Identification of Tumor and Invasion Suppressor Gene Modulators in Bladder Cancer by Different Classes of Histone Deacetylase Inhibitors Using Reverse Phase Protein Arrays

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Purpose: We assessed the ability of different classes of histone deacetylase inhibitors to target tumor and invasive suppressor genes in a panel of bladder carcinoma cell lines using reverse phase protein arrays.

Materials and Methods: Three poorly, moderately and highly invasive cell lines were exposed to histone deacetylase inhibitors, trichostatin A, apicidin, valproic acid (Sigma®) and MS-275 (AXXORA®) for 0 to 36 hours. Lysates were harvested and arrayed in a 10-fold dilution series in duplicate. Data points were collected and analyzed using a concentration interpolation methodology after normalization.

Results: Protein expression profiles revealed up-regulation of γ-catenin in highly invasive lines, and α-catenin in moderately and highly invasive lines after exposure to all histone deacetylase inhibitors, apicidin and MS-275, respectively. Gelsolin was up-regulated in poorly and moderately invasive lines after exposure to all histone deacetylase inhibitors. Desmoglein was down-regulated in poorly and moderately invasive cell lines by all 4 histone deacetylase inhibitors, in addition to decreased FAK (Transduction Laboratories™) expression in moderately and highly invasive lines exposed to valproic acid and MS-275.

Conclusions: Different histone deacetylase inhibitor classes have the potential to modulate tumor and invasive suppressor gene expression, identifying histone deacetylase inhibitors as potential therapeutic agents for bladder cancer. Reverse phase protein arrays enable high throughput screening of multiple compounds to assess the expression profile of specific protein groups targeted for therapy.

Key Words: urinary bladder; carcinoma; histone deacetylase inhibitors; genes, suppressor; protein array analysis

Abbreviations and Acronyms

DI = concentration interpolation
DMSO = dimethyl sulfoxide
DTT = dithiothreitol
FAK = focal adhesion kinase
HDAC = histone deacetylase
HDACi = HDAC inhibitor
IC_{50} = half maximum inhibitory concentration
PBS = phosphate buffered saline
RPPA = reverse phase protein array
TSA = trichostatin A

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EPIGENETIC regulation of genes in carcinogenesis is well established and controls the expression of oncogenic and tumor suppressor elements involved in different cancers. Such epigenetic induced changes[1] involve gene expression silencing by methylation[2] and acetylation/deacetylation events,[3] prompting efforts to restore normal gene expression profiles in cancer cells using demethylation agents and/or HDACis.[4] HDACis can be divided into 4 broad groups based on structure,[5] including hydroximates, cyclic peptides, aliphatic acids and benzamides.[3] TSA, valproic acid and MS-275, represent-

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ing 3 of the 4 HDACi classes, have been used in models of bladder cancer showing gene expression changes linked to the inhibition of growth and invasive behavior of bladder carcinoma cells.[6–13] Of the HDAC family 11 members show differential expression across tissues targeting different genes.[14] Members of each HDACi group interact with different HDAC family members[14,15] and are currently being evaluated in clinical trials.[5,16–21]

An alternative pathway to discover novel therapeutic approaches is to identify primary events underlying neoplastic progression and search for compounds to restore or negate the function of the specific gene(s) or proteins. We and others have identified members of the cadherin/catenin complex as altered in bladder tumorigenesis,[22] and involved in cell migration and invasion,[23,24] representing critical players in late stage, invasive bladder cancer. Such changes impact migration and invasion behavior underlying the metastatic process.

HDACis show considerable promise in the therapeutic arena. However, currently we know little of the specificity of action of different inhibitors on HDAC family members and their impact on tumor killing. We also do not know what gene(s) are best targeted for maximum therapeutic efficacy involving the restoration or suppression of different proteins linked to invasion. To search for these many different parameters we used high throughput RPPA to assess HDACis as potentially useful therapeutic agents modulating the expression of proteins involved in tumor and invasion suppression in bladder tumorigenesis.

MATERIALS AND METHODS

Cell Culture

The human bladder cell lines RT4, RT112, CUBIII, EJ, UM-UC-3, J82, HU456, 5637 (ATCC®) and BC16.1 were maintained in Dulbecco's modified Eagle's medium supplemented with 7.5% fetal bovine serum and penicillin/streptomycin. The invasive potential of the bladder carcinoma cell lines used was previously established in an in vitro invasion assay model.[25]

Histone Deacetylase Inhibitors

TSA, apicidin and MS-275 were dissolved in DMSO at stock concentrations of 1 mg/ml and diluted in medium. Valproic acid was dissolved in medium at a stock concentration of 300 mM. On clonogenic assays[26] the concentration range for each HDACi was 0 to 500 mM TSA, 0 to 3 μM apicidin, 0 to 30 mM valproic acid and 0 to 20 μM MS-275, from which we calculated the IC_{50} of each drug. From the IC_{50} a more limited range of drug concentration was selected to treat cells on RPPA analysis, including 0, 10, 50 and 100 mM TSA, 0, 0.01, 0.1, 1.0 and 3 μM apicidin, 0, 1, 5, 10 and 20 mM valproic acid, and 0, 0.1, 1.0, 5 and 10 μM MS-275. Using each concentration cells were exposed to each HDACi for 0, 4, 12, 18, 24 and 36 hours before harvest for RPPA.

Lysate RPPAs

Cells were washed in PBS, 1.5 ml ice-cold PBS were added, and cells were scraped, collected and centrifuged at 4,000 rpm for 2 minutes. After removing PBS the cell pellet was lysed in an equal volume (20 to 60 μl) of buffer containing 9 M urea, 4% CHAPS (Gentaur Laboratories, Brussels, Belgium), 2% Pharmalyte™ (pH 8.0 to 10.5) and 65 mM DTT by gentle finger tapping. After lysis the samples were centrifuged at 13,000 rpm for 30 minutes. All steps after the initial PBS wash were done at 4C. The resulting protein lysate was collected and stored at −80C.

To maintain proteins in denatured form the concentration of urea and CHAPS in the final samples was included at 6 M and 2%, respectively. Ten 2-fold serial dilutions were made from each lysate as described, including the 4-fold dilution made with buffer containing 5 M urea, 2% Pharmalyte (pH 8 to 10.5) and 65 mM DTT. The remaining dilutions were made with buffer containing 6 M urea, 1% CHAPS, 2% Pharmalyte (pH 8 to 10.5) and 65 mM DTT. Hence, only the lysate concentration changed along each dilution series.

Samples were arrayed using a 4 × 8 solid pinhead, which extracts 32 samples from a 4 × 8 well sector of a 384 microtiter plate at 1 dip. The print matrix was a nitrocellulose cast glass slide for which manufacturing conditions were optimized elsewhere for our RPPA. Each 384-well microplate contained 10-time 2-fold serial dilutions of each sample and 2 technical replicates per sample. All printing procedures were done under strict environmental control, such that microarrayer relative humidity was maintained at 80%. Resulting RPPAs were examined by microscopy for quality control.

Antibodies

Antibodies used were α, γ and β-catenin, FAK, E-cadherin (HECD-1), N-cadherin, desmoglein 2, desmocollin 2/3 (Zymed, South San Francisco, California), gelsolin, α-tubulin (Sigma) and plakophilin 3 (LifeSpan Biosciences, Seattle, Washington). RPPA antibody validation was done by Western blot analysis. Primary antibodies were screened to confirm production of a single protein band using a combination of bladder carcinoma cell lysates. Only antibodies that generated a single band on Western blot were used for signal detection on RPPA. Signals were detected by an Autostainer (Dako, Carpinteria, California) according to the manufacturer protocol.

Developing

Samples arrayed on nitrocellulose coated slides were washed 2 × 15 minutes in deionized water and blocked overnight in I-Block™ composed of 0.2% I-Block and 0.01% Tween-20 in phosphate buffered saline with continuous shaking at 4C. Microarrays were immunostained on an automated Autostainer Plus stainer (Dako) using a tyramine based catalyzed signal amplification system. A negative control was included with nonspecific mouse antibody solution (Dako) substituted for the primary antibody.