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ORIGINAL ARTICLE

Yong Ke · Keqiang Ye · Hans E. Grossniklaus
David R. Archer · Harish C. Joshi · Judith A. Kapp

Noscapine inhibits tumor growth with little toxicity to normal tissues or inhibition of immune responses

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Abstract Noscapine, a phthalideisoquinoline alkaloid derived from opium, has been used as an oral anti-tussive agent and has shown very few toxic effects in animals or humans. Recently, we reported that noscapine binds stoichiometrically to tubulin and promotes microtubule polymerization. Noscapine causes growth arrest of tumor cells in mitosis and induces apoptosis of tumor cells in vitro. Previous experiments also showed that noscapine has potent antitumor activity in mice when administered parenterally or by gastric lavage. Here, we report that the anti-mitotic effect was specific to noscapine since closely related compounds did not inhibit the growth of a lymphoma cell line. In addition, noscapine was shown to be effective in reducing the growth of the lymphoma and increasing the survival of tumor-bearing mice when administered in the drinking water. It is noteworthy that, noscapine showed little or no toxicity to kidney, liver, heart, bone marrow, spleen or small intestine at tumor-suppressive doses. Furthermore, oral noscapine did not inhibit primary immune responses, which are critically dependent upon proliferation of lymphoid cells. Thus, our results indicate that noscapine has the potential to be an effective chemotherapeutic agent for the treatment of human cancer.

Key words Drug therapy · T cells · Apoptosis

Y. Ke[1] · J. A. Kapp
Department of Pathology,
Emory University School of Medicine,
Atlanta, GA 30322, USA

K. Ye · H. C. Joshi
Department of Cell Biology,
Emory University School of Medicine,
Atlanta, GA 30322, USA

H. E. Grossniklaus · J. A. Kapp (✉)
Department of Ophthalmology,
South Clinic Bldg. B, Emory University,
1365 Clifton Rd. NE, Atlanta,
GA 30322, USA
e-mail: jkapp@emory.edu
Tel.: +1-404-778-4754,
Fax: +1-404-778-2109

D. R. Archer
Department of Pediatrics,
Emory University School of Medicine,
Atlanta, GA 30322, USA

Present address:
[1] Department of Pathology, Indiana University Medical Center,
550 N. University Blvd. Indianapolis, IN 46202-5280, USA

Introduction

Many agents currently used for cancer chemotherapy are cell-cycle-specific growth inhibitors. Some of these drugs, such as the vinca alkaloids (vincristine, vinblastine, and vindesine) [15], paclitaxel [31] and estramustine [34], interact with tubulin, the major protein of mitotic spindles, causing arrest in metaphase. These agents alter the polymerization or depolymerization of tubulin, halt mitosis of rapidly dividing cells, induce apoptosis, and are reasonably effective in cancer chemotherapy [15, 34]. However, drug resistance is a common problem with repeated and prolonged administration of chemotherapeutic agents, possibly owing to the amplification of a membrane glycoprotein involved in efflux of the drug [33]. Moreover, these anti-microtubule agents are frequently toxic to normal tissues and are effective only for certain types of cancer [19, 24, 30]. Hence, new and better chemotherapeutic drugs are needed.

To identify potential new anti-microtubule drugs, we compared the chemical structure of several microtubule inhibitors and noted that many of them contain a hydrophobic trimethoxyphenyl group and a variety of hydrophobic domains, such as lactone, tropolone or other aromatic rings [39]. Noscapine [L-α-methyl-8-methoxy-6,7-methylenedioxy-1-1(6, 7-dimethoxy-3-phthalidyl)-1,2,3,4-tetrahydroisoquinoline], an alkaloid derived from opium [17], was chosen for our studies because it possesses a lactone ring and shares some other structural features with the known anti-microtubule agents [16]. Noscapine is a non-narcotic derivative of opium that has anti-tussive activity in experimental animals [14, 38] and

in humans [2, 6]. Noscapine lacks analgesic, sedative and respiratory-depressant properties, and it does not produce euphoria or dependence [25]. Recently, we reported that noscapine binds stoichiometrically to tubulin and promotes microtubule polymerization, which causes growth arrest of tumor cells during mitosis [39]. Moreover, noscapine induces apoptosis and antitumor activity against the murine and human solid tumors that have been tested [39].

In the present study, we report that the activity of noscapine is specific in that closely related compounds are not anti-mitotic except at very high concentrations. Also, long-term survival of tumor-bearing mice is induced by noscapine delivered in the drinking water. Moreover, therapeutic doses of noscapine cause little or no toxicity to normal tissues and do not inhibit primary immune responses, which are critically dependent upon the proliferation of lymphocytes.

Materials and methods

Mice

Female C57BL/6 (H-2^b) mice, 8–12 weeks of age, were purchased from Charles River Laboratories (Raleigh, N.C.). Mice were maintained on standard laboratory chow and water ad libitum in a temperature- and light-controlled environment. All procedures were conducted according to the "principles of laboratory animal care" (NIH publication 85-23, revised 1985) under a protocol approved by the Institutional Animal Care and Use Committee.

Reagents

Noscapine hydrochloride was purchased from Sigma Chemical Co. (St. Louis, Mo.). Narceine, an achiral opium alkaloid with a structure related to noscapine, was obtained from Apin Chemicals Ltd. (Oxon, UK). Metabolic cleavage of noscapine yields two compounds, opionic acid and cotarnine [36], which were provided by Dr. M. O. Karlsson (Uppsala University, Uppsala, Sweden). Noscapine hydrochloride and related compounds were dissolved in dimethylsulfoxide (DMSO) and further diluted in saline or sterile water. Purified chicken egg ovalbumin (OVA, grade VI) and phenazine methosulfate (PMS) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT), was purchased from Diagnostics Chemicals Ltd. (Oxford, Conn.). Complete Freund's adjuvant (CFA) containing *M. tuberculosis* H37Ra was obtained from Difco Laboratory (Detroit, Mich.).

Cell lines

The T cell lymphoma cell line, EL4, arose in C57BL (H-2^b) mice after treatment with dimethylbenzanthracene [10]. EL4 has been used as a tumor model in hundreds of studies and was chosen for these studies because we wanted to compare the drug effects on normal and transformed T cells in vivo. EL4 cells transfected with the OVA cDNA gene [27], which are called E.G7-OVA, were produced and generously provided by Dr. M. J. Bevan (University of Washington, Seattle, Wash.). E.G7-OVA was used as a tumor, rather than EL4, because OVA-specific immune rejection of the tumor could serve as a positive control for tumor inhibition. EL4 and E.G7-OVA were maintained in RPMI-1640 medium supplemented with 5% fetal calf serum, 1 mM L-glutamine, 1 mM sodium pyruvate and 50 μM 2-mercaptoethanol, at 37 °C in 6% CO_2 in air and shown to be free of *Mycoplasma*.

An OVA-specific $CD4^+$ T cell clone, which is specific for the peptide $OVA_{323-339}$ in the context of H-2^d, was generated from DO11.10 T-cell-receptor-transgenic mice [28], as described previously [3]. Clones (2.5×10^5/well) were restimulated every 2 weeks in 24-well plates with 0.3 μM $OVA_{323-339}$ and irradiated BALB/c spleen cells (5×10^5/well).

In vitro proliferation assay

The growth-inhibitory effects of noscapine and related compounds were tested on tumor cells and non-transformed T cells in vitro. Serial twofold dilutions of DMSO or reagents dissolved in DMSO were made in medium in 96-well micro-titer plates. Wells with medium only were used as negative controls. E.G7-OVA cells were then added in triplicate, at 1×10^3 cells/well. Non-transformed T cells (2×10^5) were mixed with 2×10^6 irradiated, syngeneic spleen cells and 0.3 μM $OVA_{323-339}$. After incubation at 37 °C for 72 h, proliferation of E.G7-OVA and T cells was determined in a colorimetric assay by adding XTT and PMS as described by Roehm et al. [29].

Absorbance was read at 450 nm in an automatic microplate reader (Molecular Devices Corp., Menlo Park, Calif.). All experiments have been carried out at least three times with similar results and representative experiments are shown.

Tumor inoculation and treatment

C57BL/6 mice were inoculated with syngeneic E.G7-OVA T cell lymphoma by injecting 2×10^6 viable cells subcutaneously (s.c.) into the right flank [40]. Three days later, some mice were injected once in the hind footpads with 200 μg OVA emulsified in CFA [21] to induce immune rejection of the tumor. Other mice received noscapine daily at 3 mg/mouse (approx. 120 mg/kg body weight) either by intraperitoneal (i.p.) injection or by oral feeding through intragastric intubation using a 1-ml syringe fitted with a 20-gauge stainless-steel, ball-point needle (Popper & Sons Inc., New Hyde Park, N.Y.) [20]. In some experiments, noscapine was added to the drinking water at 1.5 or 3 mg/ml. Three weeks after treatment, mice were sacrificed and tumors were removed. Tumors were individually measured or weighed and comparisons between untreated controls and treatment groups were analyzed by the Student's *t*-test. Statistical differences were considered significant if *P* values were less than 0.05.

Terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL) assays

Apoptosis was determined by in situ TUNEL assays [8, 9] using an In Situ Cell Death Detection Kit (Boehringer Mannheim, Indianapolis, Ind.) according to the manufacturer's instructions. Tumors were removed from untreated or treated mice, fixed with formalin, embedded in paraffin, cut to 5-μm-thick sections and then mounted on microscope slides. Tumor tissues were dewaxed by heating at 60 °C for 15 min, rinsed in xylene, and rehydrated with a graded series of ethanol and distilled water. After incubation with proteinase K (20 μg/ml in 10 mM TRIS/HCl) at room temperature for 20 min, slides were blocked with 0.3% H_2O_2 in methanol for 30 min at room temperature and incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) at 4 °C for 2 min. TUNEL reaction mixture was added to the slides and incubated in a humidified chamber at 37 °C for 60 min. An anti-fluorescein antibody conjugated with horseradish peroxidase was added and incubated at 37 °C for 30 min. Diaminobenzidine tetrahydrochloride substrate was added and incubated at room temperature for 10 min. Slides were rinsed with phosphate-buffered saline (PBS, pH 7.4) between each step. After a final wash with PBS, cover slips were mounted and samples were analyzed under a light microscope and photographed.

Histology and hematology

Blood was collected from normal or treated mice in heparinized tubes and analyzed on a Serano Diagnostic Systems 9018 Plus hematology analyzer. Bone marrow was flushed from the tibia and femur of treated and untreated mice and 1×10^5 cells stained for the presence of phosphatidylserine in the plasma membrane using annexin V (R&D Systems). Cells were dual-labeled with lineage-specific antibodies for red blood cells (Ter119), B cells (B220), T cells (CD3) macrophages (Mac-1) and granulocytes (Gr-1) or a combination of the above. Propidium iodide was added to the samples as a marker of dead cells before annexin V staining was analyzed by flow cytometry. Various other tissues were collected from control and treated mice, including the heart, kidney, spleen, small intestines, liver and bone marrow. These tissues were fixed in formalin, embedded in paraffin, sectioned and stained with hematoxalin and eosin.

Immune responses

In some experiments, normal control or noscapine-treated C57BL/6 mice were injected in the hind footpads with 200 μg OVA emulsified in CFA. Two weeks after immunization, mice were bled and spleens were removed. Sera were tested for anti-OVA antibody by an enzyme-linked immunosorbent assay as previously described [21]. Spleen cells were stimulated with irradiated E.G7-OVA (200 Gy) at 37 °C for 1 week and then tested for cytolytic activity using ^{51}Cr-labeled EL4 or E.G7-OVA targets [20, 21].

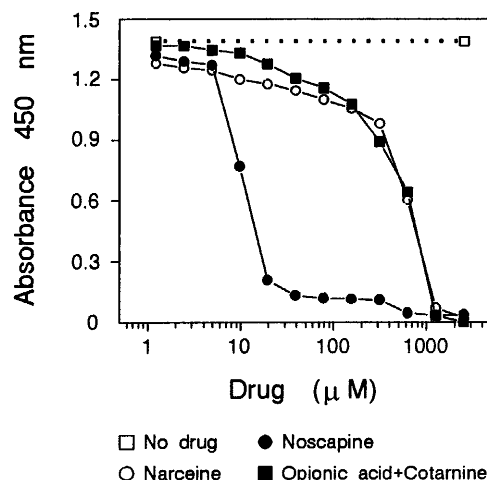

Fig. 1 Noscapine inhibits tumor cell proliferation in vitro. In 96-well microtiter plates, serial twofold dilutions of noscapine (●), narceine (○) or a mixture of opionic acid and cotarnine (■) were made in medium. Wells with medium only (□) were used as a positive control for cell growth. E.G7-OVA T cell lymphoma cells were then added at 1×10^3 cells/well and incubated at 37 °C. After 72 h, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzenesulfonic acid (XTT) and phenylmethylsulfonyl fluoride (PMS) were added. The colorimetric reaction was read at an absorbance of 450 nm and results shown are the means of triplicate samples. These results are representative of more than ten separate experiments

Results

Noscapine inhibits growth of tumor cells in vitro

We recently reported that noscapine promotes microtubule assembly and arrests the cell cycle of tumor cells in metaphase [39]. The purpose of the first experiment was to determine specificity of noscapine for E.G7-OVA T cell lymphoma cells in vitro. Growth of E.G7-OVA cells was inhibited by noscapine in a dose-dependent manner (Fig. 1). A 50% inhibition of E.G7-OVA cell proliferation was achieved at 10 μM noscapine in this particular experiment. To address whether the activity of noscapine is specific, closely related compounds were also tested. Neither narceine (an achiral opium alkaloid with a structure related to noscapine) nor the mixture of two metabolic products of noscapine (opionic acid and cotarnine) inhibited proliferation of E.G7-OVA cells at doses comparable to the noscapine dose (Fig. 1). The inhibition that was observed at doses of 500 μM and over is most likely due to non-specific cytotoxicity.

Noscapine inhibits tumor growth and induces apoptosis of tumor cells in vivo

We chose the E.G7-OVA T cell lymphoma as our model for testing the antitumor effect of noscapine for several reasons. The parental tumor, EL4, is a T-cell-derived lymphoma that has been widely studied as a tumor in syngeneic C57BL/6 mice since its description in 1950 [10]. Although EL4 grows spontaneously in syngeneic mice, it is weakly immunogenic and protection against the unirradiated tumor can be induced by injection of multiple doses of irradiated tumor cells [4]. However, the epitopes recognized by EL4-specific CTL have not been identified. EL4 cells transfected with the OVA gene (E.G7-OVA) express OVA peptides complexed to H-2K^b and can induce OVA-specific cytotoxic T lymphocytes (CTL) [27]. Like the parental EL4 cells, E.G7-OVA gives rise to tumors when injected into syngeneic mice [40]. Immunization of recipient mice with OVA incorporated into liposomes [40] retarded the growth of E.G7-OVA [40]. Thus, the OVA peptides expressed by E.G7-OVA can serve as surrogate tumor-specific antigens. In addition, we have previously shown that injection of C57BL/6 mice with OVA in CFA induces the activation of OVA-specific CTL [21]. Thus, immunization of tumor recipients with OVA can serve as a positive control for tumor inhibition.

Syngeneic mice were inoculated in the right flank with 2×10^6 viable E.G7-OVA cells. Three days later, mice were divided into four groups. The negative control group was left untreated while the positive control group was injected in the hind footpads with 200 μg OVA in CFA. Two other groups of mice were treated daily with 3 mg (120 mg/kg) noscapine either by i.p. injection or by intragastric lavage. Three weeks after tumor cell inoculation, mice were sacrificed and tumors were removed and weighed. As shown in Fig. 2, untreated mice developed solid tumors ranging in size from 4.5 g to 10.5 g (mean 7.8 ± 2.0 g). Mice immunized once with OVA in CFA developed tumors during the first few days that gradually regressed. By three weeks, five of ten mice in

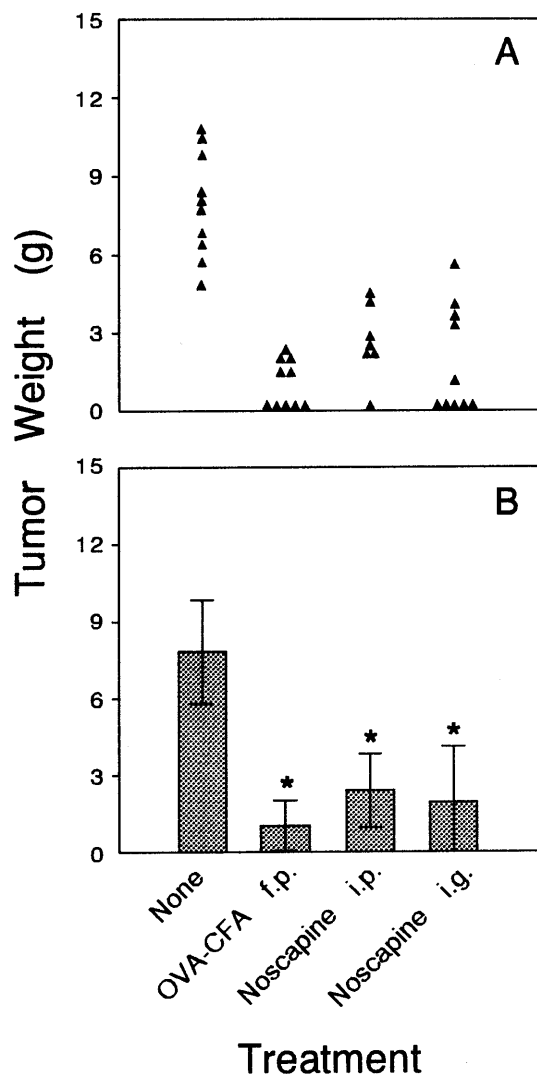

Fig. 2A, B Noscapine inhibits tumor growth in vivo. C57BL/6 mice (ten mice per group) were injected s.c. in the right flank with 2×10^6 syngeneic E.G7-OVA T cell lymphoma cells. Three days later, one group of mice was injected in the foot pads (*f.p.*) with 200 μg OVA-CFA emulsion. Two other groups of mice received 3 mg noscapine daily by i.p. injection or intra-gastric lavage (*i.g.*). Three weeks later, all mice were sacrificed and tumors were removed. Individual tumor weights are shown in **A** and averages ± SD are shown in **B**. * The results were significantly different from those of the untreated control group ($P < 0.01$) by Student's *t*-test

this group had no detectable tumors and the other mice showed only small palpable tumors (mean 1.029 ± 0.9 g).

Compared to untreated control mice, inhibition of E.G7-OVA growth by OVA-CFA immunization was statistically significant ($P < 0.001$). Some mice treated with noscapine had no palpable tumors (five of ten mice in the oral group) while others developed only small solid tumors in comparison with untreated control mice (Fig. 2). Inhibition of tumor growth was statistically significant ($P < 0.001$) in both groups treated with noscapine compared to the negative control group. None of the mice that received oral noscapine died, but 30% mortality was found after 2 weeks of treatment with noscapine i.p. The reason for this is not known, but repeated i.p. injections might have induced chronic acidosis and death of the mice. These results suggest that oral administration of noscapine is a more tolerable approach than parenteral delivery of the drug. To test the specificity of drug treatment, some mice were treated with 3.0 mg narceine i.p. However, all these mice died during the first week of treatment, suggesting that narceine is very toxic in vivo.

Although both OVA-CFA immunization and noscapine treatment completely inhibited tumor growth in some recipients, other mice developed small palpable tumors (Fig. 2). In the cases where tumors had not completely regressed by the termination of the experiment, the residual tumors were filled with holes (not shown). The remnant tumors in OVA-CFA or noscapine-treated mice were examined by TUNEL assay for signs of apoptosis. E.G7-OVA T cell lymphomas from untreated control mice showed only very few apoptotic cells and negligible TUNEL staining (Fig. 3A). By contrast, tumors removed from mice treated with OVA-CFA (Fig. 3B) or noscapine (Fig. 3C) showed many cells with apoptotic morphology throughout the tissue sections, which were stained darkly with the TUNEL agent (arrows). These in vivo results are consistent with our previous studies showing that noscapine causes apoptosis of E.G7-OVA in vitro [39].

On the basis of the observation that oral administration of noscapine inhibited tumor growth in vivo, we asked whether noscapine prolonged the survival of tumor-bearing mice. C57BL/6 mice were inoculated with E.G7-OVA and treatment was begun 3 days later. Instead of administering noscapine by gastric lavage, the drug was added to the drinking water at 1.5 mg/ml or 3 mg/ml. As shown in Fig. 4, untreated control mice all developed tumors and died within 7 weeks from invasive tumor outgrowth and metastasis into surrounding lymph nodes. Immunization with OVA-CFA protected tumor-bearing mice and 60% of these mice survived for more than 4 months ($P < 0.01$). Eighty per cent of the mice receiving either 1.5 mg/ml or 3 mg/ml of noscapine in their drinking water survived at least 4 months after tumor cell inoculation. The surviving mice did not have any detectable tumors when examined at necropsy. Thus, noscapine not only inhibited tumor progression but also prolonged survival of tumor-bearing hosts.

Effect of noscapine on normal tissues

Anti-mitotic drugs usually inhibit proliferation of nontransformed as well as transformed cells. To determine whether noscapine causes toxicity to other tissues, C57BL/6 mice were inoculated with the E.G7-OVA and 3 days later 1.5 mg/ml or 3 mg/ml noscapine was added to the drinking water. After three months of treatment, tissues were removed from tumor-free surviving mice and from normal control mice. At 1.5 mg/ml, noscapine

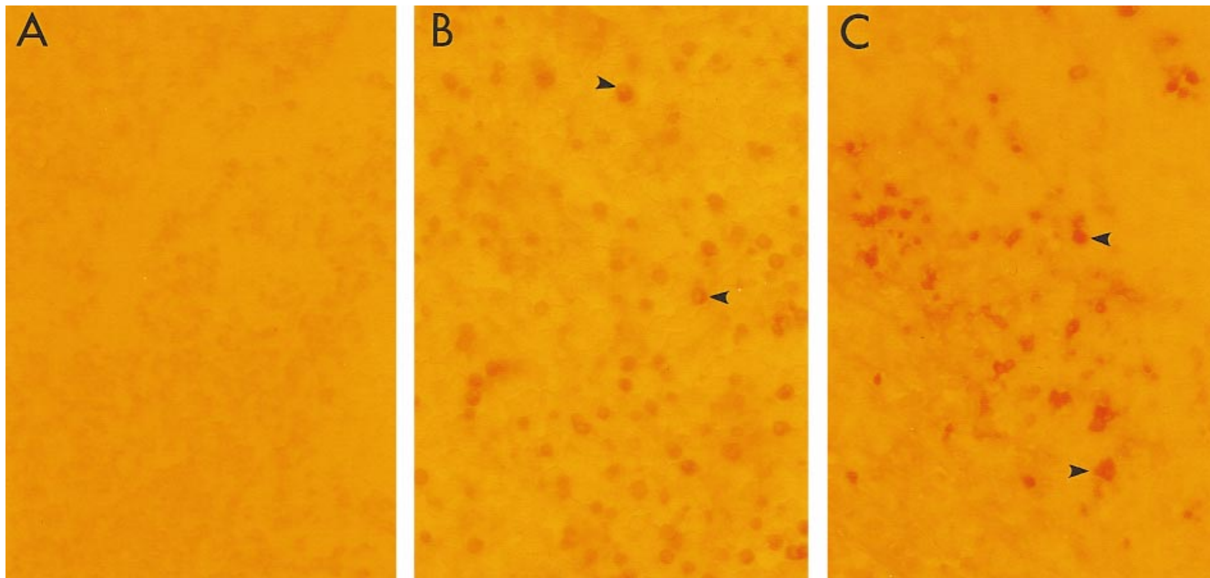

Fig. 3A–C Treatment induces apoptosis of tumor cells in vivo. C57BL/6 mice were inoculated with E.G7-OVA cells. Mice were either untreated or treated with OVA-CFA or oral noscapine as described in Fig. 2. Three weeks later, tumors were removed from untreated control mice (**A**), from mice treated with OVA-CFA (**B**) or from mice receiving noscapine orally (**C**). Tumor sections were stained by TUNEL as described in Materials and methods and photographed (40× magnification). ➤ Examples of the apoptotic cells

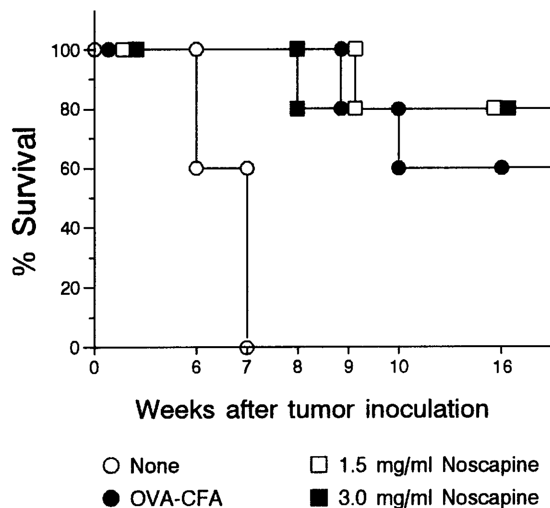

Fig. 4 Noscapine prolongs survival of tumor-bearing mice. C57BL/6 mice were injected s.c. in the flank with 2×10^6 syngeneic E.G7-OVA cells as described above. Three days later, one group of mice was left untreated (○) and another group was injected in the footpads with 200 μg OVA-CFA emulsion (●). The other two groups of mice received 1.5 mg/ml (□) or 3 mg/ml (■) noscapine in their drinking water

did not produce any obvious organ-specific toxicity in the heart, kidney or liver of treated mice compared to normal mice (not shown). Noscapine caused no toxicity in the heart or kidney; however, several isolated foci of inflammatory cell infiltrates were found in the livers of mice treated with 3 mg/ml, suggesting that noscapine was mildly hepatotoxic at higher doses (not shown). This is not particularly surprising because noscapine binds to serum albumin and α_1-acid glycoprotein [16] and is excreted via the hepatobiliary tract [36].

Rapidly dividing, normal tissues would be expected to be more sensitive to the anti-mitotic activity of noscapine than the heart, kidney, or liver. Thus, mice were treated with noscapine in the drinking water or regular water for 30 days and their spleens and small intestines were examined microscopically. No differences were seen in the histology of the spleens between control mice and those treated with noscapine (not shown). However, noscapine did induce subtle changes in the small intestine that included edema of the intestinal wall and epithelial cells, hypertrophy and hyperplasia of goblet cells, and enlargement of the crypts (Fig. 5). However, no inflammatory cell infiltration or disruption of the normal architecture was observed and treated mice did not develop diarrhea. Since noscapine dissolved in the drinking water is acidic (pH 4.5), water for the control mice was acidified to pH 4.5 with HCl in some experiments. No differences were seen in the spleen or small intestines of mice treated with regular or acidified water (not shown). In addition, similar numbers of apoptotic cells, as detected by the TUNEL assay, were found in the intestines of treated and control mice (not shown). These results suggest that noscapine is a mild irritant to the intestinal mucosa but causes no significant loss of the epithelial cells.

No consistent differences were found in the cell counts or in hematological parameters such as hematocrit, mean cell volume or mean cell hemoglobin in the peripheral blood of noscapine-treated animals compared to controls. Peripheral blood smears and the cellularity of the bone marrow were also normal. Further, no significant differences in annexin V binding were found in noscapine-treated and -untreated groups in the primitive, lineage-negative subset or in any of the mature lineages of the bone marrow (not shown).

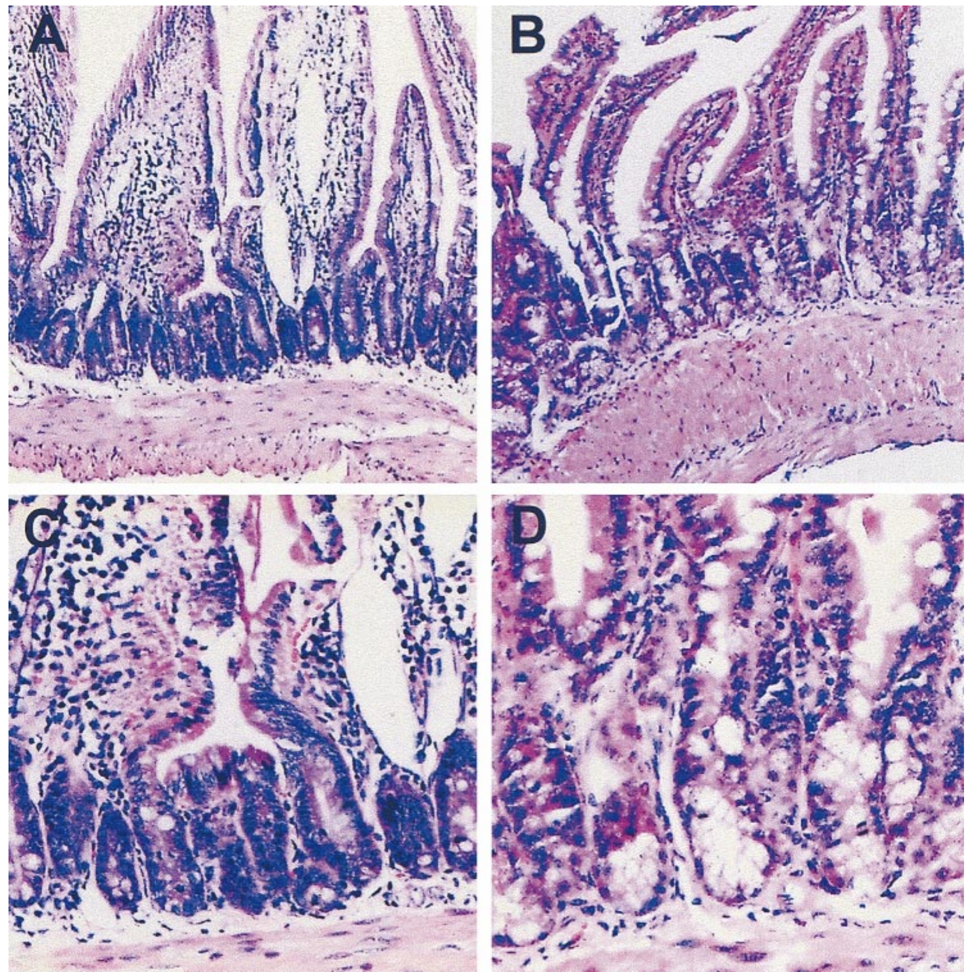

Fig. 5A–D Effect of noscapine on the small intestine. C57BL/6 mice were given acidified water (**A**, **C**) or 1.5 mg/ml noscapine (**B**, **D**) in their drinking water. They were then sacrificed after 30 days of treatment and their small intestines were processed and stained with hematoxylin and eosin. **A**, **B** 63×; **C**, **D** 160× magnification

Noscapine does not suppress immune responses

Although there was no histological evidence of damage to lymphoid organs induced by noscapine, it is possible that the drug could have a more subtle effect on the function of lymphocytes. Thus, the effects of noscapine on proliferation of transformed and non-transformed T lymphocyte cell lines were compared in vitro. Non-transformed T cell lines do not proliferate unless incubated with antigen-presenting cells plus antigen or mitogen. An OVA-specific $CD4^+$ T cell line from DO11.10 mice was incubated with irradiated spleen cells as a source of antigen-presenting cells and with $OVA_{323-339}$ plus various doses of noscapine or narceine. In a representative experiment, noscapine but not narceine (Fig. 6) inhibited the $CD4^+$ T cells and E.G7-OVA. However, about five times more noscapine was required to inhibit growth of the $CD4^+$ T cells than was needed for E.G7-OVA. In seven experiments, the average dose for 50% inhibition was 21.6 ± 3.4 μM noscapine for E.G7-OVA and 66.9 ± 26.0 μM noscapine for the $CD4^+$ T cells (P = 0.00064). Similar results were obtained with several other non-transformed T cell lines (not shown). Thus, non-transformed T cells were significantly less sensitive to the anti-mitotic activity of noscapine than the T cell lymphoma in vitro.

Lastly, we asked whether noscapine was immunosuppressive in vivo, as are many other antitumor therapeutics. C57BL/6 mice were given regular water or water containing noscapine. After 7 days, all mice were immunized in the footpad with OVA in CFA and treatment with regular water or noscapine was continued until the experiment was terminated. Three weeks after immunization, the mice were bled, sacrificed, and spleens were removed and cultured. As shown in Fig. 7A, mice treated with noscapine produced OVA-specific antibody responses comparable to those of normal control mice. Culturing spleen cells with irradiated E.G7-OVA stimulators for 1 week to activate primed precursors assessed cell-mediated immunity [27]. Stimulated spleen cells were tested for cytolytic activity using ^{51}Cr labeled EL4 or E.G7-OVA targets. It is important to note that OVA-specific responses could not be detected in such cultures unless the CTL precursors were primed in vivo [20, 27]. No inhibition of antigen-specific cytotoxicity was observed in mice treated with noscapine (Fig. 7B). Thus, noscapine caused no statistically significant inhibition of either humoral or cell-mediated immune responses, which are known to be critically dependent upon the proliferation of lymphocytes (reviewed in [18]).

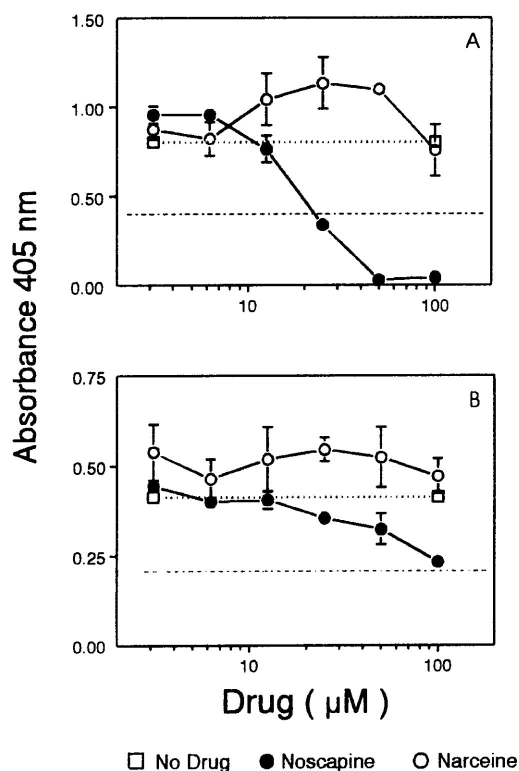

Fig. 6A, B Comparative effects of noscapine and narceine on transformed and non-transformed T cells. In 96-well microtiter plates, serial twofold dilutions of noscapine (●) or narceine (○) were made in medium. Wells with medium only (□) were used as a positive control for cell growth. E.G7-OVA T cell lymphoma cells were then added at 1×10^3 cells per well (**A**). Cloned, $CD4^+$ T cells from DO11.10 T-cell-receptor-transgenic mice were mixed with spleen cells from BALB/c mice and $OVA_{323–339}$ with or without inhibitors (**B**). After incubation for 72 h, XTT and PMS were added and the absorbance was read at 450 nm. --- 50% inhibition of the maximum response in cultures containing no drugs

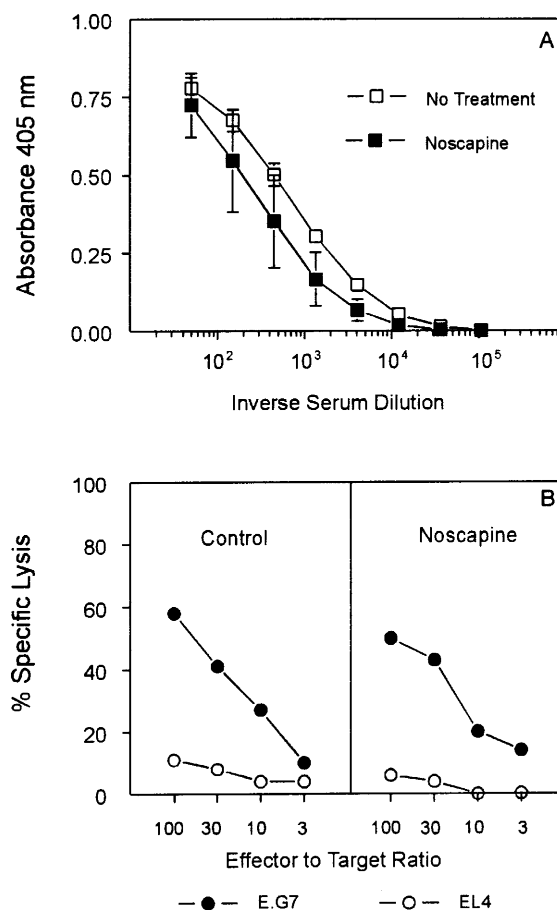

Fig. 7A, B The effect of noscapine on primary humoral and cell-mediated immunity. Three mice/group were given regular water or water containing 1.5 mg/ml noscapine. After 7 days, the mice were injected with 100 μg OVA in complete Freund's adjuvant. Treatment was continued throughout the experiment. After 3 weeks, mice were bled individually and their sera assayed for OVA-specific antibodies by enzyme-linked immunosorbent assay (**A**). Absorbance at 405 nm was read and the means ± SD calculated. Spleen cells from these mice were cultured with irradiated E.G7-OVA stimulators and assayed for cytotoxic T lymphocyte activity (**B**)

Discussion

In this study we have confirmed our recent findings that noscapine, an anti-microtubule agent, inhibits tumor cell proliferation in vitro. These results also show that neither narceine, an opium alkaloid having a structure similar to noscapine, nor a mixture of the breakdown products noscapine, opionic acid and cotarnine, inhibited proliferation of E.G7-OVA cells at doses comparable to the noscapine doses. The inhibition observed at 100-fold higher doses of these related compounds is most likely due to non-specific cytotoxicity. Thus, the antitumor activity of noscapine appears to be specific in vitro.

Our data also show that noscapine can cause tumor regression when administered in the drinking water, bypassing parenteral injections and intravenous infusions that can be complicated by infection at the injection site, pain, thrombosis of blood vessels or embolisms. Moreover, the oral route of delivery was safer than the parenteral route since about 30% of the mice treated i.p. with 3 mg/ml (approx. 120 mg/kg) died. The cause of death in these mice is unknown but acute toxicity studies have shown that the LD_{50} dose of noscapine for mice is 602 ± 31 mg/kg and transient neurotoxicity was detected at doses of about 90 mg/kg [13]. Approximately 85% of the noscapine is excreted within 24 h of i.v. injection [13], which raises the possibility that daily administration of a bolus of noscapine could lead to the accumulation of toxic quantities and death of some animals when the drug is given i.p. It is important to note that no death was observed in mice treated with 3.0 mg noscapine administered daily by gavage [39] or with 3.0 mg/ml in the drinking water (shown here). However, it is not yet known whether the bio-availability of noscapine is equivalent when the drug is administered via different routes.

The exact mechanisms by which noscapine prevents in vivo progression of malignant tumors are still not known. However, we recently reported that noscapine induces a conformational change upon binding to tubulin that promotes polymerization and assembly of microtubules. Noscapine arrested dividing cells at the metaphase of the cell cycle [39] probably as a result of spindle damage. Moreover, noscapine induces apoptosis of mammalian tumor cells as shown by (1) inter-nucleosomal DNA ladder formation, (2) condensation of chromatin, and (3) DNA fragmentation as detected by the TUNEL assay in vitro and in vivo. Whether noscapine inhibits tumor growth in vivo by cell-cycle arrest, induction of apoptosis, and/or other mechanisms is currently unknown.

In many respects, noscapine resembles paclitaxel, a taxane that has anti-microtubule and antitumor activity. Paclitaxel specifically binds to the β subunit of tubulin, which prevents depolymerization [1, 11, 32] and induces apoptosis [1, 26]. However, the use of paclitaxel is limited by adverse side-effects, including neurotoxicity [23], cardiotoxicity [31], myelosuppression [37], hypersensitivity reactions [11], alopecia [5] and gastrointestinal toxicity [12].

In contrast to the taxanes, noscapine given orally was well tolerated by mice and little or no toxicity was observed in the heart, kidney, spleen, bone marrow, peripheral blood, or liver. Mild nonspecific toxicity was observed in the small intestine but there was no evidence of apoptosis, increased mitotic figures, or gross pathology in any tissues including the small intestine. Thus, these results suggest that non-transformed host cells may be more resistant to noscapine than the tumors that were tested. Alternatively, damage to the mitotic apparatus that is induced by noscapine may be more readily repaired in non-transformed than in transformed cells. Some toxicity has been reported in experimental animals and in humans but only at much higher doses of noscapine [13, 22]. However, several studies have shown that noscapine was not mutagenic in animals [7, 35].

Although immune responses are critically dependent on proliferation of antigen-specific T cells and B cells (review in [18]), tumor-suppressive doses of noscapine did not inhibit primary humoral or cell-mediated immune responses to OVA in vivo. Antigen-induced proliferation of non-transformed T cell lines could be inhibited in vitro by noscapine but not by narceine. However, it required 3- to 5-fold higher concentrations of noscapine to inhibit proliferation of the non-transformed T cells than the T cell lymphoma. The failure of noscapine to inhibit the development of immune responses in vivo might result from limited bio-availability of the drug (sufficient for inhibition of tumor growth but too low for inhibition of T cell proliferation). Alternatively, the antitumor effect of noscapine in vivo might result from mechanisms other than its anti-mitotic activity.

Our studies also confirm the report by Zhou et al., which showed that immunization of mice with OVA (in liposomes) prolonged survival of mice inoculated with E.G7-OVA i.v. [40]. Our studies show that OVA in CFA induced regression of E.G7-OVA even when administered a few days after the subcutaneous injection of tumor. Regression was specific since injection of OVA did not cause regression of the parental, EL4, tumor (Kapp unpublished observation). Inhibition of tumor progression by noscapine treatment and inhibition by OVA-CFA immunization were qualitatively different. The T cell lymphoma did not grow significantly after noscapine treatment was started, whereas it grew for several days before regressing in mice primed with OVA-CFA. Nevertheless, both forms of treatment culminated in apoptosis and death of tumor cells. These results indicate that noscapine has the potential to be an effective chemotherapeutic agent for treatment of human cancer.

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