Intrinsic apoptotic and thioredoxin pathways in human prostate cancer cell response to histone deacetylase inhibitor

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There is a great need to develop better mechanism-based therapies for prostate cancer. In this investigation, we studied four human prostate cancer cell lines, LNCaP, DU145, LAPC4, and PC3, which differ in response to the histone deacetylase inhibitor, suberoylanilide hydroxamic acid (vorinostat), a new anticancer drug. Examining the role of intrinsic mitochondrial caspase-dependent apoptosis and caspase-independent, reactive oxygen species (ROS) facilitated cell death, has provided an understanding of mechanisms that may determine the varied response to the histone deacetylase inhibitor. We found striking differences among these cancer cells in constitutive expression and response to suberoylanilide hydroxamic acid in levels of antiapoptotic and proapoptotic proteins, mitochondria membrane integrity, activation of caspases, ROS accumulation, and expression of thioredoxin, the major scavenger of ROS. Identifying these differences can have predictive value in assessing therapeutic response and identifying targets to enhance therapeutic efficacy.

Prostate cancer is the most common cancer and the second most common cause of death in men in North America and Western Europe (1). Current therapy for metastatic prostate cancer is unsatisfactory (2). Suberoylanilide hydroxamic acid (SAHA; vorinostat) is an orally administered histone deacetylase inhibitor (HDACi) that in phase I/IIa and IIb clinical trials as monotherapy has shown significant antitumor activity in hematological and solid tumors at doses well tolerated by patients (3, 4). The first in vivo evidence of the anticancer activity of SAHA was demonstrated in nude mice bearing human prostate cancer transplants (5). Normal cells are relatively resistant to SAHA (5, 6). In preclinical studies, SAHA has been found to induce caspase-dependent and caspase-independent cell death in different transformed cells (6–9). Why different cells respond in different ways is not well understood.

An objective of the present study is to identify molecular targets mediating the anticancer activity of HDACi to increase our understanding of the mechanisms involved in response of prostate cancer cells to this agent.

The intrinsic mitochondria-mediated apoptosis pathway is the best understood induced-death pathway (10–12). Another, albeit less well studied, cellular system that plays an important role in response to stress stimuli that cause cell death is the thioredoxin (Trx)—thioredoxin-binding protein (TBP-2) redox system (13–15). Trx functions as a hydrogen donor for many protein targets, and it is the major scavenger of reactive oxygen species (ROS). TBP-2binds specifically to Trx and blocks its reducing activity (16).

This study analyzes the intrinsic mitochondrial caspase-mediated apoptotic pathway and the Trx–TBP-2 pathway that modulates ROS accumulation and caspase-independent cell death in response to SAHA (17).

Aberrant expressions of proteins of the apoptotic pathway are associated with resistance to antitumor therapy (8, 18–20). For example, a high level of Bel-2 in PC3 prostate cancer cells is associated with resistance to therapy (21). Increased expression of XIAP and survivin was found in a cisplatin-resistant LNCaP prostate cancer subline (22). It has been shown that HDACi can increase expression of proapoptotic Bel-2 family proteins as well as decrease expression of antiapoptotic proteins in various transformed cells (7, 8, 18, 22), which, in part, can account for the anticancer activity of these agents.

High levels of Trx are associated with cancers resistant to therapy (14). Further, TBP-2, which blocks Trx activity, is expressed at low levels in many human cancers (6, 23). SAHA, as well as other HDACi, radiotherapy, and chemotherapy cause the production of ROS, which can facilitate cancer cell death (6, 7, 19, 24, 25). SAHA induces TBP-2 in transformed cells, associated with a decrease in Trx activity, which can facilitate ROS-induced cell death (6).

This work used four human prostate cancer cell lines, LNCaP, DU145, LAPC4, and PC3, which differ in sensitivity to HDACi-induced cell death from very sensitive (DU145) to resistant (PC3) (26–31). We found striking differences among these prostate cancer cells in the expression of proteins regulating cell death pathways, which provide a better understanding of the molecular basis for differences in response to the HDACi among these transformed cells. This work shows that the four human prostate cancer cells have substantial differences in expression of antiapoptotic and proapoptotic proteins, mitochondrial transmembrane potential (MTP), mitochondrial protein activation, ROS accumulation, and levels of Trx and TBP-2 in culture without and with SAHA. Both caspase-dependent and caspase-independent HDACi-induced cell death occurred in these prostate cancer cells. This work indicates that SAHA-induced cell death involves different targets in the different cells, and it identifies protein markers that may be predictive of response of prostate cancer to therapy and targets for enhancing the efficacy of SAHA and other drugs in treating prostate cancers.

Results

Effect of SAHA on Growth and Death of Human Prostate Cells. SAHA (2.5 μM) completely inhibited cell growth of LNCaP, DU145, and LAPC4 after 72 h in culture (Fig. 1 A, C, and E). PC3 cell
growth was inhibited at 5 μM and completely inhibited at 10 μM (Fig. 1G). LNCaP, DU145, LAPC4, and PC3 had different sensitivity to SAHA-induced cell death: LNCaP, 50% ± 3%; DU145, 77% ± 4%; LAPC4, 40% ± 6%; and PC3, <5% cell death in culture with 5 μM SAHA for 72 h. LNCaP, 25% ± 5%; DU145, 30% ± 2%; and LAPC4, 28% ± 2% in culture with 2.5 μM SAHA for 72 h (Fig. 1B, D, and F). SAHA caused an accumulation of acetylated histone H3 and H4 in each of the four human prostate cell lines (data not shown) (17).

LAPC4 cells express wild-type androgen receptor (AR), LNCaP express a mutant AR, and both DU145 and PC3 are AR negative (28). SAHA caused a marked decrease in AR expression in LAPC4 and LNCaP cell lines. Culture of LAPC4 or LNCaP cells with 10 μM hydroxylfluoramide, an AR antagonist, did not alter the sensitivity of these cells to SAHA-induced cell death (data not shown).

Consistent with our previous reports (32), SAHA induces p21WAF1 protein in LNCaP, DU145, and PC3 cells. p21WAF1 is not detectable, and it is not induced by SAHA in LAPC4 (data not shown). SAHA induces G1 arrest in the three cell lines but not in LAPC4, consistent with p21WAF1 having an important role in SAHA-induced G1 arrest. In LAPC4 cells, SAHA induces a G2 arrest (data not shown). Expression of p21WAF1 does not protect these prostate cancer cells from HDACi-mediated cell death because SAHA induced greater cell death of LNCaP and DU145, which express p21WAF1, than LAPC4, which does not express p21WAF1.

Expression of Antiapoptotic Proteins. The mitochondrial apoptotic pathway is regulated by antiapoptotic and proapoptotic factors. Mitochondrial membrane integrity depends on the Bcl-2 family of proteins. The expression of the multidomain antiapoptotic protein Bcl-2 was assayed in the four prostate cell lines cultured without and with SAHA. Bcl-2 protein was not constitutively detectable in DU145 cells and not induced by SAHA (Fig. 2A). In PC3 cells, SAHA causes an increased accumulation of Bcl-2. Bcl-2 is expressed in LNCaP and LAPC4, and its expression is not detectably altered by SAHA (Fig. 2A). The lack of Bcl-2 in DU145 is associated with marked sensitivity and the increased levels of Bcl-2 in PC3 with resistance to SAHA-induced cell death. The data shown in Figs. 2 and 3 are studies at 5 μM SAHA. Cells cultured with 2.5 μM SAHA showed similar results with respect to each of the antiapoptotic and proapoptotic proteins (data not shown).

The antiapoptotic proteins, XIAP (inhibitor of apoptosis) and survivin, are expressed in all four cell lines (Fig. 2B). Constitutive survivin expression is greater in LAPC4 than the other three cell lines. SAHA caused a marked decrease in XIAP levels in all four cell lines and in survivin levels in LNCaP, DU145, and PC3, but not LAPC4. High levels of survivin in LAPC4 are associated with decreased sensitivity to SAHA-induced cell death (Fig. 1F). It has been reported that high levels of survivin are associated with resistance to HDACi-induced cell death of leukemia cell lines (21). A SAHA-induced decrease in the levels of XIAP and survivin does not make PC3 cells (with increased levels of Bcl-2) sensitive to HDACi-induced cell death.
respectively), whereas the addition of both HA14-1 and SAHA causes >70% PC-3 cell death within 24 h (Fig. 1J).

**XIAP Is Inhibited by Smac.** Inhibition of XIAP with Smac mimic, RMT5265 (34), does not alter PC3 resistance to SAHA-induced cell death (Fig. 1K and L). The XIAP inhibitor RMT5265 does increase the sensitivity of DU145 to SAHA-induced cell death (Fig. 1N).

**Expression of Proapoptotic Protein and Mitochondrial Membrane Permeability.** The multidomain proapoptotic proteins, e.g., Bak and Bax, and the BH3 domain-only proapoptotic proteins, e.g., Bim, Bid, Puma, Bad, Bmf, Bik, and Noxa, inactivate prosurvival proteins, like Bcl-2 (see refs. 6–10, 12, 18, 19, 34, and 35). Stress stimuli including SAHA lead to the BH3 domain-only proteins inactivating Bel-2 through direct protein–protein interaction, which can, in turn, cause decreased MTP with release of cytochrome c, which induces caspase-9 activation.

There are striking differences among the four prostate cancer cell lines in (i) the expression of proapoptotic proteins and (ii) the effect of SAHA on MTP (Fig. 3). MTP was determined with a fluorescence dye, JC-1, which aggregates in normal mitochondria and emits red fluorescence, but it cannot accumulate in mitochondria losing transmembrane potential, and it emits a diffuse cytoplasmic green signal in dead cells.

In LNCaP cells, the proapoptotic proteins Bax, Bak, Bok, Bid, Puma, Bad, Bmf, and Bik were expressed. Noxa was not expressed in LNCaP or LAPC4 cells, and it was expressed in DU145 and PC3. In LNCaP cells, SAHA increases the levels of Bak, Bmf, Bim, and Bik (Fig. 3A), it induces loss of MTP 32.8% with SAHA compared with 4.3% in cells cultured without inhibitor (Fig. 3B), and it leads to caspase-9 and caspase-3 activation, as indicated by the presence of 37-kDa caspase-9 fragment and 20-kDa and 18-kDa caspase-3 fragments (Fig. 3C).

In DU145, there was no detectable expression of Bax or Bik, and neither is induced by SAHA (Fig. 3A). The HDACi does up-regulate Bmf but none of the other proapoptotic proteins assayed. In DU145 cells, SAHA causes a marked increase in cells losing MTP [36.2% compared with 1.5% without inhibitor (Fig. 3B)] and activation of caspase-9 and caspase-3 (Fig. 3C).

LAPC4 cells had no detectable constitutive expression of Bax and Noxa. SAHA increases Bak, Bok, and Bmf expression, moderately induces loss of MTP [13.3% compared with 2.2% without inhibitor (Fig. 3B)], and causes activation of caspase-9 and caspase-3 (Fig. 3C).

PC3 cells, like LNCaP, express all of the proapoptotic proteins assayed. However, unlike LNCaP cells, SAHA does not increase the expression of these proteins in PC3 cells, and it neither significantly increases loss of MTP (4.1% compared with control.

**Fig. 3.** Expression of proapoptotic proteins in LNCaP, DU145, LAPC4, and PC3. (A) Western blot of the indicated Bcl-2 family of proapoptotic proteins in cells cultured without (−) and with (+) 15 μM SAHA for 24 and 48 h; α-tubulin is indicated as the loading control. (B) Mitochondrial transmembrane potential measured as indicated in Materials and Methods. Percentage figures indicate the proportion of cells with decreased FL-2, an indicator of mitochondrial membrane integrity. (C) Western blots of caspase-9 and caspase-3, with the activation of caspases indicated by the presence of 37-kDa fragment of caspase-9 and of the 20-kDa and 18-kDa fragments of caspase-3. α-Tubulin is indicated as the loading control. (D) Western blots of mitochondrial proapoptotic proteins AIF, cytochrome c, and Smac in cells cultured without (−) and with (+) 5 μM SAHA for 24 and 48 h; α-tubulin is indicated as the loading control.
induced cell death of LAPC4 and decreased cell death of LNCaP by 50%. These results indicate that the HDACi-induced cell death of LAPC4 and LNCaP cells (Fig. 5A) is caspase-dependent. The HDACi-induced death of LNCaP and, to a large extent, LNCaP cells, is caspase activation-independent (Fig. 4B). Increased levels of TBP-2 are associated with reduced Trx activity and increased ROS accumulation in LAPC4 and LNCaP cells (Fig. 5A and B). The increased ROS could facilitate cell death (9). The present data do not establish why SAHA-induced cell death of DU145 cells is caspase-dependent and that of LAPC4 and, to some extent, LNCaP, are not. There are several differences among these cell lines in expression of both proapoptotic and antiapoptotic proteins, but which of these differences account for cell death that is primarily caspase-dependent or caspase-independent is not clear. One can speculate that the lack of Bcl-2 expression in DU145 may be a factor making these cells particularly sensitive to SAHA-induced caspase-dependent death. In LAPC4 cells, the high constitutive levels of Bcl-2 and survivin may be factors contributing to caspase-independent cell death. It is likely that the relative importance of these cell death pathways in the anticancer activity of SAHA is cell context-dependent.

Discussion

This study has found that prostate cancer cell lines have striking differences in expression of proteins that modulate mitochondrial protein expression and release. These include increased ROS accumulation in cells cultured with SAHA relative to cells cultured without the HDACi.
dria-mediated apoptosis and ROS-facilitated cell death. These differences determine, in part, the sensitivity of these different prostate cancer cells to the HDACi SAHA-induced cell death, as well as the molecular pathways involved.

Among the four cell lines, LNCaP and DU145 are more sensitive than LAPC4 to SAHA-induced cell death, and PC3 is resistant. Bcl-2 levels play an important role in PC3 resistance because chemically blocking Bcl-2 made these cells sensitive to the HDACi. DU145, the most sensitive to SAHA-induced cell death among the four cell lines, had no detectable Bcl-2. A high level of the antiapoptotic protein, survivin, is present in LAPC4 cells, which are less sensitive than DU145 or LNCaP to the HDACi.

The four cell lines differed in constitutive expression of TBP-2. TBP-2 is not detectable in DU145, and it is relatively well expressed in LAPC4. ROS accumulated in LNCaP, DU145, and LAPC4, but not PC3, cells cultured with SAHA. SAHA-induced TBP-2 expression in all four cell lines and decreased Trx levels most markedly in LAPC4 cells. SAHA-induced LAPC4 cell death was not blocked by z-VAD, the pan-caspase inhibitor, suggesting that it was caspase-independent cell death. TBP-2 inactivation of Trx can play a role in caspase-independent cell death. By comparison, in DU145 cells, z-VAD inhibited caspase activation and blocked SAHA-induced cell death, suggesting caspase-dependent cell death.

Further evidence suggesting that the difference in the pathway of SAHA-mediated cell death is related to induced changes in the expression of proapoptotic and antiapoptotic proteins is suggested by the fact that SAHA caused more changes in proapoptotic proteins in sensitive LNCaP and DU145 cells than in the resistant PC3 cells. In LNCaP cells, SAHA increased proapoptotic proteins Bax, Bim, Bmf, Bik, cytochrome c, and Smac, decreased antiapoptotic XIAP and survivin, activated caspase-9 and -3, and increased mitochondrial transmembrane permeability. In PC3-resistant cells, SAHA did not alter MTP, increase cytochrome c or AIF levels, or activate caspase-3 or -9.

In the clinical trials with oral-administered SAHA (3), patients with significant anticancer effects had average maximum concentrations of SAHA of 2.5–3.0 μM with a range of up to 4–5 μM SAHA. It is not possible to relate directly the concentrations of SAHA to that in vivo, but the range of concentrations active in patients with solid tumors is comparable with those used in this work.

Transformed cells in culture can undergo changes in gene and protein expression from the prostate cancers from which they are derived. Accordingly, findings in cancer cells in culture may have little relevance to cancer cells in human primary tumors. In fact, there are few quantitative data to suggest this conclusion (38). Comparing the gene expression profile between tumor prostate cancer tissue (in vivo) with LNCaP cells treated with and without anticancer drugs, 1.4–3.6% of genes were differentially expressed in two sets of prostate cells. These authors (38) suggest that LNCaP cells should be a good model for many aspects of the study of prostate cancers. Several microarray studies have attempted to determine patterns of gene expression that predict therapeutic response (38–41). In general, this work has identified patterns of differentially expressed genes, but so far it has not been able to establish a set of marker genes that can reliably predict therapeutic response. It is encouraging that gene expression profiling appears to be possible with circulating tumor cells, which clearly are more accessible than pre- and posttherapy prostate biopsy material (40, 41).

The studies reported to date (40, 41) indicate that an assay for specific proteins in circulating tumor cells could be a promising technique and clearly one that requires further development. This work suggests that assays of specific proteins, such as Bcl-2, TBP-2, and cytochrome c, could be useful guides to therapeutic markers and for which immunohistochemical assay techniques are available. Further, the present findings suggest that targeting proteins, such as Bcl-2 down-regulation or TBP-2 up-regulation, could enhance the therapeutic efficacy of SAHA and other anticancer agents in the therapy of prostate cancers.

Materials and Methods

Cell Culture. Human prostate cancer cell lines LNCaP, DU145, and PC3 were obtained from American Tissue Culture Collection (Manassas, VA) and cultured in RPMI medium 1640, MEM α, and F-12K medium with 10% FBS, respectively. Human prostate cancer cell line LAPC4 was provided by Charles Sawyer (University of California at Los Angeles, CA) and cultured in Iscove’s medium with 10% FBS (29).

Cell Growth and Viability. To begin, 5 × 10^4 cells per well were seeded in a 24-well plate. After 24 h, SAHA (17) was added to cultures as indicated in the figure legends. On days 1, 2, and 3 of SAHA culture, cells were collected with trypsin digestion (6). Cell number and viability were determined by Guava PCA-96 machine with ViaCount Flex reagent (Guava Technologies, Hayward, CA), according to the manufacturer’s protocol, or by trypan blue exclusion as described in ref. 6. At least three independent analyses were performed for all determinations of cell growth and viability. The antitumor effects of combining SAHA with other reagents were examined in the prostate cancer cell lines as follows: 5 × 10^4 cells per well were seeded in a 24-well plate and cultured overnight. The cultured cells were treated with the concentration of SAHA indicated for each experiment with or without various other reagents, including Bcl-2 antagonist HA14-1 (Raylight, La Jolla, CA), or XIAP inhibitor RMT5256 [a gift from Xiaodong Wang (University of Texas Southwestern Medical Center, Dallas, TX)]. Cell growth and viability on days 1, 2, and 3 of culture were determined as described earlier.

Cell Cycle Analysis. Cell cycle analysis was performed as described in ref. (6).

Cell Fractionation and Measurement of Caspase-3 Activity. Cells were cultured with 5 μM SAHA with or without 40 μM pan-caspase inhibitor z-VAD (EMD Biosciences, San Diego, CA). z-VAD was added to cells cultured 1 h before SAHA. Cells were processed, and caspase activity was determined as described in ref. 42. Three independent experiments were performed for each analysis.

Measurement of Mitochondrial Transmembrane Potential. In a 10-cm-diameter cell culture dish, 10^6 cells were seeded. After 24 h, the cells were treated with 5 μM SAHA. Cells were collected by trypsin digestion at 48 h, centrifuged at 500 × g for 5 min at 4°C, and washed once with PBS. Cells were incubated with 5 μg/ml JC-1 (Invitrogen, Carlsbad, CA) at 37°C for 15 min. After incubation, the cells were centrifuged at 500 × g for 5 min, resuspended in PBS, and analyzed by using flow cytometry with FL-2 to detect the red fluorescence signal, which is generated from JC-1 concentrated in mitochondria in normal cells and FL-1 to measure green fluorescence, which is emitted by JC-1 diffused in the cytoplasm in cells with loss of transmembrane potential (43).

ROS Measurements. ROS measurements were performed as described in ref. 6.

Measurement of Trx. Trx was measured by using the end point insulin assay for biological samples as described in ref. 44.

Western Blotting. In a 15-cm-diameter cell culture dish, 2–3 × 10^6 cells were seeded. After 24 h, 5 μM SAHA was added to the
culture. Cells were collected by trypsin digestion at 24 and 48 h, washed with PBS, and lysed in RIPA buffer (50 mM Tris-Cl, pH 7.5/250 mM NaCl/5 mM EDTA, pH 8.0/0.5% Nonidet P-40) with protease inhibitor tablet (Roche Diagnostics, Indianapolis, IN). Protein concentration was determined by using the Bradford method. Forty micrograms of protein was resolved by SDS/PAGE and transferred onto a PVDF membrane. The membrane was blocked with 5% BSA and blotted with the following antibodies and visualized by ECL method. The antibodies against the following proteins used in the study were as follows: Bak, Bid, Bmf, Puma, cytochrome c, AIF, Smac, XIAP, and survivin (Cell Signaling, Danvers, MA); Noxa, caspase-3, and caspase-9 (EMB Biosciences, San Diego, CA); androgen receptor (DAKO, Carpinteria, CA); and p21wild (Neo-markers, Fremont, CA). The antibodies against TBP-2 and Trx were obtained from MBL (Woburn, MA) and American Diagnostic (Stamford, CT), respectively.

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