Screening Novel Agent Combinations to Expedite CTCL Therapeutic Development

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TO THE EDITOR

Cutaneous T-cell lymphoma (CTCL) is a group of non-Hodgkin lymphomas of skin-homing malignant T lymphocytes. In advanced stages, CTCL is incurable and often fatal (Arlugon et al., 2008), and blood involvement portends a poorer outcome (Agar et al., 2010). Overall response rates to systemic therapies are 30–50% and generally not durable. High-throughput screening has emerged as a rapid method for identification and prioritization of novel therapeutic compounds. Analysis of natural compounds and agents in ongoing clinical trials may accelerate opportunities for drug repurposing, synergy testing, and preclinical assessment for CTCL therapeutic potential.

Patients with CTCL at the Yale Cancer Center (New Haven, CT) were enrolled with written and informed consent in accordance with the Yale Human Investigational Review Board. For initial screening, malignant cells were isolated from the peripheral blood of four patients with CTCL, as previously reported (Cyrenne et al., 2017; Kim et al., 2018). High-throughput screening was conducted on a 1,348 agent panel rich in phosphatase and kinase inhibitors and natural compounds (Selleckchem Kinase Inhibitors, Enzo Phosphatase Inhibitors, and MicroSource Gen-Plus libraries) at the Yale Center for Molecular Discovery (New Haven, CT). The 4,000–6,000

Abbreviations: CTCL, cutaneous T-cell lymphoma

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sorted cells per well were cultured in 384-well plates (Corning 3764) and cell viability assessed (Cell Titer-Glo, Promega, Madison, WI) 72 hours following drug addition. Positive and negative controls were 20% and 0.1% DMSO, respectively, and effective kill was determined with the equation:

\[
\text{Effective Kill} = 1 - \frac{\text{luminescence of drug addition well}}{\text{luminescence of negative control well}}
\]

Mean and SD of positive and negative control wells were used to quantify signal-to-background and Z values for each screening plate to ensure assay robustness. Drug data were normalized to the mean values of the negative control (set as 0% effect) and positive control (set as 100% effect) wells within the plate. Samples from four unique patients with CTCL were used as biological replicates to minimize the risk of false positive results.

Figure 1. Patient-derived CTCL samples, normal control CD4+ samples, and CTCL cell lines demonstrate variable sensitivity to single agents. Isolated malignant cells from patient-derived CTCL samples (n = 13), normal controls (n = 8), and established CTCL cell lines (n = 5) were incubated with a range of concentrations of sanguinarine, BIIB021, pyrvinium, quinacrine, ciclopirox, salinomycin, gentian violet, NVP-BGT226, and ceritinib for 72 hours, from which IC50 values were calculated. (a) Comparison of single-agent IC50 values. Patient-derived CTCL samples and normal controls had significant differences in response to salinomycin and gentian violet. Patient samples and cell lines had significant differences in response to BIIB021 and quinacrine. (b) Specificity Indices. Salinomycin, gentian violet, sanguinarine, and NVP-BGT226 had higher specificity indices reflective of higher potency of these agents against patient-derived CTCL cells than against normal control CD4+ cells. ALK, anaplastic lymphoma kinase; C.I., confidence interval; CTCL, cutaneous T cell lymphoma; IC50, half-maximal inhibitory concentration; K+, potassium ion; Na+, sodium ion; S.I., specificity index.
of selecting agents on the basis of stochastic variation.

For confirmatory and combination assessments, selected agents that demonstrated >80% effective kill in initial screening were further assessed using 13 patient-derived CTCL cells—sorted malignant cell samples (5 female and 8 male), 8 normal (CD4+ sorted) controls (6 female and 2 male), and 5 established CTCL cell lines (HH, Hut78, Sez4, SeAx, and MyLa2059). MyLa (MyLa2059) and SeAx were provided by E. Contassot (University Hospital, Zurich, Switzerland). HH and Hut78 were purchased from American Type Culture Collection, and Sez4 was provided by A. Rook (University of Pennsylvania, Philadelphia, PA). We have previously characterized genetic alterations in HH, Hut78, and Sez4 (Lin...
et al., 2012). Cells were tested for mycobacterial contamination by PCR in November 2019.

Cells were exposed to (i) seven agents with previous Food and Drug Administration approval for non-CTCL indications or available over the counter as natural compounds (sanguinarine, pyrvinium pamoate, quinacrine, ciclopirox, salinomycin, gentian violet, and ceritinib) and (ii) two agents in phase II clinical trials for other indications (BIIB021 and NVP-BGT226) individually and in combination. Mean inhibitory concentrations were calculated using GraphPad Prism (version 8.2.0). Specificity index (half-maximal inhibitory concentration for control divided by half-maximal inhibitory concentration for patients) and synergy combination index (Chou-Talalay method [Chou, 2010]) or fold potentiation were determined using Stata/SE (version 15). Normality was formally assessed using the Shapiro–Wilk test, and on the basis of the results, either a parametric or nonparametric one-way ANOVA test (with a correction for multiple comparisons by the Benjamini–Hochberg method) was applied to assess for statistical significance of agents showing clinical promise.

The primary screen yielded 95 agents that demonstrated over 80% effective kill (available on Mendeleo). Of these, nine agents were selected with previous Food and Drug Administration approval for non-CTCL indications or in advanced clinical trials.

Single-agent titrations identified the anthelmintic pyrvinium pamoate (x = 0.027 μM), antimicrobial gentian violet (x = 0.068 μM), NVP-BGT226, a phosphoinositide 3-kinase/mTOR dual inhibitor (x = 0.101 μM), and the antibiotic salinomycin (x = 0.405 μM) as having the lowest half-maximal inhibitory concentration values (i.e., greatest per molar cytotoxicity) against patient-derived CTCL cells (Figure 1 a) and that patient-derived CTCL cells were significantly more sensitive to salinomycin (specificity index = 6.380) and gentian violet (specificity index = 3.265) than control CD4+ cells (Figure 1 b). Patient-derived CTCL cells were relatively susceptible to the antimalarial quinacrine (x = 2.662 μM) and relatively resistant to the HSP-90 inhibitor BIIB021 (x = 29.970 μM) compared with CTCL cell lines (P ≤ 0.05). When these agents were tested in combination, the average Chou-Talalay indices revealed synergy in the patient-derived CTCL cells for salinomycin plus either ciclopirox, gentian violet, or pyrvinium pamoate and for gentian violet plus sanguinarine (Figure 2 a). For gentian violet plus sanguinarine, a statistical trend was observed in patient-derived CTCL cells compared with normal CD4+ control cells (P = 0.059). Patient-derived CTCL cells also showed greater potentiation with salinomycin or gentian violet plus NVP-BGT226 than normal control cells (P = 0.022 or 0.034, respectively) (Figure 2 b).

Our results build upon preclinical data demonstrating that gentian violet enhances CTCL apoptosis and impedes tumor cell proliferation in vitro (Wu and Wood, 2018) and has shown some potential as a novel, inexpensive topical therapy for recalcitrant lesions (Cowan et al., 2019). Herein, we identify another agent, salinomycin, that demonstrates almost twice of gentian violet’s specificity for patient-derived CTCL cells as a single agent. We also observe greater synergy and potentiation in patient-derived malignant CTCL cells than in normal controls for combinations of (i) salinomycin plus NVP-BGT226 and (ii) gentian violet plus sanguinarine or NVP-BGT226. The largest animal study on gentian violet to date helps reassure us of its safety and prompted its approval as an over-the-counter agent in the USA (Arisher, 2009). Of note, both gentian violet and salinomycin are antibacterial agents with reported activity against Staphylococcus aureus. Because anti-Staphylococcus antibiotic treatment has been shown to decrease malignant T cells in the lesional skin of patients with CTCL, these agents may have clinical benefit in addition to the direct cancer cell cytotoxicity effects reported here (Lindahl et al., 2019).

The responses of five established CTCL cell lines to single and combination agents tested were diverse, confirming our earlier observation that cell lines do not consistently recapitulate the pathophysiology of patients’ CTCL cells (Yumeen et al., 2020). Overall, our results suggest several individual drugs and combinations that may warrant further preclinical and clinical investigation as novel therapies for CTCL.

Data availability statement

Datasets related to this article can be found hosted at Mendeley (https://doi.org/10.17632/hpvnkrshdg.1).

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CONFLICT OF INTEREST

FMF is a Seattle Genetics speakers bureau consultant for Acrotech, Miragen, Daiichi Sanyo, and Mallinckrodt. The remaining authors state no conflict of interest.

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Conceptualization: FNM, SY, JML, MG; Data Curation: FNM, SY, JML, MG; Formal Analysis: FNM, SY, JML, MG; Funding Acquisition: MG; Investigation: FNM, SY, JML, ALOK, SU, YVS; Methodology: FNM, SY, JML, MG; Resources: KRC, FMF, MG; Supervision: MG; Validation: FNM, SY, JML, MG; Visualization: FNM, SY, JML, MG; Writing - Original Draft Preparation: FNM, SY, JML, ALOK, SU, YVS, FMF, MG

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Map3k1 Loss Cooperates with BrafV600E to Drive Melanomagenesis

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TO THE EDITOR

Precursor melanoma lesions harbor somatic alterations that activate the MAPK signaling pathway, most commonly through the driver oncoproteins BRAF and NRAS (Shain et al., 2018). The identification of the additional genetic events required to fully trigger malignant progression has been challenging because of the high mutation burden in cutaneous melanomas (Cancer Genome Atlas Network, 2015). Large-scale sequencing studies revealed that most of the mutations present in cutaneous melanomas carry a signature of UV-induced DNA damage, consistent with the observation that cutaneous melanoma commonly arises on sun-exposed skin (Shain and Bastian, 2016). We recently described how different patterns of exposure and UVR wavelengths can affect tumor incidence and survival in a transgenic BrafV600E melanoma mouse model (Trucco et al., 2019).

Whole-exome sequencing of the tumors in our BrafV600E model revealed recurrent mutations in the Map3k1 gene across all experimental cohorts. Specifically, in 78 melanomas from Brav/;Tyr::CreERT2 (BrafV600E) mice previously reported (Trucco et al., 2019), we found 37 nonsynonymous Map3k1 mutations in 34 samples (43.6%) (Figure 1a, Supplementary Table S1), including two tumors from non--UVR-exposed mice, three from mice exposed to UVA (350–400 nm), 10 from mice exposed to UVB (310–315 nm), and 19 from mice exposed to broadband UVR (280–380 nm) (Figure 1a). Overall, the tumors with Map3k1 mutations presented a higher number of missense single nucleotide variants than those without Map3k1 mutations (mean: 395.9 vs. 204.9 single nucleotide variants, \( P = 0.0291 \); Figure 1a, Supplementary Table S2). A total of 21 Map3k1 mutations (56.8%) in 19 samples were C-to-T nucleotide transitions at dipyrimidines, characteristic of UVR signature mutations (Figure 1a), but we did not find a correlation between the presence of mutations in Map3k1 and exposure to a particular UVR wavelength (\( P = 0.0549 \); Supplementary Table S2).

Map3k1 is unique in having both serine-threonine kinase and E3-ligase functions, allowing it to regulate protein phosphorylation and ubiquitin-mediated proteasome degradation (Sudderson and Gallagher, 2015). We found that 75.7% (28/37) of the mutations in Map3k1 affected the RING domain of the protein, which contains the E2-binding site for ubiquitin-conjugating enzymes (Figure 1b). The most recurrent codon alterations were at structural residues, including conserved cysteines 438, 454, and 483, involved in the coordination of zinc, and the proline 484, which are essential for function (Figure 1c and d) (Lu et al., 2002).

To investigate the role of Map3k1 in melanomagenesis, we generated Tvr::CreERT2/;Map3k1 (Map3k1fl/fl) mice to allow conditional deletion of Map3k1 in melanocytes following topical application of tamoxifen to the dorsal skin of juvenile mice. By itself, Map3k1 deletion did not induce any pigmented lesions or skin tumors in the mice (Figure 2a), indicating that the loss of Map3k1 alone is not sufficient to induce melanocyte proliferation. However, when Map3k1fl mice were crossed to mice carrying a conditional-inducible Braf/;Tyr::CreERT2/;Map3k1fl mice were more abundant (\( P = 0.011 \)) and larger (\( P < 0.0001 \)) than the nevi in BrafV600E mice

Abbreviations: ERK, extracellular signal--regulated kinase

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