Histone deacetylase inhibitor NVP-LAQ824 sensitizes human nonsmall cell lung cancer to the cytotoxic effects of ionizing radiation

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Stage III nonsmall cell lung cancer is primarily treated with combined chemotherapy and radiation therapy. Relapses for progression of disease within irradiated sites remains a primary pattern of failure. To evaluate the interaction between histone deacetylase inhibitors and irradiation in nonsmall cell lung cancer, we studied NVP-LAQ824 in mouse models of human lung cancer. Colony formation assays were performed to determine whether LAQ824 sensitized nonsmall cell lung cancer to the cytotoxic effects of ionizing radiation. LAQ824 reduced clonogenic survival of the H23 and H460 cell lines five-fold compared with controls and four-fold compared with either agent alone ($P<0.001$). Western blot analysis of caspase cleavage, microscopic analysis of nuclei and Annexin–fluorescein isothiocyanate/propidium iodide flow cytometry assays showed that LAQ824 enhanced radiation-induced apoptosis and attenuated mitosis ($P<0.001$). Immunostaining for γ-H2AX nuclear foci was performed to determine the effect of LAQ824 on radiation-induced DNA double-strand breaks. Combined modality treatment delayed the resolution of γ-H2AX foci with over 30% of cells staining positive 6 h after treatment versus approximately 5 and 3% in cells treated with LAQ824 or radiation alone ($P<0.001$). Additionally, an in-vivo xenograft model was utilized to study the effects of fractioned irradiation and LAQ824 on tumor growth. Fractioned irradiation and LAQ824 delayed tumor growth by 19 days versus 7 and 4 days for treatment with LAQ824 and radiation alone. This study shows the effectiveness of histone deacetylase inhibitors to enhance the cytotoxic effects of radiation by attenuating DNA repair and inducing apoptosis in human nonsmall cell lung cancer. *Anti-Cancer Drugs* 18:793–800 © 2007 Lippincott Williams & Wilkins.

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Introduction

Histone deacetylases (HDACs) play a critical role in modulating chromatin structure and regulating gene expression. Transcriptionally active genes have high levels of core histone acetylation, whereas transcriptionally inactive genes have low levels. Owing to the fact that abnormal gene transcription repression is seen in many forms of cancer, HDACs have been identified as therapeutic targets for drug development. Previous reports using HDAC inhibitors have shown that these compounds influence gene expression related to cell growth, differentiation and apoptosis [1–3]. This class of drug also induces proliferating cells to arrest in the G_2/M or G_1 phase of the cell cycle [4–6] and has anticancer effects on many different types of tumor cell lines [6–10]. In this study the effects of the novel hydroxamic acid HDAC inhibitor NVP-LAQ824 and radiation were tested in human nonsmall cell lung cancer cell lines.

HDAC inhibitors are associated with cell cycle regulation and the cellular response to DNA damage. The response of proliferating cells to HDAC inhibitors or radiation is similar, and may be related to the activation of stress response signaling, cell cycle arrest, DNA damage and apoptotic cell death [11–14]. Proliferating tumor cells arrested in G_2/M phase are the most vulnerable to radiation damage and compounds that induce cell cycle arrest or attenuate DNA repair are effective radiosensitizers. These findings suggest that HDAC inhibitors enhance the cytotoxic effects of ionizing radiation.

Recent studies suggest that HDAC inhibitors may enhance the efficacy of radiation therapy in several types of tumor cell lines, activate apoptotic pathways and increase radiation-induced DNA double-strand breaks [15–19]. Although HDAC inhibitors are promising as single agents and in combined modality therapy [19], their radiosensitizing properties are cell type-specific and not fully understood [20].

Nonsmall cell lung cancer is often refractory to therapy owing to late presentation and relative resistance to

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nonsurgical treatment modalities [21]. Surgery remains the treatment of choice with 5-year survival rates of 24–64%. Unresectable nonsmall cell lung cancer shows resistance to chemotherapy and radiotherapy, and recurrences are common within the field of radiation [22]. In this study, low concentrations of LAQ824 and low-dose fractionated radiation were used in in-vitro and in-vivo human nonsmall cell lung cancer models. These studies show that HDAC inhibitor LAQ824 enhances radiation-induced apoptosis in NSCLC and thereby improves tumor growth delay in mouse models of lung cancer.

Methods

Cell culture and treatment

H23 (human lung cancer, adenocarcinoma) and H460 (human lung cancer, large cell) cell lines were obtained from American Type Culture Collection (Rockville, Maryland, USA), and maintained according to the supplier's instruction. NVP-LAQ824 provided by the Novartis Pharmaceuticals (East Hanover, New Jersey, USA) was dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 5 μmol/l. Cell cultures were incubated with LAQ824 at a concentration of 50 nmol/l unless otherwise specified. Cultures were irradiated using a Pantak (East Haven, Connecticut, USA) X-ray source at a dose rate of 2.04 Gy/min.

Clonogenic assay

To evaluate radiosensitivity, H23 and H460 cell lines were incubated with 50 nmol/l of LAQ824 for 12 h then irradiated with 3 Gy. Thirty minutes after irradiation, cultures were trypsinized into single-cell suspensions, and 500 cells were seeded onto 60-mm culture dishes and incubated for 10 days. Colonies were then stained with 1% methylene blue and the number of colonies containing at least 50 cells was counted under a dissection microscope. All of the experimental groups were repeated three times.

Immunofluorescent staining for γ-H2AX

Cells were grown on sterile histological slides in a square tissue culture plate with 15 ml of medium and treated with 50 nmol/l of LAQ824, 3 Gy of irradiation alone or LAQ824 for 12 h then 3 Gy of irradiation. Six hours after irradiation, the slides were removed and washed with cold phosphate-buffered saline (PBS), then fixed in 3.7% paraformaldehyde for 5 min at room temperature. Cells were washed in PBS three times, and anti-γ-H2AX antibody (Novus Biologicals, Littleton, Colorado, USA) was added at a dilution of 1 : 500 in antibody buffer and incubated overnight at 4°C. Cells were then washed twice in PBS and incubated with a Rhodamine red labeled secondary antibody (Molecular Probes, Carlsbad, California, USA) at a dilution of 1 : 500 in antibody buffer at room temperature for 45 min in the dark. The slides were then washed twice in PBS, incubated in the dark with 4′,6-diamidino-2-phenylindole (DAPI) (5 μg/ml) in PBS for 5 min and then washed twice with PBS. Coverslips were mounted with a glycerol/PBS (3 : 1) solution. Slides were examined with an Olympus fluorescent microscope (Center Valley, Pennsylvania, USA) and color print pictures were taken. Five fields were photographed from each slide at a × 200 magnification and prints were examined for quantitative analysis of nuclear γ-H2AX foci. Cells with more than five foci per nucleus were classified as positive for γ-H2AX staining [23].

Western blot analysis

H23 and H460 tumor cells were treated with the indicated concentration of LAQ824 for 8–12 h followed by 3 Gy. Six hours after radiation, the treated cells were lysed in 500 μl of ice-cold triple detergent buffer. Cell lysates (25 μg) were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane. The membranes were probed with anticleaved caspase-3 antibody (Cell Signaling, Danvers, Massachusetts, USA) diluted 1 : 1000, then anti-Rb antibody (Sigma, St Louis, Missouri, USA) diluted 1 : 5000. The expression of β-actin was used as a loading control.

Apoptosis assays

To quantify apoptotic cell death, two treatment protocols were used. In the first, H23 and H460 cells grown on sterile histological slides were treated with different combinations of 50 nmol/l LAQ824 and 3 Gy radiation. Twenty-four hours after irradiation, the slides were removed from culture, washed with cold PBS and fixed in 3.7% paraformaldehyde for 5 min at room temperature. Cells were washed in PBS three times, then incubated in the dark with the nucleophilic dye DAPI (5 μg/ml) in PBS for 5 min and washed twice. Coverslips were mounted with a glycerol/PBS (3 : 1) solution. Slides were examined on an Olympus fluorescent microscope. Four fields were photographed at × 200 magnification and prints were examined [24]. The experiment was repeated three times. In the second protocol similar treatments were performed. The cells were incubated in the dark for 15 min with binding buffer containing 1 μg/ml Annexin V–fluorescein isothiocyanate and 2.5-μg/ml propidium iodide (BD Bioscience Pharmingen). The number of apoptotic cells was immediately analyzed by flow cytometry.

Tumor growth delay assay

H460 cells (1.5×10^6) in 100 μl growth media were injected subcutaneously into the right hind limb of nude mice. Ten days after tumor implantation, 25 mice bearing H460 tumors were divided into five groups. An equal number of large and intermediate sized (9–12 mm diameter) tumors were present in each group. The first group received no treatment (control group) except the DMSO vehicle. The second group received 20 mg/kg of LAQ824 in 15 μl of DMSO via intravenous injection on

days 1, 3, 5, 8 and 10. The third group received radiation therapy (3 Gy × five fractions) on days 1, 3, 5, 8 and 10, and the DMSO vehicle. The fourth group received the combined treatment of group 2 (LAQ824) and group 3 (3 Gy) with irradiation 6–12 h after LAQ824 injection. Treatment groups were implanted with a jugular venous catheter for multiple intravenous injection 2–3 days before treatments started. Tumor volumes were measured three times a week using skin calipers as previously described [25,26]. The fifth group had similar treatments and tumors were sectioned for histological studies with hematoxylin and eosin staining. Institutional Animal Care and Use Committee guidelines were followed during all aspects of this study.

Statistical analysis

The mean and standard error were calculated for all quantitative experiments using Microsoft Excel software. The Student's *t*-test was used to determine statistically significance differences between groups. *P* values less than 0.05 were considered statistically significant.

Results

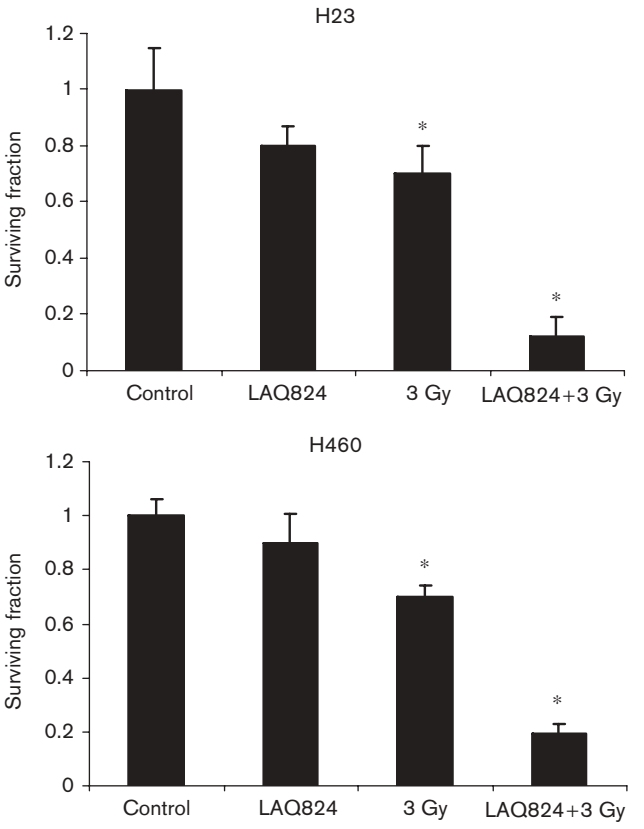
LAQ824 enhances radiation-induced cell death

The ability of LAQ824 to increase the sensitivity of human nonsmall cell lung cancer to the cytotoxic effects of ionizing radiation was studied using colony formation assays. Cells were treated with 50 nmol/l LAQ824 or control vehicle followed 12 h later by irradiation with 3 Gy. Ten days after treatment the cells were fixed and stained, and the number of surviving colonies was determined. These studies revealed that LAQ824 enhanced radiation-induced cell death in both the H23 and H460 cell lines (Fig. 1). The survival fractions for the H23 cell line were 1.00 (control), 0.82 (LAQ824), 0.70 (3 Gy) and 0.12 (LAQ824 + 3 Gy). The survival fractions for H460 cell lines were 1.00 (control), 0.91 (LAQ824), 0.71 (3 Gy) and 0.20 (LAQ824 + 3 Gy). In both cells lines combined modality treatment resulted in a statistically significant increase in cell death compared with control or either agent alone ($P < 0.001$). Furthermore, this increase was greater than what would be predicted by an additive effect of either agent. We also studied how varying the interval of LAQ824 treatment in respect to irradiation affected survival. It was determined that treatment for 8–12 h with LAQ824 before irradiation led to the best synergistic effect. At the 1- and 24-h time points the effects of combined modality treatment on tumor cell cytotoxicity were lessened in both H23 and H460 cell lines when compared with the controls and either agent alone (data not shown).

LAQ824 increases radiation-induced DNA double-strand breaks

As histone acetylation plays a critical role in modulating chromatin structure and blocks cells in the G_2/M phase of the cell cycle, we hypothesized that the HDAC inhibitor

Fig. 1



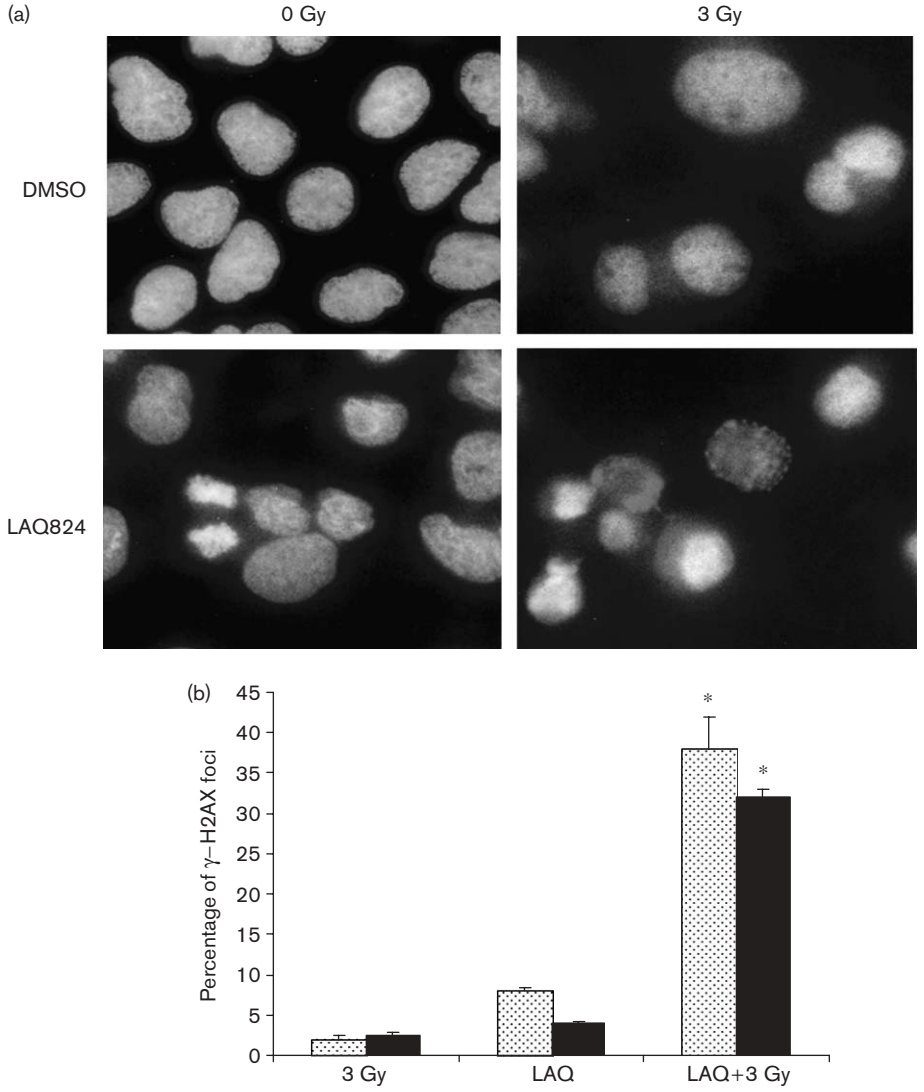
Effects of LAQ824 and ionizing radiation on clonogenic survival. Two human nonsmall cell lung cancer cell lines were treated with 50 nmol/l LAQ824 and irradiated 8–12 h later with 3 Gy. Cells were then suspended and plated in equal numbers. Ten days later, cells were fixed and stained, and the number of colonies containing over 50 cells was counted. Shown are the surviving fractions for each treatment condition. *$P<0.05$ compared with control.

LAQ824 modifies H23 and H460 cell line radiosensitivity by increasing DNA DSBs (double-strand breaks). Immunofluorescent staining for γ-H2AX showed significant enhancement of DNA DSBs for both cell lines following treatment with 50 nmol/l LAQ824 for 12 h and irradiation with 3 Gy (Fig. 2a). Quantitative analysis of γ-H2AX staining revealed a more than additive effect of irradiation and LAQ824 in inducing double-strand breaks in both the H23 and H460 cell lines. The percentages of nuclear γ-H2AX-positive cells for the H23 cell line were 2.7% (3 Gy), 7.5% (LAQ824), 37.2% (LAQ824 + 3 Gy) and for the H460 cell line were 3.1% (3 Gy), 5.4% (LAQ824), 32.4% (LAQ824 + 3 Gy) (Fig. 2b). Treatment with LAQ824 and radiation significantly enhanced the level of γ-H2AX foci in both cell lines compared with either agent alone ($P < 0.001$).

LAQ824 enhances radiation-induced apoptosis in human nonsmall cell lung cancer cell lines

Western blot analysis of caspase-3 cleavage was performed to determine the effects of LAQ824 on radiation-induced

Fig. 2

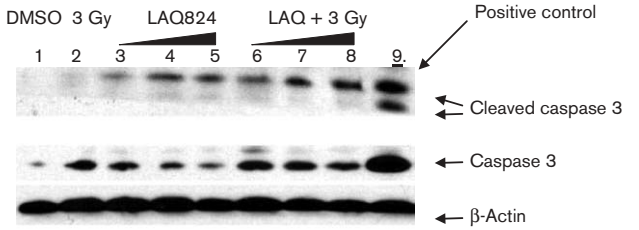


LAQ824 attenuates resolution of nuclear γH2AX foci after ionizing radiation. H23 and H460 cell lines grown on transparent slides were treated with 50 nmol/l LAQ824 for 8–12 h followed by irradiation with 3 Gy. The cells were then fixed and stained with the nucleophilic dye DAPI (blue) and anti-γ-H2AX antibodies (red). (a) Representative photographs for each treatment condition. (b) The mean and standard error of cells with positive γ-H2AX staining 12 h after radiation for each treatment condition for the H23 (stippled bars) and H460 (filled bars). *$P<0.05$. DAPI, 4′,6-diamidino-2-phenylindole dye, DMSO, dimethyl sulfoxide.

apoptosis. Treatment with 50 nmol/l LAQ824 plus 3 Gy induced caspase-3 cleavage 6 h after radiation and this response was increased in a dose-dependent manner (Fig. 3). Treatment with 3 Gy alone or 50 nmol/l LAQ824 alone did not induce substantial levels of caspase-3 cleavage. At higher concentrations of LAQ824 alone (300 nm and 1 μm), however, this pathway was activated.

To confirm the effects of LAQ824 and radiation on apoptosis, histological analysis of pyknotic nuclei using DAPI staining and flow cytometry analysis using Annexin–fluorescein isothiocyanate/propidium iodide were studied. The percentage of H23 and H460 cells demonstrating apoptotic nuclei (Fig. 4a) with DAPI staining was determined by microscopy for each cell line following treatment. Untreated control H23 cells had less than 2.5% apoptotic nuclei as compared with 3.2% for 3 Gy alone, 4.1% for LAQ824 alone and 11.0% for the combined treated group. For the H460 cell line, similar results were seen with 0.5, 1.3, 3.2 and 10.5% pyknotic nuclei for the untreated control, 3 Gy alone, LAQ824 alone, and combination of LAQ824 and 3 Gy, respectively ($n = 12$, $P < 0.05$). Additionally, combined treatment with LAQ824 and radiation completely attenuated mitosis in both cell lines ($P < 0.001$) and treatment with LAQ824 alone reduced the number of mitotic figures by

Fig. 3



LAQ824 and ionizing radiation induce caspase-3 cleavage in human nonsmall cell lung cancer. H23 cells grown to 80–90% confluency were treated as follows: (1) control vehicle; (2) 3 Gy; (3) 0.05 μmol/l LAQ824; (4) 0.3 μmol/l LAQ824; (5) 1.0 μmol/l LAQ824; (6) 0.05 μmol/l + 3 Gy; (7) 0.3 μmol/l + 3 Gy; (8) 1.0 μmol/l + 3 Gy; (9) campothecin (positive control). Six hours after radiation cells were lysed for Western blot analysis using antibodies to cleaved caspase-3, total caspase-3 and β-actin. Shown are immunoblots for each treatment condition. DMSO, dimethyl sulfoxide.

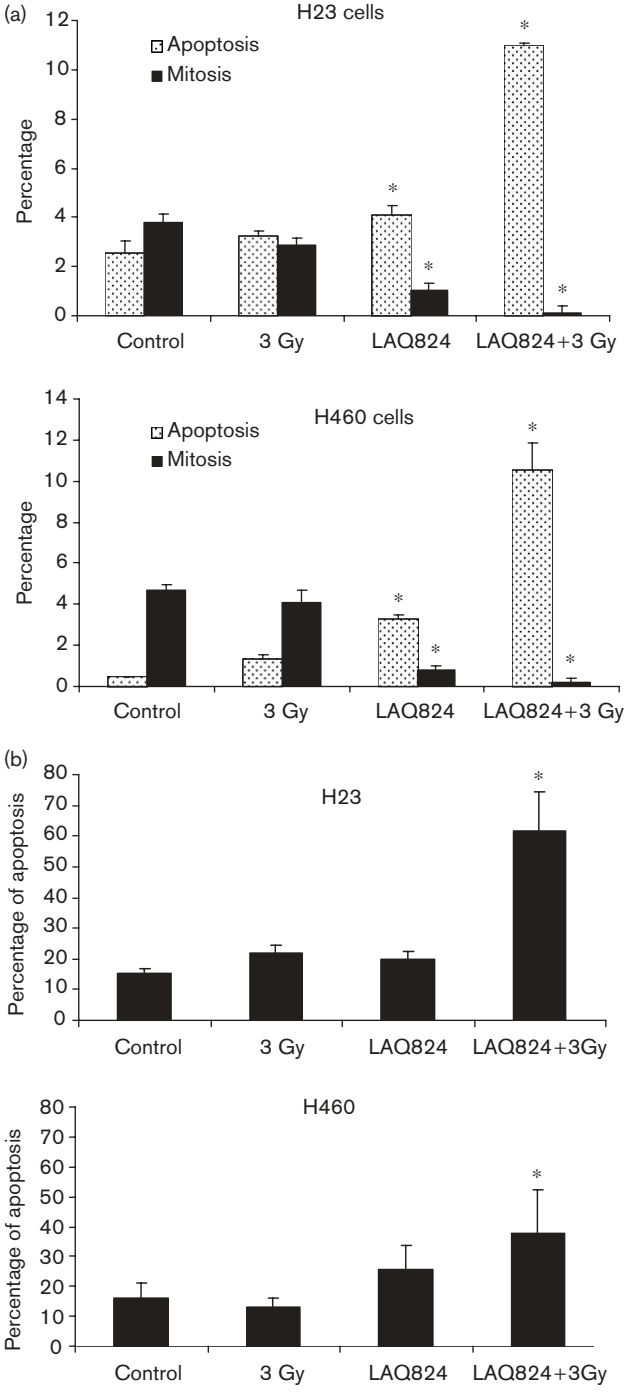
approximately 50% compared with control or radiation alone ($P < 0.05$). Flow cytometry analysis of cell cycle distribution further suggested that treatment with HDAC inhibitors leads to G_2/M arrest in these cell lines (data not shown).

Flow cytometry analysis showed an increase in the number of apoptotic cells with the combined treatment of LAQ824 and 3 Gy compared with control, LAQ824 alone and 3 Gy alone for both H23 and H460 cell lines (Fig. 4b). The apoptotic events for each treatment condition were 11.1% (H23, control), 16.5% (H23, 3 Gy), 13.8% (H23, LAQ824), 34.2% (H23, LAQ824 + 3 Gy); 5.7% (H460, control), 6.3% (H460, 3 Gy), 9.1% (H460, LAQ824) and 27.9% (H460, LAQ824 + 3 Gy). The increase in apoptosis for the combined treatment groups was statistically significant compared with controls in both cell lines ($P < 0.05$). Furthermore, this effect was greater than what would be predicted by an additive effect of radiation and LAQ824.

Effects of intravenous LAQ824 and fractioned radiation on tumor growth

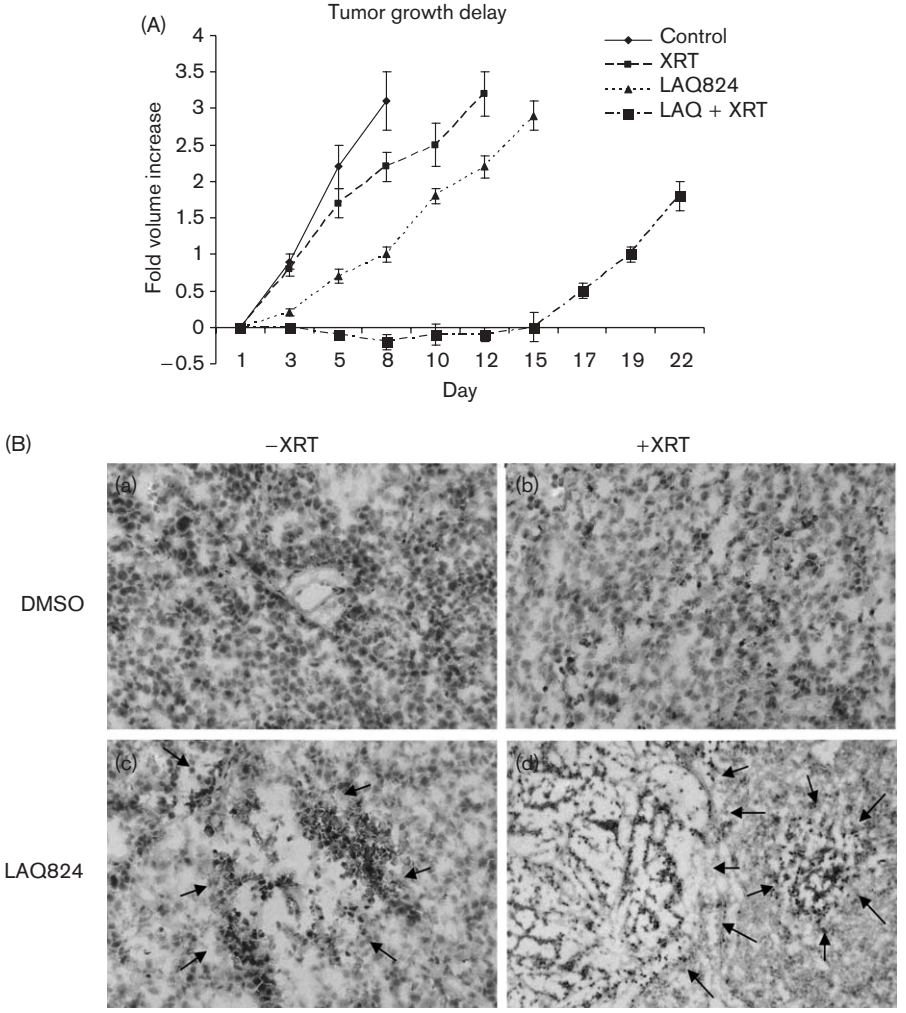
To determine the effectiveness of LAQ824 as a radiosensitizer *in vivo* a hind limb xenograft model was studied using the H460 cell line. Figure 5a shows the tumor growth curves after treatments with irradiation, LAQ824 alone or a combination of LAQ824 and irradiation. The control group tumors doubled in size in approximately 3 days. Treatment with fractionated irradiation alone delayed tumor growth by approximately 3 days and treatment with LAQ824 alone delayed tumor growth by approximately 8 days compared with the control. In comparison, the group treated with both LAQ824 and fractionated irradiation had a 19-day tumor growth delay compared with control. This increase was greater than what would be predicted by an additive effect of LAQ824

Fig. 4



LAQ824 enhances radiation-induced apoptosis. H23 and H460 (human nonsmall cell lung cancer) cell lines grown to 80–90% confluency on transparent slides or tissue culture plates where treated with 50 nmol/l LAQ824 for 8–12 h then irradiated with 3 Gy. (a) Twenty-four hours after irradiation the slides were fixed and stained with the nucleophilic dye DAPI. The percentage of apoptotic (pyknotic) nuclei and mitotic nuclei for each treatment condition as determined by microscopy is shown. (b) Twenty-four hours after irradiation the cells grown culture plates where harvested, stained with Annexin–fluorescein isothiocyanate and propidium iodide, then analyzed by flow cytometry. The percentage of cells undergoing apoptosis for each treatment conditions is shown. *P<0.05 compared with control. DAPI, 4′,6-diamidino-2-phenylindole dye.

Fig. 5



Effect of LAQ824 and fractionated irradiation (XRT) on the growth of hind limb xenografts. H460 (human nonsmall cell lung cancer) cells were injected into the hind limb of nude mice. Once tumors were visible (approximately 5 days) the mice received treatment with combinations of intravenous LAQ824 20 mg/kg and 3 Gy on days 1, 3, 5, 8, and 10. Tumor volume measurements were taken every 2–3 days during the study. (a) The growth curves for each treatment condition. (b) Representative histological tumor sections for each treatment condition stained with hematoxylin and eosin. Arrows indicate necrotic appearing cells. DMSO, dimethyl sulphoxide.

and fractionated radiation. Histological tumor sections showed that LAQ824 alone and combined treatment of LAQ824 and irradiation caused necrosis (arrows) around vasculature in the tumor tissue (Fig. 5b).

Discussion

This study demonstrates that the HDAC inhibitor LAQ824 sensitizes human nonsmall cell lung cancer to the cytotoxic effects of radiation through persistence of DNA double-strand breaks and apoptotic cell death. The clonogenic survival assays showed an optimal synergistic effect when irradiation was administered 8–12 h after treatment with LAQ824. These findings are likely associated with the effects of HDAC inhibitors on cell cycle regulation, DNA damage repair and apoptosis [1–3,11,12]. It is likely that the effects of LAQ824 on tumor cell cycle arrest provide an efficient period for an enhanced response to radiation-induced cell injury. Cell cycle analysis (data not shown) demonstrated a G_2/M phase arrest in the nonsmall cell lung cancer cell lines following treatment.

Previous reports studying different cell lines have shown that HDAC inhibitors alter the cell cycle and affect apoptotic pathways [5,19,27–30]. In this study we found that LAQ824 affects these processes in human nonsmall cell lung cancer. Caspase activation is a general feature of HDAC inhibitor-induced apoptosis. In previous reports the processing and activation of caspases 2, 3, 7, 8 and 9 were observed during HDAC inhibitor-induced apoptosis [28,31–33].

Looking at the data from this study and prior reports, a synergistic relationship between LAQ824 and radiation on cell death is a rational conclusion. Although there is no direct evidence, the formation of DNA double-strand breaks appears to enhance both apoptotic and necrotic cell death [34–36]. By attenuating mitosis and causing cell cycle arrest LAQ824 makes cells vulnerable to radiation-induced DNA double-strand breaks. Furthermore, LAQ824 has been shown to impede the repair of radiation-induced DNA double-strand breaks [16]. A report by Munshi *et al.* [37] showed that HDAC inhibitors affect nonhomologous end-joining repair, and decrease the expression of DNA repair-related molecules including Ku70, Ku80 and Rad50. Furthermore, Chinnaiyan *et al.* [18] demonstrated that treatment with HDAC inhibitors led to the differential expression of epidermal growth factor receptor, Akt, DNA-PK and Rad51. Another recent study showed that HDAC inhibitors block the nuclear translocation of HDAC4 [38], a molecule implicated in the DNA repair process [39]. These studies indicate that HDAC inhibitors affect many cellular processes involved in DNA repair and cell survival. The attenuation of these processes is likely the dominant mechanism behind the radiosensitizing properties of this class of drug.

Just as the in-vitro findings above demonstrate, the tumor growth delay assay showed a superadditive interaction between the HDAC inhibitor LAQ824 and radiation. Histological sections revealed that LAQ824 alone and treatment with LAQ824 and radiation caused apoptosis or necrosis around vasculature in the tumor tissue. Previous reports have shown a synergistic interaction between HDAC inhibitors and radiation *in vivo* [38,40]. In this study and in prior reports [38], the combined modality treatment group had few signs of systemic toxicity. HDAC inhibitors are well tolerated and preferentially target tumor cells. A question that needs to be addressed is how HDAC inhibitors alter the early and late effects of radiation in normal tissue. The tumor-specific effects of HDAC inhibitors as single agents have been attributed to preferentially targeting cells with abnormal transcription pathways. The mechanism behind their radiosensitizing ability, however, is likely related to the effect on DNA repair pathways. As these pathways are active in both tumor and normal tissue it is possible that HDAC inhibitors enhance the toxic effects of radiation in normal tissue.

In conclusion, LAQ824 is an effective radiosensitizer in human nonsmall cell lung cancer. HDAC inhibition blocks mitosis, which potentially makes cells more susceptible to radiation-induced DNA double-strand breaks and also delays the resolution of γ-H2AX foci. LAQ824 enhanced radiation-induced apoptosis in a dose-dependent manner and attenuated growth of irradiated xenografts. HDAC inhibitors are promising radiosensitizers during the treatment of nonsmall cell lung cancer.

Acknowledgement

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