

Increased Survival of Tumor-bearing Mice by the Delta Opioid SNC 80

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Abstract. Opioids represent a major source of relief from pain. However, opioid abuse may cause immunosuppression and cancer. We have recently reported results on novel non-peptidic delta- and mu-selective opioids that induced immunopotential of T cell and macrophage functions *in vitro* and *ex vivo*. In the present study, the effects of the delta-opioid receptor agonist and potent analgesic (+)-4-((alpha R)-alpha-((2S, 5R)-4-allyl-2, 5-dimethyl-1-piperazinyl)-3-methoxybenzyl)-N, N-diethyl-benzamide (SNC80) on *in vitro* and *in vivo* tumor cell growth were investigated using the L5178Y-R murine model. SNC80 marginally, but significantly ($p < 0.05$), inhibited (up to 14%) the *in vitro* growth of L5178Y-R tumor cells. However, *in vivo* intratumor administration of SNC80 (2 and 4 mg/kg) reduced up to 60% L5178Y-R tumor-bearing Balb/c mice death, and significantly ($p < 0.05$) reduced tumor weights (up to 73% reduction) in these animals. This study may support the evaluation of SNC80 in preclinical and clinical studies.

Opioids include opium, morphine, heroin, codeine, meperidine, methadone and propoxyphene. All of these relax the central nervous system and have similar sleep-inducing and narcotic (pain-relieving) effects. Heroin is the most commonly abused opioid drug in the United States; there are an estimated 400,000 to 600,000 heroin addicts in the United States alone (1, 2). Morphine and heroin produce mental clouding, drowsiness and euphoria. Some

people start out using these prescribed drugs for pain relief from an illness but gradually increase the dose on their own, thus inducing tolerance. It is known that, over time, opioid users who inject the drug may develop infections of the heart lining and valves, skin abscesses and lung congestion. Infections can lead to hepatitis, tetanus, liver disease and human immunodeficiency virus transmission. Additionally, virtually all drugs with abuse potential have central actions and many abused drugs have immunosuppressive effects leading to infectious diseases (3-8).

The role of opioids in cancer, however, is not yet clear. Carpenter *et al.* reported that acute treatment of mice with 50.0 mg/kg of morphine significantly suppressed cytotoxic T-lymphocyte activity (9), these being key cytotoxic cells against tumor cells. In addition, Zagon and McLaughlin recently showed that exposure of the human cancer cell lines MIA PaCa-2 (pancreatic adenocarcinoma), HT-29 (colon adenocarcinoma) and CAL-27 (squamous cell carcinoma of the head and neck) to [D-Ala(2),MePhe(4), Glycol(5)]-enkephalin (DAMGO), morphine, or etorphine at 10^{-6} M significantly increased cell apoptosis and necrosis (10). However, they concluded that this cytotoxic activity was weak and dose-effect relationships were not established, thus supporting the previously reported lack of growth inhibition of many of these compounds (10). Additionally, Kawase *et al.* reported that codeinone (a morphine derivative) induced cytotoxicity in the human oral tumor cells lines HSC-2 and HSG, but neither codeinone nor morphine were effective against a multidrug-resistant mouse T-lymphoma L5178 cell line transfected with the human *MDR1* gene (11). Earlier, Yeager and Colacchio (1991) showed that a high dose of subcutaneous morphine sulfate and a low dose of intrathecal morphine reduced postoperative growth of metastatic colon cancer in rats (12). In contrast, morphine was reported to stimulate tumor cell growth (13, 14) and enhance angiogenesis in a human breast

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tumor xenograft model in mice, leading to increased tumor progression (15).

The present study was undertaken to evaluate the effect of the pro-inflammatory opioid SNC80 on tumor cell growth *in vitro* and *in vivo*. Inhibition of *in vitro* L5178Y-R cell growth and a significant survival increase and tumor weight reduction in tumor-bearing mice treated with SNC80 were observed.

Materials and Methods

Reagents, culture media and cell lines. Penicillin-streptomycin solution, L-glutamine, Ficoll-hypaque solution, trypsin-EDTA solution and RPMI 1640 media were obtained from Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS), sodium dodecyl sulfate (SDS), *N,N*-dimethylformamide (DMF), phosphate-buffered saline (PBS), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The extraction buffer was prepared by dissolving 20% (wt/vol) SDS at 37°C in a solution of 50% each DMF and demineralized water, and the pH was adjusted to 4.7.

Drugs. The synthetic opioid (+)-4-((alpha R) 9-alpha-((2S, 5R)-4-allyl-2, 5-dimethyl-1-piperazinyl)-3-methoxybenzyl)-*N,N*-diethylbenzamide (SNC80) was provided by Kenner C. Rice. A stock solution of SNC80 was prepared using 10% concentrated dimethylsulfoxide and 5% 0.1N HCl.

Tumor cell line. The tumor cell line L5178Y-R (mouse DBA/2 lymphoma) was purchased from The American Type Culture Collection (Rockville, MD, USA) and was maintained in culture flasks with RPMI 1640 medium supplemented with 10% FBS, 1% L-glutamine and 0.5% penicillin-streptomycin solution (referred to as complete RPMI medium) at 37°C, in a humidified atmosphere of 5% CO₂ in air. The cellular density was kept between 10⁵ and 10⁶ cells/ml.

Cell preparation and culture. In order to determine the direct *in vitro* effect of SNC80 on tumor cell growth, cell cultures were collected, washed three times in RPMI 1640, and suspended and adjusted to 5x10⁴ cells/ml with complete RPMI medium. One hundred microliters of the cell suspensions were then added to flat-bottomed 96-well plates (Becton Dickinson, Cockeysville, MD, USA), containing triplicate cultures (100 µl) of complete RPMI (unstimulated control) or opioids at various concentrations. After incubation for 44 h at 37°C with 5% CO₂, MTT (0.5 mg/ml, final concentration) was added, and the cultures were further incubated for 4 h. Next, the cell cultures were incubated for 16 h with extraction buffer (100 µl) and the optical densities, resulting from dissolved formazan crystals, were then read in a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) at 540 nm (16). The percentage of cytotoxicity was calculated as follows:

$$\% \text{cytotoxicity} = 100 - \frac{A_{540} \text{ in opioid-treated cells}}{A_{540} \text{ in untreated cells}} \times 100$$

***In vivo* tumor growth inhibition by SNC80.** Based on our *in vitro* findings of the effects of non-peptide opioids against tumor cell

growth, we hypothesized that they could have beneficial effects against tumors *in situ*. The L5178Y-R lymphoma mouse model was used to test SNC80 antitumor activity [this opioid was reported to possess immunopotentiating properties (17, 18)]. The lymphoma was maintained by inoculating *i.p.* 0.2 ml of L5178Y-R tumor cells (5x10⁶ cells/mouse) in 4- to 6-week-old female Balb/c mice. Thirteen days after inoculation, ascites was collected from the peritoneal cavity of mice killed by cervical dislocation. After this, the ascitic suspension was placed in a 50-ml tube containing 10 ml PBS. The cell suspension was washed twice in PBS by centrifuging at 2000 rpm for 10 min and adjusted to 2x10⁷ cells/ml in complete RPMI 1640 medium. Next, female 2-month-old Balb/c mice received a subcutaneous administration (upper right thigh) of this lymphoma suspension/ml. After 7 days of tumor inoculation, L5178Y-R tumor-bearing mice were treated intratumorally with 9 doses of SNC80 at concentrations of 2 and 4 mg/kg (prepared fresh) in a volume of 0.2 ml. Controls were opioid-untreated tumor-bearing mice (untreated), vehicle (a solution containing 10% concentrated dimethylsulfoxide and 5% 0.1N HCl)-treated tumor-bearing mice (vehicle), normal untreated (untreated control w/o tumor) and SNC80 [SNC80 (4 mg/kg) w/o tumor]-treated mice. Overall survival (a) and tumor weight (b) (tumor weight was estimated by measuring the width (A) and length (B) of the tumor, and using the following equation: Weight (mg)=A(mm)xB²(mm)/2). Measures were taken on day zero (7 days after tumor administration), and on days 4 (day 11 post tumor injection), 7 (day 14 post tumor injection), 11 (day 18 post tumor injection), 14 (day 21 post tumor injection), 17 (day 24 post tumor injection), 21 (day 28 post tumor injection), 24 (day 31 post tumor injection) and 28 (day 35 post tumor injection) from the beginning of the opioid treatments which were administered using this schedule comprising a total of 9 injections. The mean tumor weight index was calculated as follows=mean final tumor weight (mg) (this represented the last record before animal death)/mean initial tumor weight (mg).

Statistical analysis. The results were expressed as mean±SEM of the response of 3 replicate determinations per treatment (*in vitro* study) or 5 mice per experimental group (6 groups as explained above) from 3 independent experiments. The level of significance was assessed by Dunnet's *t*-test.

Results

Effect of SNC80 on proliferation of L5178Y-R. The effect of SNC80 on the *in vitro* proliferation of the L5178Y-R tumor cell line was evaluated. SNC80 at concentrations ranging from 10⁻¹¹ M to 10⁻⁶ M marginally, but significantly (*p*<0.05), inhibited L5178Y tumor cell line growth (up to 14% growth inhibition), as compared with the SNC80-untreated control (Figure 1).

***In vivo* tumor growth inhibition by SNC80.** *In vivo* intra-tumor administration of SNC80 (2 or 4 mg/kg) caused 40% survival of tumor-bearing mice at 35 days post tumor injection, and they remained alive 50 days post tumor administration (Figure 2a), whereas 100% mortality was observed in opioid-untreated tumor-bearing mice (untreated) and vehicle-treated tumor-bearing mice

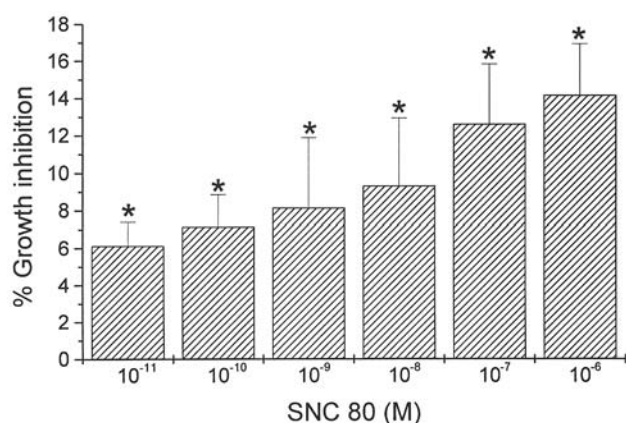


Figure 1. Tumor cell growth inhibition by SNC80. The exponentially growing L5178Y-R cell line culture was incubated for 48 h in the presence or absence of various concentrations (10^{-11} M to 10^{-5} M) of SNC80, as explained in the text. After incubation for 44 h, MTT was added to all wells and the cultures were incubated for an additional 4 h. Optical densities and percent cytotoxicity were then determined by absorption at 540 nm, as described in the text. The data represent mean \pm SEM of triplicates from three independent experiments. *, $p < 0.05$, compared with untreated control. The optical density value for proliferation of the untreated control was 0.402 ± 0.008 for the tumor cell line L5178Y-R.

(vehicle) (Figure 2a). In addition, 100% survival was observed in normal untreated and SNC80-treated mice not inoculated with tumor cells (Figure 2a). Furthermore, SNC80 caused significant ($p < 0.05$) 60 and 73% reduction in mean tumor weight index at 2 and 4 mg/kg, respectively, compared with the untreated tumor-bearing mice control (Figure 2b); the mean tumor weight index in the vehicle control animals was not significantly different from this control group (data not shown).

Discussion

The antitumor activity of opioids is a promising area of study. Besides their role in modulating the antitumoral immune host response, there is evidence that opioids might act directly on receptors on the surface of tumor cells to activate cellular pathways that lead to growth inhibition or that cause cytotoxicity. The endogenous opioid system is known to influence tumoral growth and development by modulating the host antitumoral immune response (5, 8, 19). A clear example of this is the observation that morphine, α -endorphin, met-enkephalin, leu-enkephalin, dynorphin-A and β -endorphin enhance the activity of macrophages against tumor growth (20-22). Opioids can also act directly on tumor cells to cause cytotoxicity or to inhibit proliferation (23). This possibility involves the presence of opioid receptors on the surface of tumor cells,

which suggests that exogenous alkaloid opioids and non-peptidic synthetic opioids might also have antitumoral properties. In the present study, SNC80 was observed to inhibit *in vitro* L5178Y-R lymphoma cells (Figure 1). Tumor cell growth alterations have been observed for morphine, which was shown to inhibit B-lymphoma Namalva cells and Epstein-Barr Virus-transformed lymphocytes growth, while stimulating proliferation of myeloid K562 and T-lymphoma Yurkat cells (14). The presence and type of opioid receptors on these cells may be an important factor in such a response [Reviewed in (1)]. Opioid receptors have been demonstrated in Jurkat (24), U937 (25), L929 (26) and J774 (27) tumor cell lines. No evidence, however, of the presence of such receptors has been reported for L5178Y-R cells.

Based upon our *in vitro* findings of cell growth inhibition and the reported pro-inflammatory properties of SNC80 (17, 28, 29), this opioid was tested in a murine model of tumor development. SNC80 was observed to reduce by up to 60% L5178Y-R tumor-bearing Balb/c mice death (Figure 2a) and significantly ($p < 0.05$) reduce tumor weights (up to 73% reduction) in these animals (Figure 2b). *In vivo*, the antitumor activity of SNC80 may be mediated both directly and indirectly by stimulating inflammation. In addition to its potent analgesic properties in several animal models of acute anti-nociception (30, 31), SNC80 has shown both lack of immunosuppressive effects and potentiation of immune function in the rat (18, 32). SNC80 is capable of inducing recruitment and activation of macrophages (with production of tumor necrosis factor- α (TNF- α) and nitric oxide and lymphocytes at tumor sites (17, 18, 28, 29). We have previously reported that intravenous administration of SNC80 was associated with *ex vivo* activation of TNF- α (up to 100% increase) and nitric oxide (up to 67% increase) production by peritoneal macrophages, which was potentiated by *i.v.* injection of concanavalin A (17). We also observed that pre-treatment of human peripheral blood mononuclear cells with SNC80 for 16 h significantly increased TNF- α production by resident and LPS-activated macrophages, and increased TNF- α and IL-8 mRNA signals compared with the unstimulated control (unpublished observations). TNF- α is known to cause tissue inflammation, tumor cell death and toxic side-effects such as body weight reduction (33, 34). On the other hand, SNC80-mediated activation of lymphocytes (29) may lead to the release of cytokines, such as interferon-gamma and IL-2, that are involved in pro-inflammatory processes (35). Activation of inflammatory cytokines and cytokine gene expression by the action of SNC80 on leukocytes may be associated with its capacity to regulate the development of certain types of cancer (36).

SNC80 could serve as an immunotherapeutic agent by not only increasing the pool of activated T-lymphocytes and

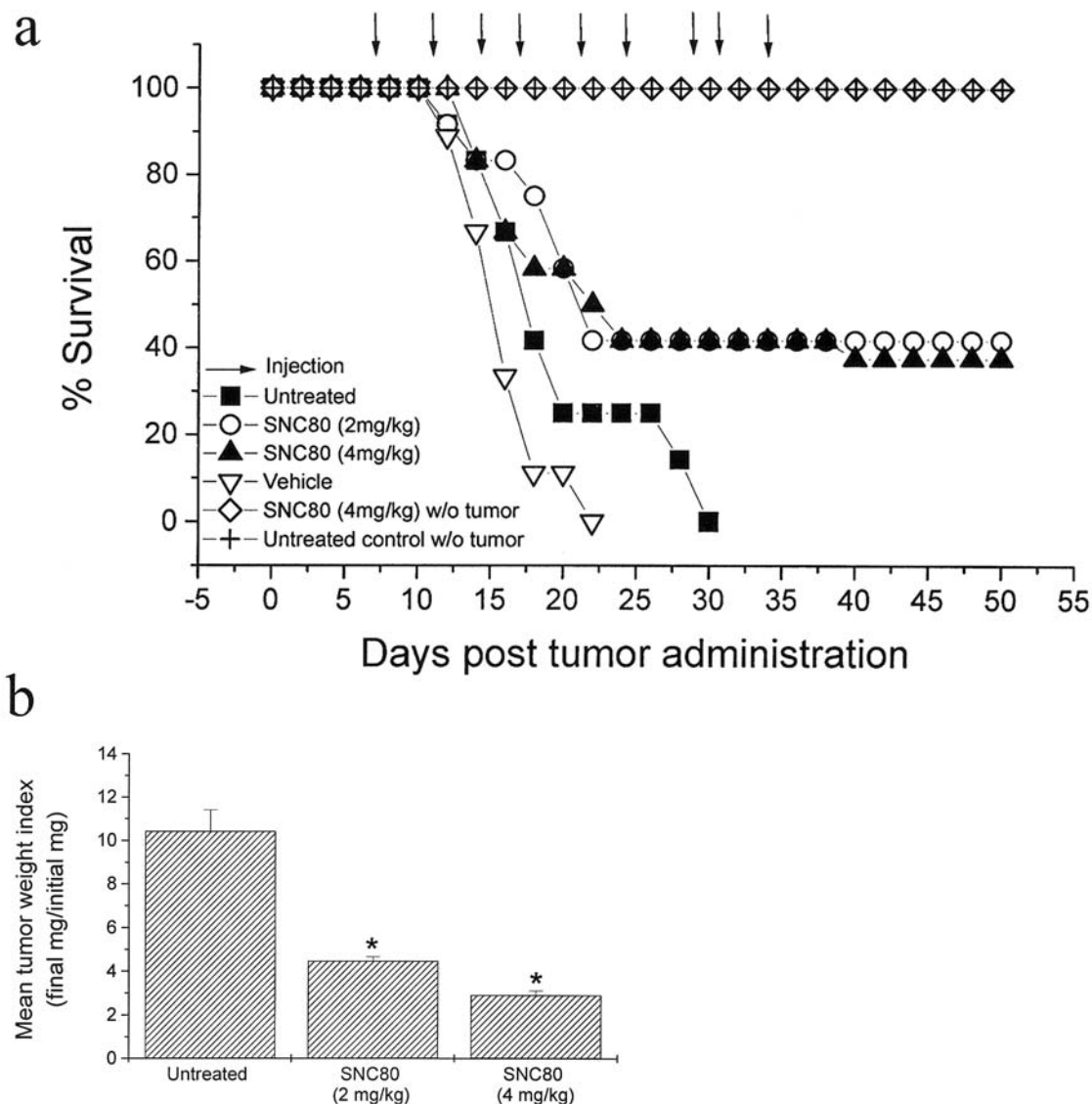


Figure 2. Increased mice survival and reduced tumor mass by SNC80. Female 2-month-old Balb/c mice received a subcutaneous administration (upper right thigh) of 2×10^7 L5178Y-R lymphoma cells/ml. After 7 days of tumor inoculation, L5178Y-R tumor-bearing mice were treated intratumorally with SNC80. Overall survival (a) and tumor weight (b) measures were taken on days 0, 4, 7, 11, 14, 17, 21, 24 and 28 (day 35 post tumor injection), as explained in the text. SNC80 was administered under this schedule (9 doses). *, $p < 0.05$ compared with the response of SNC80-untreated tumor-bearing mice.

modulating macrophage functions, but also by directly killing tumor cells. Further studies are necessary to elucidate the mechanism of action SNC80 on modulating tumor cell growth, and its role in tumor therapy.

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