Abstract. Androgen receptor (AR) expression in prostate cancer (CaP) cells varies due to the multiple changes including epigenetic modifications such as DNA methylation and histone deacylation. DNA methyltransferase and histone deacetylase inhibitors are promising for the treatment of CaP. The aim of our study was to analyze the 5-aza-2'-deoxycytidine (Aza-dC) and sodium butyrate (NaB) effects on CaP cells with modified AR gene expression. The androgen-independent human prostate cancer cell lines PC3 (lacking a functional AR) and DU145 (strongly limited expression due to methylations in the AR gene) were used. PCR of bisulfite-modified DNA and RT-PCR with bisulfite-sequencing were used for AR gene analysis of DU145 and PC3 cells following their treatment with Aza-dC and/or NaB. Re-acetylated histones around the AR gene were detected by conventional PCR of immunoprecipitated DNA obtained from treated cells. In both cell lines without the AR expression, the combined treatment was followed with significant decrease of cell viability. The co-treatment of DU145 cells caused site-specific demethylation in the AR promoter region followed by gene re-expression and increased acetylation in histones H3 and H4. The co-treatment with Aza-dC and NaB was the most effective in demethylation and re-expression of the AR gene. In the AR gene promoter, the location and density of demethylated CpGs indicated the existence of distinct promoter hot spot that could be a target of AR gene inactivation therapy of CaP patients during androgen deprivation.

Introduction

The specific causes of prostate cancer (CaP) remain unclear; however, it is known that androgens stimulate the growth of prostate cells through the androgen receptor (AR). In the treatment of CaP, androgen deprivation is one of the most effective therapeutic approaches. However, androgen deprivation therapy (ADT) results in CaP progression. Once androgen stimulation has been eliminated, either surgically or pharmacologically, the AR continues to be active through a number of different mechanisms, including increased growth factor receptor signaling (1), insulin-like growth factor-1 (2) and interleukin-6 (3) as the main drivers. Resurgent AR can promote castration-resistant CaP (CRPC) formation and it has been shown to be both causative and sufficient to the lethal form of the disease (4). Mohler et al (4,5) described that the intratumoral testosterone levels in patients with CRPC were similar to untreated benign prostatic disease. Furthermore, the CRPC tissue has levels of dihydrotestosterone which appear capable of activating the AR since prostate specific antigen (PSA) is expressed at similar tissue levels in castration-resistant and androgen-stimulated CaP. They suggest a compelling paradigm shift; CaP that recurs during ADT is not androgen-independent. However it is possible but not confirmed, that cells primarily resistant to ADT are those with stem-cell properties that never required androgens for survival and growth (6). Overall, the results suggest that prostate tumors rarely encounter a completely androgen-depleted environment (7). An early hypothesis, in which most CRPC...
bypassed AR-regulated signaling pathways, was supported by the fact that commonly studied androgen-independent CaP cell lines, such as DU145 and PC3, did not express AR. To date, little information is available about the DNA methylation pattern on the AR after the short- and long-term androgen exposure or removal (8).

Among the different types of epigenetic changes, the most studied are DNA methylation and histone modifications (8–10). DNA hypermethylation in the promoter region is an important epigenetic mechanism for the downregulation of gene expression. However, the epigenetic mechanisms that lead these changes to cancer have yet to be fully clarified.

Epigenetics is defined as the heritable change in gene expression without change in DNA sequence (9). A number of these epigenetic modifications is reversible and, therefore, offers an opportunity for epigenetically targeted antitumor therapy. Generally, acetylation of histones can be reversed by histone deacetylases (HDACs) that remove acetyl groups and finally cause transcriptional inactivation. Histone deacetylase inhibitors (HDACi) cause a return of acetylated histones with incidences in activation of gene expression. In our study, the sodium butyrate (NaB), a naturally occurring HDACi with an inhibitory effect on endometrial and ovarian cancer cells (11) and with an ability to inhibit growth of colon and prostate cancer was used. Furthermore, agent 5′-aza-2-deoxycytidine (Aza-dC) is a well-known inhibitor of DNA methyltransferases (DNMTi). The Aza-dC acts through an irreversible and covalent binding to DNA methyltransferases followed by hypomethylation of replicating DNA (12).

Several studies have focused on an effect of DNMTi in CRPC to determine whether re-expressed AR gene can lead to the decrease of aggressive CaP (13,14). These in vivo studies found that Aza-dC treatment delayed CaP progression to androgen-independent disease and prolonged survival in the TRAMP mouse model of CaP. Experiments in re-expression of AR gene using DNMTi are ongoing as epigenetically targeted antitumor therapies utilizing re-expressed AR gene in combination with other therapies could be a new promising method for patients with CRPC (15).

The aim of our study was to investigate combined effect of DNA demethylation and histone re-acetylation on re-expressed AR on AR-negative CaP cell lines PC3 and DU145 (both representing the CRPC model) and to elucidate the potential effect of the re-expressed AR in these cancer cells. The Aza-dC and NaB as a combination of DNMTi and HDACi, respectively, may be able to suppress the viability of CaP cells and may contribute to slowing down the progression.

Materials and methods

Cell culture and cell conditions. In addition to the androgen-independent human CaP cell lines PC3 and DU145, androgen-independent C4-2 cell line was used for comparison due to its AR gene expression (16). The PC3 and DU145 cell lines were purchased from the ATCC (Rockville, MD, USA), the C4-2 cell line was purchased from UroCor Labs (Oklahoma City, OK, USA). DU145 and PC3 cells were maintained in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS), 0.01% antibiotics, 2 mM L-glutamine. C4-2 cells were maintained in RPMI-1640 media (Sigma, St. Louis, MO, USA) supplemented with a final 10% concentration of FBS, 0.01% antibiotics, 2 mM L-glutamine and 1 mM sodium pyruvate. All cells were maintained at 37°C in a 5% CO₂ atmosphere.

Treatment with DNMTi and HDACi. The PC3 and DU145 cell lines were treated with NaB (Sigma-Aldrich, St. Louis, MO, USA), Aza-dC (Sigma-Aldrich) and combinations of Aza-dC and NaB for 48 h and 6 days when medium and agents were exchanged after 48 h.

Cell viability assay. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to determine the 50% inhibition of viability (IC₅₀) concentrations of the studied agents as previously described (17). Both cell lines (35–40% confluent) were treated with NaB or Aza-dC or their combinations. The concentration leading to IC₅₀ was determined by measuring absorbance at 570 nm, using a Labsystems Multiskan RC ELISA reader. The viability of treated cells was expressed as a percentage relative to the viability of control vehicle-treated cells (18). Cell viability assay was conducted in triplicate wells and represents at least three independent experiment.

Sodium-bisulfite modification and DNA methylation analysis. Genomic DNA was isolated by the Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA) and sodium-bisulfite modification was made with the EpiTect® Bisulfite kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. PCR amplification was performed with the following primers: M2/F, 5′-TGG TTT AGG AAA TTA GGA GTT ATT TAG G-3′ and M2/R, 5′-TCC CTT CGA CTC CTA TAC AAC ACT A-3′ by initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 50 sec, elongation at 72°C for 1 min, and the final extension at 72°C for an additional 15 min after the last cycle. The PCR with M4 primers/F, 5′-AGG TTT TGG TAG AGA GGT AAT TTT TGG T-3′ and M4/R, 5′-CCT AAC TAC CTT TTC ATC TAA TCT T-3′ was performed with annealing at 50°C under the same conditions as previously described. M2 primers were designed for amplification of 383 bp in the promoter region and M4 primers amplified 235 bp region of exon 1 of AR gene (Fig. 1, numbering according to the CAG-transcription initiation site II) (19). Both were proposed to amplify only unmethylated DNA after sodium-bisulfite modification. The bisulfite-modified DNA was used in 25-µl PCR reaction mixture with DyNaZyme™ EXT DNA Polymerase (Finnzymes, Espoo, Finland). The PCR products were electrophoresed in 1.0% TAE agarose gels and visualized by ethidium bromide staining.

Bisulfite-sequencing. The DU145 and PC3 samples after the bisulfite modification and methylation analysis amplified with M2 and M4 primers, respectively, were purified with QIAEX® II Gel Extraction kit (Qiagen, Hilden, Germany) or QIAQuick® PCR Purification kit (Qiagen). Genomelab™ DTCS Quick Start kit (Beckman Coulter, Brea, CA, USA) and Beckman Coulter sequence analyzer were used for sequence analysis. Bisulfite-sequenced samples were aligned with wild-type AR sequence (NC_000023.10).
cDNA analysis. Total RNA was isolated with the High Pure RNA Isolation kit and converted to cDNA using the Transcriptor First Strand cDNA Synthesis kit (Roche, Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. The CAG primers were proposed for amplification of the 476-bp polymorphic CAG region of the AR gene. The following primers were applied to detect AR expression: CAG/F, 5'-CCT GTT GAA CTC TTC TGA G-3' and CAG/R, 5'-TCT GGG ACG CAA CCT CTC-3'. PCR amplification was initially denatured at 94°C for 4 min, followed by 40 cycles of denaturation for 40 sec, annealing at 58°C for 40 sec, elongation at 72°C for 1 min and the final extension at 72°C for an additional 10 min after the last cycle. The primers were designed for amplification of wild-type human DNA without sodium-bisulfite modification. For internal control, 89-bp PCR product of the TATA-binding protein (TBP) gene was amplified. DNA isolated from a healthy man served as a positive control for expression of the AR gene. Results were confirmed by a further independent PCR amplification.

Chromatin immunoprecipitation (ChIP). ChIP was performed as described by Trtkova et al (20) with slight modifications. Briefly, cells in 100-mm dishes were cross-linked with 1 ml of 11x formaldehyde stock solution (1% final concentration of formaldehyde). Cross-linking was stopped by the addition of glycine and cells were washed with PBS. The cells were scraped and lysed in Chro-IP lysis buffer containing protease inhibitors (Complete Protease Inhibitor Cocktail, Roche Diagnostics). The lysates were reversed on a rotator for 10 min and after centrifugation at 600 g for 5 min at 4°C followed by washing. Following centrifugation (600 g for 5 min at 4°C) pellets were resuspended in 1x RIPA buffer, sonicated and then centrifuged at 16,000 g for 10 min at 4°C. Anti-acetyl-histone H3, anti-acetyl-histone H4 and normal mouse IgG antibodies (Millipore, Upstate Biotechnology, Temecula, CA, USA) were added to aliquots of 600 µg of chromatin protein lysates and incubated with rotation at 4°C overnight. Salmon sperm DNA/Protein G agarose (Upstate Biotechnology) was added to the samples and incubated with rotation at 4°C overnight. The samples were centrifuged (600 g for 3 min at 4°C) and the pellets were washed with 1x RIPA buffer containing 100 µg/ml salmon sperm DNA, then with Low salt and High salt immune complex wash buffer and finally again with 1x RIPA buffer containing 100 µg/ml salmon sperm DNA. Following centrifugation, 100 µl of elution buffer was added and samples were placed at 65°C for 20 min with occasional gentle agitation. The agarose beads were removed by centrifugation when the supernatants were moved to new tubes. The cross-linking was reverted by heating at 65°C for 6 h and then proteinase K (100 µg/ml) was added to each tube and incubated at 55°C overnight with gentle agitation. DNA was purified with QIAquick PCR Purification kit (Qiagen) according to the manufacturer's instructions. For input we added 50 µg of each chromatin lysate and 0.5 µl of proteinase K (100 µg/ml) to each tube and incubated at 55°C for 3 h with gentle agitation followed by DNA purification. PCR amplification was performed with the following primers: forward, 5'-GGT GAA ACC AGC TTC TAGA CAG GAC GGC TA-3' and reverse, 5'-GTA CAG CAC TGG AGC GGC TA-3' identical to PCR conditions for M2 primers with modification in annealing temperature (58°C) and in elongation time (80 sec). These primers were designed for amplification of 369 bp in the promoter region of wild-type AR gene and partially overlap with the sequences of M2 primers.

Statistical analysis. Multifactorial analysis of variance (ANOVA) with a post-hoc two-sided Dunnett's t-test was used to compare cell viability between treatments and the vehicle-control when a single concentration was assessed. Multifactorial ANOVA with a post-hoc Bonferroni multiple comparison test was used to compare cell viability between treatments and the vehicle-control when time points and individual cell line were assessed. Analyses were performed with the SPSS software version 15 (SPSS, Inc., Chicago, IL, USA) and statistical significance of differences was set at P<0.05 (two-sided).

Results

Cell viability assay in DU145 and PC3 cells. The DU145 and PC3 cell viabilities treated with NaB and Aza-dC (as a single agent or in combinations) were assessed by MTT assay. In comparison to the control DMSO, the NaB treatment for 48 h induced a decrease of PC3 cell viability by concentrations from 0.75 mM up to 5 mM (P=0.06 for 0.75 mM, P=0.001 for 1 mM and P<0.0001 for other concentrations) (Fig. 2A). In the case of DU145 cells, effective NaB concentration to suppress cell viability was from ≥0.5 mM (P=0.07 for 0.5 mM, P=0.004 for 0.75 mM and P<0.0001 for higher concentrations) (Fig. 2A). Differences in viability between both cell lines were significant at the following NaB concentrations: 0.025 mM (P=0.004), 2.5 mM, 4 mM (P<0.0001) and 5 mM (P=0.0003) in favor of the PC3 cells. Thus, the NaB treatment appears to be more toxic for DU145 cells compared to PC3 cell survival. Use of Aza-dC and its co-treatment with NaB for 48 h resulted in a decrease of cell viability in PC3 cells (P<0.0001...
for all concentrations) and in DU145 cells only by 5 µM Aza-dC+1 mM NaB (P=0.041) (Fig. 2B) compared to DMSO treatments. Moreover, in DU145 cells we detected increased cell viability after 5 µM Aza-dC treatment (P=0.008). Under all concentrations used in this treatment, the cell viability of both lines exhibited significantly different cytotoxicity (P=0.009 and less). Fig. 2C with 6-day exposure shows a strong decrease of viability of PC3 cells compared to DMSO (P<0.0001 for all doses), while in DU145 cells, a decrease of viability after treatments with all concentrations of Aza-dC with 5 mM NaB was apparent. Furthermore, despite the cell viability decrease after the final three co-treatments in DU145 cells, the effect of the agent used is significantly more favorable for DU145 cell viability compared to the PC3 line at all concentrations (P<0.001 for 0.2 µM Aza-dC+1 mM NaB and P<0.0001 for the others).

DNA methylation status and demethylation effect of Aza-dC and NaB on the AR gene promoter and 5'-UTR region. The M2 and M4 primers amplified unmethylated CpGs of bisulfite-modified DNA. Using M2 primers, the 383-bp PCR product obtained from PC3 cells indicated unmethylated promoter (Fig. 3A, left part) whereas in 5'-UTR we suggest methylation changes (Fig. 3A, right part). Methylation analysis of DU145 cells resulted in 235-bp PCR product amplification of the 5'-UTR and failed amplification in the promoter region (data not shown).

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The Aza-dC (0.5 and 5 µM) and combinations 0.5 µM Aza-dC with NaB (1 and 5 mM) were used for determination of DNA demethylation effect in AR-negative cell lines. Treatment of PC3 cells with Aza-dC and used combinations with NaB for 48 h (Fig. 3B, treatments marked by numbers 1-5, left part) and 6 days (right part) resulted in amplification of 235-bp PCR products. The 48-h treatment of DU145 cells with Aza-dC and its combinations with NaB is shown in Fig. 3C. Unmethylation specific M2 primers generated 383-bp PCR product in the promoter region of the AR gene (48-h treatment, left part) and only 0.5 µM Aza-dC and 5 mM NaB co-treatment generated PCR products after a 6-day exposure (right part).
In DU145 cells, we analyzed whether the demethylation effect induced by the combination of 0.5 µM Aza-dC and 5 mM NaB could result in the re-expression of the AR gene. We performed cDNAs using reverse-transcription (RT) PCR after 48 h of treatment and, using CAG primers, we amplified the CAG region in exon 1 of the AR gene. In Fig. 3D (upper panel), only the combination of 0.5 µM Aza-dC and 5 mM NaB was effective in restoring the AR gene expression. The unaffected TBP gene expression served as internal control (Fig. 3D, lower panel).

**Bisulfite-sequencing of the AR gene promoter and 5'-UTR region in cells treated with Aza-dC and NaB.** Sequencing data from DU145 cells are shown in Fig. 4. The similar demethylation pattern (-52 and +80) in sequences of all tested treatments was found in the AR gene promoter. Treatment with 5 µM Aza-dC caused partial demethylation in -126, -124 and -57 CpG sites. Combination of 0.5 µM Aza-dC and 1 mM NaB induced partial demethylation in -124 position and co-treatment with 0.5 µM Aza-dC and 5 mM NaB resulted in complete demethylation of CpGs in positions -126, -124 and partial demethylation at the -122 site. Sequence analysis in PC-3 cells treated with Aza-dC alone and with Aza-dC and NaB combinations showed demethylation of all identified CpG sites in the 5'-UTR region (data not shown). Collectively, the most effective DNA demethylation effect was evident after co-treatment of the DU145 cells with 0.5 µM Aza-dC and 5 mM NaB.

**Amplification of androgen receptor from chromatin immunoprecipitated DNA.** ChIP analysis was performed with DU145...
cell line in order to determine and compare the status of acetyl-histone H3 and acetyl-histone H4 adjacent to the AR gene. For comparison we worked with the highest concentration of NaB (5 mM) and with the combination of 0.5 μM Aza-dC and 5 mM NaB that was effective in restoring the AR gene expression. After both treatments, we found increased acetylations in histones H3 and H4 in the AR gene promoter region compared to untreated control (Fig. 5).

**Discussion**

We studied epigenetic consequences of using the combination of DNA demethylation agent and histone deacetylase inhibitor in androgen-independent cells. In the both cell lines without the AR gene expression, the co-treatment with 5-aza-2’-deoxycytidine and sodium butyrate was more effective in demethylation of specific CpG sites in the androgen receptor (AR) gene promoter than Aza-dC treatment only (Fig. 4). We found that the AR gene re-expression was accompanied by increased acetylation in histones H3 and H4 in the AR gene promoter region compared to untreated control (Fig. 5).

The CpG islands methylations in the promoter region affect gene transcription and is generally regarded as a common form of gene expression regulation. For this reason, gene promoter sites that are methylated and associated with a loss of gene transcription might be identified as the sequence important or critical for gene transcription. Evidence of the epigenetic regulation of the AR is finding that androgen-independent prostate cancer (CaP) cell lines are associated with the AR gene hypermethylation and loss of the AR gene expression (14,21). Jarrard et al (21) further demonstrated that treatment with the Aza-dC induced re-expression of the AR gene in several metastatic CaP cells followed by induction of the prostate specific antigen (PSA) gene expression, while DU145 cells remained intact to treatment even in long-term culture. Although normal prostate epithelial cells showed no DNA methylation, the AR gene hypermethylation has been detected in 20% of 10 primary and 28% of 14 hormone-refractory CaP samples (22). In our study, we found that CpG sites from -126 to -122 nucleotide positions, previously known as hot spot (23), were partially or completely demethylated in DU145 cells. Demethylated CpG sites located upstream of the core AR gene promoter (-126 to -122) could represent hot spot important for AR gene transcription. These CpGs might be targets of AR gene-inactivation therapy and may therefore be more effective compared to the current use of androgen deprivation. In addition, complete demethylation in two CpG sites (-52 and +80) (Fig. 4) adjacent to the promoter core of the AR gene (-74 to +87) was detected. However, the position -43 in promoter core, representing Sp1 binding site for Sp1 transcription factor (24), stayed methylated as was confirmed by sequencing (Fig. 4). Similarly, the study conducted by Mishra et al (25) suggested that most CpG sites remain methylated, although demethylations following Aza-dC were significant.

It is known that nucleoside analogs, Aza-dC and/or 5-azacytidine (5-Aza) are incorporated into DNA during replication and inhibit methyltransferase activity. In addition, methylation of cytosines in and around genes can result in the gene silencing. DNA methylation possibly alters chromosome structure and defines regions important for transcriptional regulation (26). Thus, DNA methylation and histone hypo-acetylation appear to be distinct but connected silencing mechanisms (26-28). Moreover, using combination of the DNA methylation and HDACi might overcome a disadvantage of the NaB instability (29) and obtains a benefit from their synergistic effect. There is also a molecular rationale for the combination of HDACi with demethylating agents since hypermethylation can lead to compact nucleosomes resistant to re-acetylation (30). The precise mode of action of HDACi in CaP cells is not completely understood. The NaB exerts an extensive inhibitory effect on HDAC activities but it is a relatively weak HDACi for millimolar concentration requirements and low specificity in inducing gene expression (30,31). In our study, the Aza-dC and NaB single and particularly combined treatment induced significant cytotoxic effects in both cell lines (Fig. 2, compared to normal prostate cells RWPE1, our unpublished results). In other studies (32,33) the NaB administration to CaP cells increased the percentage of cells in G1 phase and induced apoptosis. We observed a dose-dependent decrease of cell viability in both lines that could be a consequence of demethylation and re-expression of pro-apoptotic and anti-proliferative genes (15,32-34).

If classifications of alterations in AR signaling include AR gene amplifications and mutations, changes in the level of ligand, changes in coregulatory molecules including coactivators and corepressors, increased levels of protein due to gene amplification or altered mRNA expression, upregulation of pathways that activate AR in the absence of androgens (5,9), there are several possibilities to explain why the PC3 cells do not express functional AR despite ambiguously detected methylations in AR gene (16,35). As described by Mizokami and Chang (36), the entire sequence from +21 to +149 nucleotide positions, including a stem-loop secondary structure at +109 to +129 of AR 5’-UTR (also known as hairpin or hairpin loop) may be needed for an induction of translation. In PC3 cells, we found completely unmethylated or demethylated sequence part of 5’-UTR (Fig. 3B and D).

Finally, our results suggest the role of induced histone re-acetylation on DNA demethylation with possible respon-
sibility for epigenetic inactivation of AR gene expression that warrants further research. Our findings may help to elucidate the effects of HDAC and DNMT inhibitors on the androgen-independent CaP cells representing the CRPC model.

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