Constitutive Activation of Signal Transducers and Activators of Transcription Predicts Vorinostat Resistance in Cutaneous T-Cell Lymphoma

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Abstract

Vorinostat is a histone deacetylase inhibitor that induces differentiation, growth arrest, and/or apoptosis of malignant cells both in vitro and in vivo, and has shown clinical responses in ~30% of patients with advanced mycosis fungoides and Sézary syndrome cutaneous T-cell lymphoma (CTCL). The purpose of this study was to identify biomarkers predictive of vorinostat response in CTCL using preclinical model systems and to assess these biomarkers in clinical samples. The signal transducer and activator of transcription (STAT) signaling pathway was evaluated. The data indicate that persistent activation of STAT1, STAT3, and STAT5 correlate with resistance to vorinostat in lymphoma cell lines. Simultaneous treatment with a pan-Janus-activated kinase inhibitor resulted in synergistic antiproliferative effect and down-regulation of the expression of several antiapoptotic genes. Immunohistochemical analysis of STAT1 and phosphorylated tyrosine STAT3 (pSTAT3) in skin biopsies obtained from CTCL patients enrolled in the vorinostat phase IIb trial showed that nuclear accumulation of STAT1 and high levels of nuclear pSTAT3 in malignant T cells correlate with a lack of clinical response. These results suggest that deregulation of STAT activity plays a role in vorinostat resistance in CTCL, and strategies that block this pathway may improve vorinostat response. Furthermore, these findings may be of prognostic value in predicting the response of CTCL patients to vorinostat. [Cancer Res 2008;68(10):3785–94]

Introduction

Cutaneous T-cell lymphomas (CTCL) are a group of non–Hodgkin’s lymphoma (NHL) caused by malignant, skin-homing T cells (1–3). The most common variants are mycosis fungoides (MF) and Sézary syndrome (SS). The latter is the erythrodermic and leukemic form of the disease. The histone deacetylase (HDAC) inhibitor vorinostat (Zolinza, suberoylanilide hydroxamic acid) has been approved for the treatment of cutaneous manifestations in patients with MF/SS who have progressive, persistent, or recurrent disease on or after two systemic therapies (4–7). Vorinostat provided objective clinical and symptomatic relief in 30% of heavily pretreated patients with MF/SS (6, 7). These results prompted the search for molecular markers predictive of treatment outcome.

Preclinical studies have shown that vorinostat affects proliferation of a variety of transformed cell lines in culture and of human cancer xenografts in mice (8). In vitro, vorinostat inhibits HDACs of class I and class II, and treatment of cells with this compound at clinically achievable concentrations results in an increase in acetylation of histones and non-histone proteins. Both transcriptional and nontranscriptional effects of vorinostat have been linked to its antitumor activity. These include reexpression of tumor suppressor genes, increased oxidative stress, modulation of immune response, and inhibition of angiogenesis (9, 10). Depending on the cancer type and genetic background, response to vorinostat may be dependent on any or all of these biological responses. Likewise, resistance to this agent could arise from a variety of molecular mechanisms capable of counteracting or blocking these effects. Because vorinostat is currently being evaluated in a number of hematologic and solid tumor malignancies, further investigation of the underlying factors that determine clinical response to this compound will enhance our ability to identify cancer patients who will most likely benefit from this therapeutic modality.

To that end, pretreatment biomarkers of response to vorinostat were identified in preclinical experiments, and their relevance to clinical response was investigated. In the present study, we report the evaluation of the roles of the signal transducer and activator of transcription (STAT) proteins in the response of human lymphoma to vorinostat treatment. STAT proteins are a group of transcription factors involved in signaling from a number of growth factor and cytokine receptors, and their deregulation has been associated with malignant transformation (11–14). The STAT family comprises seven members with a high degree of homology at the amino acid level. Of these, STAT1, STAT3, STAT5a, and STAT5b are ubiquitously expressed and phosphorylated on tyrosine and serine residues in response to a variety of stimuli (15). STAT proteins are activated in lymphoid cells by cytokine receptors that engage the Janus-activated kinases (JAK; JAK1, JAK2, JAK3, and TYK2) to induce their tyrosine phosphorylation (16). More recent work has
shown that STATs can also be activated by oxidative stress, a prevalent condition in the cancer milieu (17).

Both tyrosine and serine phosphorylation are necessary for STAT nuclear localization and full transcriptional activity. Cytokine autocrine loops, tyrosine kinase activation, and silencing of STAT negative regulators, including the suppressors of cytokine signaling proteins, as well as the tyrosine phosphatases SHP-1 and SHP-2, commonly occur in cancer cells. Therefore, multiple mechanisms lead to the sustained STAT phosphorylation detected in human tumors (18, 19). Persistent activation of STATs is widely observed in hematologic malignancies including B-cell and T-cell lymphomas, as well as solid tumors, including cancers of the lung, breast, ovaries, head and neck, prostate, and skin (13, 20–23). In particular, STAT3 has been shown to be an oncogene capable of transforming immortalized mouse and rat fibroblasts (24). Constitutive STAT activation contributes to malignant transformation at least in part by promoting cell proliferation and inhibiting cell death. Indeed, the expression of several cell cycle control and antiapoptotic genes is regulated by these transcription factors (13). In addition, overexpression of STAT1 and STAT3 has been associated with resistance to platinum-based drugs (25, 26). Constitutive activation of STAT3 has also been linked with poor response to chemotherapy in breast cancer cells (27). In lymphoid malignancies, a lack of STAT3 activation correlates with a favorable clinical outcome, and inhibition of STAT3 activity sensitizes resistant NHL cells to chemotherapy (28, 29).

In light of the evidence supporting the contribution of activated STATs to the survival of cancer cells, we investigated the role of STAT pathway activation in the response of lymphoma cells to vorinostat. The results indicate that constitutive activation of STATs is involved in resistance to vorinostat across a variety of B-cell and T-cell lymphoma lines, including those of CTCL origin. Furthermore, high levels of phosphorylated tyrosine STAT3 (pSTAT3) and nuclear localization of STAT1 in malignant T cells from skin biopsies of MF/SS patients correlate with a lack of clinical response to vorinostat.

Materials and Methods

Cell lines. The cell lines used by Cell and Molecular Technologies, Inc., for this study were identified by Merck & Co., Inc., and obtained from the American Type Tissue Culture Collection and DSMZ. These cell lines were for this study were identified by Merck & Co., Inc., and obtained from the American Type Tissue Culture Collection and DSMZ. These cell lines were maintained in RPMI 1640/10% fetal bovine serum/1% glutamax/1% American Type Tissue Culture Collection and DSMZ. These cell lines were maintained in RPMI 1640/10% fetal bovine serum/1% glutamax/1% penicillin-streptomycin (Life Technologies).

Drug source. Vorinostat was produced by Merck & Co., Inc., and used for the studies conducted at Merck Research Laboratories and Cell and Molecular Technologies, Inc., JAK inhibitor I (JAKi) was purchased from Calbiochem.

Proliferation assay. CellTiter-Blue Cell Viability Assay (Promega) was performed according to manufacturer's suggested protocol. Cells were seeded in 96-well plates (Corning) and, the following day, treated with vorinostat (from maximum concentration of 30 μmol/L in 3-fold dilution) in triplicate. CellTiter-Blue reagent was added, and 4 h later, plates were read on the Flex Station II 384 (Molecular Devices). Experiments were performed in triplicate. The IC_{50} values were calculated using the Sigma Plot software. Combination index was calculated with CalcuSyn software (Biosoft).

Cell cycle analysis and apoptosis assay. For cell cycle analysis, cells were treated with vorinostat as indicated. Samples were fixed and stained with propidium iodide/RNase staining buffer according to manufacturer's recommended procedure (BD Phamingen) and subjected to flow cytometry (FACSCalibur). Data analysis was done with the aid of FlowJo 8.6 software.

ApoONE Homogeneous Caspase-3/7 Assay (Promega) was performed according to standard protocol (Promega). Cells were seeded in 96-well plates and treated with vorinostat for 24 h. The plates were read on the Flex Station II 384 (Molecular Devices). Where indicated, DNA fragmentation was assessed by terminal dUTP nick end labeling (TUNEL) assay (BD PharMingen) following standard procedure. Samples were analyzed by flow cytometry (FACSCalibur).

Cell lysate preparation and Western blot. Cells were lysed in Laemmli sample buffer (Bio-Rad) with Complete Mini protease inhibitors (Roche). Samples were sonicated, and protein concentration was determined by the BC Protein Assay (Bio-Rad). Proteins were separated using polyacrylamide criterion Tris-HCl gel (Bio-Rad) and transferred onto nitrocellulose membranes (Invitrogen) in Novex Tris-glycine transfer buffer (Invitrogen)/20% methanol/0.01% SDS. The membranes were blocked in TBS/Tween 20 (TBS-T)/5% bovine serum albumin (BSA; Sigma) and probed with anti-STAT1, anti–phosphorylated serine STAT1, anti-STAT3, anti–phosphorylated serine STAT3, anti-STAT5, or antibodies against the tyrosine-phosphorylated form of the proteins (Cell Signaling Technology), as well as anti-STAT5B (Chemicon). Anti–β-actin and anti-actin (Sigma) were used as loading controls. Membranes were washed with TBS-T and then probed in the dark with secondary antibody, Zymax goat anti-rabbit Cy5, and goat anti-mouse Cy5 (Zymed). Fluorescence emission was measured using the Typhoon 9410 (Amersham Biosciences). Signals were quantified using ImageQuant software.

Reverse-phase protein microarray analysis. Microarrays were manufactured, as previously described (30). Briefly, 20 nL of denatured protein lysates were immobilized onto nitrocellulose-coated glass slides using a GMS470 Affymetrix microarreayer in an ordered array. Printed arrays were washed with distilled H_{2}O, blocked for 2 h at room temperature with I-Block (Tropix) containing 0.1% Tween 20, followed by blocking of endogenous biotin, incubation with primary antibody at a concentration of 1:1,000 (45 min), and biotinylated secondary antibody at 1:5,000 (30 min). After secondary incubation, arrays were incubated with strepavidin-biotin complex (15 min), biotinyl tyramide (15 min), and strepavidin-peroxidase (15 min) and developed with 3,3′-diaminobenzidine tetrahydrochloride (4 min). Total protein detection was determined by SYPRO ruby blot stain (Molecular Probes), as per the manufacturer's protocol. Stained arrays were scanned on a Unimax scanner with 256 gray scale at 600 dpi and quantified using Imagequant. Staining intensities were normalized to total protein concentrations. Antibodies were purchased from Cell Signaling Technology.

Gene expression profiling and real-time PCR analysis. Total RNA was isolated from each cell line using RNeasy Midi or Mini kits (Qiagen) according to the manufacturer's protocol. For gene expression profiling studies, amplification was performed using standard protocol (31). Expression of ~25,000 human genes was determined by hybridization to 60-mer oligonucleotide arrays (Agilent Technologies, Inc.) using Stratagene Universal human reference RNA as control. Gene expression was normalized using standard methods, and statistical analysis was performed as previously described (32).

For the real-time PCR expression analysis, cDNAs were synthesized using the high-capacity cDNA archive kit, and the PCR amplification was performed in 384-well plates in triplicate using the 7900HT fast real-time PCR system following the manufacturer's suggested procedure (Applied Biosystems). Optimized primers and probes (TaqMan gene expression assays; Applied Biosystems) for Bcl-xl, survivin, Mcl-1, Bax, Bad, Osm, JunB, glicheraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin were used in these studies. The amplification protocol was as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C at 15 s and 60°C for 1 min. The data were analyzed using the comparative cycle threshold (C_{T}) method. The C_{T} values were normalized with those of actin.

Localization of STAT proteins by immunofluorescence microscopy. Lymphoma cell lines (6.25 × 10^{4}/250 μL) were spun onto coated slides using cytofumitons and cyotospin (Thermo Electron Corp.) at 1,000 rpm for 5 min. Cells were fixed and permeabilized as previously described (33). Anti-STAT1, anti-STAT3, and anti–phosphorylated serine STAT3 (Cell Signaling Technology) were added at a 1:100 dilution in TBS-T/2.5% BSA. Donkey anti-rabbit FITC-conjugated antibody (Jackson ImmunoResearch Labs.)
was used at a 1:100 dilution in TBS-T/0.25% BSA. Nuclear DNA was stained with Hoechst 33342 (Molecular Probes). Samples were imaged and photographed using a Nikon Eclipse TE2000-U microscope.

Transfection of STAT3 and STAT5 small interfering RNA oligonucleotides. HuT 78 cells were transfected with 100 nmol/L STAT3, STAT5A, and STAT5B small interfering RNA (siRNA) duplexes (SMARTpool, Dharmacon) or luciferase control duplex (negative control) using X-tremeGene transfection Reagent (Roche). Cells were seeded in 24-well plates and treated according to the manufacturer's procedure, and 48 h posttransfection, vorinostat was added to the cells as indicated. Each condition was done in triplicate. The effect on proliferation/viability was assessed using ViaLight Plus kit (Lonza).

Clinical specimens. Skin biopsies were collected from 51 MF/SS patients enrolled in the phase IIb clinical trial (7). All patients provided written informed consent before enrollment, and the study protocol was approved by the institutional review boards of all the participating academic centers. Serial 4-mm punch biopsies were taken from the skin lesion at baseline (before first vorinostat dose). Tissues were fixed in formalin and embedded in paraffin. The primary end point of the trial was the objective response rate measured by mSWAT. The objective response rate was 29.7% overall. Secondary end points included time to response, time to progression, duration of response, and pruritus relief. Median time to response was <2 mo, and time to progression was 4.9 mo. Median duration of response was not reached but estimated to be ≥185 d, and 32% of the patients had pruritus relief.

Immunohistochemical analysis of STAT1, STAT3, and STAT5 in skin biopsies. Paraffin-embedded tissues were sectioned at 5 μm and stored in nitrogen until immunostained. Sections were dewaxed in xylenes, rehydrated through graded alcohols, and then incubated in 0.3% H2O2 in methanol for 20 min at room temperature to block endogenous peroxidase activity. Antigen retrieval was performed using a commercially available citrate-based solution (Vector Laboratories) according to the manufacturer's instructions. Nonspecific binding was blocked by incubation in 10% normal goat serum for 1 h at room temperature. Sections were incubated with polyclonal antibodies directed against STAT1 (Cell Signaling Technology; 1:200 dilution), STAT5 (Cell Signaling Technology; 1:1,000 dilution), STAT3 (Cell Signaling Technology; 1:50 dilution), and pSTAT3 (Cell Signaling Technology; 1:50 dilution) followed by a biotinylated secondary
antibody (0.5 μg/mL; Jackson Immunoresearch) and ABC/DAB (Vector Laboratories) and then hematoxylin counterstaining. Sections were evaluated for nuclear and cytoplasmic staining of tumor cells and nonmalignant cells, including keratinocytes and endothelial cells, which served as internal controls. Although all portions of a given section were evaluated, scoring of 100 or more tumor cells was performed on areas selected for optimal distinction of tumor cells from nontumor cells. Photomicrographs were taken of each section and projected on a screen to validate this approach. Tumor cells were identified by a combination of criteria, including location within the epidermis (Pautrier microabscesses), nuclear size, and nuclear convolutions, often described as cerebriform nuclei. Tumor cells thus identified were scored for strong staining (2+), weak staining (1+), or no staining in nuclear and cytoplasmic compartments. The number of positively stained cells was also documented. The score recorded was the most prominent for identifiable tumor cells. The pathologist (M.E.K.) who scored the staining had no knowledge of the nature of the patient’s clinical response or whether the specimen was obtained before or after therapy.

Statistical analysis. Fisher’s exact test was used to examine the significance of the association between vorinostat response and STAT1 subcellular localization (nuclear or nuclear/cytoplasmic), as well as vorinostat response and pSTAT3 or STAT5 staining intensity (+1 or +2). The P value from the test was computed for a 2 × 2 contingency table with fixed margins (34).

Results

Differential response to vorinostat treatment in a panel of B-cell and T-cell lymphomas. A broad panel composed of approximately 40 human B-cell and T-cell lymphoma lines was used to identify potential factors involved in resistance to vorinostat treatment. First, the effects of vorinostat on proliferation/viability and apoptosis were assessed across the panel (Fig. 1A). Diversity in vorinostat sensitivity was observed among Table 1. Cell cycle analysis in CTCL cells left untreated or treated with vorinostat for 48 h

<table>
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<th>Cell line/treatment</th>
<th>%Sub-G1</th>
<th>%G1</th>
<th>%S</th>
<th>%G2-M</th>
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<tbody>
<tr>
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<td>41.5</td>
<td>37.4</td>
<td>17.9</td>
</tr>
<tr>
<td>HH VT-treated</td>
<td>41.5</td>
<td>31.2</td>
<td>12.5</td>
<td>14.8</td>
</tr>
<tr>
<td>H9 Unt</td>
<td>2.9</td>
<td>34.4</td>
<td>33.0</td>
<td>29.7</td>
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<tr>
<td>H9 VT-treated</td>
<td>4.1</td>
<td>55.9</td>
<td>13.9</td>
<td>26.1</td>
</tr>
<tr>
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<td>48.9</td>
<td>32.2</td>
<td>16.3</td>
</tr>
<tr>
<td>HuT 78 VT-treated</td>
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<td>57.2</td>
<td>12.6</td>
<td>26.9</td>
</tr>
<tr>
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<td>42.3</td>
<td>36.4</td>
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</tr>
<tr>
<td>MJ VT-treated</td>
<td>4.5</td>
<td>50.6</td>
<td>11.7</td>
<td>33.2</td>
</tr>
</tbody>
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Abbreviations: Unt, untreated; VT, vorinostat.

Figure 2. Evaluation of expression of JAK and STAT family members in human lymphoma cell lines. A, gene expression profiling results for several members of the JAK/STAT pathway, including STAT1, STAT3, and STAT5. The genes shown on the heatmap are differentially expressed based on a 0.5-fold to 0.05-fold logarithmic scale (3.2-fold to 3.2-fold linear scale). Magenta, increased gene expression; aqua, decreased gene expression. Cell lines were ranked by increasing IC50. B, reverse-phase protein microarray analysis. Arrays with the immobilized whole-cell lysates from lymphoma cell lines displaying variable vorinostat sensitivities were probed with antibodies against >50 antibodies, as described under Materials and Methods. Results for STAT1, STAT3, and caspase-3.
the cell lines. Vorinostat response was also evaluated in a panel composed of 150 human cancer cell lines from colon, breast, and lung origin (Fig. 1B). Noteworthy, compared with the colon, breast, and lung cancer cell lines, the lymphoma subset had the largest number of cell lines with the lowest half-maximal inhibitory concentration (IC50). These data are reminiscent of the spectrum of clinical responses for vorinostat as a single agent documented to date. In vitro response criteria based on the results from the two phenotypic assays were established. The available clinical data on vorinostat exposure guided the selection of the threshold vorinostat concentration (35). Lymphoma cell lines with proliferation/viability IC50 values of ≤1 μmol/L (clinically achievable) that underwent apoptosis in response to vorinostat at 1 μmol/L as assessed by activation of caspase-3,7 or DNA fragmentation were classified as sensitive. Because of the clinical activity shown by vorinostat in MF/SS, four human T-cell lymphoma lines originated from CTCL patients, including HH, HuT 78, H9, and MJ, were included in the panel (36–38). The more responsive CTCL line HH exhibited an IC50 of ~0.8 μmol/L and underwent apoptosis in response to vorinostat, as assessed by TUNEL assay. In contrast, H9, HuT 78, and MJ cells (IC50, >2 μmol/L) showed an increase in the population in the G1 or G2 phase of the cell cycle but did not show signs of DNA fragmentation upon vorinostat treatment for 48 hours (Fig. 1C; Table 1). Overall, the response to vorinostat of these CTCL lines fall within the range of the larger panel and are in agreement with results previously reported (38). The ability of the HDAC inhibitors valproate and trichostatin A to induce apoptosis in vorinostat-sensitive and vorinostat-resistant lymphoma lines was evaluated. The response pattern of valproate and trichostatin A mirrored that of vorinostat across the cell lines examined (Supplementary Fig. S1).

Detection of deregulation of JAK/STAT pathway in vorinostat-resistant lymphoma cell lines. Analysis of global gene expression profiles across lymphoma cell lines showed a differential

Figure 3. Characterization of STAT1, STAT3, and STAT5 expression, phosphorylation, and subcellular localization in lymphoma cell lines. A, whole-cell lysates from lymphoma cell lines with diverse vorinostat response were subjected to immunoblot analysis with the indicated antibodies against STAT proteins, as well as their serine-phosphorylated and tyrosine-phosphorylated forms, as indicated under Materials and Methods. B, comparison of expression and phosphorylation of STAT1, STAT3, and STAT5 across CTCL lines. C, subcellular localization of STAT1 by immunofluorescence microscopy. CTCL lines with high (HH cells) and low (MJ cells) vorinostat sensitivity were fixed, permeabilized, and stained with antibodies against STAT1, STAT3, and STAT5. Representative results for the STAT1 analysis. Nuclei were counterstained with Hoechst dye.
pattern for several members of the JAK/STAT pathway that correlated with vorinostat sensitivity (Fig. 2A). In particular, STAT1, STAT3, and STAT5 expression were significantly elevated among the cell lines with the highest vorinostat IC₅₀ values. Similar results were obtained by reverse-phase protein microarray analysis performed on whole-cell extracts prepared from a subset of the cell lines profiled (Fig. 2B). Principally, reverse-phase protein microarrays are used to assess phosphorylation changes en masse of key signaling nodes, as well as to compare expression levels of signaling proteins in cancer cells and tissues (30, 39). Briefly, protein lysates (5 nL/spot/array) are immobilized onto nitrocellulose-coated glass slides, and printed arrays are probed with antibodies against a repertoire of signaling proteins covering the major signal transduction pathways. In this study, a total of 50 proteins covering eight major signaling pathways were assayed. STAT1 and STAT3 were represented in the antibody panel. These two transcription factors emerged as the most consistently, differentially overexpressed proteins in the resistant versus sensitive cell lines examined. For a comparison, the levels of caspase-3 expression in these cell lines are shown.

Based on these observations, the expression, as well as phosphorylation, of STAT1, STAT3, and STAT5 across lymphoma cell lines were examined by Western blot analysis (Fig. 3A). Differences in STAT3 expression levels were subtle; however, the levels of STAT1 and STAT5 proteins were in general higher in the group less responsive to vorinostat. In addition, tyrosine and serine phosphorylation levels of STAT1, STAT3, and STAT5 were generally elevated in this cell line subset. Given the clinical efficacy that vorinostat has shown in patients with MF/SS CTCL, STAT activation was specifically compared among CTCL lines (Fig. 3B). This focused panel showed a similar correlation between STAT activation and low vorinostat sensitivity that recapitulated the results seen across the broad B-cell and T-cell lymphoma panel. Consistent with previous reports, tyrosine-phosphorylated STAT3 was detectable in the CTCL lines evaluated; however, higher levels were observed in the more resistant cell lines (40, 41). In the CTCL lines tested, expression and tyrosine phosphorylation of STAT1, STAT3, and STAT5 were not affected by vorinostat treatment (Supplementary Fig. S2). The differences observed in phosphorylation...
of STAT1, STAT3, and STAT5 prompted the examination of their localization by immunofluorescence microscopy. Because phosphorylated STAT proteins form dimers that translocate into the nucleus to initiate transcription, differences in their subcellular localization were investigated. Upon staining of vorinostat-sensitive HH cells with antibodies directed against STAT1, a weak signal scattered throughout the cell was observed (Fig. 3C). In contrast, in vorinostat-resistant MJ cells, the intense STAT1 signal was mainly concentrated in the nuclear compartment. Similar results were obtained with antibodies against serine-phosphorylated and tyrosine-phosphorylated STAT3, as well as STAT5 (Supplementary Fig. S3).

Elevated STAT activity is functionally linked to vorinostat resistance in CTCL cell lines HuT 78 and MJ. The role of STAT1, STAT3, and STAT5 deregulation in the response to vorinostat was further investigated. The effect of vorinostat on cell proliferation was evaluated in combination with an inhibitor of the JAK family of tyrosine kinases, upstream activators of STAT proteins. The JAKi (Calbiochem) is a potent inhibitor of JAK1, JAK2, JAK3, and Tyk2. Coincubation of HuT 78 cells with vorinostat and JAKi resulted in a mild synergistic antiproliferative effect (combination index, 0.28; Fig. 4A). Furthermore, synergistic induction of apoptosis was confirmed by TUNEL assay upon treatment with the vorinostat-JAKi combination for 48 hours (Fig. 4A). A decrease in tyrosine phosphorylation of STAT3 was detectable by immunoblot analysis after treatment with JAKi (Fig. 4A), indicating that the JAK inhibitor effectively blocked activation of STAT proteins in the CTCL HuT 78 cells. Similar results were obtained in MJ cells, wherein dephosphorylation of STAT5, the most predominantly STAT member, expressed in this CTCL line was noted (Supplementary Fig. S4).

Figure 5. Immunohistochemical analysis of STAT1 and pSTAT3 in skin biopsies from patients enrolled in the vorinostat phase IIb clinical trial. Samples were fixed with formalin, mounted onto glass slides, and probed with antibodies against STAT1 and pSTAT3 and counterstained with hematoxylin. A, representative images from the STAT1 analysis in nonresponder (NR) and partial responder (PR) patient samples. Sample scoring was done as described under Materials and Methods. Malignant T cells are shown at high magnification (100×) and marked with an arrow on the low magnification image (10×). B, representative image from pSTAT3 analysis. Arrows point to malignant T-cell lymphocytes in Pautrier microabscesses. C, summary of results from the immunohistochemical analysis of STAT1, STAT5, and pSTAT3 across the entire sample set from the phase IIb CTCL clinical trial.
lymphoma lines analyzed contributes to signaling redundancy and thus explains the effects observed upon STAT1 and STAT5a knockdown. In addition to STAT loss-of-function experiments, overexpression of constitutively active STAT3 in vorinostat-sensitive HH and SUPT1 cells resulted in an increase in vorinostat IC50 (Supplementary Fig. S5B). Overall, results from genetic and pharmacologic studies aimed at reducing activation of STAT proteins suggest that elevated STAT activation contributes to vorinostat resistance.

In addition to phosphorylation, HDACs are required for transactivation of STAT proteins (42–44). Contrasting results from studies that examined the effect of HDAC inhibitors on STAT3 phosphorylation have been reported (44, 45). In addition, work from several groups has shown that trichostatin A interferes with the transcriptional activity of STATs by (a) blocking HDAC activity and leading to their hyperacetylation, (b) inducing deacetylation of components of the STAT-associated transcription machinery like CAAT/enhancer binding protein β, and (c) interfering with the HDAC1-mediated recruitment of factors, such as RNA polymerase II, to their target promoters (43, 46, 47). Under the experimental conditions selected for this study, vorinostat treatment did not affect phosphorylation of STAT3 (Fig. 4A and Supplementary Fig. S2) or STAT5 (Supplementary Figs. S2 and S4). To determine whether vorinostat treatment resulted in an increase in STAT1, STAT3, or STAT5 acetylation, proteins were immunoprecipitated and subjected to immunoblot analysis with antibodies against acetyl-lysine. In the case of STAT3, the modest basal acetylation signal was unaffected by vorinostat treatment (Supplementary Fig. S6). No acetylation of STAT1 and STAT5 was detected pre-vorinostat or post-vorinostat treatment. It is well established that antiapoptotic genes, including Bcl-2, Bcl-xl, Mcl-1, and survivin, are transcriptionally controlled by STAT5 and/or STAT3 (48–50). Thus, modulation of the expression of these target genes, presumably involved in vorinostat resistance, was assessed. Changes in mRNA expression of relevant genes were quantified by quantitative PCR (Fig. 4C). Treatment of HuT 78 cells with the JAKi at the concentrations chosen for the combination study resulted in modest repression of the four antiapoptotic genes evaluated, similar to that achieved by vorinostat alone. Conversely, treatment of HuT 78 cells with the combination for 24 hours led to significant down-regulation (>2.4-fold) in their expression. A number of STAT5 target genes, including c-Myc and Osm, were also affected by this treatment. Of note, the expression of Bax, Bad, GAPDH, a housekeeping gene, and JunB, a cytokine-inducible gene, remained largely unchanged. Altogether, these findings raise the possibility that the synergistic antiproliferative activity of this combination results from the concerted effects of HDAC inhibition and JAK inhibition on the STAT-mediated transcriptional control of prosurvival and growth-promoting genes.

High expression and aberrant activation in skin biopsies from nonresponders of the phase IIb CTCL trial. The results gathered from the preclinical studies in human B-cell and T-cell lymphoma lines prompted the evaluation of STAT1, STAT3, STAT5, and pSTAT3 staining in skin biopsies obtained from MF/SS patients enrolled in the vorinostat phase IIb clinical trial. The description of the clinical trial design, end points, and patient population enrolled has been previously described (7). A total of 51 skin biopsies, one per patient, were collected before vorinostat treatment. Of the 51 samples stained, three low quality specimens were left out from the analysis. Immunohistochemical evaluation of the four markers was performed on 48 skin biopsies. Tumor cells were identified by a combination of criteria, including location within the epidermis (Pautrier microabscesses), nuclear size, and nuclear convolutions often described as cerebriform nuclei (Supplementary Fig. S7). The percentages of malignant T-cells stained, the predominant intensity of staining, and the subcellular localization of the signal were recorded. The results for each MF and SS patient analyzed are provided (Supplementary Table S1).

The analysis showed that STAT1 expression was detectable in malignant T cells from 43.7% of the samples (21 positively stained; 22 negatively stained; 5 poor quality specimens or no malignant cells identified). Representative examples of STAT1 staining in partial responders and nonresponders are shown (Fig. 5A). In cases where STAT1-positive cells were observed, a relationship between nuclear accumulation of STAT1 and lack of response to treatment existed (P < 0.01; Fisher’s exact test). The intensity of STAT3 staining was high (+2) in the majority of the biopsies evaluated. No correlation between vorinostat response and STAT3 expression or localization could be determined from the samples analyzed. Representative images from skin biopsy sections stained for pSTAT3 are presented (Fig. 5B). Nuclear pSTAT3 could be detected in all 48 samples evaluated at either a low level (+1 staining) or a high level (+2 staining). Statistical analysis of the immunohistochemical data indicates that patients with malignant T cells showing low nuclear pSTAT3 staining (+1) have a significantly greater chance of responding to vorinostat treatment (P < 0.001) than the patients whose samples showed high intensity (+2) nuclear staining.

In the case of STAT5, expression was detectable in malignant T cells from 41 of the 48 samples analyzed. In most cases, staining was confined to the nuclear compartment. Although STAT5 staining intensity was not significantly different between samples from nonresponder and partial responder patients (P = 0.08 > 0.05), a trend was observed between high levels of expression and lack of clinical response. A summary of the data gathered from the immunohistochemical analysis of STAT1, STAT3, and pSTAT3 is provided (Fig. 5C). When compared, the accuracy of pSTAT3 and STAT1 is similar (87.5% and 85.71%, respectively); however, the predictive power of pSTAT3 is superior. Phosphorylated STAT3 was detectable in all biopsies analyzed and thus constitutes a more robust marker across this sample set. This marker performed similarly across both MF (P < 0.023; 90% prediction accuracy) and SS (P < 0.015, 83% prediction accuracy) patient samples evaluated (Supplementary Table s1). From this analysis, 6 of 48 patients (12.5%) were misclassified as to clinical response by pSTAT3 staining. The same six patients were also either misidentified by STAT1 or did not show STAT1 staining. Therefore, combining STAT1 and pSTAT3 scores did not improve the prognostic accuracy for clinical response. Lastly, two of the four nonresponder patients misidentified by pSTAT3 and STAT1 staining were among those with stable disease (patients 6 and 19; Supplementary Table S1). These are patients with low pSTAT3 staining that did not meet the criteria of clinical response; however, they experienced some clinical benefit from vorinostat treatment. This observation may explain two of the four outlier patients and suggests that low pSTAT3 levels may identify not only responder patients but also those patients that experienced stable disease within the nonresponder population.
Discussion

The present study showed that lymphoma cell lines, including CTCL, that respond poorly to vorinostat treatment in vitro possess higher baseline levels of activated STAT1, STAT3, and STAT5 relative to the more sensitive cell lines examined. These results seem to be of clinical relevance, as they are consistent with the differential activation of STAT1 and STAT3 detected in pretreatment skin biopsies from nonresponder MF/SS CTCL patients from the phase IIb trial.

It is clear that the STAT family of transcription factors serves as an integrator of signals coming from a number of external stimuli, and their activation is a highly regulated process. Genetic or epigenetic alterations that impinge at various levels of the signaling cascade upstream of the STATs result in constitutive phosphorylation of these proteins and have been associated with increased proliferation, resistance to apoptosis, and malignancy. It is not surprising then that in some cancers the same STAT-mediated transcriptional program that sustains expression of prosurvival factors may affect the response of cancer cells to drug treatment.

Elevated expression or phosphorylation of STAT1, STAT3, and/or STAT5 has been negatively correlated with response to chemotherapy in ovarian cancer, non–small cell lung cancer cell, multiple myeloma, and NHL cells in vitro (25, 26, 29). Furthermore, high levels of pSTAT3 correlated with invasive breast cancer and resistance to combined doxorubicin/docetaxel therapy (27). In some cases, high levels of various STAT-regulated genes with antiapoptotic function, such as Mcl-1, survivin, and Bcl-xL, seem to be an underlying mechanism associated with aberrant STAT activation and poor response to chemotherapeutic agents. The present investigation failed to establish a clear relationship between activation of STATs and the mRNA expression of some of these genes. In addition to transcriptional control, posttranslational modifications that are not captured by gene expression analysis regulate the activity of proteins involved in the apoptotic cascade. It is possible that in some cases the balance between the activities of various antiapoptotic and proapoptotic factors ultimately dictates response to treatment.

It is important to emphasize that high levels of constitutively activated STAT3 in skin biopsies and cell lines derived from CTCL patients has been widely described (reviewed in refs. 1, 19). Consistent with those reports, constitutive activation of STAT3 was evident across most of the skin biopsies examined. However, our results indicate that there is a significant difference in the level of activated STAT3 across the samples analyzed. In CTCL patients, STAT3 activation has been in part attributed to production of Th2 cytokines by malignant T cells. In some cases, high levels of interleukin 2 receptor-α chain (CD25) have also been implicated in persistent STAT3 phosphorylation (22). Cytokine-independent activation of STAT3 has also been reported in CTCL cell lines. Future studies will help to address whether alterations in the positive, activating signals, including ligands (cytokines) and upstream kinases (JAKs), or defects in the activity or expression of negative regulators of the kinases that activate STATs, like SHP-1 (51), correlate with the degree of STAT phosphorylation and increased resistance to vorinostat in CTCL patients.

To gain understanding of the role of STATs as mediators of resistance to vorinostat at the molecular level, we evaluated the effect of blocking their activity in CTCL lines with poor vorinostat response. HuT 78 and MJ cells were sensitized to vorinostat by coinubcation with a JAK inhibitor. In addition, knockdown of individual STAT proteins improved sensitivity to vorinostat in HuT 78 cells. The in vitro analysis suggests that constitutively activated STATs are not only biomarkers of vorinostat resistance, but also are functionally linked to poor response to this HDAC inhibitor. These results also indicate that the synergistic inhibition of growth induced by the vorinostat/JAKi combination correlates with the decrease in expression of several antiapoptotic proteins that are STAT3 and STAT5 transcriptional targets. In the two CTCL lines tested, vorinostat treatment did not alter acetylation or phosphorylation of STAT1, STAT3, or STAT5. In addition, in HuT 78 cells, the DNA binding activity of STAT3 was not affected by vorinostat (Supplementary Fig. S8). As previously mentioned, HDACs associated with STAT1, STAT3, and STAT5 participate in chromatin remodeling, modulate the activity of coregulators, and contribute to recruit additional factors that enable transcription of a selective set of genes. Together these events may affect local chromatin structure and proper STAT transactivation. Therefore, these data are consistent with a cooperative model, whereby the JAK inhibitor mediates a decrease in STAT phosphorylation and vorinostat leads to inhibition of their HDAC-associated activity, resulting in a synergistic decrease in STAT-mediated gene transcription. One implication from the in vitro studies presented is that inhibitors of JAKs once clinically available may work well in combination with vorinostat and help overcome resistance to apoptotic death in those patients with constitutive STAT signaling.

Finally, signals triggered by an array of tyrosine kinases, including receptor tyrosine kinases, Src and JAKs, activate STAT proteins in various types of malignant cells. The mechanism of STAT-mediated drug resistance is at least in part related to the ability of these transcription factors to control the expression of general prosurvival, antiapoptotic genes. Our results add to the mounting evidence that constitutive activation of STATs may be at the core of a general mechanism of resistance to cell death. Additional studies will help to determine whether activated STATs represent tumor intrinsic determinants of clinical resistance for a broad spectrum of drugs and cancer types, similar to overexpressed ATP-binding cassette transporters or Bcl-2. Our in vitro analysis in B-cell and T-cell lymphoma lines suggests that the relationship between constitutive STAT activation and clinical response to vorinostat may not be limited to CTCL. In addition, the similarity between the sensitivity of CTCL lines to vorinostat and valproate suggests that activation of STATs are not only biomarkers of vorinostat resistance, but also are functionally linked to poor response to other HDAC inhibitors. Further clinical experience with vorinostat will provide a more detailed understanding of the role of STAT activation and resistance to vorinostat.

Disclosure of Potential Conflicts of Interest

All authors except M.E. Kadin are employees of Merck & Co.

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References

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