Mechanisms of Cell Death Induced by Histone Deacetylase Inhibitors in Androgen Receptor–Positive Prostate Cancer Cells

Oskar W. Rokhlin, Rebecca B. Glover, Natalya V. Guseva, Agshin F. Taghiyev, Karl G. Kohlgraf, and Michael B. Cohen

Department of Pathology, University of Iowa, Iowa City, Iowa

Abstract
Histone deacetylase inhibitors (HDACI) are potential therapeutic agents that inhibit tumor cell growth and survival. Although there are several publications regarding the effects of HDACIs on prostate cancer cell growth, their mechanism(s) of action remains undefined. We treated several human prostate cancer cell lines with HDACI trichostatin A and found that trichostatin A induced cell death in androgen receptor (AR)–positive cell lines to higher extent compared with AR-negative cell lines. We then discovered that trichostatin A and other HDACIs suppressed AR gene expression in prostate cancer cell lines as well as in AR-positive breast carcinoma cells and in mouse prostate. Trichostatin A also induced caspase activation, but trichostatin A–induced AR suppression and cell death were caspase independent. In addition, we found that doxorubicin inhibited AR expression, and p21 protein completely disappeared after simultaneous treatment with trichostatin A and doxorubicin. This effect may be attributed to the induction of protease activity under simultaneous treatment with these two agents. Further, simultaneous treatment with trichostatin A and doxorubicin increased cell death in AR-positive cells even after culturing in steroid-free conditions. The protease/proteasome inhibitor MG132 protected AR and p21 from the effects of trichostatin A and doxorubicin and inhibited trichostatin A–induced cell death in AR-positive prostate cells. Taken together, our data suggest that the main mechanism of trichostatin A–induced cell death in AR-positive prostate cancer is inhibition of AR gene expression. The synergistic effect of simultaneous treatment with trichostatin A and doxorubicin is mediated via inhibition of AR expression, induction of protease activity, increased expression of p53, and proteolysis of p21.


Introduction
Histone deacetylase inhibitors (HDACI) are a class of chemotherapeutic agents that have been shown to modify the expression of different genes, inhibit the cell cycle, and induce apoptosis in several cancer cell lines (1). HDACIs induce hyperacetylation of histones in both tumor and normal cells, but they are relatively nontoxic to normal cells (2, 3). Induction of histone hyperacetylation and chromatin remodeling should result in the activation of genes, but, surprisingly, gene profiling studies indicate that a similar proportion of genes are activated and repressed (4, 5). Moreover, 40% of genes within the genome were found to be affected by the HDACI suberoylanilide hydroxamic acid or depsipeptide over a 16-hour time-course treatment of CEM cells. The authors suggest that “the pleiotropic effects of HDACIs might provide an advantage as an anticancer agent because most transformed cells have multiple gene defects affecting several pathways that regulate cell proliferation and survival” (5).

HDACIs have been reported to activate both death receptor and intrinsic apoptotic pathways (1). It has recently been shown that HDACIs induced apoptosis in leukemic blasts via activation of tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL) and Fas signaling pathways, and TRAIL, TRAIL receptor 2 (TRAIL-R2), Fas ligand, and Fas were found to be up-regulated by HDACIs in the leukemic cells but not in normal hematopoietic progenitors (6). In another study, HDACIs were shown to induce the expression of p21 and TRAIL, triggering tumor-selective death in acute myeloid leukemia cells (7). As reviewed in ref. 1, the intrinsic apoptotic pathway is important for the function of HDACIs: HDACI-induced apoptosis is accompanied by up-regulation of pro-apoptotic members of Bcl-2 family, and Bcl-2 overexpression inhibits HDACI-mediated apoptosis. HDACI-induced apoptosis is usually accompanied by caspase activation, but, even in the absence of caspase activation, some HDACIs can induce cell death (8, 9).

Many different factors contribute to the development of prostate cancer, including somatic mutations of the androgen receptor (AR) or AR amplification (10). Some mutations in the AR result in altered ligand-binding specificity, permitting activation by nonandrogenic steroid hormones or even by antiandrogens. AR amplification with concomitant overexpression of AR can increase the sensitivity of prostate cancer cells to low levels of androgens that eventually result in the development of androgen-independent prostate cancer (11). It recently has been found that the AR is the main factor that determines the molecular changes required for driving prostate...
cancer cells from an androgen-dependent to an androgen-independent state (12). There are several publications that explore the effects of HDACIs on prostate cancer cell growth and cell death (3, 13-16). It has been shown that the HDACI suberoylanilide hydroxamic acid inhibited cell growth of several prostate cancer cell lines in vitro and caused significant suppression of transplanted CWR22R xenografts without detectable toxicity (3). The HDACIs sodium butyrate and trichostatin A in combination with dihydroxyvitamin D₃ synergistically inhibited the growth of several prostate cancer cell lines (13); sodium butyrate and depsipeptide decreased the level of prostate-specific antigen (PSA) in LNCaP (14), and the same effect was found after treatment of LNCaP with valproic acid (16). However, the mechanisms of HDACI effects on prostate cancer cell growth and expression of androgen-dependent genes are poorly understood and remain to be investigated.

We treated several human prostatic carcinoma cell lines with trichostatin A and found that trichostatin A induced cell death in the AR-positive cell lines LNCaP and CWR22R to higher extent compared with AR-negative cell lines PC3 and DU145. We then discovered that trichostatin A and other HDACIs suppressed AR gene expression in AR-positive human prostate cell lines as well as in AR-positive breast carcinoma cells and in mouse prostate. Trichostatin A also induced caspase activation, but trichostatin A–induced AR suppression and cell death were caspase independent. In addition, we found that doxorubicin inhibited AR expression. However, the most unexpected result was observed with p21(WAF1/CIP1) (p21) expression: trichostatin A and doxorubicin when used separately increased expression of p21, but p21 completely disappeared after simultaneous treatment with these two drugs. We then determined that simultaneous treatment with trichostatin A and doxorubicin synergistically increased cell death in AR-positive cell lines.

In summary, our data suggest that the main mechanism of trichostatin A–induced cell death in AR-positive prostate cancer is inhibition of AR gene expression. The synergistic effect of simultaneous treatment with trichostatin A and doxorubicin is mediated via inhibition of AR expression, induction of protease activity, increased expression of p53, and proteolysis of p21.

**Results**

Trichostatin A Induces Cell Death in AR-Positive Cell Lines LNCaP and CWR22R to Higher Extent Compared with AR-Negative Cell Lines PC3 and DU145

We estimated cell death using calcein assay in four different prostate cancer cell lines after treatment for 72 hours with different doses of trichostatin A. As can be seen from Fig. 1A,
the cell death of AR-positive cell lines LNCaP and CWR22R is almost twice higher compared with AR-negative cell lines PC3 and DU145. Interestingly, as we have shown previously, this correlation was not observed after treatment with TNF receptor family death ligands: LNCaP is sensitive to TNF-α-treatment (17), whereas CWR22R is resistant (data not shown); PC3 is sensitive to treatment with TNF-α, anti-Fas antibody, and TRAIL, whereas DU145 is resistant to all three death ligands (18, 19). To verify the data obtained with calcein assay, we estimated cell death in LNCaP by both calcein and CellTiter Blue assays after treatment with three different doses of trichostatin A for 24, 48, and 72 hours (Fig. 1B). As can be seen from Fig. 1, both assays gave similar results. In addition, estimation of cell death by trypan blue exclusion gave the same level of cell death as presented in Fig. 1 (data not shown). We have also analyzed cell death after 24, 48, and 72 hours of treatment with 400 ng/mL trichostatin A and did not observe a difference between 100 and 400 ng/mL trichostatin A (as can be seen from Fig. 2B for 72 hours of treatment).

Trichostatin A–Induced Cell Death Is Caspase and Androgen Independent

We found previously that cell death is androgen and caspase dependent in LNCaP after treatment with TNF receptor family ligands (20). We therefore investigated whether trichostatin A could induce caspase activity and cell death in the presence of the AR ligand dihydrotestosterone and the pan-caspase inhibitor Z-VAD-fmk. As shown in Fig. 2A, trichostatin A induced caspase activity in a dose-dependent manner; this activity was only slightly enhanced in the presence of 10 nmol/L dihydrotestosterone and, as expected, completely inhibited by Z-VAD-fmk. Surprisingly, pretreatment with Z-VAD-fmk and dihydrotestosterone did not affect cell death (Fig. 2B), which suggests that trichostatin A–induced cell death is caspase and androgen independent. In contrast, both caspase activation and cell death were caspase and androgen dependent after treatment of LNCaP with TNF-α (Fig. 2C).

We next investigated the activation of individual caspasases (2, 3, 7, 8, and 9) and poly(ADP-ribose) polymerase proteolysis by Western blot analysis (Fig. 3A). In accordance with observed DEVDase activity (Fig. 2A), all caspases were activated and this was accompanied by poly(ADP-ribose) polymerase cleavage. These data indicate that trichostatin A is able to induce apoptosis-related pathways, but the final outcome of trichostatin A treatment, cell death, can be accomplished independent of caspase activation. We then investigated how trichostatin A affects the expression of other, select proteins that have been described to play a role in cell death. Figure 3B shows that trichostatin A induced proteolysis of p21, sharply decreased the level of Akt (although it did not change the level of Akt phosphorylation), and decreased the levels of TRAIL-R1 and TRAIL-R2. Especially interesting is the effect of trichostatin A on p53 and AR expression: trichostatin A induced redistribution of p53 from the cytosol to the Triton X-100-insoluble fraction, which consists of lipid rafts, cytoskeletal proteins, and chromatin. Trichostatin A also completely eliminated AR from both cytosol and pellet fractions. These effects of trichostatin A were found to be androgen independent.

**FIGURE 2.** Trichostatin A–induced cell death is androgen and caspase independent. LNCaP was plated at a density of 7,000 cells per well in the presence of 10 nmol/L dihydrotestosterone (DHT) or 50 μmol/L Z-VAD-fmk. Trichostatin A (at the indicated concentrations) was added after 24 hours of plating. A, Caspase activity in living cells was measured with the fluorogenic substrate Ac-DEVD-AMC. The substrate (20 μmol/L final concentration) was mixed with cells in growth medium, cells were plated on 96-well plates and treated with trichostatin A, and substrate hydrolysis was monitored after 72 hours of treatment using a fluorescence reading system set to 360 nm for excitation and 460 nm for emission. B, Cell death was estimated 72 hours after trichostatin A treatment by calcein assay. Points, mean of four replicates in one of two separate experiments with similar results. C, LNCaP was treated for 48 hours with TNF-α (20 ng/mL) in the absence/presence of 10 nmol/L dihydrotestosterone or 50 μmol/L Z-VAD-fmk. Caspase activity was estimated as described in (A) and cell death was estimated by calcein assay. Caspase activity and cell death were taken as 100 in control (untreated) cells.

Inhibition of AR Is Time and Dose Dependent

Considering the very important role of AR in cell survival of androgen-dependent cells, we next investigated the dose-and
time-dependent effect of trichostatin A on expression of AR as well as genomic activity of AR using LNCaP cells with ARE(3)-Luc vector. As can be seen from Fig. 4A, 25 and 50 ng/mL trichostatin A did not change the level of AR, but starting at 100 ng/mL the expression of AR was sharply decreased after 24 hours of treatment. The level of AR was partly recovered after 48 hours of treatment and returned to normal level after 72 hours. These data indicate that trichostatin A is a reversible inhibitor of AR gene expression. However, AR transactivation was inhibited more than twice by 25 ng/mL trichostatin A after 24 hours and inhibited by 90% after 48 and 72 hours of treatment. The higher doses of trichostatin A almost completely eliminated AR transactivation potential. Given the fact that AR transactivation was inhibited while the level of AR protein remains unchanged, one may conclude that trichostatin A mediates its effect via inhibition of some AR coactivator(s).

HDACIs Suppress AR Expression in Human Breast Carcinoma Cell Lines and in Mouse Prostate

To examine the effect of other inhibitors of histone deacetylases on AR expression, we tested suberohydroxamic acid (50 µg/mL), depsipeptide (100 nmol/L), and sodium butyrate (5 mmol/L). These drugs did not induce cell death after 24 hours of treatment at the indicated doses. As shown in Fig. 5A, all HDACIs suppressed AR expression, which indicates that this effect is not trichostatin A specific. We also showed that these HDACIs did not induce cell death after 24 hours of treatment, as it was shown for trichostatin A (Fig. 1B). To address the possibility that the trichostatin A effect on AR expression is restricted to LNCaP, we treated two breast carcinoma cell lines MCF7 and T47D that were described as AR positive with trichostatin A (21, 22). As can be seen from Fig. 5B, trichostatin A inhibited AR expression in both cell lines but did not affect the expression of estrogen receptor-α. Thus, the trichostatin A effect is AR specific but not cell type specific.

The next question we asked was whether the trichostatin A effect was specific for human AR or could also affect the expression of mouse AR. We treated the mouse prostate cell line RM1 with trichostatin A; trichostatin A was also orthotopically injected twice into mouse prostates (dorsal lobe). Prostate tissues were collected 24 hours after the second injection, and expression of mouse AR was examined by Western blot analysis. Figure 5C shows that trichostatin A inhibited the mouse AR expression in both RM1 cells and normal prostate. These data indicate that HDACIs suppress human and mouse AR expression in different cancer cell lines as well as in normal tissue, affirming the generality of this response.

Trichostatin A–Mediated Inhibition of the AR Expression Occurs on the Gene Level

Two different trichostatin A–induced pathways may result in AR protein disappearance after treatment with 100 ng/mL trichostatin A: at the protein level (destabilization/proteolysis) or at the gene level (direct or indirect inhibition of the AR gene expression). To discern these alternatives, we investigated AR mRNA levels by semiquantitative PCR after treatment of LNCaP with trichostatin A for 6, 12, and 24 hours. As shown in Fig. 6A, trichostatin A inhibited AR mRNA level by 3-, 7-, and 20-fold after 6, 12, and 24 hours of treatment, respectively. This indicates that trichostatin A inhibits AR gene expression. We next investigated whether the general inhibitor of transcription,
actinomycin D, can prevent this effect of trichostatin A. As can be seen from Fig. 6B, actinomycin D prevented trichostatin A–mediated inhibition of AR expression. This fact suggests that indirect inhibition of AR gene expression is mediated by trichostatin A. At the same time, actinomycin D had a different effect on p21 expression: p21 expression was inhibited by actinomycin D even in the presence of trichostatin A, although trichostatin A itself increased p21 expression.

Because inhibition of AR expression after trichostatin A treatment is always accompanied by the increased level of p21, it is conceivable that p21 can play a role in inhibition of AR gene activity. Although p21 is not a transcription factor, some of its functions may be mediated by indirect effects of p21 on gene expression (23, 24). To test this idea, LNCaP was treated with trichostatin A, doxorubicin, and simultaneously with trichostatin A and doxorubicin (Fig. 7A). Surprisingly, we found that doxorubicin inhibited the AR expression as was observed for trichostatin A treatment. However, a most unexpected result was observed for p21 expression: trichostatin A and doxorubicin when were used separately increased expression of p21, but p21 completely disappeared after simultaneous treatment with these two drugs. The level of p21 mRNA after trichostatin A + doxorubicin treatment was unchanged after 8 hours of treatment (Fig. 7B), which indicates that trichostatin A + doxorubicin treatment induced protein degradation and did not affect the p21 gene expression.

We next asked whether this striking disappearance of p21 is caspase and/or protease/proteasome dependent. LNCaP was treated with trichostatin A and doxorubicin in the presence of either Z-V-AD-fmk or the protease/proteasome inhibitor MG132. As can be seen from Fig. 7, p21 disappearance was caspase independent, whereas suppression of protease/proteasome activity by MG132 partially protected p21 inhibition by trichostatin A + doxorubicin treatment. Interestingly, the appearance of the p14 band of p21 after MG132 treatment indicates that this proteolysis is caspase dependent, because the same band appears after treatment with trichostatin A and doxorubicin. The same pattern of Z-V-AD-fmk-independent and MG132-dependent pattern of inhibition was observed for AR expression. Z-V-AD-fmk treatment itself did not change the level of p53 and did not affect the increased level of p53 under trichostatin A + doxorubicin treatment, whereas MG132 itself increased p53 and prevented further increases with trichostatin A + doxorubicin treatment. These data suggest that it is the protease/proteasome that plays an important role in the regulation of p21, p53, and AR levels. We have also investigated the pattern of histone acetylation under trichostatin A/doxorubicin treatment and observed that only trichostatin A itself could induce histone acetylation levels independent of simultaneous treatment with doxorubicin, Z-V-AD-fmk, or MG132.

Because actinomycin D and MG132 can protect the AR from the effect of trichostatin A, we asked whether these
compounds were capable of protecting LNCaP from the death effect of trichostatin A. We pretreated LNCaP for 1 hour with 10 μmol/L MG132 or 1 μg/mL actinomycin D, treated with five doses of trichostatin A, and evaluated cell death after 48 hours of treatment. As can be seen from Fig. 8, MG132 alone at 200 ng/mL kills only 20% to 30% of cells after 48 hours treatment. Trichostatin A alone at concentrations of 12 to 25 ng/mL kill ~30% to 40% of cells and combined treatment kill 80% to 90% of cells. We then assessed the cell death in different AR-positive cell lines in comparison with AR-negative cell lines. As shown in Fig. 9C, simultaneous treatment with 50 ng/mL trichostatin A and 200 ng/mL doxorubicin completely killed AR-positive cell lines, whereas cell death in AR-negative cell lines was on the level 25% to 30%.

Because p21 disappearance under simultaneous treatment with trichostatin A and doxorubicin was caspase independent but protected by MG132, we next investigated whether this treatment could induce protease activity. Figure 10A shows that both trichostatin A and doxorubicin when used separately were not able to induce protease activity. However, LLVYase activity was readily detectable under simultaneous treatment with trichostatin A and doxorubicin. We have shown previously that after androgen withdrawal LNCaP became resistant to different types of treatment (30). To test whether androgen withdrawal affects sensitivity of LNCaP to treatment with trichostatin A + doxorubicin, LNCaP was cultured for 7 days in steroid-free condition and then treated with different doses of trichostatin A in the absence or presence of 200 ng/mL doxorubicin. As can be seen from Fig. 10B, trichostatin A–induced cell death sharply decreased in steroid-free condition (compare with Fig. 1). Doxorubicin itself also cannot induce cell death. However, together, these two agents can kill 80% of cells in steroid-free condition.

Simultaneous Treatment with Trichostatin A and Doxorubicin Increased Cell Death in AR-Positive Cell Lines

p21 is known to play an important role in apoptosis as an antiapoptotic factor (24-29). The fact that simultaneous treatment with trichostatin A and doxorubicin resulted in complete degradation of p21 protein, whereas p53 was up-regulated, as well as the fact that both drugs sharply inhibited the AR expression prompted us to examine the dose-dependent effects of simultaneous treatment with these drugs on cell death. As shown in Fig. 9, the combined treatment of LNCaP and CWR22R with trichostatin A and doxorubicin had additive effects on both caspase activation (Fig. 9A) and cell death (Fig. 9B). Doxorubicin alone at 200 ng/mL kills only 20% to 30% of cells after 48 hours treatment. Trichostatin A alone at concentrations of 12 to 25 ng/mL kill ~30% to 40% of cells and combined treatment kill 80% to 90% of cells. We then assessed the cell death in different AR-positive cell lines in comparison with AR-negative cell lines. As shown in Fig. 9C, simultaneous treatment with 50 ng/mL trichostatin A and 200 ng/mL doxorubicin completely killed AR-positive cell lines, whereas cell death in AR-negative cell lines was on the level 25% to 30%.

Because p21 disappearance under simultaneous treatment with trichostatin A and doxorubicin was caspase independent but protected by MG132, we next investigated whether this treatment could induce protease activity. Figure 10A shows that both trichostatin A and doxorubicin when used separately were not able to induce protease activity. However, LLVYase activity was readily detectable under simultaneous treatment with trichostatin A and doxorubicin. We have shown previously that after androgen withdrawal LNCaP became resistant to different types of treatment (30). To test whether androgen withdrawal affects sensitivity of LNCaP to treatment with trichostatin A + doxorubicin, LNCaP was cultured for 7 days in steroid-free condition and then treated with different doses of trichostatin A in the absence or presence of 200 ng/mL doxorubicin. As can be seen from Fig. 10B, trichostatin A–induced cell death sharply decreased in steroid-free condition (compare with Fig. 1). Doxorubicin itself also cannot induce cell death. However, together, these two agents can kill 80% of cells in steroid-free condition.
Discussion

Two recent publications strongly suggest that down-regulation of AR expression should be considered as the main strategy for the treatment of advanced prostate cancer (12, 31). Chen et al. (12) reported that an increase in AR levels was both necessary and sufficient to convert prostate cancer growth from a hormone-sensitive to a hormone-refractory stage. Overexpression of AR in LNCaP resulted in growth in at least 80% lower concentrations of the synthetic androgen R1881, and these cells were also resistant to the antiandrogen bicalutamide. Moreover, AR overexpression shortened the latency of tumor formation by 50% in castrated animals. In contrast, knockdown of AR levels by using a short hairpin RNA against AR resulted in tumors with slower growth than control cells in castrated animals. Remarkably, the tumors that did grow expressed AR, indicating selection for cells that escaped AR knockdown. Liao et al. (31) showed that knocking down AR levels by a small interfering RNA approach in AR-positive cell lines LNCaP, LAPC-4, C4-2, and CWR22Rv1 resulted in apoptotic cell death accompanied by caspase-3 and caspase-6 activation and DFF45 and poly(ADP-ribose) polymerase cleavage. At the same time, no cell death was observed in AR-negative prostate cancer cell lines or AR-positive breast carcinoma cells (31). These data show that inhibition of AR expression is itself sufficient to induce cell death in AR-positive cell lines even in the absence of additional treatment with chemotherapeutic agents or TNF receptor family death ligands.

Although some HDACIs are already being used in clinical trials as anticancer agents, their mechanism of action remains to be investigated. The main problem in elucidating the mechanisms of action of HDACIs is their pleiotropic effects that involve multiple signaling pathways. For example, gene profiling in the CEM cell line after treatment with suberoylanilide hydroxamic acid and depsipeptide revealed that >40% of genes on the array were either significantly activated or repressed (5). At the same time, the effects of HDACIs are apparently cell type specific; for example, in different cell types, HDACIs have been reported to activate either death receptor or intrinsic apoptotic pathway or induce apoptosis-independent cell death (1). There are several publications that explore the effects of HDACIs on prostate cancer cell growth and cell death (3, 13-16). It has been shown that HDACIs can

![Figure 9](https://mcr.aacrjournals.org)
inhibit cell growth and survival of several prostate cancer cell lines, but the mechanisms of HDACI effects on prostate cancer cell growth and expression of androgen-dependent genes have not been investigated.

The main finding in our study is that HDACIs suppress AR gene expression and AR transactivation, resulting in cell death in AR-positive prostate cancer cells. We found that trichostatin A induced cell death in the AR-positive cell lines LNCaP and CWR22R to higher extent compared with AR-negative cell lines PC3 and DU145. We then showed that although trichostatin A induced caspase activity in a dose-dependent manner, trichostatin A–induced cell death was caspase independent. Therefore, trichostatin A is able to induce apoptosis-related pathways, but the final outcome of trichostatin A treatment, cell death, can be accomplished independent of caspase activation. These data raised the question what are the trichostatin A targets that promote cell death in AR-positive prostate cancer cell lines.

Considering the critical role of AR in survival of androgen-dependent cells, we investigated the effect of trichostatin A on expression of AR at both protein and gene levels as well as AR transactivation. We found that low doses of trichostatin A (25-50 ng/mL) did not change the level of AR, but starting from 100 ng/mL the expression of AR was sharply diminished after 24 hours of treatment. However, even low doses, trichostatin A sharply reduced AR transactivation ability but did not change the AR protein level. These data suggest that trichostatin A at low doses can either reduce the level of AR coactivators or induce the level of AR corepressor. It remains to be investigated if the scenario is true; however, in both cases, treatment with trichostatin A results in inhibition of AR transactivation. In turn, AR silencing can result in a reduction of antiapoptotic proteins. For example, Liao et al. (31) showed that expression of Bcl-xL decreased after reduction of AR level by small interfering AR.

The dose-dependent effects on AR activity of sodium butyrate and depsipeptide were observed in LNCaP by estimation of PSA mRNA level: low doses of these agents increased the levels of PSA mRNA, whereas 5 mmol/L sodium butyrate and 100 ng/mL depsipeptide completely inhibited PSA expression (14). In another study (16), PSA expression was inhibited by treatment of LNCaP with valproic acid and trichostatin A. Therefore, HDACIs inhibit both AR expression and AR-dependent expression of PSA in a dose-dependent manner. Importantly, in accordance with caspase-independent cell death after trichostatin A treatment, the trichostatin A–mediated inhibition of AR expression was also caspase independent.

We found that all HDACIs tested suppressed AR expression. This indicates that AR inhibition is not trichostatin A specific but rather HDACI specific. To investigate whether the effect of trichostatin A on AR expression is species specific, the prostate mouse cell line RM1 was treated with trichostatin A, and trichostatin A was orthotopically injected into the mouse prostate. Trichostatin A inhibited mouse AR expression in both RM1 cells and normal mouse prostate, indicating that the effect of trichostatin A on AR is not species specific. The other key question is whether the effect of trichostatin A is tissue specific. To address this question, we treated two breast carcinoma cell lines (MCF7 and T47D) with trichostatin A and found that trichostatin A inhibited AR expression in both cell lines but did not affect the expression of estrogen receptor-α. Thus, the trichostatin A effect seems to be AR specific but not cell type specific.

Subsequent experiments were designed to determine whether trichostatin A inhibits AR expression at the protein or gene level. Two different trichostatin A–induced pathways may result in AR protein degradation: at the protein level (destabilization/proteolysis) or at the gene level (direct or indirect inhibition of the AR gene expression). To distinguish these alternatives, we investigated AR mRNA levels and found that trichostatin A inhibits AR gene expression. We next observed that actinomycin D prevented trichostatin A–mediated inhibition of AR expression. This fact suggests that indirect inhibition of the AR gene expression is mediated by trichostatin A. Trichostatin A apparently induces expression of some protein that in turn binds to the AR gene and inhibits its expression; actinomycin D, as an inhibitor of transcription,
prevents the expression of this putative inhibitor and therefore rescues the AR gene from the effect of trichostatin A. AR can also be protected from the effect of trichostatin A by the proteasome/protease inhibitor MG132 (Fig. 7). No additive effect was observed under simultaneous treatment with MG132 and trichostatin A or actinomycin D with trichostatin A (Fig. 8). On the contrary, protective effects were noticed when cells were treated with high doses of trichostatin A in the presence of MG132 or actinomycin D, suggesting that trichostatin A mediates its death effect via elimination of AR.

Doxorubicin, the most widely used chemotherapeutic agent, targets DNA topoisomerase II enzyme activity and causes DNA breaks. As expected, treatment of LNCaP with doxorubicin increased p53 expression that was accompanied by an increase in p21. p21 is well known as an inhibitor of cyclin-dependent kinases, which provides checkpoints that control entry into cell cycle. In addition, it has been shown recently that increased expression of p21 protected cells from different apoptotic stimuli, whereas decreased level of p21 facilitated apoptosis. This occurs in apoptosis induced by both chemotherapeutic drugs and TNF receptor family death ligands. It has been shown that apoptosis induced by overexpression of the cytoplasmic domain of the TRAIL-R1 in several cell lines can be suppressed by p21 through its inhibition of initiator caspases (25). In colon carcinoma cell lines, p21 was found to protect the cells from p53-dependent and p53-independent apoptosis induced by doxorubicin, etoposide, and colcemid (26). p21 also plays a protective role in apoptosis induced by Myc (27), Taxol (28), daunomycin (29), sodium butyrate (32), and cisplatin (33) and in Fas-mediated apoptosis by making complex formation between p21 and caspase-3 (34). Interestingly, p21 can be phosphorylated by either Akt (28) or protein kinase A (34), which enhances protein stability (28) or initiates complex formation between p21 and caspase-3 (34). These data indicate that p21 can directly modulate apoptotic signaling pathways independently of its cell cycle inhibitory effect.

It is interesting to note that trichostatin A and doxorubicin when used separately induced p21 expression, but under simultaneous treatment trichostatin A and doxorubicin inhibited the expression of p21, and this effect was caspase independent. We found that p21 mRNA levels did not change after trichostatin A + doxorubicin treatment, suggesting that the inhibitory effect of trichostatin A + doxorubicin treatment occurs at the protein level. To gain insight into the mechanism of p21 degradation, protease activity was investigated after treatment of LNCaP with different doses of trichostatin A + doxorubicin by using Succ-LLVY-AMC as a substrate for the proteasome and other chymotrypsin-like proteases (35). When used separately trichostatin A and doxorubicin were not able to induce protease activity, but LLVYase activity was readily detectable under simultaneous treatment with trichostatin A and doxorubicin (Fig. 10). In accordance with these data, MG132, but not Z-VAD-fmk, protected p21 from trichostatin A + doxorubicin-induced degradation. Thus, trichostatin A + doxorubicin-induced protease activity was apparently responsible for p21 degradation.

There is strong evidence that down-regulation of AR expression should be considered as the main strategy for the treatment of advanced prostate cancer (12, 31). Our data show that treatment with trichostatin A, as an example of HDACIs, induces cell death in prostate cancer cells by inhibiting AR expression. In addition, we found that simultaneous treatment with trichostatin A and doxorubicin increased expression of proapoptotic protein p53 but prevented expression of anti-apoptotic protein p21. Remarkably, simultaneous treatment with trichostatin A and doxorubicin completely killed AR-positive cell lines, whereas cell death in AR-negative cell lines was on the level of 25% to 30%. Moreover, although trichostatin A–induced cell death sharply decreased in steroid-free condition and doxorubicin itself also could not induce cell death in steroid-free condition, simultaneous treatment with these two agents killed 80% of cells in steroid-free condition.

In conclusion, our results show that combined treatment of prostate cancer with HDACIs and doxorubicin may be an effective and novel approach for the treatment of advanced AR-positive prostate cancer.

Materials and Methods

Cell Lines, Reagents, and Estimation of Cell Viability

The human prostate carcinoma cell lines LNCaP, PC3, DU145, and CWR22R as well as the breast carcinoma cell lines MCF7 and T47D were cultured in RPMI 1640 as described previously (18). To culture cells in steroid-free condition, RPMI 1640 was supplemented with 10% of charcoal-stripped serum (HyClone, Logan, UT). PC3-Bcl-2 and LNCaP-Bcl-2 transfectants with Bcl-2 overexpression, LNCaP-FADD-DN, LNCaP-si-p53, and PC3-AR cell lines were described previously (20, 36-39). To measure cell viability, we used the calcein AM assay (Molecular Probes, Eugene, OR) as described previously (20). Calcein AM is a fluorogenic esterase substrate that is hydrolyzed in live cells to a green fluorescent product (calcein), and fluorescence at 485/530 nm was measured with a FL600 fluorimeter (Bio-Tek Instruments, Inc., Burlington, VT). The level of calcein fluorescence is not dependent on the presence of multidrug resistance mechanism, because we have found previously that prostate cancer cell lines do not contain multidrug resistance–associated P-glycoprotein (36). Cells were incubated and treated in 96-well flat-bottomed plates. After incubation, medium was removed and the plates were washed with PBS and incubated with 100 μL of 2 μg/mL calcein AM solution for 30 minutes at room temperature. Fluorescence, which is proportional to cell viability, was then measured with the FL600 fluorimeter. Cell viability was also measured by CellTiter Blue cell viability assay (Promega, Madison, WI) and by trypan blue exclusion counting of live/dead cells. The CellTiter Blue assay provides a fluorometric method for estimating the number of viable cells. It uses the indicator dye resazurin to measure the metabolic capacity of cells as an indicator of cell viability. Viable cells retain the ability to reduce resazurin into resofurin, which is highly fluorescent at 530/590 nm. CellTiter Blue solution (30 μL) was added to cells in 96-well plates and incubated for 2 hours at 37°C. Cells were incubated and treated in 96- or 24-well flat-bottomed plates and cell death was estimated after 24, 48, or 72 hours. MG132 (acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin; Ac-DEVD-AMC), N-succinyl-Leu-Leu-Val-Tyr-AMC (Suc-LLVY-AMC),
and pan-caspase inhibitor Val-Ala-Asp (Z-VAD-fmk) were purchased from BioMol (Plymouth Meeting, PA). Trichostatin A, suberohydroxamic acid, and sodium butyrate were purchased from Sigma (St. Louis, MO).

Caspase and Protease Activity Measurement in Cell Lysates and in Live Cells

Caspase and protease activities in cell lysates were measured as described previously (36). Briefly, cell lysates were prepared in 1% Triton X-100 buffer (pH 7.2) containing protease inhibitors. Protein lystate (40 μg) was incubated for 60 minutes in assay buffer [20 mmol/L PIPES (pH 7.2), 100 mmol/L NaCl, 10 mmol/L DTT, 1 mmol/L EDTA, 0.1% CHAPS, 10% sucrose] with 40 μmol/L fluorescent substrate Ac-DEVD-AMC. To measure protease activity, protein lystate was incubated in assay buffer [30 mmol/L HEPES, 0.5 mmol/L EDTA, 1 mmol/L DTT (pH 7.5)] with 20 μmol/L Suc-LLVYAMC. Caspase and protease activities in intact (living) cells were measured with the fluorogenic substrates Ac-DEVD-AMC or Suc-LLVY-AMC as described previously (38). Briefly, the substrates (20 μmol/L final concentration) were mixed with cells in growth medium and cells were plated in 96-well (7,000 per well) or 24-well (50,000 per well) plates and incubated with the substrates for 24 hours to make cells adherent. Cells were then treated with death-inducing ligands or drugs, and substrate hydrolysis was monitored using a fluorescence reading system set at 360 nm for excitation and 460 nm for emission.

Western Blot Analysis

Western blot detection of proteins was done as described previously (36). Briefly, proteins (20 μg) were separated on 4% to 20% gradient SDS-PAGE and blotted onto a nitrocellulose membrane (Invitrogen, Carlsbad CA). Equal loading was controlled routinely by reversible staining of the membrane with Ponceau S solution (Sigma). Membranes were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween 20 and then incubated with the corresponding mouse monoclonal or rabbit polyclonal antibodies: anti-AR, anti-TRAIL-R1, anti-TRAIL-R2, anti-caspase-2, -7, -8, -70, and -90 (Stressgen, Victoria, British Columbia, Canada). The blots were counterstained with goat anti-mouse or goat anti-rabbit IgG conjugated with horseradish peroxidase (Pierce, Rockford, IL). The immunoreactive bands were visualized by incubation of the membrane with enhanced chemiluminescence reagent (Pierce).

Luciferase Reporter Assay

To measure AR-dependent transactivation under trichostatin A treatment, we introduced in LNCaP promoter-luciferase reporter containing three copies of ARE(3)-Luc vector with Neo gene, which was provided by Dr. Katerina Gurova (Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH) and selected permanent transfectant using G418 as selection agent. This reporter consists of a cassette of three ARE from rat probasin promoter followed by heat shock protein-70 minimal promoter, producing almost zero background expression per se in AR-negative prostate cell lines. To estimate luciferase activity, cells were harvested by trypsinization, washed in PBS, and lysed in reporter lysis buffer (200 μL; Promega). Luciferase chemiluminescence activity was measured using the luciferase assay kit (Promega). Sample aliquots (20 μL) were assayed for light emission with a plate reader luminometer (MLX Dynex Technology, Inc., Franklin, MA). The values of the luciferase assay were normalized with respect to the values of protein concentration.

Semiquantitative Reverse Transcription-PCR

Cells were treated with trichostatin A (400 ng/mL) for 6, 12, and 24 hours. RNA was purified with RNeasy Mini kit (Qiagen). To avoid contamination by DNA, samples were treated with the RNase-free DNase (Qiagen, Valencia, CA) during purification. Equal amounts (1 μg) of total RNA from control and treated cells were reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) and cDNA (5 μL) was taken for amplification with primers for p21 and AR: p21 forward primer 5'-dCGAAGTCAGTTCTTG-3' and reverse primer 5'-dAAGATGTAGACGGCCTTT-3' (bp) and AR forward primer 5'-dGTGGACGACCA-GATGCGTGT-3' and reverse primer 5'-dGAAGAGTAGCAGTGGCTTCATGC-3' (bp). All reverse transcription-PCRs were done in the linear range for each transcript compared with glyceraldehyde-3-phosphate dehydrogenase as a reference control. The PCR products were analyzed on 6% PAGE, Ethidium bromide–stained gels were scanned (Photodyne, Northbridge, CA) and analyzed with Scion imaging software (Scion Corp., Frederick, MD). Negative reverse transcription-PCR controls were done in the absence of RNA and/or reverse transcriptase. Control for contamination by DNA was prepared using RNA as a template in the PCR reaction instead of cDNA.

Intraprostate Trichostatin A Treatment

Male C57BL/6 mice were purchased from the National Cancer Institute (Bethesda, MD). Animals were maintained in the University of Iowa Animal Facility and fed sterile water and food ad libitum. Mice were treated in accordance with Institutional Animal Care and Use Committee guidelines. Animals were anesthetized with a mixture of xylazine (35 mg/kg; Phoenix Pharmaceutical, Inc., St. Joseph, MO) and ketamine (120 mg/kg; Phoenix Pharmaceutical) by i.p. injection. The abdomens of recipient and control animals were exposed by a small midline incision of the lower abdomen. Trichostatin A (500 μg/kg in 100 μL DMSO) or 100 μL of DMSO was injected into the left lateral prostatic lobe. Following trichostatin A or DMSO administration, the abdominal wall was closed using 5-0 chromic gut surgical suture (Ethicon, Somerville, NJ). Post surgery, animals were kept warm and observed until recovered from anesthesia. The procedure was repeated the following day. Animals were sacrificed 24 hours after the second trichostatin A treatment, and prostates were removed and evaluated for AR expression.
References
Molecular Cancer Research

Mechanisms of Cell Death Induced by Histone Deacetylase Inhibitors in Androgen Receptor–Positive Prostate Cancer Cells

Oskar W. Rokhlin, Rebecca B. Glover, Natalya V. Guseva, et al.

*Mol Cancer Res* 2006;4:113-123.

Updated version Access the most recent version of this article at:
http://mcr.aacrjournals.org/content/4/2/113

Cited articles This article cites 39 articles, 14 of which you can access for free at:
http://mcr.aacrjournals.org/content/4/2/113.full.html#ref-list-1

Citing articles This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/4/2/113.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.