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Novel therapeutic combination demonstrates more than additive effects in cutaneous T-cell lymphoma

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INTRODUCTION

Cutaneous T-cell lymphomas (CTCL) represent a group of non-Hodgkin's lymphomas comprised of skin-homing lymphocytes. Mycosis fungoides (MF), the most common form, is typically indolent, while Sézary syndrome (SS) is a less common, aggressive leukemic variant.¹

Early MF is often controlled with skin-directed therapies including topical steroids, chemotherapies, retinoids, phototherapy, and electron beam therapy.² Advanced MF and SS require systemic treatments including biologic response modifiers, extracorporeal photopheresis, or cytotoxic chemotherapy,² and relapses are common. Often, combination therapy is used to increase response rate and duration; however, there has been only one phase I study of combinatorial treatment in CTCL.³

Romidepsin is a histone deacetylase inhibitor (HDACi), which demonstrated a 34% response rate during a phase 2 study of CTCL patients who had received prior systemic therapy.⁴ Histone deacetylases (HDACs) modify chromatin by catalyzing the removal of acetyl moieties from lysine, producing chromatin condensation and silencing tumor suppressor genes.⁵ HDAC inhibition, thus, produces open chromatin structure and alteration of transcription resulting in cell cycle arrest, apoptosis, and cellular differentiation.⁶ Further, HDACs deacetylate structural proteins, transcription factors, and DNA repair enzymes,⁷ adding to the antitumor properties of HDACi. Though romidepsin is a promising single-

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CONFLICT OF INTEREST

The authors state no conflict of interest.

agent treatment, HDACi have also demonstrated additive or synergistic properties with other anticancer agents.⁵

Nitrogen mustard (NM, mechlorethamine hydrochloride) is a topical alkylating agent used to treat early stage CTCL with an overall response rate of up to 83%; however, this declines with disease progression.⁸ The alkylating effect of NM produces cellular DNA damage and results in apoptosis.⁹ Previous studies suggest closed chromatin structure can inhibit the formation of DNA interstrand cross-linking reactions by NM.¹⁰

We hypothesized chromatin remodeling following romidepsin treatment exposes sections of DNA that are normally protected from damage due to the effects of NM. To test our hypothesis we characterized the effects of NM and romidepsin on cell viability, apoptosis, and DNA damage. Here we present our findings demonstrating the efficacy of this combination.

MATERIALS & METHODS

Reagents

Romidepsin was provided by the UPMC pharmacy, dissolved in 80% propylene glycol and 20% dehydrated alcohol to 2mM, and stored at -20°C . Dilutions of 100 and 10uM were made in RPMI 1640 and stored at -20°C for two months. Mechlorethamine hydrochloride (Sigma Aldrich, St. Louis, MO), was diluted in sterile water to 0.1% and stored at -20°C . Fresh dilutions were made in sterile water.

Cells and cell culture

The cell line Hut78 (ATCC[®] TIB-161[™]), derived from an SS patient's peripheral blood, was obtained from American Type Culture Collection (Rockville, Maryland); analysis confirmed lack of cell line mycoplasma contamination. Four SS patients diagnosed according to WHO-EORTC criteria and three healthy controls were enrolled according to the University of Pittsburgh Institutional Review Board. Mononuclear cells were isolated from peripheral blood using Ficoll-Paque (GE Healthcare Bio-Sciences Corp, Piscataway, NJ) density centrifugation. Hut78 cells were grown in Iscove's Modified Dulbecco's Medium, and patient samples were grown in RPMI 1640 medium with interleukin-2 (BD Biosciences, San Jose, CA) 10 U/ml and interleukin-7 (BD Biosciences) 10ng/ml in a humidified incubator with 5% CO₂ at 37°C.

Cell viability

Cells were cultured overnight in a 96-well microplate divided into four groups: untreated, 1μM romidepsin, 0.0001% NM, and combination 1μM romidepsin/0.0001% NM. Each treatment was added 24 hours following culture initiation. At a predetermined endpoint (3, 6, 12, 24, or 48 hours), a CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega, Madison, WI) was conducted according to manufacturer's instructions. Plates were read using the Lmax Microplate Luminometer (Molecular Devices, Sunnyvale, CA) and SoftMax Pro Microplate Data Acquisition & Analysis Software (Molecular Devices). Each experiment was performed in quintuplicate and repeated a minimum of three times.

Annexin V binding staining

Annexin V binding analysis was carried out with an Annexin V-FITC Detection Kit (PharMingen, San Diego, California) according to the manufacturer's instructions. Patient samples were divided into four treatment groups: untreated, 1 μ M romidepsin, 0.0001% NM, and combination 1 μ M romidepsin/0.0001% NM and incubated for 24 hours. Samples were analyzed by FACScan flow cytometer (BD Biosciences), and 10,000 ungated events were acquired. Annexin V+PI- cells represent the early apoptotic populations. Annexin V+PI+ cells represent either late apoptotic or secondary necrotic populations.

Comet Assay

DNA damage following treatment was measured as described previously¹¹ using the OxiSelect™ Comet Assay Kit (Cell Biolabs, Inc., San Diego, CA). As NM is an alkylating agent, we modified the standard protocol to include gamma irradiation, inducing double strand breaks at points of alkylation. When current was applied to a single-cell agarose suspension, the damaged DNA formed a “comet tail,” visualized using the kit's Vista Green Dye. Images were captured using epifluorescence microscopy, and length and intensity of comets were assessed using the CometScore software package (TriTek Corp, Sumerduck, VA). The percent of DNA in each tail was assessed as the comet tail intensity divided by the intensity of the cell body and tail. The tail moment, the percent of DNA in the comet tail multiplied by length of the tale, indicates the degree of damage. To account for apoptotic cells included in each treatment group, the tail moment for each population was normalized to the cell viability of that population as assessed by Trypan blue staining (Life technologies, Grand Island, NY).

Statistical analysis

Unless noted, all experiments were performed in triplicate. Results were expressed as means $\pm$ SEM. Statistical significance was evaluated by Student's t test, and $p < 0.05$ was considered significant. For analysis of synergy in the viability assay, relative risk ratio (RRR) was used. Briefly, RRR is determined by calculating the ratio between the expected value (EV) of change in cell viability with two cytotoxic compounds based on their individual effects, and the actual value observed with the combination. EV is represented by formula: $EV = NA \times NB/100$, where NA and NB are the percentage of viable cells following treatment with compound A and B, respectively. RRR values less than 1 indicate a synergistic effect of the compounds; values equal to 1 indicate the mean additive effect of the compounds; while values greater than 1 represent an antagonistic effect.

RESULTS

Hut78 cell line and lymphocytes obtained from the patient and control samples were treated with romidepsin and NM alone and in combination *in vitro* at a clinically relevant concentration. Combination treatment with romidepsin and NM produced lower cell viability than either agent alone in the Hut78 cell line and all patient samples (Figure 1a, b). The relative risk ratio (RRR) of the combination treatment were 0.65 and 0.75 in the patient cohort and in Hut78, respectively, indicating the two drugs may demonstrate a synergistic effect in decreasing cell viability in culture. Treatment of control PBMCs with romidepsin

showed a mild decrease in cell viability of approximately 27%, with NM a larger decrease of 47%, and no significant difference between NM and combination treatment.

Cell viability dynamics differed between romidepsin and NM in a time-dependent fashion. NM-treated cells demonstrated reduced cell viability at 3 hours after treatment, and this reduction plateaued at 12 hours post-treatment (Figure 1c). Romidepsin treatment did not decrease cell viability until 12 hours post-treatment. This decrease continued to the 48-hour time point, resulting in a delayed but sustained effect, in agreement with romidepsin's role as an effector of gene transcription. Combination treatment produced both the early response of NM alone and the sustained response of romidepsin alone.

Moreover, combination treatment produced higher levels of apoptosis (measured with annexin V staining) than single-agent treatment (Figure 1d, e). Further, more of the apoptotic cells were both annexin V+ and PI+ in combination treated groups than in single agent groups, indicating that a larger percent of combination-treated cells were late apoptotic or secondary necrotic.

To assess severity of DNA damage due to combination therapy, we used a comet assay and measured the tail moment, an indication of the degree of damage as function of both the length and intensity of the tail. The percent of DNA in each tail was also increased with combination treatment compared with either agent alone (Figure 1f). Further, cells treated with romidepsin and NM had increased tail moments compared with no treatment in addition to either agent alone (Figure 1g).

DISCUSSION

As new therapies emerge for the treatment of CTCL, overall responses rates for new drugs remain around 30-40%.¹² To increase response, combination of agents continues to be a mainstay of CTCL treatment. However, a majority of the literature to date on combining drugs in CTCL is represented by case reports or small clinical cohorts,¹³⁻¹⁵ underscoring the need for substantiated prospective clinical trials.

Here, we provide *in vitro* evidence that combinatorial treatment with romidepsin and NM yields decreased cell viability and increased apoptosis in both primary SS cells and SS cell lines compared with either agent alone. The RRR values of combination therapy in both patient and cell line viability studies were less than 1 (0.65 and 0.75 respectively), supporting a more than additive effect. Using the comet assay, we demonstrate that combination romidepsin and NM increases DNA damage due to alkylation, supporting the hypothesis that romidepsin improves NM access to DNA.

Our study represents an important first step in rational design of the prospective clinical trial and supports further testing of combinational of HDACi and alkylating agents in CTCL.

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Figure 1. Combination therapy decreases cell viability, increases apoptosis, and increases DNA damage more than single agent therapy with NM or Romidepsin

Viability was measured following treatment with NM (0.0001%), Romidepsin (1 μ M, labeled R), or NM and Romidepsin (0.0001%, 1 μ M, labeled NM+R) at 24 hours post treatment in Hut78 cells as well as four SS patient samples and is presented as a percent of viable cells compared with untreated, control cells from each population. At 24 hours, viability was significantly decrease (* p <0.05) in both the cell line (a) and patient samples (b). Additionally, cell viability dynamics differed between treatment groups in a time-dependent fashion. Viability at 3, 6, 12, 24, and 48 hours is shown for a single patient sample performed in triplicate (c). Annexin and PI positivity were measured in four patient samples treated with NM (0.0001%), Romidepsin (1 μ M, labeled R), or NM and Romidepsin (0.0001%, 1 μ M, labeled NM+R) at 24 hours post treatment. A representative experiment from a single patient (d) as well as percent of Annexin positivity is shown (e). Cells treated with either combination or single agent NM or Romidepsin (R) therapy were used for the Comet assay in which electrophoresis creates a tail of damaged DNA that is subsequently bound by a fluorescent dye. To assess the damage in each group, percent of damaged DNA (f) and a tail moment (g) were calculated. In this figure, the percent of damaged DNA is shown as the percent above the untreated group.