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Romidepsin in Peripheral and Cutaneous T-Cell Lymphoma: Mechanistic Implications from Clinical and Correlative Data

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Conflict of Interest

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Summary

Romidepsin is an epigenetic agent approved for the treatment of patients with cutaneous or peripheral T-cell lymphoma (CTCL and PTCL). Here we report data in all patients treated on the National Cancer Institute 1312 trial, demonstrating long-term disease control and the ability to retreat patients relapsing off-therapy. In all, 84 patients with CTCL and 47 with PTCL were enrolled. Responses occurred early, were clinically meaningful and of very long duration in some cases. Notably, patients with PTCL receiving romidepsin as third-line therapy or later had a comparable response rate (32%) of similar duration as the total population (38%). Eight patients had treatment breaks of 3.5 months to 10 years; in four of six patients, re-initiation of treatment led to disease regression. Safety data show slightly greater haematological and constitutional toxicity in PTCL. cDNA microarray studies show unique individual gene expression profiles, minimal overlap between patients, and both induction and repression of gene expression that reversed within 24 h. These data argue against cell death occurring as a result of an epigenetics-mediated gene induction programme. Together this work supports the safety and activity of romidepsin in T-cell lymphoma, but suggests a complex mechanism of action.

Keywords

Epigenetic therapy; HDAC inhibitor; Romidepsin; T-cell lymphoma; Chromatin

Introduction

The epigenetic agents romidepsin (depsipeptide, FK228, FR 901228) and vorinostat are histone deacetylase (HDAC) inhibitors approved for use in T-cell lymphoma (Coiffier, *et al* 2012, Duvic, *et al* 2007, Olsen, *et al* 2007, Piekarz, *et al* 2009, Piekarz, *et al* 2011, Whittaker, *et al* 2010). HDAC inhibitors increase global histone acetylation in cancer cells and promote T-cell apoptosis (Shao, *et al* 2010, Zhang, *et al* 2005). While studies have begun to identify the molecular basis of the various subtypes of mature T-cell lymphomas (Campbell, *et al* 2010, Ferrara, *et al* 2013, Lemonnier, *et al* 2012, McKenzie, *et al* 2012, Palomero, *et al* 2014), responses to romidepsin and vorinostat have been reported across the entire disease spectrum, without regard to subtype. The precise mechanism underlying activity against T-cell lymphoma has yet to be determined, and multiple biological effects have been invoked. Leading candidate mechanisms include the induction of genes that promote apoptosis, DNA damage due to acetylation-induced double-strand breaks and reactive oxygen species release, and aberrant kinetochore assembly (Bolden, *et al* 2013, Conti, *et al* 2010, Robbins, *et al* 2005, Robert and Rassool 2012). Vorinostat and romidepsin are from different chemical classes, have different histone deacetylase affinities and are administered in different schedules - lower potency vorinostat administered on a continuous schedule and higher potency romidepsin given intermittently - yielding some differences in biological and clinical activity. Responses observed in a National Cancer Institute (NCI) phase 1 trial conducted with romidepsin provided the impetus to assess HDAC inhibitor efficacy in patients with cutaneous T-cell lymphoma (CTCL) and peripheral T-cell lymphoma (PTCL) (Piekarz, *et al* 2001); data from subsequent preclinical models supported

those observations of activity (Chakraborty, *et al* 2013, Piekarz, *et al* 2004, Tiffon, *et al* 2011).

Regulatory approval for romidepsin from the United States Food and Drug Administration (FDA) was first received for CTCL based on two clinical trials – GPI-04-0001 and NCI1312. Both trials were multi-institutional, international, non-randomized Phase II trials, with the GPI-04-0001 study sponsored by Gloucester Pharmaceuticals Inc. (GPI). Response rates were 34 and 35%, respectively, with median response durations of 15 and 13.7 months, respectively (Piekarz, *et al* 2009, Whittaker, *et al* 2010). Of potentially greater clinical significance, activity was also demonstrated in patients with PTCL, an aggressive form of lymphoma with lower response rates and higher relapse rates that traditionally has been treated with therapies developed for B-cell lymphomas. Data submitted in support of regulatory approval for PTCL were based on two trials – GPI-06-0002 and NCI1312. Response rates were 29% and 38%, respectively, with median durations of 17 and 9 months (Coiffier, *et al* 2012, Piekarz, *et al* 2011). A recent analysis of the GPI study updated the median response duration to 28 months (Coiffier, *et al* 2014). Accelerated approval was accorded in PTCL and the required follow-up trials are in progress.

Herein, we report results from the combined NCI1312 data set, focusing on analyses that were not presented in earlier publications, including long-term outcome and correlative data. The clinical data are considered together with the correlative studies to generate new insights regarding the mechanism of HDAC inhibitor action.

Patients and Methods

Study Design and Treatment Plan

NCI1312 (NCT00007345 at <http://clinicaltrials.gov/>), a prospective, non-randomized Phase II trial evaluating the safety and efficacy of romidepsin was conducted at 12 sites in the United States and Australia. The protocol underwent Institutional Review Board review at all participating sites. The trial was conducted essentially as an adaptive trial, with cohorts being added by amendment after efficacy was demonstrated, to confirm response in a broader patient population (Supplementary Figure 1). Patients with relapsed, refractory or advanced CTCL or PTCL meeting standard eligibility criteria were enrolled in one of 7 cohorts, based on subtype and extent of prior therapy. The study was initially designed as a two-cohort study of CTCL and PTCL in patients previously treated with 2 or fewer prior cytotoxic chemotherapy regimens. As the clear efficacy signal emerged, cohorts for patients with more than two prior therapies, patients with more atypical forms of T-cell lymphoma and patients previously treated with vorinostat were added. Following discussions with the FDA the decision was made to open the study at additional sites and to reproduce the efficacy data in CTCL with the addition of a replicate cohort. Subsequently, data from the various cohorts were combined for regulatory submission. Results for CTCL and PTCL were reported separately (Piekarz, *et al* 2009, Piekarz, *et al* 2011).

Administered initially on a day-1 and -5 schedule, this was amended after the first 5 patients to 14 mg/m² over 4 h on days 1, 8 and 15 of a 28-day cycle (Marshall, *et al* 2002, Sandor, *et al* 2002). Doses were reduced, per protocol guidelines, from 14 mg/m² to 10.5 mg/m² and to

8 mg/m² for patients presenting with an absolute neutrophil count less than 0.5 x 10⁹ cells/l, or platelet count between 50 and 75 x 10⁹ cells/l. Doses were held for counts less than these or for grade 3 or higher non-haematological toxicity.

Safety Assessments and Supportive Care

Toxicities were scored according to the NCI Common Toxicity Criteria version 2.0 http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcmanual_v4_10-4-99.pdf. All adverse events, regardless of clinical significance or attribution, were captured, including laboratory abnormalities. Cardiac safety evaluations led to the monitoring of potassium and magnesium during the study, with a standardized replacement schema to achieve serum magnesium and potassium levels over 0.85 mmol/l and 4.0 mmol/l, respectively, prior to romidepsin administration (Grant, *et al* 2010, Noonan, *et al* 2013, Piekarz, *et al* 2006). The protocol excluded medications known to interfere with CYP3A4 metabolism, because romidepsin is metabolized in part by CYP3A4 (Shiraga, *et al* 2005), and those known to prolong the QTc interval. Prophylactic antiemetics were administered, with granisetron the antiemetic of choice. Central lines were avoided in CTCL patients with widespread skin involvement; if required, whirlpool baths with Dakin's solution were used to reduce skin colonization prior to line placement (Frye, *et al* 2012), prophylactic antibiotics were administered while in place, and lines were discontinued between doses.

Efficacy Assessment and Response Criteria

For CTCL, a rigorous composite assessment was employed with uni-dimensional measurements of skin and visceral disease sites obtained and assessed by Response Evaluation Criteria in Solid Tumours (RECIST) (Piekarz *et al* 2009; Therasse *et al* 2000). Lymph node disease was assessed bi-dimensionally using International Working Group (IWG) criteria (Cheson, *et al* 1999). Bone marrow involvement, as recommended by IWG criteria, was scored present or absent. Generalized erythroderma was scored present or absent. Presence of circulating malignant T-cells ascertained by flow cytometry was scored present or absent. Complete response (CR) required clearing of all known disease sites. Partial response (PR) required documented response in skin (30% per RECIST) or lymph nodes (50% per IWG), with response in both compartments for a determination of PR. IWG guidelines (Cheson, *et al* 1999) were used to assess patients with PTCL.

Statistical Methods

Efficacy Data—Duration of response was determined from the date response was noted until the disease was no longer considered to be responding. Kaplan-Meier analyses provided the median duration of response.

Adverse Event Analysis—For each of the 34 toxicities observed in at least 10% of patients, the worst grade for each patient was noted (grade 0 events were calculated by subtraction for each toxicity and subtype). In cases with only two toxicity grades for comparison (five cases), Fisher's exact test was used. For comparing the distribution of toxicity grades in the majority of cases, which were constructed as 2 x C ordered tables, an exact Cochran-Armitage trend test was used to compare the distributions. In view of the

large number of tests performed, we only refer to those with p-values <0.01 as statistically significant, with those for which $0.01 < p < 0.05$ considered trends.

cdNA Array Analysis—Peripheral blood mononuclear cells from patients with circulating tumour cells were obtained before infusion (pre), and at 4 and 24 h after start of the infusion of the first cycle of treatment. Samples were hybridized on Illumina WG-8v2 human whole-genome bead arrays using a constant amount (400 ng) of total RNA at the Wistar Institute Genomics facility. Illumina BeadStudio v.3.0 software was used to export expression levels and detection P values for each probe of each sample. Array data were quantile normalized, log2 transformed and filtered to remove non-informative probes. Principal component analysis (PCA), heat maps and unsupervised hierarchical clustering were performed in Qlucore Omics Explorer v.3.0 (Qlucore AB, Lund, Sweden). Data were previously submitted to the Gene Expression Omnibus [GEO] with accession number: GSE45405 (Chakraborty, *et al* 2013).

Results

Patients and Romidepsin Administration

As shown in Table I, 131 patients were enrolled, 84 with CTCL and 47 with PTCL, with a range of mature, post-thymic lymphoma subtypes, as previously reported (Piekarz, *et al* 2011). Most patients had an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1. More men than women (57 vs. 27) with CTCL were enrolled, with a more equal distribution in those with PTCL. Median age (59 years) and age range in the two groups were comparable.

Among the 131 patients, 3404 doses of romidepsin were administered in 1214 cycles (Table II). The median number of cycles administered was 4.5 (1 – 79) for CTCL and 3 (1 – 83) for PTCL. In all, 75% of doses administered were at the protocol-prescribed dose or higher: at 14 mg/m² (67%, 2279 doses) and at 17.5 mg/m² (8%, 286 doses). The remaining 24.6% of doses were reduced (839 doses) and only 1.4% or 47 doses were held. Doses were more likely to be reduced in the PTCL population (38%) than the CTCL population (16%). We assessed the number of “reductions from the immediate prior dose” to avoid confusion with doses that were consistently reduced to maintain tolerability. At 6% (3% for CTCL and 11% for PTCL), this observation indicated that once a dose of 10.5 mg/m² was instituted, patients usually tolerated romidepsin without further need for dose reduction.

Efficacy Data for CTCL and PTCL

Updated response data for patients with CTCL are reported in Table III, including 84 patients enrolled with CTCL. The overall response rate (ORR) of 33% is comparable to that previously reported for both the NCI and the GPI trials (Piekarz, *et al* 2009, Whittaker, *et al* 2010). Patients with response had noticeable improvement after a few doses; the median time to first response was 56.5 days, or the first planned restaging cycle. Response durations are displayed graphically in Figure 1. The median duration of response (DOR) in patients with CR or PR was 13.8 months, while in patients with CR the median DOR was 19.3 months. One patient with Sézary Syndrome remains in continuous CR 10+ years after first

remission. Another patient with CTCL was declared in CR after cycle 2; therapy was discontinued after 15 cycles and the patient was followed off-therapy for 33 months before discontinuing study still in remission.

Updated response durations in patients with PTCL are also shown in Table III and Figure 1, with the subset of patients with PTCL who had received 2 prior systemic chemotherapy regimens highlighted. The ORR in the total PTCL population was 38%, with 18% achieving a CR. Patients who received romidepsin in the third line or higher setting had an ORR of 32%, with 16% achieving a CR, similar to the total population. Median durations of response were 9 and 8.4 months for all PTCL patients and for those who had received 2 prior therapies, respectively. The median DOR for patients with CR in the total PTCL population was 74.1 months (Kaplan Meier plots included in Supplementary Figure 2). Additionally, ORR among the 19 patients who had received three or more prior regimens was 38%. Responses were also observed after bone marrow transplantation. In total, 18 patients with PTCL had undergone prior bone marrow or stem cell transplant, either autologous or allogeneic; seven responses (38%) were observed in this patient subpopulation, including a CR in three patients and a PR in four patients.

Retreatment

Once the durability of responses following romidepsin treatment was recognized, 8 patients (5 CTCL, 3 PTCL) with excellent disease response had therapy with romidepsin suspended (Figure 2 and Supplementary Table 1). The patients remained on-study, but off treatment. Two patients never required retreatment and remain in CR (>10 years and >4 years). Six patients were re-treated due to disease flare occurring whilst off-therapy, after 43, 20.5 and 7.4 months in three patients with CTCL and 56.1, 3.8 and 3.5 months in three patients with PTCL. In three patients, two with CTCL and one with PTCL, re-treatment offered substantial benefit. Photographs shown in Figure 3 were obtained from one patient with stage IV disease and circulating Sézary cells at enrollment who has received 3 separate courses of re-treatment, and is currently in remission 12 years after first enrollment. Another patient with partial remission received 72 cycles of continuous therapy before a break in therapy lasting 20 months before retreatment was required. Note that second and subsequent responses were not scored or included in calculations of response duration.

Adverse Events

Romidepsin is well tolerated with few grade 3 and 4 toxicities. As shown in Supplementary Tables 2A and 2B, the toxicity profile did show some variation between the CTCL and PTCL populations. Nausea was the most common side effect and was statistically more common in CTCL, occurring at least at one point in time in 81% (68/84) of patients with CTCL and 68% (32/47) of patients with PTCL despite prophylactic administration of antiemetics. Other gastrointestinal (GI) disturbances, including dysgeusia, vomiting and anorexia, appeared to be comparable between the two groups. Although GI disturbances were a common toxicity, fewer than 10% of patients experienced any episode of grade 3. Fatigue was comparably distributed between the two disease entities, occurring in 69% (58 of 84) and 64% (30 of 47) of the CTCL and PTCL populations, respectively. Electrocardiogram (ECG) changes, including T wave flattening (grade 1) and ST segment

depression (grade 2), were commonly observed, although the clinical significance of these has not been determined (Cabell, *et al* 2009, Noonan, *et al* 2013).

We also examined the distribution of toxicities by severity grade. In this analysis, clearer differences emerged between the CTCL and the PTCL populations. A higher grade of thrombocytopenia was statistically more common in patients with PTCL, $p = 0.0091$; with a trend to greater severity in neutropenia also observed ($P=0.030$). These observations probably reflect the greater numbers of patients with prior cytotoxic therapies, including stem cell transplants. Fever was also more common in patients with PTCL, having been reported in 32% of patients with PTCL vs. 8.3% of patients with CTCL, and the severity of distribution was also greater for PTCL ($p = 0.0015$). We also noted elevated bilirubin, aspartate transaminase (AST) and alanine transaminase (ALT) more commonly in PTCL than in CTCL ($p = 0.058, 0.020$, and 0.0039 , respectively) and attributed these to a cytokine release syndrome previously reported in PTCL (Teachey *et al* 2013; Staffolani *et al* 2012; Steinhoff *et al* 2008). This could also explain the trend toward greater severity of nausea in PTCL ($p=0.045$). One example of recurrent liver function test abnormalities in a patient with PTCL is shown in Figure 4. Viral re-activation was observed in three patients (Ritchie, *et al* 2009) (2 with PTCL, one with CTCL) while on therapy; no additional cases were identified despite continued vigilance.

Off Study Reasons

Relatively few patients were removed from study for adverse events, shown in Supplementary Table 3. The most common reason for patients to be removed from study was disease progression in 63%. Adverse events accounted for 13 patients (10%) being removed from study and included fatigue, infection and viral reactivation. Seventeen patients (13%) discontinued study, six of them in order to obtain the drug in the community. There were 5 deaths (4%) on study; two with infection, one due to progression of disease and two sudden deaths within 24 h of romidepsin administration. Both of these patients had risk factors for sudden cardiac death – one with CTCL with pre-existing hypertrophic cardiac disease and significant valvular pathology, and one with PTCL with extensive atherosclerotic disease including prior myocardial infarction and vascular disease (Piekarz, *et al* 2009, Piekarz, *et al* 2011). One patient was removed from study due to worsening of pre-existing mitral valve disease that was not considered drug-related.

Correlative Studies

Correlative studies included measurement of histone acetylation and induction of the *ABCB1* (*MDR1*) gene as biomarkers of the epigenetic effect of romidepsin (Bates, *et al* 2010). There was a consistent increase in histone acetylation at 4 h, median 3-fold, which was resolving by 24 h. There was a modest correlation between serum romidepsin concentrations, persistence of histone acetylation at 24 h and response (Bates, *et al* 2010). RNA from paired samples from seven patients with significant CTCL blood involvement was analysed by cDNA microarrays; samples were obtained pre-infusion, at 4 h (at romidepsin infusion end) and 24 h (the day following romidepsin infusion) and subjected to analysis. A principal component analysis was performed on the 996 most variable genes. This analysis allowed us to see the underlying structure of the data and the major sources of variance. The gene

expression patterns were grouped by patient rather than by pre- or post-treatment sample (Figure 5A). This is further illustrated in Figure 5B, where unsupervised clustering of the 996 most variable genes results in grouping of gene expression by patient rather than by relationship to treatment. However, when variation between patients was eliminated as a nominal factor by paired analysis ($p < 0.01$, > 2 -fold change), 290 differentially expressed genes were identified: 175 at 4 h and 115 at 24 h. There was little in common (10 genes) between the data sets from the two time points, whether basal expression or gene induction was examined. Four patterns of gene expression change could be observed – “early” gene changes, with up- or down-regulation at 4 h, and “late” gene changes with up- or down-regulation at 24 h (Figure 5C). Notably, most genes meeting the significance criteria at 4 h – whether induction or repression – demonstrated reversion by 24 h; and almost all genes meeting criteria at 24 h showed little change at the earlier time point. When Ingenuity Pathway Analysis (IPA) was used to assess gene expression changes, few pathways were observed in common (Figure 5D and Supplementary Table 4). However, a similar rapid reversion was observed in the identified pathways. One of the top networks identified was nuclear factor κ B (NF κ B), which has previously been shown to be down-regulated by HDAC inhibitors (Fabre, *et al* 2008, Takada, *et al* 2006). Effects on NF κ B target genes are shown in the heat map in Figure 5E, with frequent reversal of the effect by 24 h.

Discussion

Initially identified by Fujisawa Pharmaceuticals in a screen for RAS-selective compounds, romidepsin was submitted to the NCI drug screen and selected for further development based on its unique profile in the NCI-60 cell line panel (Sandor, *et al* 2000). Although xenograft studies supported Phase I testing in solid tumours, limited activity was observed (Marshall, *et al* 2002, Sandor, *et al* 2002). However, we saw striking activity in a patient with PTCL, and then extended the trial to a cohort of 10 patients with CTCL or PTCL, where activity was again observed (Piekarz, *et al* 2001). This observation led to a Phase II trial in T-cell lymphoma conducted by the NCI’s intramural programme, as well as to the successful evaluation of other HDAC inhibitors in the treatment of T-cell lymphoma (Duvic, *et al* 2007, O’Connor, *et al* 2013, Olsen, *et al* 2007). GPI licensed romidepsin (subsequently licensed by Celgene Corporation) to complete its development, conducting separate pivotal trials for CTCL and PTCL. Data from both the NCI study and the GPI study supported regulatory submissions. The current report provides data on long-term responses in patients, the ability to retreat patients following recurrence off treatment, efficacy in the subset of patients with PTCL treated in third-line or later with romidepsin, and safety comparisons in the two populations.

Adverse event data for the entire study show that romidepsin toxicities are generally comparable in the two disease subtypes. The more frequent toxicities were fatigue, nausea and thrombocytopenia. Of note, there was a greater incidence of fever and liver enzyme and bilirubin elevation in PTCL than in CTCL, which may be related to a cytokine release syndrome that has been described in patients following T-cell activating therapies (Teachey, *et al* 2013), with chemotherapy in a patient with angioimmunoblastic T-cell lymphoma (Staffolani, *et al* 2012) and with vorinostat in a patient with CTCL (Steinhoff, *et al* 2008).

Cardiac monitoring studies were previously reported, including a consistent small increase in heart rate, but a lack of myocardial damage or significant QT prolongation (Cabell, *et al* 2009, Noonan, *et al* 2013, Piekarz, *et al* 2006). Electrolyte monitoring was added after the observation of six sudden deaths across several romidepsin trials, including two patients on the current trial. A clear relationship of romidepsin with these sudden deaths was not established because each patient had a pre-existing risk factor for sudden death. However, cardiac exclusion criteria were introduced, concomitant agents that would affect the QT interval were excluded, and potassium and magnesium monitoring was initiated. We recently reported that blood electrolyte levels met criteria for replacement in 55% of 1365 doses reviewed and hypothesized that a gradient of romidepsin across the myocardial wall could cause altered expression or function of ATP-sensitive potassium channels, thereby altering repolarization time at different levels (Noonan, *et al* 2013). Sufficient potassium and magnesium levels would work to stabilize the effects of such a gradient and potentially prevent or reduce the magnitude of the ECG changes.

Although a biomarker that allowed enrichment of a subset of patients with a high probability of response could generate an increased response rate, such a marker has not yet been identified or validated. One potential biomarker being investigated is the expression of BCL2L11 (previously termed BIM), a pro-apoptotic protein that prevents anti-apoptotic proteins from sequestering BAX and BAK1 (previously termed BAK), components of the apoptotic machinery. Laboratory models suggest that reduced levels of BCL2L11 expression or any other alteration that interferes with intrinsic apoptosis is associated with resistance to romidepsin (Chakraborty, *et al* 2013, Ellis, *et al* 2009, Ierano, *et al* 2013, Newbold, *et al* 2008) as well as other HDAC inhibitors (Lindemann, *et al* 2007). Until such a biomarker can be identified, it should be noted that the median time to response is 2 months, i.e., the first restaging visit, an indication that response does not take very long to declare itself and patients do not need to be exposed to long durations of treatment prior to determination of benefit.

PTCL is historically a disease with poor outcome, particularly in patients who relapse after initial therapy (O'Connor, *et al* 2014). An analysis of Canadian patients with PTCL receiving standard therapy after first relapse revealed a median overall survival (OS) of 5.5 months and median progression-free survival (PFS) of 3.1 months, with those receiving chemotherapy at relapse having a median OS and PFS of 6.5 and 3.7 months, respectively (Mak, *et al* 2013). To evaluate efficacy in patients with poor prognosis, we analysed the results in those with heavily pretreated PTCL for whom romidepsin was third line therapy and showed that response rate in the third line setting was comparable to that observed in the whole PTCL population (Table IIIB). Although survival data were not collected in this study, responding patients exhibited long durations of response, suggesting a possible impact on survival. Similar observations were made in the GPI study (Coiffier, *et al* 2014). An ongoing Phase III study combining romidepsin with CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) (ClinicalTrials.gov Identifier: NCT01796002) will attempt to increase both response rates and survival; a lower romidepsin dose intensity in the combination is required, based on Phase I testing (Dupuis, *et al* 2012). An early report of

vorinostat and CHOP in combination for PTCL suggested a high response rate, with 12 of 14 patients in CR and a median PFS of 31 months (Oki, *et al* 2013).

The marked benefit observed following romidepsin treatment in some patients with both CTCL and PTCL raises the inevitable question of the mechanism of action of romidepsin in T-cell lymphoma, and whether we can gain additional insights from the clinical and correlative data presented here. We can sum up the clinical data by observing that response to romidepsin occurs in just over a third of patients, and that *it does not appear to be confined to a specific subgroup*, at least based on histology. In circulating mononuclear cells containing malignant Sézary cells we clearly documented the induction of histone acetylation and gene expression - two cardinal manifestations of HDAC inhibition (Bates, *et al* 2010). However, these HDAC inhibitor effects were somewhat short-lived; acetylation was declining at 24 h. Examining the cDNA arrays obtained on samples from 7 patients, several notable features emerged. *First, PCA plots demonstrated that there is little overlap among the patient samples.* This was true not only in the post-treatment samples, but also at baseline. These baseline findings are consistent with previous reports showing differential gene expression clusters in both CTCL and PTCL (Piccaluga, *et al* 2013, Shin, *et al* 2007). *Second, of the 16,000 genes on the Illumina array, only 290 were significantly altered when significance and variance filters with a paired t-test were used to identify statistically significant genes ($p < 0.01$, > 2 -fold change), and only a few genes were altered in common between 4 h and 24 h.* Among the genes being significantly altered, we found both those that were induced in accordance with the classical understanding of HDAC inhibitor action, and also a large number of genes whose expression was repressed (Ellis, *et al* 2008, Schrupp, *et al* 2008). *Moreover, levels of almost all of the genes induced or repressed at 4 h in the clinical samples were returning to baseline by 24 h.* Among the 101 genes induced at 4 h, only 10 remained upregulated at 24 h. Among the 74 downregulated at 4 h, only 1 remained downregulated at 24 h. This was the most prominent feature of the dataset. *As seen in the PCA plots, there was little overlap among the gene expression profiles* – most of the variance was explained by patient-to-patient variation rather than by the particular genes induced. These results are consistent with what others have reported, in that there are relatively few “genes in common” when datasets have been examined and *no characteristic HDAC signature* has been found to date, in our work or in that of others (Duvic, *et al* 2007, Ellis, *et al* 2008, LaBonte, *et al* 2009, Ma, *et al* 2013). When the arrays were subjected to IPA analysis, although some common pathways were observed, the top networks among the patients were different. These were puzzling aspects of the correlative data but matched the observations we have made *in vitro*. Not surprisingly, given the T-cell origin of the disease, immune-related pathways were modulated. The MAPK pathway was identified, a pathway that we and others previously noted to be modulated following HDAC inhibition (Chakraborty, *et al* 2013, Sarkar, *et al* 2011, Wozniak, *et al* 2010).

In addition, alterations in the NF κ B pathway genes were identified, a pathway previously shown to be down-regulated by HDAC inhibitors (Bhalla, *et al* 2009, Chien, *et al* 2014, Fabre, *et al* 2008, Takada, *et al* 2006). Most studies showing reduction of NF κ B pathway genes with HDAC inhibitors have used *in vitro* exposures of 24 – 48 h. The reversion of the effects of romidepsin on the NF κ B pathway in the patient samples may be a consequence of

a shorter drug exposure due to drug clearance with a half-life of 2.5 – 3 h. Given that this pathway is known to promote survival (Van Waes 2007), a quick recovery could lead to restoration of pro-survival components. If proven valid, these observations support the development of combination approaches to sustain or extend the effects of romidepsin and possibly other HDAC inhibitors.

As a final approach to understanding the results in the clinical samples, we queried the L1000 dataset (<http://www.lincsccloud.org/l1000/>), a catalog of gene expression profiles that are part of the LINCS (Library of integrated network-based cellular signatures, <http://www.lincsproject.org/>) program and the Broad Connectivity MAP (CMAP) initiative. The LINCS data currently comprises +1.4 million gene expression profiles representing more than 20,000 small molecule compounds. Similar to the results obtained by Bolden *et al* (2013) in a gene expression profiling study of normal and transformed fibroblasts, the L1000 analysis identified multiple HDAC inhibitors with gene expression changes similar to those found in the 4-h treated patient samples (Supplementary Table 5). While the PCA plot primarily clustered samples according to patients (based on all data, not filtered for individual patient variation), removing the patient as a factor allowed the LINCS class-enrichment to emerge. Thus, an understanding of the dominant mechanisms of HDAC inhibitor action require reconciliation of both the unique gene expression changes at the patient level and those pathways observed as HDAC inhibitor effects.

Our laboratory studies have shown that histone acetylation is ubiquitous, occurs consistently at multiple residues after HDAC inhibition, and is observed whether or not cell death follows (Luchenko, *et al* 2014). Likewise, the phase in the cell cycle where arrest occurs following HDAC inhibition is particular to the cell type rather than the particular HDAC inhibitor or drug dose. Our gene expression studies, both examining candidate gene markers of pharmacodynamic effects, the cDNA data shown here and xenograft data not shown here, demonstrate a biphasic response in many genes following romidepsin. This rapid reversal of HDAC inhibitor gene induction/inhibition, combined with the observation that few genes are regulated in common, as observed in our study and others (Duvic, *et al* 2007, Ellis, *et al* 2008, LaBonte, *et al* 2009, Ma, *et al* 2013), argues against a mechanism of action dependent on a sustained gene expression programme, as classically understood for HDAC inhibitors. Conti *et al* (2010) reported that vorinostat-induced acetylation caused replication fork delays and double-strand DNA breaks. Gaymes *et al.* (2006) reported that in leukaemia cells, γ H2AX, a marker of DNA double-strand breaks, occurred simultaneously with histone acetylation. These were observed early, before the onset of apoptosis. DNA damage as a dominant mechanism of action could explain some of the clinical observations, in particular, the lack of a sensitive subset that might be expected if there were a specific epigenetic lesion that was primarily targeted (Lemonnier, *et al* 2012, Palomero, *et al* 2014). Our *in vitro* work and that of others has shown that cell death is mediated via and dependent upon the intrinsic apoptotic pathway (Chakraborty, *et al* 2013, Chen, *et al* 2009, Ierano, *et al* 2013, Jóna, *et al* 2011, Piekarz, *et al* 2004, Wiegman, *et al* 2011). If this observation is correct, the combination of romidepsin with other agents that promote cell death via apoptotic pathways (Paoluzzi and O'Connor 2010) should increase the response rates and response durations, provided increases in toxicity do not require reduction of cancer drug doses. We postulate

that cells primed for apoptosis may respond with cell death to the massive global acetylation provoked by romidepsin and read as a DNA damage signal.

Finally, it should be noted that the observation of durable responses in a subset of patients provides evidence of the long-term safety of romidepsin and the absence of cumulative toxicity, including patients who have received multiple prior therapies. The long-term responses and safety data also support the study of romidepsin at an earlier stage of disease. One patient with Sézary Syndrome remains free of disease without relapse after 10 years. Despite its widening use in the clinic, important questions remain regarding romidepsin's principal mechanisms of action and resistance, choice of agents for combination treatment and identification of a biomarker that would allow prior selection of patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Durations of response for patients with CTCL and PTCL. Durations in patients with CR are shown in dark grey, and patients with PR shown in lighter grey. *Indicates patients censored at data cut-off still in CR or censored at off study date still in response continuing romidepsin. Patients with PTCL who received romidepsin in third-line (with two or more prior chemotherapy regimens before enrollment) are indicated by the hatched bars. CTCL, cutaneous T-cell lymphoma; PTCL, peripheral T-cell lymphoma; CR, complete response; PR, partial response.

Figure 2.

Graphical display of treatment durations and intervals in patients who had treatment breaks. On-treatment interval is shown in dark grey, while the hatched areas show off-treatment interval. * Indicates that response was continuing at the time of data cut-off or study discontinuation. One patient with CTCL discontinued study to receive romidepsin at home after US Food and Drug Administration approval of the agent. CTCL, cutaneous T-cell lymphoma; PTCL, peripheral T-cell lymphoma.

Figure 3.

Patient with Stage IVA cutaneous T-cell lymphoma (CTCL) diagnosed 22 months prior to study entry. Prior therapies included 2 months of psoralen photochemotherapy, 3 months of denileukin diftitox, 2 months of pentostatin and 5 months of liposomal doxorubicin. She presented at age 64 years with stage IVA disease, with skin involvement, enlarged lymph nodes, and circulating malignant cells. After 2 cycles of romidepsin, this patient achieved a partial response, and a complete response was scored at the end of Cycle 9. At Cycle 20, a single small patch was noted on her sacral area that was biopsied and confirmed for CTCL cells. As per protocol, disease progression was called, but romidepsin was continued as compassionate use. Small patch disease waxed and waned over the next 12 cycles. A decision was made to stop romidepsin after a total 32 cycles, and the patient was followed without progression another 43 months. She then presented with generalized erythroderma, increased circulating Sézary cells in her blood, and enlargement of lymph nodes. She began a 6-cycle course of romidepsin that cleared blood and skin. She was again monitored for 34 months without treatment, after which increasing disease in skin and blood prompted another 6-cycle course of romidepsin, with clearing of disease. She was monitored off

therapy an additional 14 months until disease required re-initiation of another course of romidepsin, completed 12 years after her original enrollment on NCI1312. This course is summarized as Patient 1 in Supplementary Table 1. C, cycle; D, day

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Figure 4.

Bilirubin, ALT and AST elevation following romidepsin. Patient with PTCL (subtype PTCL not otherwise specified) previously treated with cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP). The patient's medical history was notable for fever, chills and abdominal pain, and baseline symptoms included lymphopenia, elevated alkaline phosphatase, elevated ALT and AST, hypoalbuminaemia, and hypocalcaemia. The patient started treatment with romidepsin 14 mg/m² and received a total of 39 romidepsin doses in 13 cycles with a last dose of romidepsin on C13D15, when disease progression was noted. During the early cycles, ALT and AST increased markedly after the first dose of each cycle, and increases in bilirubin were almost always noted the day after romidepsin infusion. PTCL, peripheral T-cell lymphoma; ALT, alanine transaminase; AST, aspartate transaminase

Figure 5.

cDNA microarray analysis performed on 0h, 4h, and 24h samples in 7 patients with circulating Sézary cells.

A. Principal component analysis (PCA) plot based on the 996 most variable genes (Filtered by variance: $\sigma/\sigma_{\max} > 0.3$) across all samples. The 20 samples are coloured according to patient number.

B. Heat-map and unsupervised hierarchical clustering based on the same 996 most variable genes as for the PCA plot. Again, we see clear clustering according to individual patients, rather than according to time. The 4h sample for Patient 7 was eliminated as an outlier.

Green shading indicates reduction in gene expression and red shading indicates an increase in gene expression.

C. Heat-map and one-way hierarchical clustering based on the 290 genes that differed between 4h and 0h, and between 24h and 0h identified by a paired analysis eliminating patient as a nominal factor ($p < 0.01$, > 2 -fold change). As seen, changes at 4h typically reverse by 24h, and changes at 24h were seldom seen at 4h. Only 10 genes were found in common between the two time comparisons. Expanded heat maps including gene names can be found in Supplementary Figure 3.

D. Bubble diagram showing the top-14 canonical pathways over-represented in the transcriptome of the 6 patients (P1-6) at times 4h and 24h post-treatment. The colour indicates the p-value (calculated by the right-tailed Fisher Exact Test*) for each particular pathway; from dark red ($P < 0.00001$) to pale (not significant). The size of each bubble indicates the ratio, which is the number of genes in a given pathway that meet the cut-off criteria divided by the total number of genes that make up that pathway. The larger and darker a given bubble, the more significant the finding. *The details of the calculation of the P-value can be found in this whitepaper: <http://www.ingenuity.com/wp-content/themes/ingenuity-qiagen/pdf/ipa/functions-pathways-pval-whitepaper.pdf>

E. Ingenuity Pathway Analysis identified NF κ B as one of the top altered networks at the 4 h time-point. Shown are the changes in NF κ B targets at 4 and 24 h. Heat-map and 2-way hierarchical clustering based on 29 NF- κ B target genes that differ significantly ($p < 0.05$) between time points. Patient is eliminated as a nominal factor. Target genes of NF- κ B were obtained from the NF- κ B online resource at Boston University: <http://www.bu.edu/nf-kb/gene-resources/target-genes/>.

Table 1

Patient Demographics and Clinical Characteristics

		CTCL N = 84	PTCL N = 47	%
		Patients (n)	Patients (n)	%
Sex				
	Male	57	25	53%
	Female	27	22	47%
Age, years				
	Median	59	59	
	Range	28–84	27–84	
	<50	32	8	
	50	52	39	
Disease stage at time of enrollment				
	I	7	0	0%
	IA	1		
	IB	6		
	II	21	2	4%
	IIA	3		
	IIB	18		
	III	6	11	23%
	IIIA	3		
	IIIB	3		
	IV	49	34	72%
	IVA	32		
	IVB	17		
ECOG performance status				
	0	24	20	43%
	1	49	23	49%
	2	11	4	8%

		CTCL N = 84	PTCL N = 47
		Patients (n)	Patients (n)
		%	%
Elevated LDH		35 of 81	18 of 35
		43%	51%
Low albumin		43 of 81	18 of 35
		53%	51%
No. prior therapies[*]			
	Median	3	3
	Range	0–14	1–11
Previous topical therapies			
		48	1
	PUVA	43	1
	Topical NM	17	0
	Topical bexarotene	5	0
	Topical steroids	13	0
	UVB	1	0
Previous radiation therapy[†]		44	0
	External Beam RT		17
	Localized radiotherapy	36	0
	TSEBT	13	0
Previous extracorporeal		19	0
Previous systemic, non-cytotoxic therapies		55	14
	Interferon	28	3
	Denileukin diftitox	15	1
	Alemtuzumab	4	0
	Anti-Tac antibody	5	0
	Oral corticosteroids	19	7
	Retinoid: oral bexarotene	37	3
	Retinoid: other [‡]	10	0
	Vorinostat	4	0
Previous systemic chemotherapy			
	Regimens [§]		

		CTCL N = 84	PTCL N = 47
		Patients (n)	Patients (n)
	%		
		32	0
		25	16
		15	10
		12	21
		12	45%

* Other treatments not listed include cyclosporine, tacrolimus, azathioprine, remicade, peldesine, and dendritic cell vaccine

[†] Some patients had both localized radiation therapy (RT) and total skin electron beam therapy (TSEB).

[‡] Includes oral bexarotene and interferon in combination

[§] Chemotherapies include monotherapies such as gemcitabine and methotrexate, and combination therapies such as CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone), ICE (ifosfamide, carboplatin, etoposide), CVAD (cyclophosphamide, vincristine, doxorubicin, dexamethasone), EPOCH (etoposide, vincristine, doxorubicin, cyclophosphamide, prednisone), interferon and bexarotene, methotrexate and gemcitabine, and fluorouracil with intravesical injection.

ECOG, Eastern Cooperative Oncology Group; LDH, lactate dehydrogenase; PUVA, psoralen photochemotherapy; NM, nitrogen mustard; UVB, ultra-violet B

Table II

Administered Therapy

	TOTAL	CTCL	PTCL
	N = 131	N=84	N=47
Total number of cycles	1214	749	465
Cycles per patient, number of cycles			
Median		4.5	3
Range	1 to 83	1 to 79	1 to 83
Cycles per patient, number of patients			
2	36	14	22
3 to 5	43	34	9
6	52	36	16
Doses per patient (n)			
Median		12.5	9
Range	1 to 247	2 to 158	1 to 247
Doses per patient (n)			
Total doses	3404	2068	1336
Full dose	2279	1588	691
Dose escalated	286	151	135
Reduced, total	839	329	510
First dose reduction (from immediate prior dose)	209	64	145
		3%	11%

CTCL, cutaneous T-cell lymphoma; PTCL, peripheral T-cell lymphoma.

Table III

Romidepsin efficacy in CTCL and PTCL populations

Response	Patients with CTCL		Patients with PTCL	
	(N=84)	(N=45)	2 prior therapies (N = 31)	
	n (%)	n (%)	n (%)	
CR + PR	28 (33%)	17 (38%)	10 (32%)	
CR	5 (6%)	8 (18%)	5 (16%)	
PR	23 (27%)	9 (20%)	5 (16%)	
SD	32 (38%)	5 (11%)	5 (16%)	
PD	18 (22%)	18 (40%)	12 (39%)	
NE	6 (7%)	5 (11%)	4 (13%)	
Median DOR				
CR/PR	13.8 months (1 – 127+)	9 months (2 – 76+)	8.4 months (3 – 74)	
CR	19.3 months (8 – 127+)	74 months (3 – 76+)	74 months (6 – 74)	

CTCL, cutaneous T-cell lymphoma; PTCL, peripheral T-cell lymphoma; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable; DOR, duration of response (defined as the time from first recorded response to progression of disease or censoring date)