice a week in p16-null mice, we increased the dosing frequency to thrice a week, with the goal of optimizing drug benefit. Thus, the experiments in Ink4a/Arf-null Min mice were intended to best test the efficacy of SNS-032 in this second genetic background, not to compare drug response between the respective Ink4a/Arf genotypes. We also extended our tumor analysis to the small intestine, which is largely unaffected by DSS treatment. Only the distal small intestine shows a modest increase in inflammation and Min tumorigenesis (26, 27).

SNS-032 reduced the number of intestinal tumors in Ink4a/Arf-null Min mice to 25% of that in carrier-treated mice (Fig. 3A; $P < 0.001$ for each intestinal segment and the intestine as a whole) and lower tumor burden (16% of the carrier-treated mice, $P < 0.0001$ for the intestine as a whole). The differences in tumor number and burden remained significant ($P < 0.003$ and $P < 0.005$, respectively) after exclusion of the three animals that received 4% DSS (carrier no. 1 and SNS-032–treated carrier nos. 1 and 2), which showed a preponderance of colon tumors.

![Figure 2](image-url)  
**Fig. 2.** SNS-032 reduced colon tumor number and burden in p16-null Min mice. Mice were treated as per Fig. 1 except that DSS concentration was reduced to 3% for most mice, and SNS-032 dosing frequency was increased to thrice a week. Tumor number (A) and burden (B) [total tumor area ($\pi \times r^2$)] per mouse was scored by an observer blinded to the treatment groups. Five carrier-treated (left) and nine SNS-032–treated mice (right) survived to the sacrifice point. Tumor number and burden were lower in the SNS-032–treated animals ($P < 0.003$ and $P < 0.001$, respectively).

![Figure 3](image-url)  
**Fig. 3.** SNS-032 reduced intestinal tumor number and burden in Ink4a/Arf-null Min mice. Mice were treated as per Fig. 1 except that DSS concentration was reduced to 3% for most mice, and SNS-032 dosing frequency was increased to thrice a week. Tumor number (A) and burden (B) in each intestinal segment [as designated, from proximal (jejunum) to distal (colon)] per mouse was scored by an observer blinded to the treatment groups. SNS-032–treated mice had fewer tumors in the intestine as a whole (25% of carrier-treated mice, $P < 0.001$ for each intestinal segment and the intestine as a whole) and lower tumor burden (16% of the carrier-treated mice, $P < 0.0001$ for the intestine as a whole). The differences in tumor number and burden remained significant ($P < 0.003$ and $P < 0.005$, respectively) after exclusion of the three animals that received 4% DSS (carrier no. 1 and SNS-032–treated carrier nos. 1 and 2), which showed a preponderance of colon tumors.
the small intestine (Fig. 3B), with each small intestine segment (jejunum, proximal ileum, and distal ileum) as well as colon showing a significant reduction in tumor burden (Fig. 3B; \( P < 0.001 \) for each segment).

Drug treatment was again well tolerated in the Ink4a/Arf-null Min DSS-treated mice, with all treated mice maintaining their weight (data not shown) and appearing generally healthy. At sacrifice, two drug-treated mice were noted to have pale liver lesions. Histologic analysis showed pockets of polymorphonuclear leukocytes and necrosis. It is unclear whether this pathology was related to the genetic background, the DSS treatment, the drug, or possibly infection arising from repeated i.p. injection. Similar lesions have not been noted with SNS-032 in other reported mouse or human trials (11, 12, 32).

**Cell cycle inhibition and apoptosis mediated by SNS-032**

Min mouse intestinal tissues were examined for acute effects of SNS-032. *In vitro* studies with SNS-032 have shown the accumulation of cells in G2-M phase and apoptosis (11). The G2-M accumulation is consistent with the inhibition of Cdk2, based on evidence that G2 is the cell cycle phase most sensitive to Cdk2 inhibition (33). Cdk7 and Cdk9 seem to foster the transcription of short-lived proteins such as the antiapoptotic proteins Mcl-1 and XIAP. SNS-032 may induce apoptosis in part through such targets (11). Cdk2 inhibition might also contribute to apoptotic effects. For example, administration of Cdk2 antagonist peptides (34) and sustained induction of a Cdk2-dominant negative mutant (33) can promote apoptosis in cultured tumor cells. We sacrificed p16-null and Ink4a/Arf-null Min animals, respectively, 6 hours after the third dose of carrier or SNS-032 and 4 hours after administration of BrdUrd. DNA synthesis, assayed by BrdUrd incorporation, was modestly but statistically significantly reduced by treatment with the drug (Figs. 4A and 5). However, a distinct reduction in mitotic index was confirmed by staining for PH3 (Figs. 4A and 5). A marked increase in apoptosis, assayed by aCasp3, was also noted (Figs. 4A and 5). Similar results were seen in Ink4a/Arf wild-type Min mice (data not shown).
We then extended these studies to \textit{Ink4a/Arf}-null Min tumors from SNS-032 and carrier-treated mice. Reduced BrdUrd and PH3 and increased aCasp3 staining were readily shown in these tissues (Figs. 4B and 6; data not shown). Similar results were obtained in tumor tissues from \textit{Ink4a/Arf} wild-type Min mice (data not shown). These findings validate, in intestinal tissue, the \textit{in situ} biological effects of SNS-032 seen \textit{in vitro} and in xenografts, and are consistent with inhibition of the identified targets of the drug.

**Discussion**

We found that SNS-032 strongly reduced tumor burden in highly tumor prone mouse models of intestinal tumorigenesis. The lower mitotic index and increased apoptosis observed in tumor epithelial cells are consistent with inhibition of the known drug targets Cdk2, Cdk7, and Cdk9. These results suggest the efficacy of pharmacologic Cdk inhibition for chemoprevention of intestinal tumorigenesis. The potent reduction of tumor burden observed in the small intestines of \textit{Ink4a/Arf}-null mice suggests that suppression of local inflammation is unlikely to be the principal mechanism of drug action. Inflammation is largely confined to the colon in DSS-treated mice (20, 27). To our knowledge, this is the first evidence for efficacy of SNS-032 or other small molecule Cdk inhibitors against intestinal tumors arising \textit{in situ}. \textit{In situ} models allow drug testing in immunocompetent animals, against the diversity of naturally arising tumors, and in native tumor microenvironments. Furthermore, drug efficacy can be tested in specific settings of tumor predisposition (e.g. colitis), specific genotypes generated through transgenic methods, and/or tumors generated by random mutagenesis, such as with azoxymethane (35).

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The high incidence and prevalence of colorectal cancer in the general population, combined with its still substantial
mortality and well-defined high-risk states, makes this disease particularly attractive for chemoprevention strategies. Most such efforts thus far have focused on cyclooxygenase-2 and other pathways involved in inflammation. Cdks have generally been viewed as potential targets in cancer chemotherapy but not in chemoprevention. One reason for this is that Cdks are involved in normal cell proliferation, which is required prominently for homeostasis in the bone marrow and intestinal epithelium. Thus, the “chemopreventive window”, analogous to the “therapeutic window”, for agents that target Cdks has been a concern. Indeed, we observed drug-mediated inhibition of proliferation and increased cell death of non-neoplastic intestinal epithelium. However, drug treatment was overall well tolerated. Some mice manifested diarrhea during the DSS treatment phase, but no diarrhea was noted during treatment with SNS-032. We did not assay hematopoietic variables, but clinical signs of anemia (white paws, pale intestines, and lethargy) were mild as a whole and did not increase in the drug-treated group. Further work will be required to assess whether or not the occurrence of pockets of hepatic inflammation and necrosis in two Ink4/Arf-null Min DSS-treated mice were drug-related. Similar hepatic lesions have not been reported in other preclinical models or clinical trials (11, 12, 32). Although SNS-032 has not been developed as an oral agent, humans have been given single oral doses in pilot studies (12). Bioavailability was between 4% and 33%, suggesting some potential for this route of administration. Our results establish the principle that chemoprevention with a Cdk inhibitor can be effective against intestinal tumorigenesis.

Some recent chemoprevention studies have combined agents, to achieve greater efficacy whereas minimizing side effects. The combination of difluoromethylornithine and sulindac has shown impressive efficacy in the secondary prevention of colon adenomas (36). Nonetheless, this strategy has not yet entered routine clinical use and concerns remain. Chemoprevention was incomplete, accompanied by measurable hearing loss in some patients, and may or may not translate to other high-risk groups. Cdk inhibition might eventually be considered for combination chemoprevention in high-risk groups, if buttressed by additional preclinical studies.

Our findings also support continued evaluation of SNS-032 in cancer therapy. Although early results have not been particularly promising (12), the regimens used have not always been pushed to maximum tolerated doses, have generally been quite different than the one used here, and have achieved some efficacy in hematopoietic malignancies (11). For example, some human trials have used intravenous infusion once a week or once every 3 weeks, rather than the twice to thrice a week treatment regimen in this study (12). The preclinical model used here presents an opportunity to more systematically test different regimens and the susceptibility of different tumor genotypes.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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References


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