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Original Contribution

Induction of cell cycle arrest and DNA damage by the HDAC inhibitor panobinostat (LBH589) and the lipid peroxidation end product 4-hydroxynonenal in prostate cancer cells

Piergiorgio Pettazzoni [a,b], Stefania Pizzimenti [a], Cristina Toaldo [a], Paula Sotomayor [b], Luigina Tagliavacca [b], Song Liu [c], Dan Wang [c], Rosalba Minelli [b], Leigh Ellis [b], Peter Atadja [d], Eric Ciamporcero [a], Mario Umberto Dianzani [a], Giuseppina Barrera [a,*,1], Roberto Pili [b,*,1]

[a] Department of Medicine and Experimental Oncology, Section of General Pathology, University of Turin, 10125 Turin, Italy
[b] Genitourinary Program, Roswell Park Cancer Institute, Buffalo, NY 14263, USA
[c] Department of Bioinformatics, Roswell Park Cancer Institute, Buffalo, NY 14263, USA
[d] Novartis Institute for Biomedical Research, Cambridge, MA 02139, USA

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ABSTRACT

Histone deacetylase inhibitors (HDACIs) are promising antineoplastic agents for the treatment of cancer. Here we report that the lipid peroxidation end product 4-hydroxynonenal (HNE) significantly potentiates the antitumor effects of the HDAC inhibitor panobinostat (LBH589) in the PC3 prostate cancer cell model. Panobinostat and HNE inhibited proliferation of PC3 cells and the combination of the two agents resulted in a significant combined effect. Cell cycle analysis revealed that both single agents and, to a greater extent, their combined treatment induced G2/M arrest, but cell death occurred in the combined treatment only. Furthermore, HNE and, to a greater extent, the combined treatment induced dephosphorylation of Cdc2 leading to progression into mitosis as confirmed by α-tubulin/DAPI staining and phospho-histone H3 (Ser10) analysis. To evaluate possible induction of DNA damage we utilized the marker phosphorylated histone H2A.X. Results showed that the combination of panobinostat and HNE induced significant DNA damage concomitant with the mitotic arrest. Then, by using androgen receptor (AR)-expressing PC3 cells we observed that the responsiveness to HNE and panobinostat was independent of the expression of functional AR. Taken together, our data suggest that HNE potentiates the antitumoral effect of the HDACI panobinostat in prostate cancer cells.

Prostate cancer is the most common malignancy among American men [1]. It is a heterogeneous disease, the etiology of which seems to be related to a complex range of risk factors, including lifestyle patterns and genetic and epigenetic dysregulations resulting in altered gene expression [2]. Among the epigenetic factors, both hypermethylation of CpG islands and chromatin remodeling play important roles in the repression of various tumor suppressor genes during malignant transformation of prostate epithelial cells. Moreover, histone modifications, particularly acetylation and deacetylation, are the major driving force for epigenetic gene regulation [2]. Histone deacetylases (HDACs) and histone acetyltransferases are the key enzymes responsible for these reversible modifications. Histone deacetylase inhibitors (HDACIs) are a group of structurally diverse compounds that have shown encouraging activity in solid tumor therapies [3,4]. Numerous mechanisms have been proposed to account for HDACI-mediated lethality, including oxidative damage, modulation of the proapoptotic/antiapoptotic balance, and, more recently, DNA damage induction and/or interference with DNA repair pathways [5–7].

Radiation therapy is frequently used to treat localized prostate cancer, but unfortunately this treatment is not curative in every patient. The postulated mechanism of action for radiation therapy involves the generation of reactive oxygen species (ROS). Elevated oxidative stress and aberrant redox homeostasis are frequently observed in prostate cancer cells, which often present increased ROS generation from mitochondria or NADPH oxidase [8,9] and decreased antioxidant enzymes, such as manganese superoxide dismutase (MnSOD), CuZnSOD, and catalase [10,11]. However, further exposure to exogenous ROS is hypothesized as pushing tumor cells, which already have high constitutive oxidative stress levels, to cell death, whereas normal cells may still maintain redox homeostasis through adaptive responses [12]. Reactive intermediates produced under oxidatively

* Corresponding authors. G. Barrera is to be contacted at lax: +39 011 670 7753. R. Pili, fax: +1 716 845 8232.
E-mail addresses: giuseppina.barrera@unito.it (G. Barrera), roberto.pili@Roswellpark.org (R. Pili).
[1] These authors contributed equally to this work.

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stressful conditions cause the oxidation of polyunsaturated fatty acids (PUFAs) in membrane lipidic bilayers and in adipose tissue, leading eventually to the formation of aldehydes [13]. 4-Hydroxynonenal (HNE) is the most important aldehyde generated from the lipid peroxidation of PUFAs [14]; it is able to inhibit cell proliferation and to induce apoptosis in various solid tumors [15,16]. Moreover, it has been suggested that HNE may act as a second messenger of oxidative stress, because it is more stable than free radicals and can diffuse from the site at which it is produced, reaching various intracellular and extracellular targets [17].

We postulate that HNE may contribute to controlling cancer cell proliferation and death in irradiated tissues. Because a rational drug design of novel HDACIs includes their use as sensitizing agents to radiation therapy [18], we have studied the anti-cancer effects of combined treatment with an HDACI and HNE. Panobinostat (LBH589) is a hydroxamic acid derivative with a potent HDAC inhibition activity that is currently undergoing clinical testing and has shown efficacy against prostate cancer cell growth [19].

Materials and methods

Compounds

Panobinostat (LBH589) was generously provided by Novartis Pharmaceutical and was dissolved in dimethyl sulfoxide (DMSO). HNE was purchased from Cayman Chemicals (32100) and was prepared as follows: HNE was dissolved in ethanol that was evaporated through a gentle flow of N_2 and subsequently resuspended in sterile phosphate-buffered saline (PBS). The concentration was measured by spectrophotometer recording the absorbance of an aliquot of HNE diluted 1:200 in water at 223 nm ($\varepsilon = 13{,}750$).

Primary antibodies

The antibodies against p-Cdc2 (Thr14–Tyr15) (sc-12340-R), Cdc2 (sc-747), cyclin B1 (sc-594), histone H3 (sc-10809), and androgen receptor (sc-815) were purchased from Santa Cruz Biotechnology. Additional antibodies were purchased: anti-p-H2A.X (Ser139) (05-636) and anti-acetyl H3 (06-599) from Millipore, anti-α-tubulin (CP06) from Calbiochem, anti-p21 (ab7960) and anti-pericentrin (ab4448) from ABcam, anti-phospho-H3 (Ser10) (9701) from Cell Signaling, and anti-β-actin (A1978) from Sigma–Aldrich.

Cells and culture conditions

PC3, DU145, and LNCaP cells were obtained from the American Type Culture Collection and maintained in RPMI 1640 (Gibco) supplemented with 5% penicillin–streptomycin (Gibco) and 10% fetal calf serum in a humidified atmosphere with 5% CO_2. Androgen receptor-expressing PC3 cells (PC3/AR) were kindly provided by John Isaacs (Johns Hopkins University, Baltimore, MD, USA) and were maintained under the same conditions. To test the androgen-responsiveness status, PC3/AR cells were cultured in phenol red-free RPMI 1640 (Gibco) supplemented with 5% penicillin–streptomycin, 5% L-glutamine, and 10% charcoal-stripped fetal calf serum. Androgen-supplemented conditions were obtained by supplementing the medium with 5 nM R1881 (PerkinElmer, NLP005005MG).

Colony-forming assay

Cells were trypsinized, washed in 1 × PBS, and seeded (500 cells/well) into a six-well plate and left overnight to attach. After 24 h, the cells were treated with the compounds and the medium was changed after 72 h. Cells were cultured for 9–11 days and subsequently fixed and stained with a solution of 90% crystal violet (Sigma–Aldrich), 10% methanol. The colonies were then photographed and counted with a Gel Doc equipment (Bio-Rad Laboratories).

Cell proliferation and viability

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) analysis was performed in 96-well plates. Cells were seeded (800–1500 cells/well) in 200 μl of serum-supplemented medium and treated with the compounds 24 h later. The cells were subsequently treated with 20 μl of 5 mg/ml thiazolyl blue tetrazolium bromide (M2128; Sigma–Aldrich) and, after 2 h, the medium was removed. One hundred microliters of DMSO was then added and the absorbance was recorded at 570 nm by a 96-well-plate ELISA reader. Viability was evaluated by trypan blue (T8154; Sigma–Aldrich) exclusion test.

Flow cytometry/cell cycle analysis

Adherent and nonadherent cells were harvested; washed in 1 × PBS; fixed in 70% cold ethanol; resuspended in a buffer containing 0.02 mg/ml RNase A (Worthington), 0.05 mg/ml propidium iodide (Sigma–Aldrich), 0.2% v/v Nonidet P-40 (Sigma–Aldrich), 0.1% w/v sodium citrate (Sigma–Aldrich); and analyzed with a FACScan cytometer (Becton Dickinson).

Apoptosis

Adherent and nonadherent cells where harvested, washed in 1 × PBS, and subsequently resuspended in annexin V binding buffer (556454; BD Pharmingen) supplemented with 1:100 APC-conjugated annexin V (550474; BD Pharmingen) and 1 μg/ml propidium iodide (Sigma–Aldrich). Cells were analyzed by a FACSCalibur cytometer (Becton Dickinson).

FACS analysis of H3 acetylation and H3 phosphorylation

Adherent and nonadherent cells were harvested, washed in 1 × PBS, and fixed and permeabilized with a commercially available kit (FOX P3 Fix/Perm Buffer Set 421403; Biolegend) according to the manufacturer's instructions. Cells were subsequently blocked in 5% bovine serum albumin dissolved in 1 × PBS and incubated for 1 h at room temperature with either anti-acetyl-H3 or anti-phospho-histone H3 (Ser10) antibody. After primary antibody incubation, the cells were washed three times with 1 × PBS and incubated with FITC-conjugated secondary antibody (554020; BD Pharmingen) for 1 h at room temperature guarded from light. The cells were collected, resuspended in the previously indicated buffer used for cell cycle analysis, and analyzed with a FACSCalibur cytometer (Becton Dickinson).

All the flow cytometry data were analyzed with the software FCS Express (De Novo Software).

Immunofluorescence–confocal microscopy

Cells were grown on sterile coverslips and treated as indicated, washed three times with 1 × PBS, and then fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were permeabilized through incubation with 0.1% Triton X-100 in 1 × PBS for 10 min and blocked for 1 h with 1% bovine serum albumin dissolved in 1 × PBS. Cells were incubated overnight with primary antibodies, washed three times with 1 × PBS, and incubated for 1 h at room temperature in the dark with FITC-conjugated goat anti-rabbit (554020; BD Pharmingen) and AlexaFluor 594-conjugated goat anti-mouse (A-11005; Invitrogen) secondary antibodies. Coverslips were finally mounted with a commercially available Vectashield mounting solution (H-1200; Vector Laboratories) containing 4′,6-diamidino-2-phenylindole (DAPI) and analyzed with an epifluorescence microscope (Axioskop, Carl Zeiss) or a confocal microscope (SP2, Leica).