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**Synergy of BCL2 and histone deacetylase inhibition against leukemic cells
from cutaneous T-cell lymphoma patients**

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

Benoit M. Cyrenne

2018

SYNERGY OF BCL2 AND HISTONE DEACETYLASE INHIBITION AGAINST LEUKEMIC CELLS FROM CUTANEOUS T-CELL LYMPHOMA PATIENTS.

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The presence and degree of peripheral blood involvement in patients with cutaneous T-cell lymphoma (CTCL) portend a worse clinical outcome. Available systemic therapies for CTCL may variably decrease tumor burden and improve quality of life, but offer limited effects on survival; thus, novel approaches to the treatment of advanced stages of this non-Hodgkin lymphoma are clearly warranted. Mutational analyses of CTCL patient peripheral blood malignant cell samples suggested the anti-apoptotic mediator BCL2 as a potential therapeutic target. To test this, we developed a screening assay for evaluating the sensitivity of CTCL cells to targeted molecular agents, and compared a novel BCL2 inhibitor, venetoclax, alone and in combination with a histone deacetylase (HDAC) inhibitor, vorinostat or romidepsin. Peripheral blood CTCL malignant cells were isolated from 25 patients and exposed *ex vivo* to the three drugs alone and in combination, and comparisons were made to four CTCL cell lines (Hut78, Sez4, HH, MyLa). The majority of CTCL patient samples were sensitive to venetoclax, and *BCL2* expression levels were negatively correlated ($r=-0.52$, $P=.018$) to IC50 values. Furthermore, this anti-BCL2 effect was markedly potentiated by concurrent HDAC inhibition with 93% of samples treated with venetoclax and vorinostat and 73% of samples treated with venetoclax and romidepsin showing synergistic effects. These data strongly suggest that concurrent BCL2 and HDAC inhibition may offer synergy in the treatment of patients with advanced CTCL. By using combination therapies and correlating response to gene expression in this way, we hope to achieve more effective and personalized treatments for CTCL.

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Introduction

Diagnosis, Prognosis, and Current Therapies

Cutaneous T-cell lymphoma (CTCL) is a rare form of non-Hodgkin lymphoma of the skin-homing CD4+ memory T-cells. CTCL is a group of malignancies with a variety of clinical manifestations, the majority of which may be categorized along the mycosis fungoides (MF) and Sézary syndrome spectrum. The overall annual incidence of CTCL in the United States is 6.4 cases per million persons, as calculated using Surveillance, Epidemiology, and End Results (SEER) registry data.¹ CTCL makes up 71% of all primary cutaneous lymphoma cases and the remaining 29% are predominantly B-cell derived neoplasms.^{2,3} In its early stages, mycosis fungoides (MF) is typically an indolent disease characterized by localized skin patches, plaques and tumors, comprising of 50% of all CTCL cases. In contrast, Sézary syndrome (SS) is a more aggressive form of CTCL presenting with erythroderma, and frequently, involvement of the lymph nodes, spleen, and liver. As the disease progresses, new lesions may spread, and existing lesions may enlarge in both size and thickness. In advanced disease, such as leukemic CTCL, malignant T-cells may predominate the peripheral lymphocyte compartment, or invade the viscera.⁴ In cases of advanced disease the loss of the normal T-cell receptor (TCR) diversity leads to significant immunosuppression, leading to a high incidence of opportunistic infections, and mortality.⁵

Diagnosing CTCL has been a consistent struggle for physicians and patients since it was first described in 1806. While classically MF presents as well demarcated patches involving sun-protected skin in a “bathing-suit” distribution, there is significant overlap in clinical features with benign dermatoses, such as eczema or psoriasis.^{4,6} Additionally, there may be alterations in pigmentation—either hypo- or hyper-pigmentation—and changes in hair growth patterns, such as alopecia.^{4,6} Initial diagnosis thus relies on biopsy. Histologically, MF is characterized by epidermotropism with Pautrier’s microabscesses; but much like the clinical picture, the findings on biopsy show high degree of variability, and may resemble benign inflammatory disorders such as pityriasis lichenoides, granuloma annulare, gyrate erthema, or lichen planus. This ambiguity in biopsy results frequently leads to the need for multiple biopsies and a delayed diagnosis, taking an average of 6 years from the initial presentation.⁷

This uncertainty in both clinical and histopathological diagnoses has led to the development of more sensitive and specific tests. CTCL features a T-cell clone which may be detected via polymerase chain reactions (PCR) of the T-cell receptor within the skin (i.e. fresh, frozen, or formalin-fixed paraffin-embedded biopsy specimens), the peripheral blood, or lymph node. This test, however, has limited utility due to the low concentration of malignant cells, leading to false-negatives and the frequent presence of oligoclonal expansion in other inflammatory disorders, resulting in false positives.⁷⁻¹⁰ Despite these limitations, the identification of the same clone from two biopsy sites provides a more

specific diagnostic test for MF.^{7,11} Newer techniques to assess T-cell clonality such as high throughput sequencing show improved sensitivity for detection of malignancy.^{6,7,12} The rapid identification of CTCL upon presentation is essential to improving clinical outcomes, as increased length of time to diagnosis correlates with decreased survival.¹³

The identification of the prototypical Sézary cells, with their distinct cerebriform nuclei, was initially the essential diagnostic test for blood involvement, and remains within the 2007 update of the International Society for Cutaneous Lymphomas (ISCL) and the European Organization for Research and Treatment of Cancer (EORTC) guidelines for diagnosis and staging of CTCL.¹⁴ The ability to identify the cell surface markers of circulating T-cells, specifically with the phenotype CD4+ CD7- and/or CD26-, on peripheral blood flow cytometry has improved identification of peripheral malignant T-cell populations. Flow cytometry has also proved useful in the identification of clonality via the quantification of TCR β chain variable region (TCR-V β) family usage.^{15,16} The practical application of TCR-V β analysis is hindered by the high degree of variability among expression TCRs, and the limited number of V β antibodies present in commercial assays, though the absence of V β reactivity to a given assay can also be used as an indirect marker of clonality.¹⁷ Gibson *et al.* propose new criteria for staging of blood involvement that includes tests for clonality such as the aforementioned TCR-PCR and TCR-V β flow cytometry analysis.¹⁸

The rapid and accurate diagnosis of CTCL in the skin and blood is critical for proper treatment. While patients with skin-limited patch disease, classified as

stage IA, have similar life expectancies as the general population a 5-year survival rate of 94-100%, blood and lymph node involvement are independent predictors of poor survival outcomes.¹⁹⁻²¹ In advanced stages, CTCL is a fatal disease that is incurable with conventional therapies, with a 0-18% 5- year survival rate among stage IV-B patients.^{20,22} Additional factors associated with worse prognosis include elevated lactate dehydrogenase, increased age, large cell transformation, degree of peripheral blood involvement, and the folliculotropic-MF variant.

While other lymphoproliferative diseases, such as ALL or CLL, may be risk stratified by cytogenetic findings, no similar criteria exists in CTCL. Extensive work has been performed to identify patterns in genetic alterations and expression that correlate to worse survival. Cluster analysis of genetic expression from skin biopsies of CTCL patients led to the identification of three patient clusters consisting of earliest stage disease (stages I-A and I-B), more extensive disease (stages I-B and III), and tumor type disease (stage IIB), of which the two latter clusters were associated with worse treatment response and survival.^{23,24}

Treatment strategies for CTCL are as heterogeneous as the disease itself. The site of disease and the degree of involvement, as well as patient and physician preference will largely dictate the specific prescribed course. For skin-limited disease such as MF, skin-directed treatments are preferred, such as high potency topical corticosteroids, phototherapy, nitrogen mustard-based therapies and radiation therapy. In the case of more advanced disease such as extensive

plaque, tumor, and/or leukemic CTCL, systemic therapies are indicated. These include extracorporeal photopheresis, interferon-based therapies, bexarotene, and methotrexate. With rare exceptions in cases of hematopoietic cell transplantation,²⁵ the overall response rates for novel agents including retinoids, HDAC inhibitors, and pralatrexate range from 30-50% and are generally not durable.²⁶ There remains an unmet medical need for new and more effective treatments, which the work of this thesis aims to address.

Genetic Alterations in CTCL

Recent studies^{5,27-30} have made significant strides in our understanding of the molecular pathogenesis of CTCL, most notably via exome sequencing and expression analysis. These analyses have shown a predominance of gene copy number alterations (GCNA) over single nucleotide variant (SNV) mutations, without evidence of common chromosomal translocations. The preponderance of GCNAs among CTCL patients indicates a significant degree of chromosomal instability. Although Döbbeling *et al.* found both CTCL patient samples and cell lines to lack expression of recombination-activating genes (RAG), the high frequency of focal deletions observed suggest possible involvement of RAG proteins which cause breakpoints in DNA via endonuclease-mediated cleavage, and are necessary for V(D)J (V, variability; D, diversity; J, joining) recombination.^{5,31} RAG cleavage sites can be identified by the presence of RAG heptamers, which were found adjacent to greater than 12% of CTCL deletions.⁵ Others have suggested that ectopic expression of germ cell proteins in CTCL

leads to disordered mitosis, and high rates of double-stranded DNA breaks. Several germ cell proteins were found to be highly expressed in CTCL cells relative to healthy memory T-cells, and may be responsible for or associated with severe chromosomal instability— in particular, cancer testis genes responsible for the initiation of meiosis, strand breaks and subsequent homologous recombination and the formation of synaptonemal complexes.^{32,33}

Other mutations in genes involved in DNA repair and genome maintenance—such as *POT1*, *ATM*, and *BRAC2*—have also been identified as occurring in SS patients, and may also contribute to the high degree of genetic variability in CTCL.³⁴ *ATM* and *POT1* are involved in telomere maintenance and their derangement may lead to the telomere shortening observed in CTCL. Catastrophic telomere shortening may also lead to chromothripsis, and near-global chromosomal changes, such as those observed in CTCL patient cells.^{35,36}

SNV mutations, which comprise of <10% of all driver mutations in CTCL, are also of interest in the pathogenesis of CTCL. The number of patients examined via exome sequencing and expression analysis limits power in genetic studies, which require cohorts including a minimum of 200 samples for accurate and complete identification.³⁷ Despite these limitations, point mutations have been put forth as likely driver mutations, including within *CD28*. Alternatively, genetic shuffling may result in a fusion product, for example, the CD28-CTLA4 protein that can cause constitutive activation of T-cells.³⁸ Other common mutations observed in CTCL patient cohorts include *RHOA*, *BRAF*, *STAT5B*, *PLCG1* and *NFKB2*.⁵ A new analysis of published CTCL sequencing data

suggests that the number of SNV driver mutations is greater than previously reported. Among these are 5 genes not previously associated with another malignancy: *ARHGEF3*, *CSNK1A1*, *RLTPR*, *PTPRN2*, and *RARA*.³⁷

The categories of genetic alterations include changes in the behavior of the malignant T-cell population and their imprint on the immune system, which suggest clustering under three major pathways: constitutive T-cell activation (e.g. *STAT3*, *STAT5B*, *CARD11*, *CD28*); resistance to apoptosis/cell cycle dysregulation (e.g. *MYC*, *FAS*, *CDKN2A*, *TP53*, *RB1*); and DNA structural dysregulation/chromatin remodeling or repair (e.g. *ARID1A*, *DNMT3A*) (Figure 1).

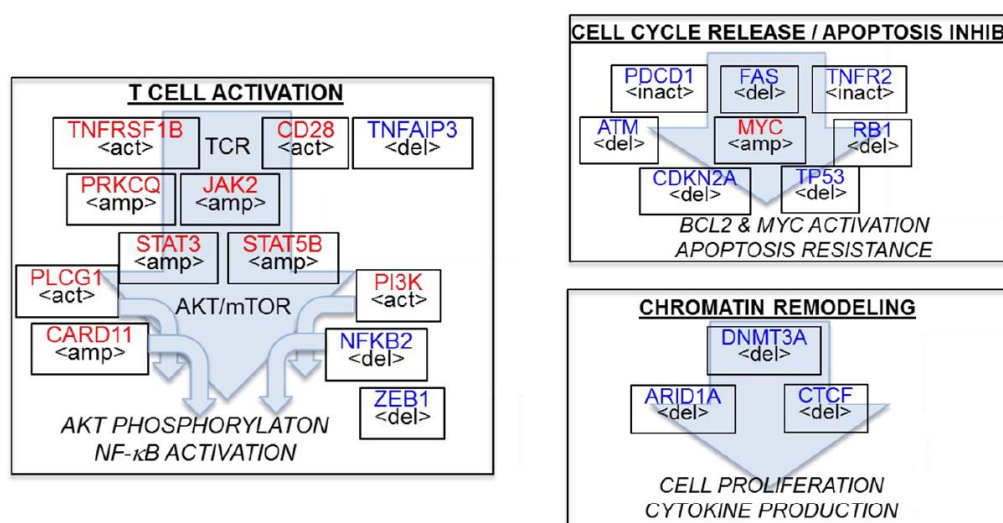


Figure 1: Pathways of the identified driver genetic alterations in CTCL identified by exome sequencing. Genetic alterations in CTCL can be grouped in three categories: T-cell activation, cell cycle release/apoptosis inhibition, and chromatin remodeling. Credit: Michael Girardi.

With this wellspring of new information, recently discovered and re-purposed agents targeting pathways or specific gene mutations may be screened as a patient-specific treatment algorithm is developed. With 30% of drugs in clinical trials failing due to lack of efficacy, a focus on expanding indications of

new molecular therapies allows us to leverage established safety profiles to fast-track new treatment options for patients.³⁹

Inhibition of Apoptosis, BCL2, and Venetoclax

One such opportunity for the repurposing of existing therapies involves the dysregulation of B cell lymphoma-2 (BCL2) driven apoptotic pathways in CTCL. The BCL2 family of proteins is a critical group of proteins involved in the regulation of apoptosis. These proteins further regulate autophagy via the binding of Beclin-1.⁴⁰ BCL2 proteins are part of the intrinsic, or mitochondrial, apoptosis pathway in which an apoptotic signal such as damage from free radicals or growth factor starvation leads to programmed cell death (i.e. apoptosis) via caspase activation. The apoptotic signal leads, via BCL2 family proteins, to the permeabilization of the outer mitochondrial membrane, the release of cytochrome c, and the subsequent activation of caspase.^{41,42} This is in contrast to the extrinsic apoptotic pathway, in which death receptors activated by signals, such as FAS-ligand or TNF- α , lead to caspase activation, or the perforin/granzyme pathway, which leads to cell death by either caspase activation (granzyme B) or, independent of caspase, via direct DNA damage (granzyme A).^{43,44}

The BCL2 family is traditionally defined by the presence of conserved residue sequences known as BCL2 homology (BH) motifs, of which there are four (BH1-4), though more recent findings suggests that BH motifs are not essential to the function or characterization of BCL2 family proteins.^{45,46} The BCL2 family can be divided up into three groups: the anti-apoptotic proteins (e.g.

BCL2, BCL-XL, MCL1, BCL-w), the multiregion pro-apoptotic proteins (e.g. BAX, BAK, BIK, BID) which are composed of multiple BH motifs, and the BH3-only pro-apoptotic proteins (e.g. BIM, BAD, NOXA, HRK, BLK, BMF, PUMA).⁴¹ The BH3-only proteins may act in two ways: as a sensitizer, in which they bind to anti-apoptotic proteins, or as activators, which may bind to either anti-apoptotic proteins or the multiregion pro-apoptotic proteins.⁴⁷ Twenty-five BCL2 family proteins have been discovered to date.⁴⁸

Anti-apoptotic proteins such as BCL2 or BCL-XL are mitochondrial-membrane bound or membrane bound upon activation. These proteins bind and sequester the BH3- only pro-apoptotic proteins such as BIM. In the presence of a death signal, the sensitizer BH3-only pro-apoptotic proteins (such as BMF) bind to anti-apoptotic proteins (such as BCL2) releasing activator BH3-only pro-apoptotic proteins (such as BIM). These proteins then in turn bind to multi-domain, pro-apoptotic proteins (such as BAX or BAK) which oligomerize to form pores in the mitochondrial membrane, resulting in the release of cytochrome c and caspase activation. The activation or inhibition of apoptosis depends on the specific ratios of pro- and anti- apoptotic proteins.⁴¹

CTCL cells exhibit a high degree of resistance to apoptosis, even in the presence of programmed-cell death inducing signals, and, as described above, CTCL cells exhibit multiple genetic alterations which explain this behavior. Four of the common gene alterations identified in CTCL are *STAT3* amplifications, *STAT5B* amplifications, and *P53* deletions; the frequency of which was previously validated by our group in the development of a new diagnostic tool, an

11-gene FISH panel.⁴⁹ Each of these mutations has been linked to the inhibition of apoptosis through the up-regulation of *BCL2* transcription, in turn leading to increased *BCL2* activity and dependence.^{50–57} Additionally point mutations have been identified in the *ITPR1* gene, which is associated with increased risk of breast cancer, and involved in *BCL2* activity and regulation.⁵⁸ The tendency of CTCL towards inhibition of apoptosis, and the putative trend towards increased *BCL2* activity in CTCL, makes inhibition of *BCL2* an attractive therapeutic target.

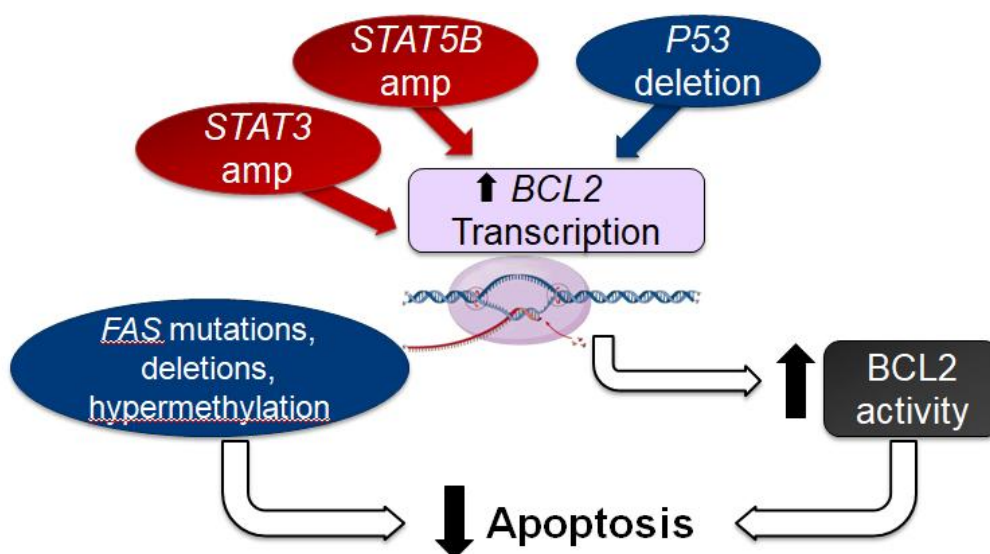


Figure 2: Genetic alterations in CTCL, leads to increased BCL2 activity, and resistance to apoptosis. *STAT3* amplifications, *STAT5B* amplifications, *P53* deletions, and *Fas* alterations result in decreased apoptosis.

Venetoclax (ABT-199) is a BH3-mimetic, *BCL2* selective inhibitor without additional cross-reactivity with *BCL-XL*, *BCL-W*, or *MCL1*.⁵⁹ Venetoclax (ABT-199) is a unique agent as a *BCL2* inhibitor due to its specificity. Other so-called “*BCL2* inhibitors” available for research purposes are not specific for *BCL2* as they have been shown to also affect *BCL-XL* and/or *MCL1*. The most promising

of these inhibitors was navitoclax (ABT-263) which showed impressive pre-clinical data in BCL2 dependent lymphoid malignancies. Further clinical development of navitoclax was restricted due to dose-limiting thrombocytopenia as a result of additional inhibition of BCL-XL, which is essential for platelet survival. ABT-263 was thus re-engineered to bind exclusively to the BCL2 binding pocket, and clinical trials have borne out its potent efficacy in the inhibition of BCL2. Venetoclax was first approved by the U.S. FDA in 2016, received accelerated approval for the treatment of relapsed or refractory CLL with 17p deletion, and is the only BCL2 inhibitor that has received approval by the U.S. FDA for clinical use.⁶⁰ Venetoclax is also currently undergoing trials for follicular lymphoma, diffuse large B cell lymphoma, acute myeloid leukemia, multiple myeloma, Waldenstrom macroglobulinemia, and NHL (excluding CTCL).

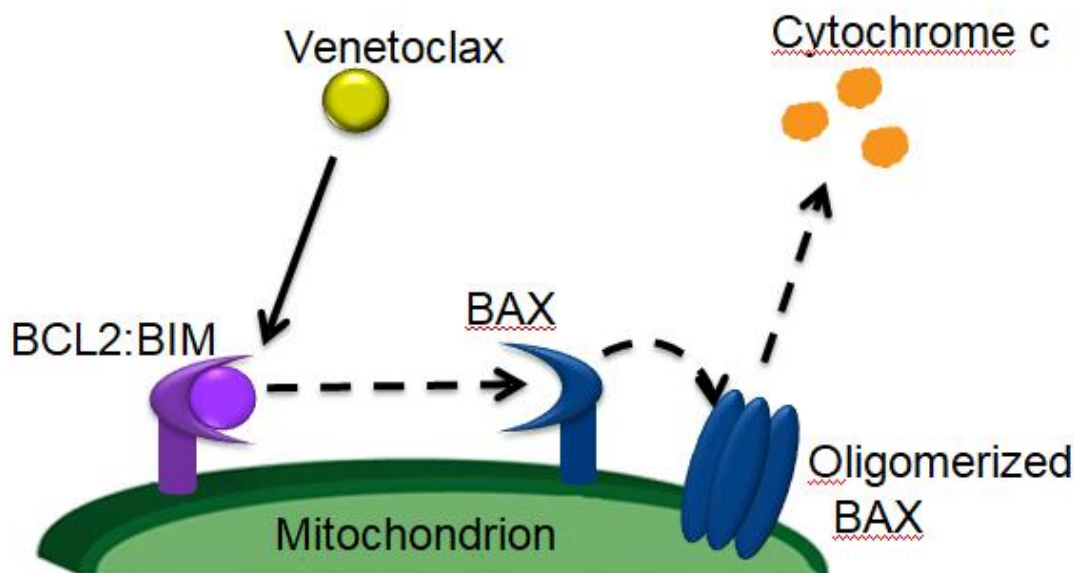


Figure 3: Mechanism of action of venetoclax (ABT-199). Venetoclax, as a BH3 mimetic, binds with high affinity to BCL2, and causing dissociation of the BCL2-BIM complex. BIM is then able to activate the pro-apoptotic protein BAX, leading to oligomerization of BAX, permeabilization of the mitochondrial membrane, and release of cytochrome c.

The potential for BCL2 inhibition in the treatment of CTCL may extend beyond its use as a monotherapy. Combined inhibition of BCL2 members and HDACs has shown synergistic effects in CTCL⁶¹ and other malignancies, including mantle cell lymphoma⁶² and glioblastoma.⁶³ Romidepsin and vorinostat are HDAC inhibitors⁶⁴ which have widespread effects including modulation of gene expression, induction of cellular differentiation, and modulation of apoptosis effector genes.⁶⁵ One mechanism of action of apoptosis is via alterations in the expression of the intrinsic apoptosis pathway, namely the BCL2 family genes,^{66–69} including upregulation of pro-apoptotic factors such as BID and BIM, and down regulation of the anti-apoptotic factors such as BCL-XL and MCL1.^{70–72}

Through in vitro testing of CTCL patient-derived blood samples, we demonstrate the potential of BCL2 inhibition by venetoclax as a novel treatment for CTCL. Furthermore, we reveal that this effect is synergistically potentiated via HDAC inhibition afforded by romidepsin or vorinostat. These results suggest that BCL2 inhibition, alone or in combination with HDAC inhibition, represents a viable novel strategy in the treatment of advanced CTCL with blood involvement.

Statement of Purpose

Validate BCL2 as a potential target for the treatment of CTCL

Prior to my arrival in the Girardi Lab, Jason Weed (YSM 2017) led efforts in designing and implementing an *in vitro* targeted dry testing platform for leukemic CTCL, leading to the identification of several candidate drugs for further testing, the most promising among which was venetoclax. We aim to test the *in vitro* sensitivity of venetoclax on an expanded patient cohort in order to validate BCL2 as a potential therapeutic target.

Associate expression of BCL2 family genes to the in vitro patient sample sensitivity to venetoclax

In order to validate the putative mechanism of action of venetoclax in the killing of CTCL cells—inhibition of BCL2—we sought to associate genetic expression of *BCL2* and other BCL2 family members to the sensitivity of venetoclax. Additionally we sought to identify ratios of expression of BCL2 family members that predict sensitivity to venetoclax.

Identify synergistic combinations of therapies

We hypothesize that drugs which alter genetic expression of BCL2 family members, such as the HDAC inhibitors, vorinostat and romidepsin, will synergize with venetoclax for more efficient killing of CTCL cells, with the hope that in clinical practice, such combinations will limit both resistance and systemic toxicities.

Methods

Patient Samples

Blood samples were obtained from CTCL patients at the Yale Cancer Center, receiving treatment either at the Yale Photopheresis Center or at the Yale-New Haven Hospital Smilow Cancer Hospital. Written informed consent was obtained from all subjects in accordance with the Yale Human Investigational Review Board. Approximately 45 mL of peripheral blood was obtained from consenting patients and healthy donors and collected in lithium heparin tubes (BD Vacutainer). The whole blood was diluted in a 3:1 ratio with phosphate-buffered saline (PBS), and peripheral blood mononuclear cells (PBMC) were separated from whole blood using SepMate™. 15 mL of Ficoll (GE Healthcare Ficoll-Paque Premium) was placed at the bottom of SepMate™-50 tubes and diluted blood was layered on top. The SepMate™-50 tubes were then centrifuged for 20 minutes at 1200g at room temperature. Buffy coats were isolated and washed with PBS with 2% fetal bovine serum (FBS) in 15 mL conical tubes, centrifuged for 8 minutes at 300g at room temperature, resuspended in PBS with 2% FBS, rewashed in PBS with 2% FBS and centrifuged again for 8 minutes at 300g at room temperature to minimize residual Ficoll. The pellet was then re-suspended in RPMI and counted using hemocytometer. CD4+ T-cells were selected by Miltenyi Biotec (Bergisch Gladbach, Germany) MACS CD4+ negative selection kit per the manufacturer's protocol and supplemented with antibodies to select for CD26- and/or CD7- cells,

depending on the known aberrant phenotype of the patient, as measured by the patient's most recent peripheral blood flow cytometry.

Aliquots of unfractionated PBMC and purified samples were obtained to assess purity and verify expression of phenotypic markers. Additional aliquots of purified samples (minimum 500 000 cells) were obtained and lysed for RNA analysis using Qiagen RNEasy (see below). Following isolation, cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, glutamine (2mM), penicillin (100 μ /mL), and streptomycin (100 μ g/mL), as well as IL2 (10ng/mL), IL7 (5ng/mL), IL15 (10ng/mL), and IL13 (10ng/mL; all cytokines from R&D Systems) at 37°C, 5% CO₂, and 95% humidity.

Cell Viability Assay

Following isolation of the desired cell population, cells were incubated in quadruplicate at a density of 100cells/ μ L in 100 μ L of media, for a total of 10⁵ cells/well, in a black optical 96-well plate. In order to limit effects from uneven evaporation across the plate, cells were not plated in the exterior two rows of wells, and instead filled with 100 μ L of media. In order to ensure an equal concentration of cells across the plate, cells were continuously mixed using gentle pipetting throughout the plating process.

Cells were incubated for 72 hours with venetoclax (3 nM to 100 μ M), romidepsin (0.1 nM to 10 nM), and/or vorinostat (0.1 μ M to 10 μ M). The CellTiter-Glo® Luminescent Cell Viability Assay (Promega, WI, USA) was used to measure the number of viable cells in culture based on quantitation of the ATP

present. Plates were read using the Perkin Elmer Victor Light Luminescence Counter (Waltham, MA, USA). Drug concentrations were applied in approximate half-log₁₀ increments to patient samples, and in 2-fold increments for cell lines. Cell luminescence was normalized to a vehicle control containing 0.2% DMSO and corrected for media.

Stocks of 50nM venetoclax, 20mM vorinostat, and 20nM romidepsin from ApexBio (Houston, TX, USA) were prepared in DMSO and stored at -20°C. Camptothecin (10µM) from Sigma (St. Louis, MO, USA) was used a positive control for both the Viability Assay and the Caspase-3/7 Assay.

Caspase-3/7 Assay

Patient samples were incubated for 1-48 hours as described above in the Cell Viability Assay. Following incubation, the Promega Caspase-Glo® 3/7 assay (Madison, WI, USA) was used to quantitate caspase activity, as per the manufacturer's protocol. Plates were read using a Perkin Elmer Victor X Light Luminescence Counter.

Flow Cytometry Analysis

Unfractionated PBMC and isolated malignant T-cell populations were analyzed using flow cytometry. All samples were matched with an isotype control. The following monoclonal antibodies were included: CD3 from BD Biosciences (San Jose, CA, USA); CD4, CD7, and CD26 from eBioscience (San Diego, CA, USA); and TCR-Vβ from Beckman Coulter (Brea, CA, USA). Cells

were blocked with human TruStain FcX™ from BioLegend (San Diego, CA, USA) for 10 minutes then incubated with selected antibodies for 20min at 4°C. Cells were then washed three times and fixed in 1% paraformaldehyde. Flow cytometry was conducted on the Stratified-13 from Stratified Inc. (San Jose, CA, USA). All flow cytometric data were analyzed using FlowJo software (v10; FlowJo, LLC).

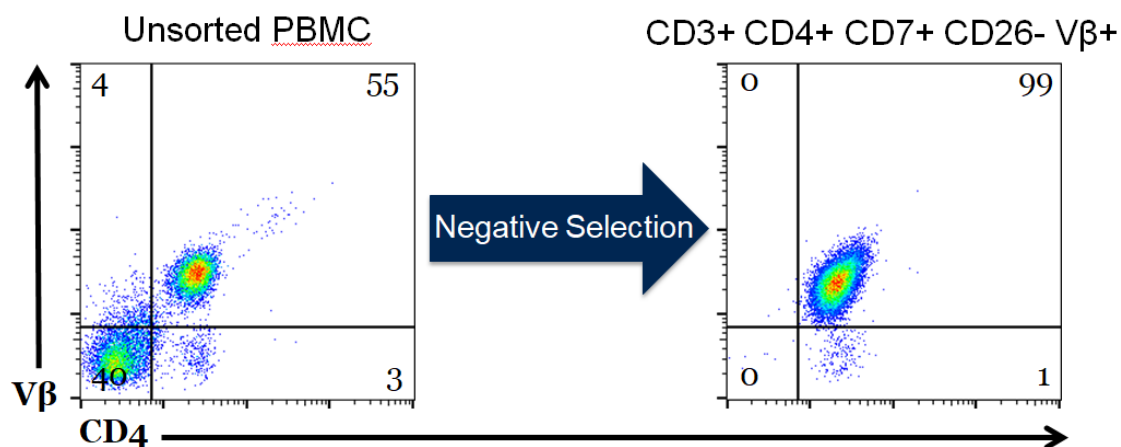


Figure 4: Malignant cells negatively selected by magnetic bead sorting based on the patient's malignant phenotype

Cell Lines

MyLa 2059,⁷³ Sez4,⁷⁴ HH,⁷⁵ and Hut78⁷⁶ are well established CTCL cell lines. MyLa 2059 and HH were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, glutamine (2mM), penicillin 100μ/mL, and streptomycin (100μg/mL) at 37°C, 5% CO₂, and 95% humidity. Sez4 was cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, glutamine (2mM), penicillin 100μ/mL, and streptomycin (100μg/mL), as well as IL2 (20ng/mL; R&D Systems) at 37°C, 5% CO₂, and 95% humidity. Hut78 was cultured in IMDM medium supplemented with 20% heat-inactivated FBS,

glutamine (2mM), penicillin 100 μ /mL, and streptomycin (100 μ g/mL) at 37°C, 5% CO₂, and 95% humidity. All media and supplements were obtained from Invitrogen (Carlsbad, CA, USA).

Statistical analysis

For the Cell Viability Assay and Caspase-3/7 assays, each drug concentration was performed in quadruplicate, and the mean values were plotted with their respective standard deviation. The mean inhibitor concentration was determined using GraphPad Prism (Version 7.01). Patient samples were grouped using hierarchical clustering with Ward's method⁷⁷ in R (Version 3.0.1). Combination index (CI) values and standard errors were calculated using the Chou-Talalay method⁷⁸ in Microsoft Excel.

Quantitative Real-Time Reverse-Transcriptase-PCR

RNA was extracted using the All-Prep DNA/RNA Micro Kit for patient samples, or with the All-Prep DNA/RNA Mini Kit for cell lines from Qiagen (Hilden, Germany) as per the manufacturer's specifications. RNA was quantified using the BioTek Epoch spectrophotometer. 2 μ g of RNA was converted to cDNA using the High Capacity Reverse Transcription Kit from Applied Biosystems (Foster City, CA, USA) per the manufacturer's instructions. Patient cDNA samples were then pre-amplified using the Applied Biosystems TaqMan® Pre-Amplification Master Mix as per the manufacturer's instructions. Gene expression was quantified using the TaqMan® primers and Gene Expression Master Mix

and the Applied Biosystems 7500 Real Time PCR System. Samples were characterized in duplicates. Obtained Ct values were normalized to HPRT1 expression and expression differences relative to healthy controls were calculated using $RQ = 2^{-\Delta\Delta Ct}$.

Results

CTCL patient population and peripheral blood analysis

To begin to assess the potential of the BCL2 inhibitor venetoclax in the treatment of CTCL with blood involvement (B1 or B2), peripheral blood samples were obtained from 25 patients diagnosed with CTCL who have a malignant cell population measured by flow cytometry. Patients were classified by their initial diagnosis as erythrodermic/Sézary syndrome (13/25), mycosis fungoides (10/25), or folliculotropic MF (2/25) (Table 1). Patient treatment statuses ranged from extracorporeal photopheresis (ECP) with or without adjunct therapies (18/25), to multimodal therapies (6/25), to cytoreductive therapy (1/25). Each patient sampled underwent clinical flow cytometry, from which blood parameters were calculated (Table 2). Patients were additionally tested for clonality by either PCR based T-cell receptor (TCR) gene rearrangement studies or the presence of a clonal V β antigen detectable by flow cytometry, with 25/25 patient samples positive for clonality based on one or both tests. Eight patients were classified as B1, and the remainder were classified as B2 based on the 2007 ISCL classification¹⁴ and the 2016 Gibson et al. criteria.¹⁸

Table 1. Summary of CTCL patients' peripheral blood flow cytometric parameters. Abnormal cell phenotype identified by flow cytometry as phenotypically atypical: TCR-V β positive if >50% of the population of atypical cells express a single V β , or by indirect evidence, if there is less than 20% expression of the entire 27 V β antibody panel.¹⁸ PCR positive if ≥ 1 of 3 PCR reactions identifies a clone. B-stage based on ISCL classification¹⁴ and the 2016 criteria proposed by Gibson et al.¹⁸ Patients staged as B2 have elevated absolute CD3+ or CD3+ CD4+ cell counts, with the upper limits of normal defined as greater than 2245 cells/ μ l and greater than 1612 cells/ μ l, respectively. CD4+CD7- and CD4+ CD26- percent specifications are defined as percent of peripheral blood mononuclear cells (lymphocytes and monocytes).

| Pt ID | Sorted Abnormal Cell Population | TCR-V β + | V β gene identified | PCR + | CD4/CD8 Ratio | CD4+ CD7- % | CD4+ CD26- % | Stage |
|-------|---------------------------------|-----------------|---------------------------|-------|---------------|-------------|--------------|-------|
| 1 | CD3+ CD4+ CD7- CD26- | Yes | 20 | Yes | 8.37 | 19.7 | 14.1 | B1 |
| 2 | CD3+ CD4+ CD26- | Yes | 2 | Yes | 179.0 | 8.6 | 80.2 | B2 |
| 3 | CD3+ CD4+ CD26- | Yes | 12 | Yes | 2.12 | 8.9 | 34.3 | B2 |
| 4 | CD3+ CD4+ CD7- CD26- | Yes | Indirect | No | 8.11 | 14.3 | 38.5 | B2 |
| 5 | CD3+ CD4+ CD26- | Yes | 16 | Yes | 15.6 | 10 | 71.5 | B2 |
| 6 | CD3+ CD4+ CD7- CD26- | Yes | 1 | Yes | 28.69 | 86.2 | 84.5 | B2 |
| 7 | CD3+ CD4+ CD7- CD26- | Yes | 13.6 | None | 56.81 | 83.9 | 58.7 | B2 |
| 8 | CD3+ CD4+ CD7- CD26- | Yes | 12 | Yes | 24.78 | 54.9 | 71.4 | B2 |
| 9 | CD3+ CD4+ CD7- CD26- | N/A | N/A | Yes | 10.22 | 38.9 | 36.5 | B2 |
| 10 | CD3+ CD4+ CD7- CD26- | Yes | 13.1 | Yes | 7.06 | 44.3 | 25.5 | B2 |
| 11 | CD3+ CD4+ CD26- | Yes | 13.6 | Yes | 9.66 | 11.4 | 46 | B2 |
| 12 | CD3+ CD4+ CD26- | Yes | Indirect | Yes | 5.18 | 1.9 | 48.5 | B2 |
| 13 | CD3+ CD4+ CD7- CD26- | Yes | 21.3 | Yes | 59.63 | 89.8 | 88.7 | B2 |
| 14 | CD3+ CD4+ CD26- | No | None | Yes | 4.45 | 16.2 | 4.8 | B1 |
| 15 | CD3+ CD4+ CD7- CD26- | Yes | Indirect | Yes | 6.95 | 63.9 | 62.6 | B2 |
| 16 | CD3+ CD4+ CD7- CD26- | Yes | Indirect | Yes | 20.02 | 87.2 | 76.3 | B2 |
| 17 | CD3+ CD4+ CD7- CD26- | Yes | 7.2 | Yes | 4.7 | 30.4 | 33.8 | B2 |
| 18 | CD3+ CD4+ CD7- CD26- | Yes | 7.2 | Yes | 3.74 | 27.4 | 26.0 | B1 |
| 19 | CD3+ CD4+ CD7- CD26- | No | None | No | 1.21 | 20.6 | 22.5 | B1 |
| 20 | CD3+ CD4+ CD7- CD26- | Suspicion | Indirect | Yes | 1.63 | 22.5 | 24.5 | B1 |
| 21 | CD3+ CD4+ CD7- CD26- | Suspicion | 5.3 | None | 4.75 | 69.6 | 71.8 | B2 |
| 22 | CD3+ CD4+ CD7- CD26- | Suspicion | 17 | Yes | 1.75 | 19.9 | 18.5 | B1 |
| 23 | CD3+ CD4+ CD26- | Yes | 2 | Yes | 27.91 | 51.1 | 89.5 | B2 |
| 24 | CD3+ CD4+ CD7- CD26- | Suspicion | Indirect | Yes | 1.3 | 23.7 | 23.8 | B1 |
| 25 | CD3+ CD4+ CD7- CD26- | No | None | Yes | 8.87 | 24.9 | 19.1 | B1 |

Table 2. Summary of patient characteristics.

| Pt ID | Sex | Age | CTCL Subtype | Current Therapy | Previous Therapy |
|-------|-----|-----|--------------|-----------------------------------------------------|-------------------------------------------------------------------------------------------------------------|
| 1 | M | 73 | FMF | ECP | None |
| 2 | M | 84 | SS | Brentuximab, vorinostat | PUVA, ECP, vorinostat, MTX, bexarotene, IFN α -2b, romidepsin, gemcitabine/doxorubicin, alemtuzumab, |
| 3 | M | 66 | SS | ECP | NB-UVB, topical steroids |
| 4 | M | 85 | SS | ECP | Topical steroids |
| 5 | F | 71 | SS | Gemcitabine | ECP, bexarotene, topical steroids, IFN α -2b, vorinostat, romidepsin, belinostat |
| 6 | M | 73 | SS | Pralatrexate | Romidepsin, ECP, bexarotene, topical steroids |
| 7 | F | 61 | SS | ECP, bexarotene | Topical steroids, phototherapy |
| 8 | M | 77 | SS | Romidepsin, vorinostat | ECP, NB-UVB |
| 9 | M | 70 | SS | Pralatrexate | Romidepsin, vorinostat, mechlorethamine, ECP |
| 10 | M | 71 | MF | ECP, bexarotene | NB-UVB, topical steroids |
| 11 | F | 79 | SS | ECP, bexarotene, vorinostat, IFN α -2b | Topical steroids |
| 12 | F | 64 | SS | ECP, bexarotene | Topical steroids |
| 13 | F | 76 | SS | ECP | None |
| 14 | M | 61 | MF | ECP, NB-UVB | None |
| 15 | M | 67 | SS | ECP, bexarotene | None |
| 16 | F | 40 | FMF | ECP, bexarotene | NB-UVB, TSEBT |
| 17 | M | 66 | SS | ECP, bexarotene, NB-UVB, topical steroids | None |
| 18 | F | 64 | MF | ECP, bexarotene, IFN α -2b, IFN γ -1b | None |
| 19 | M | 87 | MF | ECP | MTX, bexarotene, topical steroids |
| 20 | M | 85 | MF | ECP, bexarotene, NB-UVB | Topical steroids |
| 21 | M | 70 | MF (V) | EPOCH | None |
| 22 | M | 77 | MF | ECP, bexarotene | NB-UVB, topical steroids |
| 23 | M | 52 | MF | Gemcitabine | NB-UVB, bexarotene, vorinostat, ECP, IFN α -2b, Romidepsin, |
| 24 | F | 62 | MF | ECP, bexarotene, NB-UVB, mechlorethamine | Topical steroids |
| 25 | M | 62 | MF | ECP, bexarotene | Topical steroids |

Abbreviations: ECP, extracorporeal photopheresis; EPOCH, etoposide, prednisone, vincristine, cyclophosphamide, doxorubicine; F, female; FMF, folliculotropic mycosis fungoides; IFN, interferon; M, male; MF, mycosis fungoides; MTX, methotrexate; NB-UVB, narrow band ultraviolet-B; PUVA, psoralen ultraviolet light A; SS, Sézary syndrome; TSEBT, total body electron beam therapy; V, visceral involvement.

CTCL patient samples show highly variable sensitivity to venetoclax in vitro

We observed the cytotoxic/cytostatic effects of venetoclax in all 25 CTCL patient samples after 72 hours of incubation as measured by the change in cell viability via media ATP quantitation. Patient samples demonstrated a wide range of sensitivities to venetoclax, with mean 50% inhibitory concentrations (IC₅₀) ranging from picomolar (85 pM) to micromolar (6.6 μM) levels. The observed heterogeneity in sensitivity to venetoclax is not dissimilar to the variation observed in T-ALL⁷⁹ or CLL.⁵⁹ The most sensitive samples compare favorably to pre-clinical studies in B-cell lymphomas, the results of which have since been validated via clinical trial.⁶⁰ In order to better study the significance and origins of the observed variation, we used hierarchical clustering based on their responsiveness to venetoclax to divide the tested samples into two groups, low and high responders. The majority of tested samples (20/25) were defined as high responders with a group mean IC₅₀ of 79.0 nM compared to the low responders (5/25) with a group mean IC₅₀ of 3.2 μM. Dose response curves for three representative samples: the most sensitive, the median, and the most resistant sample, are shown in Figure 5Bi.

Four established CTCL cell lines—MyLa 2059, Sez4, HH, and Hut78—were also analyzed for sensitivity to venetoclax (Figure 5Bii). All four cell lines demonstrated relatively low sensitivity to venetoclax with IC₅₀s of 2.47 μM, 7.4 μM, 10.2 μM, and 33.6 μM respectively. In addition to the differences in IC₅₀, cell lines and patients were observed to have different patterns in their dose response curves. Cell lines demonstrated a significantly higher Hill slope relative

to patient samples ($P < .0001$) suggesting distinct mechanisms of action between cell lines and patients (Figure 5C). Among patient samples, there was a statistical trend noted between response status and Hill slope ($P = .07$; data not shown). Hypothesizing an association between tumor burden and increased sensitivity to induction of apoptosis via inhibition of BCL2, we compared the mean inhibitory concentration of venetoclax between patients classified as B1 versus B2 (ISCL Classification)¹⁴, as well as between patients classified as mycosis fungoides (non-erythrodermic) versus Sézary syndrome (erythrodermic), and found a higher sensitivity in patients with more advanced disease, and in patients with Sézary syndrome (Figure 5D,E).

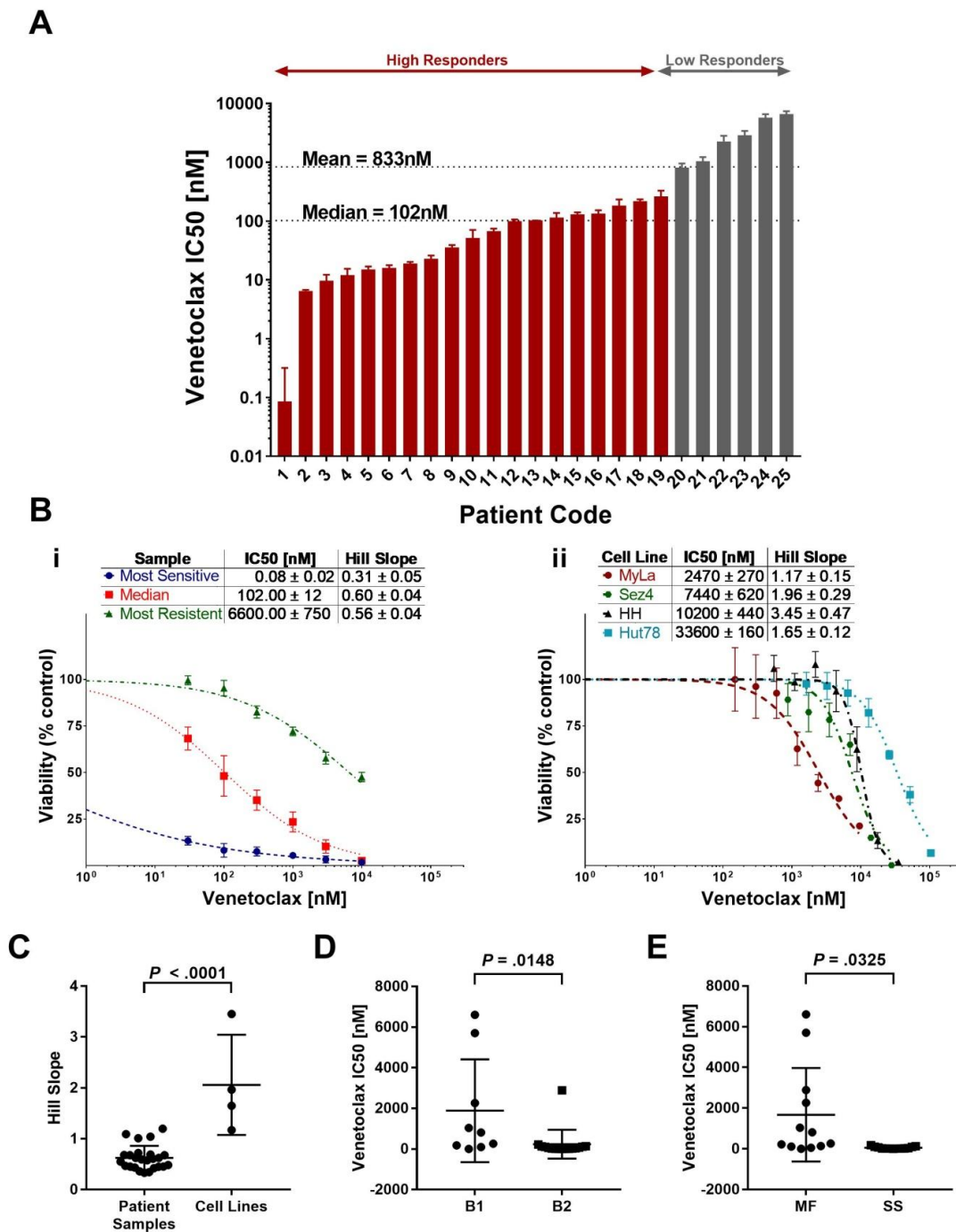


Figure 5. Cell line and patient samples demonstrate variable sensitivity to venetoclax. Isolated malignant cells from patient samples and 4 human-derived CTCL cell lines were incubated with a range of concentrations of venetoclax for 72 hours from which IC50s and Hill slopes were calculated. The median and mean IC50 for patient samples were 102nM and 833nM, respectively. (A) Patient samples in order of IC50. Patients were grouped into high responders and low

responders using hierarchical cluster analysis. (B) Representative dose response curves for patient samples (Bi), and CTCL cell lines (Bii). (C) Comparison of Hill slopes between patient samples and cell lines. The difference between them would suggest distinct methods of actions. (D) Patient samples are most likely to be sensitive to venetoclax in more advanced disease. Stage based on ISCL classification.¹⁴ (E) Patient samples classified as Sézary syndrome (SS) are more likely than mycosis fungoides (MF) patients to be sensitive to venetoclax.

Baseline BCL2 mRNA expression predicts in vitro sensitivity to venetoclax

To study the differences in response to venetoclax among both primary patient samples and cell lines, the baseline genetic expression of four BCL2 family members was measured using quantitative RT-PCR and expressed relative to the mean expression of the healthy controls (Figure 7A). Significant differences were noted in *BCL2* expression between high and low responders as well as high responders and cell lines, while none were observed between low responders and cell lines. Patient samples expressed lower amounts of *BCL2* than healthy controls, on average 29% less but this difference was not statistically significant ($P=0.19$). Also notable was the similar expression of *BCL2L1* (the gene encoding BCL-XL) among patient samples and cell lines, with both sets demonstrating no difference relative to the healthy controls. Significant differences were noted in the expression of *BCL2L11* (the gene encoding BIM) among cell lines, with high expression noted in Hut78, and low or undetectable expression of *BCL2L11* mRNA in the MyLa, Sez4, and HH lines (Figure 6A).

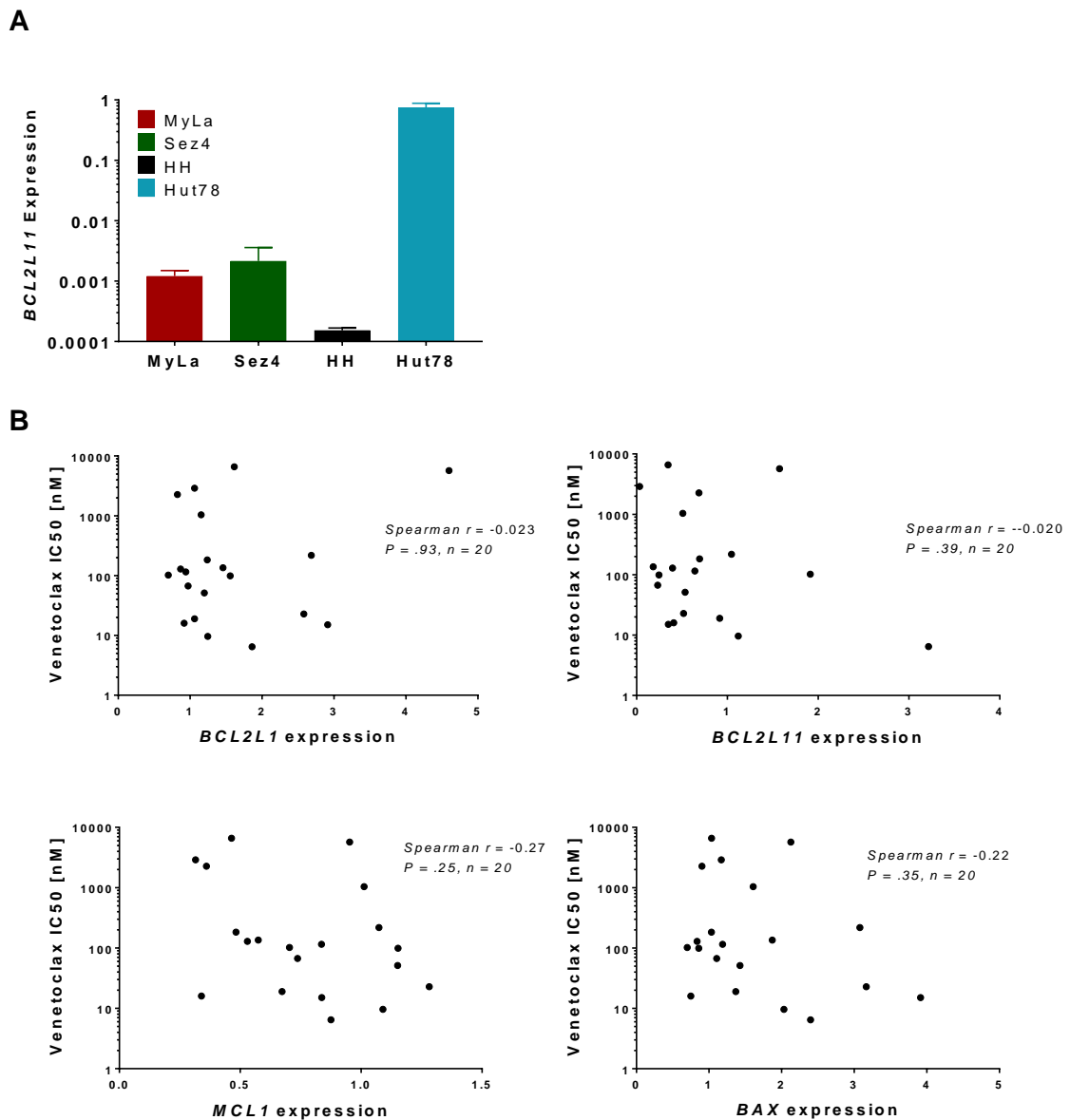


Figure 6. Baseline Gene Expression. (A) Baseline *BCL2L11* expression in the four tested CTCL cell lines, MyLa, HH, and Hut78, measured as a fraction of healthy controls. *BCL2L11* expression was not detected in the HH cell line after 40 cycles of RT-PCR. (B) Baseline expression of *BCL2L1*, *BCL2L11*, *MCL1* and *BAX* in primary patient samples did not correlate to sensitivity to venetoclax.

For the 20 patient samples examined, IC50 values were plotted as a function of relative expression of *BCL2* member mRNA. While a negative correlation was found between baseline expression of *BCL2* and IC50

(Spearman $r = -0.52$, $P = .018$) (Figure 7B), mRNA expression of other BCL2 family members (*BCL2L1*, *MCL1*, *BCL2L11*, *BAX*) did not correlate with the IC50 values (Figure 6B). Furthermore, we examined ratios of expression of *BCL2* to the other tested BCL2 family members and found that higher ratios of *BCL2* to *BCL2L1* correlate with greater sensitivity to venetoclax *in vitro*, while ratios of *BCL2* to *MCL1* and *BCL2* to *BCL2L11* do not (Figure 7C). These results point to the importance of both *BCL2* and *BCL2L1* expression, and indeed relative expression of one another, to the survival of malignant T-cells. Additionally, the limited correlations of both *BCL2* and the *BCL2/BCL2L1* ratio to sensitivity suggest that a more complex relationship between *BCL2* expression and inhibition of apoptosis may exist.

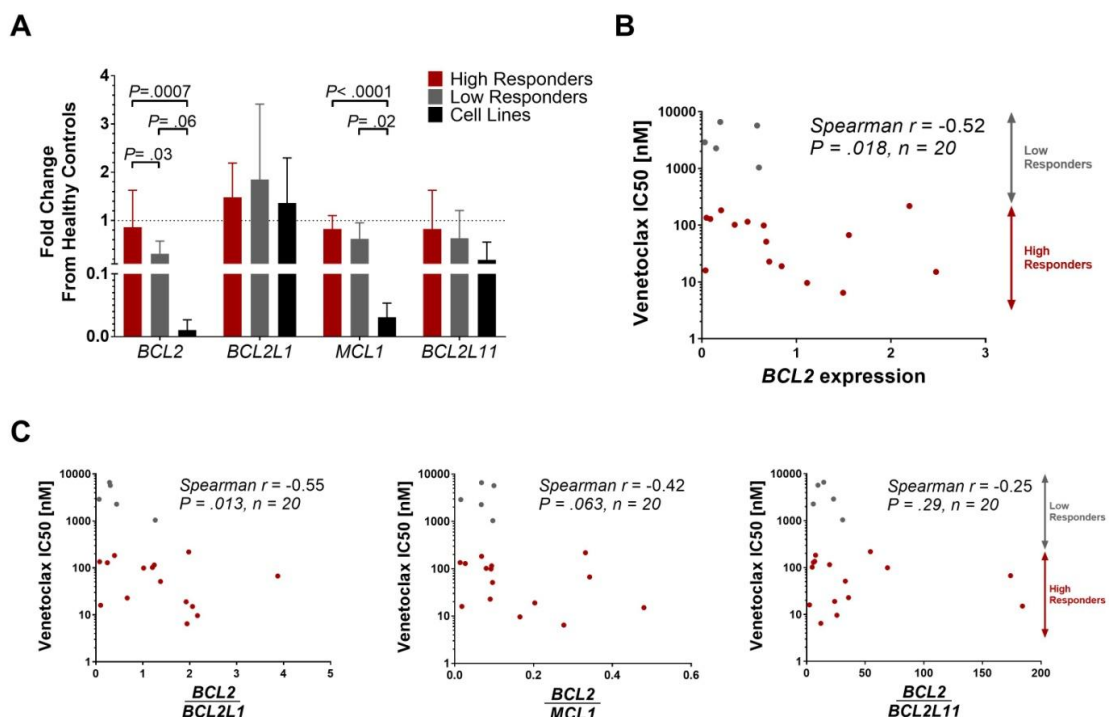


Figure 7. Baseline gene expression of BCL2 family members and correlation to IC50. *BCL2*, *BCL2L1*, *MCL1*, and *BCL2L11* were measured in 20 patient samples, 4 cell lines and 3 healthy controls. Results are expressed as a

fraction of the mean of the healthy controls. (A) Baseline expression of *BCL2* was found to differ significantly between high responders and low responders. No difference was found between low responders and cell lines. *BCL2L1* was expressed similarly among all four groups. Decreased *MCL1* expression was noted in cell lines compared to patient samples. (B) *BCL2* mRNA expression may predict response to venetoclax *in vitro*, with higher expression correlating to increased sensitivity. (C) Increased ratios of expression of *BCL2* to *BCL2L1* predicts higher sensitivity to venetoclax.

Combination inhibition of BCL2 and histone deacetylase results in synergistic killing of CTCL cells from patient samples

Fifteen of the CTCL patient samples were tested for synergy between venetoclax and two HDAC inhibitors, vorinostat and romidepsin. Each of the three drugs was tested individually and in combination and IC₅₀ values and Hill slopes were calculated. From these dose response curves, the combination index (CI) was calculated using the Chou-Talalay method in Microsoft Excel, with a CI < 1 indicating a synergistic interaction⁸⁰. For combinations of venetoclax with either HDAC inhibitor, synergy was observed at the higher dose range (Figure 8A,B).

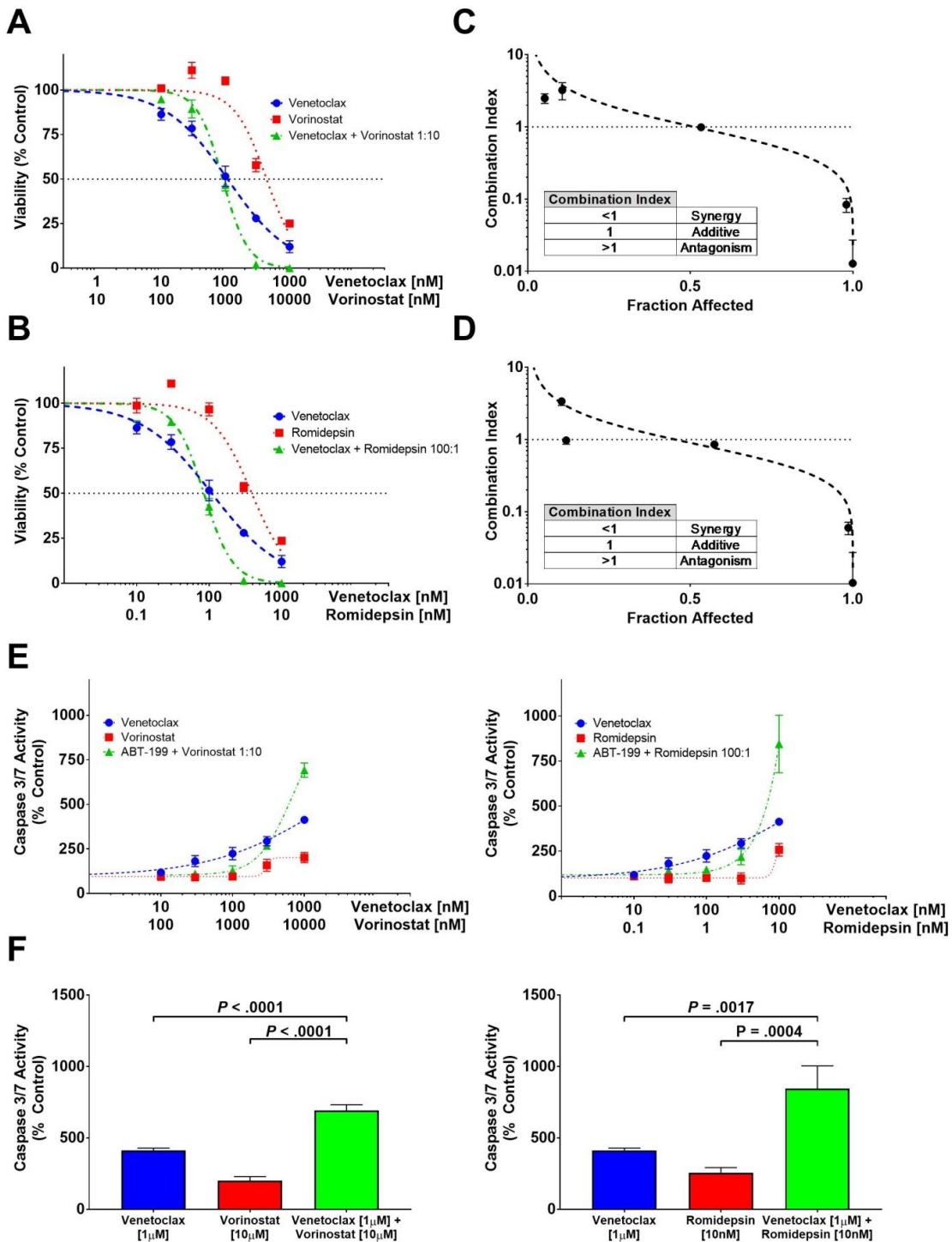


Figure 8. Combinations of venetoclax with either romidepsin or vorinostat demonstrate synergy in patient derived cultures. (A) Viability curves for venetoclax, vorinostat and their combination for Patient 11. After 72 hours of incubation the viability curves and the combination index were calculated using the Chou-Talalay median-effect equation. Means of the quadruplicates are

shown with standard deviations. (B) Viability curves for venetoclax, romidepsin and their combination for Patient 11. (C) CI and fitted curves for the combination of venetoclax and vorinostat for Patient 11. (D) CI and fitted curves for the combination of venetoclax and romidepsin for Patient 11. (E) Caspase-3/7 activation after 24 hours of incubation of the cell culture derived from Patient 11 with venetoclax, vorinostat, romidepsin and their combinations. All concentrations were studied in quadruplicate and plotted are the means with their respective standard deviations. (F) A significant increase in activation of caspase is seen in higher concentration combinations. Effects are similar for combinations of venetoclax with either vorinostat (Fi), and romidepsin (Fii).

93% of tested samples demonstrated synergy of response with combinations of venetoclax and vorinostat at 10% viability (0.9 fraction affected), while 73% demonstrated synergy of response between venetoclax and romidepsin (Figure 8C,D; Figure 9A; Table 3). No correlation was found between either expression of BCL2 or sensitivity to venetoclax and the degree of synergy (data not shown). Additionally, no differences were found between mycosis fungoides patients or Sézary syndrome patients and degree of synergy, for combinations of venetoclax with either HDAC inhibitor (Figure 9Bi,ii).

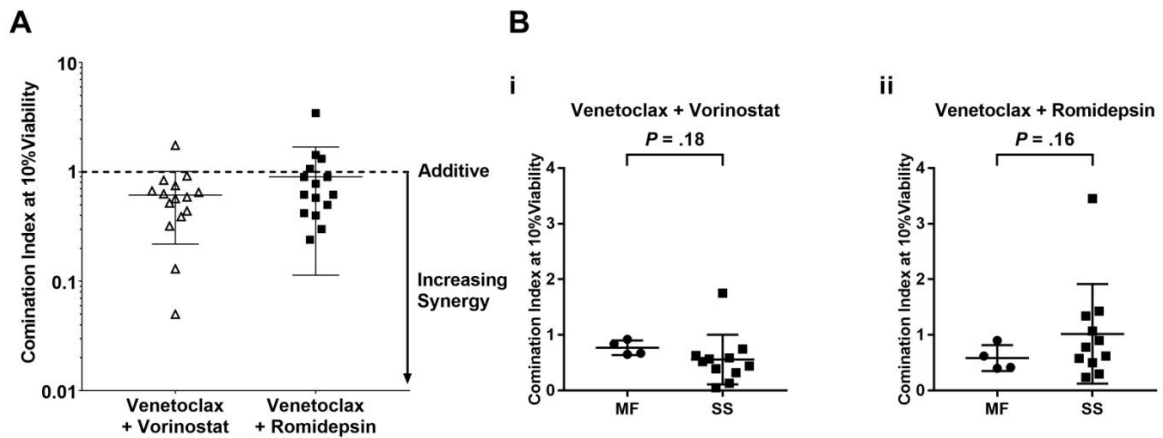


Figure 9. Summary of Combination Index data. For each of the 15 patient samples tested for synergy between venetoclax and either vorinostat or romidepsin the combination index was calculated at 10% viability (0.9 fraction affected). No correlation was found between the combination index and the diagnosis at original presentation. Each patient was classified as either Sez ar Syndrome (erythrodermic) or mycosis fungoides (non-erythrodermic).

In order to investigate the mechanism of action, the induction of apoptosis at 24 hours was measured by caspase 3/7 activation. Dose response curves were again produced for venetoclax, vorinostat, and romidepsin individually and in combination. The effect of combination therapy upon caspase 3/7 activity displayed a dose-dependent action (Figure 8E). Our measurements of caspase activation support the observations made with the cell viability assay regarding the form of interaction between venetoclax and the HDAC inhibitors, in which there are disparate effects at low and high drug concentrations. At low doses of venetoclax and either HDAC inhibitor, antagonism was observed. In contrast, we observed a significant increase in caspase 3/7 activity with combination therapy compared to either drug alone at high doses (Figure 8F).

Table 3: Calculated combination index at 10% viability (0.9 fraction affected) for each tested patient sample. Synergistic interactions are noted in green, while antagonistic interactions are noted in red, as per the legend (Adapted from Chou⁷⁸). 93% of samples displayed synergy between venetoclax and vorinostat. 73% of samples demonstrated synergy between venetoclax and romidepsin.

| | |
|---------------------|----------|
| Strong Synergy | <0.3 |
| Moderate Synergy | 0.3-0.7 |
| Weak Synergy | 0.7-0.9 |
| Additive | 0.9-1.1 |
| Weak Antagonism | 1.1-1.45 |
| Moderate Antagonism | 1.45-3.3 |
| Strong Antagonism | >3.3 |

| Pt # | Drug IC50 [nM] | | | Combination Index at 10% Viability (± SEM) | |
|------|----------------|------------|---------------|--------------------------------------------|-------------------------|
| | Venetoclax | Vorinostat | Romidepsin | Venetoclax + Vorinostat | Venetoclax + Romidepsin |
| 2 | 6.467 | 2569 | 2.310 | 0.75 ± 0.09 | 0.50 ± 0.05 |
| 3 | 9.607 | 1254 | 1.515 | 0.05 ± 0.24 | 0.24 ± 0.06 |
| 4 | 12.11 | 57.17 | 7.927 | 0.13 ± 0.05 | 0.90 ± 4.10 |
| 5 | 15.06 | 1813 | 6.759 | 0.63 ± 0.09 | 1.34 ± 0.20 |
| 6 | 15.98 | 2032 | 15.15 | 0.39 ± 0.06 | 0.62 ± 0.07 |
| 7 | 18.95 | 2202 | 2.066 | 1.75 ± 0.16 | 1.43 ± 0.29 |
| 8 | 22.86 | 1531 | 1.245 | 0.52 ± 0.07 | 0.78 ± 0.20 |
| 10 | 51.25 | 1468 | 1.764 | 0.92 ± 0.38 | 0.90 ± 0.27 |
| 11 | 67.19 | 4761 | 4.163 | 0.32 ± 0.03 | 0.30 ± 0.03 |
| 12 | 99.1 | 1486 | 8.556 | 0.59 ± 0.14 | 1.07 ± 6.28 |
| 13 | 102 | 2260 | 2.138 | 0.44 ± 0.10 | 0.58 ± 0.21 |
| 14 | 115.1 | 465.7 | 1.867 | 0.84 ± 0.22 | 0.40 ± 0.08 |
| 15 | 128.9 | 1266 | 3.884 | 0.57 ± 0.46 | 3.45 ± 29.51 |
| 21 | 1040 | 2160 | 1.357 | 0.67 ± 0.24 | 0.42 ± 0.10 |
| 23 | 2891 | 5387 | 2.577 | 0.65 ± 0.21 | 0.62 ± 0.49 |
| | | | % Synergistic | 93 | 73 |

Synergy studies were similarly performed on the cell lines (Figure 10). The only cell line to display significant synergy was MyLa 2059 when venetoclax was combined with vorinostat. In both the HH and Hut78 cells, venetoclax and

vorinostat had antagonistic responses. In combinations of venetoclax and romidepsin, only the HH cell line demonstrated synergistic sensitivity. Unlike the response of patient cells which are well modelled by the Hill and median-effect equations, the cell lines display significant deviations from the expected responses, especially at high doses. This may be attributed to the heterogeneity of the cell lines, which are not necessarily clonal, or to the highly cytotoxic effects of drugs such as romidepsin at higher doses.^{26,81} Thus, our ability to detect synergy at these dose ranges is limited.

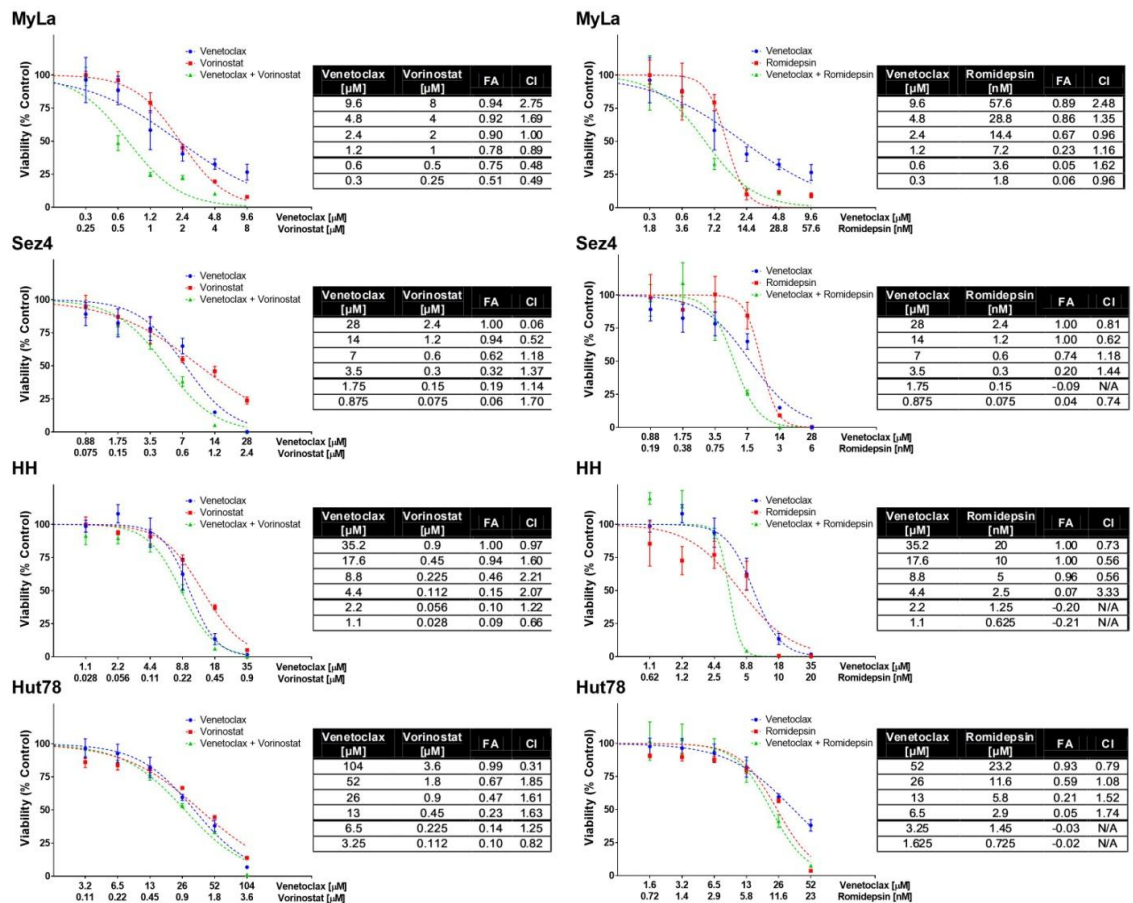


Figure 10. Searching for synergy in CTCL cell lines. Four CTCL cell lines were tested for synergy between venetoclax and vorinostat, and between venetoclax and romidepsin. Cell lines were first incubated with incremental increase of each of the three drugs individually to calculate IC50 values. Next cell

lines were incubated with combinations of each drug in an approximate 1:1 ratio of the calculated IC50 values. Synergy was noted between venetoclax and vorinostat in the MyLa cell line and between venetoclax and with high doses of romidepsin in the HH cell line.

BCL2 family member expression changes in response to HDAC inhibition

To provide insight into the mechanism of action of synergy and antagonism between venetoclax and the HDAC inhibitors romidepsin and vorinostat, we incubated the four CTCL cell lines for 24 hours with 5 μ M vorinostat or 5nM of romidepsin before isolation of RNA and measured the relative expression of 7 BCL2 family members compared to a DMSO vehicle control via qRT-PCR (Figure 11A). Following exposure to vorinostat there was a 40-fold increase in *BCL2L11* expression and a 12-fold increase in *BMF* expression. The dramatic increase in *BCL2L11* mRNA is critical to the function of BCL2, which acts to inhibit apoptosis via direct binding to BIM. This shift in balance towards pro-apoptotic factors is not only one of the methods of action of vorinostat, but sheds light on interactions between venetoclax and HDAC inhibition. Furthermore, we were able to detect a statistically significant trend between the change in expression of *BCL2* in response to vorinostat and the calculated combination index at a fraction affected of 0.9 (Figure 11B). MyLa, the only cell line in which *BCL2* expression was increased, was the only cell line to demonstrate significant synergy. Romidepsin caused a 13-fold increase in *BMF* and 4-fold increase in *BCL2L11* expression. No significant changes in expression of *BCL2* were noted following exposure to romidepsin in any of the three cell lines.

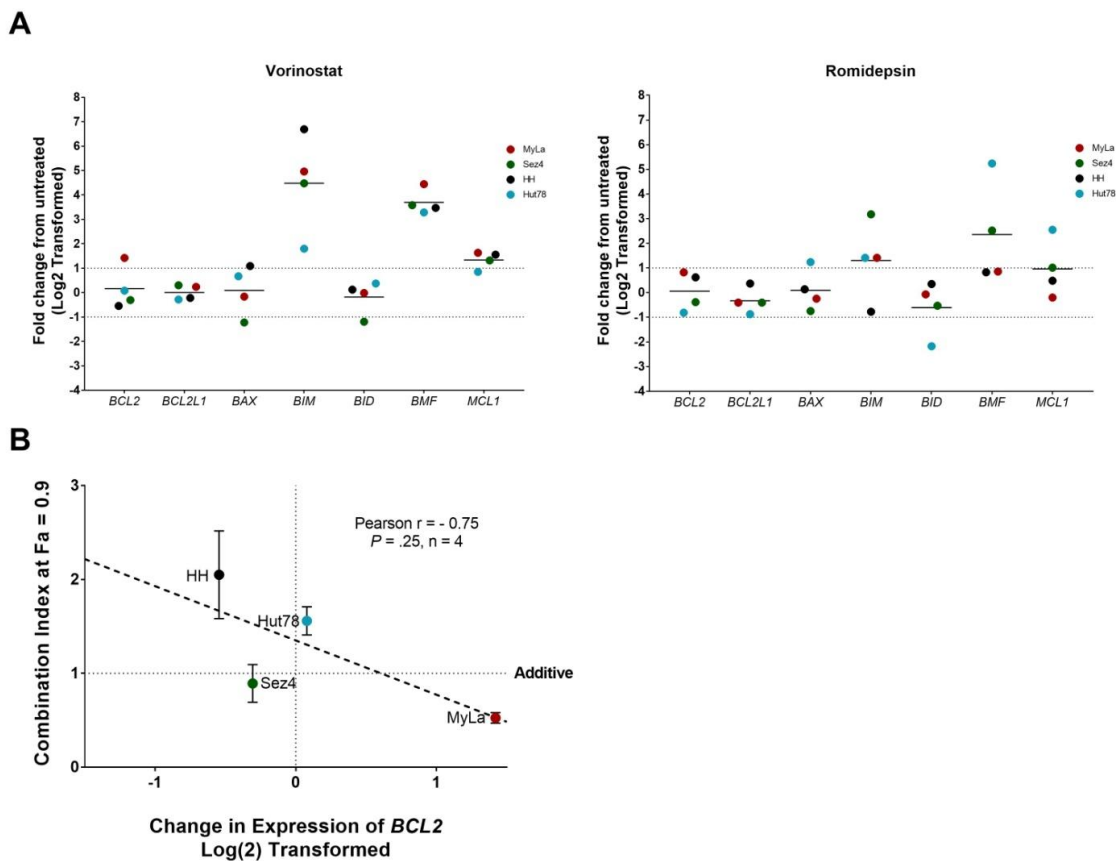


Figure 11. Changes in genetic expression of BCL2 family members following incubation with vorinostat or romidepsin. (A) Significant changes include the increased expression of *BCL2L1*, *BMF*, and *MCL1*. Notable is the 2.7-fold increase in *BCL2* expression in the MyLa cell line with vorinostat. (B) The amount of calculated synergy, represented by the combination index at 0.9 fraction affected (10% viability) was correlated to change in expression of *BCL2* after 24 hours of incubation with 5 μ M vorinostat, a strong correlation was noted, but only limited conclusions can be drawn, due to the small sample size.

Discussion

Guided by recent studies^{5,27,82-85} that suggest that increased resistance to apoptosis in CTCL is at least partly due to gene alterations that may increase the transcription of *BCL2*, and encouraged by the success of venetoclax in the treatment of CLL,⁸⁶ we sought in this study to explore the potential of venetoclax for the treatment of CTCL. We identified a group of patients, the high responders, with exquisite sensitivity to venetoclax at a mean IC50 of 79.0 nM. This range is comparable to the range of sensitivities, <300 nM previously observed in BCL2 dependent immature CD4- CD8- T-ALL subtypes. Three clinical trials⁸⁷⁻⁸⁹ investigating the treatment of CLL and NHL found the maximum plasma concentrations for 400mg, 800mg, and 1200mg to be 2.2 μ M, 3.5 μ M and 4.0 μ M. These values far exceeded the *in vitro* mean inhibitory concentrations observed in our study. We are further encouraged as the maximum tolerated dose of venetoclax has not yet been well established, necessitating dose escalation beyond 1200mg.^{90,91} Indeed, the most significant adverse events in the trials for CLL were deaths due to tumor lysis syndrome.⁹²

Having established the response of primary patient samples to venetoclax *in vitro*, we next searched for a biological assay that would predict sensitivity and distinguish high responders from low responders. BCL2 protein and mRNA expression have been found to have an inverse relationship with sensitivity to venetoclax *in vitro* CLL,⁹³ NHL,⁵⁹ and T-ALL.⁷⁹ Our data indicates that a similar relationship exists in CTCL, additionally supported by the significant difference in expression of BCL2 among high and low responders. Other work has shown that

expression of *BCL2L1* and *MCL1* contribute to resistance to venetoclax,^{60,70,72,94} but our work suggests that higher expression of either at baseline does not correlate with sensitivity in the patient samples tested.

Previous work^{79,95,96} with T-ALL has identified a wide variation in response to venetoclax among cell lines and primary patient samples, wherein T-ALL cells displaying the immature phenotypes CD4⁻ CD8⁻, such as LOUCY cell line, exhibit BCL2 dependence, and correspondingly submicromolar sensitivity *in vitro* to venetoclax. In contrast, cell lines with mature phenotypes demonstrate dependency for survival on the protein BCL-XL, and decreased sensitivity to venetoclax with greater than 1 μ M IC50 values. CTCL itself has been characterized by TCR sequencing⁹⁷ as a disease derivative of mature memory T-cells, from which one would predict dependency on BCL-XL. Direct measurements of mitochondrial membrane depolarization^{98,99} have highlighted the stark differences in responses to BH3 mimetics between BCL2 and BCL-XL survival-dependent cells. A recent study¹⁰⁰ looked at the BH3 profiles of T-cell lymphomas cell lines, including two examined in our study, MyLa and Hut78. The two cell lines were identified as BCL-XL dependent, which was confirmed in our study, as we measured similar expression of *BCL2L1* in all three cells as well as significantly decreased expression of *BCL2* and *MCL1*, relative to normal controls. These findings, along with our observation of poor sensitivity of the three cell lines to the BCL2 selective inhibitor venetoclax, suggest that some CTCL cases are BCL-XL dependent. This interplay of BCL2 and BCL-XL was additionally significant in primary patient samples where the majority of samples

had a higher expression of *BCL2* than *BCL2L1*, and a larger ratio predicted of sensitivity to venetoclax.

Synergy has been described between BCL2 inhibitors with a number of different therapies and in numerous malignancies.^{62,63,79,95,101–104} Here, we show evidence of synergy between venetoclax and two HDAC inhibitors vorinostat and romidepsin. Synergy was consistently observed at the higher dose ranges of either HDAC inhibitor indicating a dose-dependent mechanism. Not only did we detect antagonism at low doses, but there was some evidence of direct inhibition of caspase 3/7 activity with administration of either HDAC inhibitor alone. This is consistent with reports on both romidepsin⁶⁶ and vorinostat¹⁰⁵ which showed the effects of either drug differ dramatically at low and high dose, including one study where romidepsin was found to inhibit the production of reactive oxygen species at low doses.⁶⁸

The lack of synergy observed in the CTCL cell lines suggests, perhaps unsurprisingly, that expression of and dependence on BCL2 is required for synergistic interactions with other therapies. While in the cell lines the observed synergy can be linked directly to changes in BCL2 expression, the preferential effect observed with vorinostat and venetoclax is likely due to the greater degree of change in the expression of *BCL2L11*. Dissociation of BCL2-BIM complexes have been implicated as the primary mechanism of induction of apoptosis by venetoclax and other BCL2 inhibitors.^{21, 46, 68} The low baseline expression of *BCL2L11* in the HH and MyLa cell lines further supports the proposed theory that

change in expression of *BCL2L11* is critical to apoptosis by romidepsin, while baseline expression is non-essential to therapeutic effects.^{107,108}

In summary, this study supports the clinical implementation of venetoclax as a novel therapy for leukemic CTCL, and further demonstrates a synergistic effect when venetoclax is combined with the HDAC inhibitors romidepsin and vorinostat. The potential for an effective combination oral therapy with venetoclax and vorinostat is also intriguing. Further clinical trials are planned to explore this potential synergy in vivo.

Appendix

Transplantation in the Treatment of Primary Cutaneous Aggressive

Epidermotropic Cytotoxic CD8 Positive T-Cell Lymphoma

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Abstract

MicroAbstract: Several studies have suggested that hematopoietic stem cell transplant (HSCT) may provide a cure for Primary Cutaneous Aggressive Epidermotropic Cytotoxic CD8 positive T-Cell Lymphoma (CD8+ PCAETL). We summarize published literature on this disease and present outcomes of 8 patients with CD8+ PCAETL treated at our institution. In our experience, allogeneic HSCT and the novel agents brentuximab and pralatrexate demonstrate substantial activity against this disease.

Structured Abstract:

Background: Primary Cutaneous Aggressive Epidermotropic Cytotoxic CD8 positive T-Cell Lymphoma (CD8+ PCAETL) is a rare subtype of peripheral T-cell lymphoma with poor outcomes and without a standardized treatment strategy. Allogeneic hematopoietic stem cell transplantation (HSCT) has been suggested as a potential curative therapy.

Methods: We conducted a retrospective case series. We identified eight patients with the diagnosis of CD8+ PCAETL, four of whom also underwent allogeneic HSCT.

Results: Eight patients were treated at our center with combination chemotherapy and several novel agents, including HDAC inhibitors, Brentuximab, and Pralatrexate. Patients underwent a median of 8.5 treatments prior to HSCT. Six of the eight patients examined, including all four who received a HSCT, were alive at their last follow up.

Conclusion: Allogeneic HSCT is a promising treatment modality for CD8+ PCAETL. Given the aggressive nature of this disease and lack of sustained remission with currently available therapies, HSCT should be considered early in the course of treatment. Two novel agents, brentuximab and pralatrexate, demonstrated significant activity against CD8+ PCAETL, and may be incorporated earlier in the treatment course.

Introduction

Primary Cutaneous Aggressive Epidermotropic Cytotoxic CD8 positive T-Cell Lymphoma (CD8+ PCAETL) comprises 1% of all cases of cutaneous T cell lymphomas. The disease was given provisional status under the umbrella category Peripheral T-Cell Lymphoma Not Otherwise Specified (PTCL-NOS), in the 2005¹⁰⁹, 2008¹¹⁰, and 2016¹¹¹ WHO-EORTC classifications. The disease remains a diagnostic challenge as subtypes may lack markers such as CD8 and the $\alpha\beta$ -TCR^{112–114} originally thought to be essential to the diagnosis, and due to histopathologic mimickers of the disease such as lymphomatoid papulosis Type D¹¹⁵. PCAETL is characterized by disseminated ulcerative lesions, frequent extra-cutaneous involvement, and a high mortality rate. There exists no standardized treatment algorithm and treatment strategies vary widely among centers. Several studies and case reports have suggested that hematopoietic

Abbreviations: AE, adverse effects; AML, acute myeloid leukemia; ATG, *anti-thymocyte globulin*; AWD, alive with disease; AWOD, alive without disease; BEAM, carmustine, cytarabine, etoposide, melphalan; BMT, bone marrow transplant; BTX, bexarotene; Bu, busulfan; CD8+ PCAETL, Primary Cutaneous Aggressive Epidermotropic Cytotoxic CD8 positive T-Cell Lymphoma; CHASE, cyclophosphamide, cytosine arabinoside, etoposide, and dexamethasone; CHOEP, cyclophosphamide, adriamycin, vincristine, etoposide, prednisone; CHOP, cyclophosphamide, adriamycin, vincristine, prednisone; CR, complete response; D, dead; *DICE*, *dexamethasone, ifosfamide, cisplatin, etoposide*; DHAP, dexamethasone, cytarabine, cisplatin; DLI, donor lymphocyte infusion; DOL, death of lymphoma; ECP, extracorporeal photopheresis; EORTC, European Organisation for Research and Treatment of Cancer; EPOCH, etoposide, prednisone, vincristine, cyclophosphamide, doxorubicine; F, female; Flu, fludarabine; Gem, gemcitabine; GVHD, graft versus host disease; HDAC, histone deacetylase; HSCT Hyper-CVAD, cyclophosphamide, vincristine, doxorubicine hydrochloride, dexamethasone; ICE, ifosfamide, carboplatin and etoposide; M, male; MCEC, ranimustine, carboplatin, etoposide, cyclophosphamide; MIDDLE, methotrexate, etoposide, ifosfamide, mesna, l-asparaginase; MTX, methotrexate; mo, months; N/A, not applicable; ND: Not Documented; NM, nitrogen mustard NR: No Response; PD, progressive disease; PPX, prophylaxis; PR, partial response; ProMACE-CytaBOM, cyclophosphamide, doxorubicin, etoposide cytozar, bleomycin, vincristine, methotrexate and prednisone; PTX, pralatrexate; PUVA, psoralen ultraviolet light A; Romi, romidepsin; SCT, stem cell transplant; SMILE, dexamethasone, methotrexate, ifosfamide, L-asparaginase, etoposide; TBI, total body irradiation TSEBT, Total skin electron beam therapy; UCTH1, anti-human CD3; WHO, World Health Organization; XRT; radiation therapy; y, years.

stem cell transplant may represent a definitive, and potentially curative therapy^{112,116,117}. In this review we present our single-center experience of eight cases of CD8+ PCAETL, including 4 who underwent allogeneic stem cell transplantation, and a review of 18 published cases describing the use of transplant in these patients.

Methods

We performed a retrospective analysis of all patients with the diagnosis of CD8+ PCAETL treated at the Yale-New Haven Hospital between 2007 and 2017. Patients were included in the analysis if they met the clinical, histopathologic, and molecular criteria for CD8+ PCAETL defined by the 2016 WHO classification, including an epidermotropic proliferation of abnormal cytotoxic CD8+ lymphocytes. Two patients were previously reported as a part of a larger case series¹¹². Immunohistochemistry was obtained and included antibodies against the following: CD2, CD3, CD4, CD5, CD7, CD8, CD20, CD30, CD56, β -F1 TIA-1, and/or granzyme B. Demographic information, symptoms, treatment, outcome, and follow-up information were obtained and documented in Tables 1, 2, and 3. Yale University's Institutional Review Board granted exempt status for this retrospective case series.

We conducted a review of the literature to identify previously reported cases where bone marrow or stem cell transplants were used for the treatment of CD8+ PCAETL. A MEDLINE (Ovid), Embase (Ovid), and Web of Science search was performed using the search words "Primary Cutaneous Aggressive Epidermotropic Cytotoxic CD8 positive T-Cell Lymphoma", and the key words

“Cutaneous T-Cell Lymphoma”, along with “bone marrow transplant”, “allogeneic stem cell transplant”, “autologous stem cell transplant”, and “stem cell transplant”. Additional cases were identified using citation searches. A total of 18 cases were identified (Table 5).^{112,118–128}

Results

Patient Characteristics

We identified eight cases with a diagnosis of CD8+ PCAETL. A complete list of patient characteristics can be found in Table 1. The median age at time of diagnosis was 53 years (range 20 to 77 years). The most common clinical skin presentation was with multiple ulcerated lesions, with one patient unique in only having a single lesion, and another with no ulceration. Lesions were either patch/plaque or tumors, with two patients presenting with both. PET scanning demonstrated FDG avid lymphadenopathy in 5 of 8 patients. One patient had lymph node involvement confirmed by biopsy. A single patient presented with visceral involvement, with diffuse metastasis, including the brain. Bone marrow involvement was done in 5 patients and none showed involvement. Three of eight patients had presence of circulating neoplastic cells on flow cytometry.

Pathology

Pathology was consistent among the 8 patients and included presence of epidermitropism and atypical lymphocytes with hyperchromatic nuclei. Invasion of hair follicles was noted in 2 of 8 patients, though hair follicles were not present in all samples. Acanthosis was noted in 2 of 8 biopsies. Necrotic keratinocytes

with frank ulceration were seen in 4 of 8 samples. Infiltrates extended into the dermal papillae (3 of 8), superficial (5 of 8), mid (3 of 8), deep dermis (2 of 8), and superficial panniculus (1 of 8). Red cell extravasation and mild vascular ectasia were seen in 3 of 8 biopsies. Both small/medium lymphocytes (4 of 8) and large lymphocytes (4 of 8) were noted.

Immunophenotyping was performed on all samples (Table 3, 4). All 8 samples were found to be CD3+, and the majority (7/8) were CD8+ as well. This includes one sample that was found to be positive for both CD4 and CD8. One patient was CD4- and CD8-, though otherwise pathologically identical to the other samples. Samples showed variable expression of CD2, CD5, and CD7. In all samples TIA-1 (7/7) and granzyme (2/2) were expressed. All samples that were tested were positive for the cytotoxic marker β -F1, and negative for $\gamma\delta$ surface markers.

Treatment and Outcome

Treatment and outcomes for the 8 patients are summarized in Table 1 and 3, and more detailed information can be found for each individual patient in Table 4. Systemic chemotherapy was used in all but one patient. The only patient not to receive systemic therapy was the only patient without multiple, disseminated lesions. The tumor in this patient regressed following radiation therapy to the area. Other complete responses were achieved with total skin electron beam therapy (TSEBT) (1/4), allogeneic stem cell transplantation (SCT) (3/4), adriamycin, vincristine, prednisone (CHOP) chemotherapy (1/2), vincristine, doxorubicin, cyclophosphamide, and prednisone (EPOCH) chemotherapy (1/4),

gemcitabine based chemotherapy (1/5) and brentuximab therapy (1/4); however, durable responses were observed only with allogeneic SCT or brentuximab therapy. Of the 4 patients who were treated with brentuximab vedotin, 3 had CD30 staining in their tumor cells and the fourth was CD30 negative. Of the four patients who received an allogeneic SCT, two received a matched-related donor (MRD) transplant, one received an unrelated donor (URD) transplant, and one received a double unrelated umbilical cord blood transplant. One patient received two donor lymphocytes infusions. Conditioning among the four patients was either reduced intensity conditioning with fludarabine and buslfan (1/4), or standard myeloablative therapy with total body irradiation (1/4) with either pentostatin (1/4) or fludarabine and cyclophosphamide (1/4). Six patients remain alive, including all four who received a SCT. Four patients with active disease are on salvage chemotherapy and two are without evidence of disease. Of the two patients who died, one died as a result of complications from lymphoma with diffuse visceral involvement, and one due to sepsis. The median follow-up time was 40 months (range 6 to 57 months).

Tables

Table 1: Patient Cohort Characteristics

| Variable | n (%) |
|------------------------------------|------------|
| Sex (female, male) | 5, 3 |
| Median age, y (range) | 53 (20-77) |
| Presentation | |
| Single Lesion | 1 (12.5%) |
| Multiple Lesions | 7 (87.5%) |
| Patch/plaque | 6 (75%) |
| Tumor | 4 (50%) |
| Ulceration | 7 (87.5%) |
| Lymphadenopathy | 5 (62.5%) |
| Visceral Involvement | 1 (12.5%) |
| Bone marrow involvement | 0 (5) (0%) |
| Circulating tumor cells | 3 (37.5%) |
| B symptoms | 0 (0%) |
| LDH elevated | 1 (12.5%) |
| Therapies | |
| Median number of therapies (range) | 8.5 (1-13) |
| Chemotherapy | 7 (87.5%) |
| CHOP | 2 (25%) |
| EPOCH | 4 (50%) |
| DICE | 1 (12.5%) |
| Hyper CVAD | 1 (12.5%) |
| DHAP | 2 (25%) |
| SMILE | 1 (12.5%) |
| MIDLE | 1 (12.5%) |
| Gemcitabine | 5 (62.5%) |
| Pralatrexate | 5 (62.5%) |
| Bendamutine | 2 (25%) |
| Vinorelbine | 2 (25%) |
| HDAC inhibitors | 6 (75%) |
| Brentuximab | 4 (50%) |
| UCHT1 | 1 (12.5%) |
| Denileukin Diftitox | 3 (37.5%) |
| Alemtuzumab | 1 (12.5%) |
| Pembrolizumab | 1 (12.5%) |
| TSEBT | 4 (50%) |
| Radiation | 2 (25%) |
| Allogeneic Transplant | 4 (50%) |
| DLI | 1 (12.5%) |
| Status at Last Follow-up | |
| Alive without disease | 2 (25%) |
| Alive with disease | 4 (50%) |
| Dead of lymphoma or complications | 2 (25%) |
| Median follow-up time, mo (range) | 40 (6-171) |
| Overall Survival | 75% |

Table 2: Patient Cohort Pathology

| Immunophenotype Markers | Number positive (n) | % |
|--------------------------------|----------------------------|----------|
| CD2 | 2 (7) | 29 |
| CD3 | 8 (8) | 100 |
| CD4 | 1 (6) | 17 |
| CD5 | 4 (6) | 67 |
| CD7 | 3 (6) | 50 |
| CD8 | 7 (8) | 88 |
| CD30 | 2 (6) | 33 |
| TIA-1 | 7 (7) | 100 |
| Granzyme B | 2 (2) | 100 |
| $\gamma\delta$ -TCR | 0 (6) | 0 |
| β -F1 | 6 (6) | 100 |

Table 3: Summary of Treatment Response

| Treatment | Number of patients with Documented Response, either SD, PR, or CR (n) | % Response |
|------------------|------------------------------------------------------------------------------|-------------------|
| EPOCH | 3 (4) | 75% |
| Gemcitabine | 4 (5) | 80% |
| Pralatrexate | 3 (5) | 60% |
| HDAC Inhibitors | 0 (6) | 0% |
| Brentuximab | 4 (4) | 100% |
| TSEBT | 3 (4) | 75% |
| Allogeneic SCT | 2 (4) | 50% |

Table 4: Individual patient immunophenotype, treatment, outcome, current status

| Pt | Age at diagnosis, y | Immunophenotype | Treatment and Response | BMT | Conditioning | Current Status |
|----|---------------------|--------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------|---------------------------|----------------|
| 1 | 36 | <i>Positive:</i> CD3, CD4, CD8, CD30, TIA-1, Granzyme-B <i>Negative:</i> ND | CHOP (PR), bexarotene (PR), Vorinostat (PD), TSEBT (PR), Denileukin Diftitox (PR), gem/vinorelbine/doxorubicin (PR, d/c due to AE), EPOCH (PD), Romidepsin (PD), PTX (PD), Brentuximab/cyclophosphamid/adriamycin/prednisone (PR), HSCT (CR) | Allogeneic | Flu/BU | AWD |
| 2 | 77 | <i>Positive:</i> CD3, CD8, TIA-1, β -F1 <i>Negative:</i> CD2, CD4, CD5, CD7, CD20, CD30, CD56 | XRT (CR) | | | AWD |
| 3 | 52 | <i>Positive:</i> CD3, CD5, CD8, TIA-1, β -F1 <i>Negative:</i> CD2, CD4, CD7, CD30 | PTX (PR), XRT (PR), gem/doxorubicin (PR), TSEBT(PD), romidepsin (PD), ICE (PD), CHOP (CR), HSCT (relapse), bendamustine and brentuximab (SD) | Allogeneic | TBI | AWD |
| 4 | 54 | <i>Positive:</i> CD3, CD8 <i>Negative:</i> CD2, CD5 | TSEBT (PR), ECP (PD), Hyper-CVAD (PD), DICE (PD), XRT (PD) | | | DOL |
| 5 | 61 | <i>Positive:</i> CD2, CD3, CD5, CD7, CD45RO, CD56, TIA-1 <i>Negative:</i> CD4, CD8 | TSEBT (PR), PTX (PD), EPOCH (PR) | | | AWD |
| 6 | 50 | <i>Positive:</i> CD2, CD3, CD8, TIA-1, β -F1 <i>Negative:</i> CD4, CD7, CD30 | Vorinostat (PD), romidepsin (PD), denileukin diftitox/bexarotene (PR), gem (PD), alemtuzumab (PD), brentuximab (PR, d/c due to AE), peg-interferon/mechlorethamine (PD), PTX (PR), pembrolizumab (PD) | | | D, sepsis |
| 7 | 20 | <i>Positive:</i> CD3, CD5, CD7, CD8, TIA-1, β -F1 <i>Negative:</i> CD2, CD4, CD30 | PTX (PR), romidepsin (PD), gem (PD), UCHT1 (PD), EPOCH (PR), bendasmutine (PD), DHAP (SD), TSEBT (CR), MIDDLE (PR), gem/vinorelbine/doxorubicin, HSCT (CR) | Allogeneic | Cyclophosphamide /flu/TBI | AWOD |
| 8 | 65 | <i>Positive:</i> CD3, CD5, CD7, CD8, CD30, TIA-1, Granzyme B, <i>Negative:</i> CD2, CD4 | EPOCH (CR), romidepsin (PD), cyclophosphamide (PD), gem/oxaliplatin (CR), HSCT (relapse), EPOCH (PD), DHAP (PD), DLI (PD), brentuximab (CR) | Allogeneic, DLI | Pentostatin/TBI | AWOD |

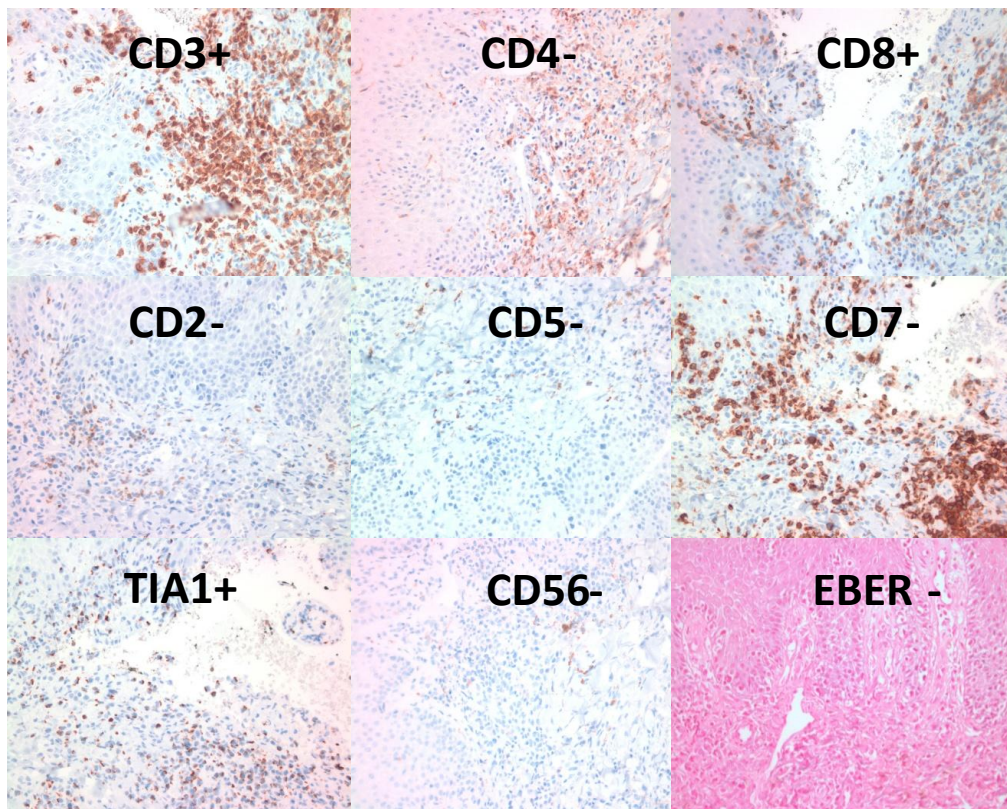
Table 5: Summary of cases from literature

| Reference | n | Type of transplantation | Age, years | Sex | Sx if specified | Immunophenotype | Previous Treatment | Conditioning | RR | Response duration, months | GVHD PPx | Current Status/Follow-up Information |
|--------------------------------|---|-------------------------|------------|-----|-----------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------|---------------------------------------|-------------|---------------------------|--------------------------------------------|-----------------------------------------------------------------------------------------------|
| Agnarsson, 1990 ¹¹⁸ | 2 | Autologous | 38 | F | Widespread papulonodules; chronic patches and psoriasiform plaques | <i>Positive:</i> CD3, CD5, CD7, CD8, Ia <i>Negative:</i> CD2, CD4, CD25, CD30, T9 | Interferon alfa 2b, topical NM, MTX | ND | CR, relapse | ND | ND | Relapsed, treated with PUVA, alive with stable disease 36mo after diagnosis |
| | | Autologous | 62 | F | Widespread papulonodules; chronic patches and psoriasiform plaques | <i>Positive:</i> CD3, CD5, CD7, CD8, <i>Negative:</i> CD2, CD4, CD25, CD30, T9, Ia | Topical NM, TSEBT, isotretinoin, interferon alfa-2b | ND | CR, relapse | ND | ND | Died 50mo after diagnosis |
| Berti, 1999 ¹¹⁹ | 1 | BMT | 31 | M | Widespread patches, plaques, papulo-nodules, tumors, necrotic lesions | <i>Positive:</i> CD3, CD7, CD8, CD45RA, TIA-1, TCR- β <i>Negative:</i> CD1a, CD2, CD4, CD5, CD25, CD30, CD45RO, Granzyme B, Perforin | Interferon alfa, ProMACE-CytaBOM | ND | NR | 0 | ND | Following BMT treated with TSEBT without response. Succumbed to disease 20mo after diagnosis |
| Liu, 2007 ¹²⁰ | 1 | Allogeneic | 44 | F | Generalized ulcerative lesions | <i>Positive:</i> CD3, CD7, CD8, CD25, TIA1, perforin, TCR- β <i>Negative:</i> CD2, CD4, CD20, CD30, CD56 | CHOP | Cyclophosphamide, TBI | CR | 17 mo | Tacrolimus, MTX | GVHD with total-body desquamation, resolved with oral prednisone |
| Webber, 2010 ¹²¹ | 1 | Autologous | 38 | M | Extensive ulcerative plaques and tumors | <i>Positive:</i> CD8, CD45A, β -F1 <i>Negative:</i> CD2, CD4, CD45RO | PUVA, interferon alpha, acitretin, XRT, TSEBT, gem | flu/ melphalan, alemtuzumab | CR | 2 mo | ND | Treated with lenalidomide following relapse without remission. Patient succumbed to infection |
| Kikuchi, 2011 ¹²² | 1 | Allogeneic, DLI | 6 | F | Disseminated skin lesions and left inguinal lymph node metastasis | <i>Positive:</i> CD2, CD3, CD5 (very weak), CD8, CD45RO (very weak), TIA, granzyme B <i>Negative:</i> CD4, CD7, CD20, CD30, CD45RA, CD56, TDT | CHOP, nelarabine containing chemotherapy with cyclophosphamide, vincristine, Ara-C, L-asparaginase and 6-mercaptopurine | etoposide, melphalan, TBI | NR | 0 | ND | Lesion remained followed transplant, treated with TSEBT, DLI, before succumbing to disease |
| Moritz, 2014 ¹²³ | 1 | Allogeneic | 27 | M | Generalized skin involvement and extensive lymph node infiltration | ND | PUVA, LD-MTX, acitretin, CHOEP, pentostatin, alemtuzumab, XRT | Fludarabine, melphalan, thiotepa, ATG | CR | 14 mo | Cyclosporine A, MTX/ mycophenolate mofetil | Complicated by bacterial colitis, EBV activation, sepsis |
| Wehkamp, 2015 ¹²⁴ | 1 | Allogeneic | 48 | M | Extensive ulcerative plaques and patches | <i>Positive:</i> CD8, TCR- β <i>Negative:</i> ND | CHOP | TBI, cyclophosphamide, ATG | CR | 36 mo | Cyclosporine | GVHD |
| Robson, 2015 ¹²⁵ | 1 | BMT | 31 | M | Ulcerated patches, plaques, nodules | <i>Positive:</i> CD7, CD8, CD45RA, MIB-1, β -F1 <i>Negative:</i> CD2, CD3, CD4, CD45RO | PUVA, chemotherapy | ND | ND | ND | ND | Dead |

| | | | | | | | | | | | | |
|-------------------------------------|---------------------------------------|------------|------------------------|----|-----------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------|------------------|-------------------|--------|------|-------------------------------------------------------------------------------------------------------------------------------------------------|
| Paton, 2016 ¹²⁶ | 1 | Autologous | 57 | M | Ulcerated plaques and tumors | <i>Positive:</i> CD3, CD8, CD7, CD56, β -F1 <i>Negative:</i> CD4, CD30 | Systemic chemotherapy | ND | ND | ND | ND | Dead |
| Guitart, 2017 ¹¹² | 6 | Allogeneic | 19 | F | Plaque, platch | <i>Positive:</i> CD3, CD5, CD7, CD8, CD45RA, TIA-1, Granzyme B, β -F1 <i>Negative:</i> CD2, CD4, CD30, CD56 | NM, TSEBT, romi, EPOCH, Gem, UCTH1, bendamustine, | ND | 5/6 CR | 46 mo | ND | AWOD |
| | | Allogeneic | 28 | M | Psoriasiform dermatitis | <i>Positive:</i> CD3 (partial), CD7, CD8, CD30+ in Large Cells, TIA-1, Granzyme B, β -F1 <i>Negative:</i> CD2, CD4, CD5, CD56 | TSEBT, BXT, romi, Liposomal doxorubicin, vorinostat, PTX, brentuximab, CHOP, EPOCH, gem, denileukin diftitox | ND | | 168 mo | ND | AWD |
| | | Allogeneic | 46 | F | Ulcerated nodule | <i>Positive:</i> CD3, CD5, CD8, TIA-1, β -F1 <i>Negative:</i> CD2, CD4, CD7, CD30, CD56 | Phototherapy, TSEBT, liposomal doxorubicin, PTX, brentuximab, gem, bendamustine | ND | | 24 mo | ND | AWD |
| | | Allogeneic | 31 | M | Necrotic ulcerations | <i>Positive:</i> CD3, CD7+ CD8, CD45RA, TIA-1, β -F1 <i>Negative:</i> CD4, CD5, CD30, CD56, Granzyme B | Hyper-CVAD, BXT, PUVA, | ND | | 84 mo | ND | AWD |
| | | Allogeneic | 63 | M | Patches, tumor | <i>Positive:</i> CD3, CD7, CD45RA, TIA-1 subset, Granzyme B, β -F1 <i>Negative:</i> CD4, CD5, CD8, CD56 | CHOEP, TSEBT, | ND | | 10 mo | ND | Dead |
| | | Allogeneic | 21 | F | Ulcerated plaque | <i>Positive:</i> CD2, CD3, CD7, CD8 (weak), CD45RA, CD56, TIA-1, Granzyme B, β -F1 (weak) <i>Negative:</i> CD4, CD5, CD30 | EPOCH, bendamustine, DHAP, SMILE, Gem, PTX, romi, XRT | ND | | 16 mo | ND | AWOD |
| | Plachouri, 2017 ¹²⁷ | 1 | Autologous, allogeneic | 47 | M | Disseminated ulcerative plaques | <i>Positive:</i> CD3, CD8, TIA-1, granzyme B <i>Negative:</i> CD4, CD30, CD45RO, CD45RA, CD56 | MTX, CHOEP, DHAP | BEAM, alemtuzumab | CR | 60mo | ND |
| Saruta, 2017 ¹²⁸ | 1 | Autologous | 38 | M | Multiple ulcerated erythematous lesions | <i>Positive:</i> CD3, CD8, TIA-1 <i>Negative:</i> CD4, CD20, CD30 | Etoposide, interferon gamma, CHOP | CHASE, MCEC | CR | 46 mo | N/A | Developed treatment related AML, treated with umbilical cord blood transplant, followed by relapse, allogeneic SCT, patient succumbed to sepsis |

Table 6: Treatment Algorithm for CD8+ PCAETCL

| |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Initial therapy for disseminated disease |
| CHOP, EPOCH, or other aggressive lymphoma regimen |
| If no complete remission achieved, second line regimens |
| Gemcitabine regimens Pralatrexate Brentuximab vedotin (if CD30+) Nelarabine, alemtuzumab, bendamustine Total skin electron beam radiotherapy |
| If complete remission achieved |
| Allogeneic stem cell transplant if patient is a candidate and has a donor (consider total skin electron beam radiotherapy prior to transplant) Minimal evidence to support role of autologous transplantation |

Figures**Figure 1:** Cytotoxic T cells are CD8+ with expression of cytotoxic markers such as TIA-1

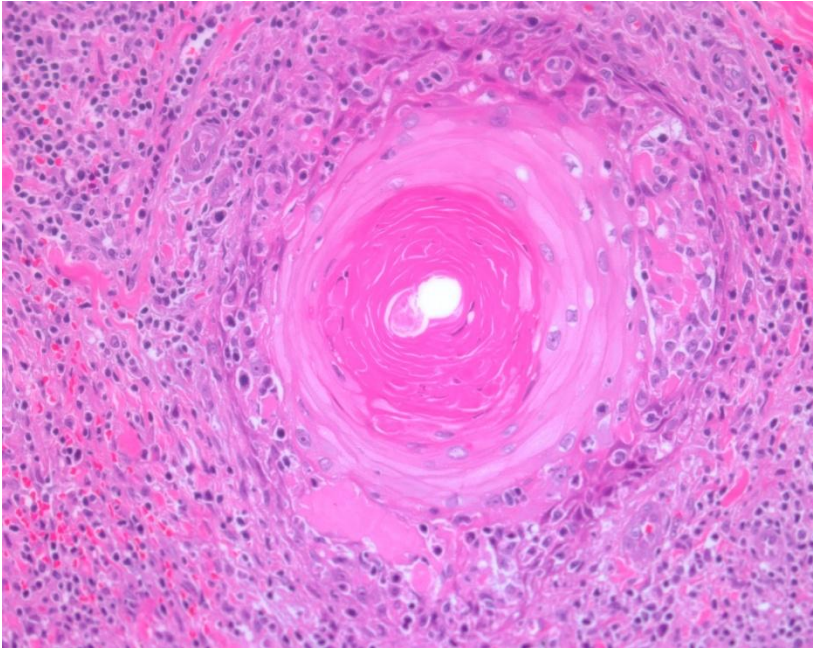


Figure 2: CD8+ PCAETL atypical lymphocytes involving hair follicle

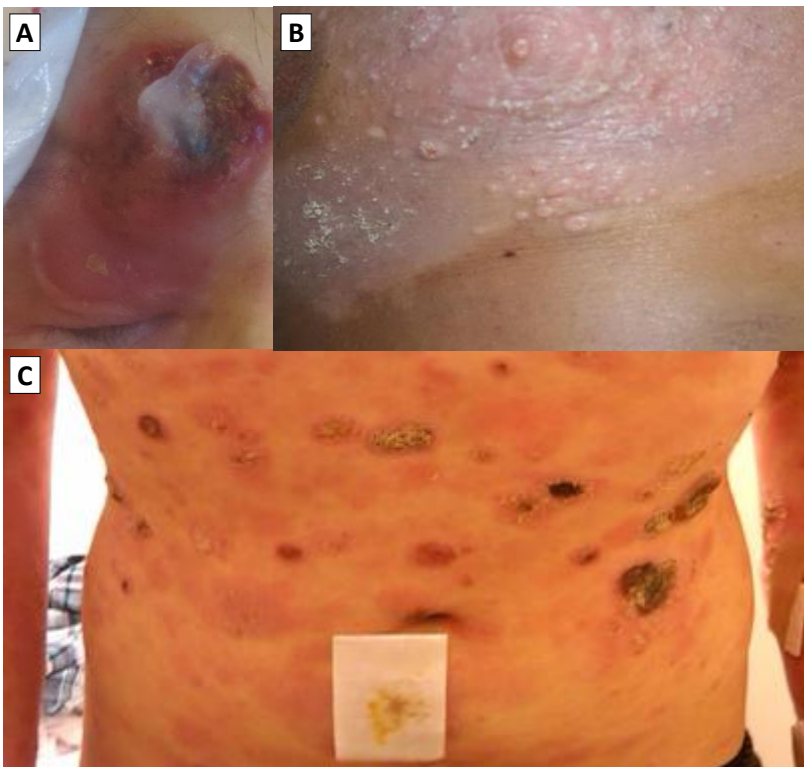


Figure 3: Variable presentations of CD8+ PCAETL (A) focal ulcer (Patient #2), (B) patch/plaque (Patient #1), and (C) erosive, extensive (Patient #7)

Discussion

A rapidly progressing form of cytotoxic CD8 positive T-Cell Lymphoma was first described in 1990 by Agnarsson et al¹¹⁸, and named formally by Berti et al¹¹⁹ in 1999. In the 1999 case series, 17 patients with CD8+ cutaneous lymphomas were described, including a subset of patients with epidermotropic, rapidly progressing, treatment resistant, and ultimately fatal disease. Since then, there have been over 100 cases described in the literature including the largest case series to date published in 2017^{112,129}. Difficulty in distinguishing CD8+ PCAETL from its more indolent imitators can lead to delayed diagnosis and poor outcomes, including published reports of patients dying prior to initiation of treatment^{112,116}. Additionally, the progressive nature and extreme severity of the disease has led to a paucity of data regarding effective treatments. There is little data documenting the response to novel agents such as HDAC inhibitors and targeted antibody toxins.

In the largest case study to date, Guitart et al. observed that allogeneic stem cell transplantation was the sole therapy that resulted in sustained response of any type¹¹². In order to define the role of transplantation in the treatment of CD8+ PCAETL we identified 18 published cases of stem cell transplantation. A summary of the findings can be found in Table 5. Of these, 3 occurred prior to the 2005 WHO classification. Of these 18 cases, 6 were autologous bone marrow or peripheral blood stem cell transplants, 11 were allogeneic stem cell transplants, and 2 were bone marrow transplants of undocumented origin. There was one published report of two transplantations in

the same patient, an autologous transplantation followed by an allogeneic transplant. The number of prior therapies ranged from 1-11, with a median of 3 prior therapies. Systemic chemotherapy was utilized in 16/18 cases, including all cases after 1990. Complete or partial response was achieved in 13 patients, 3 had no response, while 2 patients lacked documentation of response. Of the 13 responding patients, only 2 had a reported relapse, though follow up information was not reported or incomplete in some studies^{112,118,125,126}. Autologous transplantation resulted in durable response in 2/6 cases, while allogeneic transplantation resulted in durable response in 9/11 cases. One case was complicated by treatment related acute myelogenous leukemia which was treated with an umbilical cord blood transplant and a second allogeneic peripheral blood HSCT. The overall survival among patients receiving any type of transplant was 55.6% at a median follow up of 24 months.

In our series of 8 patients with CD8+ PCAETL, 4 underwent allogeneic stem cell transplantation, including one case of umbilical cord blood transplant. One patient received infusion of donor lymphocytes for post transplant relapse, and achieved remission with Brentuximab. All patients who received a transplant were alive at the last follow up. No patients achieved durable remission with any single or multi-agent systemic chemotherapy. Single agent HDAC inhibitor therapy did not result in responses. A notable finding was the relative activity of the novel agents pralatrexate and brentuximab vedotin in relapsed and refractory patients. Brentuximab vedotin, an antibody-drug-conjugate directed against the CD30 antigen, caused a partial or complete response in all three of the CD30

positive patients and resulted in stable disease in a CD30 negative patient by conventional immunohistochemistry.

Our limited series and review of the literature outline clinical challenges in the treatment of CD8+ PCAETL with conventional chemotherapeutic approaches. Unfortunately, many patients demonstrate resistance to multiple chemotherapy agents and regimens; however, the advent of new therapy modalities and the increased use of hematopoietic stem cell transplant has led to improved outcomes and survival. Table 6 outlines a treatment algorithm for PCAETCL based on available limited evidence. Our findings support implementation of an aggressive strategy early in the course of treatment, including the consideration of allogeneic stem cell transplant and donor search at the time of diagnosis. Further, the use of novel agents earlier in the course of therapy remains to be defined. Future studies should include molecular and genetic profiling of the tumor in an effort to identify mutations and altered pathways that may lead to a precision medicine approach for these patients.

Clinical Practice Points:

- CD8+ PCAETL is a rare, rapidly progressive, and deadly disease, for which no formalized treatment strategy exists, though HSCT have been suggested a potential curative therapy
- In this case series , four of eight patients underwent an allogeneic stem cell transplant, resulting in a complete and durable remission in two patients. All patients who received a transplant were alive at their last follow up (median 45.5 months).
- Brentuximab and pralatrexate demonstrated significant anti-disease activity in relapse and resistant cases of CD8+ PCAETL, with overall response rates of 100% and 60% respectively.

- Our study suggests that more aggressive use of HSCT, including early consideration and donor search at time of diagnosis, may result in better outcomes.
- Further research is necessary to better understand the activity of novel agents such as brentuximab and pralatrexate early in disease course.

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