Noscapine Crosses the Blood-Brain Barrier and Inhibits Glioblastoma Growth

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ABSTRACT

The opium alkaloid noscapine is a commonly used antitussive agent available in Europe, Asia, and South America. Although the mechanism by which it suppresses coughing is currently unknown, it is presumed to involve the central nervous system. In addition to its antitussive action, noscapine also binds to tubulin and alters microtubule dynamics in vitro and in vivo. In this study, we show that noscapine inhibits the proliferation of rat C6 glioma cells in vitro (IC50 = 100 μM) and effectively crosses the blood-brain barrier at rates similar to the ones found for agents such as morphine and [Met]enkephalin that have potent central nervous system activity (P ≤ 0.05). Daily oral noscapine treatment (300 mg/kg) administered to immunodeficient mice having stereotactically implanted rat C6 glioblastoma into the striatum revealed a significant reduction of tumor volume (P ≤ 0.05). This was achieved with no identifiable toxicity to the duodenum, spleen, liver, or hematopoietic cells as determined by pathological microscopic examination of these tissues and flow cytometry. Furthermore, noscapine treatment resulted in little evidence of toxicity to dorsal root ganglia cultures as measured by inhibition of neurite outgrowth and yielded no evidence of peripheral neuropathy in animals. However, evidence of vasodilation was observed in noscapine-treated brain tissue. These unique properties of noscapine, including its ability to cross the blood-brain barrier, interfere with microtubule dynamics, arrest tumor cell division, reduce tumor growth, and minimally affect other dividing tissues and peripheral nerves, warrant additional investigation of its therapeutic potential.

INTRODUCTION

Patients diagnosed with glioblastoma (WHO grade IV) have a median survival of 9–12 months despite surgical resection, radiation therapy, and/or chemotherapy (1, 2). The infiltrative nature of astrocytic tumor growth rarely allows complete surgical resection, and more than 90% of tumors recur within 2 cm of the primary tumor site. Postoperative radiotherapy prolongs survival, but the prognosis is still less than 2 years. Intrinsic chemoresistance and poor penetration of drugs through the blood-brain barrier remain significant challenges for the chemotherapeutic treatment of gliomas (3). Given the limited efficacy of existing therapy, even when combined, there is a considerable need to direct research efforts to develop more effective treatments for brain tumors.

Malignant gliomas develop in part as a result of genetic mutation(s) in checkpoint genes resulting in deregulation of the cell cycle. Abrogation of the G1-S checkpoint is a frequent event in the development of gliomas (4–7), implying a role for cyclin-dependent kinases cyclin-dependent kinase 4/6 and their catalytic partners and α-type cyclins that are required for progression through the G1-S phases of the cell cycle. The cyclin-dependent kinase/cyclin D complex is inhibited in response to DNA damage or inadequate cell growth by p16INK4 and CIP/KIP, resulting in the activation of the G1-S checkpoint and arrest of normal cells in G1 (8–13). Homozygous deletions of G1-S checkpoint genes have been found in 41% of glioblastomas, suggesting that checkpoint mutations may contribute to the uncontrolled cell proliferation of glioblastoma (14). Other mutations have also been well documented including mutations in p53 (15) or in the retinoblastoma gene (16), each of which is found in nearly one-half of all gliomas.

Our laboratory has identified a microtubule-interacting chemotherapeutic agent that overcomes many of the limitations associated with other tubulin-binding drugs. This agent, noscapine, is an antitussive opium alkaloid that lacks sedative, euphoric, analgesic, and respiratory depressant properties (17). The precise mechanism for the antitussive effects of noscapine is unknown, although it appears centrally mediated. Noscapine can reduce electrically induced cough, characteristic of drugs affecting the autonomic nervous system (18), and radiolabeled noscapine binds the central nervous system (19, 20). Cough suppression was the only pronounced pharmacological effect of noscapine known for more than 30 years. In the last 5 years, we demonstrated that noscapine: (a) binds to tubulin and alters its conformation and assembly properties; (b) interferes with microtubule dynamics both in vitro and in living cells; (c) arrests a variety of mammalian cells in mitosis and targets them for apoptosis; and (d) inhibits growth of murine thymoma cells,
human breast carcinoma, and melanoma cells in mice by inducing polyplody and apoptosis (21–23). Furthermore, in contrast to other microtubule-interacting agents such as paclitaxel, nocodazole, and vinblastine, noscapine modifies microtubule dynamics without affecting total tubulin polymer mass in reconstituted systems and without altering the steady-state monomer/polymer equilibrium of microtubule assembly in cells (23).

In this study, we show that glioma cell treatment with noscapine induces polyplody. Noscapine-treated cells undergo excessive DNA synthesis and atypical nuclear divisions in the absence of cytokinesis, resulting in multinucleated cells. We further show that noscapine crosses the blood-brain barrier and inhibits the growth of subcutaneous (s.c.) and intracranially (i.c.) implanted rat C6 glioma cells in immunocompromised mice without apparent toxicity to organs with rapidly proliferating tissues or induction of neurological symptoms.

MATERIALS AND METHODS

Mice and Cell Lines. Eight-week-old athymic female mice (nu/nu) were purchased from the National Cancer Institute (Bethesda, MD). The rat C6 glioma cell line (American Type Culture Collection) was maintained in DMEM supplemented with 10% fetal bovine serum and passaged no more than 10 times. Primary glial cells were isolated as follows. Cells from the mouse subventricular zone of C57BL/6 mice were dissected under a dissecting microscope, manually dissociated using a flame-polished pipette, and grown in DMEM containing 20% fetal bovine serum. Glial cells used for experiments were identified as cells that contain glial fibrillary acidic protein, an intermediate filament subunit found exclusively in glial cells. These cells did not express neuronal markers such as Tuj-1, an antibody that is specific for neuronal β-tubulin.

Cell Density Assay. Cell proliferation was determined by the WST-1 tetrazolium salt assay (Boehringer Mannheim), which quantifies the amount of formazan dye formed when tetrazolium salt is cleaved by cellular mitochondrial enzymes present in viable cells. Cells were plated at a density of 1 × 10^4/well in 96-well microtiter plates in 0.2 ml of culture medium. Cells were allowed to adhere overnight and then incubated with 0, 0.1, 1, 2, 10, 50, 100, or 1000 μM noscapine (97% purity; Aldrich; 100× stock in DMSO) for 0, 12, 24, 48, 72, or 96 h. The final concentration of DMSO in medium never exceeded 1%. Five hours before the end of the specified incubation periods, 50 µl of WST reagent were added to the cells. At the end of the incubation, cell density was estimated by measuring the absorbance of the colored formazan reaction product at 450 nm using a microtiter plate reader (Molecular Devices Ltd., Crawley, West Sussex, United Kingdom).

Tubulin, DNA, and Bromodeoxyuridine Staining. Rat C6 glioma cells and primary glial cells were cultured on polyl-ornithine-coated glass coverslips and allowed to adhere for 24 h. To examine how noscapine affects microtubule morphology and DNA content, noscapine was dissolved in DMSO, and cells were then incubated at 37°C with 0, 25, 50, 250, 500, and 1000 μM noscapine for 24, 48, 72, or 96 h. Cells were fixed in methanol at −20°C for 5 min, incubated with anti-α-tubulin antibody (DM1A; 1:500 dilution; Amersham Biosciences) for 2 h at room temperature, washed and incubated with a goat antimouse secondary antibody (1:200 dilution; Jackson) for 1 h at room temperature, and visualized using immunofluorescence. To visualize DNA, tubulin-stained cells were further incubated with 20 μg/ml propidium iodide (Boehringer Mannheim) for 30 min before washing with PBS. Cells were subsequently mounted on coated glass slides and analyzed using confocal microscopy for microtubule morphology, DNA content, and the number of cells in mitosis (at least 100 cells were examined per condition). In a separate experiment, to examine whether noscapine-treated cells go through multiple rounds of DNA synthesis, cells plated as described above were treated with noscapine for 72 h. After 24 h, 30 μM bromodeoxyuridine (BrdU; Sigma Chemical Co.) was added for the remaining 48 h. Cells were fixed and stained with anti-BrdU antibody (1:200 dilution; Boehringer Mannheim) and stained with FITC-labeled secondary antibody (1:100; Jackson), mounted on glass slides, and analyzed for BrdU incorporation by confocal microscopy.

Flow Cytometric Analysis of Cell Cycle Status. Cell cycle status was determined by measuring cellular DNA content after staining with propidium iodide by flow cytometry (21). Cells (1 × 10^6) were plated on 10-cm dishes and incubated for 24 h before the addition of 0, 50, 250, 500, or 1000 μM noscapine in 1% DMSO for 0, 6, 12, 24, 48, 72, or 96 h. Staurosporine (100 nM), a well-known cytotoxic agent, was used as a positive control. In an independent study, cells were treated with noscapine at the same dosages and durations specified as above, washed three times with PBS at 37°C, and allowed to recover for 96 h in fresh medium without noscapine. Cells from both experiments were removed with trypsin, collected, washed twice in ice-cold PBS, fixed overnight in 70% ethanol at −20°C, and centrifuged at 1000 × g for 10 min. Cells were then resuspended in 30 μl of phosphate/citrate buffer [0.2 M Na_2HPO_4/0.1 M citric acid (pH 7.5)] and incubated with propidium iodide (20 μg/ml) and RNase A (20 μg/ml) in PBS for 30 min. The propidium iodide fluorescence was measured using a Becton Dickinson flow cytometer. Data were analyzed using Winlist software (Verity Software House, Topsham, ME).

In Vitro Bovine Brain Microvessel Endothelial Cell Assay. Bovine brain microvessel endothelial cells were isolated from the cerebral cortex as described previously (24) on polycarbonate membrane filters. In brief, bovine brain microvessel endothelial cells were isolated from the gray matter of the bovine cerebral cortex by enzymatic digestion followed by subsequent centrifugations and seeded into primary culture. Polycarbonate membranes (13 mm; pore size, 3.0 μm; diffusion area, 0.636 cm²) were placed in tissue culture dishes (100 mm; Corning, Corning, NY) and coated with rat-tail collagen and bovine fibronectin (Sigma). Isolated brain microvessel endothelial cells were seeded onto the prepared tissue culture dishes at a density of 5 × 10^4 cells/cm² in a culture medium consisting of 45% MEM, 45% Ham’s F-12 nutrient mixture (Life Technologies, Inc., Grand Island, NY), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 13 mM sodium bicarbonate, 10% plasma-derived equine serum, 100 mg/ml heparin, 100 mg/ml streptomycin, 100 mg/ml penicillin G, 50 mg/ml polymyxin B, and 2.5 mg/ml amphotericin B (Sigma Chemical Co.). The cells were cultured at 37°C with 5% CO₂. Medium was replaced on the 3rd day after seeding, and then every 2 days until confluent monolayers were formed (10–14 days). Conflu-
ence was determined by inspecting the areas around the polycarbonate membranes with an inverted microscope.

Bovine brain microvascular endothelial cell monolayers cultured on a polycarbonate membrane were placed in a Side-Bi-Side diffusion cell (Crown Glass Co., Somerville, NJ) containing 3 ml of continuously stirred physiological assay buffer (122 mM NaCl, 3.0 mM KCl, 1.2 mM MgSO₄, 25 mM NaHCO₃, 0.4 mM K₂HPO₄, 1.4 mM CaCl₂, 10 mM d-glucose, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) on each side at 37°C (Fig. 5). At time 0, noscapine (500 μM) was added to the donor chamber, and 100-μl aliquots were removed from the receptor chamber at various time points (15, 30, 60, 90, and 120 min) and stored for high-performance liquid chromatography (HPLC) analysis. An equal volume of assay buffer was added to replace the aliquots removed. [14C]sucrose (10.44 Ci/mmol; NEN Research Products, Boston, MA), a molecule that does not cross the membrane, served as a negative control and [Met]enkephalin (DPDPE; Tyr-Gly-Gly-Phe-Met), which is known to permeate the blood-brain barrier, was used as a positive control. Background leakiness was monitored and corrected for by determining the levels of [14C]sucrose in the samples via scintillation spectrometry (efficiency, 93% for [14C]sucrose; Beckman LS 5000 TD counter; Beckman Instruments Inc., Fullerton, CA). Passage of the test solute across the in vitro blood-brain barrier monolayer was determined by reverse phase-HPLC as described previously (25). Reverse phase-HPLC values were obtained in moles and used to determine permeability coefficients found by using the following equation:

\[ PC = \frac{X}{t(C_d - C_r)} \]

where \( PC \) is the permeability coefficient (cm/min), \( X \) is the amount of substance in moles in the receptor chamber at time \( t \) (min), \( A \) is a constant diffusion area (0.636 cm²), and \( C_d \) and \( C_r \) are the concentration of the substance in the donor chamber and receptor chamber (in mol cm⁻²).

**In Vivo Tumorigenicity Assays.** Immediately before surgery, rat C6 glioma cells were washed twice with PBS and placed into serum-free DMEM medium. Thirty anesthetized 8-week-old athymic nude mice received 1 × 10⁶ rat C6 glioma cells stereotactically implanted into the left striatum (coordinates: anterior-posterior, +2.5; medial-lateral, +3.5; dorsal-ventral, −2 mm; from the Bregma). C6 glioma cells in a volume of 2 μl were slowly injected over a time span of 15 min (for illustration, see Fig. 7A). Due to the age of the animals, the needle easily penetrated the skull. Sham-operated animals (\( n = 15 \)) received an identical intracranial injection of serum-free medium alone. After injection, the needle track was sealed with bone wax, and the incision was closed with Ethicon staples (Endo Surgery, Inc.). An independent group of 30 animals received 1 × 10⁶ rat C6 glioma cells in a volume of 0.2 ml s.c. into the right flank. Six days after s.c. injection when s.c. tumors were palpable or six days after stereotactic injection, animals were divided into two groups (\( n = 15 \) / group). One group received noscapine hydrochloride by daily gavage (300 mg/kg dissolved in de-ionized water (dH₂O; pH 4.5)), and the other group received the vehicle solution alone gavage (dH₂O; pH 4.5). Tumor volumes were recorded biweekly for animals in the s.c. group. On day 21 (15 days of noscapine treatment), animals with intracranial tumors were anesthetized with 4% chloral hydrate and then perfused intracardially with phosphate-buffered saline followed by 2.5% glutaraldehyde and 2.5% paraformaldehyde in phosphate buffer. At necropsy, the following tissues were taken for analysis: spleen; duodenum; liver; sciatic nerve; sural nerve; dorsal and ventral roots; and brain. Before perfusion, blood from the heart was taken for a complete blood count using a complete blood count instrument (CDC Technologies, Oxford, CT), and bone marrow was removed from the right femur and tibia bones for WBC analysis using a 25-gauge needle. Brain weight was obtained upon sacrifice for both s.c. and intracranial tumor groups. On day 21 (15 days of noscapine treatment), animals in the s.c. tumor group (\( n = 30 \)) received one final dose of noscapine (\( n = 15 \)) or vehicle solution (\( n = 15 \)) 2 h before sacrifice to determine noscapine concentrations in the brain at the reported half-life of noscapine (26). Animals in the s.c. group were sacrificed by cervical dislocation, and the brains were retained for HPLC detection of noscapine as described below.

**Determination of Noscapine Concentration in Animal Tissues by High-Performance Liquid Chromatography.** Two h after the final noscapine administration (\( n = 15 \)) or vehicle solution (\( n = 15 \)), animals were anesthetized and then sacrificed by cervical dislocation. This timing was selected based on the reported half-life of noscapine (154 min; Ref. 27). Blood was collected before animal sacrifice directly from the heart and centrifuged, and plasma was removed and stored at −80°C for HPLC analysis of noscapine and its metabolites. Brains were removed without perfusion; meninges and exterior blood vessels were dissected including the middle cerebral artery, the arteries forming the circle of Willis, superior sagittal sinus, and the transverse sinus. Dissected brains were homogenized and centrifuged to remove cell debris, and supernatants were collected and stored at −80°C for HPLC analysis. HPLC analyses were performed in a double-blind fashion according to a previously published method (28). In brief, samples were analyzed on a reverse-phase-HPLC system consisting of a WISP 710B Autoinjector, two model 6000A Solvent Delivery Pumps, Automated Gradient Controller (Waters Associates, Milford, MA), LC-65T Detector/Oven (210 nm; Perkin-Elmer, Norwalk, CT), 3390A Integrator (Hewlett-Packard Co., Avondale, PA), and a 218TP54 column (4.6 × 250 mm; Vydac, Hesperia, CA). Samples were eluted using a linear gradient of acetonitrile against 0.1 M NaH₂PO₄ buffer (pH 2.4). The flow rate was maintained at 1.5 ml/min, and the column temperature at 40°C. The capacity factor was defined as follows: \( k = (t_r - t_o)/t_o \), where \( t_r \) is the retention time of the retained peak and \( t_o \) is the retention time of an unretained peak.

**Image Analysis.** Brains from animals that received i.c. injections were fixed by perfusion as described above and sectioned into 1-mm-thick slices using a brain matrix (for illustration, see Fig. 8B; Ted Pella, Redding, CA). Each 1-mm-thick section was then embedded in paraffin blocks maintaining proper orientation so that the anterior-most side would be sectioned first. A single 5-μm section was then cut from each block and stained with H&E for three-dimensional reconstruction. Slides were coded, and each section was captured at an identical magnification using a digital camera (SPOT camera; Diagnostic Instruments Inc., Sterling Heights, MI). Tumor cells were identified because they stained with greater intensity than surrounding normal striatal cells as shown in Fig. 7A (arrow). The differences in staining intensity were detected using AIS image.
analysis software (Imaging Research, Inc., St. Catherine’s Heights, Ontario, Canada). The system was calibrated using a micrometer and selecting the appropriate number of pixels equal to 1 mm. Tumor cross-sectional area was determined by using an auto-sampling tool, which bases its selection on staining intensity. The autoscan tool uses an algorithm to select the target region based on H&E-staining intensity differences between the selected target gray value and background gray value identified. The autoscan feature searches in concentric circles seeking a continuous boundary. This boundary is defined by an average threshold level of staining intensity that circumscribes the target area pixel by pixel, radiating outward from the first darkly stained pixel selected. Adjacent tumor regions in the same section were identified as separate targets. This procedure enabled us to quantify infiltrative tumor regions consisting of normal and tumor tissue within a boundary because normal tissue had lower than threshold-staining intensity and thus was excluded from the cross-sectional tumor area measurements. The autoscan feature also excluded open spaces such as vessel lumens (for illustration, refer to Fig. 7, C and D). Tumor regions were verified by manual microscopic inspection by a pathologist (B. H. W.). The three-dimensional tumor volume was computed using cross-sectional tumor area × 1 mm (the distance between 5-μm sections). If more than one tumor target site was present in a cross-sectional tumor area, targets were added for that section. The blind code was broken after all of the sections had been scanned and tumor volumes obtained.

**Toxicity Evaluation.** After animal perfusion and sacrifice on day 21, liver, duodenum, and spleen were sectioned, stained with H&E, and analyzed by two pathologists (B. H. W. and D. L. D.) for microscopic evaluation. Bone marrow was removed before fixation from the femur and tibia bones and analyzed by flow cytometry following antibody lineage-markers: CD3 (T cells); B220 (B cells); MAC-1 (macrophages); and Gr-1 (granulocytes; PharMingen, San Diego, CA). Cells were also incubated with 20 μg/ml propidium iodide to determine the percentage of dead cells. Sciatic nerve, dorsal root ganglion, dorsal and ventral roots, and sural nerves were removed from four animals from each group, embedded in plastic, cut at 1 μm, and stained with 0.5% toluidine blue for microscopic analyses. Sections were evaluated blindly for evidence of sensory and motor neuropathies by a neuropathologist (J. D. G.).

**Dorsal Root Ganglion Cultures and Evaluation of Neuropathy.** Dorsal root ganglion neurons were cultured as described previously (29). In brief, dorsal root ganglia were dissected from newborn mice. Ganglia were transferred into L-15 medium (Life Technologies, Inc.), separated from roots and connective tissue sheaths, pooled, dissociated, and washed twice with PBS (pH 7.4). Dorsal root ganglia were then plated (five per dish) in MEM supplemented with 1% N2 (Life Technologies, Inc.), 10 ng/ml 7S nerve growth factor (Sigma), and 1.4 nm l-glutamine (Sigma) and incubated at 37°C in a 5% carbon dioxide atmosphere. Next, cultures were permitted to mature for 5 days to allow a lush halo of neurites around the explants to develop. Neuritic extensions were allowed to proceed to evaluate the effect of noscapine on established neurites as opposed to the effect on primary neurite outgrowth. After 5 days in the culture medium, the medium was changed and 25, 50, or 250 μM noscapine or DMSO vehicle solution alone (final concentration 1%) was added. Cultures were monitored and imaged on 0, 4, 8, and 10 days post noscapine treatment. The diameter of the circular halo of neurites was measured on the initial day of noscapine exposure (day 0), and on days 4, 8, and 10. Axonal survival was quantified by the longest remaining axon and the area of the remaining dorsal root ganglion halo (Fig. 10B). The axonal length was measured from the center of the halo to the visible distal ends of the axon in the periphery of the halo. Halo areas were calculated by tracing the outside circumference of the remaining culture halo. Because there was variability in the physical characteristics of individual cultures, each dorsal root ganglion served as its own control by normalizing data at days 4, 8, and 10 to the condition before noscapine exposure. Data were analyzed as a percentage change from day 0 before noscapine treatment. Normalized data were then ex-
amined for statistical significance by ANOVA, with post-test correction for multiple comparisons (29).

RESULTS

Noscapine Inhibits Rat C6 Glioma Cell Proliferation. To determine whether noscapine could inhibit glioma cell growth in vitro, we chose the aggressive and rapidly dividing rat C6 glioma cell line. Using the tetrazolium salt (WST-1) cell viability assay (see “Materials and Methods”), we generated a dose-response curve by incubating cultures of rat C6 glioma cells and primary mouse glial cells (as normal tissue control) with noscapine for 72 h and observed a dose-dependent inhibition of cell viability (Fig. 1A). Noscapine inhibited the viability of rat C6 glioma cells with an IC50 of 250 μM at 72 h. Primary glial cells were less sensitive, having an IC50 of 500 μM (at 72 h). Manual counts of cell numbers verified this finding (not shown). Because 250 μM noscapine exposure resulted in a significant inhibition of glioma cells while primary cells were nearly unaffected, we selected this dose to examine how this concentration affects cell viability over time (24–96 h; Fig. 1B). We conclude that 250 μM noscapine exposure for 72 h is an optimal dose to inhibit C6 glioma cell viability without significantly reducing primary cell viability.

Noscapine Exposure Causes Abnormal S-Phase Re-entry and Results in Excessive DNA Accumulation. The mechanism of the decreased cell viability observed in C6 cells exposed to 250 μM noscapine for 72 h was evaluated by analyzing the cell cycle distribution by flow cytometry with propidium iodide (Fig. 2). Cultures of C6 cells or primary murine glial cells were treated with noscapine, fixed, and stained with the DNA intercalating fluorescent dye propidium iodide. Untreated C6 cells (Fig. 2C) and primary cells (Fig. 2B) exposed to 250 μM noscapine for 72 h had normal cell cycle distributions. The number of cells with 2N DNA content (peak in green) was 72% and 69%, respectively. The number of cells with 4N DNA content (peak in blue) was 14% and 18%, respectively. Approximately 50% of primary cells incorporated BrdUrd (inset in green) compared with the nuclear staining shown in red. B, 24% of primary cells exposed to 250 μM noscapine for 72 h had 2N DNA content. Note the increase in the number of cells (37%) with 4N DNA content after noscapine exposure (peak in blue). Fifty percent of noscapine-treated primary cells incorporated BrdUrd (insets). C, vehicle-treated glioblastoma cells contained 1% of cells with 2N DNA content and 9% of cells with 4N DNA content. Note the position of the peak showing cells with abnormal ~8N-16N DNA content (yellow peak). This phenomenon is not seen in untreated C6 glioma cells (C) or in primary glial cells (A and B). Bar = 5.0 μM.

Fig. 2. Noscapine induces polyploidy in rat C6 glioma cells. Rat C6 glioma cells and murine primary glial cells were treated with 250 μM noscapine in DMSO or vehicle solution alone (DMSO) for 72 h and then fixed and stained with the DNA-binding fluorescent dye propidium iodide (PI) and with BrdUrd (BrdU). A, 72% of vehicle-treated primary glial cells contain 2N DNA content (peak in green), and 14% of cells contain 4N DNA content (peak in blue). Approximately 50% of primary cells incorporated BrdUrd (inset in green) compared with the nuclear staining shown in red. B, 24% of primary cells exposed to 250 μM noscapine for 72 h had 2N DNA content. Note the increase in the number of cells (37%) with 4N DNA content after noscapine exposure (peak in blue). Fifty percent of noscapine-treated primary cells incorporated BrdUrd (insets). C, vehicle-treated glioblastoma cells had a DNA content similar to vehicle-treated primary cells with 69 and 18% of cells containing 2N and 4N DNA content, respectively. BrdUrd-positive vehicle-treated glioma cells were observed in approximately 60% of cells. D, noscapine-treated glioma cells contained 1% of cells with 2N DNA content and 9% of cells with 4N DNA content. Note the position of the peak showing cells with abnormal ~8N-16N DNA content (yellow peak). This phenomenon is not seen in untreated C6 glioma cells (C) or in primary glial cells (A and B). Bar = 5.0 μM.
profiles with approximately 70% of cells in G_0-G_1 phase with 2N DNA content (Fig. 2, left peaks in green) and 16% of cells in G_2-M phase containing 4N DNA content (Fig. 2, right peaks in blue). In primary glial cells, noscapine exposure (250 μM for 72 h) resulted in a decrease to 24% of G_0-G_1 phase cells with 2N DNA content and an increase of cells in G_2-M with 4N DNA content to 37% (Fig. 2B). In contrast, only 1% of rat C6 glioma cells contained 2N DNA content, and 9% of cells contained 4N DNA (Fig. 2D, blue peak) content after 72 h of continuous noscapine exposure. In addition, 44% of cells contained 8N-16N DNA (Fig. 2D, yellow peak). Primary glial cells (Fig. 2B) did not accumulate enhanced DNA content at identical doses, but rather, we observed reversible mitotic arrest after continuous noscapine exposure for up to 6 h (not shown). Flow cytometric data suggest that noscapine exposure causes 4N DNA accumulation in primary glial cells, suggesting G_2-M arrest; whereas it results in 8N-16N DNA accumulation in C6 cells, suggesting continuous DNA synthesis.

To determine whether C6 cells exposed to noscapine accumulate DNA by inappropriately reentering multiple rounds of S phase, immunofluorescence was performed to detect BrdUrd incorporation (Fig. 2, inset in green), a thymidine analog incorporated into cells during DNA synthesis (S phase). Nuclear staining with propidium iodide (Fig. 2, inset in red) was used to detect all nuclei. Anti-BrdUrd staining was apparent in about 50% of the primary glial cells treated with vehicle alone (Fig. 2A, inset in green) or with noscapine (Fig. 2B, inset in green). Anti-BrdUrd staining was also present in approximately 50% of C6 cells treated with vehicle (Fig. 2C, inset in green), indicating that cell division occurred during the course of the experiment. In contrast, manual cell counts revealed that 97% of C6 cells incubated with noscapine for 72 h incorporated BrdUrd under these conditions, indicating that most cells were in S phase. These cells showed multiple discrete BrdUrd-positive micronuclei (Fig. 2D, inset in green and nuclear staining in red) and had DNA content between 8N and 16N, suggesting that noscapine-treated cells were undergoing multiple rounds of DNA replication in the absence of cytokinesis resulting in cells with

Fig. 3 Noscapine exposure results in multinucleated cells with abnormal mitotic figures in rat C6 glioma cells. Double-labeling immunofluorescence is shown with an anti-α-tubulin antibody that stains microtubules in green and propidium iodide nuclear staining that is shown in red. Noscapine-treated rat C6 glioma cells have large, abnormal, multilobed nuclei and intact microtubule arrays (E); and mitotic figures, when observed, were abnormal with multiple microtubule asters and misaligned chromosomes (F). These effects were not apparent in noscapine-treated primary glial cells (B and C) or in vehicle-treated primary glia (A) or vehicle-treated rat C6 glioma cells (D). Note that noscapine exposure did not alter microtubule morphology in primary glial cells (B) or in rat C6 glioma cells (E). Bar = 5.0 μm.
Noscapine treatment does not perturb the morphology of microtubule arrays and results in multinucleated glioma cells with abnormal mitoses. To examine microtubule morphology, cellular microtubule arrays were observed by immunofluorescence (Fig. 3). We used an antibody against α-tubulin (Fig. 3, green) to stain microtubules and the DNA-specific stain, propidium iodide (Fig. 3, red), to stain chromosomes. In vehicle-treated C6 glioma and primary glial cells, microtubule arrays were localized throughout the cytoplasm of interphase cells (Fig. 3, A and D). Microtubule arrays in vehicle-treated cells appeared similar to those in untreated control cells (not shown), indicating that the vehicle solution (DMSO final concentration was less than 1% in cell medium) had no effect on microtubule arrays. Microtubule morphology of interphase primary glial cells in the presence or absence of 250 μM noscapine for 72 h was similar (Fig. 3, compare A and B). Mitotic figures of normal glial cells were not visibly affected by noscapine, as shown by chromosomes properly aligned on the metaphase (Fig. 3C). In contrast, as already observed in Fig. 2, C6 glioma cells treated with noscapine revealed large, abnormal, multiple nuclei (Fig. 3E). Their microtubule structure was similar to that of vehicle-treated cells (Fig. 3, compare E with D). However, the spindle structure of mitoses observed in noscapine-treated glioma cells were abnormal with misaligned chromosomes and multiple microtubule asters (Fig. 3, compare F with C). These data suggest that noscapine treatment causes abnormal mitoses and accumulation of micro-nuclei in rat C6 glioma cells, whereas cultures of primary glial cells do not accumulate DNA, and normal mitoses are observed when exposed to noscapine.

**Noscapine Exposure Causes Increased Mitotic Arrest.** To quantify the number of cells in M phase of mitosis in the presence or absence of noscapine, cells were treated with 250 μM noscapine for incubation periods ranging from 0 to 96 h and then fixed and stained with an anti-α-tubulin antibody and propidium iodide, and the number of cells in mitosis counted (Fig. 4). Noscapine treatment (250 μM) resulted in an increase in the number of mitotic figures observed in a time-dependent manner up to 24 h. Mitotic figures were observed in 68% of noscapine-treated primary glial cell cultures at 24 h (E). In contrast, only 36% of noscapine-treated rat C6 glioblastoma cells were observed in mitosis at 24 h (F). The number of cells observed arrested in mitosis after 48, 72, or 96 h of noscapine exposure decreased in a time-dependent manner with 42% primary glial cells and 20% glioma cells arrested at 48 h and 12 and 5%, respectively, at 72 h. At 96 h, approximately 5% of both cell types were observed in mitosis. In the absence of noscapine, the number of cells in mitosis at any given time was approximately 5% (G and H, primary glia and rat C6 glioma, respectively).

Noscapine was deposited at a concentration of 500 μM in the donor chamber, and aliquots were removed from the receiver chamber at 15, 30, 60, 90, and 120 min. The noscapine concentration was determined by HPLC, and the apparent permeability coefficient (in cm/min) calculated as described in “Materials and Methods.” Passage of noscapine across the barrier was determined by the concentration detected by HPLC in the donor chamber compared with the receiver chamber. Noscapine was detected in the receiver chamber at a concentration of 10.21 μM (0.2%) after 15 min that increased to 96.6 μM after 120 min (19%; Fig. 5B). Noscapine was found to cross the simulated blood-brain barrier with a permeability coefficient of 21.7 × 10⁻⁴ cm/min. This rate is 31.8% more efficient than morphine (14.8 × 10⁻⁴ cm/min), an opiate known to possess lipophilic character and permeate the barrier (Fig. 5B; P ≤ 0.05; Student’s t test), and similar to the positive control, [Met]enkephalin (DPDPE; 24.24 × 10⁻⁴ cm/min). Although this model does not account for drug metabolism that occurs in vivo, it provides evidence that noscapine efficiently crosses the blood-brain barrier compared with other agents known to permeate well.

Next, we determined whether noscapine was transported across the blood-brain barrier in vivo. We homogenized and centrifuged whole brains of noscapine-treated animals and then determined noscapine concentration by HPLC in supernatants. The average noscapine concentration obtained from brain homogenates of noscapine-treated animals was 18.2 ± 3.7 μM (±SD). The animals used for this study were not perfused, so the values obtained include the amount of noscapine present in brain tissue and the vascular network. These results are also consistent with the radioactive data describing noscapine accumulation in rat brain. 
(19). Our in vitro and in vivo results suggest that noscapine is capable of efficiently crossing the blood-brain barrier.

**Noscapine Inhibits Rat C6 Glioma Growth in Vivo.** To examine the ability of noscapine to inhibit the growth of tumors in vivo, we first injected rat C6 glioma cells s.c. into immunodeficient mice. 15 days of noscapine administration beginning 6 days after s.c. implantation of $1 \times 10^6$ C6 glioma cells significantly inhibited tumor growth (Fig. 6, $P \leq 0.01$; Student’s $t$ test). On day 21, s.c. tumor volume was reduced by 60% in the noscapine-treated group compared with the vehicle-treated group ($1510 \pm 237$ and $3739 \pm 586$ mm$^3$, respectively; $n = 12$ and 11 respectively). As a result of the large s.c. tumors, overall animal body weight significantly increased in untreated animals (mean weight at the time of sacrifice of vehicle-treated animals was $28.61 \pm 2.02$ g, and the mean weight of noscapine-treated animals was $23.36 \pm 1.49$ g; weight $\pm$ SE; $P \leq 0.01$; Student’s $t$ test). The weight of the resected tumors could partially account for this observation. The average s.c. tumor mass taken from vehicle-treated animals was $2.56 \pm 1.62$ g compared with $0.79 \pm 0.44$ g removed from noscapine-treated animals.

To demonstrate that noscapine can treat gliomas in their orthotopic brain location despite the blood-brain barrier, we next injected $1 \times 10^3$ C6 cells i.c. into the striatum of immunocompromised mice ($n = 30$). Noscapine daily by gavage (300 mg/kg; $n = 15$) or vehicle solution alone ($n = 15$) was administered to animals for 15 days beginning on day 6, and animals were euthanized 21 days after tumor implantation. The daily noscapine dosage of 300 mg/kg (corresponding to approximately six times the in vitro concentration) was chosen based on noscapine solubility and the favorable IC$_{50}$ of 250 $\mu$M at 72 h. Intracraniatal tumor volumes were analyzed as follows: perfused brains were cut into 1-mm-thick sections (Fig. 7B) and embedded into paraffin. The first 5-$\mu$m section from each block was cut, stained (H&E), and examined for cross-sectional tumor area. Representative brain sections of mice treated or not with noscapine are shown in Fig. 8, A and B, respectively. The
sections revealed extensive migration of tumor cells (identified by their darker staining) in the injected hemisphere in both groups (Fig. 8, A and B, and insets E and F). Brains of untreated animals showed a dense twirling pattern of tumor cells that largely replaced normal brain tissue in the tumor center while infiltrating extensively normal brain at the periphery (Fig. 8E). Lumen of large blood vessels were much smaller than in the contralateral tumor-free hemisphere, possibly suggesting compression by interstitial pressure (Fig. 8E). Brains of noscapine-treated animals showed clearly reduced numbers of tumor cells. Tumor cells in noscapine-treated tissue extensively infiltrated the normal brain and tended to cluster around blood vessels without altering lumen size. In some cases, small areas of hemocyanin were noted, an observation that usually reflects subsided hemorrhage. Noscapine may have induced death of some rapidly proliferating endothelial cells in tumor vasculature, leading to vessel leakage. To try to quantify the difference in tumor burden between both groups while accounting for the intermixing of normal and tumor cells, we used digital imaging. Cross-sectional tumor area was determined for each 5-μm slice and representative striatal sections from vehicle- and noscapine-treated animals are shown (Fig. 8, C and D, blue). Using three-dimensional image analysis reconstruction of brain tumor volume, we found that 15 days of noscapine treatment significantly inhibited intracranial brain tumor growth by 78% (Fig. 8G, P ≤ 0.01; Student’s t test). In addition, we found a trend toward increased brain weight in vehicle-treated animals compared with noscapine-treated animals receiving intracranial tu-

**Fig. 6** Time course of s.c. rat C6 glioma tumor growth. Palpable tumors were established 6 days after injecting 1 × 10⁶ rat C6 glioma cells s.c. in mice. Mice were treated beginning on day 6 with 300 mg/kg noscapine in acidified water by gavage daily for 15 days (●), whereas vehicle-treated animals received acidified water alone by gavage (▲). Tumor volume shown is ± SE. Day 21, P ≤ 0.01 (Student’s t test).

**Fig. 7** Rat C6 glioma cells were stereotactically implanted into the striatum. A, striatal section depicting india ink stereotactically injected using the coordinates shown. Rat C6 glioma cells (1 × 10⁶) in serum-free DMEM (vehicle solution) or vehicle solution alone were precisely implanted into the striatum of nude mice using a 10-μl Hamilton syringe. AP, anterior-posterior; ML, medial-lateral; DV, dorsal-ventral. B, after 15 days of 300 mg/kg noscapine treatment in vehicle solution (dH₂O; pH 4.5) or vehicle solution alone administered by gavage, animals were euthanized and perfused, and brains were removed and cut into 1-mm-thick macrosections as shown here.
tumors (median brain weight, 0.50 ± 0.06 and 0.42 ± 0.09 g for untreated and noscapine-treated, respectively, ± SE). This is suggestive of increased brain density as a result of the tumor tissue. The striking inhibition of tumorigenicity observed in the intracranial tumor models suggest that noscapine may be effective for the management of some types of gliomas.

Toxicity Evaluation. Given the efficiency of noscapine to reduce tumor growth, the next concern was to examine its...
potential toxicity in a variety of tissues. Extending our previous findings (22), we show that noscapine had no apparent systemic toxicity, even in animals carrying a heavy tumor burden and brain tumors known to cause overall anergy (30). Treated animals did not show any signs of behavioral or neurological deficit and were equally active as untreated animals and gained weight. However, unexplained blood vessel dilation in noscapine-treated brain tissue was observed in both the tumor-infiltrated and contralateral hemispheres compared with untreated tissue (Fig. 8F).

Hematological toxicity was absent as determined by complete blood count (Fig. 9B). No significant toxic side effects could be detected by histopathology in sites of rapidly dividing tissues such as spleen, duodenum, and liver as revealed by histopathology (Fig. 9, A–C). Given that the principal toxicity of existing microtubule-targeting agents is peripheral neuropathy, we examined peripheral motor and sensory nerves for evidence of neuropathy. We did not find evidence of either tubulovesicular accumulations, as may be seen with impaired axonal transport, or axonal degeneration in either sensory or motor fibers (Fig. 10A). These types of pathological changes have been reported with other agents that disrupt microtubule function. The absence of such pathology suggests that noscapine may be less toxic to peripheral nerves than other reported tubulin-binding agents.

To further evaluate any potential toxic effects of noscapine on peripheral nerves, we examined dorsal root ganglion cultures...
Fig. 10 Daily oral noscapine treatment results in minimal evidence of peripheral neuropathy. A, representative ventral root sections stained with toluidine blue from vehicle- and noscapine-treated animals. B, cultured dorsal root ganglion cells (DRG) in the absence and presence of noscapine. Total axonal length and the dorsal root ganglion cell halo area were the quantitative parameters used to measure neurotoxicity. Dorsal root ganglion cells were cultured for 5 days and then incubated with noscapine for up to 10 days. Percent change in axonal length (C) and percent change of dorsal root ganglion cell halo area (D) after 0 μM (■), 25 μM (□), 50 μM (■), or 250 μM (□) noscapine treatment for 4, 8, or 10 days are shown.
DISCUSSION

Antineoplastic agents that interact with microtubules represent an important group of drugs that disrupt mitosis and particularly mitotic spindle activity by interfering with microtubule dynamics. Microtubule-targeting drugs currently in use either promote excessive stability of microtubules, such as the taxane family, or induce depolymerization of microtubules like the Vinca alkaloids (33). Our prior results suggest that the most prominent effect of noscapine is on microtubule dynamics, significantly enhancing the percentage of time microtubules spend idle or in a paused state (22). In this study, we show that noscapine significantly reduces the mitotic index of rat C6 glioma cells. Mitotic C6 glioma cells but not normal glial cells became polyploid after nuclear endoreplication. Mitotic cells showed abnormal spindle formation with excessive duplication (38). p53 prevents cell cycle progression when p53 null fibroblasts (40).

Because cell cycle checkpoint mechanisms in tumor cells are frequently faulty (34–36), cancer cells may be more susceptible than normal cells to noscapine. Our data support the hypothesis that transformed cells proceed improperly through the cell cycle resulting in abnormal mitoses and ultimately undergo cell death. Evidence of apoptosis was not observed in these experiments. Abrogation of the G1-S checkpoint is a frequent event in the development of gliomas (4–6), and this is known to cause failure of arrest of division (continued replication) in response to treatment with microtubule-targeting drugs (37). p53 interacts with the centrosome and regulates centrosome duplication (38). p53 prevents cell cycle progression when spindle assembly is blocked by antimicrotubule agents (39), and abnormal centrosome amplification and unbalanced chromosome segregation are observed in p53 null fibroblasts (40). Consistent with these findings, p53 was found to prevent hyperploidy in human glioma cells exposed to nocodazole (41). Whether a similar mechanism operated in C6 glioma cells in response to noscapine is unclear. C6 glioma cells have been reported to contain wild-type TP53 alleles, but the p53 pathway might be defective through alternative means such as p14ARF deletion, a common feature in gliomas (42, 43, 44).

Our observation that noscapine crosses an experimental blood-brain barrier efficiently led us to study whether noscapine could inhibit the tumorigenicity of the rapidly dividing rat C6 glioma cell line in vivo. We found that noscapine showed greater than 78% inhibition of the growth of intracranial glioma in immunocompromised mice. Histopathology of noscapine-treated brain tissue revealed a dramatically reduced number of malignant cells and an increase of dilated blood vessels surrounded by a layer of neoplastic cells. In contrast, we observed a massive infiltration of tumor growth and an absence of dilated vessels in vehicle-treated animals. Although noscapine treatment resulted in a marked reduction of tumor cells and tumor infiltration, there remains a significant need for additional treatments that will target residual infiltrated tumor cells. Additional studies are warranted to examine whether noscapine will prove equally efficient in the treatment of mice carrying human tumors that exhibit a slower mitotic rate or for the treatment of spontaneously occurring gliomas in transgenic mice.

The use of any chemotherapeutic agent that affects microtubule structure or dynamics raises a concern of neurotoxicity, particularly in regard to the peripheral nervous system. These types of drugs are thought to disrupt normal axonal transport, leading to axonal degeneration and clinical symptoms of numbness and/or weakness (45). It is encouraging that doses of noscapine that show efficacy against brain tumors did not cause any overt pathological changes in the peripheral nervous system. Additionally, sensory neurites died only with prolonged exposure to high doses of noscapine. There are no human reports of peripheral neuropathy with the use of low dose noscapine as an antitussive, however, the assessment of neurotoxicity in the setting of treatment for brain tumors requires human clinical trials.

We observed blood vessel dilation in noscapine-treated tissue in both the tumor-infiltrated and contralateral brain hemispheres. Additional studies are warranted to examine whether dilation is present in other organs, to determine whether the dilation observed relates to the antitussive effects of noscapine, and to assess whether the effects observed constitute a clinical risk at the noscapine doses required to achieve antitumor effects.

Noscapine only affects microtubule dynamics (22) rather than changing the net equilibrium between the monomer and the polymer distribution of tubulin within the cell (23). Normal cells with intact checkpoint proteins could conceivably tolerate the relatively less disruptive microtubule effects of noscapine compared with other known antimitotic agents. Normal microtubule morphology is retained in nontumor cells after noscapine exposure, and cells resume cell division upon noscapine removal. Upon in vivo treatment, noscapine levels rise only transiently in plasma; pharmacokinetic studies in mice and humans reveal peak concentration at 3 h after oral ingestion and a relatively fast clearance thereafter. This suggests that normal cells likely resume cell division after the noscapine concentration decreases below the threshold level in a few hours (27). In support of this hypothesis, BrdUrd measurements in duodenum, spleen, and liver did not show changes in cell division rates between treated and untreated animals (data not shown). These properties of noscapine might explain the absence of toxicity at sites of...
Noscapine Crosses Blood-Brain Barrier, Inhibits Glioblastoma

Noscapine should be further tested in humans to confirm a positive adverse event profile and to examine its ability to inhibit the growth of the subset of central nervous system tumors that show rapid proliferation rates such as glioblastoma. Perhaps, this could best be achieved in conjunction with other therapies because noscapine alone did not eradiclate the tumor type tested in this study. In conclusion, noscapine was able to significantly reduce the growth of a very aggressive experimental mouse glioma and therefore is a promising anticancer agent that provides novel hope for the treatment of malignant gliomas that have a less than 20% response rate to conventional chemotherapy (3) and for which existing treatments are associated with debilitating toxic side effects (46).

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