Histone deacetylase inhibitor NVP-LAQ824 has significant activity against myeloid leukemia cells in vitro and in vivo

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NVP-LAQ824 is a novel potent hydroxamic acid-derived histone deacetylase inhibitor that induces apoptosis in nanomolar concentrations in myeloid leukemia cell lines and patient samples. Here we show the activity of NVP-LAQ824 in acute myeloid leukemia cells and BCR/ABL-expressing cells of mouse and human origin, both sensitive and resistant to imatinib mesylate (Gleevec, STI571). Whereas imatinib inhibited overall cellular tyrosine phosphorylation in Ba/F3.p210 cells, NVP-LAQ824 did not inhibit tyrosine phosphorylation, and did not affect BCR/ABL or ABL protein expression. Neither compound was able to inhibit cellular tyrosine phosphorylation in the imatinib-resistant Ba/F3.p210-T315I cell line. These data taken together suggest that BCR/ABL kinase activity is not a direct target of NVP-LAQ824. Synergy between NVP-LAQ824 and imatinib was demonstrated against BCR/ABL-expressing K562 myeloid leukemia cell lines. In addition, we show that NVP-LAQ824 was well tolerated in vivo in a pre-clinical murine leukemia model, with antileukemia activity resulting in significant prolongation of the survival of mice when treated with NVP-LAQ824 compared to control mice. Taken together, these findings provide the framework for NVP-LAQ824 as a novel therapeutic in myeloid malignancies.

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Introduction

Most human leukemias have multiple genetic mutations that lead to dysregulated cell cycle control, a block in differentiation, or enhanced viability, and it is highly probable that more than one of these effects is necessary for malignant transformation to occur. Each genetic mutation typically leads to altered expression of specific sets of genes. Both the leukemia-specific oncogenes as well as downstream signaling pathways are attractive targets for therapy.

Within the nucleus, double-stranded DNA is closely associated with histones to form structurally important nucleosome units. Nucleosome formation permits the accommodation of large quantities of compact DNA, and also serves to regulate gene transcription and DNA replication. The histones possess polypeptide amino-terminal tails rich in positively charged lysine residues that are attracted to the negatively charged DNA. Neutralization of the positive charges by acetylation provides a reversible mechanism for regulating gene transcription. Increased histone acetylation by histone acetyltransferases is a key feature of transcriptionally active chromatin, and affects regulation of the expression of approximately 2% of all cellular genes in both normal and malignant cells. Histone deacetylation is also integral to the histone assembly and cell replication mechanisms. Disturbing the balance between histone acetylation and deacetylation may therefore disturb gene transcription as well as DNA replication.

Histone deacetylation is believed to suppress the expression of several genes that regulate cell growth and differentiation in leukemia cells. For example, induction of p21, a key mediator of G1 arrest and differentiation, has been observed when leukemia cells are treated with the HDAC inhibitors trichostatin a and suberoylanilide hydroxamic acid (SAHA). The HDAC inhibitor trichostatin A (TSA) and phenylbutyrate have been shown to be effective against Class I HDACs and can be inactivated by the HDAC inhibitors trichostatin A (TSA) and phenylbutyrate. Similarly, aberrant acute promyelocytic leukemia (APL)-associated fusion proteins of retinoic acid receptor-α (RARα) associate with a corepressor complex containing HDAC activity. The transforming potential of these chimeric proteins is manifested by the repressed transcription that results from this association. HDAC inhibitors like TSA have been shown to enhance the responsiveness of all-trans retinoic acid (ATRA)-resistant cells harboring mutant PML/RAR fusion genes in lymphoblastic leukemia (T-ALL), associates with a member of the Class I HDACs and the corepressor mSin3A, thereby restricting its function in erythroid differentiation. Transcriptional repression could be relieved by TSA. Finally, several genes relevant to cancer, including MYC and MYB, have been found to be highly sensitive to histone hyperacetylation in leukemic cells.

Thus, transcriptional repression mediated by the association of leukemia oncogenes with HDACs provides a rationale for the use of HDAC inhibitors in the treatment of various types of leukemia. Recent studies suggest that HDAC inhibitors may offer a novel approach to the treatment of leukemia and other malignancies. In acute leukemia, there is growing evidence to support this mechanism in the context of t(15;17)- and t(8;21)-positive AML, in which the abnormal transcription factor protein products form a co-repressor complex with HDAC to block cellular differentiation. The combination of phenylbutyrate and ATRA has induced a complete clinical remission in a patient with APL resistant to ATRA alone.

We now report the effects of the cinnamic acid hydroxamate NVP-LAQ824, a novel HDAC inhibitor with activity in nanomolar concentrations on leukemia cells. We show that this agent induces cell cycle arrest and programmed cell death in both acute and chronic myeloid leukemia cells in vitro. In addition, NVP-LAQ824 has significant activity in vivo, as...
demonstrated by its ability to prolong the survival of mice injected with myeloid leukemia cell lines. NVP-LAQ824 also overcomes resistance in myeloid leukemia in vitro to the ABL inhibitor imatinib and displays synergy with imatinib. The results support the hypothesis that HDACs may be an excellent therapeutic target for myeloid leukemias, and suggest that NVP-LAQ824 and related agents are worthy of further investigation in clinical trials.

Materials and methods

Reagents

NVP-LAQ824 and imatinib were obtained from Novartis Pharma AG, Basel, Switzerland. Sodium butyrate (NaButyrate) was purchased as a 1 M solution in sterile distilled water from Upstate Biotechnology (Lake Placid, NY, USA). Serial dilutions were then made of each compound, in their respective vehicles, to obtain final dilutions for cellular assays. GM-CSF was purchased from Calbiochem (San Diego, CA, USA). Steel factor was purchased from PeproTech (Rocky Hill, NJ, USA).

Isolation of primary cells from AML patients and normal primary bone marrow cells

Mononuclear cells were isolated from peripheral blood samples obtained from AML patients with greater than 70% myeloblasts, as well as normal bone marrow, by density gradient centrifugation through Ficoll-Paque Plus (Amersham Pharmacia Biotech AB, Uppsala, Sweden) at 2000 rpm for 30 min, followed by two washes in 1× PBS. Cells were then cultured in cellogro IMDM media (Mediatech, Inc., Herndon, VA, USA) supplemented with 20% fetal calf serum, 1% glutamine, penicillin/streptomycin, 20 ng/ml GM-CSF and 1.5 μg/ml steel factor.

Synergy studies

For synergy studies, imatinib and NVP-LAQ824 were added simultaneously at fixed ratios. Statistical significance of differences observed in drug-treated vs control cultures was determined using Student’s t-test. The minimal level of significance was P<0.05. For drug combination studies, cell viability was determined by trypan blue, and was expressed as the fraction of growth-affected (FA) drug-treated vs control cells; data were analyzed by Calculsyst software (Biosoft, Ferguson, MO, USA; Cambridge, UK), using the Chou-Talalay method.11 Briefly, the combination index = (D1)/[(D1)1 + (D2)/[(D2)2] where (D1) and (D2) are the concentrations required by each drug in combination to achieve the same effect by concentrations (D1)1 and (D2)2 of each drug alone. Values less than one indicate synergy, whereas values greater than one indicate antagonism.

In vivo murine studies

32D.p210 cells used in animal studies were free of Mycoplasma contamination and viral contamination. Cells were washed once with 1× Hank’s balanced salt solution (HBSS) (Mediatech, Inc., Herndon, VA, USA), and then resuspended in 1× HBSS prior to administration to animals. Female BALB/c mice initially weighing 15–18 g and 6–7 weeks of age at delivery (Harlan Sprague-Dawley, Indianapolis, IN, USA) were administered cell suspensions containing 1×10^5 32D.p210 cells via tail vein injection. After 3 days, mice were administered either vehicle (D5W, 5% dextrose) or 25 mg/kg NVP-LAQ824 (diluted in D5W) once daily via intraperitoneal administration. Mice were then monitored for signs of leukemia and were killed when they became moribund, according to ethical protocol. The experimental group consisted of 13 animals, and the control group consisted of 12 animals.

Results

NVP-LAQ824 induces hyperacetylation of histone H4 in HL60 cells

As a positive control for histone acetylation, strong induction of acetylation of histone H4, significantly above baseline levels, was observed in HL60 cells treated with 5 mM NaButyrate. By comparison, a similar dose-dependent increase in acetylation of histone H4 was observed with NVP-LAQ824 in nanomolar concentrations (Figure 1a). The concentration of NVP-LAQ824 needed to induce hyperacetylation of histone H4 was closely correlated to that needed to induce cell death (Figure 1b and c).

NVP-LAQ824-induced inhibition of growth

HL60 cells were treated with either NVP-LAQ824 or vehicle (sterile water) for 0–72 h. NVP-LAQ824 induced cell death within 24–48 h (Figure 1b). HL60 cells were then treated for 24 h with increasing concentrations of NVP-LAQ824 (up to 0.1 μM) in parallel with 5 mM of the HDAC inhibitor NaButyrate (Figure 1c and d). Both compounds caused a dose-dependent decrease in cell viability, as measured by trypan blue exclusion. Moreover, these results indicate that the cytotoxic effects of NVP-LAQ824 are approximately 50 000 times more potent than NaButyrate.

Minimal toxicity was observed at 24 h against CD34+ selected bone marrow cells from a normal donor, as assessed by trypan blue viability assays (Figure 1e). A 24 h exposure of normal CD34+ selected bone marrow cells to 100 nM NVP-LAQ824 resulted in only a 7.3% increase of dead cells (from 18% in controls to 25.3% in treated), as determined after staining with annexin-V and propidium iodide (PI) and assessment by flow cytometry (Figure 2a). However, by 72 h, there was an increased toxicity against normal CD34+ selected bone marrow cells, which resulted in a 16% increase of dead cells (from 9.5 to 25.5%) with 10 nM NVP-LAQ824, and an 84% increase of dead cells (from 9.5 to 93.6%) with 100 nM NVP-LAQ824 (data not shown). These viability assays and annexin-V/PI staining performed after 72 h have been confirmed by CFU-GM colony-forming assays performed over 72 h (data not shown), and suggest that more prolonged exposure to NVP-LAQ824 could be toxic to hematopoietic progenitor cells and possibly stem cells.

By comparison, annexin-V/PI staining at 24 h demonstrated a 62.1% increase in the apoptotic and necrotic fractions of HL60 cells (from 7.9% in controls to 70% in treated) (Figure 2b), demonstrating increased toxicity against leukemia cells. A dose-dependent induction of programmed cell death accompanied the inhibition of the growth of AML cells and normal bone marrow mononuclear cells within 24–72 h of treatment with NVP-LAQ824 (Figure 2c). Whereas the extent to which NVP-LAQ824 induced apoptosis in normal bone marrow vs AML patient cells was similar following 24 h of treatment (Figure 2c: left upper and lower panels), AML patient cells show increased sensitivity to drug concentrations.
0.1 μM or higher, compared to normal bone marrow cells. Following 72 h of drug treatment, AML cell viability was between 21 and 25% compared to normal marrow cells at 0.1–10 μM after 72 h (P < 0.001 for concentrations 0.1–10 μM; Figure 2c right upper and lower panels).

Effects of NVP-LAQ824 on the growth, cell cycle progression and viability of cells derived from AML patients and normal bone marrow cells

Viability growth curves were generated for AML patient cells treated with increasing concentrations of NVP-LAQ824 for 24, 48 and 72 h. NVP-LAQ824 inhibited the growth of primary cells from patients with AML in a dose-dependent manner in nanomolar concentrations (Figure 3). NVP-LAQ824 was also found to cause cell cycle arrest in malignant and normal cells (Table 1, Supplementary Information). Cell cycle G1 arrest of K562 cell lines was observed in a dose-dependent fashion following 24–48 h of treatment with NVP-LAQ824. Similarly, the percentage of AML patient cells and normal bone marrow mononuclear cells cycling in G1 increased in a dose-dependent fashion following 24–48 h of treatment with NVP-LAQ824. However, G1 arrest in the patient cells was not as striking as that observed in K562 cells because the patient cells’ baseline G1 percent was significantly lower.
higher than that of the highly proliferating K562 cell line. Variable degrees of G2M arrest have been reported in previous publications of other HDAC inhibitors. Although the mechanism of G2M arrest has not been fully elucidated, the inhibitory activity of p21 against cdc2 and cyclin B during G2M may be contributing. However, the cell cycle responses vary significantly between cells. In our current experiments, a minor increase in G2M was observed in the K562 cells, but
was overshadowed by an increase in G1. Furthermore, G1 arrest was accompanied by a loss of G2M in patient AML cells and normal bone marrow cells. Our current data therefore indicate that G1 arrest is the dominant cell cycle effect of NVP-LAQ824 in leukemia, as was previously reported in multiple myeloma.26

In vivo effects of NVP-LAQ824

Viability growth curves were generated for 32D.p210 cells treated with either NVP-LAQ824 or vehicle (sterile water) for 24–72 h. The trypan blue exclusion assay demonstrated that, within 48–72 h, 32D.p210 cells were completely killed with 0.1 μM NVP-LAQ824 (Figure 4a).

BALB/c mice administered 32D.p210 cells via tail vein injection were given intraperitoneal injection of either 25 mg/kg NVP-LAQ824 or D5W vehicle on a daily basis, beginning 3 days after introduction of the leukemic cells. Treatment of mice with NVP-LAQ824 delayed the onset of symptoms of leukemia and lethality, as compared to mice treated with the vehicle control (Figure 4b). All mice included in this analysis showed evidence of 32D.p210 leukemic cells in their blood and tissue samples. The log-rank test for differences in survival time between NVP-LAQ824-treated mice and vehicle control-treated mice was statistically significant (log rank \( P = 0.002 \) and Wilcoxon \( P = 0.006 \)). Median survival times were 20 days in the HDAC mice and 15.5 days in the vehicle control mice.

NVP-LAQ824 treatment of imatinib-resistant K562 cells

We were interested in investigating whether or not the use of an inhibitor of histone deacetylation could override drug resistance to an oncogene-specific small-molecule inhibitor. We looked at NVP-LAQ824 in the myeloid cell line K562, made resistant to the small-molecule tyrosine kinase inhibitor imatinib. To assess the potency of NVP-LAQ824 in the imatinib-responsive BCR/ABL-positive erythroleukemia cell line K562, viability growth curves were generated for cells treated with either NVP-LAQ824 or sterile water for 24–72 h. The trypan blue exclusion assay demonstrated that, within 48–72 h, K562 cells were completely killed with 0.1 μM NVP-LAQ824 (Figure 5a). Whereas imatinib was unable to kill imatinib-resistant cells at concentrations that were toxic toward nonresistant cells, NVP-LAQ824 was observed to kill nonresistant K562 and imatinib-resistant K562 cells with similar potency (Figure 5b and c). Similar to these findings, a recent study has shown that NVP-LAQ824 can override imatinib resistance in T315I-BCR/ABL-expressing HL60 cells and in primary CML-BC cells.29

Drug combination studies with NVP-LAQ824

Since NVP-LAQ824 showed an ability to override imatinib resistance, we investigated the potential use of NVP-LAQ824 in combination with imatinib. Imatinib has been shown to act additively or synergistically with a variety of standard chemotherapeutic agents.39,40 The HDAC inhibitor, SAHA, a structural
analog of NVP-LAQ824, has been demonstrated to enhance the effects of imatinib in BCR/ABL-positive cells. More recently, NVP-LAQ824 has been shown to enhance the effects of imatinib at single doses in BCR-ABL-expressing cells. In order to determine whether or not an additive or synergistic effect would result from simultaneous administration of a range of doses of NVP-LAQ824 and imatinib, K562 cells were treated with NVP-LAQ824 and imatinib alone and in combination (Figure 6a). The results demonstrate that co-treatment of NVP-LAQ824 and imatinib across a range of concentrations results in significantly more cell
Ba/F3.p210 and Ba/F3.p210-T315I cells are responsive to toward BCR/ABL as its target (Figure 7b). In contrast, both imatinib, which supports the notion that imatinib is selective (T315I) are unresponsive to even high concentrations of IL3-dependent signaling. BCR/ABL-expressing Ba/F3 cells in addition affect signaling components necessary for selectively toxic toward BCR/ABL as a target and does not IL3-reversible effect (Figure 7a). This suggests that imatinib is imatinib induces BCR/ABL upregulation while inhibiting phosphorylation. In contrast, NVP-LAQ824 has minimal effects on BCR/ABL levels and does not inhibit phosphorylation.

**Comparison of effects of NVP-LAQ824 and imatinib on viability and tyrosine phosphorylation of BCR/ABL expressing cells**

We were interested in investigating the mechanism whereby NVP-LAQ824 kills BCR/ABL-expressing cells, and in determining whether or not this mechanism depends on BCR/ABL kinase activity as a target. Ba/F3.p210 cells treated with imatinib for 72 h are killed by 1 μM imatinib, an IL3-reversible effect (Figure 7a). This suggests that imatinib is selectively toxic toward BCR/ABL as a target and does not in addition affect signaling components necessary for IL3-dependent signaling. BCR/ABL-expressing Ba/F3 cells harboring a point mutation in the imatinib-binding domain (T315I) are unresponsive to even high concentrations of imatinib, which supports the notion that imatinib is selective toward BCR/ABL as its target (Figure 7b). In contrast, both Ba/F3.p210 and Ba/F3.p210-T315I cells are responsive to NVP-LAQ824 (Figure 7c) and do not display IL-3 rescue or the differential responsiveness seen by both cell lines to imatinib (Figure 7d and e). NVP-LAQ824 inhibits the growth of both Ba/F3.p210 and Ba/F3-T315I, with 40% viable cells at 10 nM NVP-LAQ824 for Ba/F3.p210 and at 1 nM for Ba/F3-T315I, and complete inhibition of cell viability at 50 and 30 nM, respectively. Thus, both cell line derivatives were highly sensitive to NVP-LAQ824.

Ba/F3.p210 cells were treated for 1 h with either 1 μM NVP-LAQ824 or 1 μM imatinib, and the protein lysates extracted from cells were analyzed by immunoblotting (Figure 8). A representative immunoblot is shown in Figure 8a, and densitometry analyses of two separate experiments are presented in Figure 8b. These experiments demonstrate the differential effects of NVP-LAQ824 and imatinib on BCR/ABL phosphorylation. In particular, after 1 h, imatinib induces BCR/ABL upregulation while inhibiting phosphorylation. In contrast, NVP-LAQ824 has minimal effects on BCR/ABL levels and does not inhibit phosphorylation.

**Discussion**

Genetic defects that lead to deregulation of such processes as differentiation, apoptosis and cell cycle progression are trademarks of myeloid leukemia. In AML, both a differentiation block, associated with chimeric transcription factor oncogenes resulting from chromosomal translocations, and accelerated proliferation, associated with constitutive activation of tyrosine kinases, appear to be necessary events.42–44 In CML, patients progress through a 3–5-year `chronic' phase to an acute leukemia-like, ‘’blast crisis’ phase, characterized by a block in differentiation and growth factor independence. The oncogene BCR/ABL, a chimeric fusion protein resulting from the Philadelphia (Ph) chromosome translocation t(9;22),45–47 is characterized by elevated Ab1 tyrosine kinase activity,48–50 and has antiapoptotic function.

Compounds that inhibit HDACs represent a promising novel class of anticancer agents that affect the regulation of genes associated with cell growth, differentiation, and apoptosis, and therefore should be potentially valuable therapeutic tools for myeloid leukemia. Inhibition of HDACs would lead to increased histone acetylation, and consequently would be expected to activate genes responsible for inducing programmed cell death or blocking the growth of malignant cells.

However, while numerous HDAC inhibitors have been shown to display varying degrees of activity against different types of leukemia, the majority that are in late-stage development offer less than ideal potency and/or stability. Short-chain fatty acids, including the butyrates and valproic acid, comprise the first of four classes of HDAC inhibitors. Butyrate and analogs of butyrate have shown activity against the myelomonocytic leukemia (U937) cells and HL60 cells,51 erythroleukemia cells52 and acute T lymphoblastic leukemia cells.53 However, butyrates generally inhibit HDAC activity and cell growth at millimolar concentrations. More recently, pivaloyloxymethyl butyrate (AN-9), a novel prodrug of butyric acid, was shown to be toxic toward acute leukemia and doxorubicin-resistant acute leukemia, with a high IC50 in the range of 45–50 μM.54 AN-9 has undergone evaluation as an intravenously administered agent in a Phase I dose escalation study on patients with advanced solid malignancies.55 Some common side effects, including gastrointestinal distress and fever, were observed, and dose escalation was limited by the volume of the intralipid formulation of vehicle for AN-9 that could be safely administered. The widely prescribed anticonvulsant valproic acid induces differentiation
of leukemic blasts from acute myeloid leukemia patients; yet it only displays HDAC inhibitor activity at relatively high concentrations.

Cyclic tetrapeptides are a second class of HDAC inhibitors, and include such agents as apicidin and FK-228 (FR901228, depsipeptide). Apicidin was shown to induce apoptosis in HL60

Figure 7 Comparison of effects of NVP-LAQ824 and imatinib on viability of BCR/ABL-expressing cells. (a, b) Effects of imatinib on cellular proliferation and viability of Ba/F3.p210 cells and Ba/F3.p210-T315I cells, respectively. Cells were cultured in the presence of increasing concentrations of imatinib in the presence and absence of IL-3. Ba/F3.p210 cells were inhibited by imatinib, and cells were rescued in the presence of IL-3. Ba/F3.p210-T315I cells were not inhibited by imatinib. Data for experiments are shown as the percentage of untreated (control) cells. (c, d) Effects of NVP-LAQ824 and imatinib, respectively, on cellular proliferation and viability of Ba/F3.p210 and Ba/F3.p210-T315I cells. Cells were cultured in the presence of increasing concentrations of NVP-LAQ824 or imatinib in the absence of IL-3. Both Ba/F3.p210-T315I cells and Ba/F3.p210 cells were inhibited by NVP-LAQ824. In contrast, only the Ba/F3.p210 cells were sensitive to imatinib. Data for experiments are shown as the percentage of untreated (control) cells. (e) Effects of NVP-LAQ824 on cellular proliferation and viability of Ba/F3 cells, Ba/F3.p210 cells and Ba/F3.p210-T315I cells, in the presence of IL-3. Cells were cultured in the presence of increasing concentrations of NVP-LAQ824. There was no rescue of cell viability in the presence of IL-3. Data for experiments are shown as the percentage of untreated (control) cells.
Depsipeptide induces apoptosis in lymphoma cells \textit{in vitro} with an IC$_{50}$ of approximately 6 nM, and prolonged survival of mice in lymphoma and promyelocytic leukemia models.\textsuperscript{57–59} FK228 has been evaluated as an intravenously administered agent in a Phase I clinical trial involving patients with advanced, incurable cancer, with common side effects, such as gastrointestinal distress, observed.\textsuperscript{60} Another Phase I trial showed grade-4 thrombocytopenia and grade-4 cardiac arrhythmia among several dose-limiting toxicities.\textsuperscript{61} Although pre-clinical toxicity data on FK228 suggested cardiac toxicity as a side effect, no myocardial damage was noted in the study. However, reversible ECG changes with ST/T wave flattening were regularly observed in FK228-treated patients.

Representatives of a third class of HDAC inhibitors are benzamides MS-275 and CI-994, both of which are presently in clinical trials. MS-275, like SAHA and oxamflatin, generally works at micromolar concentrations \textit{in vitro}.\textsuperscript{62} In comparison to a novel non-hydroxamate sulfonamide under pre-clinical investigation, MS-275, itself an anilide-based HDAC inhibitor, exhibited notable hematological toxicity, including a lowering of peripheral white and red blood cell counts, in mice.\textsuperscript{63} CI-994 has been evaluated in a Phase I study involving patients with...
solid tumors.\(^6^4\) Thrombocytopenia was the dose-limiting toxicity, and common side effects such as gastrointestinal distress were observed. There was also evidence of efficacy, as several patients achieved stable disease. The mechanism of action of CI-994 is unclear, as it inhibits histone deacetylation, but is not a direct inhibitor of HDAC.\(^4^4\)

The fourth class of HDAC inhibitors is the hydroxamic acids, which include compounds like oxamflatin, SAHA and TSA. TSA, originally developed as an antifungal agent, has high instability and has been primarily useful as a research tool.\(^5^6\) Oxamflatin shows antiproliferative activity against HeLa cells by inducing histone hyperacetylation and, in effect, changing the expression of certain endogenous genes involved in cell cycle control and cell morphology.\(^6^6\) SAHA has been shown to act in synergy with the cyclin-dependent kinase (CDK) inhibitor flavopiridol (FP) in inducing apoptosis of U937 cells.\(^6^7\) A Phase 1 clinical trial of the novel hydroxamic acid-derived HDAC inhibitor SAHA has recently been reported.\(^6^8\) Of 29 patients evaluated, four patients (two with lymphoma and two with bladder cancer) had objective tumor regression with clinical improvement. SAHA was well tolerated, although treatment delay was necessary in the lymphoma patients due to leukopenia and thrombocytopenia, likely resulting from the limited marrow reserve in these heavily pretreated patients. An accumulation of acetylated histones in peripheral blood cells as well as tumor tissue post-treatment was observed. However, SAHA is generally active only at micromolar concentrations, and to improve efficacy modifications of the structure of hydroxamic acid-based compounds have led to the development of derivatives with subnanomolar activities.\(^6^9,7^0\) NVP-LAQ824 is a hydroxamic acid-derived HDAC inhibitor, which differs from other clinically active compounds in this class in its ability to induce apoptosis and cause cell cycle arrest in myeloid cell lines and primary patient samples with an IC\(_{50}\) in the nanomolar range.\(^7^6^-7^0\) Normal bone marrow CFU-GM were approximately two-fold less sensitive to NVP-LAQ824 in terms of induction of apoptosis, as compared to AML patient cells, and, in vivo, the agent was generally well-tolerated and prolonged survival in a mouse model. It has previously been shown that NVP-LAQ824 kills multiple myeloma cells resistant to conventional chemotherapy at nanomolar concentrations, and shows efficacy in a murine myeloma model.\(^7^6\)

For some AML patient samples, we observed a slightly higher cell number at lower concentrations of NVP-LAQ824, suggesting that low concentrations of NVP-LAQ824 may sometimes increase cell proliferation. Previously, DiGiuseppe \(et\) \(al\)\(^7^1\) published that low doses of phenylbutyrate (<1 mm) consistently led to a slight transient increase in \(S\)-phase cells on days 2–3 of incubation. The increase in \(S\) phase was <10\%, but was not necessarily associated with increased cell number, on days which was not examined. Yu \(et\) \(al\)\(^7^2\) showed that, in response to very low concentrations of phenylbutyrate, there was a 5% increase in \(S\) phase in one figure, but no increase in cell numbers as determined by colony formation. In contrast, we have only observed a decrease in \(S\) phase in K562 cells using low concentrations of NVP-LAQ824.

Our finding of modestly enhanced cell number in AML samples at a low concentration of NVP-LAQ824 was not a consistent phenomenon. Not all of the patient samples showed this effect, and this may be due to intra-experimental variability that occurs between control samples and low-dose drug samples. Taken together, the data suggest that while it is not completely outside of the realm of possibility that low concentrations of NVP-LAQ824 (≤10 \(\mu\)M) increase cell growth, there is not enough evidence at present to comment on whether this is a generalized phenomenon for this class of agents. Additional studies will need to be performed to determine the significance, if any, of these observations.

Significantly, NVP-LAQ824 was able to override resistance to the small-molecule inhibitor imatinib, established to block the activity of the kinase (BCR/ABL) known to play a key role in the pathogenesis of CML. Imatinib is a particularly promising new oncogene-specific drug that induces hematologic remissions in virtually all stable-phase CML patients and most blast crisis-phase patients.\(^7^1\) However, there are reports of drug resistance in patients in the chronic phase, and most imatinib-treated patients in blast crisis relapse in a few months.\(^7^4\) A recent study has shown synergistic effects of the proteasome inhibitor bortezomib used against BCR-ABL-expressing human leukemia cells in combination with either SAHA or NaButyrate.\(^7^5\) In addition, this combination was able to override imatinib resistance in imatinib-resistant K562 cells and CD34+ mono-nuclear cells obtained from an imatinib-resistant patient.

NVP-LAQ824 was able to inhibit the cell growth of imatinib-resistant K562 cells with a similar potency to that seen in non-resistant K562 cells. These cells display a two-fold increase in BCR/ABL protein, as compared to nonresistant cells, and show elevated cellular tyrosine phosphorylation.\(^7^6\) In our present studies, we observed upregulation of BCR/ABL protein levels, but inhibition of BCR/ABL phosphorylation after treatment of imatinib-sensitive cells with imatinib, consistent with our previous findings.\(^7^6\) One hypothesis is that imatinib forms a complex with the inactive form of BCR-ABL, and there may be a compensatory stabilization or upregulation of BCR/ABL on the part of the cell in response to inhibition of tyrosine phosphorylation by imatinib. It could be upregulation of the inactive form of the protein, or stabilization of the inactive form of BCR/ABL that is due to its complexing with imatinib. In drug-resistant cells, BCR/ABL protein upregulation has correlated with enhanced expression of BCR/ABL mRNA and BCR/ABL gene amplification. Drug-resistant cells, as a consequence, show elevated cellular pTYR levels, and are able to survive the presence of drug. In nonresistant cells, we do not observe changes in BCR/ABL DNA levels or RNA expression. However, the exact mechanism of imatinib resistance in these cells is unclear. The ability of NVP-LAQ824 to override resistance in imatinib-resistant cells, coupled with a demonstration of synergy between imatinib and NVP-LAQ824 in non-resistant K562 cells, suggests a potential for effective combination therapy in CML patients. These findings are supported by similar results obtained in a recent study investigating the effects of NVP-LAQ824 in BCR/ABL-positive imatinib-resistant and nonresistant cells.\(^7^9\)

Ba/F3.p210 cells treated with 1 \(\mu\)M imatinib for 72 h were rescued by IL-3, suggesting that imatinib is selectively toxic toward BCR/ABL as a target and does not in addition affect IL-3-dependent signaling. This concept that imatinib is selective toward BCR/ABL as its target is supported by our experimental results showing that BCR/ABL-expressing Ba/F3 cells harboring a point mutation in the imatinib-binding domain (T315I) are unresponsive to even high concentrations of imatinib. Importantly, both Ba/F3.p210 and Ba/F3.p210-T315I cells are responsive to NVP-LAQ824, and do not display IL-3 rescue or the differential responsiveness seen by both cell lines to imatinib. These results indicate that other alternative targets are likely involved in NVP-LAQ824-induced cytotoxicity. For example, given that histone hyperacetylation affects the transcription of thousands of genes,\(^7^7\) as well as the acetylation of nonhistone targets such as Hsp90 and Ku,\(^7^9\) it is likely that many potentially important targets are affected by.
NVP-LAQ824. Future experiments designed to identify these targets will give better insight into the mechanism of action of NVP-LAQ824 in BCR/ABL-positive myeloid leukemia.

We have found that NVP-LAQ824 does not affect the intrinsic tyrosine kinase activity of BCR/ABL, and does not induce downregulation of BCR/ABL in Ba/F3-p210 cells or Ba/F3-p210-T3151 cells. These are cell lines stably transected with a BCR/ABL-expressing plasmid not associated with histones, and we therefore did not expect that a HDAC inhibitor would directly affect BCR/ABL expression. Nimmanapalli et al. reported downregulation of BCR/ABL in K562 cells, which have a genomic rearrangement of BCR/ABL and would be regulated by histone acetylation status. However, Nimmanapalli et al. also observed downregulation of BCR/ABL in p210-T3151 cells, and suggested that NVP-LAQ824 induced proteasomal degradation of BCR/ABL due to hyperacetylation of heat shock protein 90, which plays a role in the stabilization of the BCR/ABL protein.

In our current set of experiments, we demonstrate the differential effects of NVP-LAQ824 vs imatinib on BCR/ABL phosphorylation status in K562 cells. After 1 h of exposure to imatinib, BCR/ABL protein levels are upregulated, while phosphorylation is inhibited. In contrast, NVP-LAQ824 has minimal effects on BCR/ABL and does not inhibit phosphorylation. These data support the concept that BCR/ABL kinase activity is not a direct early target of NVP-LAQ824. These results are consistent with results presented by Nimmanapalli et al. who show that BCR/ABL mRNA levels do not fall until after 4 h, and there are no changes in BCR/ABL protein levels at 4 h but downregulation at 8 and 24 h. Neither NVP-LAQ824 nor imatinib was able to inhibit cellular tyrosine phosphorylation in the imatinib-resistant Ba/F3-p210-T3151 cell line.

We have previously demonstrated upregulation of BCR/ABL with imatinib in BCR/ABL-expressing cells, but the significance remains unclear. Imatinib is well known to bind to the inactive form of BCR/ABL. One hypothesis is that there may be a modest upregulation of the inactive form of the BCR/ABL protein, possibly as a result of stabilization of BCR/ABL due to its complex formation with imatinib. Another hypothesis is that there may be a compensatory stabilization or upregulation of BCR/ABL on the part of the cell in response to inhibition of tyrosine phosphorylation by imatinib.

The results of this study suggest that NVP-LAQ824 should be tested in clinical trials for patients with advanced myeloid leukemia, as demonstrated by its high potency in vitro, as well as efficacy in vivo. Furthermore, the ability of NVP-LAQ824 to override resistance to promising small-molecule tyrosine kinase inhibitors like imatinib, and its impressive synergism with imatinib, highlight the potential of this compound to strengthen already existing, promising therapies.

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Supplementary Information

Materials and methods for cell culture, cell cycle, cytotoxicity assays and Western blot are listed in Supplementary Information on the Leukemia website (http://www.nature.com/leu).

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