Enhanced Antitumor Activity Induced by Adoptive T-Cell Transfer and Adjunctive Use of the Histone Deacetylase Inhibitor LAQ824

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Abstract

Tumors grow in the presence of antigen-specific T cells, suggesting the existence of intrinsic cancer cell escape mechanisms. We hypothesized that a histone deacetylase (HDAC) inhibitor could sensitize tumor cells to immunotherapy because this class of agents has been reported to increase tumor antigen expression and shift gene expression to a proapoptotic milieu in cancer cells. To test this question, we treated B16 murine melanoma with the combination of the HDAC inhibitor LAQ824 and the adoptive transfer of gp100 melanoma antigen-specific pmel-1 T cells. The combined therapy significantly improved antitumor activity through several mechanisms: (a) increase in MHC and tumor-associated antigen expression by tumor cells; (b) decrease in competing endogenous lymphocytes in recipient mice, resulting in a proliferative advantage for the adoptively transferred cells; and (c) improvement in the functional activity of the adoptively transferred lymphocytes. We confirmed the beneficial effects of this HDAC inhibitor as a sensitizer to immunotherapy in a different model of prophylactic prime-boost vaccination with the melanoma antigen tyrosinase-related protein 2, which also showed a significant improvement in antitumor activity against B16 melanoma. In conclusion, the HDAC inhibitor LAQ824 significantly enhances tumor immunotherapy through effects on target tumor cells as well as improving the antitumor activity of tumor antigen-specific lymphocytes. [Cancer Res 2009;69(22):8693–9]

Introduction

Metastatic melanoma occasionally responds to immune-based therapies, with durable response rates ranging from 5% to 15% (1, 2). The reasons for these low response rates include suboptimal immune system activation, tumor-derived local immunosuppressive factors, immunosuppressive cells, decreases in tumor antigen and MHC expression, and intrinsic tumor antiapoptotic mechanisms in cancer cells (3). Tumor cell antiapoptotic factors can be pharmacologically modulated, which may lead to improvement in the response rates to tumor immunotherapy (3). Histone deacetylase (HDAC) inhibitors seem particularly suited for this purpose because they have multiple effects on gene transcription and protein function resulting in a proapoptotic phenotype in malignant cells with a favorable therapeutic window compared with normal cells (4–6). In particular, increased histone acetylation induced by HDAC inhibitors results in the activation of genes responsible for generating an intracellular proapoptotic milieu (7–9), increasing surface expression of tumor necrosis factor superfamily death receptors such as Fas, tumor necrosis factor–related apoptosis-inducing receptor, and tumor necrosis factor receptor (8, 10, 11), increased expression of MHC molecules and other molecules involved in antigen processing and presentation (12–15), and increased expression of tumor antigens recognized by CTLs and ligands for NK activating receptors (16, 17).

The class I and II HDAC inhibitor suberoylanilide hydroxamic acid (vorinostat) has been approved by the Food and Drug Administration for the treatment of cutaneous T-cell lymphomas. LAQ824, similar to suberoylanilide hydroxamic acid, is also a pan-HDAC inhibitor hydroxamic acid derivative. LAQ824 has been tested in animal models for its direct antitumor effects mainly against hematopoietic lineage cancer cells (18–20). This agent and a related compound (LHB 589) are in clinical testing in patients with cancer (21). We hypothesized that LAQ824 would render melanoma cells more susceptible to immunotherapy. To test this hypothesis, we developed two models in which LAQ824 was administered together with tumor antigen-specific immunotherapy. Our data show that in vivo treatment with LAQ824 increases the efficacy of antigen-specific immunotherapy in the pmel-1 model and in a vaccine prime-boost model mediated by an improvement in antigen presentation by tumor cells and enhanced function of immune cells, both resulting in increased antitumor activity.

Materials and Methods

Mice, cell lines, and reagents. C57BL/6 (The Jackson Laboratory) and pmel-1 (kind gift from Dr. Nicholas Restifo, Surgery Branch, National Cancer Institute) mice were bred and kept under defined-flora pathogen-free conditions at the American Association for Laboratory Animal Care–approved Animal Facility of the Division of Experimental Radiation Oncology, University of California-Los Angeles, and used under the University of California-Los Angeles Animal Research Committee protocol no. 2004-159. The B16 murine melanoma (American Type Culture Collection) was maintained in DMEM (Mediatech) with 10% FCS (Omega Scientific) and 1% (v/v) penicillin, streptomycin, and amphotericin (Omega Scientific). The HDAC inhibitor LAQ824 was provided by Dr. Peter Atadja (Novartis Pharmaceuticals).
Institute for Biomedical Research) through a material transfer agreement as a lyophilized powder. LAQ824 was resuspended in 5% tartaric acid and injected i.p. daily at 25 mg/kg for in vivo studies. For in vitro experiments, LAQ824 was resuspended in DMSO at a 10 mmol/L stock solution.

Bone marrow–derived dendritic cells. The generation of dendritic cells from murine bone marrow in cultures of granulocyte-macrophage colony-stimulating factor and interleukin (IL)-4 was described previously (22). Day 8 dendritic cell cultures were pulsed for 2 h with either gp10025-33 peptide for the pmel-1 adoptive transfer model or TRP2280-288 peptide for the prime-boost vaccine model. Between 5 × 10^5 and 1 × 10^6 cells per mouse were injected s.c. in the right flank of mice.

**pmel-1 adoptive transfer therapy in vivo model.** B16 tumors were implanted s.c. as described previously (22). When tumors reached 5 to 8 mm in diameter, mice received a lymphodepleting regimen of 500 cGy total body irradiation. The following day, 1 × 10^5 activated pmel-1 splenocytes were adoptive transfer into experimental mice via a lateral tail vein. pmel-1 splenocytes had been activated with 100 units/ml IL-2 (a kind gift from Novartis-Chiron), 10 ng/ml IL-15 (a kind gift from Amgen), and 1 μg/ml gp10025-33 peptide (Beckman Coulter) to 2 for 4 days before i.v. injection. Subsequently, gp10025-33 peptide-pulsed dendritic cells were given s.c. on the day of adoptive transfer and 1 week later, in both cases, followed by 3 days of daily i.p. administration of 50,000 IU IL-2. LAQ824 was given i.p. daily at 25 mg/kg starting on the day of pmel-1 adoptive transfer.

Dendritic cell prime/Listeria monocytogenes–boost animal model. C57BL/6 mice received TRP2280-288 peptide-pulsed dendritic cells prime s.c. 1 week later with the L. monocytogenes–TRP2 i.v. boost. Recombinant L. monocytogenes expressing a murine TRP2 transgene were prepared as described previously (23, 24). Mice received s.c. B16 tumor challenge 3 weeks later followed by 25 mg/kg LAQ824 i.p. injection five times weekly.

**Flow cytometry analysis.** Splenocytes and tumor-infiltrating lymphocytes, obtained from enzymatically digested B16 tumors harvested from mice as described previously (25), were stained with antibodies to CD8αβ–, (Caltag), CD3ε,γ,δ, Thy1L,γ,c–,γ,c–, and Thy1L,2αβ–,γ,c– (BD Bioscience), and/or gp10025-33/D1 tetramer (Beckman Coulter) and analyzed with a FACS-Calibur machine using FCS Express software (DeNovo Software). Intracellular IFN-γ staining was done as described previously (26).

**Immunohistochemistry.** Immunohistochemical staining was done as described previously (26). Briefly, sections of cryopreserved tissues were incubated with primary antibodies to CD3 (500A2) and CD8 (Ly-2; both from BD Bioscience) and/or gp10025-33 peptide-pulsed dendritic cells were given i.p. 3 days of daily i.p. administration of 50,000 IU IL-2. LAQ824 was given i.p. daily at 25 mg/kg starting on the day of pmel-1 adoptive transfer.

**Results**

**LAQ824 enhances the antitumor activity of pmel-1 adoptive transfer immunotherapy.** Mice with 12-day established s.c. B16 tumors (mean diameter 5-8 mm) received a 500 cGy lymphodepleting dose of total body irradiation and complete pmel-1 adoptive transfer therapy, consisting of 1 × 10^6 activated pmel-1 T cells adoptively transferred i.v. into B16 tumor-bearing mice along with gp10025-33 peptide-pulsed dendritic cell vaccination and high-dose IL-2 therapy (Fig. 1A). The addition of LAQ824 improved the antitumor activity compared with pmel-1 adoptive transfer therapy alone (Fig. 1B). Combined data from two replicate experiments showed that mice receiving both LAQ824 and pmel-1 adoptive transfer had a statistically significant prolongation in survival (Fig. 1C).

**Improvement of antitumor activity in a prime-boost vaccination model with the addition of LAQ824.** We tested if LAQ824 could sensitize in a different immunotherapy model. Wild-type C57BL/6 received TRP2280-288 peptide-pulsed dendritic cell prime and recombinant L. monocytogenes–TRP2 boost alone or together with LAQ824 treatment, and mice were then challenged with B16 melanoma (Fig. 2A). Mice given TRP2 prime-boost along with LAQ824 had a statistically significant improvement in tumor protection, with ~80% of mice remaining tumor-free at the end of the study (Fig. 2B). These results were consistent in two replicate studies. Therefore, the administration of a HDAC inhibitor also improved the antitumor activity in a prophylactic vaccine model against B16 melanoma.

**Enhanced susceptibility of normal lymphocytes to LAQ824 provides a therapeutic benefit for adoptively transferred T cells.** To study the direct effects of LAQ824 exposure, B16 tumor cells, wild-type splenocytes, and activated pmel-1 splenocytes were incubated ex vivo with increasing concentrations of LAQ824 for 2 h and then allowed to rest overnight before being analyzed with a
MTT cell viability assay. Activated pmel-1 cells had a significant survival advantage over naive wild-type splenocytes at low concentrations of LAQ824 (1-100 nmol/L), with at least 1 log lower sensitivity (Fig. 3A). Contrary to our expectations, both naive and activated splenocytes were more sensitive to LAQ824-mediated cytotoxicity than B16 when tested in vitro. To test whether a similar survival benefit existed with LAQ824 treatment in vivo, pmel-1 T cells were isolated from C57BL/6 mice after adoptive transfer and the percentage of gp100 25-33/Db tetramer-positive cells was quantified over time. Mice treated with pmel-1 adoptive transfer and LAQ824 had a significantly higher percentage and absolute number of gp100 25-33/Db tetramer-positive cells in the spleen (Fig. 3B; data not shown), which was consistent for the duration of the experiment (Fig. 3C). We then assessed the ratio of adoptively transferred Thy1.1+ pmel-1 cells to that of endogenous T cells in lymphodepleted, congenic Thy1.2 recipient C57BL/6 mice. In the group of mice treated with both pmel-1 adoptive transfer therapy and LAQ824, the ratio for pmel-1 cells to endogenous T cells was significantly higher than that of mice treated with pmel-1 adoptive transfer alone (Fig. 3D). These in vivo studies confirmed the in vitro results and suggest that one mechanism by which LAQ824 acts is by providing a survival advantage to the adoptively transferred activated T cells.

Treatment with LAQ824 induces an increased intratumoral infiltration by adoptively transferred pmel-1 cells. Similar experiments were conducted to analyze the intratumoral infiltration by pmel-1 cells. LAQ824 induced a significant increase in both the percentage (Fig. 4A) and the total number of pmel-1 cells per gram of tumor tissue (Fig. 4B) as measured by gp100 25-33/Db tetramer staining. Results were corroborated with immunohistochemical staining of CD8+ cells in excised tumors (Fig. 4C and D). The pictures were taken from the tumor periphery to avoid large areas of central necrotic tissue in both groups. Therefore, in vivo treatment with LAQ824 increases B16 intratumoral infiltration by adoptively transferred pmel-1 cells.

LAQ824 increases expression of MHC class I both in vivo and in vitro. To determine if the HDAC inhibitor increases MHC and/or antigen expression by B16 tumor cells, as has been suggested previously (14, 31), single-cell suspensions of B16 tumor cells from treated and untreated mice were stained for H2-Kb and H2-Db and the gp100 melanoma antigen (HMB45). In two replicate experiments, administration of LAQ824 to B16 tumor-bearing mice induced an increase in expression of MHC class I molecules by surface staining and the gp100 tumor antigen by intracellular staining (Fig. 4E). Therefore, LAQ824 likely increases antigen-driven expansion of pmel-1 cells by increasing MHC and antigen expression.

LAQ824 enhances the functional activity of adoptively transferred pmel-1 cells in vivo. We then analyzed the effects of LAQ824 on the ex vivo effector functions of pmel-1 cells.
pmel-1 cells were restimulated in vitro for 6 h with the cognate gp100<sub>25-33</sub> peptide, compared with an irrelevant control peptide (AH1), in low concentrations of IL-2. IFN-γ was measured by intracellular cytokine staining. Splenocytes from mice that had received the combined therapy had higher production of IFN-γ when stimulated ex vivo with cognate gp100<sub>25-33</sub> peptide (Fig. 5A). These findings were reproducible among replicate mice and specific for the gp100<sub>25-33</sub> peptide (Fig. 5B). We then tested the cytotoxic activity of adoptively transferred pmel-1 cells with or without the addition of LAQ824 treatment in a CFSE-based in vivo cytotoxicity assay (32). As targets, wild-type splenocytes were pulsed with either control OVA<sub>257-264</sub> or the cognate gp100<sub>25-33</sub> peptide and differentially stained with CFSE before being injected into mice that had previously been given pmel-1 adoptive transfer. pmel-1-reconstituted mice treated with LAQ824 showed increased cytotoxic activity against gp100<sub>25-33</sub> peptide-pulsed splenocytes compared with mice receiving pmel-1 adoptive transfer alone, although there was no significant killing of splenocytes pulsed with OVA<sub>257-264</sub> control peptide (Fig. 5C). The results were consistent in assays repeated weekly after pmel-1 adoptive transfer until study day 28 (Fig. 5D). Taken together, these experiments suggest to us that this HDAC inhibitor provides a functional benefit to pmel-1 cells shown by increased antigen-specific cytokine production and cytotoxic activity in vitro. Alternatively, the preferential depletion of naive endogenous lymphocytes may increase the ratio of pmel-1 effector cells leading to an apparent improvement in functionality.

LAQ824 effect on adoptively transferred pmel-1 cells is independent of B16 tumor presence. To further determine if HDAC inhibitor treatment had direct effects on immune effector cells or its effects were indirectly mediated by HDAC inhibitor-modulated B16 cells, we tested the effects of LAQ824 when administered to non–tumor-bearing mice reconstituted with pmel-1 adoptive transfer. In the absence of gp100 antigen presented by B16 tumor cells, the addition of LAQ824 to pmel-1 adoptive transfer still resulted in a significantly increased percentage of gp100<sub>25-33</sub>/<sup>Db</sup> tetramer-positive cells (Fig. 5E) as well as IFN-γ-producing CD8<sup>+</sup> cells at each time point evaluated (Fig. 5F). Therefore, the HDAC inhibitor benefits antigen-specific immune effector cells independent of antigen presentation by the tumor.

Gene expression profiling confirmed the modulation of antigen, MHC, and immune gene expression by HDAC inhibitor therapy. In an effort to identify gene targets modulated by the combination of pmel-1 adoptive transfer and HDAC inhibitor treatment, we performed whole-genome expression profiling comparing untreated and LAQ824-treated B16 and pmel-1 samples both in vitro and in vivo (study groups are detailed in Supplementary Table S1). The whole-genome profiling was analyzed using unsupervised clustering, and the replicate samples from each of the 10 groups clustered together (data not shown). To eliminate tissue or sample type-specific gene signatures, we compared LAQ824-treated samples versus control samples directly. We noted marked differences in gene expression profiling B16 and pmel-1 cells exposed in vitro or in vivo to LAQ824 (Supplementary Fig. S1). A consistent finding in the in vivo experiments was that silver (the gene denomination of the gp100 melanoma tumor antigen) was one of the genes with the most significant decrease in B16 tumors (likely containing tumor cells, immune cells, and stromal cells) harvested from mice that had received the complete pmel-1 adoptive transfer with LAQ824 (Supplementary Fig. S1). It was not restricted to this tumor-associated antigen because expression of other melanosomal antigens, such as the murine homologue of Melan-A, also decreased after combined therapy. Given that these expression data were taken from late time points (2 weeks after pmel-1 adoptive transfer), the results strongly suggest that strong, targeted-specific elimination of tumor-associated antigen occurred in B16 melanomas in vivo when combined with HDAC inhibitor treatment.
Multiple lines of evidence, including our flow cytometry studies, suggest that HDAC inhibitors modulate MHC molecules and antigen processing machinery. Most MHC molecules selected from differentially expressed transcripts had low expression in vitro regardless of LAQ824 exposure but high expression in the in vivo harvested samples only after pmel-1 adoptive transfer therapy (Supplementary Fig. S1B compared with Supplementary Fig. S1C). This gene expression pattern followed closely the gene expression pattern of IFN-related genes such as IFN-inducible GTPase 1 and 2, IFN-γ–induced GTPase, and IFN-regulatory factor 1; other immune cytokines and signaling genes such as signal transducer and activator of transcription 1 and 2, Janus kinase 2, IL-33, and granzyme B; other immune activating genes such as T-cell receptor-β, Fc receptors, and CD52; and chemokines and their receptors such as chemokine (C-C motif) ligands 5, 7 to 9, and 12. From these studies, we concluded that the effects of LAQ824 on the different cell types that contribute to this combined therapy are markedly different depending on the analysis context, with a final overall effect of favoring an effective antitumor response when analyzing samples treated in vivo with LAQ824.

**Discussion**

In these studies, we show that the HDAC inhibitor LAQ824 improves the antitumor activity of two tumor immunotherapy strategies in the B16 murine melanoma model. In the pmel-1 adoptive transfer model, we defined three main mechanisms of this beneficial effect. One is an increase in MHC and antigen expression by tumor cells, which was shown when tumors were treated with single-agent LAQ824 therapy in vivo. The combined adoptive transfer pmel-1 and HDAC inhibitor therapy resulted in markedly decreased expression of gp100 and other tumor-associated antigen expression, which we have interpreted as representing tumor cell killing. However, the effects on antigen presentation alone are
unlikely to explain the beneficial effects of the combined therapy, because agents such as IFN-γ induce increases in MHC class I and antigen expression at much higher levels than this HDAC inhibitor (33). A second mechanism is a decrease in competing endogenous lymphocytes in recipient mice, resulting in a selective advantage for the repopulation with adoptive transfer pmel-1 cells. Our in vivo studies confirmed the results of in vitro differential sensitivity in MTT assays and showed a selective repopulation of lymphodepleted mice with pmel-1 cells compared with congenic endogenous lymphocytes. These results suggested to us that the effects of LAQ824 may work both by enhancing antigen-driven T-cell expansion through increased tumor-associated antigen presentation by tumor cells in vivo and by enhancing homeostatic proliferation mediated by the differential sensitivity of naive recipient splenocytes and the relative resistance of the adoptive transfer activated pmel-1 cells. This hypothesis is further supported by a comparable pmel-1 expansion in both the presence and the absence of B16 tumors in recipient mice. A third mechanism is an improvement in the functional activity of the adoptively transferred antitumor CTLs. We acknowledge that the data could also be interpreted as a quantitative increase pmel-1 cells in the combined therapy groups without increase in functionality. The interpretation is supported by the high mean fluorescence intensity for IFN-γ intracellular cytokine staining in the combined therapy group when restimulated with cognate peptide as well as increased lytic activity in vivo, suggesting increased functionality in addition to an absolute increase in number.

There is a body of recent literature suggesting that HDAC inhibitors have immunosuppressive effects (34, 35), which includes seemingly discordant results of effects on T regulatory cells (36, 37). However, our data add to the previously reported findings of HDAC inhibitor-induced immune potentiation, including the
MS-275 increasing the antitumor activity of high-dose IL-2 against the Renca murine kidney cancer model (36), the HDAC inhibitor trichostatin A against the modified lung cancer cell line TC-1 (15), and the HDAC inhibitor depsipeptide (FK228) also against B16 melanoma as in our experience (31). Therefore, it is likely that the immunomodulating effects of HDAC inhibitors on T regulatory cells and other immunosuppressive cell subsets are not dominant in the setting of powerful tumor immunotherapy strategies.

A major benefit of the gene expression profiling in this model is to provide evidence that the effects of the HDAC inhibitor treatment in vitro had markedly different effects compared with the analysis of gene expression profiling in the same cell subsets (pmel-1 and B16) when analyzed in vivo. Comparison of the same gene expression patterns in vitro and in vivo suggested to us that the effects of HDAC inhibitor therapy was likely a combined effect that includes interactions between the adoptively transferred cells, the tumor microenvironment and the tumor cells, as opposed to a single effect in one of these cell subsets.

In conclusion, our studies provide insights on how a pan-HDAC inhibitor can improve vaccine-based and adoptive cell therapy-based immunotherapies. Given the reproducibility of our findings in two different tumor immunotherapy models as well as data from others (15, 31, 36), we believe that HDAC inhibitors of the same class as LAQ824, such as the Food and Drug Administration–approved vorinostat or the related compound LBH 889 currently in clinical testing, could improve tumor immunotherapy in translational clinical studies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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