5-Aza-2′-Deoxycytidine Delays Androgen-Independent Disease and Improves Survival in the Transgenic Adenocarcinoma of the Mouse Prostate Mouse Model of Prostate Cancer

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Abstract

Purpose: We have previously shown that 5-aza-2′-deoxycytidine (5-aza) is an effective chemopreventive agent capable of preventing early disease progression in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model. The purpose of this study was to determine the effect of 5-aza on preexisting TRAMP prostate cancers and prevention of androgen-independent prostate cancer.

Experimental Design: TRAMP mice with established prostate cancers were treated with 5-aza, castration, castration + 5-aza, or vehicle control (PBS). One cohort of 22 mice per treatment was euthanized after 10 weeks of treatment, whereas a second cohort of 14 mice per group was followed until death to determine survival. Histologic sections of prostate, pelvic lymph nodes, lung, and liver were blinded and analyzed by a certified genitourinary pathologist (K.J.W.).

Results: Combined treatment (castration + 5-aza) provided significant survival benefits over either single treatment (combined versus castration $P = 0.029$, combined versus 5-aza $P = 0.036$). At 24 weeks of age, 86% of mice within the PBS cohort exhibited histologic evidence of prostate cancer, whereas only 47% of the combined cohort exhibited malignant disease ($P < 0.0001$). Additionally, whereas 43% of the PBS treatment group exhibited lymph node metastases, these were only observed in 21% of the combined treatment mice.

Conclusions: This is the first study to examine the effect of 5-aza and combined castration + 5-aza on preexisting prostate cancer in an animal model. Based on these preclinical findings, we suggest that 5-aza treatment may prolong the time to an androgen-independent status and thus survival in a hormone-deprived setting in prostate cancer.

The process of prostate tumorigenesis is supported, in part, by epigenetic inactivation of genes required for normal regulation of cellular growth. Aberrant methylation of CpG dinucleotide–rich regions of gene promoters drives transcriptional silencing and is frequently observed in human tumorigenesis (1). Microarray screening studies in prostate cancer have identified several genes, such as *GSTP1* and *TGFBR2*, as frequently hypermethylated in prostate cancer cells (2). *TGFBR2* in particular has been shown to be hypermethylated in 98% of patients with localized prostate cancer compared with only 13% of patients with benign prostatic hyperplasia (3).

The enzymes that are necessary to actively maintain methylation after each cell division are the DNA methyltransferases (DNMT-1, DNMT-3A, and DNMT-3B; ref. 4). DNMTs covalently attach methyl groups at cytosine rings within the so-called CpG islands (5, 6). DNMT-1 maintains methylation patterns, whereas DNMT-3a and DNMT-3b are thought to be responsible for *de novo* methylation and embryonic imprinting; however, it has been shown that all three enzymes can facilitate both roles (6–8) and a more recent study showed that DNMT-1 and DNMT-3b cooperatively maintain DNA methylation and gene silencing in human cancer cells (6–9). In human prostate cancer, DNMT-1, DNMT-3a, and DNMT-3b have been shown to be significantly up-regulated in normal prostate tissue versus localized and metastatic prostate cancers (10). Aberrant DNA methylation is a hallmark of cancer—mostly displaying global hypomethylation and at the same time hypermethylation of CpG-rich promoter regions (11, 12). In this way, cancers present unique methylation profiles that distinguish them from benign tissue and other cancer types through their silencing of tumor suppressors (e.g., GSTP1, APC, RASSF1a, PTGS2, and MDR1; refs. 13, 14).

Research using cell culture models has shown that it is possible to reverse promoter methylation and restore transcription with 5-aza-2′-deoxycytidine (5-aza; a potent DNA

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methyltransferase inhibitor; ref. 3). 5-Aza (decitabine; Dacogen, MGI Pharma, Inc., Bloomington, MN) is a nucleotide analogue of cytosine and a prodrug of the phosphorylated version that is integrated into DNA during S phase (15). Phosphorylated 5-aza gets integrated into DNA instead of cytosine where it can covalently bind to the DNMTs, inhibit their catalytic activity, and ultimately lead to demethylation.

The retinoblastoma protein (pRb) plays an important role in cell cycle regulation through its ability to regulate G_1-S phase transition; however, the loss of Rb has consequences other than the deregulation of the cell cycle (16). We have recently shown that DNMT-1 is transcriptionally regulated by E2F-1 after the disruption of pRb, thus linking pRb loss to aberrant DNA hypermethylation (17). Continuing with this line of investigation, we examined the effects of 5-aza on prostate tumor formation in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model. The TRAMP model is based on prostate-specific rat probasin promoter–driven expression of SV40 early-region genes (T/t antigens; Tag; ref. 18). Therefore, this model is particularly relevant to Rb-based disruption because large T antigen inhibits the pocket protein family, p107, p130, and Rb, which results in increased E2F-1 activity (17). We found that 5-aza inhibited aberrant *de novo* DNA methylation in the TRAMP animals and completely prevented prostate cancer development for as long as the drug was administered (19).

Gingrich et al. (20) have shown that after castration, TRAMP mice will develop androgen-independent prostate cancer in >80% of animals after a period of prostate tumor involution. We hypothesized that the emergence of androgen-independent disease may result in part from aberrant hypermethylation that may be prevented or delayed by 5-aza treatment. To test this hypothesis, we used cancer-related death as an end point for the survival cohort and prostate tumor weight and histology as an outcome measure.

Once patients undergo androgen deprivation as primary or secondary treatment, remission usually lasts for 2 to 3 years (21). During that time, androgen-independent disease commonly emerges, which ultimately leads to metastasis-associated death. Therefore, a treatment is needed for these patients that either prevents or delays the development of androgen-independent prostate cancer. This therapy should target the subpopulation of androgen-independent cells in residue or recurrent disease before they progress to highly aggressive androgen-independent cancer cells. We propose a strategy to prevent or delay the emergence of hormone-independent prostate cancer at the time of androgen withdrawal in a mouse model that develops androgen-independent prostate cancer in response to castration. Results of this study are the first to report a positive effect of 5-aza on delaying the development of lethal androgen-independent disease and serve as a proof-of-principle study to justify further research on the effects of DNA methylation inhibitors in human prostate cancer.

Materials and Methods

Mice. Male C57BL/6 mice hemizygous for the rPB-Tag transgene (derived from TRAMP founder 8247; ref. 18) were mated with wild-type female FVB mice (Charles River, Wilmington, MA) to generate both wild-type and hemizygous TRAMP mice. All mice were maintained under specific pathogen-free conditions at 72°F in a 12 h light/12 h dark cycle with *ad libitum* food (chow 5001, LabDiet, Richmond, IN) and water in University of Michigan animal housing facilities.

Genotyping. Tail tissue (3 mm) obtained from 15-day-old pups was incubated overnight at 57°C in 600 μL TNES [10 mmol/L Tris (pH 7.5), 400 mmol/L NaCl, 100 mmol/L EDTA, and 0.6% SDS] and 35 μL proteinase K (10 mg/mL). Following the addition of NaCl to 1.25 mol/L, samples were centrifuged and genomic DNA was precipitated from the supernatant with 1 volume of 100% ethanol. DNA was then spooled onto a closed-ended capillary tube, washed thrice in 70% ethanol, and dissolved in TE buffer. The resulting DNA was used in a genotyping PCR reaction with the following primers TAg forward 5′-CCGGTCGACCG-GAAGCTTCCACAAGTGCATTTA-3′ and TAg reverse 5′-AGGCATTCCAC-CACTGCTCCCATTCATC-3′.

Maximum tolerable dose determination. As DNA hypomethylation is time dependent and requires two to three cell cycles to be effective and 5-aza is known to have a short half-life in serum, we were aiming at three 5-aza doses on 3 consecutive days per week to achieve a maximum effect (22). Therefore, and based on the findings of McCabe et al. (19), a range of 5-aza doses (0.25-1.0 mg/kg) were tested by i.p. injection thrice weekly in wild-type male C57BL/6 × FVB mice starting at 15 weeks of age for toxicity over a maximum 20-week treatment period. Toxicity was primarily detected as significant weight loss but also as altered gait, lethargy, ruffled coat, and hindered breathing.

Treatments. At 15 weeks of age, mice were randomized to receive either control (PBS), 5-aza (Sigma-Aldrich, St. Louis, MO), castration, or castration + 5-aza. Treatments were administered thrice weekly on consecutive days as 300 μL i.p. injections of either PBS or 0.25 mg/kg 5-aza dissolved in PBS. Treatment was continued until 24 weeks of age when mice were either euthanized as part of the 24-week cohort or left untreated until they died spontaneously or were euthanized due to marked morbidity, including, but not limited to, progressive weight loss, altered gait or significant lethargy.

Surgery. After initial treatment with 5-aza for 3 days, castrations were carried out under ketamine (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA)/xylazine (AnaSed, Lloyd Laboratories, Shenandoah, IA) anesthesia. Testes were removed through a scrotal midline incision after clamping testicular blood supply with Micro-serrefines (Fine Science Tools, San Francisco, CA). Surgical staples (Michel Wound Clip, Medicon, Tuttlingen, Germany) were used to close the incision. Mice were monitored postsurgery for mortality and morbidity and staples were removed after 7 days.

Necropsy, tissue procurement, and statistical analysis. Mice were weighed and euthanized at 24 weeks, or upon signs of morbidity by CO_2 inhalation followed by induction of a bilateral pneumothorax. A ventral midline incision was made from the groin to the suprasternal notch and from the groin to the knee to allow for examination of the internal organs. The genitourinary tract, including the bladder (minus urine), prostate, urethra, and seminal vesicles, was excised and weighed. The ventral, lateral, and dorsal prostate were separated from the remaining genitourinary tract (including urethra) and weighed because we believe pure prostate weight (excluding the anterior prostate that usually does not develop cancer) is a better indicator for tumor load. Otherwise, seminal vesicles, which can be obstructed due to a prostate tumor or shrunk due to castration, would influence the weight and not reflect tumor load. The lumbar and sacral lymph nodes were harvested and analyzed for enlargement (potential metastatic spread). Next, intestines, kidneys, liver, spleen, and lungs were removed and examined for macroscopic metastases. At a minimum, samples of the prostate, seminal vesicles, lumbar and sacral lymph nodes, lung, and liver were fixed in formalin overnight, paraffin embedded, sectioned at 5 μm, and stained with H&E. All histologic samples were blinded and evaluated by a genitourinary pathologist (K.J.W.). We used the previously described grading system from Kaplan-Lefko that identified tissue either as normal, prostatic intraepithelial neoplasia, well-differentiated, moderately differentiated, or poorly differentiated adenocarcinoma (23). Statistical power calculations were used to plan the cohort numbers: To estimate power, we used a 4-week difference for survival, and for

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calculations of histologic differences we estimated to see a 50% change in tissue grading. Kaplan-Meier methods were used for survival analysis among the PBS-, 5-aza–, castration-, and castration + 5-aza–treated groups, and statistical significance was tested using the log-rank test. Fisher's exact test was used to compare histology between treatment groups; for the analysis of prostate weights, we applied the Mann-Whitney test. All analysis were done using SAS software version 9.12 (SAS Institute, Cary, NC) with $P < 0.05$ considered significant.

Results and Discussion

The current study used an F_1 TRAMP male cohort, exhibiting heterozygous transgene expression, derived from male C57BL/6 TRAMP mice and female FVB wild-type mice. F_1 TRAMP males and their wild-type counterparts underwent castration, received i.p. 5-aza injections, received a combined treatment of castration + 5-aza, or were sham operated and received vehicle injections at 15 weeks of age. Not only was this age chosen due to reports of 100% of mice shown to have established prostate cancer (23), but our consulting pathologist (K.J.W.) confirmed in pilot studies that these TRAMP males exhibit established disease at this time. Treatment continued for 10 weeks at which time 19 to 22 mice per group were euthanized at the age of 24 weeks and their histopathology was compared. The remaining 12 to 14 mice in the survival groups did not receive further treatment after 24 weeks of age. Ten animals in total were lost in both the survival and the 24-week cohort due to surgery and injection related infections of the peritoneal cavity. Therefore, there were 81 animals left for analysis in the 24-week cohort and 53 animals in the survival cohort.

We determined the maximum tolerable dose of 5-aza with a range of doses (0.25-1.0 mg/kg) thrice weekly. The drug was administered by i.p. injection for 10 weeks starting at 15 weeks of age. Treatments were discontinued at 24 weeks of age as many of the PBS-treated TRAMP mice will die around that age due to prostate cancer (24). Mice that were injected with ≥0.5 mg 5-aza per kilogram of body weight on 3 consecutive days of the week did not survive beyond 10 weeks of treatment, whereas mice treated with 0.25 mg 5-aza per kilogram of body weight thrice weekly did not exhibit any adverse effects when treated as long as 20 weeks (when dose testing was stopped; data not shown).

The 5-aza doses used in animal studies are usually calculated in mg/kg of body weight, whereas clinical trials in humans usually calculate the necessary dose of the drug in mg/m^2. To compare dosages from these mice to reported human dosages, the amount of 5-aza used in this study (0.25 mg/kg) compared with doses used in recent clinical trials is very low as clinical trials use 0.13 to 53 mg/kg (15). The best way to compare treatment in animal studies and human studies would be to compare plasma concentrations as 5-aza is given i.v. in humans but through i.p. injection in the animals in this study; i.v. infusions can administer the drug more slowly than a single i.p. injection can.

Combined treatment mice received their first 5-aza treatments for the 3 days before castration to minimize negative effects on wound healing. Because 5-aza has a short half-life *in vivo*, the drug should be cleared by the time of surgery 24 h later. Another reason to start 5-aza treatment before castration is the sensitizing effect that 5-aza has on other chemotherapeutics when administered first (25–29; Fig. 1). Fang et al. (30) investigated the effect of 5-aza on DU 145 cells when treated with cisplatinum. DU 145 cells were grown in the presence of 5-aza for 120 h before treatment with cisplatinum, which significantly increased the apoptotic response of DU 145 cells.

Prostate weights. In a previous study, we saw that 5-aza prevents prostate enlargement compared with PBS-treated mice (19). In the current study, we noticed that the prostate weights within the 5-aza–treated group differed from all other treatment groups. Seventy-one percent of these prostates were of normal size and weighed <1.0 g, whereas in the PBS group 71% of the prostates of the survival cohort were >1.0 g (Mann-Whitney test $P = 0.017$). The same trend was seen when castration (67% >1.0 g) or castration + 5-aza (54% >1.0 g) were compared with 5-aza alone but this was not statistically significant (Fig. 2). However, a smaller prostate size, as indicator of tumor load, did not correlate with a survival benefit for the 5-aza–treated mice. Surprisingly, mice with near normal-sized prostates still had poorly differentiated cancers and had a higher likelihood of an early, cancer-related death. It seems that in the 5-aza group, larger tumors portend a better survival, whereas this was not observed in the other treatment groups; for the combined treatment group, even the opposite seems to be true—larger tumors in this group were an indicator for an early death (Fig. 2).

Animals were palpated for prostate tumors starting at 15 weeks of age. PBS-treated animals had small detectable prostate tumors by 18.6 weeks, which grew over an average of 12 weeks before the animals succumbed to tumor burden. In the castrated group, detectable tumors were delayed by 2.5 weeks on average when compared with the PBS group and the time from detection to death was only 8.5 weeks. This pattern likely represents tumor involution and the outgrowth of the more aggressive androgen-independent tumor. For the combined

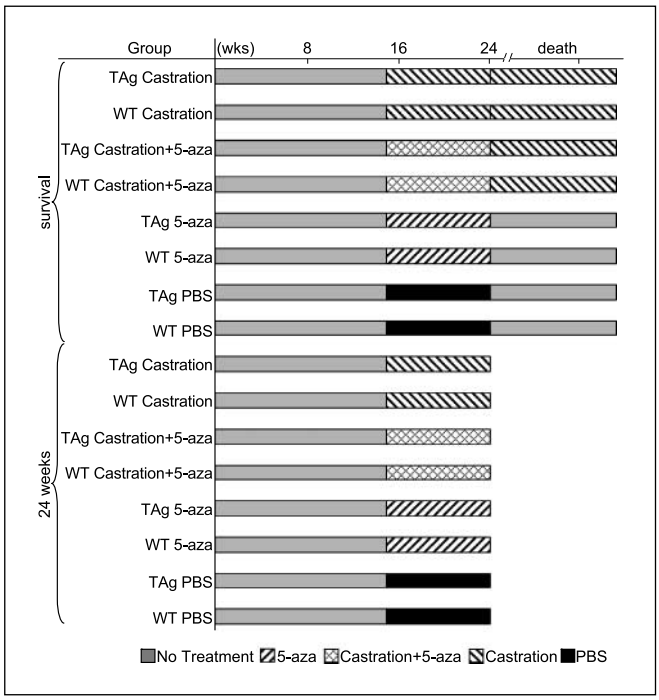

Fig. 1. Treatment schedule. Treatment for all treatment groups was started at 15 wk of age. Vehicle control (PBS) or 5-aza injections were administered thrice a week for 10 wk for all groups. Mice were either euthanized at 24 wk or allowed to survive until death. For every TRAMP treatment group, there was a wild-type (*WT*) control group.

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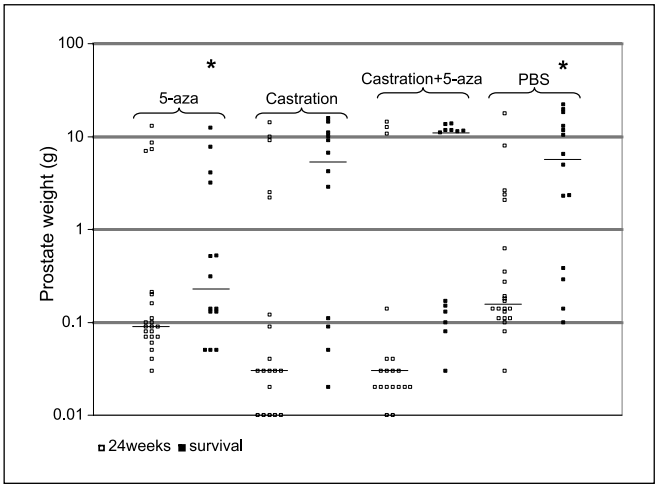

Fig. 2. Weight of the prostate (dorsal, lateral, and ventral lobe) at 24 wk and survival. *, $P = 0.017$ using the Mann-Whitney test between 5-aza survival and PBS survival. Bar, median for each group.

treatment, the delay to a palpable tumor was even longer (first palpation of a tumor occurred at 29.7 weeks on average); however, once established, those tumors expanded even faster than from the castrates with an average time from first palpation to death being 6.5 weeks (data not shown).

Body weights. We also noticed a dramatic weight loss (−31.31% average) in 8 of 14 animals treated with 5-aza within the 2 weeks before cancer-related death. This weight loss was not observed in the combined treatment. We do not believe that this wasting is due to 5-aza toxicity (as we saw with the high doses during dosage testing) as 5-aza wild-type control mice neither displayed any symptoms during and after treatment nor lost any weight. Comparing weight changes using the Mann-Whitney test, it seems that 5-aza induces a significant weight change compared with combined treatment, castration, and 5-aza–treated wild-type mice ($P = 0.0006$, $P = 0.003$, $P = 0.015$; data not shown). We believe that the weight loss in the 5-aza–treated TRAMP animals is due to metastasis to the brain as we noticed seizures and detected brain metastasis in some of these animals. Such an effect of 5-aza has not been reported and warrants further investigation as it would be of significant importance in human cancers that are being treated with 5-aza. No seizures were observed in any of the other treatment groups.

Histology. All tissue sections were graded as normal; prostatic intraepithelial neoplasia; or well-differentiated, moderately differentiated, or poorly differentiated adenocarcinoma by a trained genitourinary pathologist (K.J.W.). We investigated whether treatment effects were associated with a detectable histology and whether histology was significantly different between treatment groups. We observed a significant difference between the frequency of normal and precancerous/cancerous histology (including prostatic intraepithelial neoplasia and well-differentiated, moderately differentiated, and poorly differentiated cancers) in the combined treatment over PBS ($P < 0.001$; Fisher's exact test) at the 24-week time point. Eighty-six percent of the PBS cohort exhibited prostate cancer (well differentiated, moderately differentiated, or poorly differentiated); however, 63% of the combined treatment cohort exhibited normal/prostatic intraepithelial neoplasia prostate histology (Table 1). In addition, we saw a significant difference between combined treatment with 58% normal histology compared with 5-aza ($P = 0.007$) with only 14% normal histology. No significant difference was observed when comparing combined treatment to castration alone with 30% normal histology ($P = 0.19$).

Histologic examination of prostates from all four survival cohorts revealed poorly differentiated prostate cancer in 100% of mice upon death. Additionally, 100% of mice exhibited lymph nodes containing poorly differentiated metastatic deposits. Metastatic deposits were also detected in lung, liver, kidney, spleen, and brain. However, there were no statistically significant differences between any of the cohorts at the survival time point (data not shown). Besides the 100% lymph node metastasis in every group, we found 54% lung metastasis in the combined treatment group compared with 50% in the castrated and 43% in the 5-aza as well as PBS-treated mice. The number of liver metastasis was even lower with 15% in the combined treatment, 17% in the castrated group, 21% in the PBS group, and 38% of animals in the 5-aza group, which was not statistically significant. Figure 3 shows some of the histology encountered in the various treatment animals, including histology of brain metastasis.

Although previous studies reported more than twice the number of lymphatic metastases in castrated versus intact mice (45% versus 19%), we did not make the same observation (20).

Table 1. Effect of treatments on histologic distribution of lesions in TRAMP mice at 24 wks

Treatment group	*n*	Histology					*P*	LN Mets (%)	*P*
		Normal	PIN	WD	MD	PD			
Castration + 5-aza	19	58	5	0	0	37		21	
PBS	21	0	14	14	5	67	<0.0001*	43	0.19†
5-Aza	22	14	41	5	0	41	0.007‡	18	0.10§
Castration	19	30	0	0	0	70	0.19‖	37	0.29¶

Abbreviations: PIN, prostatic intraepithelial neoplasia; WD, well-differentiated prostate cancer; MD, moderately differentiated prostate cancer; PD, poorly-differentiated prostate cancer; LN, lymph node; Mets, metastasis.
*Fisher's exact test comparing the frequency of normal pathology between castration + 5-aza and PBS groups.
†Fisher's exact test comparing the frequency of lymph node metastasis between castration + 5-aza and PBS groups.
‡Fisher's exact test comparing the frequency of normal pathology between castration + 5-aza and 5-aza groups.
§Fisher's exact test comparing the frequency of lymph node metastasis between 5-aza and PBS groups.
‖Fisher's exact test comparing the frequency of normal pathology between castration + 5-aza and castration groups.
¶Fisher's exact test comparing the frequency of lymph node metastasis between castration and 5-aza groups.

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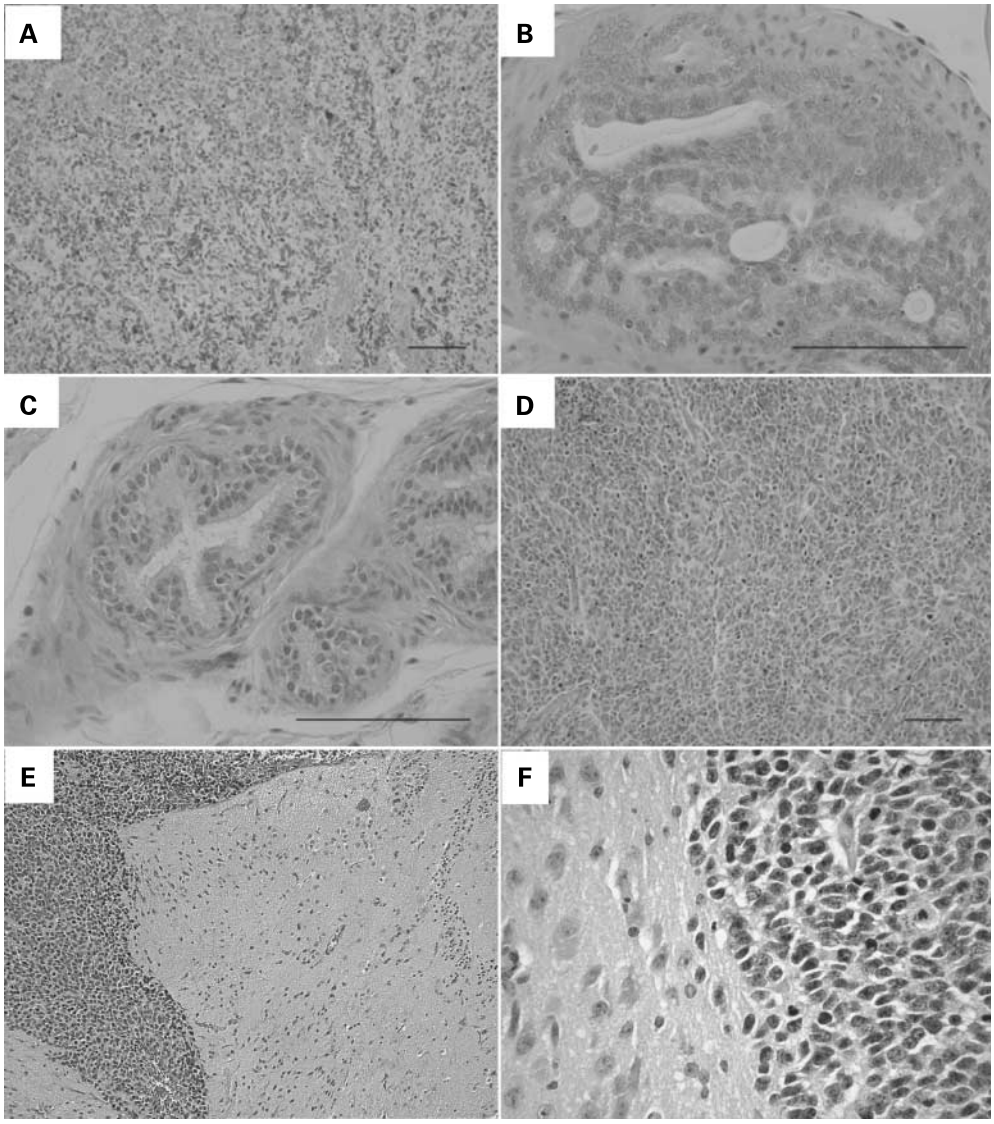

Fig. 3. Representative H&E staining of TRAMP tumors from all treatment groups at 24 wk. *A,* poorly differentiated adenocarcinoma of the prostate from a PBS-treated animal (×20). *B,* prostatic intraepithelial neoplasia in a 5-aza – treated animal (×60). *C,* normal prostate from a combined treatment mouse (×60). *D,* poorly differentiated adenocarcinoma from the castration group (×20). Bar, 100 μm. *E* and *F,* metastatic deposits of prostate cancer in the brain.

In fact, the number of lymph node metastases at 24 weeks in our study was lower for the castrated group (37%) versus the PBS group (48%). This difference may be due to the fact that we castrated our mice at 15 weeks of age, whereas the previous study castrated at 12 weeks of age. According to another study that investigated different time points for castration (15 and 20 weeks of age), survival and histology were related to the time of castration (24).

These results show that at 24 weeks, there are substantial differences in tissue histology in all treatment groups and combined treatment is superior to other treatments considered in this study. Although not statistically significant, the incidence of malignant disease (well differentiated-poorly differentiated) in the castrated group (70%) compared with the combined treatment group (37%) indicates a favorable trend. When interpreted together with the survival data, this finding supports the hypothesis that 5-aza delays the emergence of androgen-independent prostate cancer in castrated TRAMP mice (Fig. 3; Table 1).

Survival. Prostate cancer in C57BL/6 × FVB F_1 TRAMP mice is a rapidly progressing disease with 50% of PBS-treated transgenic mice dying of their disease by 30.6 weeks of age and 100% dead by 42 weeks (Fig. 4A and D). Treatment of these mice with 5-aza or castration alone did not significantly extend survival with the average survival time 30.6 and 29.1 weeks, respectively (Fig. 4A, B, and D). Combined treatment of castration + 5-aza, however, did extend survival by 5.1 weeks to an average survival time of 35.7 weeks (Fig. 4D). Statistical analysis revealed that survival of the castration + 5-aza cohort was significantly greater when compared with 5-aza treatment alone (P = 0.0363; log-rank test) or castration alone (P = 0.0285; log-rank test).

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We believe the castration alone cohort to be the best comparison for the combined treatment as castration in the TRAMP will not control disease and even has a tendency to worsen outcome compared with no treatment (24). Similar to the findings of Gingrich and Kaplan (20, 31) who reported that 20% to 35% of castrated mice remained tumor-free at 24 weeks of age, we observed 30% of 24-week-old castrated mice remaining disease-free. Although pathology at 24 weeks is benign in a subset of castrated mice, 100% of the castrated survival cohort died due to poorly differentiated prostate cancer. These castrated mice did not have a better survival than PBS-treated controls. No statistical significance was found for survival between 5-aza and PBS (P = 0.9304), 5-aza and castration (P = 0.8548), or castration and PBS (P = 0.6704; Fig. 4D).

This is the first study to evaluate the effect of 5-aza on the emergence of androgen-independent prostate cancer in mice. In this proof-of-principle study, we evaluated treatment with a drug (5-aza) that affects the epigenome, which could be beneficial to prostate cancer patients that have a high likelihood of recurring after primary or secondary treatment for their cancer. 5-Aza is a very potent DNA methyltransferase inhibitor and it therefore offers the possibility for epigenetic cancer therapies. 5-Aza has been extensively studied in clinical trials of acute lymphoid leukemia, acute myeloid leukemia, chronic myeloid leukemia, myelodysplastic syndrome, head and neck cancer,

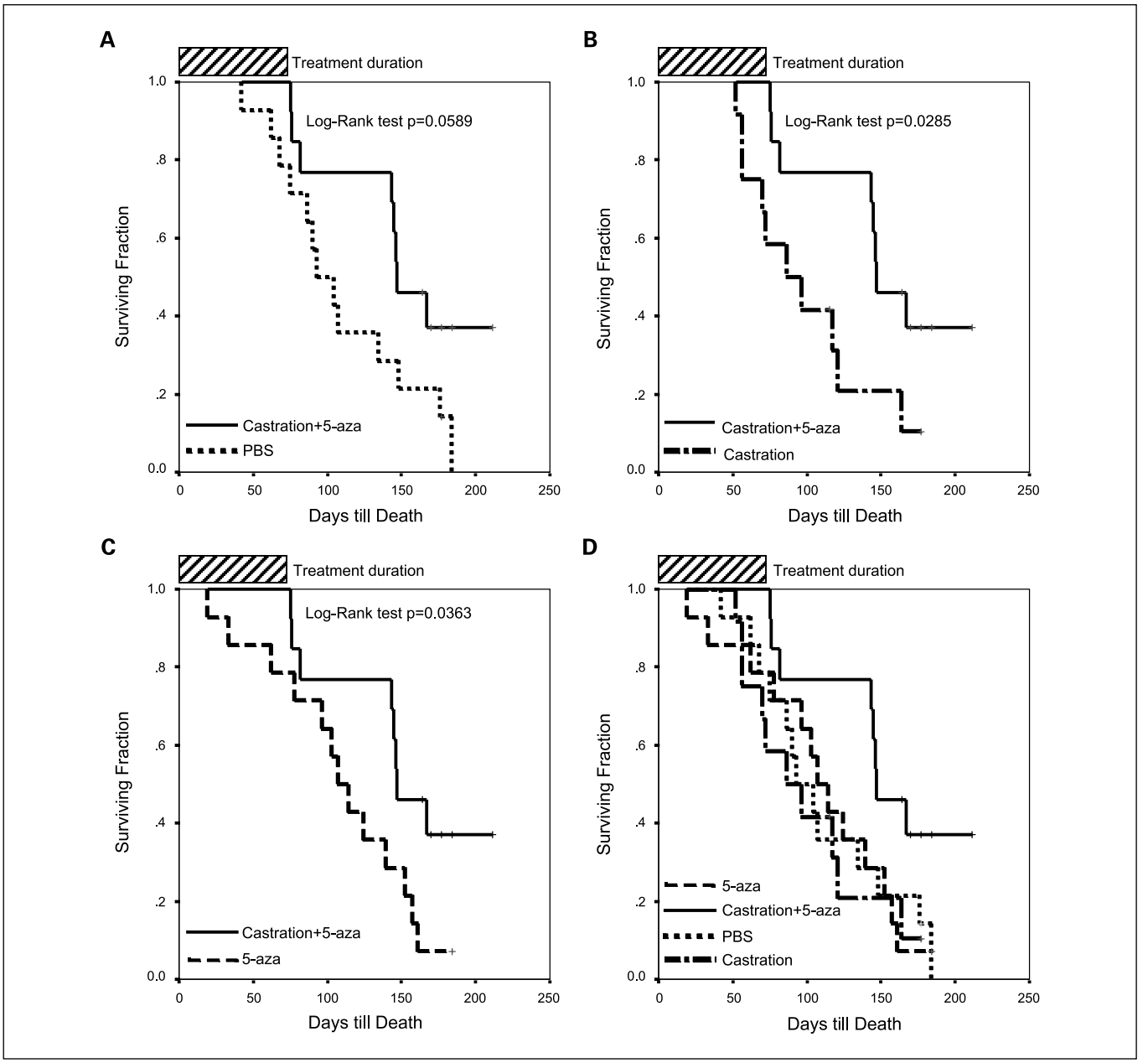

Fig. 4. Treatment with castration + 5-aza significantly increases survival of TRAMP mice. Treatment duration is indicated. *A,* Kaplan-Meier analysis for PBS and combined treatment. *B,* Kaplan-Meier analysis for castration and combined treatment. *C,* Kaplan-Meier analysis for 5-aza and combined treatment. *D,* Kaplan-Meier analysis of all treatment groups.

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colorectal cancer, melanoma, non–small cell lung cancer, renal cancer, cancer of the testes, and metastatic tumors (15) and has recently been approved by the Food and Drug Administration for the treatment of myelodysplastic syndrome (32). Currently, clinical trials are investigating its usefulness in the treatment of solid tumors. In cell culture studies, 5-aza has been found to be useful as a sensitizer for other chemotherapies as it reverses drug resistance (25–29). To get a benefit from chemosensitization, it seems necessary that 5-aza is administered before any other chemotherapeutic as 5-aza may function to demethylate proapoptotic genes (33).

We hypothesized that the emergence of androgen-independent disease may result in part from aberrant hypermethylation, which could be prevented or delayed by the addition of 5-aza in combination with androgen ablation. In this scenario, 5-aza would target hormone-refractory cells and restore expression of tumor-suppressor genes, proapoptotic genes, and most importantly androgen-responsive genes. Jarrard et al. (34) showed that whereas the androgen receptor promoter is hypermethylated in some androgen receptor–negative cell lines, its expression can be restored by treatment with 5-aza *in vitro*. An independent study also showed that 5,6-dihydro-5′-azacytidine, another DNA methyltransferase inhibitor, was capable of restoring androgen receptor responsiveness in DU 145 cells (35).

5-Aza has been evaluated in hormone-independent metastatic prostate cancer patients in a phase II trial by Thibault et al. (36). The study found a modest clinical activity although the patient cohort consisted of men well past the onset of androgen-independent prostate cancer. In this study, patients received 5-aza infusions for 1 h every 8 h for 24 h; however, 5-aza has a very short half-life (15-25 minutes) and more durable effects might be achieved through a low-dose pulse (1 h) schedule as described by Issa in patients with myelodysplastic disease (37, 38). Because of these results, we believe the effect of 5-aza in addition to castration can be improved by an optimized administration of the drug. As 5-aza is S-phase specific (39) and knowing that prostate cells have a relatively slow cell cycle, it would be preferable to increase the stability of the drug or find a drug that is more stable but capable of inducing the same effects. A possible alternative is zebularine [1-(β-D-ribofuranosyl)-1,2-dihydropyrimidin-2-one], which is another cytidine analogue able to inhibit DNMT function and is more stable than 5-aza. Zebularine can be administered orally and is less toxic. Although zebularine seems to be less potent than 5-aza at restoring methylated p16 in human cancer cell lines (5-aza doses were 10- to 100-fold lower), its greater stability allowed for continuous administration, which did result in the reactivation of p16 (40, 41). In a different study, zebularine was shown to be as effective as 5-aza when comparing demethylated global DNA (42). According to another study, zebularine was not efficient at demethylating tissue inhibitor of metalloproteinase-3 in HCT116 cells compared with 5-aza (56% versus 84%; ref. 32).

In conclusion, the combined treatment of castration + 5-aza significantly prolongs survival, and 5-aza seems to delay the onset of androgen-independent disease in the TRAMP mouse model. Although our study showed an overall survival benefit compared with individual treatments, further research is necessary to delineate the precise mechanism of 5-aza action (specific gene targets) and to exploit its full potential for prostate cancer therapeutics.

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