Chemical ablation of androgen receptor in prostate cancer cells by the histone deacetylase inhibitor LAQ824

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Abstract

Androgen receptor plays a critical role in the development of primary as well as advanced hormone-refractory prostate cancer. Therefore, ablation of androgen receptor from prostate cancer cells is an interesting concept for developing a new therapy not only for androgen-dependent prostate cancer but also for metastatic hormone-refractory prostate cancer, for which there is no effective treatment available. We report here that LAQ824, a cinnamyl hydroxamatic acid histone deacetylase inhibitor currently in human clinical trials, effectively depleted androgen receptor in prostate cancer cells at nanomolar concentrations. LAQ824 seemed capable of depleting both the mutant and wild-type androgen receptors in either androgen-dependent and androgen-independent prostate cancer cells. Although LAQ824 may exert its effect through multiple mechanisms, several lines of evidence suggest that inactivation of the heat shock protein-90 (Hsp90) molecular chaperone is involved in LAQ824-induced androgen receptor depletion. Besides androgen receptor, LAQ824 reduced the level of Hsp90 client proteins HER-2 (ErbB2), Akt/PKB, and Raf-1 in LNCaP cells. Another Hsp90 inhibitor, 17-allyamino-17-demethoxygeldanamycin (17-AAG), also induced androgen receptor diminution. LAQ824 induced Hsp90 acetylation in LNCaP cells, which resulted in inhibition of its ATP-binding activity, dissociation of Hsp90-androgen receptor complex, and proteasome-mediated degradation of androgen receptor. Consequently, LAQ824 blocked androgen-induced prostate-specific antigen production in LNCaP cells. LAQ824 effectively inhibited cell proliferation and induced apoptosis of these prostate cancer cells. These results reveal that LAQ824 is a potent agent for depletion of androgen receptor and a potential new drug for prostate cancer. [Mol Cancer Ther 2005;4(9):1311–9]

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Introduction

Androgen receptor is a nuclear transcription factor of the steroid hormone receptor superfamily (1). It is activated by androgens to regulate prostate gland development and prostate cancer growth (1, 2). Surgical ablation of androgens and antiandrogen therapy are two of current therapeutic approaches for patients with locally advanced or metastatic prostate cancer (3–5). These treatments are based on deprivation of ligand and blockage of ligand-receptor interaction. Although most patients initially respond to these therapies, the disease eventually progresses to hormone-refractory state (1, 3, 5). Studies have shown that androgen refractory in prostate cancer is caused mainly by androgen receptor overexpression, mutations, or posttranslational activation of the receptor or its coactivators (1, 3, 5, 6). These changes allow androgen receptor to be activated by low levels of adrenal androgens, other steroids, and even antiandrogens or by ligand-independent mechanisms. In other words, most androgen-refractory prostate cancer cells still depend on androgen receptor for their growth and survival (1, 3). Therefore, ablation of androgen receptor from prostate cancer cells would be a better therapeutic strategy for advanced prostate cancer (4, 7).

Androgen receptor is bound to the heat shock protein-90 (Hsp90) molecular chaperone complex, which is essential for androgen receptor stability and maturation (8–13). The Hsp90 chaperone activity is ATP dependent (10, 12). It has been shown that interrupting ATP binding to Hsp90 with nucleotide mimetics such as geldanamycin or 17-allyamino-17-demethoxygeldanamycin (17-AAG) destabilizes the Hsp90-client protein complexes that results in degradation of Hsp90 client proteins, including androgen receptor, ErbB2 (HER-2), and Akt in prostate cancer cells (8, 12–17).

Histone deacetylase (HDAC) inhibitors have been recognized for their antiproliferative and apoptotic effects on cancer cells, which are mediated in part by selective alternation in genes expression, such as induction of p21[waf] expression (18–21). However, because the spectrum of relevant HDAC inhibitor targets in various types of human cancer has yet to be determined, it is not entirely clear which types of cancer are good candidates for further development of HDAC inhibitor therapy.

Previous studies on HDAC inhibitors have focused mostly on their effects on regulation of gene transcription through acetylation of histones and other transcription regulators. However, transcription regulation alone does not seem to account for all of their antiproliferative/apoptotic effects in cancer cells (18, 22). Indeed, proteins besides histones and transcription factors and coregulators can be modified by acetylation, these includes the androgen receptor chaperone Hsp90 (22–24). Modification of Hsp90 by acetylation was first reported by Yu et al. (22). It was observed that a depsipeptide HDAC inhibitor, FK228, could deplete Hsp90 client proteins Raf-1, ErbB1, ErbB2, and mutant p53 in H322 lung cancer cells and SK-Br-3 breast cancer cells. These investigators went on to show that FK228 induced Hsp90 acetylation in SK-Br-3 cells, which correlated with the loss of its binding activities to ATP and client proteins (22).

LAQ824 is a cinnamyl hydroxamatic acid derivative that inhibits HDAC at nanomolar concentrations (23–28). LAQ824 is currently in clinical trials as an anticancer drug (25). Prompted by the observation that FK228 could affect Hsp90 client proteins, we have found that LAQ824 induces Hsp90 acetylation in chronic myeloid leukemia cells and breast cancer cells (23, 24). Hsp90 acetylation induced by LAQ824 resulted in the loss of Hsp90 ATP-binding activity and degradation of Hsp90 client proteins Bcr-Abl and ErbB2, respectively, in these cells (23, 24). Significantly, LAQ824 can deplete Bcr-Abl containing the T315I mutation and induce apoptosis of leukemic cells harboring the T315I mutant Bcr-Abl (23). The T315I mutant Bcr-Abl is resistant to the Bcr-Abl tyrosine kinase inhibitor imatinib (Gleevec) as well as the second-generation dual Src/Abl inhibitors currently in clinical and preclinical development for chronic myelogenous leukemia (29, 30). Therefore, due to its ability to deplete Bcr-Abl, HDAC inhibitors like LAQ824 offer a potential solution for drug resistance to Bcr-Abl tyrosine kinase inhibitors in chronic myelogenous leukemia.

Another hydroxamate-based HDAC inhibitor is super-oylanilide hydroxamic acid (SAHA; refs. 19, 20). It has been reported that SAHA suppresses prostate cancer cell growth *in vitro* and *in vivo* (31). Interestingly, although SAHA induces growth inhibition in both androgen receptor–positive (LNCaP) and androgen receptor–negative (PC-3 and TSU-Pr1) prostate cancer cells, the androgen receptor–positive LNCaP cells are much more sensitive to SAHA for inhibition of cell proliferation (31). Furthermore, SAHA induces apoptosis of LNCaP cells but not PC-3 and TSU-Pr1 cells, suggesting that SAHA has a greater effect on androgen receptor–positive prostate cancer cells than on androgen receptor–negative prostate cancer cells. Whether SAHA inactivates Hsp90 and affects androgen receptor level in prostate cancer cells has not been reported.

Based on observations that androgen receptor is an Hsp90 client protein, that the Hsp90 inhibitor 17-AAG can induce androgen receptor degradation, and that LAQ824 can inactivate the Hsp90 chaperone in leukemia and breast cancer cells, we postulated that LAQ824 is a potential agent for chemical ablation of androgen receptor in prostate cancer cells. Using LNCaP cells as a primary model for androgen receptor–positive human prostate cancer cells, we provide evidence here that LAQ824 is able to deplete androgen receptor in prostate cancer cells. Whereas LAQ824 may deplete androgen receptor through multiple mechanisms, our data suggest that inactivation of Hsp90 is involved in LAQ824-induced androgen receptor depletion in prostate cancer cells. Androgen receptor depletion by LAQ824 results in inhibition of androgen-stimulated secretion of prostate-specific antigen (PSA) and cell proliferation. Furthermore, LAQ824 induces apoptosis of prostate cancer cells.

Materials and Methods
Materials
LAQ824 (NVP-LAQ824) was prepared by Novartis Pharmaceuticals, Inc. (East Hanover, NJ). 17-AAG was from the Developmental Therapeutics Branch of Cancer Therapy Evaluation Program, National Cancer Institute (Bethesda, MD). Sodium butyrate, actinomycin D, and cyclohexamide were from Sigma (St. Louis, MO). The synthetic androgen methyltrienolone (R1881) was from NEN Life Sciences Products (Boston, MA). Z-VAD-FMK, a cell-permeable pan-caspase inhibitor, was from Calbio-chem (La Jolla, CA). MG-132, a cell-permeable proteasome inhibitor, was from Calbiochem. ATP-Binders Resin[6], in which ATP is bound to polyacrylamide-based resin by linking its γ-phosphate to a 13-atom linker, was from Novagen (Madison, WI). The human PSA Elisa kit was from Anogen (Mississauga, Ontario, Canada).

Sources of antibodies to following antigens are acetylated-lysine and Akt were from Cell Signaling (Beverly, MA); Hsp90 was from Stressgen (Victoria, British Columbia, Canada); androgen receptor and Raf-1 were from Santa Cruz Biotechnology (Santa Cruz, CA); HER-2 was from Calbiochem; β-actin was from Sigma; poly(ADP-ribose) polymerase (PARP) was from PharMingen (San Diego, CA); and p21[waf] (p21) was from NeoMarkers (Fremont, CA).

Cell Culture
LNCaP cells (American Type Culture Collection, Manassas, VA) and C4-2 cells (32, 33) were grown in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS) at 37°C/5% CO_2. For experiments involving R1881 treatment, LNCaP cells were plated in phenol red–free RPMI 1640 plus 10% charcoal/dextran-treated FBS (Hyclone, Logan, UT). LAPC-4 cells (34) were grown in Iscove's medium (Invitrogen) containing 15% FBS as described (35).

Preparation of Cell Lysate, Immunoblotting, and Immunoprecipitation
Cells were treated with various agents as indicated in figure legends. After treatment, cells were placed on ice,

washed with cold PBS, and lysed in lysis buffer [1% Triton X-100, 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 5 mmol/L sodium pyrophosphate, 25 mmol/L NaF, 0.5 mmol/L sodium orthovanadate, 1 mmol/L DTT, 1 μg/mL pepstanin, 2 μg/mL leupeptin, 2 μg/mL aprotinin, 0.1 mg/mL phenylmethylsulfonyl fluoride]. Cell lysates were centrifuged twice at 24,000 × *g* for 15 minutes at 4°C. Protein concentrations in cell lysate supernatants were determined using the Bio-Rad (Bradford) protein assay reagent. For immunoblotting analysis (36, 37), 40 μg of each sample was used. For immunoprecipitation, 1.3 mg of each sample was mixed with 4 μg antibody and 25 μL Protein G-agarose for 3 hours at 4°C. Immunoprecipitates were washed five times with lysis buffer, heated (95°C) in 1× SDS loading buffer for 5 minutes, and processed for SDS-PAGE and immunoblotting analysis.

For preparation of Triton X-100 soluble and insoluble fractions, cells grown in six-well plates were treated and lysed with lysis buffer containing 1% Triton X-100 as described above. After removal of Triton X-100 soluble cell lysate supernatants by centrifugation, the pellets were washed once with the lysis buffer and 1× SDS loading buffer (50 μL) was then added to the pellets and heated at 95°C for 15 minutes to dissolve the Triton X-100-insoluble proteins.

Measurement of PSA Secretion

LNCaP cells (1 × 10⁵ cells per well) were plated in 24-well plates in phenol red–free RPMI 1640 containing 10% charcoal/dextran-treated FBS. Twenty-four hours after plating, the medium was replaced with 0.5 mL fresh medium containing 0 to 0.25 μmol/L LAQ824 plus 1 nmol/L R1881 or solvent control. The incubation was continued for 24 hours. After which, the media were collected and centrifuged at 3,000 rpm for 10 minutes in a microcentrifuge. The cleared media were used for determination of PSA concentrations using the Human PSA Elisa Kit (Anogen) according to the supplier's instruction.

ATP Bead Pull-Down Assay

LNCaP cells were treated with or without LAQ824. Cells were lysed as described above for immunoprecipitation. Cell lysate supernatants (1 mg each) were incubated with 20 μL (3 mg) ATP-Binders Resin at 4°C for 2 hours. ATP beads were washed five times with the lysis buffer. Bound proteins were eluted with 1× SDS gel loading buffer and heat denaturation and then processed for immunoblotting analysis.

Cell Proliferation Assay

LNCaP cells (6 × 10⁴ cells per well) were plated into 12-well plates in RPMI 1640 containing 10% charcoal/dextran-treated FBS. After 24 hours, the medium was replaced with fresh medium with or without 0.2 nmol/L R1881 and 0.1 μmol/L LAQ824, 1 mmol/L sodium butyrate, or solvent control (day 0). Viable cell numbers were determined by the trypan blue exclusion assay (38). LAPC-4 cells (6 × 10⁴ cells per well) were plated as above in Iscove's medium containing 15% charcoal/

dextran-treated FBS. After attachment, cells were cultured in Iscove's medium/5% charcoal/dextran-treated FBS plus test agents. Viable cell numbers were determined as above.

Results

LAQ824 Depletes Androgen Receptor in Prostate Cancer Cells

To determine whether LAQ824 affects androgen receptor in prostate cancer cells, we treated LNCaP cells with various concentrations of LAQ824 for 24 hours. Cell lysates were prepared and analyzed by immunoblotting (Fig. 1A). LAQ824 decreased the androgen receptor level in LNCaP cells at the lowest concentration tested (0.1 μmol/L). The androgen receptor depletion effect was increased as the LAQ824 concentration increased. The control immunoblot indicated that a similar amount of β-actin was present in each sample. Similar to a previous observation in breast cancer cells (24), LAQ824 also decreased the HER-2 protein level in LNCaP cells (Fig. 1A). In cells undergoing apoptosis, the 110-kDa PARP is specifically cleaved into an 85- and a 25-kDa fragment. As shown in Fig. 1A, apoptotic PARP cleavage was detected in LNCaP cells treated with ≥0.25 μmol/L concentrations of LAQ824.

Androgen receptor in LNCaP cells contains a point mutation (T877A). To evaluate whether LAQ824 selectively affects mutated androgen receptor, we determined the effect of LAQ824 on androgen receptor in LAPC-4 cells, which is a human prostate cancer cell line expressing the wild-type androgen receptor (34). LAPC-4 cells were treated with various concentrations of LAQ824 for 24 hours, whereas LNCaP cells were treated with LAQ824 (0.25 μmol/L, 24 hours) as the positive control. Figure 1B shows that the wild-type androgen receptor was depleted in LAPC-4 cells after LAQ824 treatment as was the mutant androgen receptor in LNCaP cells.

C4-2 is an androgen-independent cell line derived from LNCaP cells (32). To assess whether LAQ824 is able to deplete androgen receptor from androgen-independent prostate cancer cells, LNCaP and C4-2 cells were treated with LAQ824 as above and the effect on androgen receptor was analyzed. As shown in Fig. 1C, LAQ824 effectively depleted androgen receptor in C4-2 cells. Thus, LAQ824 is able to deplete both mutant and wild-type androgen receptors in both androgen-dependent and androgen-independent prostate cancer cells.

HDAC inhibitors like LAQ824 may exert their effects through transcriptional and posttranslational mechanisms. To assess whether androgen receptor degradation is accelerated by LAQ824, we treated LNCaP cells for various time with transcription and translation inhibitors actinomycin D and cyclohexamide (39) or with these two inhibitors plus LAQ824 and then measured the relative androgen receptor protein level in these cells. As shown in Fig. 1D, the androgen receptor level decreased faster in cells treated with actinomycin D and cyclohexamide
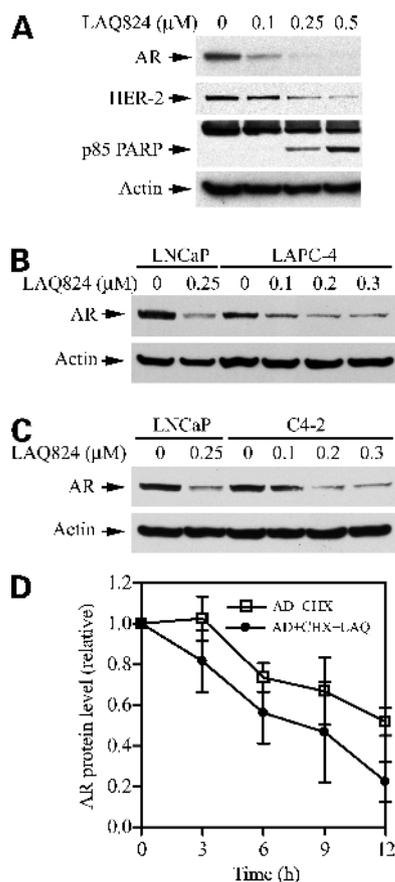
Figure 1. LAQ824 decreases the androgen receptor (*AR*) protein level in prostate cancer cells. **A,** LNCaP cells were treated with indicated concentrations of LAQ824 for 24 h. Cell lysates (40 μg each) were separated on a 7.5% SDS-polyacrylamide gel and analyzed by immuno-blotting with antibodies to androgen receptor, HER-2, PARP, and β-actin, respectively. **B** and **C,** LNCaP, LAPC-4, and C4-2 cells were treated for 24 h with LAQ824 as indicated. Cell lysates were prepared and analyzed by immunoblotting with antibodies to androgen receptor and β-actin. **D,** LNCaP cells were pretreated with actinomycin D (2 μmol/L) and cyclo-hexamide (140 μmol/L). After 30 min (0 h), LAQ824 (0.3 μmol/L), or solvent was added and cells were harvested at various time as indicated. Cell lysates were analyzed by immunoblotting with antibodies to androgen receptor and β-actin. The androgen receptor protein level was normalized to the corresponding β-actin level at each time point. *Points,* averages of two experiments.

plus LAQ824 than in cells treated with actinomycin D and cyclohexamide alone. This result suggests that a posttranslational mechanism contributes to LAQ824-induced androgen receptor depletion in LNCaP cells.

Comparison of the Effects of LAQ824 and 17-AAG on LNCaP Prostate Cancer Cells

17-AAG, a nucleotide mimetic inhibitor of Hsp90, binds to the Hsp90 chaperone protein and induces degradation of Hsp90 client proteins such as Akt, Raf-1, HER-2, and steroid receptors. We compared the effects of LAQ824 and 17-AAG on LNCaP cells. Cells were treated either with 0 to 1.0 μmol/L LAQ824 or 17-AAG. Both LAQ824 and 17-AAG decreased the amounts of androgen receptor, Akt, and Raf-1 in LNCaP cells, whereas extracellular signal-regulated

kinase 1/2 protein levels were not affected by either LAQ824 or 17-AAG (Fig. 2). Thus, inactivation of Hsp90 with 17-AAG could lead to depletion of androgen receptor. Data shown in Figs. 1 and 2 also show that LAQ824 could deplete Hsp90 client proteins HER-2, Akt, and Raf-1 in LNCaP cells, suggesting that LAQ824 could inactivate Hsp90 in prostate cancer cells.

Both LAQ824 and 17-AAG increased the p21 protein level in LNCaP cells. However, LAQ824 had a biphasic effect on the p21 level, which peaked at 0.25 μmol/L LAQ824, but the p21 level decreased at higher concentrations of LAQ824. Another difference between LAQ824- and 17-AAG-induced responses is that PARP cleavage was detected in LNCaP cells treated with LAQ824 but not with 17-AAG at concentrations used in this study.

LAQ824 Induces Hsp90 Acetylation and Hsp90-Androgen Receptor Dissociation in LNCaP Cells

To further examine whether Hsp90 inactivation is involved in LAQ824-induced androgen receptor depletion, we analyzed Hsp90 acetylation in LNCaP cells. Hsp90 acetylation is linked to the inactivation of its chaperone activity in leukemia and breast cancer cells (22–24). LNCaP cells were treated with or without LAQ824, Hsp90 was immunoprecipitated, and Hsp90 acetylation was probed by immunoblotting with an antibody to acetylated lysine. Figure 3A shows that LAQ824 induced Hsp90 acetylation in LNCaP cells.

The ATP-binding activity is essential for the Hsp90 chaperone activity. To determine whether LAQ824 alters the ATP-binding activity of Hsp90, we did the ATP bead pulldown assay. LNCaP cells were treated with or without LAQ824 and cell lysate supernatants were incubated with ATP beads. Hsp90, β-actin, and 14-3-3 bound to the ATP beads were analyzed by immunoblotting. As shown in Fig. 3B, a smaller amount of Hsp90 from LAQ824-treated LNCaP cells than that from control
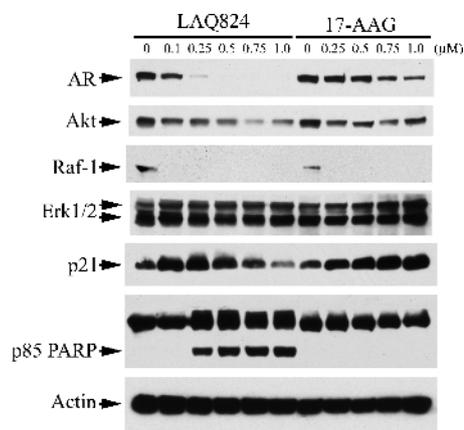

Figure 2. Hsp90 inhibitors lower the androgen receptor (*AR*) level in LNCaP cells. LNCaP cells were treated for 24 h with 0 to 1.0 μmol/L LAQ824 or 17-AAG as indicated. Cell lysates (40 μg each) were analyzed by immunoblotting with antibodies to androgen receptor, Akt, Raf-1, Erk1/2, p21, PARP, or β-actin.
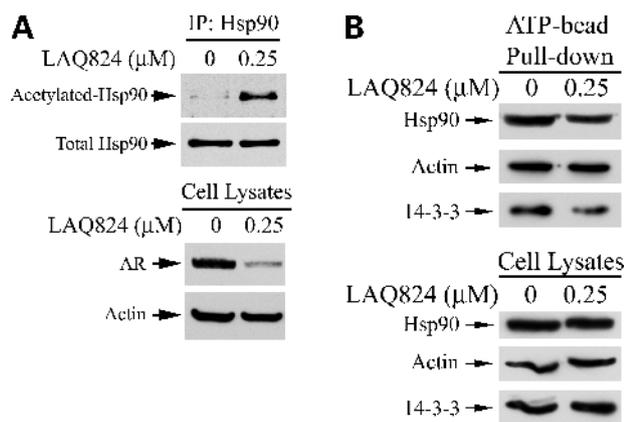
Figure 3. LAQ824 induces Hsp90 acetylation and inactivation. **A,** LNCaP cells were treated with LAQ824 for 24 h as indicated, Hsp90 was immunoprecipitated from cell lysates (1 mg each), and immunoprecipitates were analyzed by immunoblotting with an antibody to acetylated lysine. After analysis of Hsp90 acetylation, the nitrocellulose filter was stripped of antibody and the total Hsp90 protein on the filter was determined by immunoblotting with an anti-Hsp90 antibody (*top*). An aliquot of each cell lysate (40 μg) from a parallel experiment was analyzed by immunoblotting with antibodies to androgen receptor (*AR*) and β-actin (*bottom*). **B,** cell lysates (1 mg each) from LNCaP cells were incubated with ATP-Binders resin. Proteins pulled down by the ATP beads were separated on a SDS-polyacrylamide gel and the amounts of Hsp90, β-actin, and 14-3-3 bound to the ATP beads were examined by immunoblotting (*top*). Aliquots of cell lysate supernatants (30 μg each) were also examined by immunoblotting with antibodies as indicated (*bottom*).

cells was pulled down by the ATP beads, indicating that Hsp90 from LAQ824-treated cells has lower ATP-binding activity. β-Actin is an ATP-binding protein that can be pulled down by the ATP beads.[6] No difference was found in the amount of β-actin bound to the ATP beads between LAQ824-treated cells and control cells. 14-3-3 is a scaffolding protein that binds to Hsp90 client proteins such as Raf-1. Interestingly, less 14-3-3 was pulled down by the ATP beads from LAQ824-treated cells than that from the control cells (Fig. 3B).

Hsp90-androgen receptor coimmunoprecipitation assay was subsequently done to determine whether LAQ824 treatment resulted in dissociation of the Hsp90-androgen receptor complex. To minimize the difference in the androgen receptor protein level among samples used for immunoprecipitation, LNCaP cells were treated with 0.3 μmol/L LAQ824 for short durations (3–8 hours). As shown in Fig. 4A, whereas androgen receptor was clearly depleted in LNCaP cells treated with 0.3 μmol/L LAQ824 for 8 h, a similar amount of androgen receptor was immunoprecipitated from control cells and cells that had been treated for 3 or 5 hours with LAQ824. Hsp90 was coimmunoprecipitated with androgen receptor from control cells but not from LAQ824-treated cells (Fig. 4A). Analysis of cell lysate supernatants used for the immunoprecipitation indicated that the same level of Hsp90 was present in all samples (Fig. 4B). Thus, LAQ824 treatment resulted in dissociation of the Hsp90-androgen receptor complex.

Sensitivity of LAQ824-Induced Androgen Receptor Degradation to Protease Inhibitors

Data presented in Figs. 1 and 2 show that LAQ824 could induce PARP cleavage, which is an indication of caspase activation and apoptosis. To assess whether LAQ824-induced androgen receptor degradation is a consequence of caspase activation, LNCaP cells were treated with LAQ824 in the presence or absence of a cell-permeable pan-caspase inhibitor, Z-VAD-FMK. Z-VAD-FMK alone had no apparent effect on the androgen receptor level in LNCaP cells. Whereas incubation of cells with Z-VAD-FMK inhibited LAQ824-induced PARP cleavage, this did not prevent LAQ824-induced androgen receptor degradation (Fig. 5A). This result suggests that androgen receptor degradation induced by LAQ824 is not due primarily to caspase activation or apoptosis.

The proteasome pathway is recognized for its role in degradation of unfolded proteins. To determine whether proteasome activity is involved in LAQ824-induced androgen receptor degradation, we treated LNCaP cells with LAQ824 together with or without the proteasome inhibitor MG-132. Unexpectedly, MG-132 treatment alone reduced the level of androgen receptor in Triton X-100 soluble cell lysate. Nevertheless, LAQ824 did not further reduce the androgen receptor level in the presence of MG-132 (Fig. 5B). Subsequently, we analyzed both Triton X-100 soluble and insoluble cell lysates for the presence of androgen receptor. As shown in Fig. 5C, in the absence of MG-132, LAQ824 reduced androgen receptor in both Triton X-100-soluble and -insoluble cell lysates. MG-132 treatment led to accumulation of androgen receptor in the Triton X-100-insoluble cell lysates (Fig. 5C). However, LAQ824 did not reduce the amount of androgen receptor
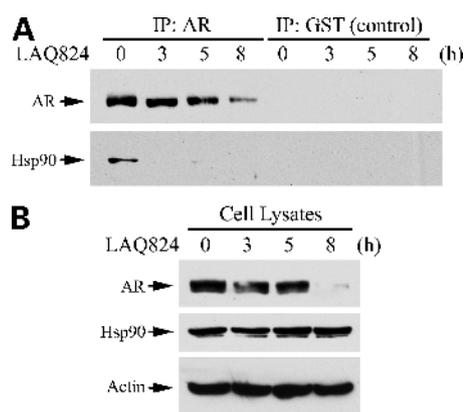

Figure 4. Dissociation of Hsp90-androgen receptor (*AR*) complex by LAQ824 in LNCaP cells. **A,** cells were treated with 0.3 μmol/L LAQ824 for the duration indicated. Cell lysates (1.3 mg each) were subject to immunoprecipitation with a mouse monoclonal anti-androgen receptor antibody or with a mouse monoclonal antibody to glutathione S-transferase (*GST*, used as a control for potential nonspecific binding). Immunoprecipitates were analyzed by immunoblotting with a polyclonal antibody to androgen receptor or an anti-Hsp90 antibody. **B,** an aliquot of each cell lysate (30 μg) used for the immunoprecipitation was examined by immunoblotting with antibodies to androgen receptor, Hsp90, and β-actin.
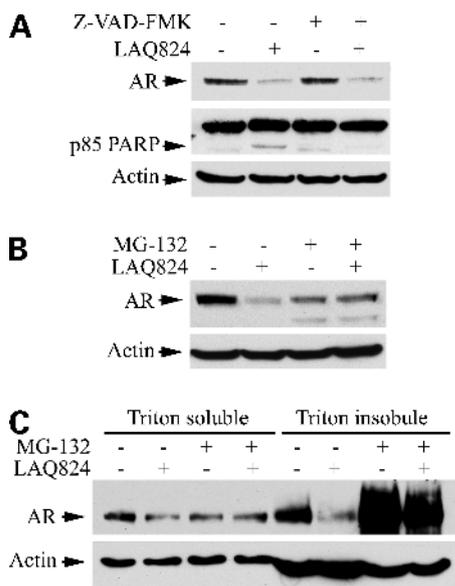
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Figure 5. Effects of protease inhibitors on LAQ824-induced androgen receptor (*AR*) degradation. **A,** LNCaP cells were pre-incubated with (+) or without (−) the caspase inhibitor Z-VAD-FMK (20 μmol/L) for 20 min and the incubation was continued in the presence or absence of LAQ824 (0.25 μmol/L) as indicated for 24 h. Cells were lysed in lysis buffer containing 1% Triton X-100. Cell lysates were analyzed by immunoblotting with antibodies to androgen receptor, PARP, and β-actin. **B** and **C,** LNCaP cells were pretreated with MG-132 (20 μmol/L) for 30 min followed by addition of LAQ824 (0.25 μmol/L) as indicated. After 24 h of treatment, Triton X-100 soluble (**B – C**) and insoluble (**C**) cell lysates were prepared and analyzed by immunoblotting with antibodies to androgen receptor or β-actin.

in LNCaP cells treated with MG-132 in both fractions of cell lysates (Fig. 5C). These results suggest that proteasome activity is involved in LAQ824-induced androgen receptor degradation.

Sodium Butyrate Has Minimal Effect on Androgen Receptor Protein Level in LNCaP Cells

It is not yet clear which HDAC is responsible for deacetylation of Hsp90. However, cytoplasmic deacetylases HDAC6 and HDAC10 are the most likely candidates. HDAC6 and HDAC10 are resistant to the HDAC inhibitor sodium butyrate (40). We reasoned that if Hsp90 inactivation by acetylation plays a significant role in LAQ824-induced androgen receptor depletion, a HDAC inhibitor that does not affect Hsp90 acetylation would have a smaller effect on androgen receptor depletion. LNCaP cells were treated for 24 hours with sodium butyrate or with LAQ824 as indicated in Fig. 6A. Up-regulation of p21[waf] was observed in cells treated with sodium butyrate and with LAQ824 (Fig. 6A), indicating the effectiveness of these HDAC inhibitors in regulation of gene expression. Whereas LAQ824 (0.2 and 0.3 μmol/L) markedly reduce the androgen receptor protein level, sodium butyrate at 1 and 2 mmol/L either had no effect or had a minimal effect, respectively, on androgen receptor level (Fig. 6A). To determine if sodium butyrate affects Hsp90 acetylation, Hsp90 was immunoprecipitated from LNCaP cells treated

with sodium butyrate or LAQ824 and acetylation was analyzed by immunoblotting with an antibody to acetylated lysine. As shown in Fig. 6B, LAQ824 increased Hsp90 acetylation, whereas sodium butyrate did not. These results support the notion that inhibition of Hsp90 by LAQ824 plays a significant role in depletion of androgen receptor in prostate cancer cells.

LAQ814 Inhibits Androgen-Stimulated PSA Production and Cell Proliferation

We next examined if LAQ824 treatment impairs androgen receptor–dependent activities in LNCaP cells. The serum marker for prostate cancer, *PSA*, is an androgen-responsive gene. Incubation of LNCaP cells with 1 nmol/L of the synthetic androgen R1881 stimulated PSA production by 3.2-fold (Fig. 7). Incubation of LNCaP cells with 0.05 to 0.25 μmol/L LAQ824 resulted in inhibition of R1881-stimulated PSA production in a concentration-dependent manner. At 0.25 μmol/L LAQ824, R1881-stimulated PSA production was inhibited by 94%.

Proliferation of LNCaP cells was stimulated about 2-fold in 4 to 6 days by 0.2 nmol/L R1881 in the medium containing charcoal/dextran-treated serum. To examine the effect of LAQ824 on androgen-stimulated LNCaP cell proliferation, we added a low concentration of LAQ824 (0.1 μmol/L) to the medium. LAQ824 at 0.1 μmol/L was able to induce androgen receptor degradation (Fig. 1A) and inhibit androgen receptor–stimulated PSA production (Fig. 7) but did not induce PARP cleavage (Figs. 1 and 2) in our assays. As shown in Fig. 8, LNCaP cell proliferation ceased after 1 day in the presence of 0.1 μmol/L LAQ824. There was no difference between viable cell numbers in LNCaP cells treated with or without R1881 in the presence of 0.1 μmol/L LAQ824 (Fig. 8). Thus, LAQ824 is very effective in inhibiting LNCaP cell proliferation. Similarly, LAQ824 effectively blocked R1881-dependent cell proliferation of LAPC-4 cells (Fig. 8C).

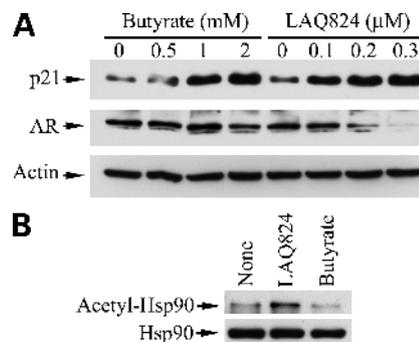It was observed previously that HDAC inhibitor treatment could induce androgen receptor acetylation and

Figure 6. Comparison of the effects of sodium butyrate and LAQ824 on androgen receptor (*AR*) and Hsp90. LNCaP cells were treated with sodium butyrate or LAQ824 for 24 h as indicated. **A,** cell lysates were analyzed by immunoblotting with antibodies to p21[waf], androgen receptor, and β-actin. **B,** LNCaP cells were treated with LAQ (0.3 μmol/L) or sodium butyrate (2 mmol/L) for 24 h and Hsp90 was immunoprecipitated and analysis for acetylation by immunoblotting.
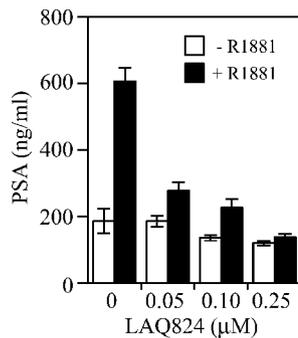
Figure 7. LAQ824 inhibits androgen-stimulated PSA secretion. LNCaP cells were incubated in medium containing steroid-free FBS in the presence or absence of 1 nmol/L R1881 and LAQ24 at indicated concentrations for 24 h. Media were collected and PSA concentrations in the media were determined. From three experiments done in duplicates.

expression of acetylation mimics of androgen receptor mutants in DU145 cells, which lack endogenous androgen receptor, increased cell survival and growth (41). This raises the speculation that HDAC inhibitors that do not induce androgen receptor depletion may promote prostate cancer cell growth. To assess this possibility, we compared effects of LAQ824 and sodium butyrate on LNCaP cell proliferation. Cells were cultured in medium containing LAQ824 (0.1 μmol/L) or sodium butyrate (1 mmol/L) for 4 days and viable cell numbers were determined. The result shows that both LAQ824 and sodium butyrate inhibited LNCaP cell proliferation, although LAQ824 was much effective (Fig. 8B).

Discussion

It is well known that androgen receptor–mediated androgen signaling is essential for the growth and survival of primary prostate cancer cells. Whereas no longer depending on testicular androgens, most of hormone-refractory (androgen independent) prostate cancer cells continue to express androgen receptor (3, 6). Accumulated evidence has shown that a major mechanism for progression to hormone-refractory (androgen independent) prostate cancer is the reactivation of androgen receptor (1, 3, 42). Therefore, ablation of androgen receptor from prostate cancer cells is a rational therapeutic strategy not only for primary androgen-dependent prostate cancer but also for recurrent metastatic prostate cancer that is resistant to androgen ablation therapy.

This innovative concept has been tested previously with positive outcomes. One approach was to directly target androgen receptor mRNA to inhibit its translation or to degrade the messenger thus blocking synthesis of new androgen receptor protein. It was reported that antisense oligonucleotide and hammerhead ribozyme to the androgen receptor mRNA inhibit androgen receptor expression and prostate cancer cell growth (4, 7, 43). Another approach is to induce androgen receptor degradation by

targeting the Hsp90 chaperone (12). It was shown that the Hsp90 inhibitors geldanamycin and its analogue 17-AAG could induce androgen receptor degradation in prostate cancer cells and inhibit prostate cancer xenograft growth (13, 14).

We show here that LAQ824 (25), a small-molecule HDAC inhibitor currently in human clinical trials, is very effective in depleting androgen receptor in prostate cancer
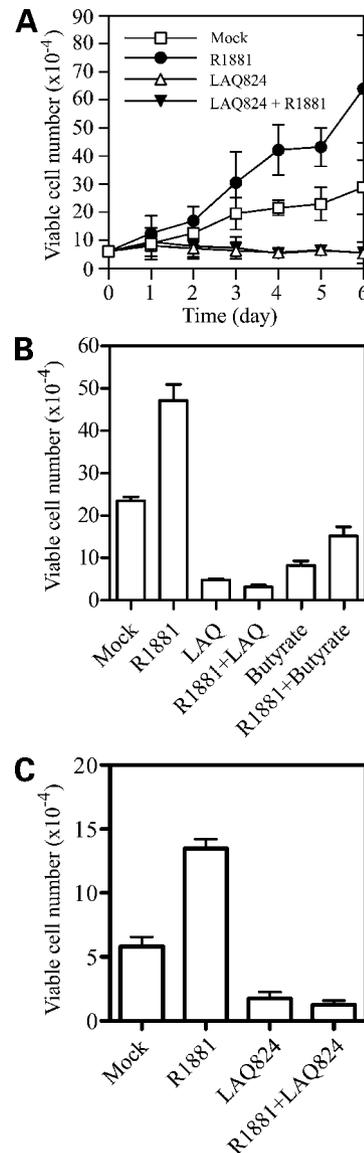
Figure 8. LAQ824 blocks LNCaP and LAPC4 cell proliferation. **A,** LNCaP cells were cultured with medium containing steroid-free FBS with or without LAQ824 (0.1 μmol/L) as well as R1881 (0.2 nmol/L) as indicated. Viable cell numbers were determined every day by trypan blue exclusion assay. From two experiments done in duplicates. **B,** LNCaP cells were cultured as above for 4 d in the presence or absence of LAQ824 (0.1 μmol/L) or sodium butyrate (1 mmol/L) and viable cell numbers were determined. **C,** LAPC-4 cells were cultured with or without R1881 (0.2 nmol/L) and LAQ824 (0.1 μmol/L) for 6 d and viable cell numbers were determined by the trypan blue exclusion assay.

cells. LAQ824 seems able to deplete both wild-type and mutant androgen receptors in both androgen-dependent (LNCaP and LAPC-4) as well as androgen-independent (C4-2) prostate cancer cells. Consistently, 17-AAG was reportedly able to induce degradation of both wild-type and mutant androgen receptors in prostate cancer cells (14).

It is possible that LAQ824 may deplete androgen receptor through multiple mechanisms. Indeed, in a real-time PCR analysis, we found that LAQ824 treatment (0.25 μmol/L, 24 hours) could reduce relative androgen receptor mRNA level to 43% using β-actin mRNA as the reference. Therefore, inhibition of androgen receptor mRNA synthesis or stability contributes to the overall effect of LAQ824 on androgen receptor level. Consistent with this notion, the loss of an uncharacterized androgen receptor transcription suppressor was observed in a LNCaP-derived cell line that overexpresses androgen receptor (44). Expression of the androgen receptor suppressor complex could be partially induced by HDAC inhibitors (44).

Our current study, however, has focused on evaluation of the involvement of the posttranslational mechanism in LAQ824-induced androgen receptor depletion. Several lines of evidence suggest that inactivation of the Hsp90 molecular chaperone is involved in the androgen receptor depletion effect of LAQ824 in prostate cancer cells. First, besides androgen receptor, other Hsp90 client proteins, such as HER-2, Akt, and Raf-1, are reduced by LAQ824. This observation suggests that LAQ824 does not specifically affect androgen receptor. Rather, it affects all Hsp90 client proteins that we have examined. Second, the Hsp90 inhibitor 17-AAG also depletes androgen receptor in LNCaP cells. Thus, inactivation Hsp90 will lead to androgen receptor depletion in prostate cancer cells. Third. LAQ824 induces Hsp90 acetylation, which results in inhibition of its ATP-binding activity. Therefore, LAQ824 is able to inactivate Hsp90. Fourth, LAQ824 induces dissociation of Hsp90-androgen receptor complex. Hsp90-androgen receptor interaction is known to maintain androgen receptor stability. Thus, dissociation of Hsp90-androgen receptor complex will result in androgen receptor degradation. Fifth, whereas 17-AAG does not induce PARP cleavage in LNCaP cells at the concentration used in our study, it reduces androgen receptor level in these cells, suggesting that depletion of androgen receptor is not a secondary response to caspase activation and apoptosis. Sixth, similar to geldanamycin-induced androgen receptor degradation (13), proteasomal activity is involved in LAQ824-induced androgen receptor degradation, suggesting that degradation of denatured androgen receptor is a necessary step in androgen receptor depletion in cells treated with LAQ824. Seventh, a HDAC inhibitor (sodium butyrate) that does not affect Hsp90 acetylation has little effect on androgen receptor level in LNCaP cells.

It has been observed that androgen receptor could be acetylated, which increased the androgen receptor transcription activity in transfected DU145 cells (41). Our data indicate that LAQ824 inhibits androgen receptor–mediated gene expression in LNCaP cells that contain the endogenous androgen receptor. Conceivably, depletion of androgen receptor by LAQ824 overrides the potential positive effect of androgen receptor acetylation on androgen receptor–dependent transcription. One might predict that an HDAC inhibitor that does not induce androgen receptor degradation will lead to androgen-dependent cell proliferation because of the potential induction of androgen receptor acetylation and transcription activity. This does not seem to be the case in sodium butyrate–treated LNCaP cells (Fig. 8B). This is likely because sodium butyrate induces expression of p21^{waf}, which is an inhibitor of cell proliferation.

Both LAQ824 and SAHA are hydroxamic acid–derived HDAC inhibitors that are currently in clinical trials. Although it was reported that SAHA inhibited LNCaP cell growth and induced apoptosis of LNCaP cells (31), the underlying mechanism has not been reported. It is conceivable that SAHA may also induce Hsp90 acetylation and androgen receptor depletion. However, micromolar concentrations are required for SAHA to inhibit cell growth and induce apoptosis in LNCaP cells (31), whereas LAQ824 is effective at nanomolar concentrations. Therefore, LAQ824 seems to be a more potent drug than SAHA for prostate cancer cells.

Although there are conflicting reports in clinical studies about whether HER-2 overexpression plays a major role in progression of prostate cancer to hormone-refractory disease, many prostate cancer specimens are HER-2 positive (45, 46). Laboratory studies have shown that HER-2 and Akt activate androgen receptor signaling to regulate growth and survival of prostate cancer cells (47–49). In addition, loss of PTEN function is frequently observed in high grade and metastatic prostate cancer (50). In addition to depleting androgen receptor, LAQ824 is also able to induce HER-2 and Akt depletion in prostate cancer cells. Furthermore, LAQ824 up-regulates the cyclin-dependent kinase inhibitor p21, inhibits cell proliferation, and induces apoptosis of prostate cancer cells. Taken together, these findings reveal that LAQ824 is potentially a promising novel therapeutic agent for prostate cancer, particularly for hormone-refractory prostate cancer that no longer responds to androgen ablation and antiandrogen therapies. In a larger context, our study suggests that prostate cancer is a good candidate for development of HDAC inhibitor therapy.

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Chemical ablation of androgen receptor in prostate cancer cells by the histone deacetylase inhibitor LAQ824

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