Dinitroazetidines Are a Novel Class of Anticancer Agents and Hypoxia-Activated Radiation Sensitizers Developed from Highly Energetic Materials

Shoucheng Ning¹, Mark Bednarski², Bryan Oronsky³, Jan Scicinski³, Gordon Saul³, and Susan J. Knox¹

Abstract

In an effort to develop cancer therapies that maximize cytotoxicity, while minimizing unwanted side effects, we studied a series of novel compounds based on the highly energetic heterocyclic scaffold, dinitroazetidine. In this study, we report the preclinical validation of 1-bromoacetyl-3,3-dinitroazetidine (ABDNAZ), a representative lead compound currently in a phase I clinical trial in patients with cancer. In tumor cell culture, ABDNAZ generated reactive free radicals in a concentration- and time-dependent manner, modulating intracellular redox status and triggering apoptosis. When administered to mice as a single agent, ABDNAZ exhibited greater cytotoxicity than cisplatin or tirapazamine under hypoxic conditions. However, compared with cisplatin, ABDNAZ was better tolerated at submaximal doses, yielding significant tumor growth inhibition in the absence of systemic toxicity. Similarly, when combined with radiation, ABDNAZ accentuated antitumor efficacy along with the therapeutic index. Toxicity studies indicated that ABDNAZ was not myelosuppressive and no dose-limiting toxicity was apparent following daily administration for 14 days. Taken together, our findings offer preclinical proof-of-concept for ABDNAZ as a promising new anticancer agent with a favorable toxicity profile, either as a chemotherapeutic agent or a radiosensitizer.

Introduction

Cancer is a leading cause of death worldwide. An estimated 1.4 million people will be diagnosed with cancer each year in the United States and the number of predicted cancer deaths will be more than half a million (1). Radiotherapy and chemotherapy are two main modalities of cancer treatment but often fail to cure many patients. The key factors limiting the therapeutic efficacy are the dose-limiting toxicities on normal tissues and the existence of hypoxic tumor cells (2, 3). Chemotherapy has been used with radiotherapy to treat the radioresistant tumors but is often associated with significantly increased toxicity (4). The identification of agents that specifically target and radiosensitize hypoxic tumor cells is a promising area of investigation. A number of different radiosensitizers have been developed, such as hyperbaric oxygen (5), electron affinity nitroimidazole compounds (6, 7), thiol depletion agents (8–10), metal tetrahexylin (11), and bioreductive compounds (12). However, the normal tissue toxicity and the formation of toxic byproducts have been the significant problems for clinical use with these radiosensitization approaches (13). For these reasons, it is important to develop new therapeutic agents with improved efficacy, minimal toxicity to normal tissues, and an enhanced therapeutic window.

Energetic materials are chemical compounds that contain significant quantities of potential energy that is released upon activation and breakage of chemical bonds. These materials can be designed for controllable storage and release of chemical energy under specific conditions (14). In an effort to develop unique targeted cancer therapies, which maximize cytotoxicity to cancer cells, while minimizing unwanted side effects to normal tissue, we have studied a series of novel compounds based on the highly energetic heterocyclic dinitroazetidine scaffold, which can be selectively activated in tumors in a controlled manner. Our research has resulted in the selection of a lead compound, 1-bromoacetyl-3,3-dinitroazetidine (ABDNAZ), for clinical development both as a stand-alone chemotherapeutic agent and as a radiation sensitizer. Activation of ABDNAZ resulted in selective targeting of hypoxic tumors and enhancement of the effect of radiation by locally releasing highly energetic free radicals in tumors as well as by increasing tumor blood flow. Our data show that ABDNAZ is a promising novel anticancer drug with a very favorable toxicity profile that is efficacious in preclinical tumor models as both a stand-alone chemotherapeutic agent and as a radiosensitizer.

Materials and Methods

Materials

ABDNAZ used in these studies was obtained from ATK Aerospace Systems. ABDNAZ is a cyclic nitro compound with...
The cellular structure of ABDNAZ has regularly been confirmed in vitro and cell morphology in vitro and in vivo. All experiments were carried out on exponentially growing cells with cell population doubling times of approximately 20 to 36 hours.

**Cell lines**

Eleven human cancer cell lines and a murine squamous cell carcinoma (SCC) cell line (Table 1) were grown and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen) supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 100 µg/mL streptomycin in a 37°C humidified incubator with a mixture of 95% air and 5% CO₂. The identity of cells has regularly been confirmed throughout the course of the studies by observation of the growth pattern and cell morphology in vitro and in vivo. All experiments were carried out on exponentially growing cells with cell population doubling times of approximately 20 to 36 hours.

**In vitro cytotoxicity assay**

Cell counting kit-8 (CCK-8), a modified MTT assay with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (Dojindo Molecular Technologies), was used to measure the cytotoxicity of ABDNAZ. Cells were inoculated into 96-well plates and incubated overnight. Freshly prepared solutions of ABDNAZ were added in a final concentration ranging from 0.1 to 1,000 µmol/L in 200 µL of loading volume. Three days later, plates were washed with PBS and refilled with 100 µL of phenol red–free growth medium. CCK-8 solution was added and after 2-hour incubation at 37°C, the optical absorbance of wells was measured at a wavelength of 450 nm with a microplate reader ( Molecular Devices). Cell viability was calculated as the percentage of the optical density of the treated cells to that of untreated control cells.

**In vitro clonogenic assay**

Survival curves were generated with an in vitro clonogenic assay as previously described (17). Briefly, cells were inoculated in Petri dishes and irradiated in the presence or absence of ABDNAZ with a 137Cs source at a dose rate of 300 cGy/min. After irradiation, cells were incubated for 10 to 14 days and stained with crystal violet for colony count.

For in vitro clonogenic assays after exposure to hypoxia, cells were inoculated in 60-mm glass dishes with medium containing freshly prepared ABDNAZ and loaded into gas-tight aluminum gassing chambers on a shaking table. Hypoxia was achieved in the chambers through a series of 5 alternate evacuations followed by gassing with 95% nitrogen and 5% CO₂ to 20 psi at 37°C. After the last round of gassing, the chambers were incubated on the shaking table at 37°C for 2 to 4 hours and then irradiated. Cells were rinsed, trypsinized, counted, and plated in Petri dishes in triplicate for colony formation as described earlier.

**Measurement of intracellular reactive oxygen species**

Intracellular reactive oxygen species (ROS) was measured with a fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Invitrogen Molecular Probes) as previously described (18). Briefly, cells were grown in a black 96-well plate overnight, incubated with DCFH-DA probe for 1 hour, and treated with ABDNAZ and/or irradiation. Immediately following addition of ABDNAZ or radiation, the green fluorescence was observed under a fluorescence microscope (Leica Microsystems) and quantitated with a spectrofluorometer ( Molecular Devices). The green fluorescence was measured again at 10 minutes, 30 minutes, and 1 to 24 hours after treatment.

**DNA damage assay**

DNA damage was assayed by immunocytochemical staining of γH2AX. Cells were grown overnight on glass coverslips in a 60-mm dish. After exposure to ABDNAZ, cells were washed with PBS, fixed in ice-cold methanol for 10 minutes at −20°C, permeabilized with 0.2% Triton X-100 for 10 minutes, and blocked with 3% bovine serum albumin for 60 minutes. Cells were incubated with a primary rabbit anti-human phospho-histone γH2AX antibody (Cell Signaling Technology) for 60 minutes and then incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Santa Cruz Biotechnology) for 60 minutes in dark. After washing with PBS, coverslips were mounted on slides with a drop of mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI). Slides were visualized and photographed by a Leica fluorescence microscope.

---

**Table 1.** IC₅₀ of AB DNAZ against cancer cell lines in vitro

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>IC₅₀, µmol/L</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCC VII</td>
<td>SCC</td>
<td>1.8</td>
<td>0.3</td>
</tr>
<tr>
<td>22B</td>
<td>Oral SCC</td>
<td>2.3</td>
<td>0.5</td>
</tr>
<tr>
<td>PANC-1</td>
<td>Pancreatic carcinoma</td>
<td>2.3</td>
<td>0.7</td>
</tr>
<tr>
<td>M21</td>
<td>Melanoma</td>
<td>2.6</td>
<td>0.5</td>
</tr>
<tr>
<td>U87</td>
<td>Glioblastoma</td>
<td>2.7</td>
<td>0.6</td>
</tr>
<tr>
<td>RKO</td>
<td>Colon carcinoma</td>
<td>3.0</td>
<td>0.4</td>
</tr>
<tr>
<td>HT29</td>
<td>Colorectal cancer</td>
<td>3.4</td>
<td>0.3</td>
</tr>
<tr>
<td>SNB75</td>
<td>Glioblastoma</td>
<td>3.8</td>
<td>1.4</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Breast adenocarcinoma</td>
<td>4.0</td>
<td>0.5</td>
</tr>
<tr>
<td>A498</td>
<td>Renal cell carcinoma</td>
<td>4.9</td>
<td>1.3</td>
</tr>
<tr>
<td>IMR32</td>
<td>Brain neuroblastoma</td>
<td>5.1</td>
<td></td>
</tr>
<tr>
<td>AG49</td>
<td>Non–small cell lung carcinoma</td>
<td>6.0</td>
<td>1.8</td>
</tr>
</tbody>
</table>

NOTE: The SCC VII is a murine cancer cell line. All others are of human origin.
Apoptosis analysis
Apoptotic cell death was quantitated by measuring the number of subdiploid (sub-G1) cells stained with propidium iodide (PI) as described previously (18). Briefly, ABDNAZ-treated and untreated control cells were collected and fixed with ethanol, treated with RNase A, and stained with PI. The DNA content was analyzed with a FACScan flow cytometer (Becton, Dickinson and Company). The percentage of cells in the sub-G1 phase was calculated as previously described.

Tumor model and therapy
Male C3H mice, 7 to 8 weeks old and 20 to 25 grams in body weight, were purchased from Charles River Laboratories. Mice were normally bred and maintained under specific pathogen-free conditions and sterilized food and water were available ad libitum. Mice were injected s.c. in the right flank with 5 × 10^5 SCC VII tumor cells in 0.05 mL Hank solution. When tumors reached an average size of 150 mm^3 (100–300 mm^3), mice were randomly assigned to the treatment groups. ABDNAZ was injected i.p. or i.v. at doses as specified in each experiment. For irradiation, the unanesthetized tumor-bearing mice were placed in individual lead boxes with tumors protruding through a cutout window at the rear of each box. Radiation was delivered by a Philips RT-250 200 kVp X-ray unit (12.5 mA; half value layer, 1.0 mm Cu) at a dose rate of 140 Gy/min. The length and width of the tumors were measured with calipers before treatment, and 3 times a week thereafter until the tumor volume reached at least 4 times (4×) the pretreatment volume. The tumor volume was calculated with the formula: tumor volume = π/6 × length × width^2. The tumor volume quadrupling time (TVQT) was determined by a best-fit regression analysis. The tumor growth delay (TGD) time is the difference between the TVQT of treated tumors compared with that of untreated control tumors. Both the TVQT and TGD time were calculated for each individual animal and then averaged for each group. The data are presented as percentage of the pretreatment volume on day 0. Body weight of animals was measured 3 times a week. The animal experiments described herein were approved by the Stanford University (Stanford, CA) Administrative Panel for Laboratory Animal Care.

Microbubble-enhanced ultrasound image
Blood perfusion and blood volume in tumors were assessed by microbubble-enhanced ultrasound imaging as previously reported (19). Briefly, tumor-bearing mice were treated with ABDNAZ and radiation and then imaged at various time points on a Vevo770 system (VisualSonics Inc.). Mice were anesthetized with a 3% isoflurane oxygen and imaged with a 6 × 6 mm^2 view field. A baseline image was acquired before microbubble injection. The contrast-enhanced image was acquired 5 seconds after a bolus injection of the nontargeted microbubble contrast agent (1.0 × 10^7 microbubbles/0.1 mL) via a tail vein catheter. Images were acquired at a frame rate of 18 frames per second for a total of 800 frames. The image data were analyzed with Vevo770 Contrast Mode software by comparing the contrast cine loop with the baseline cine loop to generate a contrast overlay to identify the differences in image intensity between these 2 loops.

Intestinal crypt stem cell assay
The detailed procedure for intestinal crypt cell survival following irradiation has been described elsewhere (20). Briefly, one group of mice was irradiated with total body irradiation (TBI). Another group of mice was injected i.p. with 10 mg/kg ABDNAZ 10 minutes before TBI. Three days after radiation, the duodenum, jejunum, and ileum were removed, fixed, and stained with hematoxylin and eosin (H&E). Surviving crypts (>10 cells) for each cross-section were counted under microscopy.

Statistics
Data were statistically analyzed with a 2-tailed Student t test. The dose-modifying factor (DMF) attributable to the radiosensitization effect of ABDNAZ was calculated by dividing the radiation dose without ABDNAZ by the radiation dose required to produce the same survival in the presence of ABDNAZ (21).

Results

ABDNAZ is a potent chemotherapeutic agent
The cytotoxic antiproliferative effects of ABDNAZ were studied on a panel of 12 cancer cell lines in vitro with a CCK-8 cell counting kit. The IC50 values (concentration required for 50% inhibition of cell growth) ranged from 1.8 to 6.0 μmol/L (Table 1). Comparison with cisplatin (CDDP) showed that ABDNAZ had similar activity to CDDP against 4 human cancer cell lines and SCC VII cells, with IC50 values of 2.6 ± 1.6 μmol/L and 4.4 ± 2.2 μmol/L for ABDNAZ and CDDP (P > 0.05), respectively (Fig. 2A).

Cytotoxic activity of ABDNAZ is enhanced under hypoxia
The cytotoxicity of ABDNAZ under hypoxic condition compared with normal aerobic condition was examined in SCC VII cells with a clonogenic assay. Cells were exposed to ABDNAZ for 2 hours in a hypoxia chamber or in a cell culture incubator. In addition, tirapazamine, a hypoxic cytotoxin (22), was included in the study as a positive control. As shown in Fig. 2B, SCC VII cells were more sensitive to ABDNAZ under hypoxia than under normoxia. The IC50 values were 0.14 and 0.66 μmol/L for cells under hypoxia and normoxia, respectively, representing a 4.7-fold increase in cytotoxicity under hypoxic condition. In a separate experiment, ABDNAZ was found to be even more potent than tirapazamine under hypoxia (Fig. 2C). The IC50 values for ABDNAZ and tirapazamine were 0.14 and 0.96 μmol/L (P < 0.01), respectively.

ABDNAZ increases intracellular ROS generation, γH2AX expression, and apoptosis
To test the initial hypothesis that ABDNAZ would generate ROS that would have downstream cytotoxic effects on tumor cells, levels of intracellular ROS, DNA damage (γH2AX expression), and apoptosis of tumor cells following exposure to ABDNAZ were measured. Data showed that ABDNAZ induced intracellular ROS generation in a dose- and time-dependent manner in HT29 and SCC VII cell lines (Fig. 3A). The levels of intracellular ROS increased and peaked at 6 hours after
Figure 2. Cytotoxic activity of ABDNAZ in vitro. A, concentration–survival curves of 5 cancer cell lines treated with either ABDNAZ or CDDP. There were no statistically significant differences in IC50 values between ABDNAZ and CDDP for each cell line. B and C, survival curves of SCC VII cells treated with ABDNAZ (B) under normoxia or hypoxia or either ABDNAZ or tirapazamine (TPZ; C) under hypoxia. Data are mean ± SD from 3 independent experiments.

Figure 3. ABDNAZ induced ROS generation, DNA damage, and apoptosis of tumor cells. A, intracellular ROS in HT29 and SCC VII cells. O.D., optical density. B, γH2AX expression in HT29 cells. Left, the γH2AX fluorescence in HT29 cell treated with 0 to 10 μmol/L ABDNAZ for 2 hours. Right, the quantification of γH2AX-positive cells as percentage of total cells counted in a time course. *, P < 0.01 versus 0 hour. C, apoptosis for SCC VII, HL60, and Daudi cells after exposure to ABDNAZ for up to 72 hours. D, apoptosis of HL60 and Daudi cells treated with ABDNAZ in the presence or absence of NAC (10 mmol/L) or BSO (200 μmol/L). *, P < 0.01 versus ABDNAZ alone.
addition of ABDNAZ in HT29 cells and at 2 hours in SCC VII cells. For HT29 cells, the fluorescence signals increased from a baseline of 1.3 ± 2.1 measured before ABDNAZ treatment to 6.5 ± 1.0, 27.5 ± 3.0, and 69.2 ± 17.5 at 6 hours after exposure to 1, 10, and 100 μmol/L of ABDNAZ, respectively. For SCC VII cells, the fluorescence signals increased from 8.7 ± 2.4 at baseline to 19.1 ± 7.6, 63.2 ± 25.7, and 160.6 ± 29.8 at 6 hours for 1, 10, and 100 μmol/L ABDNAZ, respectively.

ABDNAZ-induced γH2AX expression in HT29 cells and SCC VII cells was found to increase in a dose- and time-dependent manner (Fig. 3B). The γH2AX-positive HT29 cells increased from 0.5% ± 0.4% before ABDNAZ treatment to 49.4% ± 9.5% after 2 hours of exposure to 10 μmol/L ABDNAZ, representing a 99-fold increase in unrepaired DNA damage. Corresponding induction of apoptosis also increased over 72 hours (Fig. 3C). At 72 hours, 5 μmol/L ABDNAZ induced 60% apoptosis in SCC VII cells and 96% in promyelocytic leukemia HL60 cells (P < 0.01 vs. baseline). At the same time point, ABDNAZ at 4 μmol/L induced 45% apoptosis in B-cell lymphoma Daudi cells. ABDNAZ at 2 μmol/L also induced a significant level of apoptosis compared with the baseline (Fig. 3C).

To examine whether ABDNAZ-induced apoptosis was due to the generation of ROS, we treated HL60 cells and Daudi cells with a glutathione (GSH) precursor, N-acetylcysteine (NAC), and a GSH synthesis inhibitor, buthionine-(S,R)-sulfoximine (BSO), and measured the level of apoptosis (Fig. 3D). Pretreatment with NAC was found to completely inhibit ABDNAZ-induced apoptosis. The level of apoptosis decreased from 21% after ABDNAZ exposure to 1% baseline in HL60 cells and from 11% for ABDNAZ to 5% of baseline in Daudi cells. In contrast, pretreatment with BSO increased ABDNAZ-induced apoptosis from 21% to 33% in HL60 cells and from 11% to 25% in Daudi cells, suggesting that intracellular redox status and ROS generation impact ABDNAZ-induced apoptotic cell death.

Next, we treated tumor-bearing mice with one i.v. dose of 10 mg/kg ABDNAZ with or without coadministration of one dose of 500 or 1,000 mg/kg NAC. NAC alone had a minor effect on tumor growth (P = 0.3 vs. control). When given in combination with ABDNAZ, NAC slightly reduced the ABDNAZ-induced TGD time of 1.7 ± 1.4 days to 1.3 ± 0.3 days and 1.1 ± 0.5 days for 500 and 1,000 mg/kg NAC (P > 0.05, n = 6–8), respectively. Although these differences in TGD time were small, these data do suggest that NAC decreased the cytotoxic effect of ABDNAZ in vivo (Fig. 4C).

**ABDNAZ inhibits tumor growth in vivo**

The in vivo antitumor effect of ABDNAZ was studied in mice bearing SCC VII tumors. This is an aggressive tumor with a volume doubling time of approximately 2 days. ABDNAZ was prepared as a solution in 5% dimethyl sulfoxide (DMSO)/water and administered i.p. with either 5 mg/kg body weight daily for 5 days or 10 mg/kg every other day for 3 doses or 15 mg/kg 2 doses in the first week. All 3 ABDNAZ regimens inhibited tumor growth and resulted in a significant increase in TGD time (1.8–4.5 days, P < 0.01, compared with untreated control tumors; Fig. 4A). ABDNAZ at 15 mg/kg for 2 doses total was more effective than other dose regimens tested (P < 0.01). None of the ABDNAZ regimens caused a significant decrease in animal body weight when compared with untreated control mice (Supplementary Fig. S1A).

Next, we studied the antitumor activity of oral administration of ABDNAZ. Tumor-bearing mice were orally gavaged with ABDNAZ at doses of either 12 mg/kg for 5 days or 20 mg/kg every other day for 3 doses or one dose of 60 mg/kg. All 3 dose regimens delayed tumor growth (P < 0.01 vs. control) and did not cause significant loss of body weight (Supplementary Fig. S1B).

We also compared the antitumor efficacy of ABDNAZ and cisplatin. Mice with SCC VII tumors were injected i.p. with a single dose of either 15 mg/kg ABDNAZ or 10 mg/kg CDDP (MTD at 12 mg/kg). As shown in Fig. 4B, ABDNAZ was as potent as CDDP at inhibiting tumor growth, and produced a TGD time of 4.5 ± 1.1 days for ABDNAZ compared with 4.3 ± 0.9 days for CDDP (P = 0.7). In addition, observations of the animals during the study suggested that the ABDNAZ-treated mice appeared healthier than the cisplatin-treated mice.

Taken together, our data showed that ABDNAZ is a potential anticancer agent without obvious systemic toxicities in terms of body weight loss.
ABDNAZ is a potent radiation sensitizer

Because ABDNAZ is activated both by hypoxia and ionizing irradiation (15), we hypothesized that ABDNAZ could be used in combination with radiation therapy as a radiosensitizer to selectively enhance the radiosensitivity of tumor cells, especially in hypoxia. We initially studied the effect of ABDNAZ on the radiation response of HT29 cells and SCC VII cells in vitro under normal conditions. ABDNAZ alone reduced survival by 50% at 0.1 μmol/L, 80% at 1 μmol/L, and 99% at 2 μmol/L in both cell lines. When combined with radiation, treatment with ABDNAZ shifted the radiation survival curves downward (Fig. 5A). The radiation survival at 2 Gy (SF2) was decreased from 72% to 12% in HT29 cells and from 89% to 1.4% in SCC VII cells. At 10 Gy, ABDNAZ further decreased the radiation survival from 1.3% to 0.07% for HT29 cells and from 3.3% to 0.03% in SCC VII cells, respectively. The radiation DMFs of ABDNAZ were 1.7 and 1.6 at 10% survival for HT29 cells and SCC VII cells, respectively.

We next studied the effect of ABDNAZ on radiation survival under hypoxia. SCC VII cells were preexposed to 1 μmol/L ABDNAZ for 2 hours in a hypoxia chamber and then irradiated. As expected, ABDNAZ increased the radiation sensitivity of radioresistant hypoxic cells and reduced their radiation survival to levels close to that observed under normoxia (Fig. 5B). Meanwhile, the radiosensitivity of normoxic cells was also enhanced by ABDNAZ, albeit to a lesser degree than observed under hypoxia. The DMFs of ABDNAZ at 10% survival were 1.6 and 1.9 for normoxia and hypoxia, respectively, suggesting that ABDNAZ more preferentially radiosensitized SCC VII tumor cells under hypoxia.

The level of intracellular ROS in HT29 cells and SCC VII cells was also increased in a dose-dependent manner following exposure to ABDNAZ and radiation (Fig. 5C). ABDNAZ (1–100 μmol/L) alone and radiation (2–10 Gy) alone induced ROS generation in these cells. When used in combination, the levels of ROS were significantly higher than that observed with either ABDNAZ alone or radiation alone (P < 0.01).

We then studied the in vivo therapeutic efficacy of combining ABDNAZ with radiotherapy. Tumor-bearing mice were treated with either 5 mg/kg ABDNAZ i.p. daily for 5 days, or local tumor radiation of 250 cGy daily for 5 days, or both ABDNAZ and radiation. Results showed that either radiation alone or ABDNAZ alone inhibited tumor growth and produced TGD times of 3.2 ± 1.8 days and 1.8 ± 0.6 days, respectively (P < 0.01, compared with untreated control; Fig. 6A). The combination treatment of radiation and ABDNAZ resulted in a TGD time of 7.0 ± 2.1 days (P < 0.01 vs. ABDNAZ or radiation alone). We also studied the effect of using higher daily doses of radiation (400 cGy) and ABDNAZ (6 mg/kg; Fig. 6B). Again, ABDNAZ was able to enhance the therapeutic efficacy of radiotherapy and prolong the TGD time from 7.9 ± 2.4 days for radiation alone to 13.9 ± 1.9 days for combination therapy (P < 0.01 vs. ABDNAZ alone or radiation alone). All of the combination regimens did not cause a significant decrease in animal body weight compared with untreated control mice or radiation-treated mice (Supplementary Fig. S2).

To optimize the treatment regimen, we studied the sequence and timing effects of administration of ABDNAZ with radiation. Figure 6C graphically shows the effect of sequence and timing between administration of ABDNAZ (10 mg/kg) and radiation (7 Gy) on TGD. Results showed that the synergistic antitumor effect of combining ABDNAZ with radiation was sequence- and time-dependent. The maximum TGD occurred when ABDNAZ was given 0 to 2 hours before radiation, with 4x TGD times of 4.5–4.0 days for ABDNAZ given 0 to 2 hours before radiation compared with 2.8 to 3.3 days for ABDNAZ given at 2 or 24 hours after radiation (P < 0.05).

Because the metabolism of ABDNAZ involves the loss of nitro groups leading to the formation of nitric oxide (NO; ref. 23), experiments were carried out to determine whether the observed radiosensitization effect of ABDNAZ could be due, in part, to changes in tumor blood flow arising from the effect of nitric oxide on blood vessels in tumors. We therefore measured the blood perfusion rate and blood volume in tumors before and after treatment with ABDNAZ by microbubble-enhanced ultrasound imaging. Results showed that treatment with ABDNAZ either alone (Fig. 7A) or in combination with radiation (Fig. 7B) dramatically increased the rates of blood perfusion and blood volume of SCC VII tumors in a dose- and time-dependent manner. The microbubble signals peaked 6 hours after ABDNAZ administration.
after administration of ABDNAZ, maintained at the highest level for approximately 48 hours, and then gradually decreased to its base level (Fig. 7C).

Toxicity study
Detailed preclinical toxicity studies of ABDNAZ in mice, rats, and dogs will be reported elsewhere. Briefly, we found that the maximum tolerated dose in mice was 10 to 12 mg/kg for a single-dose i.v. bolus injection and 15 to 20 mg/kg for a single-dose i.p. injection. A daily i.p. dose of 6 mg/kg for 14 days was well-tolerated and there were no unexpected deaths, and no obvious change in the general appearance, skin reaction, or daily activity of mice. Cumulative toxicity was not observed. There were no signs of myelosuppression following a 15 mg/kg dose or 6 mg/kg daily for 14 days. All hematologic parameters and chemistry panel analyses were in the reference range. Also, ABDNAZ did not increase radiation-induced myelosuppression (Supplementary Fig. S3).

Gastrointestinal toxicity caused by radiation limits the therapeutic dose that can be used for treating abdominal and pelvic tumors with radiotherapy. Therefore, we conducted experiments to study the effect of ABDNAZ on intestinal crypt survival following a TBI. Results showed that administration of ABDNAZ did not increase the TBI radiation–induced cell killing of crypt stem cells in the duodenum, jejunum, and ileum. Rather, it actually protected the crypt cells against TBI and increased the number of viable crypt cells (Supplementary Fig. S4).

Discussion
ABDNAZ is a small-molecule compound that has been modified from energetic materials that originated in the defense and aerospace industry. This compound contains a unique high energetic organic nitro functional group, a gem dinitroazetidine that has not been used to date for medical and...
pharmaceutical applications. In an aerospace setting, compounds containing this energetic functionality, such as trinitrotetroazetidine, are designed to fragment explosively (14, 24). Modification of this structure resulted in ABDNAZ, a nonexplosive, selectively activated compound with the potential for use in the treatment of cancer (15).

The preclinical in vitro and in vivo studies reported herein show that ABDNAZ administered as a single agent is cytotoxic for a broad spectrum of human cancer cell lines in vitro and has efficacy for inhibiting growth of established tumors in vivo. The IC<sub>50</sub> dose of ABDNAZ is similar to that of cisplatin under normoxia. However, ABDNAZ is more cytotoxic under hypoxic conditions to tumor cells than both cisplatin and the bioreductive cytotoxin tirapazamine. ABDNAZ efficiently radiosensitizes tumor cells under both normoxic and hypoxic conditions in vitro. Studies in tumor-bearing mice showed that ABDNAZ effectively inhibits tumor growth without causing systemic toxicity. When combined with conventional radiation therapy, ABDNAZ enhanced the radiation response of tumors and significantly improved the therapeutic index. ABDNAZ can be administered daily, every other day, or weekly by i.v. or i.p. injection, or oral administration. ABDNAZ is well tolerated, is not myelosuppressive, has no effect on hepatic or renal function, and has no apparent dose-limiting toxicity when administered daily for 14 days. Our data show that ABDNAZ is a promising novel anticancer drug with a very favorable toxicity profile that is efficacious in preclinical tumor models as a stand-alone chemotherapeutical agent and as a radiosensitizer.

The mechanism of action of ABDNAZ has not been fully elucidated and is the subject of ongoing studies. Our current results reported here show that ABDNAZ induced intracellular ROS generation and increased the expression of the DNA damage marker γH2AX in tumor cells in a concentration- and time-dependent manner. Modulation of intracellular redox status with the GSH precursor NAC or the GSH synthesis inhibitor BSO significantly affected the magnitude of ABDNAZ-induced DNA damage and apoptosis, suggesting that the ROS generation is one of the underlying mechanisms by which ABDNAZ exhibits its cytotoxic antiproliferative effect on tumor cells. Our data also show that combined use of ABDNAZ and radiation augmented the generation of ROS and apoptosis. Because ionizing irradiation produces free radicals mainly via indirectly ionizing reactions with water molecules, combined use of ABDNAZ and radiation could act synergistically through different mechanisms and pathways to produce enhanced levels of cytotoxic free radicals.

Our preliminary data suggest that ABDNAZ also results in nitric oxide release via decomposition of conjugates formed in vivo, which has effects on tumors as well. We have recently reported that after i.v. administration, ABDNAZ binds rapidly to hemoglobin and GSH, decomposing ultimately to soluble metabolites in the blood (23). The major soluble metabolites are the GSH and cysteine adducts. In addition, species derived from ABDNAZ that had released one or both nitro groups were detected by liquid chromatography/mass spectrometry or by proteomic studies, suggesting that ABDNAZ itself might act as an NO donor. These findings well explain the results from our tumor-imaging studies that ABDNAZ markedly increased regional blood perfusion and blood volume in tumors. NO is an active cellular signaling molecule and well known as the endothelium-derived relaxing factor (EDRF). The blood vessel endothelium uses NO to signal the surrounding smooth muscle to relax, thus resulting in vasodilation effect and increasing blood flow in tissues. Taken together, these data support the initial hypothesis that ABDNAZ releases NO radicals that affect tumor blood flow and would be expected to increase the intratumoral concentration of ABDNAZ or its metabolites and thereby enhance its therapeutic efficacy. The effect of ABDNAZ on blood perfusion could theoretically affect tumor oxygenation and will be investigated in the future.

Normal tissue toxicity and the presence of hypoxic cells in human tumors are the principal reasons for the frequent failure of radiotherapy to cure or control cancer. An ideal radiosensitizer should enhance the effect of radiation on tumors and sensitize the hypoxic tumor cells without sensitizing normal tissues to radiation or adding other toxicity to the treatment regimen. Such an agent has yet to be identified and to date there are no U.S. Food and Drug Administration-approved radiosensitizers for clinical use. Results obtained with the radiosensitizing compounds in clinical trials have been discouraging in terms of their efficacy and toxicity profiles (25). Bioreductive agents, tirapazamine in particular, have shown great promise preclinically and in early clinical trials, results from phase III trials have been disappointing and conflicting, in part, due to suboptimal tumor penetration and activation outside of the targeted hypoxic regions of tumors (12, 26). Several chemotherapeutic agents have been used to enhance the effect of radiation therapy (27, 28). However, these drugs have overlapping toxicities and can significantly augment radiation effects in normal tissues. In addition, these chemotherapeutic drugs do not have selective activity against hypoxic tumor cells. Because ABDNAZ has activity under both normoxic and hypoxic conditions, this drug has promising potential for the treatment of a variety of cancers either as a stand-alone single agent or in combination with radiation therapy. Furthermore, ABDNAZ did not sensitize normal tissue, such as intestinal crypt stem cells, to radiation and has a favorable toxicity profile at therapeutic doses. The efficacy and toxicity profile of ABDNAZ warrant further investigation and development as a novel anticancer agent.

Disclosure of Potential Conflicts of Interest
All authors have ownership interest in RadioRx, Inc. (including patents and stock options). B. Oronsky is an employee of RadioRx, Inc. as CMO; J. Scicinski is an employee of RadioRx, Inc. as VP Research and Development; and G. Saul is an employee of RadioRx, Inc. as CEO. M. Bednarski, G. Saul and S.J. Knox are the founders of RadioRx, Inc.

Acknowledgments
The authors thank Donna Peehl, Professor of Urology at Stanford University Medical Center, for helpful discussion and thoughtful review of the article. The authors also thank Junqiang Tian for assistance with intestinal crypt cell experiments. Collaboration with ATK Aerospace Systems on the development of ABDNAZ and other novel dinitroazetidine compounds is also acknowledged.

Received July 11, 2011; revised February 13, 2012; accepted March 2, 2012; published OnlineFirst May 16, 2012.


References


Dinitroazetidines Are a Novel Class of Anticancer Agents and Hypoxia-Activated Radiation Sensitizers Developed from Highly Energetic Materials
Shoucheng Ning, Mark Bednarski, Bryan Oronsky, et al.