Biological Evaluation of a Potential Anticancer Agent Methyl *N*-[5-(3'-Iodobenzoyl)-1*H*- Benzimidazol-2-yl]Carbamate

Zbigniew P. Kortylewicz,¹ Don W. Coulter,² and Janina Baranowska-Kortylewicz¹

Abstract

Background: Resistance of cancer to chemo- and radiotherapy remains a major clinical problem. This study contributes to the ongoing search for agents that can bypass this resistance by developing a novel antimitotic theranostic.

Materials and Methods: Methyl *N*-[5-(3'-iodobenzoyl)-1*H*-benzimidazol-2-yl]carbamates **1** and **2** were synthesized from a common precursor **3** or its 3'-stannylated derivative. The cytotoxicity of compound **1** was evaluated in several neuroblastoma and glioblastoma cell lines and in the NCI 60-cell assay. Biodistribution was conducted in mice after oral administration of compound **2** to determine tissue and brain uptake.

Result: Lethal concentrations (LC₅₀s) of compound **1** in neuroblastoma and glioblastoma are >15× lower compared with compound **3**, a drug currently tested in clinical studies in pediatric and adult brain tumors. Growth inhibition concentrations (GI₅₀) are in the nanomolar range in 60 cancer cell lines. When compound **1** is combined with a 4-Gy dose of radiation, <0.5% of cells retain their reproductive integrity. Increased hydrophobicity of new agents greatly enhances their brain uptake after oral administration.

Conclusions: Compound **1** is potently cytotoxic in a wide range of human cancer cell lines. Its structure allows incorporation of imaging and therapeutic radionuclides. It is therefore expected that compound **1** can be developed into a novel theranostic modality across a wide range of malignancies.

Keywords: benzimidazole carbamates, cancer, glioblastoma, neuroblastoma, radiosensitizer, theranostics

Introduction

Microtubules play a fundamental role in diverse cellular functions. In cancer cells, polymerization of tubulin to form microtubules is essential for proliferation, invasion, and metastases.^{1–3} Microtubules have long been recognized as useful targets for some of the most successful anticancer drugs.³ Most microtubule-targeted agents bind to one of four main sites within microtubules: taxane, laulimalide, vinca alkaloid, and colchicine sites.⁴ Drugs that bind to the colchicine domain also inhibit angiogenesis.⁵ Given the clinical success of vinca alkaloids and taxanes, it is evident that microtubules represent an excellent target for cancer therapy and

continue to be a promising target for development of new agents. However, the utility of these drugs is frequently limited by the primary or acquired resistance and serious side-effects. Development of novel microtubule-targeted drugs that can evade resistance mechanisms and are nontoxic is clinically important. A significant research effort is focused on drugs to the colchicine binding site as a means to overcome resistance. This emerging category of antimitotics comprises structurally diverse small molecules^{6,7} and includes benzimidazole carbamates (BCars), a class of broad-spectrum anthelmintic compounds.^{8–12} Clinical studies indicate that BCars have minimal and manageable toxicities.^{13,14} Two BCars, albendazole (ALB) and mebendazole (**3**), developed

¹J. Bruce Henriksen Cancer Research Laboratories, Department of Radiation Oncology, University of Nebraska Medical Center, 986850 Nebraska Medical Center, Omaha, Nebraska.

²Division of Hematology/Oncology, Department of Pediatrics, University of Nebraska Medical Center, 982168 Nebraska Medical Center, Omaha, Nebraska.

Address correspondence to: Janina Baranowska-Kortylewicz; College of Pharmacy, Department of Pharmaceutical Sciences; University of Nebraska Medical Center, 986120 Nebraska Medical Center; Omaha, NE 68198-6120
E-mail: jbaranow@unmc.edu

nearly 50 years ago were recently reintroduced as potential oral therapeutics for glioblastoma.^{15–17} However, systemic bioavailability and brain uptake of these drugs after oral administration are low.¹⁸ Maximum plasma concentrations of compound **3** are ~ 100 nM.¹⁷ In the case of ALB, only its sulfoxide metabolite can be detected in plasma and cerebrospinal fluid.¹⁹ The anticancer activity of ALB sulfoxide is significantly lower compared with the parent drug.²⁰ New drugs must address these deficiencies.

Tubulin-targeted drugs arrest cancer cells in the G2/M phase, admittedly the most radiosensitive cell cycle phase, and can synergize with ionizing radiation.²¹ For this reason, multimodality treatments combining BCars with ionizing radiation, either external beam radiotherapy (XRT) or molecular radiotherapy, are expected to be effective. The authors report biological evaluation of BCar that has significant antiproliferative and radiosensitizing properties and improved brain uptake. The new BCar was designed to function as a theranostic, that is, it can be used with stable isotopes as a chemotherapeutic or radiosensitizer or can be radiolabeled without reshaping the chemical structure with positron- and γ -emitting radiohalides for imaging. In the age of individualized treatments, it is important to quantify the uptake of a drug by the tumor as well as normal tissues to assess the efficacy and potential off-target toxicities.

Materials and Methods

Cell lines

Cell lines were purchased from ATTC. U-87 MG (U87), U-138 MG (U138), and SK-N-SH (SK) cells were maintained in ATCC-formulated EMEM. BE(2)-C (BE) cells were maintained in a 1:1 mixture of EMEM and F12 medium. Media were supplemented with 10% heat-inactivated fetal bovine serum (Rockland Immunochemicals) and antibiotics (penicillin/streptomycin, 100 U/mL). Cells were enumerated using the Cellometer[®] Auto 1000 (Nexcelom Bioscience).

Buffers and chemicals

All reagents were purchased from commercial suppliers in the highest purity available and used without further purification. Ethanol and phosphate-buffered saline (PBS) were of USP grade. Chemical structures of compounds evaluated in this study are shown in Figure 1 and they were synthesized as described previously.²² Mebendazole **3** was purchased from Sigma-Aldrich. Physicochemical parameters of evaluated compounds are listed in Supplementary Table S1. HPLC purification and the analytical traces are shown in Supplementary Figures S1 and S2.

FIG. 1. Chemical structures of methyl *N*-[5-(3'-halobenzoyl)-1*H*-benzimidazol-2-yl]carbamates used in this study.

Cell survival and LC₅₀ determination

Compound **1** or **3** dissolved in dimethyl sulfoxide (DMSO) was added to the appropriate cell culture medium in concentrations ranging from 0 to 1.5 μ M. Higher concentrations were not evaluated as the amount of DMSO required to maintain the drug's solubility was not compatible with cell culture. The final concentration of DMSO was maintained at $\leq 0.5\%$ in all flasks, including controls. With each treatment group ($n=4-6$ per concentration), duplicate flasks of control cells were harvested alongside treated cells. Live cells were enumerated using the trypan blue exclusion assay. LC₅₀s were estimated using the sigmoidal dose-response curve with variable slope (GraphPad Prism). The reproductive integrity was evaluated using a clonogenic survival assay.

Compound **1** was also evaluated by the NCI Developmental Therapeutics Program using a 60-cell one-dose screen and the NCI 60-cell five-dose screen. The methodology is described at https://dtp.cancer.gov/discovery_development/nci-60/methodology.htm

Concentration- and time-dependent uptake and subcellular distribution

Cell uptake was tested as a function of the radioactive concentration of compound **2** and time of exposure. With each treatment group ($n=3$), control cells were grown without radioactivity and harvested alongside. Cells for the subcellular distribution experiments were harvested by scraping. The subcellular distribution of radioactivity was determined as described before.^{23–25} Supplementary Figure S3 shows the results of these experiments.

Ionizing radiation (XRT) treatment

Compound **1** or **3** dissolved in DMSO was added to cells at a concentration of 1 μ M. Treatment groups included drug ($n=4$), 4 Gy XRT ($n=4$), drug +4 Gy XRT ($n=4$), and untreated controls (NT, $n=4$). The concentration of DMSO was 0.5% in all flasks. Cells grown as monolayers ($\sim 60\%$ confluent) were irradiated with a 4-Gy dose at the rate of 1.36 Gy/min in a Mark I 68A irradiator (JL Shepherd and Associates). At designated times after treatment, cells were harvested, enumerated, their viability determined, and plated at 200 cells and 500 live cells per flask ($n=2$ or $n=4$). Cells were left undisturbed for ~ 7 d, fresh medium was added, and colonies were allowed to grow for up to 3 weeks. Colonies were processed and counted using ImageJ software (<http://rsbweb.nih.gov/ij/>, accessed May 31, 2019), as described previously.^{24,25}

Effects of the treatment sequence were measured in treatment groups A, B, C, and NT ($n=4$ in each group). On day 0, all cells were given media with 0.5% DMSO; groups A and C were also given 1 μ M compound **3**; and groups A and B were irradiated at 2 Gy. On day 1, group A and its matching controls were harvested, counted, their viability determined, and surviving fractions (SFs) calculated; group B was given fresh medium with 1 μ M compound **3** and its controls received fresh media with 0.5% DMSO; and group C and its controls received fresh media containing 0.5% DMSO and group C was irradiated at 2 Gy. On day 2, groups B and C alongside their controls were harvested. Cells were counted, their viability determined, and SFs calculated.

Cell cycle analyses

Control and treated cells were harvested, washed with PBS, 2×10^6 aliquots were transferred into test tubes, centrifuged at 1000 rpm, and pellets were resuspended in 70% ethanol and kept at 4°C overnight. Just before analyses, cells were centrifuged to remove ethanol and cell pellets were washed with PBS and centrifuged again. Telford reagent was added, gently vortexed, and cells were incubated at 4°C for 1 h. Cell cycle analyses were done using the FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems).

Microarray and Western immunoblot analyses

Human apoptosis array C1 was purchased from RayBiotech. Cell lysates were prepared using provided standard lysis buffers with protease and phosphatase inhibitors. Protein concentration was measured using the micro-BCA method (Pierce Biotechnology). For Western immunoblot, aliquots of 80 μ g total protein/well were used. Protein samples in sample buffer were denatured at 95°C for 5 min, cooled, and loaded onto Any kD™ Mini-PROTEAN® TGX™ precast protein minigels (BioRad). Proteins were transferred overnight and membranes blocked in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween-20 buffer containing 5% nonfat dry milk. Blocked membranes were incubated with anti-phospho-H3 antibodies at 1:1200 dilution (mAb 53348) or anti-H3 antibodies at 1:2400 dilution (mAb 4499). Horseradish peroxidase-linked goat anti-rabbit immunoglobulin G secondary antibody 7074 was used at 1:5000 dilution (Cell Signaling Technology). Antigens were detected with ChemiGlow® (Alpha Innotech Corporation). The protein load was measured with anti- β -actin antibody sc-47778 (Santa Cruz Biotechnology). For the human apoptosis array analysis, lysates were diluted with the blocking buffer from the array kit to a final concentration of 0.5 mg total protein/mL. The protocol provided with the array was followed without modifications.

Biodistribution studies

Research involving animals was performed in accordance with the institutional guidelines, as defined by the Institutional Animal Care and Use Committee. Athymic NCr nude mice (spontaneous mutant) were purchased from Taconic. Experiments were conducted in mice of both genders (6–8 weeks old). Groups of mice ($n=4$ per time) received an average oral dose (PO) of 0.34 ± 0.03 MBq compound **2**. Whole-body radioactivity was measured longitudinally in a dose calibrator (Capintec CRC-15R). Mice were euthanized at 1, 3, and 20 h after drug administration. Blood was collected through cardiac puncture. Tissues were dissected, rinsed in PBS, and patted dry. The radioactive content was measured using the 15–80 keV channel of the Cobra II model 5003 γ -counter (PerkinElmer) alongside the aliquots of the dose formulation. Uptake values were calculated in terms of the percent injected dose per gram of tissue.

Statistical analyses

Data are expressed as the mean $\pm$ standard deviation or mean $\pm$ standard error and analyzed for statistical significance using a two-sided, unpaired Student's *t*-test with a

significance level of $p=0.05$. Cell survival data were evaluated using one-way analysis of variance with Tukey–Kramer or Dunnett multiple comparison tests using GraphPad software.

Results

Cytotoxicity studies

The cytotoxicity of compounds **1** and **3** was tested in U87,²⁶ U138,²⁷ SK,²⁸ and BE²⁹ cell lines. Compound **1** was also evaluated using the NCI 60-cell five-dose screen. LC₅₀ values were determined after 24 h of exposure to compound **1** or **3** at concentrations from 0 to 1 μ M. BE cells were most sensitive to compound **1** with LC₅₀ of 18.9 nM (Table 1). LC₅₀ values of compound **3** in glioblastoma cells were estimated to be $>3 \mu$ M. Both glioblastoma lines were more sensitive to compound **1** than compound **3** (Table 1), especially U87, for which LC₅₀ for compound **1** is 200 ± 60 nM, at least $\sim 15 \times$ lower compared with compound **3**. Similarly to survival evaluated at 24 h, the reproductive integrity of cells depended on the drug and its concentration (Figs. 2 and 5). SFs of BE and SK cells treated with 0.07 μ M compound **3** and measured in a clonogenic assay were 0.19 ± 0.03 and 0.31 ± 0.04 , respectively. Compound **1** was significantly more effective in eradicating chemoresistant BE cells compared with compound **3**. When BE cells were treated with compound **1** and their reproductive integrity evaluated, the SF was 0.07 ± 0.01 .

Concentrations required for 50% growth inhibition (GI₅₀) and total growth inhibition (TGI) were also determined. In glioblastoma and astrocytoma cell lines, SF-268, SF-295, SF-539, SNB-75, SNB-19, and U251, GI₅₀ values ranged from 23 to 69 nM (Supplementary Table S2). With the exception of SK-MEL-2 (0.95 μ M), OVCAR-4 (1.2 μ M), and TK-10 (11 μ M), remaining 57 cell lines have GI₅₀ values in the nanomolar range (Fig. 3A). All TGI concentrations are $<10 \mu$ M. In several cell lines, TGI was observed at concentrations <100 nM (Fig. 3B), suggesting the potential utility of this novel BCar in a wide range of malignancies.

Cell cycle analyses and cellular morphology

Flow cytometric analyses of the cell cycle after exposure to 1 μ M compound **1** or **3** showed accumulation of cells in the G2/M phase with a concomitant decrease of cells in G1

TABLE 1. LC₅₀ VALUES FOR COMPOUNDS **1** AND **3** IN HUMAN NEUROBLASTOMA AND GLIOBLASTOMA CELL LINES

Cell line	Compound 1 LC ₅₀ μ M $\pm$ SE	Compound 3 LC ₅₀ μ M $\pm$ SE
BE(2)-C	0.02 ± 0.01	0.97 ± 0.15
SK-N-SH	1.29 ± 0.24	0.98 ± 0.16
U-87 MG	0.20 ± 0.06	Not determined
U-138 MG	2.62 ± 1.35	Not determined

SE, standard error; LC₅₀, concentration required to kill 50% of cancer cells; BE(2)-C, neuroblastoma cell line; SK-N-SH, neuroblastoma cell line; U-87 MG, cell line derived from malignant glioma; U-138 MG, glioblastoma cell line.

FIG. 2. Effects of compounds **1** and **3** on cell survival, cell cycle, and morphology of SK-N-SH and BE(2)-C human neuroblastoma cells. (A) Concentration-dependent survival of SK-N-SH and BE(2)-C cells treated for 24 h with compound **1**. (B) Concentration-dependent survival of SK-N-SH and BE(2)-C cells treated for 24 h with compound **3**. (C) Reproductive integrity (clonogenic survival) of SK-N-SH and BE(2)-C cells treated with 1 μ M compound **3**. (D) Cell cycle analyses of BE(2)-C cells. **cpd 1**: cells treated with 1 μ M compound **1**. (E) Distribution of the cell cycle phases in BE(2)-C cells derived from the flow cytometry data. (F) Morphology of BE(2)-C cells. (G) Morphology of SK-N-SH cells. **cpd 1**: cells treated for 24 h with 1 μ M compound **1**. **cpd 3**: cells treated for 24 h with 1 μ M compound **3**. (500- μ m bar is shown in the left-hand corner). NT, untreated cells.

and S phases. Responses to these drugs were strikingly different. Compound **1** arrested $\sim 70\%$ of BE in G2/M compared with $\sim 53\%$ G2/M arrest in response to compound **3** (Figs. 3 and 4). A large subdiploid population was detected in BE, indicating apoptotic cells. Significant response differences were also noted between cell lines. Over 50% of BE cells treated with compound **3** were in the G2/M phase compared with just 35% of SK cells, after the identical treatment. Morphological changes were significant. Treatment of SK with compound **1** produces giant cells with multiple aberrant nuclei, increase in nuclear size heterogeneity, and a significant disruption of the nuclear envelope (Fig. 2G). Similar changes in the SK cell sizes are also observed after treatment with compound **3** and ionizing radiation (Fig. 4). Increased cell diameters are indicative of DNA tetraploidy and a G2/M cell cycle arrest.³⁰ A few BE cells

remaining and still adhering to the flask surface after treatment with compound **1** are also larger compared with untreated cells. The mitotic cell rounding of BE is apparent with evidence of nuclear condensation (Fig. 2F). To further corroborate the mitotic arrest, Western immunoblot analyses of phospho-histone H3 (Ser10) were conducted (Fig. 4H). Phosphorylation of H3 at Ser10 is a reliable mitosis-specific marker as it occurs during the early prophase and is negligible during the interphase.^{31–33} The mitotic arrest of BE is maintained even at 24 h after treatment with compound **1**, as indicated by high expression of phosphorylated H3 confirming the flow cytometry results.

To determine the combined effect of ionizing radiation and BCar, asynchronous BE and SK cells, $\sim 60\%$ confluent, were exposed to compound **1** or **3**, a bolus dose of 4 Gy XRT, or a combination of a drug and 4 Gy XRT. The effect

FIG. 3. Inhibition of cellular growth by compound 1 evaluated in 60 human cancer cell lines with the NCI 60-cell five-dose screen. (A) Concentrations required for 50% growth inhibition (GI₅₀, nM). (B) Concentrations required for TGI (μM, logarithmic scale). TGI, total growth inhibition.

of these treatments on distribution of the cell cycle phases was analyzed (Fig. 4). Cell cycle profiles of both lines indicate that XRT treatment with a dose of 4 Gy resulted in a significant G2/M arrest, as was the exposure to 1 μM compound 1 or 3. Additionally, 4 Gy XRT totally inhibited DNA synthesis in SK. Only 2.9% of these cells were in the S phase (Fig. 4C). A combined treatment of SK with 4 Gy XRT and a drug produced G1/G0 and G2/M arrests with <1% cells in the S phase. SK cells after combined treatments were ~3× larger compared with untreated cells (Fig. 4E–G). BE cells are radio- and chemoresistant.²⁵ These cells did not arrest in the G1/G0 phase; however, treatment with compound 1 or 3 arrested BE in the G2/M phase. Combined treatments also produced a significant subdiploid population.

Cell survival and reproductive integrity

Cell death was measured 24 h after treatment. Live cells were enumerated using a trypan blue exclusion assay (Fig. 5A). Because all media contained 0.5% DMSO, which attenuates cellular radiosensitivity,³⁴ a dose of 4 Gy was used instead of a typically employed 2 Gy. Approximately 40% of cells in both lines were killed by 4 Gy XRT alone 24 h after irradiation. Compound 1 alone or in combination with XRT was 4.7× and 8.7× more effective in BE than SK

cells, respectively (Figs. 3 and 5). Combination of compound 1 with XRT was also more effective compared with 3 + XRT.

Cell survival was also measured using a clonogenic assay. BE cells appear to completely lose their reproductive integrity after exposure to 1 μM compound 1 in combination with 4 Gy XRT, that is, clonogenic cells are not detected even after 3 weeks in culture (Fig. 5B, C). SK cells resume proliferation after removal of the drug from the media and their SFs are higher. The treatment sequence did not appear to have a major influence on cell killing (Supplementary Table S3). Only a borderline difference ($p=0.06$) was noted between SFs of SK cells treated with XRT 24 h before the drug treatment and SK cells treated concurrently with both modalities.

Apoptosis

Treatment of BE with compound 1 in combination with XRT activates several proapoptotic proteins, including caspase-3, Fas, hsp60, HTRA2, and IGFBP-2, with their respective expression amplified by 950%, 360%, 480%, 140%, and 180%, in cells treated with compound 1 + XRT compared with untreated controls (Fig. 5D–F). When used as a single modality, compound 1 downregulates several antiapoptotic proteins. It also reduces the expression of these proteins in

FIG. 5. Survival of neuroblastoma cells treated with either benzimidazole **1** or **3** without and with ionizing radiation. **(A)** Surviving fractions of SK-N-SH and BE(2)-C cells 24 h after indicated treatments. **(B)** and **(C)** Clonogenic survival of BE(2)-C cells. NT: untreated control cells. XRT: cells treated with a 4-Gy dose of ionizing radiation, harvested 24 h later, and plated for the clonogenic assay. **cpd 1**: cells treated for 24 h with 1 μ M compound **1**, harvested, and plated for the clonogenic assay. **cpd 1 + XRT**: cells treated with 1 μ M compound **1** for 24 h, given fresh medium, irradiated at a dose of 4 Gy, harvested 24 h later, and plated for the clonogenic assay. **cpd 3**: cells treated for 24 h with 1 μ M compound **3**, harvested, and plated for the clonogenic assay. **cpd 3 + XRT**: cells treated with 1 μ M compound **3** for 24 h, given fresh medium, irradiated at a dose of 4 Gy, harvested 24 h later, and plated for the clonogenic assay. **(D)** Lysates obtained from untreated control BE(2)-C cells analyzed with the human apoptosis array. **(E)** Lysates obtained from BE(2)-C cells treated with 1 μ M compound **1** and 4 Gy ionizing radiation analyzed with the human apoptosis array. 1=Caspase-3; 2=FAS (**apo-1**); 3=HSP27; 4=HSP60; 5=HSP70; 6=HTRA2; 7=IGFBP2; 8=SMAC; 9=survivin; 10=XIAP. **(F)** Changes in the expression of several pro- and antiapoptotic proteins in BE(2)-C cells after the indicated treatments, detected with human apoptosis array membranes. The intensity of all readings is normalized to the expression of analyzed proteins by untreated control cells.

cells treated with compound **1** + XRT compared with XRT alone. For example, XRT treatment of BE upregulated survivin by >210% compared with untreated cells, while compound **1** reduced the levels of survivin by ~40%. Combined treatment with compound **1** + XRT reduced survivin by 50% (Fig. 5F).

Biodistribution and brain uptake

BCar¹ is more hydrophobic compared with other BCars; logP of compound **1** is 4.19 compared with compound **3** logP=2.89, ALB logP=2.75, and ALB sulfoxide logP=1.15, suggesting a possibility of improved penetration through the blood-brain barrier. To corroborate this notion, biodistribution of compound **2** after PO administration was conducted in healthy mice (Fig. 6A). The whole-body clearance of compound **2** is monophasic (Fig. 6B) with the biological half-life of 4.27 ± 0.31 h after a single bolus PO

dose of compound **2**. The estimated whole body mean residence time is ~9 h, which compares favorably with 16.7 h for ALB sulfoxide in patients treated with eight daily doses of ALB at 15 mg/kg.³⁵ The brain uptake of compound **2** at 1 h after dosing was >3000 higher compared with reported values (Fig. 6C). Taking into consideration that all glioblastoma and astrocytoma cell lines evaluated using the 60-cell screen with compound **1** had GI₅₀ values of ~20–70 nM, a single nontoxic dose of 15 mg/kg, assuming a toxicity profile of compound **1** similar to compound **3** or ALB, should result in drug levels sufficient to produce GI₅₀, that is, concentrations of compound **1** in the brain ranging from 12,000 nM at 1 h to 120 nM at 20 h.

Discussion

Disruption of microtubule function is a clinically important mechanism of action of many anticancer drugs. A serendipitous

FIG. 6. *In vivo* evaluation of orally administered ¹²⁵I-labeled benzimidazole carbamate **2** in mice. (A) Tissue distribution at three times after administration. (B) Whole-body clearance curve. (C) Brain uptake of compound **2** at 1, 3, and 20 h after oral dose of 0.38 MBq/mouse (lit indicates data derived from Biedler et al.²⁸).

observation that microtubule-targeted fenbendazole, an anthelmintic used to treat pinworm infections, has anticancer properties in laboratory mice^{36,37} sparked renewed interest in this class of agents and subsequently resulted in several clinical trials of compound **3** in glioma and pediatric brain tumors. The absence of side-effects of orally administered BCars in adults and children, even very young (<24 months of age),³⁸ is a major driving force in attempts to repurpose compound **3** for the treatment of cancer. The challenge of compound **3** and other oral BCars is their limited and variable bioavailability after oral administration. At the level of C_{max} , the cerebrospinal fluid of patients receiving ALB daily at 15 mg/kg for 8 d contained <0.0003% of the dose as ALB sulfoxide, a metabolite with minimal anticancer activities. BCars¹ is more hydrophobic and its brain uptake after PO administration is far better compared with compound **3** or ALB, indicating that orally administered compound **1** has a potential to outperform current BCars in the treatment of brain malignancies.

Motivated by the likelihood that the combination of ionizing radiation and BCars may be safer and more effective than conventional antimetabolic drugs, the authors have designed 3'-iodo-BCars to function as theranostics. When ¹²⁷I is in the 3'-position, compound **1** can serve as an oral chemotherapeutic and radiosensitizer. The same platform can be used to produce BCars radiolabeled with either positron- and γ -emitting radiohalides for imaging or α - and β -particle-emitting radiohalides for molecular radiotherapy. Drugs that disrupt microtubule function are regarded as radiosensitizers because many of them arrest cancer cells in the G2/M phase, the most radiosensitive phase of the cell cycle. Combined treatments with taxanes and radiation are common. However, the utility of taxanes is often limited by primary or acquired resistance. Patupilone, developed to overcome these shortcomings, also synergizes with ionizing radiation therapy.³⁹ The authors hypothesized that BCars will exhibit similar potentiating effects in combination with ionizing radiation, their data support this notion. At concentrations easily achievable in solid tumors, compound **1** in combination with 4 Gy XRT diminishes the reproductive potential of cancer cells by >99.5%. The authors' microtubule-targeted agent is expected to be particularly

useful in pediatric patients as a replacement for some chemotherapeutics, which can be carcinogenic, mutagenic, and clastogenic. BCars such as compound **3** or ALB, commonly used in children, showed no evidence of an increased incidence of tumors in mice and rats. These drugs were also negative in various genotoxicity tests. Compound **3** does not induce any cytologically detectable chromosome damage in pre- and postmeiotic germ cells of the mouse.⁴⁰ By analogy, compound **1** is expected to have a similar toxicity profile.

Conclusions

Microtubule-targeted *N*-[5-(3'-iodobenzoyl)-1*H*-benzimidazol-2-yl]carbamate **1** is highly cytotoxic in a wide range of human cancer cell lines. This drug's efficacy in reducing the reproductive potential of cancer cells is significantly amplified when combined with ionizing radiation, suggesting its utility as a radiosensitizer. Compound **1** is a prototypal microtubule-disrupting theranostic. It can be radiohalogenated for imaging or molecular radiotherapy. *In vitro* data and documented low toxicities of other BCars support the authors' decision to proceed toward preclinical evaluation of the efficacy in animal models.

Acknowledgments

The authors are grateful to Ms. Victoria B. Smith, BSc, Facility Manager of the UNMC Flow Cytometry Research Facility, for her assistance with the cell cycle analyses. The authors are also grateful to Drs. T. McGuire, J.G. Sharp, and Y. Yan for their advice and support.

Disclosure Statement

There are no existing financial conflicts.

Funding Information

These studies were funded, in part, by the National Cancer Institute Grant R21CA187548 (J.B.-K.) and the State of Nebraska LB 905 Pediatric Cancer Research Program (D.W.C.).

Supplementary Material

Supplementary Figure S1
 Supplementary Figure S2
 Supplementary Figure S3
 Supplementary Table S1
 Supplementary Table S2
 Supplementary Table S3

References

- Himes RH. Interactions of the catharanthus (*Vinca*) alkaloids with tubulin and microtubules. *Pharmacol Ther* 1991; 51:257.
- Yadav S, Narasimhan B, Kaur H. Perspectives of benzimidazole derivatives as anticancer agents in the new era. *Anticancer Agents Med Chem* 2016;16:1403.
- Jordan MA, Wilson L. Microtubules as a target for anticancer drugs. *Nat Rev Cancer* 2004;4:253.
- Akhmanova A, Steinmetz MO. Control of microtubule organization and dynamics: Two ends in the limelight. *Nat Rev Mol Cell Biol* 2015;16:711.
- Tozer GM, Kanthou C, Parkins CS, et al. The biology of the combretastatins as tumour vascular targeting agents. *Int J Exp Pathol* 2002;83:21.
- Torres FC, García-Rubiño ME, Lozano-López C, et al. Imidazoles and benzimidazoles as tubulin-modulators for anti-cancer therapy. *Curr Med Chem* 2015;22:1312.
- Patil R, Patil SA, Beaman KD, et al. Indole molecules as inhibitors of tubulin polymerization: Potential new anticancer agents, an update (2013–2015). *Future Med Chem* 2016;8:1291.
- Actor P, Anderson EL, DiCuollo CJ, et al. New broad spectrum anthelmintic, methyl 5(6)-butyl-2-benzimidazolecarbamate. *Nature* 1967;215:321.
- Brugmans JP, Thienpont DC, van Wijngaarden I, et al. Mebendazole in enterobiasis. Radiochemical and pilot clinical study in 1,278 subjects. *JAMA* 1971;217:313.
- Ireland CM, Gull K, Gutteridge WE, et al. The interaction of benzimidazole carbamates with mammalian microtubule protein. *Biochem Pharmacol* 1979;28:2680.
- Laclette JP, Guerra G, Zetina C. Inhibition of tubulin polymerization by mebendazole. *Biochem Biophys Res Commun* 1980;92:417.
- Lacey E, Watson TR. Structure-activity relationships of benzimidazole carbamates as inhibitors of mammalian tubulin, in vitro. *Biochem Pharmacol* 1985;34:1073.
- Rafi S, Memon A, Billo AG. Efficacy and safety of mebendazole in children with worm infestation. *J Pak Med Assoc* 1997;47:140.
- Silber SA, Diro E, Workneh N, et al. Efficacy and safety of a single-dose mebendazole 500 mg chewable, rapidly-disintegrating tablet for *Ascaris lumbricoides* and *Trichuris trichiura* infection treatment in pediatric patients: A double-blind, randomized, placebo-controlled, phase 3 study. *Am J Trop Med Hyg* 2017;97:1851.
- Popovic DJ, Posa M, Popovic KJ, et al. Application of a widely-used tropical anti-worm agent, mebendazole, in modern oncology. *Trop J Pharm Res* 2017;16:2555.
- De Witt M, Gamble A, Hanson D, et al. Repurposing mebendazole as a replacement for vincristine for the treatment of brain tumors. *Mol Med* 2017;23:50.
- Čáňová K, Rozkydalová L, Rudolf E. Anthelmintic flubendazole and its potential use in anticancer therapy. *Acta Medica (Hradec Kralove)* 2017;60:5.
- Münst GJ, Karlaganis G, Bircher J. Plasma concentrations of mebendazole during treatment of echinococcosis: Preliminary results. *Eur J Clin Pharmacol* 1980;17:375.
- Takayanagui OM, Bonato PS, Dreossi SA, et al. Enantioselective distribution of albendazole metabolites in cerebrospinal fluid of patients with neurocysticercosis. *Br J Clin Pharmacol* 2002;54:125.
- Králová V, Hanušová V, Staňková P, et al. Anti-proliferative effect of benzimidazole anthelmintics albendazole, ricobendazole, and flubendazole in intestinal cancer cell lines. *Anticancer Drugs* 2013;24:911.
- Rohrer Bley C, Furmanova P, Orlowski K, et al. Microtubule stabilising agents and ionising radiation: Multiple exploitable mechanisms for combined treatment. *Eur J Cancer* 2013;49:245.
- Kortylewicz ZP, Baranowska-Kortylewicz J. Radio-synthesis of microtubule-targeted theranostic methyl *N*-[5-(3'-radiohalobenzoyl)-1H-benzimidazol-2-yl]carbamates. *J Label Compd Radiopharm* 2018;61:749.
- Kortylewicz ZP, Nearman J, Baranowska-Kortylewicz J. Radiolabeled 5-iodo-3'-*O*-(17beta-succinyl-5alpha-androstan-3-one)-2'-deoxyuridine and its 5'-monophosphate for imaging and therapy of androgen receptor-positive cancers: Synthesis and biological evaluation. *J Med Chem* 2009;52:5124.
- Kortylewicz ZP, Kimura Y, Inoue K, et al. Radiolabeled cyclosaligenyl monophosphates of 5-iodo-2'-deoxyuridine, 5-iodo-3'-fluoro-2',3'-dideoxyuridine, and 3'-fluorothymidine for molecular radiotherapy of cancer: Synthesis and biological evaluation. *J Med Chem* 2012;55:2649.
- Han G, Kortylewicz ZP, Enke T, et al. Co-targeting androgen receptor and DNA for imaging and molecular radiotherapy of prostate cancer: In vitro studies. *Prostate* 2014;74:1634.
- Allen M, Bjerke M, Edlund H, et al. Origin of the U87MG glioma cell line: Good news and bad news. *Sci Transl Med* 2016;8:354re3.
- Ponten J, Macintyre EH. Long term culture of normal and neoplastic human glia. *Acta Pathol Microbiol Scand* 1968;74:465.
- Biedler JL, Helson L, Spengler BA. Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer Res* 1973;33:2643.
- LaQuaglia MP, Kopp EB, Spengler BA, et al. Multidrug resistance in human neuroblastoma cells. *J Pediatr Surg* 1991;26:1107.
- Tahara M, Inoue T, Miyakura Y, et al. Cell diameter measurements obtained with a handheld cell counter could be used as a surrogate marker of G2/M arrest and apoptosis in colon cancer cell lines exposed to SN-38. *Biochem Biophys Res Commun* 2013;434:753.
- Hendzel MJ, Wei Y, Mancini MA, et al. Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. *Chromosoma* 1997;106:348.

32. Paulson JR, Taylor SS. Phosphorylation of histones 1 and 3 and nonhistone high mobility group 14 by an endogenous kinase in HeLa metaphase chromosomes. *J Biol Chem* 1982;257:6064.
33. Gurley LR, D'Anna JA, Barham SS, et al. Histone phosphorylation and chromatin structure during mitosis in Chinese hamster cells. *Eur J Biochem* 1978;84:1.
34. Chapman JD, Doern SD, Reuvers AP, et al. Radio-protection by DMSO of mammalian cells exposed to X-rays and to heavy charged-particle beams. *Radiat Environ Biophys* 1979;16:29.
35. Jung H, Hurtado M, Sanchez M, et al. Clinical pharmacokinetics of albendazole in patients with brain cysticercosis. *J Clin Pharmacol* 1992;32:28.
36. Gao P, Dang CV, Watson J. Unexpected antitumorogenic effect of fenbendazole when combined with supplementary vitamins. *J Am Assoc Lab Anim Sci* 2008;47:37.
37. Duan Q, Liu Y, Booth CJ, et al. Use of fenbendazole-containing therapeutic diets for mice in experimental cancer therapy studies. *J Am Assoc Lab Anim Sci* 2012;51:224.
38. Montresor A, Stoltzfus RJ, Albonico M, et al. Is the exclusion of children under 24 months from anthelmintic treatment justifiable? *Trans R Soc Trop Med Hyg* 2002;96:197.
39. Oehler C, von Bueren AO, Furmanova P, et al. The microtubule stabilizer paclitaxel (epothilone B) is a potent radiosensitizer in medulloblastoma cells. *Neuro Oncol* 2011;13:1000.
40. Leonard A, Vandesteen R, Marsoom R. Mutagenicity tests with mebendazole in the mouse. *Mutat Res* 1974;26:427.