The Histone Deacetylase Inhibitor NVP-LAQ824 Inhibits Angiogenesis and Has a Greater Antitumor Effect in Combination with the Vascular Endothelial Growth Factor Receptor Tyrosine Kinase Inhibitor PTK787/ZK222584

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ABSTRACT

Chromatin remodeling agents such as histone deacetylase inhibitors have been shown to modulate gene expression in tumor cells and inhibit tumor growth and angiogenesis. Vascular endothelial growth factor (VEGF) and VEGF receptors represent critical molecular targets for antiangiogenesis therapy. In this study, we investigated the biological effect of the histone deacetylase inhibitor NVP-LAQ824 in combination with the VEGF receptor tyrosine kinase inhibitor PTK787/ZK222584 on tumor growth and angiogenesis. We report that treatment with NVP-LAQ824 inhibited tumor and endothelial cells and was associated with increased histone acetylation, p21 up-regulation, and growth inhibition. In addition, NVP-LAQ824 treatment inhibited the expression of angiogenesis-related genes such as angiopoietin-2, Tie-2, and survivin in endothelial cells and down-regulated hypoxia-inducible factor 1-α and VEGF expression in tumor cells. Combination treatment with NVP-LAQ824 and PTK787/ZK222584 was more effective than single agents in inhibiting in vitro and in vivo VEGF-induced angiogenesis. Endothelial cell proliferation, tube formation, and invasion into the Matrigel plugs were reduced. In mouse models with established subcutaneous prostate (PC3) and orthotopic breast tumors (MDA-MB321), this combination treatment induced 80 to 85% inhibition of tumor growth without overt toxicity. These results suggest that the combination of histone deacetylase inhibitors and VEGF receptor inhibitors may target multiple pathways in tumor progression and angiogenesis and represents a novel therapeutic approach in cancer treatment.

INTRODUCTION

Uncontrolled tumor cell proliferation and new blood vessel formation (angiogenesis) are two major targets in the current cancer therapeutics. In most forms of solid tumors, genetic and epigenetic alterations are initially responsible for the deregulated tumor cell expansion and selection (1, 2). After the tumor reaches a certain size, autocrine and paracrine signaling between various growth factors and endothelial receptors are responsible for endothelial cell survival, proliferation, differentiation, and angiogenesis to facilitate additional growth of the tumor (3, 4).

Among the various factors responsible for promoting angiogenesis, great interest has been focused on the family of vascular endothelial growth factors (VEGF) and its endothelial receptors (ref. 5). Oncogenic mutations and hypoxic pressure cause tumor cells to secrete VEGF (6, 7). The interaction between VEGF and endothelial VEGF receptors, especially VEGF receptor 2 (KDR/Fkrl), activates receptor associated kinase activity and initiates critical signaling pathways leading to tumor angiogenesis (8). During this multistep angiogenesis process, endothelial cells also require the expression of various proteins such as survivin for cytoprotection and inhibition of apoptosis (9, 10) and angiopoietin (Ang)-1 and Ang-2 along with their receptor Tie-2 for blood vessel stabilization and sprouting (11, 12). Attempts to target these critical processes involved in tumor angiogenesis have led to the clinical development of angiogenesis inhibitors. PTK787/ZK222584 is a potent and selective inhibitor of the VEGF receptor tyrosine kinase, specifically targeting the VEGF receptor 2 (KDR/flk-1; ref. 13). In addition, this agent also inhibits other kinases such as the platelet-derived growth factor receptor β tyrosine kinase and the c-Kit protein tyrosine kinase (14, 15). PTK787/ZK222584 has been shown to modulate VEGF-induced gene expression in endothelial cells and to inhibit cell proliferation and survival (14). In several preclinical animal models, PTK787/ZK222584 has been shown to inhibit tumor growth and angiogenesis at the dose of 50 to 100 mg/kg/d without overt toxicity (14, 16). Clinical data suggest that PTK787/ZK222584 can be administered safely on a continuous daily dosing schedule with achievable plasma concentrations in the low micromolar range (17).

Chromatin remodeling agents like histone deacetylase inhibitors represent an emergent class of therapeutic agents that induce tumor cell cytostasis, differentiation, and apoptosis in various hematologic and solid malignancies (18, 19). The targets of these agents are the histone deacetylases, which induce nucleosomal histone deacetylation, euchromatic chromatin condensation, and gene expression silencing (20, 21). Altered histone deacetylation activity is associated with cancer. Five classes of histone deacetylase inhibitors have been characterized and include short-chain fatty acids (i.e., sodium butyrate and phenylbutyrate); hydroxamic acids (i.e., suberoylanilide hydroxamic acid and trichostatin A); cyclic tetrapeptides containing a 2-amino-8-oxo-9, 10-epoxy-decanoyl moiety (i.e., trpacon A); cyclic peptides without the 2-amino-8-oxo-9, 10-epoxy-decanoyl moiety (i.e., FK228); and benzamides (i.e., MS275). Unlike several chemotherapeutic drugs that induce arrest of cell growth and apoptosis through DNA damage and cytoskeleton toxicity, histone deacetylase inhibitors may exert their antitumor activity through chromatin remodeling and gene expression modulation that affect the cell cycle and survival pathways. Recently, our group reported that the histone deacetylase inhibitor phenylbutyrate has a direct inhibitory effect on endothelial cell proliferation and angiogenesis in vivo (22). Other reports have also indicated that histone deacetylase inhibitors reduce new blood vessel formation by down-regulating angiogenesis-related gene expression in endothelial and tumor cells (23–25). In particular, histone deacetylase inhibitors such as trichostatin A, suberoylanilide hydroxamic acid, and FK228 have been shown to inhibit gene or protein expression related to angiogenesis such as hypoxia-inducible factor 1-α (HIF-1α); VEGF, VEGF receptor, and eNOS. NVP-LAQ824 is a structurally novel hydroxamic acid derivative in the early stages of clinical development. It inhibits histone deacetylase at concentrations below 0.15 μmol/L, suppresses the growth of various cancer cell lines in vitro by inducing either G1 or G2 arrest, and has antitumor activity against human lung, colon, breast, and multiple myeloma xenografts.
in vivo (26–27). In a preliminary pharmacokinetic study in mice, a daily dosing of NVP-LAQ824 (35 mg/kg/d) achieved a peak plasma concentration greater than 2.0 µmol/L (28).

Tumor progression is a process involving multiple stages and pathways, and a rational antitumor strategy should be directed toward multiple independent or interacting targets. A growing body of evidence suggests that angiogenesis inhibitors may have limited therapeutic efficacy as single agents (29, 30). On the other hand, several preclinical reports have shown a greater antitumor effect of angiogenesis inhibitors in combination with radiation and chemotherapy (31, 32). In this study, we investigated the antiangiogenic and antitumor effect of the combination between histone deacetylase inhibitor NVP-LAQ824 and VEGF receptor 2 kinase inhibitor PTK787/ZK222584. Our results indicate that whereas the effect of PTK787/ZK222584 on endothelial cells, NVP-LAQ824 treatment targets endothelial cells and epithelial tumor cells. The combination of these two agents resulted in a greater antitumor and antiangiogenic effect in vitro and in vivo compared with single agents.

MATERIALS AND METHODS

Cell Lines, Reagents, and Animals. Human prostate (PC3), human renal cell (RCC118), and human breast (MDA-MB231) carcinoma cell lines and bovine aorta endothelial cells were obtained from American Type Culture Collection, Manassas, VA [except for the RCC118 cell line, which was kindly provided by Dr. Elisabeth Jaffe (Johns Hopkins University, Baltimore, MD)]. The cell lines were maintained in RPMI 1640 with l-glutamine supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and cultured in complete EMEM medium containing EMEM bullet kit (Cambrex, Walkersville, MD) with growth factors and 5% fetal bovine serum supplied by the vendor, and all experiments were performed using endothelial cells between passage 3 and passage 8. Stock solutions of NVP-LAQ824 and PTK787/ZK222584 (kindly provided by Novartis Pharma AG, East Hanover, NJ) for in vitro assays were prepared in DMSO. For the in vivo animal experiments, NVP-LAQ824 was dissolved in saline solution, and PTK787/ZK222584 was dissolved in saline solution with 5% DMSO and 1% Tween 80% (14). Six-week-old male athymic nude mice (National Cancer Institute, Bethesda, MD) were housed under pathogen-free conditions. All animal studies were performed according to the protocol approved by the Animal Care and Use Committee at Johns Hopkins University.

Cell Proliferation Assay. The proliferation assay was run as described previously (22). In brief, cancer and endothelial cells were seeded (1 × 10⁴ cells/well) in 24-well plates and incubated at 37°C, 5% CO₂, for 24 hours. The medium was then replaced with varying concentrations of NVP-LAQ824 (0, 10 nmol/L, 100 nmol/L, 1 µmol/L, and 10 µmol/L) in triplicates.

2,3-Bis[2-Methoxy-4-Nitro-5-Sulfophenyl]-2H-Tetrazolium-5-Carboxanilide Inner Salt Assay. The effects of drug exposure on endothelial cell growth and survival were assessed by 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay (Roche, Indianapolis, IN) based on the manufacturer’s protocol. The XTT reagent can be metabolized to a formazan dye by metabolically active cells. In brief, 10,000 cells were seeded into a 96-well plate. After overnight incubation, the medium was aspirated and replaced with growth factor reduced medium containing only 0.5% fetal bovine serum to keep the cell viable but not proliferating. Twenty-four hours later, cells were washed with cold PBS and replenished with medium containing 0.5% fetal bovine serum and 50 ng/mL VEGF with various concentrations of NVP-LAQ824 (2, 10, 50, 150, 250, and 1250 nmol/L), PTK787/ZK222584 (4, 20, 100, 300, 500, and 2500 nmol/L), or combination (in fixed ratio of 1:2). Seventy-two hours later, viable cell numbers were quantitated by XTT.

Median Dose Effect/Isobologram Analysis. To calculate the combination effect of NVP-LAQ824 and PTK787/ZK222584, the combination index isobologram method of Chou and Talalay (33) was used. This method involves plotting dose–effect curves for each agent and combinations in multiplet dilutions concentrations by using the median–effect equation and the combination index equation. Combination index values, <1, and >1 indicate an additive effect, synergism, and antagonism, respectively. The combination index values can be determined at different effect levels and different dose levels, and the isobologram can be automatically generated by using the computer software CalcuSyn (Biosoft, Cambridge, United Kingdom).

Reverse Transcription-PCR. Total mRNA from cells was isolated with Trizol reagent (Invitrogen). Total mRNA (1000 ng) was subjected to semi-quantitative reverse transcription-PCR for VEGF, Ang2, Tie2, and HIF-1α gene expression using a Mastercycler (Eppendorf, Westbury, NY). PCR primers were synthesized based on published sequences. Reverse transcription-PCR of β-actin transcripts was used as an internal control to normalize for loading differences between samples. Primer sequences for β-actin, 5′-ATGATGATATCGCCGGCGC-3′ and 5′-CTCCCTTAAGTTGACCGATTCT-3′; p21, 5′-GGCAAGATCAGTCTCT-3′ and 5′-TCATGTTGTGTCGGCC-3′; Tie2, 5′-ATGGTCGAAGAGCCGAGGAC-3′ and 5′-TATCCGCGTCTGTGGACGTTG-3′; VEGF, 5′-CCCTGTGCCTCT-CTCCAC-3′ and 5′-TGTTGATTTGACCGCCTCTCA-3′; Ang2, 5′-ATTGCAACTGCGTGGATT-3′ and 5′-AAATGGAAGAACACCATGC-3′; survivin, 5′-GGCAGCCTTTTCTCAAGGACCAACC-3′ and 5′-CAGAGGCTTACATCATGCGACGG-3′. Results were reproducible in repeated experiments.

Western Blot Analysis. To evaluate the effect of NVP-LAQ824 on Ang-2, Tie-2, survivin, and HIF-1α protein expression, cells were plated in T75 flasks and treated with different doses of NVP-LAQ824 for 24 hours. The cells were then washed twice with PBS and homogenized with M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). Proteins (30 µg/lane) from the cell lysates were separated on 4 to 15% Tris-HCl gel (Bio-Rad, Hercules, CA) and blotted with primary antibodies anti-Ang-2, Tie-2, HIF-1α, survivin, or β-actin. The antibody binding was revealed using horseradish peroxidase-conjugate secondary antibodies and an enhanced chemiluminescence blot detection system (Amersham Biosciences, Piscataway, NJ). All antibodies for Western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) except for the anti-β-actin antibody (Sigma, St, Louis, MO). Results were reproducible in repeated experiments.

Enzyme-Linked Immunoabsorbant Assay. VEGF protein released by PC3 cells was quantitated by human a VEGF₆₅ enzyme-linked immunoabsorbant assay (ELISA) kit (R&D Systems, Inc., Minneapolis, MN) based on the manufacturer’s instructions. The values from ELISA were adjusted to the total protein concentration in the cell culture media. The experiment was repeated three times with similar results.

Tube Formation Assay. HUVECs were cultured in complete EGM medium until 60 to 70% confluency, followed by 24-hour starvation in basal EGM (absence of growth factors). Cells were then plated in 24-well plates (5 × 10⁴ cells/well) in 300 µL of growth factor-reduced Matrigel (Becton Dickinson, Franklin Lakes, NJ), in the presence of 0.2 µmol/L NVP-LAQ824, 0.2 µmol/L PTK787/ZK222584, or a combination of both. Cells were cultured in basal EGM medium containing 50 ng/mL human recombinant VEGF₆₅. Each condition was performed in triplicate. Phase microscopic analysis of the morphology of capillary-like structures formed by HUVECs 15 hours after culturing. For each individual well, four digitized pictures were taken from different locations within the well to cover the central area. The digitized pictures were processed using ImagePro Plus software (Media Cybernetics, Silver Spring, MD), and the tube network was quantitated and expressed as the percentage of tube formation per field (×100 = ×10 objective lens and ×10 ocular lens). The final results were pooled from two separate experiments.

Matrigel Plug Angiogenesis Assay In vivo. The original Matrigel assay was partially modified to enhance the VEGF-induced neo-vascularization (34). Four- to six-week-old athymic nude mice received subcutaneously injections in the abdomen of 500 µL of Matrigel (Becton Dickinson) supplemented with recombinant mouse VEGF₆₅ (R&D Systems) and bovine aorta endothelial cells (1 × 10⁷ cells/plug). Two injections per animal were performed. Matrigel without VEGF was injected as a negative control. The animals were then randomly divided in four groups (five animals per group) and treated with vehicle (saline solution with 5% DMSO and 1% Tween 80, PTK787/ZK222584 (100 mg/kg/d, by gavage), NVP-LAQ824 (40 mg/kg/d, by intraperitoneal injections), or a combination of PTK787/ZK222584 and NVP-LAQ824. These doses were based on prior published reports (16, 26).
Treatment continued for 10 days after Matrigel injection. The mice were then sacrificed, and plugs were retrieved for immunohistochemical analysis of angiogenesis. The plugs were fixed in PBS-buffered 10% formalin containing 0.25% glutaraldehyde and were processed for Masson’s Trichrome staining. The ImagePro Plus software (Media Cybernetics) was used to quantify vascularization in the histologic sections. The mean area per field from eight to 10 fields per section per plug \((\times 100 = \times 100\) objective lens and \(\times 10\) ocular lens) was calculated and expressed as mean percentage microvessel area occupied by blood vessels per field with \(\pm SEM\). The experiment was repeated twice with similar results.

**Tumor Growth In vivo.** Tumor cells were resuspended in Hank’s solution and mixed with Matrigel (1:1) in a final volume of 0.1 mL. PC3 cells \((2 \times 10^5)\) were injected bilaterally and subcutaneously into male athymic mice to form two tumors per mouse. MDA-MB231 cells \((2 \times 10^6)\) were injected orthotopically into the mammary fat pad of female athymic mice. As tumors became established \((50–100 \text{ mm}^3)\), mice were randomly assigned to the control group \((n = 7)\) and to experimental groups \((n = 7\) each group). Each experimental group was treated with NVP-LAQ824 \((40\ \text{mg/kg/d, i.p.)}, \ PTK787/ZK222584 \((100\ \text{mg/kg/d, by gavage}), \) or a combination of both. Control animals were given vehicle consisting of saline solution with 5% DMSO and 1% Tween 80. Mice were treated 7 d/week for 7 each 0.5% serum \((\times 100\) versus untreated controls). MDA-MB231, PC3, and dermal-HMVEC cells \((0.01–10\ \text{ng/mL})\) were treated with 1 \(\text{ng/mL rhVEGF}\) \((0.01\ \text{ng/mL rhVEGF} \) for 48 hours. Viable cell numbers were assessed by Coulter counter at different time points. Results are expressed as means \(\pm SD\). \(*, P < 0.05\) versus untreated controls. B. Renal carcinoma \((\text{RCC118}), \) dermal endothelial \((\text{dermal-HMVEC}), \) and breast carcinoma \((\text{MDA-MB231})\) cells were treated with 0.25 \(\text{ng/mL NVP-LAQ824}\) for 48 h, and the viable cells were counted. Results are expressed as means \((\% \text{ of control})\) \(\pm SD\). \(*, P < 0.05\) versus untreated controls. C. MDA-MB231, PC3, and HUVEC cells were starved overnight and then stimulated with 0.5% serum with 50 \(\text{ng/mL rhVEGF (VEGF)}, \) or 0.5% serum with 50 \(\text{ng/mL rhVEGF and treated with 1 \(\mu\text{g/mL PTK787/ZK222584 (VEGF + PTK787)}\) for 48 hours. Viable cells were counted, and results are expressed as means \((\% \text{ of control})\) \(\pm SD\). \(*, P < 0.05\) versus untreated controls; **, \(P < 0.05\) versus VEGF-treated cells.

**RESULTS**

**Effect of NVP-LAQ824 on Tumor and Endothelial Cell Proliferation.** NVP-LAQ824 treatment of either prostate tumor \((\text{PC3})\) or endothelial cells \((\text{HUVECs})\) induced a dose-dependent inhibition of cell proliferation and more than 70 to 90% reduction of cell number \((\text{Fig. 1A})\). Under similar experimental conditions, NVP-LAQ824 also effectively inhibited the growth and survival of breast cancer \((\text{MDA-MB231}), \) kidney cancer \((\text{RCC118}), \) and dermal microvascular endothelial cells by 65 to 85% compared with the controls \((\text{Fig. 1B})\). In contrast to NVP-LAQ824, PTK787/ZK222584 inhibited only the VEGF-induced proliferation of endothelial cells and had no effect on cancer cell lines, which did not respond to VEGF stimulation \((\text{Fig. 1C})\).

**Examination of Angiogenesis-Related Gene Expression in NVP-LAQ824-Treated Endothelial Cells.** The growth inhibitory effect of histone deacetylase inhibitors in tumor cells is associated with an increase of histone acetylation and p21 induction, as confirmed by our results with MDA-MB231 cells treated with increasing doses of NVP-LAQ824 \((\text{Fig. 2A})\). Thus, we determined whether a similar association exists in endothelial cells. Treatment of HUVECs with NVP-LAQ824 induced histone H3 acetylation and up-regulated p21 mRNA and protein expression \((\text{Fig. 2A})\). However, when we tested the effect of NVP-LAQ824 on the apoptosis inhibitor \(\text{survivin} \) gene, we observed different results between endothelial and tumor cells. NVP-LAQ824 treatment inhibited survivin expression in proliferating endothelial cells but had no significant effect on either PC3 or MDA-MB231 carcinoma cells \((\text{Fig. 2B})\). To further explore the direct antiangiogenic effect of NVP-LAQ824 on endothelial cells, we measured the expression of endothelial-specific genes such as Ang-2 and Tie-2. Reverse transcription-PCR and Western blot results indicated significant expression of both genes at baseline, but NVP-LAQ824...
induced a dose-dependent down-regulation of mRNA and protein levels of Ang-2 and Tie-2 after 24 hours of treatment (Fig. 2C). A similar observation was made with dermal microvascular endothelial cells (data not shown). In contrast, NVP-LAQ824 had no significant effect on the expressions of Tie-1 receptor (data not shown).

Effect of Combination of NVP-LAQ824 and PTK787/ZK222584 on Angiogenesis In vitro and In vivo. Based on the results of angiogenesis-related gene modulation by NVP-LAQ824 in endothelial cells, we investigated the antiangiogenic effect of NVP-LAQ824 and PTK787/ZK222584 as single agents and in combination in vitro and in vivo. Proliferation and survival in VEGF-stimulated endothelial cells was assessed by XTT assay in the presence of increasing concentrations of single drugs or combination in a fixed ratio (2:1; Fig. 3A). Both drugs exhibited a dose-dependent inhibitory effect in endothelial cells (i.e., 26% inhibition with 150 nM NVP-LAQ824 and 37% inhibition with 300 nM PTK compared with control; $P < 0.001$). The combination was more effective than single agent (i.e., 51% inhibition for 150 nM NVP-LAQ824 + 300 nM PTK; $P < 0.02$; Fig. 3A). To determine whether the inhibitory effect of NVP-LAQ824 and PTK787/ZK222584 was additive, the mean value of each dose-response curve in Fig. 3A was subjected to median dose effect/isobologram analysis. Three isobolograms for effective 25, 50, and 75% inhibition doses were plotted (Fig. 3B). By conservative estimation (mutually nonexclusive), the relationship between NVP-LAQ824 and PTK787/ZK222584 resulted to be additive at all three effective levels. Similar analysis using data from multiple experiments resulted in a combination index $\pm 95\%$ confidence interval equals to 1.0 or slightly less than 1.0, which indicates additive or slightly synergistic effect (Fig. 3C). As expected, the additive effect was not observed in either PC3 or MDA-MB231 cancer cells because PTK787/ZK222584 had no direct effect on tumor cell proliferation (data not shown).

Thus, we assessed whether these agents impaired endothelial cell differentiation in the tube formation assay. As single agents, PTK787/ZK222584 and NVP-LAQ824 resulted in substantially less tube formation, and the combination was more effective than single agents (Fig. 3D). Finally, we performed the Matrigel plug angiogenesis assay to further assess the effect of NVP-LAQ824 and PTK787/ZK222584 on new blood vessel formation in vivo. Matrigel plugs retrieved from the control animals had an extensive vascularization 10 days after implantation (Fig. 3E). In contrast, plugs from mice treated daily with NVP-LAQ824, PTK787/ZK222584, or the combination had significantly reduced vascularization by quantitative imaging analysis. As a single agent, NVP-LAQ824 and PTK787/ZK222584 provided $\sim 50\%$ inhibition of new blood vessel formation, whereas the drug combination was statistically more effective inducing $\sim 60\%$ inhibition.

Examination of Hypoxia-Inducible Factor-1α and Vascular Endothelial Growth Factor Expression in NVP-LAQ824-Treated Tumor Cells. A critical switch in tumor-related angiogenesis is the ability of cancer cells to secrete VEGF into the surrounding environment for the recruitment of blood vessels under normal and hypoxic conditions. The antitumor effect of NVP-LAQ824 has been associated with its ability to acetylate and destabilize heat shock protein 90, a chaperone for many oncogenic proteins (35, 36). As a result of this inhibition, we hypothesized that NVP-LAQ824 may inhibit HIF-1α expression and down-regulate VEGF production. In PC3 and MDA-MB231 tumor cell lines, NVP-LAQ824 was very effective in inhibiting HIF-1α expression under normal and hypoxic conditions (Fig. 4A). Such inhibition occurred at the protein level only because HIF-1α mRNA was not altered (Fig. 4A).

Next, we measured VEGF mRNA and protein levels. In MDA-MB231 cells, induction of HIF-1α increased VEGF mRNA and secreted VEGF protein as indicated by reverse transcription-PCR (Fig. 4B) and ELISA (Fig. 4C), respectively. NVP-LAQ824 induced
Fig. 3. Effect of combination of NVP-LAQ824 and PTK787/ZK222584 on VEGF-stimulated angiogenesis in vitro and in vivo. A. In vitro angiogenesis was measured by endothelial cell proliferation and survival using XTT assay. HUVECs were starved overnight, then stimulated with rhVEGF (50 ng/mL), and treated with solvent (control) or increasing concentrations of NVP-LAQ824 (2–1250 nmol/L), PTK787/ZK222584 (4–2500 nmol/L), or a combination of the two (fixed ratio 1:2) for 72 hours in quadruplicates. Results are expressed as the percentage of solvent-treated control (mean ± SD). * P < 0.02 versus single agents. Similar results were obtained from two independent experiments. B. The mean values in A were subjected to isobologram analysis. The effective 25, 50, and 75% inhibition doses (ED25, ED50, and ED75, respectively) were plotted. Curved lines indicate conservative estimation (mutually nonexclusive). Points below the line indicate synergistic effect and points on the line indicate additive effect. C. Similar to B, the combination indexes at effective 20, 50, 75, and 90% inhibition doses were plotted (mean ± 95% confidence interval) from the results of multiple experiments (n = 3). The dotted line indicates the combination index range of 0.9 to 1.1, which indicates additive effect. D. In vitro angiogenesis was assessed by measuring the extensiveness of endothelial cells forming a capillary tube-like network structure. HUVECs were starved overnight, then plated on Matrigel, and treated with 0.2 μmol/L NVP-LAQ824, PTK787/ZK222584 or combination in the presence of 50 ng/mL rhVEGF. Tube formation was assessed after 15 hours. Results are expressed as percentage tube formation means ± SD. Similar results were obtained from two independent experiments. *, P < 0.05 versus untreated controls; **, P < 0.05 versus single agent NVP-LAQ824 or PTK787/ZK222584. E. In vivo angiogenesis was measured by Matrigel plug assay in nude mice. Matrigel mixed with 150 ng/mL VEGF was injected subcutaneously into the abdomen of nude mice. Mice were treated with vehicle control, NVP-LAQ824 (40 mg/kg/d), PTK787/ZK222584 (100 mg/kg/d), or a combination of both for 10 days. Representative photos taken from Matrigel plugs show blood vessels represented in red and collagen background represented in blue. Approximately 10 fields were taken for each plug, and five plugs from each treated or control group were analyzed by ImagePro software. Results are expressed as percent microvessel area means ± SEM. Similar results were obtained from two independent experiments. *, P < 0.05 versus untreated controls; **, P < 0.05 versus single agent NVP-LAQ824 or PTK787/ZK222584.
Figure 4. NVP-LAQ824 down-regulates HIF-1α and VEGF expression in cancer cells. A. PC3 and MDA-MB231 were treated with the indicated concentrations of NVP-LAQ824 for 24 hours. For the hypoxic condition, cells were incubated in a hypoxic incubator (O2 < 0.5%) for the final 5 hours. Reverse transcription-PCR for gene expression and immunoblots for protein expression were used to assess HIF-1α. B. Cells were treated under the same condition as in A, and reverse transcription-PCR was used to assess the VEGF gene expression. C. Cells were treated with NVP-LAQ824 for 24 hours as in A, and the secreted VEGF protein concentrations in the conditioned cell culture media were measured by ELISA and normalized by the total protein concentrations. Results are expressed as means (micrograms per milligram of protein) ± SD. Similar results were obtained from two independent experiments. * P < 0.05 versus untreated controls.

Effect of Combination of NVP-LAQ824 and PTK787/ZK222584 on Tumor Growth In vivo. To determine the effect of NVP-LAQ824 and PTK787/ZK222584 on tumor growth in vivo, we established subcutaneous PC3 and orthotopic MDA-MB231 tumor xenografts. Once tumors became palpable (50–100 mm³), mice were randomly divided into four groups, and treated with vehicle, NVP-LAQ824 (40 mg/kg/d), PTK787/ZK222584 (100 mg/kg/d), or a combination of both. In the subcutaneous PC3 model, PTK787/ZK222584 and NVP-LAQ824 treatment induced ~35 and ~75% tumor growth inhibition, respectively. Combination treatment had a greater effect than single agent with ~85% tumor growth inhibition compared with the controls (Fig. 5A). In the orthotopic MDA-MB231 tumor model, PTK787/ZK222584 and NVP-LAQ824 exhibited ~54 and 60% growth inhibition as single agent, respectively. Combination treatment was more effective with a ~80% inhibition compared with the controls (Fig. 5B). There was no significant body weight difference among all groups of mice, and no signs of overt toxicity were related to drug treatment.

To further assess the inhibitory effect induced by NVP-LAQ824 and PTK787/ZK222584, CD31 staining of blood vessels was performed in the retrieved MDA-MB231 tumor samples. PTK787/ZK222584 and NVP-LAQ824 induced ~50% inhibition of microvessel density, and the combination treatment resulted in a ~75% inhibition (Fig. 5C). Similar effects were obtained with PC3 tumors (data not shown). Finally, Western blot analysis of tumor samples revealed that in mice treated with NVP-LAQ824, there was reduced HIF-1α protein expression and increased acetylated histone H3 protein expression compared with the vehicle-treated controls (Fig. 5D).

DISCUSSION

In the current study, we have identified a novel combination strategy targeting tumor and endothelial cells. A combination of the histone deacetylase inhibitor NVP-LAQ824 and the VEGF receptor kinase inhibitor PTK787/ZK222584 was more effective than single agents in the inhibition of prostate and breast tumor growth in vivo. The NVP-LAQ824 antitumor and antiangiogenesis effect was associated with the down-regulation of angiogenesis-related genes such as HIF-1α, VEGF, Ang-2/Tie-2, and survivin. NVP-LAQ824 modulated the expression of multiple genes playing important roles in tumor progression and angiogenesis. Cotreatment with PTK787/ZK222584 induced an additional inhibition of VEGF signaling and angiogenesis. Combination of NVP-LAQ824 and PTK787/ZK222584 resulted in an additive inhibitory effect on VEGF-induced endothelial cell proliferation and survival in vitro and was more effective than single agents in inhibiting angiogenesis and tumor growth in vivo.

Two general mechanisms are potentially responsible for the antitumor effect of this combination therapy (Fig. 6): (1) NVP-LAQ824 and PTK787/ZK222584 affected tumor growth by acting on inde-
pendent and parallel pathways. The chromatin remodeling activity of NVP-LAQ824 induced cell cycle arrest by gene expression modula-
tion (such as p21) in tumor and endothelial cells. PTK787/ZK222584,
at the same time, affected endothelial cells and angiogenesis by
blocking the VEGF signaling pathway. (2) The combination therapy
may also target converging pathways. Both drugs act as independent
roadblocks on the VEGF pathway. Concomitant treatment with LAQ24 and PTK787/ZK222584 may result in a “vertical” inhibition
of the VEGF pathway by affecting VEGF tumor production and
endothelial cell VEGF receptor kinase activity. The down-regulation
of VEGF signaling may also work in concert with the NVP-LAQ824-
induced survivin and Tie-2 inhibition and consequent impairment
of blood vessel integrity. In addition, PTK787/ZK222584 may also
inhibit c-Kit and platelet-derived growth factor receptor β kinases or
other unknown kinases (15). Although in the bioassays we targeted
specifically VEGF-induced proliferation and differentiation, the in

Fig. 5. Effect of combination of NVP-LAQ824 and PTK787/ZK222584 on tumor growth and angiogenesis in vivo. A and B, growth of subcutaneous PC3 and orthotopic MDA-MB231 tumors. PC3 and MDA-MB231 cells were injected into male or female athymic mice to form subcutaneous or orthotopic tumors, respectively. When the tumors measured 50 to 100 mm³, the animals were randomly divided into control and experimental groups. Mice were treated daily with vehicle (control), NVP-LAQ824 (40 mg/kg/d by i.p.), PTK787/ZK222584 (100 mg/kg/d, by gavage), or combination of both. Results are expressed as tumor volume means ± SEM. Arrows indicate the time when treatments began. *, P < 0.05 versus untreated controls; **, P < 0.05 versus single-agent NVP-LAQ824 or PTK787/ZK222584; C, effect of NVP-LAQ824/PTK787/ZK222584 treatment on microvessel density. MDA-MB231 tumors were harvested from control vehicle, NVP-LAQ824, PTK787/ZK222584, or combination-treated mice, sectioned, and immunostained with rat anti-mouse endothelial cell antigen CD31 antibody to visualize the endothelial cells. Representative photos of tumors after immunostaining for endothelial cells are shown (blood vessels are in red). Quantitative analysis of microvessel density was performed using the ImagePro software. Results are expressed as percent microvessel area means ± SEM. *, P < 0.05 versus untreated controls; **, P < 0.05 versus single-agent NVP-LAQ824 or PTK787/ZK222584. D, effect of NVP-LAQ824 on protein expression in vivo. Sections of MDA-MB231 tumors were homogenized and immunoblotted with antibodies for HIF-1α, acetyl-histone H3, and β-actin.

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NVP-LAQ824 treatment has been previously shown to cause either apoptosis after 48 hours of drug exposure, whereas cancer cells were associated with the induction of p21 gene expression and seems to be mediated through cell cycle arrest rather than apoptosis. 

The antiproliferative effect of NVP-LAQ824 on different cancer cell lines was associated with the induction of p21 up-regulation; HIF-1α and VEGF inhibition in tumor cells; and survivin, Ang-2, and Tie-2 down-regulation in endothelial cells. PTK787/ZK222584 affects endothelial cells by inhibiting VEGF signaling. Combination treatment results in a vertical impairment of the VEGF pathway and in inhibition of parallel survival mechanisms.

Histone deacetylase inhibitors mediate their antitumor activity by chromatin remodeling and subsequent gene expression modulation. These mechanisms are critical to tumor cell proliferation and survival. The antiproliferative effect of NVP-LAQ824 on different cancer cell lines was associated with the induction of p21 gene expression and seems to be mediated through cell cycle arrest rather than apoptosis. 

NVP-LAQ824 treatment has been previously shown to cause either G1 or G2 arrest depending on the cancer cell lines tested. In our study, NVP-LAQ824 treatment induced G2 arrest in both in PC3 cells and HUVECs (data not shown). However, endothelial cells underwent apoptosis after 48 hours of drug exposure, whereas cancer cells presented a persistent G2 arrest (data not shown).

NVP-LAQ824 may also indirectly limit tumor growth by inhibiting tumor-related angiogenesis. Treatment with NVP-LAQ824 inhibited VEGF expression in prostate (PC3) and breast (MDA-MB231) cancer cell lines under normoxic and hypoxic conditions. Reduction of tumor VEGF secretion had a direct effect on blood vessel recruitment. NVP-LAQ824 also had a direct inhibitory effect on endothelial cells. Induction of p21 and inhibition of survivin, Ang-2, and Tie-2 expression may compromise the proliferation and differentiation of endothelial cells. Survivin is a member of the inhibitor of apoptosis protein family that is important for cancer cells (9) and endothelial cell survival response to cytotoxic agents (10, 39). Thus, by targeting endothelial cell survival pathways, histone deacetylase inhibitors may improve the antiangiogenesis activity of molecular targeted drugs and antineoplastic agents. In our experiments, we observed a down-regulation of survivin expression in endothelial but not in tumor cells after NVP-LAQ824 treatment. This difference suggests that survivin gene and protein expression may be regulated by different mechanisms in “normal” cells compared with epithelial carcinoma cells.

The mechanism underlying the modulation of angiogenesis-related gene by histone deacetylase inhibitors (23–25) is not clearly understood. One possibility is that histone deacetylase inhibitor may repress gene expression by inducing protein acetylation (40). Transcription factor Ying-Yan 1 can act as a transcriptional suppressor once it is acetylated (41), and it has been implicated in the down-regulation of eNOS transcription in endothelial cells treated by the histone deacetylase inhibitor trichostatin A (25). Recent reports have indicated that the histone deacetylase inhibitors trichostatin A and FK228 down-regulate VEGF expression by suppressing HIF-1α under hypoxic conditions in Lewis lung carcinoma cell lines via up-regulation of p53 and von Hippel-Lindau gene transcription (23, 42). In our study, PC3 cells have inactivated p53 due to deletion, whereas MDA-MB231 has mutated p53. Up-regulation of p53, therefore, could not explain the VEGF transcriptional suppression. There was also no clear von Hippel-Lindau gene transcriptional induction by NVP-LAQ824 in the cell lines tested in our study (data not shown). The observed differences may be related to the different cell types and histone deacetylase inhibitors used. One possibility for the VEGF transcriptional suppression is that NVP-LAQ824 acetylates and destabilizes heat shock protein 90 (35), which consequently is unable to properly perform its chaperone function to protect HIF-1α from degradation (36). Another possibility is that NVP-LAQ824 may indirectly increase the acetylation and the subsequent degradation of HIF-1α mediated through the acetylase ARD1 (43). Thus, inhibition of deacetylation and subsequent increase of nonhistone protein acetylation induced by histone deacetylase inhibitors may cause the degradation of critical regulatory factors.

The role of microvessel density as an indicator of antiangiogenic treatment efficacy remains controversial (44). In our study, the CD31 staining of the tumor samples was performed at a relatively late time point when there was a significant difference in tumor burden among the control and experimental groups. Thus, we cannot rule out the possibility that the reduction in microvessel density observed in the combination-treated tumors may not be due to a direct antiangiogenic effect but to tumor burden suppression and indirect reduction of pro-angiogenic cytokines secreted by the tumor cells. In the Matrigel plug assay, where angiogenesis was driven by exogenous VEGF, we observed that NVP-LAQ824 and PTK787/ZK222584 inhibited new blood vessel formation and drug combination had a greater inhibitory effect (Fig. 3E).

Subcutaneous and orthotopic human xenotransplants are recognized as reasonable models to test the antitumor effects of a specific drug or drug combination. However, preclinical studies involving models with either experimental or spontaneous metastases may represent a more clinically relevant approach. We are currently testing this combination treatment in metastatic models and investigating the potential effect of chromatin remodeling agents on the biological mechanisms underlying the metastatic process.

In summary, this report provides evidence that a histone deacetylase inhibitor may exert its antiangiogenic activity by impairing critical gene expression in tumor and endothelial cell compartments. Our results demonstrate the efficacy of a novel therapy that combines the histone deacetylase inhibitor NVP-LAQ824 and the VEGF receptor–targeting angiogenesis inhibitor PTK787/ZK222584. The greater in vivo antitumor effect of this drug combination compared with single agents is the result of simultaneous targeting of multiple independent and converging pathways. VEGF-targeted therapy alone may cause tumor hypoxia and, in theory, drive tumor cell adaptation and progression. Agents that target HIF-1α may prevent the compensatory activation of cancer cells and selection for cells with an overexpressed HIF-1α (45). Combination therapies with agents that target endothelial cells to block angiogenesis and histone deacetylase inhibitors to prevent tumor adaptation to the resulting hypoxia by down-regulating angiogenesis-related gene expression represent an effective strategy in cancer treatment that warrants clinical testing.
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The Histone Deacetylase Inhibitor NVP-LAQ824 Inhibits Angiogenesis and Has a Greater Antitumor Effect in Combination with the Vascular Endothelial Growth Factor Receptor Tyrosine Kinase Inhibitor PTK787/ZK222584

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