Azacitidine improves antitumor effects of docetaxel and cisplatin in aggressive prostate cancer models

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*(C Festuccia and G L Gravina contributed equally to this work)

Abstract

One of the major obstacles in the treatment of hormone-refractory prostate cancer (HRPC) is the development of chemoresistant tumors. The aim of this study is to evaluate the role of azacitidine as chemosensitizing agent in association with docetaxel (DTX) and cisplatin using two models of aggressive prostate cancer, the 22rv1, and PC3 cell lines. Azacitidine shows antiproliferative effects associated with increased proportion of cells in G0/G1 and evident apoptosis in 22rv1 cells and increased proportion of cells in G2/M phase with the absence of acute cell killing in PC3 cells. In vivo, azacitidine (0.8 mg/kg i.p.) reduced tumor proliferation and induced apoptosis in both xenografts upmodulating the expression of p16INKA, Bax, Bak, p21/WAF1, and p27/KIP1, and inhibiting the activation of Akt activity and the expression of cyclin D1, Bcl-2, and Bcl-XL. In vitro treatments with azacitidine lead to upregulation of cleaved caspase 3 and PARP. BCl2 antagonists, such as HA-14-1, enhanced the effects of azacitidine in these two prostate cancer models. In addition, azacitidine showed synergistic effects with both DTX and cisplatin. In vivo this agent caused tumor growth delay without complete regression in xenograft systems. Azacitidine sensitized PC3 and 22rv1 xenografts to DTX and cisplatin treatments. These combinations were also tolerable in mice and superior to either agent alone. As DTX is the standard first-line chemotherapy for HRPC, the development of DTX-based combination therapies is of great interest in this disease stage. Our results provide a rationale for clinical trials on combination treatments with azacitidine in patients with hormone-refractory and chemoresistant prostate tumors.

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Introduction

Prostate cancer represents a global public health problem. Worldwide, it is the second most common noncutaneous cancer in men, accounting for ~10% of male cancers (Haas et al. 2008). In recent years, 5 year survival rates for prostate cancer have been ranked the third highest of all cancers (Desireddi et al. 2007, Svatek et al. 2008). Although chemotherapy historically has had limited utility in treating advanced prostate cancers, the results from two recent randomized clinical trials indicated that docetaxel (DTX)-based chemotherapy improved survival in patients with hormone-refractory prostate cancer, HRPC (Patel et al. 2005, Armstrong et al. 2007). Docetaxel and mitoxantrone are two food and drug administration – approved chemotherapeutic agents for clinical treatment of advanced HRPC and DTX is the standard first-line chemotherapy in this disease stage.
Thus, the development of DTX-based combination therapies is of great interest for HRPC. Some clinical studies supported taxane-based therapy, a promising platform to develop new combinational schedule, which remains a high priority for many ongoing studies (Patel et al. 2005, Armstrong et al. 2007 and for a review, see Calabro` and Stemberg 2007). In addition a recent report shows that single-agent satraplatin improves progression-free survival (PFS) as second-line chemotherapy for patients with HRPC and provides further evidence that platinum salts may have a role in the treatment of HRPC (Nakabayashi et al. 2008, Ross et al. 2008). Nevertheless, improvement in the efficacy of chemotherapy is urgently needed for patients with chemotherapy-resistant HRPC.

It is now well established that cancer cells exhibit a number of genetic and epigenetic defects in the machinery that governs programmed cell death and that sabotage of apoptosis is one of the principal factors aiding in the progression and treatment of neoplastic diseases. Epigenetic modifications, specifically DNA hypermethylation, are believed to play an important role in the downregulation of genes important for protection against apoptosis. In addition to classical genetic abnormalities, epigenetic modifications have emerged as a central driving force in the molecular pathology of prostate cancer (McCabe et al. 2006, Murphy et al. 2008). Several years ago, Plumb et al. (2000) showed that resistance to carboplatin in ovarian cancer cells was mediated by hypermethylation and loss of function of the MLH1 mismatch repair gene. Of interest, decitabine, a deoxyderivative ofaza-CR, a cytosine analogue with hypomethylating properties, reverses the downregulation of expression of some membrane transporters in vitro (Plumb et al. 2000), raising the untested possibility that repeated treatment could lead to a progressive increase in efficacy.

In this study, we used azacitidine and its pharmacological formulation (Vidaza) to verify if this agent was able as single agent to alter tumor cell proliferation and apoptosis, as well as to improve the sensitivity against DTX and cisplatin in vitro and in vivo using two models of aggressive prostate cancer. 22Rv1 is an androgen-responsive human prostate carcinoma cell line derived from a primary prostate tumor that expresses mutant (H874Y) androgen receptors (AR), whereas PC3 is an androgen irresponsive, AR negative prostate carcinoma cell line. Our results indicate that azacitidine has antiproliferative effects in both cell lines and showed pro-apoptotic effects in 22rv1 cells. In vitro showed synergistic effects with DTX and cisplatin. Its pharmacological preparation (Vidaza) caused tumor growth delay without complete stasis or regression as single agent, whereas improved anti-tumor effects of DTX and cisplatin resulting these combinations well tolerable in mice. Therefore, use of DNA methylation inhibitors might be a suitable therapeutic tool for the amplification of pharmacologic responses of HRPC versus first- and second-line chemotherapies.

Materials and methods

Reagents

All the materials for tissue culture were purchased from Hyclone (Cramlington, NE, USA). Antibodies when not otherwise specified were purchased from Santa Cruz (Santa Cruz, CA, USA). Plasticware was obtained from Nunc (Roskilde, Denmark). Azacitidine (Vidaza) was obtained in collaboration with Celgene Corporation (Summit, NJ, USA). DNA methyltransferase activity was evaluated in nuclear cell extracts by a colorimetric EpiQuik DNA methyltransferase Activity Assay Kit (BioVision, Mountain View, CA, USA).

Cell lines

The two aggressive prostate cancer models (22rv1 and PC3 cell lines) were obtained from American Tissue Culture Collection (ATCC, Rockville, MD, USA) and grown as recommended.

Cell proliferation inhibition assay

Cell proliferation was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) assay. Briefly, cells were seeded in 96-well tissue culture plates (Costar, Corning, NY, USA) at 3000 cells per well per 150 μl. At 24 h after seeding, cells were treated with 50 μl serial concentrations of azacitidine, DTX, cisplatin or the combination of azacitidine plus DTX and cisplatin. After 72 h of drug exposure, MTT (100 μl) solution (2 mg/ml) was added to each well to incubate for 2 h. Absorbance at 570 nm was recorded using a 96-well plate reader. Inhibition curves were drawn by means of values obtained by OD percentages versus control for each concentration and IC₅₀ values were calculated by the GraFit method (Erithacus Software Limited, Staines, UK). The Chou and Talalay (1984) combination index (CI) analysis is a well-established index to determine the pharmacologic interaction of two drugs. When CI = 1 (represents an additive effect of the two drugs), the CI formula is in the same form as a traditional isobologram equation. Synergism is defined as less than the expected additive effect with CI < 1 and antagonism is defined as CI > 1.
Apoptosis analysis by flow cytometry

Treated and untreated cells were collected and fixed for 30 min by the addition of 1 ml 70% ethanol. After 30 min, the cells were pelleted by centrifugation (720g; 5 min), and resuspended in 1 ml DNA staining solution (PBS containing 200 mg/ml RNase A, 20 mg/ml propidium iodide plus 0.1% Triton X-100) and stained by incubation at room temperature for 60 min. All cells were then measured on a FACScan flow cytometer (Becton Dickinson, Bedford, MA, USA) and analyzed using Cell Quest software (Becton Dickinson). Resulting DNA distributions were analyzed by ModFit (Verity Software House, Topsham, ME, USA) for the proportion of cells in sub-G0 (apoptotic cells), G1, S, and G2-M phases of the cell cycle.

In vivo treatments

Male CD1 nude mice (Charles River, Milan, Italy) were maintained under the guidelines established by our Institution (University of L’Aquila, Medical School and Science and Technology School Board Regulations, complying with the Italian government regulation no. 116, January 27 1992, for the use of laboratory animals). All mice received s.c. flank injections of 1×10^6 PC3 and 22v1 cells. Tumor growth was assessed by bi-weekly measurement of tumor diameters with a Vernier caliper (length × width). Tumor weight was calculated according to the formula: \( TW (mg) = \text{tumor volume} (\text{mm}^3) = \frac{d^2 	imes D}{2} \), where \( d \) and \( D \) are the shortest and the longest diameters respectively. Treatments used in our in vivo study were started when tumor volumes reached about 80 mm^3 (Day 0) and were stopped after 28 days. Group study were started when tumor volumes reached about diameters respectively. Treatments used in our in vivo treatments

1: 10 mice received i.p. injections of 100 l PBS for consecutive 7 days. Group 2: 10 mice received i.p. injections of 100 l of aza-CR (0.8 mg/kg) for consecutive 7 days. Group 3: 10 mice received i.p. injections of DTX (7.5 mg/kg per week) every week for a maximum of four administrations. Group 4: 10 mice received i.p. injections of 100 l of aza-CR (0.8 mg/kg) for consecutive 7 days followed by a dose of DTX (7.5 mg/kg per week) every week for a maximum of three administrations. Group 5: 10 mice received i.p. injections of 5.0 mg/kg per day cisplatin for 10 consecutive days. Group 6: mice received i.p. injections of 100 l of aza-CR (0.8 mg/kg) for consecutive 7 days followed by i.p. injections of 5.0 mg/kg per day cisplatin for 10 consecutive days. Experiments were stopped at 28th day. We replicate the experiments in order to analyze a total of 20 animals for group. The effect of azacitidine alone or in combination with cisplatin (CP) or DTX on tumor growth was evaluated measuring, for all groups, the mean tumor weight at the end of experiments. Fractional tumor volume (FTV) for each treatment group was calculated as the ratio between the mean tumor volumes of treated and untreated animals. This was performed for treatment a (FTVa), for treatment b (FTVb), and for treatment a+b (FTV a+b). Combination indices were assayed as described above. Animals were killed by spinal cord dislocation and tumors were subsequently removed surgically. A part of the tumor was directly frozen in liquid nitrogen for protein analysis and the other part was fixed in paraformaldehyde overnight for histochemical evaluations and immunohistochemical analyses including endothelial (CD31 and vWF; Dako, Glostrup, Denmark), proliferative (Ki67; Dako), apoptosis (bcl2, p21, p27, death receptors and their ligands Santa Cruz), and signaling (p-Akt; Rockland Immunochemicals, Gilbertsville, PA, USA) marker analysis. Anti-DNMT1, DNMT3a, and DNMT3b antibodies were purchased from Biocarta (Hamburg, Germany). Indirect immunoperoxidase stain of tumor xenografts was performed on Biocarta (Hamburg, Germany). Indirect immunoperoxidase stain of tumor xenografts was performed on paraffin embedded with four micrometer tissue sections. Briefly, sections were incubated with primary antibodies for a night at 4°C. Next, avidin–biotin assays were done using the Vectastain Elite kit obtained from Vector Laboratories. Mayer’s hematoxylin was used as nuclear counterstain. Slides were analyzed separately from CF and PM.

Martius yellow-brilliant crystal scarlet blue technique

Stains for these techniques were purchased from HD Supplies (Aylesbury, UK). This technique was used to analyze the presence of red cells dispersed in tumor tissue and present in blood vessels as well as to evaluate better the presence of micro-thrombi and bleeding zones. Martius yellow, a small molecule dye, together with phosphotungstic acid in alcoholic solution stains red cells. Early fibrin deposits may be colored, but the phosphotungstic acid blocks the staining of muscle, collagen, and connective tissue fibers. Brilliant crystal scarlet, a medium-sized molecule, stains muscle, and mature fibrin. Phosphotungstic acid removes any red stain from the collagen. The large molecule dye aniline blue stains the collagen and old fibrin.

Hemoglobin assay

Tumor hemoglobin levels were quantified as described elsewhere (14). Tumors were homogenized in double-distilled water. Eighty microliters of homogenates were mixed with 1 ml Drabkin’s solution and
incubated for 15 min at room temperature. After centrifugation at 400 g for 5 min, the supernatants were subjected to absorbance measurement at 540 nm. The absorption that is proportional to hemoglobin concentration, was divided by tumor weight.

Assessment of global methylation levels

Proteins were extracted from untreated and in vivo Aza-CR-treated tumors and cells at 7, 14, 21, and 28 days and DNMT activity were assayed. As described, frozen tumors were pulverized in a liquid nitrogen-cooled Thermovac pulverizer. The resulting powders were homogenized in 10 volumes of a 10 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA, 0.5 mM dithiothreitol, 10 mM sodium molybdate, phosphatase inhibitor cocktail 2 with a dilution 1/100, and protease inhibition cocktail 2 with a dilution 1/100, both from Sigma-Aldrich. The homogenates were centrifuged for 1 h at 105 000 g (4 °C) and the supernatants (cytosols) were used for protein determination by immunoblotting. Total protein content was measured using the bicinchoninic acid assay.

Western blot analysis

Cells were washed with cold PBS and immediately lysed with 1 ml lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 1 mM sodium orthovanadate, 30 mM p-nitrophenyl phosphate, 10 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Flash-frozen tissue samples were crushed using liquid nitrogen pre-chilled mortar and pestles. Upon addition of lysis buffer, tissues were further homogenized with an electric homogenizer and centrifuged in a microfuge a top spin. Supernatants were collected and analyzed. Cell lysates and tissue extracts were electrophoresed in 7% SDS-PAGE, and separated proteins transferred to nitrocellulose and probed with the appropriate antibodies using the conditions recommended by the antibody suppliers.

Statistics analysis

Continuous data were expressed as mean and s.d. and were compared using an unpaired Student’s t-test. Categorical data were analyzed by the exact Fisher’s test. P value less than 0.05 were considered statistically significant.

Results

In vitro experiments: effects of azacitidine as single agent

Initially, we analyzed the effects of azacitidine on DNA methylation and expression of DNMT1, DNMT3a, and DNMT3b. After 48 h of treatment with azacitidine, the decrease in the DNMT activity was maximal after 10 days and it was maintained up to 2 weeks (Fig. 1A). Simultaneously, a significant decrement in DNMT1 and DNMT3b expression levels occurred with a peak observed at 5–10 days (Fig. 1B). Conversely, azacitidine treatment did not affect the protein expression levels of DNMT3a. Antiproliferative effect of azacitidine treatment was higher in 22rv1 cells than in PC3 cells with IC50 of 0.5 and 2.5 μM respectively. Treatment of with azacitidine resulted in a dose- and time-dependent inhibition of growth with G1 phase arrest associated to apoptosis in 22rv1 cells, whereas this drug arrested PC3 cells in G2/M phase without acute cell killing. Also, azacitidine increased the levels of p21, p27, p53, Bcl-Xs, and Bax and to reduce Bcl2 and Bcl-Xl expression in 22rv1 cells (Fig. 2A). This was also associated with an increased expression of cleaved caspase 3 and PARP. The expression of p21, Bcl2, caspase 3, and PARP was
not changed in PC3 cells following azacitidine treatment, whereas the expression of protein associated to G2/M arrest such as p16INKA, cyclin B and cdk1, cdk2 and cdk4 were differentially modulated (Fig. 2B). The treatment with HA-14-1, a Bcl2 antagonist, was able to induce more apoptotic events when co-administrated with Aza-CR in PC3 (Fig. 3A) and 22rv1 cells (Fig. 3B). These results suggest that the amount of apoptosis is related to Bcl2 levels and the low levels of apoptosis observed in PC3 cells might be partially associated with the higher expression of Bcl2. Aza-CR treatment in PC3 cells was also associated with the phosphorylation of p38 MAP kinase and with the reduced expression of activated Erk (Fig. 5B) which is required for the induction of cell cycle arrest in the G2/M phase. However, the induction of p38 MAPK activity was able to start the apoptotic program by downmodulation of Bcl2 when Aza-CR treated PC3 cells were grown in polyhema-covered Petri dishes (Fig. 3C). The apoptosis mediated by absence of adhesion (anoikis) was induced by a p38 MAPK-dependent Bcl-2 downmodulation (Fig. 3D) because SB203580, a p38 MAPK inhibitor, treatment caused a significative slow down in Aza-CR/polyhema-dependent Bcl2 downmodulation.

**In vitro effects of azacitidine in combination with DTX or cisplatin**

We analyzed the effects of combined treatments with azacitidine (0.5–1 μM for PC3 and 0.1–0.5 μM for 22rv1), DTX (0.5, 1.0, and 10 nM for both cells) and cisplatin (1, 2.5, and 10 μg/ml for both cells). Azacitidine sensitized versus DTX and cisplatin in PC3 and 22rv1 cells (Tables 1 and 2). In PC3 cells, the stronger antitumor effect was observed with azacitidine (1.0 μM) and DTX (1.0 nM (CI = 0.29)) and with azacitidine (1.0 μM) and cisplatin (10 μg/ml (CI = 0.27)). In 22rv1 cells, the highest antitumor effects were observed with azacitidine (0.5 μM) and cisplatin (10 μg/ml (CI = 1.0)).

![Figure 2](image-url) Western blot analysis on the expression of pro-apoptotic and antiapoptotic elements in 22rv1 (A) and PC3 (B) cells treated with 1, 2.5, and 5.0 μM aza-CR. In 22rv1 cells, aza-CR increased the expression of pro-apoptotic elements such as p21 (twofold with 2.5 and 5.0 μM aza-CR), p53 (four- to five-fold with 2.5 and 5 μM aza-CR), Bax (two- to four-fold with 2.5 and 5 μM aza-CR) and Bcl-XS (twofold with both 2.5 and 5 μM aza-CR) as well as the activity of caspase 3 (four- to six-fold with 2.5 and 5 μM aza-CR). The antiapoptotic element Bcl2 and Bcl-XL was reduced (about tenfold with both 2.5 and 5 μM aza-CR). In PC3 cells, no caspase 3 and PARP activity was showed and this was associated to no significant modulation of Bcl2 and p21 expression. Modulation in check point proteins of cell cycle was, however, shown. P16INK4A levels were significantly increased (about twofold with 5.0 μM aza-CR), Cdk1 levels were increased of about two with both 1.0 and 2.5 μM aza-CR and of about fourfold with 5.0 μM aza-CR, Cdk2 levels were weakly reduced (1.5-fold with 5.0 μM aza-CR), Cdk4 levels were significantly reduced of about fourfold with 2.5 and 5.0 μM aza-CR and cyclin B1 levels were not modified. Each lane was loaded with 40 μg proteins and normalized versus actin. This experiment is representative of three individual experiments.
DTX (1.0 nM (CI = 0.50)) and with azacitidine (1.0 μM) and cisplatin (2.5 μg/ml (CI = 0.44)).

**In vivo effects of azacitidine as single drug or followed by DTX or cisplatin**

The i.p. administration of Vidaza (0.8 mg/kg for 7 consecutive days) on intact nude male mice inoculated subcutaneously with 22rv1 and PC3 cells resulted in a statistically significant tumor growth decrement ($P < 0.001$) in both xenograft models (Fig. 4). In PC3 xenografts (Table 3), following azacitidine treatment we demonstrated a 36.7% of reduction in tumor weight respect to controls ($P < 0.001$) with a tumor growth delay (TGD) of 5.5 days. The proliferation index was also significantly reduced to 42.6% in azacitidine treated tumors ($P < 0.001$) with tumor apoptosis of about 5%. The vessel count was also reduced to 18.7% ($P < 0.05$). In 22rv1 xenografts (Table 4), we demonstrated a 65.5% reduction of tumor weight in aza-CR treated mice respect to controls ($P < 0.001$), with a tumor growth delay (TGD) of 25.5 days. The proliferation index was also reduced to 74.3% ($P < 0.001$) and apoptosis was evident in about 15% of cells. The vessel count was reduced to 70.4%

![Figure 3](image-url) Role of Bcl2 in the absence of aza-mediated PC3 cell apoptosis. We used the Bcl2 inhibitor HA-14.1 to demonstrate that bcl2 overexpression in PC3 cells could be responsible for the absence of acute apoptosis induced by aza-CR treatment. HA-14.1 (10 μM) triggered apoptosis of PC3 cells both in basal culture condition and in the presence of aza-CR pretreatment (1.0 μM). HA-14.1 was synergistic with aza-CR in PC3 cells, whereas this drug was only additive in 22rv1 cells. These results are representative of three individual experiments. Each lane was loaded with 40 μg proteins.

**Table 1** Pharmacological parameters of combination treatment with azacitidine and docetaxel in PC3 and 22rv1 cells

<table>
<thead>
<tr>
<th>Treatment A (DTX)</th>
<th>Treatment B (aza-CR)</th>
<th>Combination treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cells</strong></td>
<td><strong>Dose (nM)</strong></td>
<td><strong>GI</strong></td>
</tr>
<tr>
<td>PC-3</td>
<td>0.5</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.75</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.75</td>
</tr>
<tr>
<td>22rv1</td>
<td>0.5</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.55</td>
</tr>
</tbody>
</table>

GI = inhibition (percent versus control).
Similarly to that observed in vitro, the levels of DNMT1 and DNMT3b were also lower in azacitidine-treated mice when compared with controls both in PC3 and 22rv1 xenograft models (data not shown).

The treatment with azacitidine followed by DTX or CP was also studied in vivo. An increase in the effectiveness of DTX and cisplatin was observed after 7 days of co-administration with azacitidine both in PC3 and 22rv1 xenograft models (Fig. 4 and Tables 3 and 4). The results are as follows:

(i) Docetaxel and cisplatin alone reduced tumor weight of PC3 xenografts by 41.0% ($P<0.001$) and 22.2% ($P<0.01$), with a tumor growth delay of 5.1 and 2.1 days respectively when compared with controls. The proliferation index was also reduced to 49.9% ($P<0.001$) and 23.6% ($P<0.01$) respectively in DTX- and cisplatin-treated tumors. Apoptosis was evident only in DTX-treated tumors at levels of about 7% ($P<0.01$). In this xenograft model, 7 days of treatment with azacitidine followed by DTX or cisplatin resulted in a significant decrease in tumor weight of 76.4%

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**Table 2** Pharmacological parameters of combination treatment with azacitidine and cisplatin in PC3 and 22rv1 cells

<table>
<thead>
<tr>
<th>Treatment A (CP)</th>
<th>Treatment B (aza-CR)</th>
<th>Combination treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cells</strong></td>
<td><strong>Dose (µg/ml)</strong></td>
<td><strong>GI</strong></td>
</tr>
<tr>
<td>PC-3</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.80</td>
</tr>
<tr>
<td>22rv1</td>
<td>2.5</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>0.85</td>
</tr>
</tbody>
</table>

GI = inhibition.

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**Figure 4** Tumor proliferation in PC3 and 22rv1 xenografts in the presence of azacitidine alone or in combination with docetaxel (DTX) and cisplatin (CP). Group 1: 10 mice received (i.p.) injections of 100 µl PBS for consecutive 7 days. Group 2: 10 mice received i.p. injections of 100 µl of aza-CR (0.8 mg/kg) for consecutive 7 days. Group 3: 10 mice received i.p. injections of docetaxel (7.5 mg/kg per week) every week for a maximum of four administrations. Group 4: 10 mice received i.p. injections of 100 µl of aza-CR (0.8 mg/kg) for consecutive 7 days followed by a dose of docetaxel (7.5 mg/kg per week) every week for a maximum of three administrations. Group 5: 10 mice received i.p. injections of 5.0 mg/kg per day cisplatin for 10 consecutive days. Group 6: mice received i.p. injections of 100 µl of aza-CR (0.8 mg/kg) for consecutive 7 days followed by i.p. injections of 5.0 mg/kg per day cisplatin for 10 consecutive days. Proliferation was measured considering the variation of tumor volume (mm$^3$) in the time (days). Controls were administered with saline and treated xenografts were administered with 0.8 mg/kg for the first 7 days of treatment. Animals were killed at 28th day of treatment.
control animals. PI (proliferation index) was measured considering the mean of Ki67 positive cell percentage. TGD (tumor growth delay) was determined by the difference in time taken for the mean tumor volume to double in treated versus control animals. Pi (proliferation index) was measured considering the mean of Ki67 positive cell percentage.

Table 3 Antitumor activity of Vidaza in PC-3 tumors alone or in combination with docetaxel (DTX) or cisplatin (CP)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Mice</th>
<th>Weight of mice (g ± S.D.)</th>
<th>Tumor weight (g ± S.D.)</th>
<th>TGD (days)</th>
<th>PI (Ki67 %)</th>
<th>Apoptosis</th>
<th>Vessels</th>
<th>Tumor-free mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vidaza</td>
<td>0.8</td>
<td>20</td>
<td>25.3 ± 1.1</td>
<td>652 ± 220</td>
<td>25.5</td>
<td>11.7 ± 0.7</td>
<td>15.4 ± 3.0</td>
<td>11.4 ± 0.8</td>
<td>1/20</td>
</tr>
<tr>
<td>DTX</td>
<td>7.5</td>
<td>20</td>
<td>25.0 ± 1.7</td>
<td>305 ± 121</td>
<td>17.3</td>
<td>15.4 ± 5.1</td>
<td>10.2 ± 1.5</td>
<td>17.5 ± 3.0</td>
<td>0/20</td>
</tr>
<tr>
<td>Vidaza + DTX</td>
<td>0.8 + 7.5</td>
<td>20</td>
<td>21.8 ± 2.5</td>
<td>205 ± 125</td>
<td>28.0</td>
<td>9.3 ± 1.4</td>
<td>18.2 ± 2.5</td>
<td>9.5 ± 1.0</td>
<td>2/20</td>
</tr>
<tr>
<td>CP</td>
<td>5</td>
<td>20</td>
<td>25.1 ± 1.5</td>
<td>450 ± 101</td>
<td>7.5</td>
<td>21.3 ± 3.9</td>
<td>25.5 ± 2.5</td>
<td>7.5 ± 1.5</td>
<td>1/20</td>
</tr>
<tr>
<td>Vidaza + CP</td>
<td>0.8 + 5</td>
<td>20</td>
<td>20.8 ± 2.8</td>
<td>147 ± 66</td>
<td>30.2</td>
<td>7.5 ± 2.0</td>
<td>34.3 ± 8.0</td>
<td>7.4 ± 4.4</td>
<td>6/20</td>
</tr>
</tbody>
</table>

TGD (tumor growth delay) was determined by the difference in time taken for the mean tumor volume to double in treated versus control animals. PI (proliferation index) was measured considering the mean of Ki67 positive cell percentage ± S.D. measured on five random fields at ×100. Apoptosis was measured as the percentage of tunnel positive cells ± S.D. measured on five random fields (×400). Vessels were measured as the vessel number per field at ×100.

(P < 0.001 versus both Vidaza and DTX single treatments) and of 58.3% (P < 0.01 versus Vidaza and P < 0.001 versus cisplatin) respectively. Moreover, sequential regimens of azacitidine + DTX or azacitidine + cisplatin were respectively associated with tumor growth delay values of 25.5 and 15.2 days: reductions in the proliferation index of 96.5% (P < 0.001 versus both single treatments) and 81.5% (P < 0.001 versus both single treatments); significant increases in apoptosis of 18% (P < 0.01 versus both single treatments) and 14.3% (P < 0.01 versus both single treatments); and reductions in vessel counts of ~75% (P < 0.001 versus both single treatments) and 55% (P < 0.001 versus Vidaza and P < 0.01 versus cisplatin treatments). Additionally, 15.0% (3/20, NS) and 25.0% (5/20, P = 0.056, NS) of mice were tumor-free after 28 days of treatment.

(ii) In 22r1 xenografts, DTX or cisplatin alone reduced tumor weight of 46.8% (P < 0.001) and 30.9% (P < 0.001), with a tumor growth delay of 17.3 and 7.5 days respectively when compared with controls. The proliferation index was also reduced of 66.1% (P < 0.001) and 53.2% (P < 0.001) respectively in DTX- and cisplatin-treated animals. Apoptosis was evident in 10% (P < 0.05) and 25% (P < 0.01) of cells in DTX- and cisplatin-treated animals respectively. In this xenograft model, 7 days of treatment with azacitidine followed by DTX or cisplatin resulted in a significant decrease in tumor weight of 68.5% (P < 0.05 versus DTX treatment, whereas not significant versus Vidaza single treatment) and 77.5% (P < 0.001 versus both single treatments) respectively. Additionally, sequential regimens of azacitidine + DTX or azacitidine + cisplatin were respectively associated with tumor growth delay values of 28.0 and 30.2 days; reductions in the proliferation index of 79.6% (P < 0.01 versus Vidaza and P < 0.001 versus DTX single treatments) and 83.5% (P < 0.001 versus both single treatments); significant increases in apoptosis of 18.2 and 34.3% (P < 0.001 versus all single treatments); reductions in vessel counts of about 75% (P < 0.05 versus Vidaza and P < 0.001 versus cisplatin treatments) and 58.3% (P < 0.001 versus cisplatin treatments) respectively.

Table 4 Antitumor activity of Vidaza in 22r1 tumors alone or in combination with docetaxel (DTX) or cisplatin (CP)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>Mice</th>
<th>Weight of mice (g ± S.D.)</th>
<th>Tumor weight (g ± S.D.)</th>
<th>TGD (days)</th>
<th>PI (Ki67 %)</th>
<th>Apoptosis</th>
<th>Vessels</th>
<th>Tumor-free mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vidaza</td>
<td>0.8</td>
<td>20</td>
<td>24.1 ± 2.0</td>
<td>225 ± 55</td>
<td>25.5</td>
<td>11.7 ± 0.7</td>
<td>15.4 ± 3.0</td>
<td>11.4 ± 0.8</td>
<td>1/20</td>
</tr>
<tr>
<td>DTX</td>
<td>7.5</td>
<td>20</td>
<td>25.0 ± 1.7</td>
<td>305 ± 121</td>
<td>17.3</td>
<td>15.4 ± 5.1</td>
<td>10.2 ± 1.5</td>
<td>17.5 ± 3.0</td>
<td>0/20</td>
</tr>
<tr>
<td>Vidaza + DTX</td>
<td>0.8 + 7.5</td>
<td>20</td>
<td>21.8 ± 2.5</td>
<td>205 ± 125</td>
<td>28.0</td>
<td>9.3 ± 1.4</td>
<td>18.2 ± 2.5</td>
<td>9.5 ± 1.0</td>
<td>2/20</td>
</tr>
<tr>
<td>CP</td>
<td>5</td>
<td>20</td>
<td>25.1 ± 1.5</td>
<td>450 ± 101</td>
<td>7.5</td>
<td>21.3 ± 3.9</td>
<td>25.5 ± 2.5</td>
<td>7.5 ± 1.5</td>
<td>1/20</td>
</tr>
<tr>
<td>Vidaza + CP</td>
<td>0.8 + 5</td>
<td>20</td>
<td>20.8 ± 2.8</td>
<td>147 ± 66</td>
<td>30.2</td>
<td>7.5 ± 2.0</td>
<td>34.3 ± 8.0</td>
<td>7.4 ± 4.4</td>
<td>6/20</td>
</tr>
</tbody>
</table>

TGD (tumor growth delay) was determined by the difference in time taken for the mean tumor volume to double in treated versus control animals. PI (proliferation index) was measured considering the mean of Ki67 positive cell percentage ± S.D. measured on five random fields at ×100. Apoptosis was measured as the percentage of tunnel positive cells ± S.D. measured on five random fields (×400). Vessels were measured as the vessel number per field at ×100.
DTX single treatments) and 81% ($P < 0.001$ versus Vidaza and NS versus cisplatin single treatments). Moreover, 10.0% (2/20) and 30.0% (6/20) of mice treated with the Vidaza plus DTX and Vidaza plus cisplatin combined treatments were tumor-free after 28 days, although only the second combined treatment was statistically significant versus control ($P < 0.05$) and the statistic analysis showed not significant values for the comparisons versus Vidaza or cisplatin ($P = 0.096$ for both comparisons).

(iii) Sequential treatments with either azacitidine + DTX or azacitidine + cisplatin resulted in a significant reduction in tumor blood vessels compared with controls (Tables 3 and 4) both in PC3 and 22rv1 xenografts. This antiangiogenetic effect was further supported by quantification of hemoglobin content in

![Figure 5](A) Western blot analysis showing the increment of DNMT1 and DNMT3b expression without modulation of Akt activity after CP treatment in PC3 and 22rv1 tissues. Forty micrograms of proteins were loaded in each lane. (B). Immunohistochemical appearance of PC3 tumors harvested from control and CP treated mice showing increased DNMT1 expression after CP treatment. (C). Microscopic appearance of PC3 and 22rv1 tumors stained with Martius yellow-brilliant crystal scarlet blue technique. We show the presence of large blood vessels (stained in yellow/orange) near to cancer cell nests of untreated 22rv1 tumors. Untreated PC3 tumors show the presence of numerous and small blood vessels scattered in the tumor mass and delineating single tumor cell nests. Vidaza reduces blood vessel number both in 22rv1 and PC3 xenografts. Vessels appear dilated with unstructured capillary bed dispersed in collagen I deposits (azure staining) primarily in 22rv1 tumors. DTX treated 22rv1 tissues appear with large unstructured capillary bed and abundant hemorrhagic areas. The presence of numerous phagocytes (neutrophilic granulocytes and monocytes) dispensed in collagen I deposit suggests that a previous coagulative necrosis was present. In the combined treatment (Vidaza plus DTX), the above-mentioned appearance was amplified with deposition also of fibrin clots (pale pink-staining) evident in 22rv1 tissues and formation of dense collagen I deposits (fibrosis) which envelop tumor cell nests. Single pictures are at $\times 400$. (B) Angiogenesis was evaluated measuring hemoglobin presence in tissue extracts or counting CD34/von Willebrand double positive endothelial cells forming blood vessels. Full colour version of this figure available via [http://dx.doi.org/10.1677/ERC-08-0130](http://dx.doi.org/10.1677/ERC-08-0130).
tumor tissues, used as a marker of the number of blood vessels. To specifically assess vascular density within the tumors, sections were stained with Masson trichromic and BMBS. Blue-stained spiral strands of collagen were detected in peripheral areas, whereas PC3 cells grow to form large cell masses that were surrounded by red-stained blood vessels that often seemed dilated. Control xenografts exhibited numerous dilated and even sinusoid-looking vessels as compared with treated xenografts. The antiangiogenic effects of azacitidine followed by either DTX or cisplatin were further confirmed by immunohistochemical localization of von Willebrand factor that is a marker for endothelial cells and the presence of microvessels. However, a reduction in microvessel density was only observed in tumors treated with azacitidine + cisplatin compared with controls or with azacitidine alone. Results also showed antiangiogenic effects with the combination of azacitidine and DTX, including significant reductions in the vessels analyzed with the presence of fibrin clots and red dispersion in the tumor parenchyma, and the amount of hemoglobin analyzed in tissue extracts (Fig. 5A and B).

(iv) In addition, we found that treatments with cisplatin were able to increase the levels of DNMT1 in PC3 but not in 22rv1 cells. This is in agreement with the higher effects of combination azacitidine plus cisplatin in PC3 when compared with those observed in 22rv1. In Fig. 5C, we show western blot analysis performed on PC3 and 22rv1 tissue extracts whereas in Fig. 5D, we show the expression of DNMT1 in PC3 xenografts with or without cisplatin treatment. DTX was not able to modify DNMT expression in PC3 and 22rv1 xenografts (data not shown) supporting the high effects of combination treatment with azacitidine and cisplatin observed in both PCA models.

Discussion
One of the major obstacles in curing locally advanced and metastatic prostate cancer is the development of resistance to therapy. Historically, chemotherapy has had limited utility in treating castration-resistant locally advanced and metastatic prostate cancer. Nevertheless, DTX is the standard first-line chemotherapy for this disease. The responses to DTX are, however, partial and associated with increased resistance to apoptosis, thus indicating a clear need for new therapies in HRPC patients. A recent report shows that single-agent satraplatin improves PFS as second-line chemotherapy for patients with HRPC (Nakabayashi et al. 2008, Ross et al. 2008). The resistance of tumor cells to anticancer agents remains a major cause of failure in the treatment of patients with cancer. The classic mechanism for the evolution of a multidrug-resistant phenotype was believed to involve a single molecular mechanism, such as overexpression of P-glycoprotein. However, it now seems that the multidrug-resistant phenotype represents a complex, multifactor process in which epigenetic mechanisms may play a crucial role. Epigenetic alterations, including aberrant promoter methylation, are responsible for the silencing of tumor suppressor genes and for increased resistance to chemotherapy. This highlights a potential role for DNMT as potential molecular targets in cancer therapy (Mishra et al. 2008). Exploiting gene reactivation by epigenetic acting agents in combination with cytotoxic therapies has been a strategy that holds much clinical promise (for review, see Lu et al. 2006). Although azacitidine has shown significant promise in the treatment of hematopoietic malignancies, and is approved for the treatment of myelodysplastic syndrome (MDS; Issa et al. 2005, Abdulhaq & Rossetti 2007), research into its activity in solid tumors has been less impressive and have been similarly disappointing (Schwartzmann et al. 2000, Pohlmann et al. 2002, Appleton et al. 2007). However, it is important to note that the majority of these studies used dose and schedule combinations that are now recognized to be suboptimal. In addition, the major issue involves either drug uptake or tumor cell proliferation rate because the analysis of tumor tissues showed a disappointingly small decrease of methylation (3%). This has been shown to be below the threshold necessary for resensitization to chemotherapy (Appleton et al. 2007). Our research demonstrates that tumor levels of DNMT1 and DNMT3b were lower in azacitidine-treated conditions both in vitro and in PC3 and 22rv1 xenograft models. Our in vitro data indicate that DNA methylation activity reaches maximum inhibition of inhibition at 10 days after a 48 h of azacitidine treatment. This observed inhibition remained at high levels for a maximum 2 weeks. In parallel, a significant downregulation in DNMT1 and DNMT3b expression occurred and peaked at 5–10 days, and persisted even after 14 days. This reduction of DNMT activity was associated with the in vitro and in vivo re-expression of markers of differentiation, including AR (Jarrard et al. 1998, Gravina et al. 2008), and is responsible for the restoration of responsiveness to hormonal therapy (Izbicka et al. 1999, Sonpavde et al. 2007, Gravina et al. 2008). This reduction in DNMT activity is also likely responsible for the delay in progression to
androgen independent disease and for the improvement in survival in the transgenic adenocarcinoma of mouse prostate mouse model of prostate cancer (Zorn et al. 2007). In the present report, we observed also that DNMT inhibition with azacitidine was able to induce the in vitro and in vivo expression of p16INKA, Bax, Bak, p21/WAF1, and p27/KIP1. This was also responsible for inhibiting the activation of Akt, the expression of cyclin D1, Bcl-2, and Bcl-XL associated with the processing of caspases-3 with increased apoptosis. Nevertheless, we observed an increased Bcl2 mediated apoptosis by culturing aza-CR treated cells in presence of polyhema which impedes cell adhesion. This condition induces a specialized apoptotic mechanism termed anoikis that is normally suppressed in tumor cells (Diaz-Montero et al. 2006, Glinsky 2006, Kupferman et al. 2007) including PC3 cells. Although the reactivation of anoikis by aza-CR can represent an important therapeutic approach to increase tumor cell death present in the blood stream and could slow down the metastatic process and contribute to antimetastatic treatments of advanced tumors, ad hoc experiments are required.

In addition, the downmodulation of p-Akt and members of Bcl-2 family could indeed sensitize PC3 and 22rv1 cells versus DTX and cisplatin increasing the antiproliferative and pro-apoptotic effects of single drugs both in vitro and in vivo. The in vivo experiments revealed synergistic effects with DTX and cisplatin both in PC3 and 22rv1 xenografts. Immunocytochemistry and enzymatic assays revealed also that in vivo DNMT1 expression was significantly increased in cisplatin treated 22rv1 and PC3 xenografts, whereas the induction of DNMT1 levels was lower in DTX treated tumors. This is in agreement with previous reports showing that an increased DNMT1 expression is a common event in a multimodality-resistant phenotype in tumor cells (Misrha et al. 2008), in cisplatin and taxane (Segura-Pacheco et al. 2006) resistance mechanisms. Taken together, our study indicate the DNMT downmodulation represents an useful therapeutic approach for enhancing chemosensitivity of prostate cancers to DTX and cisplatin and provide a rationale for clinical trials on combination treatments with azacitidine in patients with advanced prostate tumors.

**Declaration of interest**

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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