Published OnlineFirst July 19, 2018; DOI: 10.1158/1535-7163.MCT-17-1192

Small Molecule Therapeutics

Molecular Cancer Therapeutics

Inhibition of BMI1, a Therapeutic Approach in Endometrial Cancer

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Abstract

With rising incidence rates, endometrial cancer is one of the most common gynecologic malignancies in the United States. Although surgery provides significant survival benefit to early-stage patients, those with advanced or recurrent metastatic disease have a dismal prognosis. Limited treatment options include chemotherapy and radiotherapy. Hence, there is a compelling need for developing molecularly targeted therapy. Here, we show that the polycomb ring finger protein BMI1, also known as a stem cell factor, is significantly overexpressed in endometrial cancer cell lines, endometrial cancer patient tissues as well as in nonendometrioid histologies and associated with poor overall survival. PTC-028, a second-generation inhibitor of BMI1 function, decreases invasion of endometrial cancer cells and potentiates caspase-dependent apoptosis, while normal cells with minimal expression of BMI1 remain unaffected. In an aggressive uterine carcinosarcoma xenograft model, single-agent PTC-028 significantly delayed tumor growth and increased tumor doubling time compared with the standard carboplatin/paclitaxel therapy. Therefore, anti-BMI1 strategies may represent a promising targeted approach in patients with advanced or recurrent endometrial cancer, a population where treatment options are limited. *Mol Cancer Ther; 17(10); 2136–43. ©2018 AACR.*

Introduction

Endometrial cancer is expected to be diagnosed in approximately 61,000 new patients this year in the United States, making it the most common gynecologic malignancy (1, 2). Whereas type I is characterized by low grade, has endometrioid histology, and is hormonally driven, type II cancers have a higher grade, are aneuploid, and have a high frequency of TP53 mutations. These consist of histologies such as serous, carcinosarcoma, and clear cell as well as some high-grade endometrioid tumors (2, 3). Five-year overall survival for type I endometrial cancers is approximately 85%, whereas type II cancers range from 50% to 60%, demonstrating an urgent need to improve treatment options for the more aggressive subtypes of endometrial cancer (4). Research focused on genetic abnormalities in endometrial cancer has led to the development of clinical trials exploiting targeted alterations in the PI3K/AKT and mTOR pathways (2). However, despite our increasing knowledge, there are currently no approved molecularly targeted therapies. Therefore, research into potential targets for therapeutic treatment of endometrial cancer is of utmost importance.

BMI1, a member of the Polycomb Repressor Complex 1 (PRC1), mediates gene silencing by regulating chromatin structure and is frequently upregulated in major types of cancer where its expression correlates with poor prognosis (5–10). However, there has been limited research into the role of BMI1 in endometrial cancer to date. A previous study reported that in addition to breast, ovarian, and cervical cancer, BMI1 was also significantly overexpressed in endometrial cancer (11). Inhibiting BMI1 levels by miR-194 reverted highly invasive endometrial cancer cell lines from a mesenchymal to a more epithelial phenotype (12). The presence of miR-194, which is known to inhibit BMI1 levels, was inversely related to tumor stage in type I endometrial cancer samples (13). Realizing the pathologic significance, chemical inhibitors against BMI1 were developed very recently. The first-generation BMI1 inhibitor PTC-209 was tested in colorectal and ovarian cancer models showing promising results; however, intratumoral administration was required (10, 14). Here, we demonstrate that BMI1 levels were significantly elevated in endometrial cancer cell lines as well as in endometrial cancer patient tumors. PTC-028 (15), a second-generation inhibitor of BMI1 function with optimized pharmaceutical properties, depleted BMI1 levels that led to decreased cellular invasion and reduced cellular viability by potentiating caspase-dependent apoptosis. In a mouse model of endometrial cancer, treatment with PTC-028 significantly increased the tumor doubling time (DT) and delayed tumor growth compared with the standard-of-care carboplatin and paclitaxel. Therefore, targeting BMI1 may serve as a promising therapeutic approach in endometrial cancer.

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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Materials and Methods

Cell culture and chemicals

AN3CA (16), HEC1A, and HEC1B cells (17) authenticated by the ATCC were purchased by our laboratory and used within 6 to 8 months. RL95-2 (18) originally from ATCC was kindly provided by Dr. Danny Dhanasekaran, University Of Oklahoma Health Sciences Center (OUHSC, Oklahoma City, OK) and authenticated by short tandem repeat (STR) profiling. D6B is a primary culture of the human endometrium that was established and kindly provided by Dr. Doris Benbrook, OUHSC (19). CS99 was established from a uterine carcinosarcoma by Schulten and colleagues (20) and further characterized and provided to us by Dr. Jason Somarelli, Duke University (Durham, NC; ref. 21). Ishikawa cells (22) were authenticated by the European Collection of Authenticated Cell Cultures (ECACC) and were purchased by our laboratory. MFE296 (23) originally from ECACC was provided by Dr. Jie Wu, OUHSC, and authenticated by STR profiling. AN3CA cells were cultured in EMEM + 10% FBS. HEC1A was cultured in McCoy's modified media + 10% FBS and HEC1B was cultured in EMEM + 10% FBS. RL95-2 was cultured in DMEM and F12 (1:1) + 10% FBS. D6B and CS99 cells were cultured in DMEM + 10% FBS. Ishikawa and MFE296 cells were cultured in RPMI + 10% FBS. All the cells were cultured with 1× penicillin–streptomycin in a 5% CO_2 humidified atmosphere. PTC-028 was provided by PTC Therapeutics.

Cell lysis, cell fractionation, SDS-PAGE, and Western blotting

Total cellular lysate was prepared in RIPA (Boston Bioproducts) and protein concentration was measured using a BCA Assay Kit (Pierce, 23225). A standard protocol was used for immunoblotting. The cell lysates were separated on 10% or 15% glycine SDS-PAGE gel and transferred to PVDF membrane. Membranes were blocked in 5% BSA in TBS with 0.1% TWEEN-20 (TBST) for 1 hour at room temperature. Primary antibodies were prepared in TBST with 5% BSA and membranes were incubated in primary antibody at 4°C for overnight. Antibodies were purchased from the following venders; BMI1 from Invitrogen (37–5,400); β-actin (4970), PARP (9542), cleaved caspase-3 (9664), cleaved caspase-9 (7237) from Cell Signaling Technology and secondary antibodies conjugated with horseradish peroxidase IgG rabbit (A6154) and mouse (A4416) from Sigma. Primary antibodies were used in dilutions recommended by the manufacturer. Secondary antibodies were used at a concentration of 1:10,000.

IHC and tissue microarray

A tissue microarray (TMA) containing 203 patients samples consisting of all endometrial cancer histologies was used for detection of BMI1 expression by IHC. The patient samples were collected at the OUHSC. Written informed consent was obtained from all women enrolled into the study and Institutional Review Board approval was provided by OUHSC. IHC was performed using BMI1 (1:200) antibody [Bethyl Laboratories (IHC-00606)] and based on immunoreactivity and intensity, staining was graded as 1 (low), 2 (intermediate), and 3 (high). IHC of BMI1 was scored using the standardized H-score, which takes into account both intensity and percentage of cells stained. The difference in H-score between groups were assessed by Wilcoxon rank-sum or Kruskal–Wallis test. For ease of interpretation, the H-score, range 0 to 240, was analyzed as a binary variable (sample median, 40 as the cutoff) and a 3-level categorical variable (sample tertiles as cutoffs), when assessing its association with clinical factors (stage, grade, histology, LVSI, and DOI) using χ^2 test and survival outcomes [progression-free survival (PFS) and overall survival (OS)]. Kaplan–Meier method and log-rank test were used to examine the unadjusted association between H-score and survival outcomes. Adjusted analyses were also performed using the Cox regression, controlling for histology, age at diagnosis, and stage. Statistical significance was defined as a two-sided P value of < 0.05. SAS v. 9.3 was used for all the analyses.

Determination cell viability and of apoptosis

We used the ApoTox-Glo Triplex Assay Kit (G6321) from Promega to determine apoptosis in various endometrial cancer cell lines as well as normal endometrial cells. Cells were treated with PTC-028 at various concentrations. The cells were incubated simultaneously to measure two protease activities; one is a marker of cell viability, and the other is a marker of cell death. The live- and dead-cell proteases produced different products, AFC and R110, which had different excitation and emission spectra, allowing them to be detected concurrently. The second part of the assay utilized a luminogenic caspase-3 to caspase-7 substrate (the tetrapeptide sequence DEVD), in a reagent optimized for caspase activity, luciferase activity, and cell lysis. Luminescence was proportional to the amount of caspase activity present. In addition, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positivity was assessed in PTC-028–treated and untreated CS99, and Ishikawa cells. We utilized the TUNEL Apoptosis Detection Kit (DeadEnd Fluorometric TUNEL System, Promega) according to the supplier's instructions. They were then analyzed by fluorescence microscopy. The number of TUNEL-positive nuclei was counted from approximately 300 cells per treatment group. For caspase rescue, CS99 and Ishikawa cells were treated with the pan-caspase inhibitor z-VAD-fmk (10 μmol/L; Selleckchem) for 3 hours with or without PTC-028 (50 nmol/L) for 48 hours, and analyzed cell viability using the MTS assay.

Gelatin degradation assay

Acid-washed coverslips were first coated with 50 μg/mL poly-L-lysine for 20 minutes at room temperature, and then fixed with 0.5% glutaraldehyde for 15 minutes. Gelatin matrix was prepared by mixing 0.2% gelatin and Oregon Green 488 Gelatin Conjugate (Life Technologies) at an 8:1 ratio. After coating for 10 minutes, coverslips were washed with PBS and self-fluorescence was quenched with 5 mg/mL sodium borohydride for 15 minutes followed by washing with PBS. For degradation assay, 25,000 cells for CS99 and 30,000 cells for Ishikawa were seeded in each well of a 12-well plate containing 488 Gelatin Conjugate cover slips. Twelve hours after plating, cells were treated with PTC-028 (20 nmol/L) or vehicle for 24 hours and 36 hours for CS99 and Ishikawa, respectively. Next, cells were fixed in 4% paraformaldehyde and stained with Alexa Flour 555 Phalloidin (Life Technologies) for 15 minutes at room temperature. The cells were washed with PBS and mounted with VECTASHIELD-mounting medium containing DAPI (Vector Laboratories). Images were acquired at 40× (with 1.6× Optovar) using the Zeiss Axio-Observer Z1. Cells that degraded the extracellular matrix (ECM) at focal adhesions (FA) sites were scored positive and approximately 100 random cells were quantified. The percentage of cells showing degradation was plotted.

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Mouse xenograft models

Female athymic nude mice (NCr-nu; 6–8 weeks old) were purchased from Harlan Laboratories. All mice were housed and maintained under specific pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and NIH (Bethesda, MD). All studies were approved and supervised by the University of Oklahoma Animal Facility under the guidance of the IACUC #16-004-SSHIC. CS99 cells (1×10^6/100 μL) were injected subcutaneously into the flanks of female athymic nude mice and randomized when the tumor reached the volume of approximately 100 mm^3. Mice were randomized to three different groups receiving vehicle, PTC-028, or carboplatin (Hospira, Inc.) and paclitaxel (Actavis Pharma, Inc.; C+T), which is the standard of care for advanced or recurrent endometrial cancer. PTC-028 was administered orally at 15 mg/kg twice weekly. Carboplatin and paclitaxel were administered weekly by intraperitoneal route at 50 and 15 mg/kg, respectively. After 2 cycles of treatment, mice were followed for tumor growth and euthanized when their tumor volume exceeded 1,500 mm^3 as per the IACUC limit. Tumor DT was calculated according to Mehrara and colleagues (24) using the equation DT = LN(2)/SGR, where SGR (specific growth rate) = ln $(V_2/V_1)/(t_2-t_1)$. In this experiment, we have utilized 7 mice in the vehicle-treated group, 8 mice in PTC-028 group, and 7 mice in C + T group, respectively.

Data analysis and statistics

All the experiments were repeated independently three times unless otherwise noted. Data are expressed as means ± SD unless otherwise noted. One-way ANOVA was performed to compare the mean among three or more groups, and Student *t* test was performed to compare the mean between two groups. Survival analysis was performed by the Kaplan–Meier method and log-rank analysis. Statistical significance was set at $P < 0.05$, using GraphPad Prism 6 software.

For the TMA

The descriptive statistics (mean, SD, count, and percentage) were reported. The difference in H-score between groups were assessed by Wilcoxon rank-sum or Kruskal–Wallis test. For ease of interpretation, the H-score was analyzed as a binary variable (sample median as the cutoff), when assessing its association with survival outcomes (PFS and OS). Kaplan–Meier method and log-rank test were used to examine the unadjusted association between H-score and survival outcomes. Adjusted analyses were also performed using the Cox regression, controlling for histology, age at diagnosis, and stage. Statistical significance was defined as a two-sided *P* value of < 0.05. SAS v. 9.3 was used for all analyses.

Results

BMI1 is elevated in endometrial cancer

The expression levels of BMI1 were determined in the immortalized normal endometrial cells (D6B), as well as in both type I and type II endometrial cancer cell lines. Type I was represented by endometrioid adenocarcinoma cell lines, that is, HEC1A, HEC1B, ISHIKAWA, MFE296, and RL95-2. Type II was represented by AN3CA (dedifferentiated adenocarcinoma) and CS99 (carcinosarcoma). Compared with normal endometrial cells, BMI1 levels were elevated in all the cancer cell lines (Fig. 1A). To evaluate whether this finding translated to endometrial patient samples, a TMA, representing 203 patients consisting of all endometrial cancer histologies was developed from previously archived patient samples at the OUHSC. IHC for BMI1 was performed and scored using the standardized H-score, which takes into account both intensity and percentage of cells stained (25, 26). When present, predominant nuclear staining for BMI1 was observed. Representative high, intermediate, and low staining

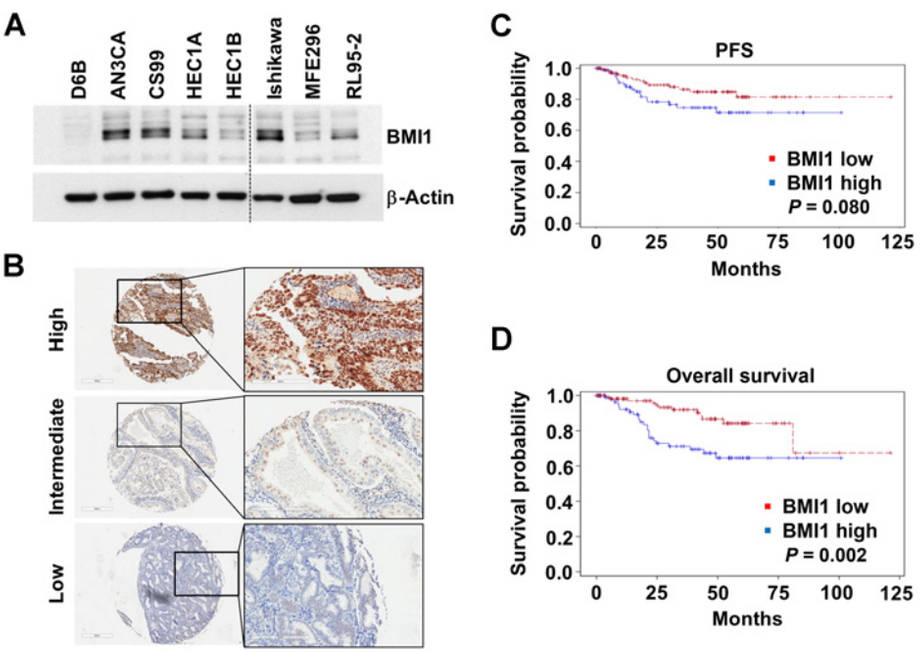

Figure 1.

BMI1 levels are elevated in endometrial cancer. **A,** Expression of BMI1 in normal and malignant endometrial cells. **B,** IHC staining of endometrial cancer patient TMA. Representative images at ×4 magnification are shown of (i) high, (ii) intermediate, and (iii) low staining tissues. Inset shows magnified areas at ×20. **C** and **D,** Kaplan–Meier analysis of progression-free survival (**C**) and overall survival (**D**) based on high and low BMI1 expression levels utilizing the median H-score. The proportion survival is plotted versus time since diagnosis in months. Kaplan–Meier curves with a log-rank test where $P < 0.05$ was considered significant.

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Table 1. Demographic variables and association with BMI1

	All patients ($n = 203$)	BMI1 low ($n = 114$)	BMI1 high ($n = 89$)	*P*
Median age at diagnosis (range)	61 (35–90)	61 (38–88)	62 (35–90)	0.81
FIGO stage				
IA	129 (64%)	71 (62%)	58 (65%)	
IB	45 (22%)	26 (23%)	19 (21%)	
II–IV	29 (14%)	17 (15%)	12 (14%)	0.91
Histology				
Endometrioid	158 (78%)	95 (83%)	63 (71%)	
Nonendometrioid	45 (22%)	19 (17%)	26 (29%)	0.03
Depth of invasion				
None	48 (24%)	24 (21%)	24 (27%)	
<50%	90 (44%)	51 (45%)	39 (44%)	
>50%	63 (31%)	38 (33%)	25 (28%)	
Unknown	2 (1%)	1 (1%)	1 (1%)	0.55
LVSI				
Yes	38 (19%)	21 (18%)	17 (19%)	
No	156 (77%)	88 (78%)	68 (76%)	
Unknown	9 (4%)	5 (4%)	4 (5%)	0.90
Recurrence				
Yes	32 (16%)	14 (12%)	18 (20%)	
No	171 (84%)	100 (88%)	71 (80%)	0.12

tissues are shown in Fig. 1B. Utilizing the median H-score as a cutoff, we categorized the samples into low or high staining cohorts (Table 1). The median age of the cohort was 61 (range, 35–90). The majority of the patients had disease confined to the uterus, defined as stage IA and IB ($n = 174$, 86%) and endometrioid histology ($n = 158$, 78%). There was no association between age, depth of invasion, lymphovascular space invasion (LVSI), and BMI1 level. There was a statistically significant association between nonendometrioid histology and high BMI1 ($P = 0.03$) as well as a trend toward an increased recurrence rate in patients with high BMI1 (20%) versus low BMI1 (12%). Both PFS (Fig. 1C) and OS (Fig. 1D) were compared between these two cohorts. Patients with high BMI1 expression had worse OS ($P = 0.002$; Fig. 1D) and a trend toward worse progression-free survival (PFS; $P = 0.080$; Fig. 1C). On the basis of these results, a multivariate Cox proportional hazards model was designed controlling for age, stage, and histology, which are all known to impact prognosis in endometrial cancer (27–29). As with univariate analysis, the HR for PFS was not significant ($P = 0.40$). However, there was a significant association of BMI1 with OS (HR, 2.4; 95% CI, 1.1–5; $P = 0.021$). Together, these results indicate that BMI1 is elevated in endometrial cancer cell lines and patient tissues and suggest that high BMI1 levels may be an independent prognostic marker for OS in patients with endometrial cancer.

PTC-028 attenuates endometrial cancer cell viability and invasion

Among the endometrial cancer cells tested, significant expression of BMI1 was observed in Ishikawa (type I), CS99 and AN3CA (type II) lines (Fig. 1A). To determine the effect of BMI1 depletion by PTC-028, normal and malignant endometrial cells were treated with increasing concentrations of PTC-028 for 48 hours followed by the ApoTox-Glo Triplex assay that simultaneously determines cell viability, cytotoxic cell death, and caspase-3/7 activity by utilizing two different protease markers and a luminogenic caspase substrate, respectively (14). PTC-028 did not affect viability, cytotoxic cell death, or caspase activity in normal endometrial cells (Fig. 2A), which have minimal BMI1 expression (Fig. 1A), but dose dependently inhibited cell viability of Ishikawa, AN3CA, and CS99 (Fig. 2B–D). The IC_{50} value of PTC-028 in Ishikawa, AN3CA, and CS99 lines was approximately 50 nmol/L (Fig. 2B–D). In the low BMI1–expressing HEC1A, IC_{50} was approximately 100 nmol/L and in the least BMI1–expressing HEC1B, IC_{50} could not be reached up to a concentration of approximately 100 nmol/L (Supplementary Fig. S1). Although a dose-dependent increase in caspase-3/7 activity was observed, there was no effect on cytotoxic cell death (Fig. 2B–D). We previously established that a cytotoxic cell death signal is generated in this assay only by a disruption of the cellular membrane (Triton-X) but not by apoptotic stimuli such as cisplatin (14). Importantly, treatment with PTC-028 at 50 nmol/L for 48 hours significantly depleted BMI1 levels in AN3CA, Ishikawa, and CS99 cells (Fig. 2E). These results suggest that inhibition of BMI1 by PTC-028 significantly attenuates endometrial cancer cell viability by triggering the apoptotic mode of cell death.

Because the depth of myometrial and cervical invasion of the endometrium is recognized as a prognostic factor for lymph node metastasis and OS (30–32), the phenotypic effects of PTC-028 treatment on cellular invasion was investigated. A representative endometrial cancer cell line each of type I (Ishikawa) and type II (CS99) served as models. The degradation of ECM components by matrix metalloproteases is a hallmark of cellular invasion; therefore, the FITC-gelatin degradation assay was performed previously (33). Interestingly, treatment with PTC-028 at sub-IC_{50} concentrations significantly reduced the number of cells invading the matrix by 6.2-fold and 3.5-fold in Ishikawa and CS99, respectively, compared with control (Fig. 2F and G). Together, these results suggest that inhibition of BMI1 by PTC-028 decreases viability and invasive potential of endometrial cancer cells.

PTC-028 induces endometrial cancer cell death via caspase-dependent apoptosis

Results from the Apotox-Triplex assay (Fig. 2B–D) suggested that the activation of caspase-mediated apoptosis may be responsible for decreased cellular viability. To confirm the apoptotic mode of cell death, TUNEL positivity was evaluated in the endometrial cancer cells that were treated with 50 nmol/L

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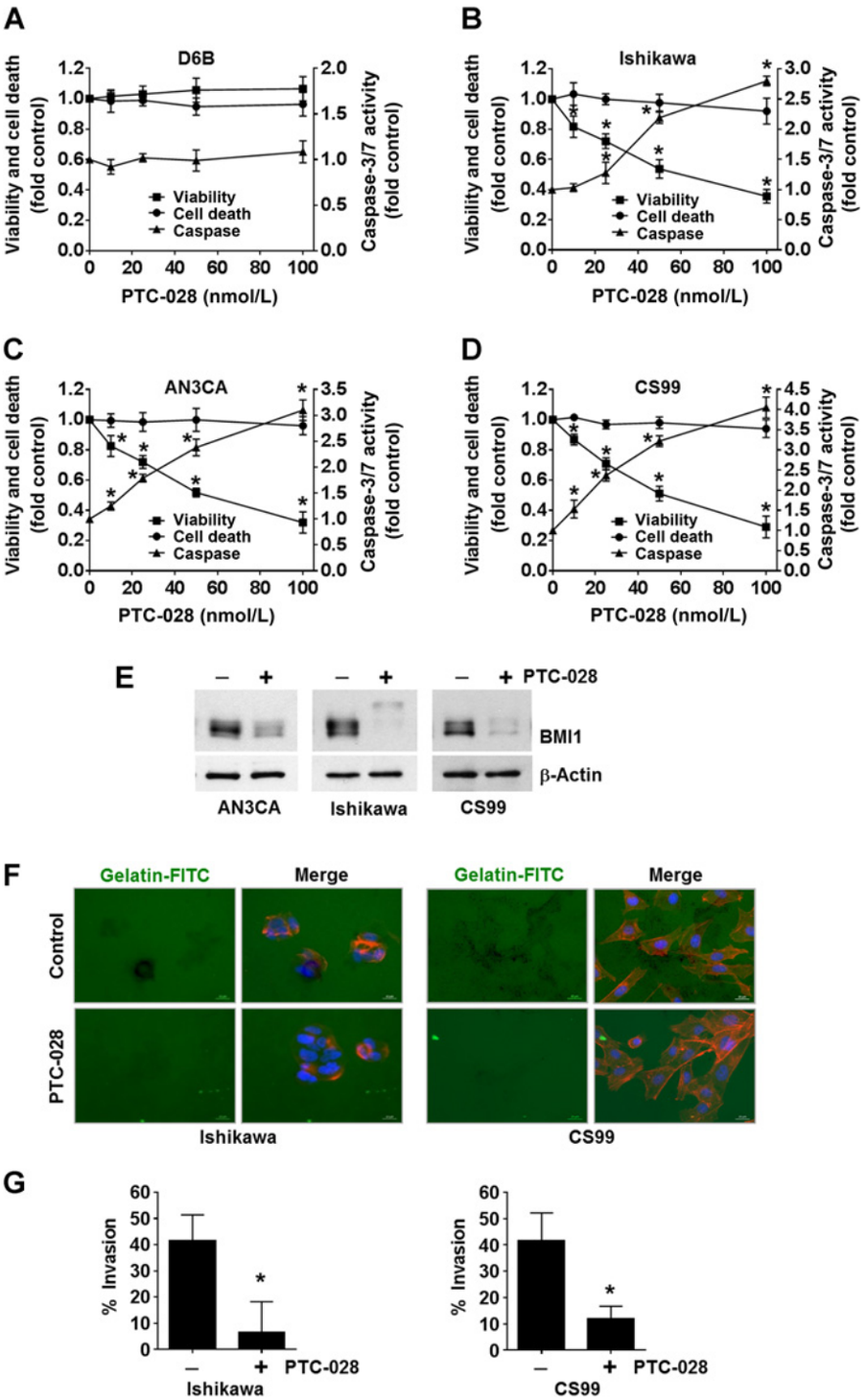

Figure 2.

PTC-028 inhibits endometrial cancer cell viability and invasion. **A–D,** D6B (**A**), Ishikawa (**B**), AN3CA (**C**), and CS99 cells (**D**) were treated with increasing concentrations of PTC-028 for 48 hours and cell viability, cytotoxic cell death, and caspase-3/7 activity were evaluated using the ApoTox-Glo Triplex assay. Data are mean ± SD of three independent experiments performed in triplicate. *, $P < 0.05$ when comparing with respective vehicle-treated control by two-way ANOVA. **E,** AN3CA, Ishikawa, and CS99 cells were treated with 50 nmol/L PTC-028 for 48 hours. Expression of BMI1 was determined by immunoblotting. **F,** Representative images of Ishikawa and CS99 cells plated on Oregon Green 488 Gelatin–coated coverslips and treated with 20 nmol/L PTC-028 for 24 hours (CS99) or 36 hours (Ishikawa). The cells were fixed and stained with Alexa Fluor 555 Phalloidin and mounted in VECTASHIELD mounting medium containing DAPI. **G,** Quantification of invaded cells was counted from approximately 100 cells per treatment group as represented in **F**. Data are mean ± SD of three independent experiments performed in triplicate. *, $P < 0.05$ when comparing with respective vehicle-treated control by Student *t* test.

PTC-028 for 48 hours. Compared with the untreated control, significant TUNEL positivity was observed in the PTC-028–treated Ishikawa (∼48%) and CS99 (∼42%) cells, respectively (Fig. 3A). To further corroborate these results, both the cell lines were treated with PTC-028 and BMI1 levels and apoptotic markers evaluated by immunoblotting. A significant decrease in levels of BMI1 and increase in cleavage of PARP, caspase-3, and caspase-9 were observed in the PTC-028–treated endometrial cancer cells (Fig. 3B). These results indicate that depletion of BMI1 by PTC-028 induces caspase-mediated apoptotic cell death. To further confirm that PTC-028–mediated decrease in cell viability was due to apoptosis, we treated the cells with the pan-caspase inhibitor

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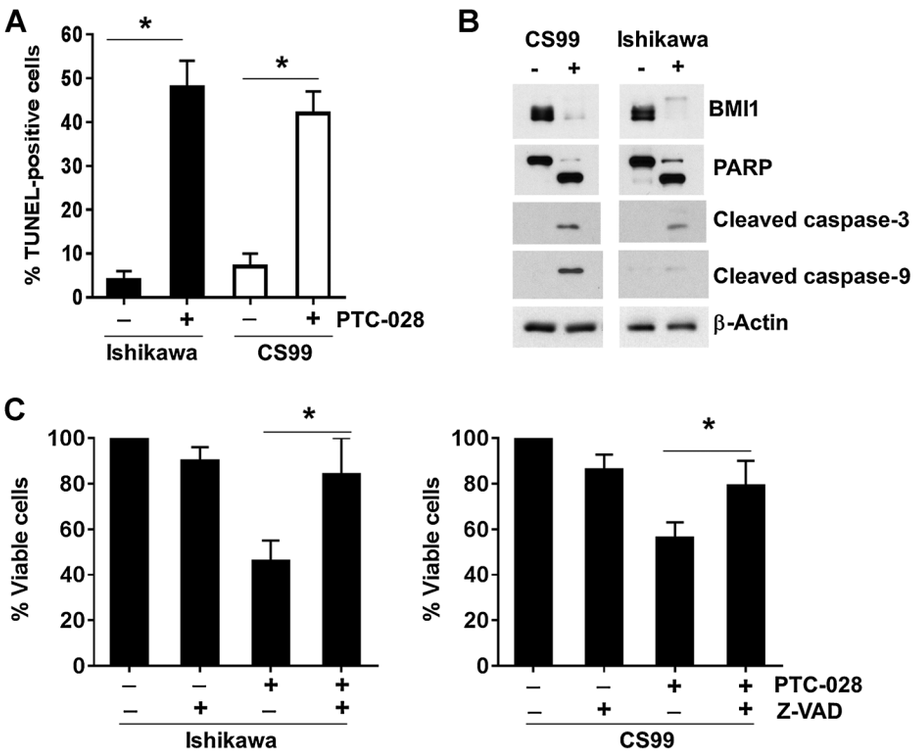

Figure 3.

PTC-028 induces caspase-dependent apoptosis in endometrial cancer cells. **A,** Ishikawa and CS99 cells were treated with 50 nmol/L PTC-028 for 48 hours; cells were subjected to the TUNEL assay and analyzed by fluorescence microscopy. The number of TUNEL-positive nuclei was counted from approximately 400 cells per treatment group. Data represent the mean ± SD of three independent experiments performed in triplicate. *, $P < 0.05$ when comparing with respective control by Student *t* test. **B,** CS99 and Ishikawa cells were treated with 50 nmol/L PTC-028 for 48 hours. Expression of BMI1, PARP, cleaved caspase-3, cleaved caspase-9, and β-actin was determined by immunoblotting. **C,** Ishikawa and CS99 cells were pretreated with or without the pan-caspase inhibitor z-VAD-fmk (z-VAD) at 10 μmol/L for 3 hours followed by PTC-028 at 50 nmol/L for 48 hours. Cell viability was assessed by the MTS assay. Vehicle-treated control cells were set to 100%. Data are mean ± SD of three independent experiments performed in triplicate. *, $P < 0.05$ when comparing with respective control by a one-way ANOVA.

z-VAD-fmk (10 μmol/L) for 3 hours with or without PTC-028 (50 nmol/L) for 48 hours and analyzed cell viability using the MTS assay. Compared with PTC-028 only, dual treatment with z-VAD-fmk significantly rescued cell viability by approximately 38% in Ishikawa and approximately 23% in CS99 cells, respectively (Fig. 3C). These results confirm that PTC-028–mediated depletion of BMI1 induces caspase-dependent apoptosis in endometrial cancer cells.

PTC-028 delays *in vivo* tumor growth

To evaluate the efficacy of PTC-028 *in vivo*, a mouse xenograft model was developed using CS99 cells (34). When tumor volume reached approximately 100 mm^3, mice were randomized into three groups. The first group received PTC-028 at 15 mg/kg twice weekly by oral administration. The second group received intraperitoneal injections of carboplatin and paclitaxel at 50 and 15 mg/kg weekly for two cycles (35). The third group received vehicle only. After two cycles of treatment, mice were followed for tumor growth and euthanized when their tumor burden reached IACUC approval limits. Because of tumor burden, all animals in the vehicle group were euthanized by approximately day 8, while those in the carboplatin/paclitaxel group were euthanized by approximately day 18 and those in the PTC-028 group were euthanized by approximately day 24 after initiation of treatment (~24 days; Fig. 4A). To compare tumor growth rates over the entire experimental period, tumor DT was calculated according to Mehrara and colleagues (Fig. 4B; ref. 24). Compared with the vehicle (~1.9 days) and carboplatin/paclitaxel (~3.3 days) treated group, the tumor DT was significantly delayed in the PTC-028 (~4.9 days) treated group (Fig. 4B). These results indicate that single-agent PTC-028 provides significantly delayed tumor growth compared with the standard-of-care chemotherapy in an endometrial cancer mouse model.

Discussion

Although majority of endometrial cancers are effectively treated with surgery, those patients with advanced or recurrent disease have limited treatment options and a high rate of chemoresistance. To improve response rates, strategies for development of more effective targeted therapy are needed in this patient population.

Although the expression of BMI1 has been linked with poor prognosis in other cancers (5, 6, 9–11, 36, 37), there have been limited studies in endometrial cancer. It was previously reported that inhibiting BMI1 levels by miR-194 reverted highly invasive endometrial cancer cell lines from a mesenchymal to a more epithelial phenotype (12). We find that BMI1 levels were significantly elevated in both type I and type II models of endometrial cancer cell lines. These observations were corroborated by the TMA, where 91% of the endometrial cancer patient tumor tissues expressed BMI1 compared with 9% that did not. Within the TMA, expression of BMI1 significantly correlated with type II histologies. However, a significant number of type I endometrial cancers also had elevated BMI1 levels (40%). In addition, elevation of BMI1 led to worse OS independent of histology. This suggests that elevated BMI1 may be an independent prognostic marker and could be utilized moving forward to help potentially identify patients in both types of endometrial cancers that have worse survival and will benefit from adjuvant treatment, potentially targeted at BMI1 elevation.

The first-generation BMI1 inhibitor, PTC-209, inhibited self-renewal of cancer-initiating cells, causing irreversible impairment

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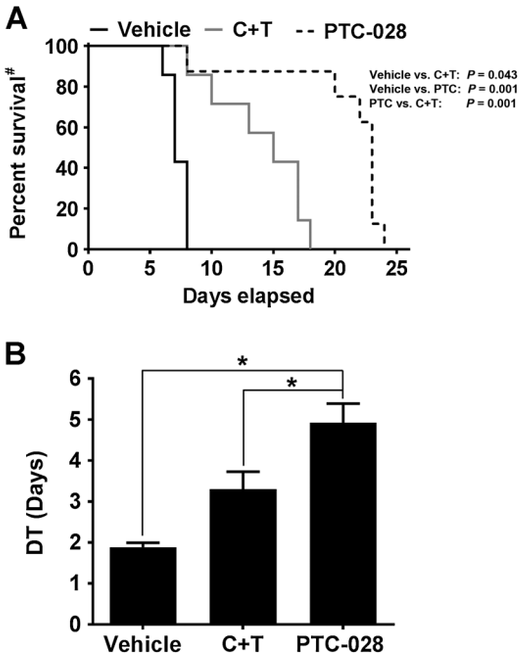

Figure 4.

Treatment with PTC-028 decreased tumor DT and delayed tumor growth in mouse xenograft model. **A,** CS99 cells were injected into 6-week-old female athymic nude mice. Mice were randomized into 3 groups of 7 to 10 each and treatment initiated when tumor volume exceeded approximately 100 mm^3. PTC-028 was administered orally at 15 mg/kg twice weekly. Carboplatin at a dose of 50 mg/kg/weekly and paclitaxel at 15 mg/kg/weekly (C + T) were administered by intraperitoneal injections and vehicle control given orally and intraperitoneally. Treatment continued for two cycles (14 days), and mice were followed for survival. #Percent survival was calculated by Kaplan–Meier method and *P* values determined by log-rank test based on the number of days the animals survived before the euthanization as per IACUC limits. **B,** Tumor DT was calculated for each treatment groups according to Mehrara and colleagues (24). Data are mean ± SD and *, $P < 0.05$ when comparing between indicated groups by one-way ANOVA.

in primary colorectal tumor growth (10). However, to inhibit subcutaneous xenograft tumors, intratumoral administration of PTC-209 at 60 mg/kg/day for 10 days was required (10), which is not feasible from a therapy perspective. Here, we utilized the second-generation inhibitor of BMI1, PTC-028, which with superior pharmacologic properties (15) potentiates tumor inhibition upon oral administration. *In vitro*, PTC-028 decreased the expression of BMI1 and dose dependently decreased the viability of endometrial cancer cells. Importantly, PTC-028 had no effect on normal endometrial cells (D6B) that have minimal BMI1 levels, demonstrating specificity of the inhibitor against BMI1. Furthermore, at sub-IC_{50} concentrations, PTC-028 inhibited invasion consistent with reversal of EMT and invasion by BMI1-shRNA in endometrial cancer cells (12). We further confirmed that PTC-028 led to a caspase-dependent apoptotic mode of cell death in endometrial cancer cells.

Importantly, carcinosarcoma represents one of the most aggressive subsets of endometrial cancer with a 5-year survival of less than 10% in patients presenting with advanced stage disease (38). Platinum-based chemotherapy is the standard for both local and advanced disease; however, response rates of only approximately 50% necessitate the development of other treatment approaches (39). Interestingly, in a carcinosarcoma xenograft model, orally administered PTC-028 demonstrated impressive single-agent activity compared with intraperitoneally delivered carboplatin and paclitaxel. This was exemplified by a significant increase in tumor DT and delayed tumor growth of mice bearing CS99 xenograft tumors. Taken together, these results indicate that anti-BMI1 strategies might provide an opportunity to improve outcome in women with advanced or recurrent endometrial cancer and also provide a rationale for further studies to determine its therapeutic benefit along with combination therapy in a clinical setting. Several proteins that are clinically relevant are directly or indirectly linked with expression of BMI1, for example, c-Myc, MDR1, PTEN, and BID (40–43). Therefore, the BMI1 and associated protein scores can be stratified on basis of their expression from tumor tissues versus adjacent nontumor tissue. It may be expected then that patients with a higher BMI1 score would respond favorably to anti-BMI1 therapy. However, the efficacy of this strategy can only be appreciated in the context of clinical outcome that is linked to the BMI1 score in pre- versus post-treated tumor tissues.

Disclosure of Potential Conflicts of Interest

K.N. Moore reports receiving a commercial research grant from PTC Therapeutics and is a consultant/advisory board member for Astra Zeneca, Clovis, Tesaro, Genentech/Roche, Janssen, and Immunogen. L. Cao has ownership interest (including stock, patents, etc.) in PTCT. A. Branstrom is the vice president (Biology) at and has ownership interest (including stock, patents, etc.) in PTC Therapeutics, Inc. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments

This study was supported by the NIH CA 157481 and by a Presbyterian Health Foundation Seed grant to R. Bhattacharya. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We thank an Institutional Development Award (IDeA) grant (P20 GM103639) from the National Institute of General Medical Sciences of the NIH supporting the use of the Histology and Immunohistochemistry Core.

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Received December 4, 2017; revised March 20, 2018; accepted July 10, 2018; published first July 19, 2018.

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Published OnlineFirst July 19, 2018; DOI: 10.1158/1535-7163.MCT-17-1192

Molecular Cancer Therapeutics

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Mol Cancer Ther 2018;17:2136-2143. Published OnlineFirst July 19, 2018.