Treatment of hormone-refractory breast cancer: apoptosis and regression of human tumors implanted in mice

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Abstract
Following surgery, the hormone dependence of breast tumors is exploited for therapy using antagonists such as tamoxifen, although occasional hormone-resistant clones do appear. Another chemotherapeutic strategy uses microtubule inhibitors such as taxanes. Unfortunately, these agents elicit toxicities such as leukocytopenia, diarrhea, alopecia, and peripheral neuropathies and are also associated with the emergence of drug resistance. We have previously described a tubulin-binding, natural compound, noscapine, that was nontoxic and triggered apoptosis in many cancer types albeit at 1 µmol/L or higher concentrations depending on the cell type. We now show that a synthetic analogue of noscapine, 9-bromonoscapine, is ~10-fold to 15-fold more potent than noscapine in inhibiting cell proliferation and induces apoptosis following G2-M arrest in hormone-insensitive human breast cancers (MDA-MB-231). Furthermore, a clear loss of mitochondrial membrane potential, release of cytochrome c, activation of the terminal caspase-3, and the cleavage of its substrates such as poly(ADP-ribose) polymerase, suggest an intrinsic apoptotic mechanism. Taken together, these data point to a mitochondrially mediated apoptosis of hormone-insensitive breast cancer cells. Human tumor xenografts in nude mice showed significant tumor volume reduction and a surprising increase in longevity without signs of obvious toxicity. Thus, our data provide compelling evidence that 9-bromonoscapine can be useful for the therapy of hormone-refractory breast cancer. [Mol Cancer Ther 2006;5(9):2366–77]

Introduction
Breast cancer is one of the most frequent malignancies among women in the U.S. and is the leading cause of death worldwide between the ages of 40 and 55 years (1, 2). This disease is controlled by surgery and radiotherapy, and is commonly supported by adjuvant chemotherapies or hormone therapies (3). It is well established that the ovarian hormones, estrogen and progesterone, are essential for the growth and maintenance of the mammary ductal tissue (4). During postlactational regression of breasts, extensive apoptosis of ductal cells is required, whereas myoepithelial cells and basal lamina persist, and are reused during the resumption of extensive cell proliferation (5, 6). Likewise, breast tumor cells are also heavily dependent on estrogen and progesterone hormones for their maintenance and growth (4). Fortunately, very effective antagonists for these hormones exist, such as tamoxifen, which is widely used for the treatment of these tumor types usually subsequent to surgical resection (7). However, occasional loss of receptors due to genetic lesions in tumor cells and overexpression of drug efflux pumps lead to resistance towards hormone-mimetic drugs as well as other chemotherapeutic agents. Although second-generation selective estrogen receptor modulators such as raloxifene and second-line treatment options such as the aromatase inhibitors (letrozole and anastrazole) are somewhat effective, they are primarily useful against tumors that have a positive hormone receptor status. The challenge, thus, lies in the emergence of hormone-refractory tumors which no longer respond to the antihormone therapy.

Another effective target for the treatment of these hormone-insensitive breast tumors is the microtubule cytoskeleton (8–12). Microtubules are ubiquitous polymers assembled from the noncovalent head-to-tail association of α-tubulin and β-tubulin (13–15). The process of microtubule assembly is highly dynamic, consequently, microtubules extend and shorten continually within the cellular milieu (16–20). This dynamic property is critical for microtubules to carry out many of their cellular functions (20–23). Especially, accurate chromosome segregation during mitosis, which requires an exquisite regulation of spindle microtubule dynamics, even a minor alteration of the microtubule dynamics could halt mitotic progression (21, 24–28). This is probably the primary basis for the use of microtubule-interfering agents in cancer chemotherapy (29, 30). Small molecules that stabilize and bundle microtubules such as taxanes are currently used to treat hormone-refractory breast tumors (31). However, due to their toxicity in normal healthy cells as well as cancer cells, they display several side effects such as leukocytopenias, alopecia, diarrhea, and peripheral neuropathies (30, 32).
Furthermore, currently available antimitotic drugs have to be infused i.v. over long periods of time in the clinic, necessitating the use of vehicles such as cremophor, which can cause complications due to hypersensitivity reactions. Chemotherapy by microtubule-interfering agents is also limited by the emergence of drug resistance owing to mutations in the target, microtubules/tubulin, overexpression of drug efflux pumps, and many other mechanisms (33–37). Taken together, despite the currently used treatment modalities, there is still no effective cure for patients with advanced stages of breast cancer, especially in cases of hormone-refractory cancer (38). Therefore, the discovery and/or the development of drugs that combat hormone-insensitivity and display better therapeutic indices would have an important effect on breast cancer morbidity and mortality.

Our laboratory recently discovered the tubulin-binding property of noscapine (39), a naturally occurring, orally available, anti-cough drug that is widely used in Asia, South America, and Western Europe (40). Noscapine shows antitumor activity against a variety of cancer types both in vitro and in vivo in xenograft models (41–47). To augment its anticancer activity, we synthesized a brominated analogue of noscapine, 9-bromonoscapine (9-Br-nos), that is ~12-fold more potent than the founding compound noscapine against the hormone-insensitive breast cancer cells (MDA-MB-231), and significantly regresses human breast xenograft tumors implanted in nude mice. This is due to the extensive apoptosis induced both in cells in vitro and in tumor xenografts in vivo. The molecular events associated with apoptosis involve the decrease of Bcl2/Bax ratio, disruption of transmembrane mitochondrial potential, release of cytochrome c from the mitochondria, loss of plasma membrane lipid asymmetry followed by changes in the cellular morphology, activation of caspase-3, cleavage of caspase-3 substrates such as poly(ADP-ribose)polymerase (PARP), and the appearance of terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL)–positive apoptotic bodies. We further show that 9-Br-nos treatment causes a significant inhibition and regression of tumor volume without any detectable toxicity in tissues and organs such as the brain, heart, lung, and the sciatic nerve. More importantly, we see no evidence of toxicity in the liver, kidney, spleen, duodenum, and frequently dividing cells like the spleen and duodenum, thereby increasing the longevity of treated mice. In addition, we see no evidence of toxicity in the liver, kidney, brain, heart, lung, and the sciatic nerve. More importantly, the immunosurveillance system is not compromised, as evidenced by the unaltered B and T cell counts among the untreated and the 9-Br-nos–treated groups. Additionally, 9-Br-nos does not affect hematopoiesis and the levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and blood urea nitrogen. Our preclinical data thus show that 9-Br-nos may be a safe and effective drug for the treatment of human hormone-refractory breast cancer.

Materials and Methods

Chemicals and Cell Culture

Noscapine was purchased from Sigma (St. Louis, MO). The brominated analogue of noscapine, 9-Br-nos, was prepared as described previously (48). The compounds were dissolved in DMSO (Sigma) and stored as 10 and 100 mmol/L stock solutions at −20°C until use. For all in vitro assays, the compounds were diluted to concentrations ranging from 10 nmol/L to 1,000 μmol/L. The human breast cancer cell lines, MDA-MB-231 and MCF-7, were obtained from American Type Culture Collection (Manassas, VA). MCF-7/Adr human mammary carcinoma cells were obtained from Dr. S. Misra, University of South Carolina (Charleston, SC). MTR-3 cells (49) were from Dr. Maricarman Panigone-Silva, Penn State College of Medicine (Hershey, PA). MCF-7, MDA-MB-231, and MCF-7/Adr cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). MTR-3 cells were grown in phenol red–free DMEM supplemented with 4 mmol/L L-glutamine, 5% charcoal-cleared fetal bovine serum (HyClone, Logan, UT) and 1 μmol/L tamoxifen (Sigma). Cells were maintained in a humidified incubator at 37°C with a supply of 5% CO2/95% air atmosphere.

In vitro Cell Proliferation Assay

Cells were seeded in 96-well plates at a density of 5 × 103 cells per well. They were treated with increasing gradient concentrations ranging from 0.01 to 1,000 μmol/L of 9-Br-nos the next day while in the log-phase growth. After 72 hours of drug treatment, cells were fixed with 50% trichloroacetic acid and stained with 0.4% sulforhodamine B dissolved in 1% acetic acid. Cells were then washed with 1% acetic acid to remove the unbound dye. The sulforhodamine B assay measures cell density by quantitating colored sulforhodamine B bound to cellular proteins fixed to the plates by trichloroacetic acid (50). The protein-bound dye was extracted with 10 mmol/L Tris base to determine the absorbance at 564 nm wavelength. The percentage of cell survival as a function of drug concentration was then plotted to determine the IC50 value (the drug concentration needed to prevent cell proliferation by 50%).

Cell Cycle Analysis

Cells were seeded in culture dishes and grown until 70% confluent. The medium was then replaced with a new medium containing either vehicle (0.1% DMSO) or 10 μmol/L 9-Br-nos for 24, 48, and 72 hours. After the incubation period, cells were centrifuged, washed twice with ice-cold PBS, and fixed in 70% ethanol. Tubes containing the cell pellets were stored at 4°C for at least 24 hours. Cells were then centrifuged at 100 × g for 10 minutes, and the supernatant was discarded. The pellets were washed twice with 5 mL of PBS and then stained with 0.5 mL of propidium iodide (PI; 0.1% in 0.6% Triton-X in PBS) and 0.5 mL of RNase A (2 mg/mL) for 45 minutes in dark. Samples were then analyzed on a FACSCalibur flow cytometer (Beckman Coulter, Inc., Fullerton, CA).

Immunofluorescence Microscopy

Cells were grown on poly(l-lysine)-coated glass coverslips for immunofluorescence microscopy as described previously (48). After treatment, cells were fixed with cold (−20°C) methanol for 5 minutes and then washed with PBS for 5 minutes. Nonspecific sites were blocked by incubating with 100 μL of 2% bovine serum albumin in PBS at 37°C for 30 minutes.
15 minutes. A mouse monoclonal antibody against α-tubulin (DM1A, Sigma) was diluted 1:500 in 2% bovine serum albumin/PBS (100 μL) and incubated with the coverslips for 2 hours at 37°C. Cells were then washed with 2% bovine serum albumin/PBS for 10 minutes at room temperature before incubating with a 1:200 dilution of a FITC-labeled goat anti-mouse IgG antibody (Jackson ImmunoResearch, Inc., West Grove, PA) at 37°C for 1 hour. Coverslips were then rinsed with 2% bovine serum albumin/PBS for 10 minutes and incubated with PI (0.5 μg/mL) for 15 minutes at room temperature before they were mounted with Aquamount (Lerner Laboratories, Pittsburgh, PA) containing 0.01% 1,4-diazobicyclo(2,2,2)octane (Sigma). Cells were then examined using confocal microscopy for microtubule morphology and the number of cells in mitosis (at least 100 cells were examined per condition). PI staining of the nuclei was used to visualize the apoptotic bodies.

Evaluation of the Mitochondrial Transmembrane Potential

The ampholytic cationic fluorescent probe, DiOC6, was used to monitor the 9-Br-nos–induced changes in the mitochondrial transmembrane potential. Briefly, cells were seeded at a density of 10^6 and treated with 10 μmol/L 9-Br-nos for 0, 24, 48, and 72 hours. After drug treatment, cells were loaded with the probe, DiOC6 (50 nmol/L), for 30 minutes at 37°C before flow cytometric analysis. The supernatant was removed, and the cells were harvested and resuspended in PBS. Measurement of the retained DiOC6 in 20,000 cells of each sample was done in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). DiOC6 was excited at 488 nm, and fluorescence was analyzed at 525 nm (FL-1) after logarithmic amplification.

Annexin V Staining for Apoptosis

Cells were grown in culture dishes, and 24 hours after seeding, they were exposed to 10 μmol/L of 9-Br-nos for 48 hours. After the incubation period, adherent cells were harvested by mild trypsinization and were pooled together with detached cells. Cells were then stained with Alexa-Fluor 488–conjugated Annexin V and PI using the Vybrant Apoptosis Assay Kit from Molecular Probes (Eugene, OR) as per the manufacturer’s protocol. Two-color flow cytometric analyses were done on a FACSCalibur equipped with a single argon-ion laser. The density plots illustrate four cell populations (live, apoptotic, necrotic, and late apoptotic/dead) defined by their fluorescence characteristics. Live cells are Annexin V– and PI-negative. In these cells, phosphatidylyserine translocation to the outer leaflet of the plasma membrane has not occurred and the integrity of the plasma membrane is still intact. Thus, early apoptotic cells are Annexin V–positive and PI-negative, and their membranes are impermeable to the DNA-binding red dye, PI. Necrotic cells appear red due to the access of PI through their damaged plasma membranes. Thus, the late apoptotic and dead cells are both Annexin V–positive and PI-positive.

TUNEL Assay for Apoptosis

DNA strand breaks were identified using the TUNEL assay as described (48). Cells treated with 10 μmol/L 9-Br-nos for 72 hours were washed with ice-cold PBS, fixed in 1% paraformaldehyde, and 3’-DNA ends were detected using the APO-BrdU TUNEL Assay Kit (Molecular Probes). This assay was run on a flow cytometer equipped with a 488 nm argon laser as the light source. PI (total cellular DNA) and anti–bromodeoxyuridine-labeled Alexa-Fluor 488 (apoptotic cells) were the two dyes used. PI fluoresces at 623 nm, whereas Alexa-Fluor 488 fluoresces at 520 nm, sufficient to clearly distinguish the two separate peaks, when excited at 488 nm. Single and dual parameter displays were created using the Cell Quest Data Acquisition Software (Becton Dickinson). The gating display was the standard dual parameter DNA doublet discrimination display with the DNA area signal on the y axis and the DNA width signal on the x axis. From this display, a gate was generated around the nonclumped cells and the second gated dual parameter display was generated with the DNA (linear red fluorescence) on the x axis and Alexa-Fluor 488 (log green fluorescence) on the y axis. Apoptotic cells were subsequently counted as those expressing high Alexa-Fluor 488 fluorescence.

Determination of Caspase-3 Activity

Cells (10^6) were incubated with 10 μmol/L of 9-Br-nos for 0, 12, 24, 48, and 72 hours. Caspase-3 activity was measured by the cleavage of the small synthetic substrate Z-DEVD-aminoluciferin (CaspaseGlo 3/7 Assay System Kit; Promega, Madison, WI) that becomes luminogenic upon cleavage. The luminescent signal, which is directly proportional to the amount of caspase-3 activity, was measured in a luminescence plate reader.

Immunoblot Analysis

Proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked for 2 hours in Tris-buffered saline containing 0.2% Tween 20 and 5% fat-free dry milk, and then incubated first with primary antibodies for 2 hours, and finally with horseradish peroxidase–conjugated secondary antibodies for 1 hour. All primary antibodies against human proteins were from Cell Signaling Technology (Beverly, MA; Bcl2, BAX, cytochrome c, cleaved caspase-3, and cleaved PARP). The human β-actin antibody was from Santa Cruz Biotech., Inc. (Santa Cruz, CA). All secondary antibodies were from Jackson ImmunoResearch Laboratories. For cytochrome c release assays, cells were lysed in lysis buffer [10 mmol/L HEPES (pH 7.5), 10 mmol/L KCl, and 1 mmol/L EDTA] with protease inhibitor cocktail (Sigma), frozen and thawed thrice, and spun at 2,000 × g for 5 minutes, and the supernatant was further centrifuged at 60,000 × g for 30 minutes at 4°C. The supernatant was analyzed for cytochrome c content by Western blot analysis. All Western blot analysis experiments were repeated at least twice with similar results.

In vivo Analysis of Hormone-Refractory Breast Carcinoma Progression

Six- to 8-week-old female BALB/c athymic (nu/nu) nude mice were obtained from Harlan-Sprague Labs, (Indianapolis, IN) and were housed in the Emory University Animal Care Facility. Susensions of 10^6 MDA-MB-231

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Results and Discussion

9-Br-nos Inhibits the Proliferation of Hormone-Insensitive Breast Cancer Cells Including Drug-Resistant Tumor Cells

9-Br-nos was synthesized in our laboratory from the founding anticancer compound, noscapine (Fig. 1A; ref. 48). Noscapine is comprised of two flat ring systems, isoquinoline and benzofurananone linked by a rotatable C—C bond including two chiral centers. However, only one out of the four noscapine stereoisomers is biologically active (39). Unlike noscapine, 9-Br-nos possesses a bromine atom at position 9 of the isoquinoline ring system, replacing an acidic proton (Fig. 1A). We have reported earlier that 9-Br-nos binds tubulin with a higher affinity as compared with noscapine (48), and it does not perturb the total cellular microtubule mass like noscapine (40). In this study, we investigated whether 9-Br-nos can manage the hormone-resistant phenotype of recurrent breast tumors. By using sulforhodamine B staining–based cytotoxicity assay, we found that 9-Br-nos significantly inhibited cell proliferation in the hormone-insensitive MDA-MB-231 cells in a concentration-dependent manner (Fig. 1B). The median inhibitory concentration (IC50) of 9-Br-nos in MDA-MB-231 cells was 3.3 μmol/L (Fig. 1B). This is ~12-fold lower than the IC50 value for noscapine, 36.3 μmol/L (Fig. 1B). We also evaluated the sensitivity of hormone-sensitive (estrogen receptor–positive breast cancer cells, MCF-7) towards 9-Br-nos. Our results show an IC50 of 1.0 μmol/L for 9-Br-nos in MCF-7 cells, thus demonstrating that 9-Br-nos can inhibit the cellular proliferation of breast cancer cells irrespective of their receptor status. Surprisingly, drug-resistant variants of MCF-7 cells, MCF-7/Adr (Adriamycin-resistant) and MTR-3 (tamoxifen-resistant) also responded to the antiproliferative effects of 9-Br-nos with low IC50 values (see Table 1). This conforms to the broad usage of 9-Br-nos over a wider range of cell types regardless of their receptor status and efflux pump overexpression.

9-Br-nos Induces Spindle Abnormalities

We then examined the effect of 9-Br-nos on the spindle architecture using confocal microscopy. We found that whereas untreated MDA-MB-231 cells exhibited normal radial microtubule arrays, cells treated with 9-Br-nos for 24 hours showed pronounced multipolar spindles and...
condensed chromosomes that did not complete congression to the metaphase plate indicating mitotic arrest (Fig. 1C). This is probably due to the activation of the spindle assembly checkpoint, a cellular surveillance mechanism that monitors the integrity of the mitotic spindle. After 48 to 72 hours of treatment, numerous micronucleated and multinucleated cells were evident (Fig. 1C). Both the accumulation of polyploid DNA and multiple poles suggest abortive progression of the cell cycle without cell division.

9-Br-nos Induces G2-M Arrest in Hormone-Refractory Breast Cells

To investigate the precise mechanisms responsible for the 9-Br-nos–mediated abortive cell divisions, we sought to examine the cell cycle distribution profile of 9-Br-nos–treated cells. A representative cell cycle progression profile over the time of 9-Br-nos treatment in a three-dimensional disposition is shown in Fig. 1D. These data reveal that the cell cycle progressively deteriorates from time 0 of treatment to 24 to 72 hours, and that there is an abnormal accumulation of cells either in polyploid state in the beginning, progressing towards the sub-G1 amounts of DNA indicating apoptosis. This is clearly in line with the concomitant appearance of apoptotic bodies as shown by immunofluorescence experiments (Fig. 1C). The quantitation of cells in G2-M and sub-G1 phases is shown in Fig. 1E. Our results indicate that 9-Br-nos treatment caused significant perturbations of cell cycle progression in MDA-MB-231 cells at 24 hours resulting in a clear increase.
of the percentage of cells in the G2-M phase (~40%) compared with control cells (Fig. 1E). This increased population of cells with 4N DNA correlated with concomitant losses from G0/G1 phases (Fig. 1D). Following this, we observed the disappearance of the G2-M population and the appearance of a characteristic hypodiploid DNA content peak (sub-G1), indicative of apoptotic cells (Fig. 1D), perhaps, due to genotoxicity caused by the overaccumulation of DNA. This can be seen as an increase of apoptotic index at 72 hours of 9-Br-nos treatment (Fig. 1E). These results show that 9-Br-nos–treated cells arrest in G2-M phase preceding cell death.

9-Br-nos Induces Apoptosis through the Activation of Mitochondrial Pathway

9-Br-nos Decreases Bcl2/BAX Ratio and Disrupts Transmembrane Mitochondrial Potential. One of the main regulatory steps of apoptotic cell death is controlled by the ratio of antiapoptotic and proapoptotic members of the Bcl2 family of proteins (51, 52), which determines the susceptibility to apoptosis. For example, overexpression of antiapoptotic Bcl2 family members can tip the delicate balance in favor of survival, thereby conferring drug resistance, at least in some cellular tumor model systems (53, 54). On the other hand, overexpression of proapoptotic BAX or BAK is sufficient to increase the sensitivity of malignant cancer cells to apoptosis and to overcome drug resistance (55, 56). To investigate the mitochondrial apoptotic events involved in 9-Br-nos–induced apoptosis, we first analyzed the changes in the levels of proapoptotic protein, BAX, and antiapoptotic protein, Bcl2. Western blot analysis showed that treatment of MDA-MB-231 cells with 10 µmol/L of 9-Br-nos increased BAX protein levels in a time-dependent manner (Fig. 2A). In contrast, 9-Br-nos decreased Bcl2 levels, which led to a decrease in the antiapoptotic/proapoptotic Bcl2/BAX ratio as a function of time of treatment (Fig. 2A). Thus, we sought to determine whether alterations in the proapoptotic/antiapoptotic balance at the mitochondrial membrane reflected a perturbation of the mitochondrial membrane integrity and transmembrane potential. Collapse of ΔΨm is associated with a loss of mitochondrial membrane integrity and is an early event in the initiation and activation of apoptotic cascades (57). We monitored this collapse of ΔΨm as a reduction in the uptake of the fluorochrome DiOC6. As shown in Fig. 2B, a substantial reduction in the uptake of fluorochrome DiOC6 was observed when MDA-MB-231 cell populations were treated with 10 µmol/L 9-Br-nos, and this phenomenon was not seen with untreated control cells. The percentage of depolarized cells increased as a function of time of treatment, beginning with 20% at 24 hours, and reaching a maximum of ~73% at 72 hours (Fig. 2C).

9-Br-nos Treatment Causes the Release of Cytochrome c from the Mitochondria. The role of mitochondrial damage in apoptosis was suggested to be mediated by the release of cytochrome c (58). Therefore, we prepared cytosolic extracts under conditions that preserve the mitochondria and measured cytosolic cytochrome c levels by immunoblot analysis. Figure 3A shows that the cytosolic fraction from untreated cells contained no detectable amounts of cytochrome c, although it did become detectable after 48 hours of 9-Br-nos treatment. Results showing an increase in the level of cytosolic cytochrome c suggested that its release from mitochondria to the cytosol could be an important event in the 9-Br-nos–induced apoptosis. There is a likelihood that cytochrome c release to the cytoplasm might be the result of an increase in the proapoptotic Bcl2 members or a decrease in prosurvival Bcl2 family members followed by disruption of mitochondrial membrane integrity. Overall, these observations suggested the involvement of mitochondrial membrane potential dissipation and cytochrome c release from the mitochondria into the cytosol in 9-Br-nos–induced apoptosis in MDA-MB-231 cells. Cytochrome c release from the mitochondria into the

### Table 1. \textit{In vitro} tumor cell killing activity of 9-Br-nos in drug-resistant cell lines and their normal counterpart

<table>
<thead>
<tr>
<th>Tumor cell line</th>
<th>Tumor type</th>
<th>Drug resistance</th>
<th>IC50 (µmol/L)</th>
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<tr>
<td>MCF-7</td>
<td>Breast</td>
<td>Parental</td>
<td>1.0</td>
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<tr>
<td>MTR-3</td>
<td>Breast</td>
<td>Tamoxifen</td>
<td>2.3</td>
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<tr>
<td>MCF-7/Adr</td>
<td>Breast</td>
<td>Adriamycin</td>
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Figure 2. 9-Br-nos induces apoptosis in MDA-MB-231 cells through the mitochondrial pathway. \textbf{A}, Western blot analysis of Bcl2 and BAX protein upon treatment of cells with 9-Br-nos for the indicated times. \(\beta\)-Actin was used as a loading control. \textbf{B}, 9-Br-nos disrupts mitochondrial transmembrane potential. MDA-MB-231 cells were treated with 9-Br-nos for 0, 24, 48, and 72 h. Cells were then incubated with 50 nM/L of dihydroxyanthracene iodide (DiOC6), and analyzed by flow cytometric analysis. \(x\) axis, the DiOC6 fluorescence intensity; \(y\) axis, the number of cells. Results are representative of three experiments. \textbf{C}, quantitation of time-dependent increase in the number of depolarized cells upon 9-Br-nos treatment.
cytosol has been shown to be a key event taking place in the apoptosome in the initiation and activation of the downstream caspase cascades involving caspase-3/caspase-7 for the execution of apoptosis (57, 59), which was investigated next.

**9-Br-nos Causes the Activation of Caspase-3 and PARP Cleavage.** Based on increased apoptosis and dissipation of mitochondrial membrane potential and cytochrome c release into the cytosol in 9-Br-nos–treated cells, our next aim was to examine the involvement of caspsases that play a major role in cleaving a variety of substrates. Thus, the activation of caspase-3, upon its cleavage by upstream proteases, is considered as a hallmark of the apoptotic process. Therefore, we monitored the active form of the cysteine protease using a small conserved modified peptide substrate which becomes luminogenic upon cleavage. Our results show that 9-Br-nos treatment caused a time-dependent activation of caspase-3 in MDA-MB-231 cells (Fig. 3B). We also investigated the levels of cleaved active subunits of procaspase (executioner caspase-3) by immunoblotting cell lysates following 9-Br-nos treatment for 0, 24, 48, and 72 hours. Our results show that 9-Br-nos caused a prominent increase in the activated caspase-3 following 72 hours of 9-Br-nos exposure (Fig. 3A). Next, we examined the effect of 9-Br-nos–induced caspase activation on PARP cleavage, which is one of the downstream substrates of the caspase cascade and is a reliable marker of apoptosis. Caspsases separate the NH2-terminal DNA-binding domain of PARP from its COOH-terminal catalytic domain (89 kDa) by their cysteine protease activity (60). As expected, we found a time-dependent increase in cleaved PARP after probing with cleaved PARP-specific antibody (Fig. 3A). Overall, these results suggested the activation of caspase-3 and PARP cleavage together with mitochondrial damage and cytochrome c release in 9-Br-nos–induced apoptotic death of MDA-MB-231 cells.

**Figure 3.** 9-Br-nos induces apoptosis in MDA-MB-231 cells as examined by Annexin V staining, TUNEL assay, caspase-3 activity, and cleavage of caspase-3 and PARP. A, representative immunoblots of cleaved caspase-3 and cleaved PARP along with the loading control, β-actin. B, quantitation of time-dependent increase in caspase-3 activity upon 9-Br-nos treatment. Cells were treated with 10 μmol/L of 9-Br-nos for 0, 12, 24, 48, and 72 h, and caspase-3 activity was analyzed using the luminogenic substrate Z-DEVD-aminoluciferin (*, P < 0.01). C, quantitative flow cytometry analysis of phosphatidylserine exposure in MDA-MB-231 cells treated with 10 μmol/L of 9-Br-nos for 0, 24, 48, and 72 h. Alexa-Fluor 488 conjugate of Annexin V was used along with PI to distinguish among three subpopulations: PI-negative and Alexa-Fluor 488–negative viable cells with intact membrane and preserved amino-phospholipid asymmetry (bottom left quadrant), PI-negative and Alexa-Fluor 488–positive early apoptotic cells with intact cellular membrane exposing phosphatidylserine (bottom right quadrant) and PI-positive and Alexa-Fluor 488–positive late apoptotic cells with compromised asymmetry and membrane permeability (top right quadrant). Representative results of three independent experiments. D, TUNEL analysis in cells incubated with 10 μmol/L 9-Br-nos for 0, 24, 48, and 72 h. After the indicated incubation periods, cells were processed for a flow cytometry–based terminal deoxynucleotidyl transferase–mediated BrdUTP reaction. The addition of BrdUTP to the terminal deoxynucleotidyl transferase reaction provides a means to label the DNA strand breaks and is detected by an Alexa-Fluor 488–labeled anti-bromodeoxyuridine antibody. DNA content was determined by the binding of DNA-specific dye, PI (x axis). Apoptotic cells were determined by the incorporation of bromodeoxyuridine at the 3′-OH ends of the fragmented DNA as measured by anti-bromodeoxyuridine Alexa-Fluor 488–labeled antibody on the y axis. The number of apoptotic cells is indicated by the number of Alexa-Fluor 488–positive cells of the total gated cells. The values presented on cytograms are the percentage of apoptotic cells (top) and normal cells (bottom). Data from a representative experiment of two experiments.
9-Br-nos Induces Loss of Plasma Membrane Asymmetry and the Appearance of TUNEL-Positive Cells

Biochemically, the early apoptotic process is characterized by loss of lipid asymmetry between the two leaflets of plasma membrane, consequently, phosphatidylserine translocates from the inner leaflet and is displayed on the outer leaflet (61). To quantitate the population of both the early and late apoptotic cells, we used Annexin V, a protein with high-affinity binding to phosphatidylserine, labeled to Alexa-Fluor 488 (green staining), along with an impermeant red DNA-binding dye, PI, in a flow cytometric analysis. Figure 3C shows the density plots of PI versus Annexin/Alexa-Fluor 488 fluorescence obtained from untreated control cells. The untreated cell cultures contained very few apoptotic cells (~0.3%) and as evident from Fig. 3C, cells treated with 9-Br-nos for 48 hours showed both early and late apoptotic cells (22.2% and 24.1%, respectively) suggesting continued initiation and execution of apoptosis.

The termination of apoptosis is characterized by changes in the cellular morphology, including membrane blebbing, formation of apoptotic bodies, and disruption of cytoskeleton, hypercondensation, and fragmentation of chromatin. We quantified the increase in the concentration of 3’-DNA ends due to fragmentation using a flow cytometry–based TUNEL assay. We showed that 9-Br-nos–treated MDA-MB-231 cells revealed ~73% TUNEL-positive cells (Fig. 3D) at 72 hours of exposure as compared with untreated control cells, suggesting extensive DNA cleavage.

9-Br-nos is Effective Against Hormone-Refractory Tumor Xenografts and Prolongs Animal Survival

We next asked if 9-Br-nos was effective against human tumors implanted in nude mice s.c. as xenografts of hormone-refractory MDA-MB-231 cells. When well-established xenografts were palpable with a tumor size of ~100 mm³, mice were randomized into vehicle control and treatment groups of eight animals each. The treatment group received a therapeutic dose of 9-Br-nos (300 mg/kg orally) by gavage. The control untreated group received the vehicle alone (water, pH 4.0). Oral administration of 9-Br-nos significantly reduced tumor volume in hormone-refractory xenografts (Fig. 4A). The reduction in tumor volume on days 16, 22, and 30 were ~45%, 59%, and 74%, respectively, for the 9-Br-nos–treated group compared with animals receiving only the vehicle solution (Fig. 4B). All animals in the control group had to be euthanized by day 30 post-inoculation due to tumor overburden (in compliance with the Institutional Animal Care and Use Committee guidelines). To evaluate the general health and systemic homeostasis of untreated and treated groups of tumor-bearing mice, we monitored the progression of body weights of these animals during the course of 9-Br-nos treatment. Our results indicate that 9-Br-nos–treated animals did not show any apparent weight loss compared with the control vehicle–treated animals during the course of treatment indicating no overall toxicity. 9-Br-nos–treated animals, however, showed normal weight gain during the treatment period (data not shown). Kaplan-Meier analysis revealed a significantly increased survival time with 50% 9-Br-nos–treated animals surviving until day 100 (~4-fold; from 26 to 100 days). The 75%
quartile survival was increased by ~3-fold (from 24 to 76 days; Fig. 4C). Surviving mice were followed for 120 days without any signs of recurrent tumors and were sacrificed.

**Tumor Reduction in Xenografts Is Also a Result of Potent Apoptosis**

We next asked if 9-Br-nos regressed the xenografted tumors by triggering apoptosis. Consistent with our results from the *in vitro* caspase-3 activation and TUNEL assays, we observed an immunohistochemically widespread expression of cleaved caspase-3 (Fig. 5V), cleaved PARP (Fig. 5X), and TUNEL-positive cells (Fig. 5T) in the remaining small regressed tumor sections of 9-Br-nos treatment groups. We conclude that the regression of tumor xenografts is a result of 9-Br-nos–induced apoptosis.

**EM011 Therapy Does Not Cause Histologic and Hematologic Toxicity**

Chemotherapy still remains the standard treatment of choice for cancer, although the benefits are modest and toxicity is substantial. Because anticancer drugs are cytotoxic for normal as well as neoplastic cells, the range of unwanted effects that accompanies their use is broad. Many of the toxic effects are potentially life-threatening or seriously debilitating. Therefore, a safe, well-tolerated regimen is highly desirable and has been a goal of clinical research. To determine whether 9-Br-nos treatment results in toxicities to normal tissues, we examined tissue sections of the liver, kidney, spleen, brain, heart, lung, gut, and the sciatic nerve of tumor-bearing mice by H&E staining at the end points of control and 9-Br-nos treatment groups (30 and 120 days, respectively; Fig. 5A–P). Our results show that 9-Br-nos did not cause any detectable pathologic abnormalities in the mice. There was a complete absence of any metastatic lesions in these organs in 9-Br-nos–treated groups.

We also did a complete blood count analysis and found that 9-Br-nos treatment did not alter RBC, WBC counts, hemoglobin concentrations, and hematocrit in animals bearing hormone-refractory tumor xenografts (Fig. 6D). In addition, we evaluated the organ-associated toxicity and our results show that the levels of alanine aminotransferase, aspartate aminotransferase, and blood urea nitrogen

**Figure 5.** 9-Br-nos (300 mg/kg) administered daily does not cause any detectable pathologic abnormalities in normal tissues. A to P, H&E staining of paraffin-embedded 5-µm-thick tissue sections of the liver, kidney, spleen, brain, heart, lung, gut, and sciatic nerve from untreated and 9-Br-nos–treated groups of mice (magnification, ×200). These tissues were indistinguishable between treated and untreated control groups. Q and R, tumor micrographs from untreated and 9-Br-nos–treated mice, respectively. The 9-Br-nos–treated tumor microsections reveal large areas of tumor cell death, consistent with the therapeutic effects of 9-Br-nos. Microsections from untreated tumor tissue show sheets of tumor cells with high-grade pleomorphic nuclei with minimal cell death. Massive angiolymphatic invasion typical of aggressive tumors was also observed in the untreated tumor sections, but not in the treated mice. S to X, immunohistochemical staining of paraffin-embedded tumor sections from the control group (S, U, and W) and the 9-Br-nos treatment group (T, V, and X) for TUNEL, cleaved caspase-3, and cleaved PARP, respectively, as indicators of apoptosis. T, numerous TUNEL-positive cells (seen as apoptotic brown nuclei) as compared with untreated control (S). V and X, extensive cleaved caspase-3 and cleaved PARP staining, respectively, as compared with the control group (U and W).
were comparable between the 9-Br-nos–treated and the untreated mice bearing tumor xenografts (Fig. 6E). Tumor-suppressive doses of 9-Br-nos did not cause alterations in the B220+, CD4+, and CD8+ cell counts as compared with the untreated controls (Fig. 6A–C).

Although microtubule agents such as the taxanes and Vincas have been effective against hormone-resistant disease, they eventually present resistance. This is primarily due to three major mechanisms that give rise to drug resistance to antimicrotubule agents (see for example, ref. 62). Besides, the overexpression of efflux pumps, tubulin mutations also tend to account for resistance to antimicrotubule drug therapy (37). In addition, tubulin is a multi-gene family, and taken together, α-tubulin and β-tubulin subunits are encoded by at least 12 different genes in humans. Different tumor types express these isotypes of tubulin to different extents. Microtubule drugs interact differently to different isotypes. Therefore, the isotypic composition of tubulin determines the therapeutic outcome of a particular tumor (63–65). We believe that the invention of 9-Br-nos as a nontoxic anticancer agent, particularly for hormone-resistant breast tumors, is a major advancement.

We thus conclude that 9-Br-nos clearly inhibits the cellular proliferation of both hormone-insensitive and hormone-sensitive breast cancer cells. 9-Br-nos is also active against the Adriamycin- and tamoxifen-resistant breast cancer phenotypes. Furthermore, it significantly inhibits tumor growth in hormone-resistant human breast tumor xenografts by inducing extensive apoptosis and prolonging longevity. Although the founding compound, noscapine, is already in clinical trials, 9-Br-nos represents an additional edge over noscapine because of its higher potency, by at least an order of magnitude, without compromising the nontoxic profile of noscapine, including the unaltered immunosurveillance system. Therefore, our preclinical data qualifies 9-Br-noscapine to undergo clinical trials.

Acknowledgments
We thank members of the Joshi laboratory for discussions.

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Figure 6. 9-Br-nos does not have an obvious hematologic toxicity. A to C, flow cytometry–based immunophenotyping assays showing no alterations in the B220+, CD4+, and CD8+ cell counts from untreated and 9-Br-nos–treated groups of mice. D, complete blood count analysis of tumor-bearing 9-Br-nos–treated mice compared with untreated mice. No significant difference could be detected between the two groups in the WBC count (WBC), RBC count (RBC), hemoglobin concentration (HB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean platelet volume (MPV), and red cell distribution width (RDW). E, the organ-associated toxicity was also assessed for both groups and the levels of alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, and creatinine were comparable for the treated and the untreated groups.
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