Brominated Derivatives of Noscapine Are Potent Microtubule-interfering Agents That Perturb Mitosis and Inhibit Cell Proliferation

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ABSTRACT
Noscapine, a microtubule-interfering agent, has been shown to arrest mitosis, to induce apoptosis, and to have potent antitumor activity. We report herein that two brominated derivatives of noscapine, 5-bromonoscapine (5-Br-nosc) and reduced 5-bromonoscapine (Rd 5-Br-nosc), have higher tubulin binding activity than noscapine and affect tubulin polymerization differently from noscapine. In addition, they are able to arrest cell cycle progression at mitosis at concentrations much lower than noscapine. Interestingly, whereas noscapine-arrested cells have nearly normal bipolar spindles, cells arrested by 5-Br-nosc and Rd 5-Br-nosc form multipolar spindles. Nevertheless, noscapine and the two derivatives all affect the attachment of chromosomes to spindle microtubules and they impair the tension across paired kinetochores to similar degrees. 5-Br-nosc and Rd 5-Br-nosc are also more active than noscapine in inhibiting the proliferation of various human cancer cells, including those that are resistant to paclitaxel and epothilone. Our results thus indicate a great potential for the use of 5-Br-nosc and Rd 5-Br-nosc both as biological tools for studying microtubule-mediated processes and as chemotherapeutic agents for the treatment of human cancers.

Microtubules are helical polymers assembled from the heterodimer of α- and β-tubulin. They play important roles in shaping cells and directing intracellular motility. During cell division, microtubules form a bipolar apparatus called the mitotic spindle, which mediates chromosome distribution into two daughter cells (McIntosh, 1994). Microtubules are intrinsically dynamic and they alternate abruptly and stochastically between periods of growth and shortening. The dynamic nature is crucial for the organization and function of microtubules, especially for spindle morphogenesis and chromosome movement during mitosis (Desai and Mitchison, 1997). Eukaryotic cells have evolved a surveillance mechanism called the spindle assembly checkpoint, which blocks cell cycle progression at mitosis when the spindle has a defect or when chromosomes are not properly aligned at the equatorial plane (Zhou et al., 2002d). Not surprisingly, chemical compounds that target microtubules can arrest cells at mitosis, a property attributed to the use of microtubule-interfering agents in cancer chemotherapy (van Tellingen et al., 1992; Rowinsky, 1997; Crown and O’Leary, 2000).

There are two classes of microtubule-interfering agents: those that inhibit microtubule polymerization (such as colchicine, nocodazole, and the vinca alkaloids) and those that promote microtubule polymerization, such as the taxoids and epothilone (Jordan and Wilson, 1999). It is now clear that although all of these agents can efficiently block cell cycle progression, only a select few have been used clinically for the treatment of human cancers. Even for drugs that are currently in clinical use (e.g., paclitaxel and vinblastine), although patients have impressive initial response, many of them relapse after treatment because of the development of drug resistance. Thus, the development of more potent and selective antimicrotubule drugs are greatly needed, especially for the treatment of human cancers resistant to currently used drugs.

We have recently identified noscapine (Fig. 1A), a phthaldieisoquinoline alkaloid from opium, as a microtubule-interfering agent.
ferring agent that binds stoichiometrically to tubulin and alters tubulin conformation (Ye et al., 1998). Like many other antimicrotubule agents, noscapine suppresses the dynamics of microtubule assembly, blocks cell cycle progression at mitosis, and then causes apoptotic cell death in many cancer cell types (Ye et al., 1998, 2001; Zhou et al., 2002a, 2002b). Noscapine inhibits the progression of murine lymphoma, melanoma, and human breast tumors implanted in nude mice with little or no toxicity to the kidney, heart, liver, bone marrow, spleen, or small intestine and does not inhibit primary humoral immune responses in mice (Ye et al., 1998; Ke et al., 2000; Landen et al., 2002). The water solubility and feasibility for oral administration are also very valuable advantages of noscapine over many other antimicrotubule drugs (Dahlstrom et al., 1982; Haikala et al., 1986; Karlsson et al., 1990). In this study, we demonstrate that two brominated noscapine derivatives, 5-bromonoscapine (5-Br-nosc; Fig. 1D) and reduced 5-bromonoscapine (Rd 5-Br-nosc; Fig. 1G), are more potent microtubule-interfering agents that arrest mitosis and inhibit cell proliferation with much higher efficiency than noscapine.

Materials and Methods

Materials. Goat brain microtubule proteins were isolated in the presence of 1 M glutamate by two cycles of polymerization and depolymerization (Hamel and Lin, 1981). Tubulin was purified from the microtubule proteins by phosphocellulose chromatography as described previously (Panda et al., 2000; Joshi and Zhou, 2001), and the concentration of tubulin was determined by the method of Bradford (1976) using bovine serum albumin as a standard. The tubulin solution was stored at −80°C until future use. Paclitaxel and nocodazole were purchased from Sigma (St. Louis, MO), and noscapine (97% purity) was from Aldrich (Milwaukee, WI).

Preparation of Brominated Derivatives of Noscapine. 5-Br-nosc (1,3-dihydro-4,5-dimethoxy-1-[1,2,3,4-tetrahydro-5-bromo-8-methoxy-2-methyl-6,7-methylendioxy-isoquinolyl-1]isobenzofuran-

Fig. 1. Fluorescence quenching of tubulin by noscapine and its two derivatives, 5-Br-nosc and Rd 5-Br-nosc. A, D, and G show the molecular structures of noscapine, 5-Br-nosc, and Rd 5-Br-nosc, respectively. The tubulin fluorescence emission spectrum is quenched by noscapine (B), 5-Br-nosc (E), and Rd 5-Br-nosc (H) in a concentration-dependent manner. C, F, and I are double reciprocal plots showing a dissociation constant (K_d) of 144 ± 2.8 μM for noscapine binding to tubulin (C), 54 ± 9.1 μM for 5-Br-nosc binding to tubulin (F), and 106 ± 4.2 μM for Rd 5-Br-nosc binding to tubulin (I).
3-one) and Rd 5-Br-nosc (1,3-dihydro-4,5-dimethoxy-1-[1,2,3,4-tetrahydro-5-bromo-8-meth-ox2-2-methyl-6,7-methylenedioxy)isoquinolinyl]-1-benzofuran) were prepared as described previously with minor modifications (Urushitani, Washida, and Srinivasan, 1993). Briefly, noscapine was dissolved in 48% HBr solution, and Br2/H2O was added to this solution until no semisolid precipitate formed. The supernatant liquid was decanted to another flask, and NH3 solution was added to the semisolid precipitate to form solid precipitate. The supernatant liquid was also neutralized to pH 10 to form solid precipitate. The two solid precipitates were combined and recrystallized with ethanol to produce 5-Br-nosc.

Yield, 75%; melting point; 169 to 170°C; calculated percentage (found percentage), C, 53.67 (53.62); H, 4.50 (4.44); N, 2.85 (2.71). IR, 2945 (m), 2800 (m), 1759 (s), 1612 (m), 1500 (s), 1443 (s), 1263 (s), 1091 (s), 933 (w) cm⁻¹. 1H NMR (CDCl3, 300 MHz), δ 7.04 (d, J = 7 Hz, 1H), 6.32 (d, J = 7 Hz, 1H), 6.03 (s, 2H), 5.51 (d, J = 4 Hz, 1H), 4.28 (d, J = 4 Hz, 1H), 1.40 (s, 3H), 1.38 (s, 3H), 2.8 (2.8 to 1.93, 4H). Mass spectrometry: fast atom bombardment ions, 3H), 2.52 (s, 3H), 2.8 to 1.93 (s, 4H). Mass spectrometry: fast atom bombardment ions, m/z (relative abundance percentage), 494 (93.8), 492 (100), 300 (30.5), 298 (35.4); matrix-assisted laser desorption ionization ions, m/z 491.37 (M⁺), 493.34; electrospray ionization/tandem mass spectrometry, parent ion masses, 494, 492; daughter ion masses (intensity, percentage): 433 (51), 431 (37), 300 (100), 298 (93.3). Rd 5-Br-nosc was prepared by using the same procedure as for 5-Br-nosc, except that the reduced form of noscapine was used as the starting material. Yield, 70%; melting point, 113 to 114°C; calculated percentage (found percentage), C, 55.24 (55.19); H, 5.05 (5.25); N, 2.93 (2.88). 1H NMR (CDCl3, 300 M Hz), δ 6.73 (d, J = 8Hz, 1H), 6.11 (d, J = 8Hz, 1H), 6.08 (s, 2H), 5.78 (s, 2H), 5.52 (s, 4 = 12Hz, 1H), 5.05 (dd, J = 12Hz, 1H), 4.90 (s, 1H), 3.86 (s, 6H), 3.83 (s, 3H), 3.42 to 3.19 (m, 2H), 2.95 (s, 3H), 2.82 to 2.80 (m, 2H). Infrared, 2950 (m), 2852 (m), 1635 (w), 1616 (m), 1450 (s), 1267 (s), 1226 (s), 1078 (s), 1035 (s) cm⁻¹. Mass spectrometry: fast atom bombardment ions, m/z (relative abundance %): 480 (100), 478 (100), 462 (8), 460 (8.3), 300 (18), 298 (19), 179 (12.5); matrix-assisted laser desorption ionization ions, m/z 478.5 (M⁺), 480.5; electrospray ionization/tandem mass spectrometry, parent ion mass: 480, 478; daughter ion masses (intensity, percentage): 462 (74), 460 (52.5), 447 (21), 445 (16.6), 431 (83.3), 429 (66.6), 300 (79), 298 (74.7), 193 (11), 191 (23.5), 179 (100).

**Cell Culture.** HeLa cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) (the medium and serum were both purchased from Invitrogen (Carlsbad, CA), as were the following media and sera). MCF-7, DU 145, and Caco-2 cells were grown in Eagle’s minimum essential medium supplemented with 10% fetal bovine serum (FBS) (the medium and serum were both purchased from Invitrogen (Carlsbad, CA). Equivalent amounts for each treatment group were equivalent volumes for each treatment group were loaded for SDS/polyacrylamide gel electrophoresis. The proteins were then electrophoretically transferred for Western blot analysis using a mouse monoclonal anti-α-tubulin antibody (DM1A; Sigma) and a horseradish peroxidase-conjugated anti-mouse secondary antibody (Jackson Immunoresearch, West Grove, PA). Tubulin bands were visualized using enhanced chemiluminescence following the manufacturer’s instructions (Amersham Biosciences, Piscataway, NJ), and their relative levels were determined by densitometric analysis using a Lynx video densitometer (Biological Vision Inc., San Mateo, CA).

**Tubulin Polymerization Assay.** Spectrophotometer cuvettes (0.4-cm path length) held a solution consisting of microtubule assembly buffer (100 mM PIPES, 2 mM EGTA, 1 mM MgCl₂, 1 mM GTP, pH 6.8) and 10 or 100 μM noscapine, 10 or 100 μM 5-Br-nosc, 10 or 100 μM Rd 5-Br-nosc, 10 μM paclitaxel, 10 μM nocodazole, or the solvent DMSO. Cuvettes were kept at room temperature before the addition of 10 μM purified tubulin and shifted to 37°C in a temperature controlled Ultratoc 3000 spectrophotometer (Pharmacia BioTech, Cambridge, UK). The assembly was monitored by measuring the changes in absorbance (350 nm) at 0.5-min intervals.

**Measurement of Insoluble and Soluble Tubulin.** Cells were washed with phosphate-buffered saline (PBS), and soluble proteins were then extracted under conditions that prevent microtubule depolymerization (0.1% Triton X-100, 0.1 M N-morpholinoethanesulfonic acid, pH 6.75, 1 mM MgSO₄, 2 mM EGTA, 4 M glycerol). The remaining cytoskeletal fraction in the culture dish was dissolved in 0.5 ml of 0.5% SDS in 25 mM Tris, pH 6.8. Total protein concentration was then determined in each fraction by BCA reagents (Pierce, Rockford, IL). Equivalent amounts for each treatment group were loaded for SDS/polyacrylamide gel electrophoresis. The proteins were then electrophoretically transferred for Western blot analysis using a mouse monoclonal anti-α-tubulin antibody (DM1A; Sigma) and a horseradish peroxidase-conjugated anti-mouse secondary antibody (Jackson Immunoresearch, West Grove, PA). Tubulin bands were visualized using enhanced chemiluminescence following the manufacturer’s instructions (Amersham Biosciences, Piscataway, NJ), and their relative levels were determined by densitometric analysis using a Lynx video densitometer (Biological Vision Inc., San Mateo, CA).

**Flow Cytometric Analysis.** The flow cytometric evaluation of the cell cycle status was performed as described previously (Zhou et al., 2002b). Briefly, 2 × 10⁶ HeLa cells were centrifuged, washed twice with ice-cold PBS, and fixed in 70% ethanol. Tubes containing the cell pellets were stored at −20°C for at least 24 h. After this, the cells were centrifuged at 1000 g for 10 min and the supernatant was discarded. The pellets were resuspended in 30 μl of phosphate/citrate buffer (0.2 M Na₂HPO₄/0.1 M citric acid, pH 7.5) at room temperature for 30 min. Cells were then washed with 5 μl of PBS and incubated with propidium iodide (PI, 20 μg/ml)/RNase A (20 μg/ml) in PBS for 30 min. Samples were analyzed on a Coulter Elite flow cytometer (Beckman Coulter, Inc., Fullerton, CA).

**Immunofluorescence Microscopy.** Cells were grown on poly(t-lysine)-coated glass coverslips for immunofluorescence microscopy as described previously (Zhou et al., 2002b, 2002c). To visualize microtubules, cells were fixed with methanol for 5 min at −20°C, processed with a mouse monoclonal anti-α-tubulin antibody (DM1A diluted 1000-fold, Sigma) followed by a fluorescein isothiocyanate (FITC)-conjugated anti-mouse secondary antibody (diluted 100-fold; Jackson ImmunoResearch). Cells were stained with PI (0.5 μg/ml) for 30 s at room temperature to visualize DNA. Coverslips were
and Rd 5-Br-nosc have higher tubulin binding activity than noscapine. We also examined the tubulin binding activity of reduced noscapine as a control for Rd 5-Br-nosc and could not obtain a dissociation constant because the effect of reduced noscapine on tubulin fluorescence was minimal (data not shown).

Effects of 5-Br-nosc and Rd 5-Br-nosc on the Assembly of Tubulin Subunits. We have previously shown that noscapine, although binding to tubulin, does not significantly promote or inhibit microtubule polymerization, even at a concentration as high as 100 μM; instead, it alters the steady-state dynamics of microtubule assembly primarily by increasing the amount of time that the microtubules spend in an attenuated (pause) state (Zhou et al., 2002b). This unique property of noscapine has led to its successful use in exploring the role of microtubule dynamics during the spindle assembly checkpoint signaling (Zhou et al., 2002b). In this study, we examined the effects of 5-Br-nosc and Rd 5-Br-nosc on the assembly of tubulin subunits into microtubules in vitro by measuring the changes in the turbidity produced upon tubulin polymerization (Fig. 2). As expected, 10 μM paclitaxel strongly promoted tubulin polymerization into microtubules, and 10 μM nocodazole strongly inhibited tubulin polymerization (Fig. 2). In addition, consistent with our previous observation (Zhou et al., 2002b), 10 or 100 μM noscapine did not have a significant effect on tubulin polymerization. 10 μM 5-Br-nosc or Rd 5-Br-nosc did not have a significant effect on tubulin polymerization, either. However, at 100 μM, both 5-Br-nosc and Rd 5-Br-nosc inhibited tubulin polymerization, although the effect of Rd 5-Br-nosc was not as obvious as that of 5-Br-nosc (Fig. 2).

Effects of 5-Br-nosc and Rd 5-Br-nosc on the Cellular Levels of Insoluble versus Soluble Tubulin. To test the effects of 5-Br-nosc and Rd 5-Br-nosc on tubulin polymerization in cells, we prepared cell extracts that contains insoluble and soluble tubulin, respectively, from HeLa cells treated with these agents (Fig. 3). An equivalent amount of the solvent DMSO was used as a control, and 10 or 100 μM noscapine, 10 μM paclitaxel, and 10 μM nocodazole were also used as comparisons. We found that the percentage of insoluble tubulin in cells treated with the solvent DMSO was 61.5% (control, Fig. 3). For cells treated with 10 μM paclitaxel, 99.2% of tubulin was in the insoluble form, and for

![Image](http://molpharm.aspetjournals.org)

**Fig. 2.** Effects of noscapine, 5-Br-nosc, and Rd 5-Br-nosc on the assembly of tubulin into microtubules in vitro. The assay was based on the light scattering ability of tubulin polymer, reflected as the absorbance at 350 nm wavelength. An equivalent amount of the solvent DMSO was used as a control, and 10 μM paclitaxel and nocodazole were also used for comparisons.

Results

5-Br-nosc and Rd 5-Br-nosc Have Higher Tubulin Binding Activity than Noscapine. Tubulin, like many other proteins, contains considerable aromatic amino acids, such as tryptophan, which can quench an intrinsic fluorescence. Tubulin-binding agents typically quench the fluorescence emission spectrum of tubulin in a concentration-dependent manner (Peyrot et al., 1992; Panda et al., 1997; Ye et al., 1998). This provides a basis for using the fluorescence titration method to determine the dissociation constant (Kd) between tubulin and its binding agents. We found that noscapine, 5-Br-nosc, and Rd 5-Br-nosc all quenched tubulin fluorescence in a concentration-dependent manner (Fig. 1, B, E, and H). The dissociation constant (Kd) was determined by the formula: 1/B = Kd/[free ligand] + 1, where B is the fractional occupancy, and [free ligand] is the concentration of free noscapine, 5-Br-nosc, or Rd 5-Br-nosc (Fig. 1, C, F, and I). This analysis gave a dissociation constant (Kd) of 144 ± 2.8 μM for noscapine binding to tubulin, 54 ± 9.1 μM for 5-Br-nosc binding to tubulin, and 106 ± 4.2 μM for Rd 5-Br-nosc binding to tubulin. These results thus indicate that 5-Br-nosc...
those cells treated with 10 μM nocodazole, only 9.8% of the

tubulin was insoluble (Fig. 3). However, the percentage of

insoluble tubulin in cells treated with 10, 100 μM noscapine, 10

μM 5-Br-nosc, and 10 μM Rd 5-Br-nosc was 60.5, 59.8, 60.2, and 60.9%, respectively, which were very similar to that

in the control cells (Fig. 3). Consistent with the inhibitory

effect of high-dose 5-Br-nosc on tubulin polymerization (Fig.

2), the percentage of insoluble tubulin in cells treated with

100 μM 5-Br-nosc was 37.6%. In contrast, the percentage of

insoluble tubulin in 100 μM Rd 5-Br-nosc treated cells was

59.7%, which was only slightly lower than that in the control

cells (Fig. 3).

5-Br-nosc and Rd 5-Br-nosc Are More Active than

Noscapine in Arresting Mitosis. Because all known mi-
crotubule-interfering agents, including noscapine (Ye et al.,

1998, 2001; Zhou et al., 2002b, 2002a), are able to arrest

mitosis in mammalian cells (Jordan and Wilson, 1999), we

examined the effects of 5-Br-nosc and Rd 5-Br-nosc on cell

cycle progression and compared with the effect of noscapine.

We used HeLa cells because they are known to have a tight

spindle assembly checkpoint (Skoufias et al., 2001; Zhou et

al., 2002b). We found that the percentage of mitotic cells (the

mitotic index) was increased in a concentration-dependent

manner upon treatment with noscapine, 5-Br-nosc, and Rd

5-Br-nosc for 24 h (Fig. 4A). The concentration needed to

arrest 50% of HeLa cells at mitosis was 18.4 μM for noscapine,
as determined by the curve of mitotic index versus drug

dose. In contrast, only 7.7 and 3.6 μM were re-

quired, respectively, for 5-Br-nosc and Rd 5-Br-nosc to arrest

50% of cells at mitosis (Fig. 4A), indicating that they are more

active than noscapine in arresting mitosis. The higher activ-

ity of 5-Br-nosc and Rd 5-Br-nosc in arresting mitosis was

further confirmed by flow cytometric analysis of DNA con-

tent. For example, the fraction of cells in G2/M, which have
duplicated (4N) DNA content, was 15.2, 51.8, and 72.7%,

respectively, upon treatment with 6.4 μM noscapine, 5-Br-

nosc, and Rd 5-Br-nosc for 24 h (Fig. 4B).

We examined the morphology of HeLa cells arrested by

5-Br-nosc and Rd 5-Br-nosc and compared it with noscapine-
arrested cells as well as normal interphase, prometaphase,

and metaphase cells. Microtubules existed as a radial array

in normal interphase cells and they formed a bipolar appa-
ratus, the mitotic spindle, in mitosis (Fig. 4C). Consistent

with our previous observation (Zhou et al., 2002b), noscapine-
arrested HeLa cells had nearly normal bipolar spindles but
did not complete chromosome alignment; some chromosomes

were aligned at the equatorial plane, whereas the rest re-

mained near the spindle poles. In contrast, cells arrested by

5-Br-nosc and Rd 5-Br-nosc had multipolar spindles (Fig.

4C). Furthermore, the multipolar spindle morphology existed
even in cells arrested by high concentrations (e.g., 100 μM of

5-Br-nosc and Rd 5-Br-nosc (data not shown).

Impairment of Chromosome Attachment to Kinetochore

Microtubules by Noscapine, 5-Br-nosc, and Rd

5-Br-nosc. The abnormal spindle morphology in cells ar-
arrested by noscapine, 5-Br-nosc, and Rd 5-Br-nosc suggested

that the attachment of chromosomes to kinetochore microtu-

bules might be disrupted. To test this, we examined the

localization patterns of Mad2, a spindle assembly checkpoint

protein known to be essential for sensing the attachment of

chromosomes to kinetochore microtubules in human cells (Li

and Benezra, 1996). In prometaphase, Mad2 was localized to

the kinetochore region, whereas in metaphase, it was no

longer detectable at kinetochores (Fig. 5). In noscapine-ar-

rested mitotic cells, Mad2 was present at the kinetochores on

chromosomes that were near the spindle poles but was not
detectable on chromosomes aligned at the equatorial plane

(Fig. 5; also see Zhou et al., 2002b). In cells arrested by

5-Br-nosc and Rd 5-Br-nosc, Mad2 signal could be seen at the

kinetochores on most chromosomes (Fig. 5). These results

indicate that noscapine, 5-Br-nosc, and Rd 5-Br-nosc disrupt

the attachment of chromosomes to kinetochore microtubules.

Comparable Impairment of Kinetochore Tension by

Noscapine, 5-Br-nosc, and Rd 5-Br-nosc. When the sister

chromatids become attached to kinetochore microtubules

from two opposite spindle poles, tension develops across the

sister kinetochores because of the mitotic force that tends to

pull the chromatids toward two opposite spindle poles

against the glue (cohesin) that holds the sister chromatids

together (Mitchison and Salmon, 1992; Rieder and Salmon,

1994; Nicklas, 1997). The tension across sister kinetochores,

apparent as a visible increase in the distance between them,
is believed to be crucial for the spindle assembly checkpoint

signaling in organisms from yeast to humans (Nicklas, 1997;

Zhou et al., 2002d). We thus asked whether the spindle

Fig. 3. Effects of noscapine, 5-Br-nosc, and Rd 5-Br-nosc on the levels of insoluble versus soluble tubulin in HeLa cells. A, Western blot analysis

showing the levels of insoluble (I) and soluble (S) tubulin in cells treated for 4 h with 10 or 100 μM noscapine, 10 or 100 μM 5-Br-nosc, 10 or 100

μM Rd 5-Br-nosc, 10 μM paclitaxel, 10 μM nocodazole, or an equivalent amount of the solvent DMSO as a control. The preparation of cell extracts

that contain insoluble and soluble tubulin, respectively, was described under Materials and Methods. B, quantitation of the percentage of insol-

uble tubulin in cells treated as described above by densitometry. Values and error bars shown in this graph represent the averages and standard

deviations, respectively, of three independent experiments.
defects caused by noscapine, 5-Br-nosc, and Rd 5-Br-nosc were associated with impaired kinetochore tension.

We examined kinetochore tension by measuring the distance between paired kinetochores. Immunofluorescent staining followed by confocal microscopy allowed us to clearly resolve the paired kinetochores on sister chromatids (Fig. 6A, insets). The distance between sister kinetochores in normal metaphase HeLa cells was measured to be 1.91 ± 0.41 μm on average (Fig. 6B), which was in agreement with those reported previously (Skoufias et al., 2001; Zhou et al., 2002b). In contrast, in cells arrested by noscapine, 5-Br-nosc, and Rd 5-Br-nosc, the average sister kinetochore distance was measured to be 1.32 ± 0.39 μm (30.9% reduction), 1.28 ± 0.36 μm (33.0% reduction), and 1.26 ± 0.41 μm (34.0% reduction), respectively (Fig. 6B). The comparable extents of reduction in the sister kinetochore distance by noscapine, 5-Br-nosc, and Rd 5-Br-nosc indicate that they impair kinetochore tension to similar degrees.

5-Br-nosc and Rd 5-Br-nosc Are More Potent than Noscapine in Inhibiting the Proliferation of Human Cancer Cells. We then performed in vitro cell proliferation assays to examine the effects of noscapine, 5-Br-nosc, and Rd 5-Br-nosc on the proliferation of a series of human cancer cell lines. They included human breast cancer cell lines MCF-7 and MDA-MB-231, cervical cancer cell lines HeLa and Ca Ski, colon cancer cell lines Caco-2 and T84, ovarian cancer cell lines SK-OV-3 and SigC, and a prostate cancer cell line, DU 145 (Fig. 7A). We found that 5-Br-nosc and Rd 5-Br-nosc inhibited the proliferation of these human cancer cell lines more efficiently than noscapine, as reflected by the much lower IC$_{50}$ values, the drug concentrations needed to prevent cell proliferation by 50% (Fig. 7A). For example, the IC$_{50}$ values of noscapine, 5-Br-nosc, and Rd 5-Br-nosc were 33.4 ± 3.7, 5.8 ± 1.1, and 3.8 ± 0.5 μM, respectively for MCF-7 cells, and 34.8 ± 3.1, 3.9 ± 0.8, and 2.2 ± 0.7 μM, respectively for DU 145 cells (Fig. 7A). The significantly lower IC$_{50}$ values suggest that 5-Br-nosc and Rd 5-Br-nosc are more potent than noscapine in inhibiting the proliferation of human cancer cells.

We also examined the effects of noscapine, 5-Br-nosc, and Rd 5-Br-nosc on the proliferation of a set of human ovarian cancer cells sensitive or resistant to paclitaxel and epothilone (Fig. 7B). These included the parent cell line 1A9 (a clone of line A2780) and four drug-resistant cell lines, including two paclitaxel-resistant cell lines (1A9/PTX10 and 1A9/PTX22), an epothilone-resistant cell line (1A9/A8), and a multidrug-resistant cell line (A2780/AD10). 1A9/PTX10, 1A9/PTX22, and 1A9/A8 were derived from 1A9 and they harbor β-tubulin mutations that confer resistance to paclitaxel or epothilone (Giannakakou et al., 1997, 2000). A2780/AD10 cells were also derived from A2780 and they overexpress P-glycoprotein producing a multidrug resistance phenotype (Pryor...
et al., 2002). We found that noscapine effectively inhibited the proliferation of 1A9, 1A9/PTX10, 1A9/PTX22, 1A9/A8, and A2780/AD10 cells (Fig. 7B). Furthermore, 5-Br-nosc and Rd 5-Br-nosc were more active than noscapine in inhibiting the proliferation of these drug-resistant cancer cell lines, as indicated by their much lower IC50 values (Fig. 7B).

Discussion

Microtubule-interfering agents have played an important role in early experiments studying the basic mechanisms of mitosis. This is primarily because of their ability to interfere with spindle microtubules and halt cell cycle progression at mitosis. In fact, the subunit of microtubules, tubulin, was first identified as a high-affinity colchicine receptor located on the mitotic apparatus (Weisenberg et al., 1968). In addition, the major components involved in the spindle assembly checkpoint were identified in budding yeast for mutants that fail to arrest in mitosis in the presence of microtubule-interfering agents (Li and Murray, 1991; Hoyt et al., 1991). We have recently found that noscapine is a unique antimicrotubule agent that alters microtubule dynamics without affecting the total polymer mass of tubulin and have successfully used it in probing the spindle assembly checkpoint mechanisms (Zhou et al., 2002b). In this study, we demonstrate that two brominated derivatives of noscapine, 5-Br-nosc and reduced Rd 5-Br-nosc, have higher tubulin binding activity than noscapine and affect tubulin polymerization differently from noscapine. To facilitate the use of 5-Br-nosc and Rd 5-Br-nosc in studying mitotic processes, it will be of great importance to investigate their effects on the dynamic instability parameters of microtubule assembly.

In this study, we demonstrate that 5-Br-nosc and Rd 5-Br-nosc are able to arrest cell cycle progression at mitosis at concentrations much lower than noscapine. In addition, although noscapine-arrested cells have nearly normal bipolar spindles, cells arrested by 5-Br-nosc and Rd 5-Br-nosc form multipolar spindles. The multipolar spindle morphology is intriguing, because it suggests that 5-Br-nosc and Rd 5-Br-nosc might also affect the centrosome, which tethers the minus ends of spindle microtubules and is critical for spindle morphogenesis in most cells. We show that the inhibitory effect of 5-Br-nosc on tubulin polymerization is greater than that of Rd 5-Br-nosc. However, Rd 5-Br-nosc is in turn more active than 5-Br-nosc in arresting mitosis and inhibiting cell proliferation. These results thus support the hypothesis that the mechanisms by which microtubule-interfering agents arrest cells at mitosis and inhibit cell proliferation might lie in the suppression of spindle microtubule dynamics instead of their action on microtubule polymerization or depolymerization (Wilson and Jordan, 1995).

The spindle assembly checkpoint, as a molecular safeguard, is essential for faithful transmission of chromosomes during mitosis. The spindle assembly checkpoint examines whether prerequisites for chromosome segregation have been satisfied and thereby determines whether to execute or to delay chromosome segregation (Zhou et al., 2002d). Only when all the chromosomes are attached by kinetochore microtubules from two opposite poles and proper tension is

Fig. 6. Noscapine, 5-Br-nosc, and Rd 5-Br-nosc impair the tension across paired kinetochores. A, immunofluorescence micrographs showing paired kinetochores (green) and microtubules (red) in control metaphase HeLa cells and in cells arrested by 18.4 μM noscapine, 7.7 μM 5-Br-nosc, and 3.6 μM Rd 5-Br-nosc. Insets are magnified images of representative kinetochore pairs. Scale bar, 10 μm. B, noscapine and its two derivatives cause the reduction of sister kinetochore distances to similar extents, indicating that they impair kinetochore tension to similar degrees. Sister kinetochore distances were measured as described under Materials and Methods.
placed on the paired kinetochores does anaphase take place, allowing the physical splitting of sister chromatids. Microtubule-interfering agents, although acting on microtubules with different mechanisms, all disrupt microtubule dynamics (Jordan and Wilson, 1999), which may affect both the attachment of chromosomes to kinetochore microtubules and the tension exerted on kinetochore. It is thus not surprising that all the known microtubule-interfering agents are able to halt mitotic progression by activating the spindle assembly checkpoint (Jordan and Wilson, 1999). Consistently, noscapine, 5-Br-nosc, and Rd 5-Br-nosc act on tubulin differently and cause distinct spindle defects; however, they all affect chromosome attachment to kinetochore microtubules, as indicated by localization of Mad2 at the kinetochore region. In addition, they impair kinetochore tension to similar degrees, as indicated by similar extents of reduction in the distance between sister kinetochores. The potential of 5-Br-nosc and Rd 5-Br-nosc as biological tools for studying mitotic processes thus merits thorough evaluation.

Microtubule-interfering agents, such as paclitaxel, doxetaxel, and the vinca alkaloids, have proven effective for chemotherapeutic management of human cancers (van Tellingen et al., 1992; Rowinsky, 1997; Crown and O’Leary, 2000). Unfortunately, these microtubule drugs produce a variety of side effects (Rowinsky, 1997). This is mainly because microtubules perform many other functions, such as cytoplasmic organization and axonal transport, besides their function in chromosome movement during mitosis. In addition, the clinical use of the taxoids and vinca alkaloids has been limited by the development of drug resistance contributed by P-glycoprotein overexpression (Gottesman and Pastan, 1993; Bradley and Ling, 1994), altered expression of tubulin isoforms (Burkhardt et al., 2001), and other unknown mechanisms. We show that 5-Br-nosc and Rd 5-Br-nosc are more potent than noscapine in inhibiting the proliferation of a series of human breast, cervical, colon, ovarian, and prostate cancer cells. Moreover, these two brominated derivatives are also more potent than noscapine in inhibiting the proliferation of human ovarian cancer cells resistant to paclitaxel and epothilone. These findings thus indicate a great potential for the use of 5-Br-nosc and Rd 5-Br-nosc as chemotherapeutic agents for the treatment of human cancers, especially for those that are resistant to currently used microtubule drugs.

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