Cancer Letters 288 (2010) 236–250

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Troglitazone inhibits histone deacetylase activity in breast cancer cells

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ARTICLE INFO

Article history:
Received 6 May 2009
Received in revised form 9 July 2009
Accepted 10 July 2009

Keywords:
Troglitazone
Breast cancer
Histone post-translational modifications
Cell killing
PI3K/AKT inhibition

ABSTRACT

We previously demonstrated that the PPARγ agonist Troglitazone (TRG), a potent antiproliferative agent, in combination with the anthracycline antibiotic Doxorubicin (DOX), is an effective killer of multiple drug resistant (MDR) human cancer cells. Cell killing was accompanied by increased global histone H3 acetylation. Presently, we investigated the epigenetic and cell killing effects of TRG in estrogen receptor (ER) positive MCF7 breast cancer cells. MCF7 cells were treated with the Thiazolidinediones (TZDs) TRG and Ciglitazone (CIG), the non-TZD PPARγ agonist 15PGJ2, and the histone deacetylase inhibitors (HDACi's) Trichostatin A (TSA), sodium butyrate and PXD101. Using MTT cell viability assays, Western analyzes and mass spectrometry, we showed a dose-dependent increase in cell killing in TRG and HDACi treated cells, that was associated with increased H3 lysine 9 (H3K9) and H3K23 acetylation, H2AX and H3S10 phosphorylation, and H3K79 mono- and di-methylation. These effects were mediated through an ER independent pathway. Using HDAC activity assays, TRG inhibited HDAC activity in cells and in cell lysates, similar to that observed with TSA. Furthermore, TRG and TSA induced a slower migrating HDAC1 species that was refractory to HDAC2 associations. Lastly, TRG and the HDACi's decreased total and phosphorylated AKT levels. These findings suggest that TRG's mode of killing may involve downregulation of PI3K signaling through HDAC inhibition, leading to increased global histone post-translational modifications.

1. Introduction

Inhibition of histone deacetylase (HDAC) activity, resulting in increased global acetylation of specific histone lysine residues, is a potent means of killing cancer cells [1,2]. All cells encode Class I, II and III HDAC enzymes that work together to ensure that specific lysines within histones are deacetylated at precisely the correct moment in time and space. It is currently held that histone acetylation drives transcription and that histone deacetylation mediates gene silencing. Thus, increased global histone acetylation resulting from HDAC inhibition leads to increased global transcription. Many important genes involved in cellular quality control are upregulated by HDAC inhibitors (HDACi's), including genes required for cell cycle inhibition, DNA damage repair, free radical scavenging and apoptosis [3–5]. Thus, HDACi's generate a situation that is conducive to eliminating damaged and potentially tumorigenic cells.

Early studies identified Trichostatin A (TSA; a hydroxamate) and sodium butyrate (a bacterial derived short-chain fatty acid) as potent HDACi's and antiproliferative agents [6–8]. Various studies have suggested TSA could be either a competitive or a noncompetitive inhibitor of HDAC activity [8–13]. The bulk of the evidence supports the idea that TSA acts as a competitive inhibitor by binding

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doi:10.1016/j.canlet.2009.07.011

to the catalytic site of HDAC1, 2 and 3 [9,12,13]. However, short *in vivo* half-lives and potential mutagenic byproducts have rendered some hydroxamic acids as ineffective therapeutic agents [5,14,15]. New HDACi's have been developed and are currently approved or in clinical trials, such as SAHA (suberoylanilide hydroxamic acid/Vorinostat [16–18]). The novel hydroxamate-type HDACi PXD101 (belinostat), for example, has been shown to be an effective antiproliferative agent, alone or in combination with other anti-cancer agents, against cutaneous T-cell lymphoma (CTCL), multiple myeloma (MM), osteoclastogenesis and colon cancer [19–21]. The specific mechanisms employed by HDACi's to kill tumor cells remain unclear. In this study, we will investigate the effects of the HDACi's PXD101, Trichostatin A (TSA) and sodium butyrate on histone post-translational modifications (PTMs).

Over the past several years another class of drug has been shown to be effective against cancer. The insulin-sensitizing Thiazolidinedione (TZD) drugs act as PPARγ receptor agonists, and have been found in multiple studies to have potent antiproliferative activity in cancer cells [22–24]. A member of this class, Troglitazone (TRG), was originally used as an oral anti-hyperglycemic agent in Diabetes Type 2 (DM2 [25,26]), but rare and severe hepatotoxicity resulted in the drug being withdrawn from the market [27]. Single agent chemotherapeutic clinical trials using TRG in patients with prostate cancer and liposarcoma had modest effects only [28,29]. Furthermore, phase II clinical trials showed that TRG monotherapy in chemotherapy-resistant metastatic colorectal cancer and refractory breast cancer also had little benefit [30,31], leading to the conclusion that TRG is an ineffective treatment for these cancers. It is now recognized that combination therapy utilizing TRG with additional cancer agents yields benefits not observed with TRG monotherapy [32–34]. Interestingly, a retrospective analysis of 87,678 DM2 individuals treated with TZDs obtained from the Veterans Integrated Services Network 16 (VISN 16) over a 6 year period showed a 33% decreased risk of developing lung cancer, but no significant reduction of colorectal or prostate cancer [35]. In a separate study, a meta-analysis of DM2 trials using the TZD Rosiglitazone (ROS) demonstrated a significantly decreased risk of developing cancer, of any form, as compared to controls, suggesting a primary preventative effect [36].

Consistent with observations that TRG is more potent against various cancers when used in combination therapy, we demonstrated that TRG, together with DOX, was a potent killer of drug resistant K562 leukemia cells, but alone had little effect [37]. Furthermore, TRG had a significant effect on histone H3 levels and PTMs in both selected and unselected K562 cells. Here, we investigated the effects of TRG on histone metabolism in MCF7 breast cancer cells. We show that the TZDs TRG and Ciglitazone (CIG) increased histone H3K9 acetylation in a dose-dependent manner that correlated with cell killing, with TRG far more effective than CIG. We compared the epigenetic profile of MCF7 cells generated after TRG treatment with that of three HDACi's, TSA, sodium butyrate and PXD101. Significantly, it was observed that these HDACi's affect the epigenome in a manner similar to TRG. An estrogen receptor (ER) negative breast cancer cell line, MDA-MB-231, exhibited the same effects as the ER positive MCF7 cells, indicating the effects of TRG are independent of the ER status of these cells. Importantly, TRG, like TSA, inhibits HDAC activity in cells and in lysates, indicating that TRG binds to HDAC enzymes. Lastly, TRG appears to induce a slower migrating HDAC1 band that does not interact with HDAC2. We conclude that the antiproliferative effect of TRG is linked to its ability to influence the epigenetic profile of cells.

2. Materials and methods

2.1. Cell culture

MCF7 human breast cancer cells (obtained from American Type Culture Collection) were cultured in 75 cm^2 tissue culture flasks (Nalgene) in Dulbeccos Modified Eagles medium containing 10% fetal bovine serum (Invitrogen) and 1X antibiotic/antimycotic solution (Gibco-Invitrogen). Cells were cultured in a humidified atmosphere (5% CO_2) at 37 °C and were incubated for 48 h in the presence or absence of the drugs at the indicated concentrations. All treatment compounds were reconstituted in Dimethylsulfoxide (DMSO) and control cells were incubated with an equivalent volume of DMSO vehicle only.

2.2. Western analysis and protein coimmunoprecipitation

MCF7 cells were removed with a rubber policeman, collected by centrifugation at 4 °C, and resuspended in ice-cold lysis buffer (20 mM HEPES (pH 7.5), 50 mM KCl, 10% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT plus 1X-Sigma mammalian cell anti-protease cocktail). The cells were lysed using multiple freeze–thaw cycles followed by pulse sonication and high-speed centrifugation at 4 °C to remove cell debris. For Western analysis equivalent amounts of protein (assessed by Bradford protein assay [38] using Biorad Protein Reagent) were resolved by SDS–PAGE. An aliquot of the protein lysate was set aside for analysis of HDAC activity. Following electrophoresis the proteins were trans-blotted onto nitrocellulose membranes (Pall-VWR). The membranes were blocked overnight on a gyratory plate at 4 °C with 5% molecular grade fat free skim milk powder (Biorad Laboratories, Mississauga ON Canada) in phosphate-buffered saline (PBS) containing 0.1% Tween-20. Primary antibody incubations were overnight at 4 °C and secondary HRP antibodies were applied for 30 min, also at 4 °C. Subsequent washes were carried out in the same buffer. An enhanced chemiluminescence (ECL) detection system (Dupont-NEN) was used to detect the antigen/antibody complexes. Blots were exposed to BioMax chemiluminescent X-ray film (Kodak) and target signals were scanned and quantified using a HP scanner (Scanjet G4010) and ImageJ software.

For coimmunoprecipitation experiments, 750 μg of each lysate was resuspended in a final volume of 300 μl with IP buffer (25 mM Tris–HCl pH 7.5, 125 ml NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.01% Tween-20, 0.25% glycerol, protease inhibitors (Sigma) and 1 mM DTT). Protein concentrations were determined by Bradford quantitation. Lysates were then precleared with 25 μl protein A Sepharose for 90 min