AURORA A KINASE INHIBITION SELECTIVELY SYNERGIZES WITH HISTONE DEACETYLASE INHIBITOR THROUGH CYTOKINESIS FAILURE IN T-CELL LYMPHOMA

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Running title: Alisertib synergizes with romidepsin in TCL

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Abstract
**Purpose**: Aurora A kinase (AAK) is expressed exclusively during mitosis, and plays a critical role in centrosome duplication and spindle formation. Alisertib is a highly selective AAK inhibitor that has demonstrated marked clinical activity of alisertib across a spectrum of lymphomas, though particularly in patients with T-cell lymphoma (TCL). We sought to compare and contrast the activity of alisertib in preclinical models of B- and T-cell lymphoma, and identify combinations worthy of clinical study. High throughput screening of pralatrexate, the proteasome inhibitor (ixazomib) and the histone deacetylase (HDAC) inhibitor (romidepsin) revealed that only romidepsin synergized with alisertib, and only in models of TCL. We discovered that the mechanism of synergy between AAK inhibitors and HDAC inhibitors appears to be mediated through cytokinesis failure.

**Experimental Design**: A high throughput screening approach was employed to identify drugs that were potentially synergistic in combination with alisertib. Live cell imaging was used to explore the mechanistic basis for the drug: drug interaction between alisertib and romidepsin. An *in-vivo* xenograft TCL model was used to confirm *in-vitro* results.

**Results**: *In vitro*, alisertib exhibited concentration-dependent cytotoxicity in B- and TCL cell lines. Alisertib was synergistic with romidepsin in a T-cell specific fashion which was confirmed *in vivo*. Live cell imaging demonstrated that the combination treatment resulted in profound cytokinesis failure.
Conclusions: These data strongly suggest that the combination of alisertib and romidepsin is highly synergistic in TCL through modulation of cytokinesis and merits clinical development.
**Translational Relevance**

AAK overexpression has been demonstrated in hematological malignancies including TCL. Recently, selective inhibitors of AAK have been studied in the clinic and have been shown to be active across a spectrum of lymphomas, particularly in peripheral T-cell lymphomas (PTCL). Capitalizing on the activity in PTCL requires a systematic evaluation of alisertib’s activity with other T-cell active drugs, given none of these agents will be used exclusively as single agents in the future. Using a high throughput screening approach we demonstrated a highly selective synergistic interaction between alisertib and romidepsin in preclinical and clinical models of TCL. We show that the combination of alisertib and romidepsin synergize through induction of complete cytokinesis failure, generating a compelling rational for the clinical evaluation of this combination in patients with PTCL.

**Introduction**
The TCLs are a heterogeneous subset of non-Hodgkin’s lymphoma that exhibit a poor prognosis. Present treatment options for patients with relapsed/ refractory (RR) PTCL are largely palliative. Recently, a retrospective study of patients (N=153) with PTCL after first relapse or progression, who were not candidates for stem cell transplant, reported a median overall survival (OS) of only 5.5 months and a median progression free survival (PFS) of only 3.1 months (1). These findings underscore the need to not only identify novel drugs active in PTCL, but to think about how these agents might be configured in rational combination regimens. Since 2009, the U.S. FDA has approved pralatrexate, two HDAC inhibitors and the CD30 targeted immunoconjugate Brentuximab vedotin (Bv) for patients with RR PTCL and anaplastic large T-cell lymphoma (ALCL) (2). An intriguing feature of these drugs is their ‘apparent’ lineage specific activity, as pralatrexate and the HDAC-inhibitors have shown significant activity in patients with PTCL. Bv was approved in ALCL, the prototypical disease expressing CD30. Bv has also demonstrated activity in other malignancies known to express CD30, albeit not to the same extent seen in ALCL (3). While the activity of these agents in heavily treated patients is impressive, their lineage specific activity offers the prospect that they can form the basis of novel drug regimens with improved activity in PTCL.

Aurora kinases are a family of serine-threonine kinases (AAK, Aurora B and Aurora C kinases) that are highly expressed during mitosis, with very specific functions in cell signaling and mitotic division. AAK plays a critical role in chromosome maturation and separation as well as bipolar spindle assembly during G2/M phase of mitosis (4). High level expression of AAK is associated with centrosome amplification, mitotic
abnormalities, chromosomal instability, and malignant transformation (5). It has been shown that overexpression of AAK plays a role in the pathogenesis of various hematologic malignancies. Kanagal-Shamanna et al found that over-expression of AAK was detected in 68% of TCL cases including ALK+ and ALK- ALCL, PTCL-not otherwise specified (PTCL-NOS), cutaneous T-cell lymphoma (CTCL), T-cell lymphoblastic lymphoma/leukemia, and T-cell prolymphocytic leukemia, providing support for its role in T-cell lymphomagenesis (6). AAK inhibitors have been shown to exhibit unique activity in TCL and do not produce the neurotoxicity seen with other M-phase specific agents (7-9). Alisertib is a highly selective competitive inhibitor of the ATP binding site on AAK. The inhibition of AAK causes a mitotic spindle defect which leads to abnormal mitosis, initiating an accumulation of cells in G2/M and the development of polyploidization. Preclinical studies of alisertib in models of B-cell lymphoma (BCL) have demonstrated that a dose of 20 and 30 mg/kg administered daily for three weeks exhibited 100% inhibition of tumor growth (10). While these models support the broad activity of alisertib across many subtypes of BCL, virtually no preclinical data exist in TCL, where clinical development is largely focused.

We sought to systematically compare and contrast the activity of alisertib in panels of B- and TCL lymphoma, and screen for its potential synergy with other drugs active in PTCL, including ixazomib, pralatrexate, and romidepsin. We demonstrate that alisertib and romidepsin appear to exhibit a remarkably restricted pattern of synergy only in models of TCL, but not in BCL. To clarify the mechanistic basis for the synergy we developed a novel live suspension cell imaging technique to demonstrate that the combination of alisertib and romidepsin induces cytokinesis failure. These data report
for the first time that this combination exhibits lineage specific activity, which is confirmed in a novel cell based in vitro imaging assay. We believe these findings may create prospects for biomarker discovery efforts in the clinic.

**Materials and Methods**

**Cells and Cell lines.**

H9, HH, C5MJ, J.Cam 1.6, SUP-T1, Tib152, and CCL119 are TCL cell lines purchased from ATCC (Manassas, VA). SU-DHL6, SU-DHL2, Jeko-1, JVM-2, Z-138, Rec-1 are characterized BCL lines purchased from ATCC. DND41 is a T-cell line purchased from Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH (DSMZ) (Braunschweig, Germany). OCI-LY10 and OCI-LY7 are BCL cell lines from DSMZ. All cell lines were grown in RPMI 1640 (H9, HH, J. Cam 1.6, SUP-T1, Tib-152, CCL119, DND41, Jeko-1, JVM-2, Z-138, Rec-1, SU-DHL6, SU-DHL2) or IMDM media (C5MJ, OCI-LY10, OCI-LY7) with 10% Fetal bovine serum (FBS) (Life Technology, Grand Island, NY) and maintained at a concentration of 0.3 x 10⁶ cells/mL. All cell lines were authenticated from a hematopathologist including verification of morphology and immunophenotype (11-13).

**Materials.**

Reagents for western blotting were obtained from Bio-Rad laboratories and Invitrogen Life Technologies (Grand Island, NY). Dimethyl sulfoxide (DMSO) was obtained from Sigma Aldrich (St. Louis, MO). Drugs were obtained as follows: alisertib and ixazomib (MLN-2238) were provided by Takeda Pharmaceuticals, pralatrexate, and
romidepsin were obtained from the institutional pharmacy. All reagents for cell cycle and apoptosis analysis were obtained from Invitrogen Life Technologies (Grand Island, NY).

**Cytotoxicity Assays.**

For all in-vitro assays, cells were counted, incubated, and processed as previously described (11-15). Alisertib and ixazomib were diluted in DMSO to a final concentration of ≤ 0.01%. Romidepsin was diluted in 2 mL of 80% propylene glycol (USP) and 20% dehydrated alcohol (USP). Pralatrexate was diluted in 1 mL of diluent consisting of sodium chloride, sodium hydroxide, and hydrochloric acid to achieve an isotonic solution. For combination experiments, the final concentration of all drugs was selected to approximate the IC_{10}-IC_{30}. For all cytotoxicity experiments, Cell-Titer-Glo Reagent (Promega Corp, Madison, WI), a Synergy HT Multi-Detection Microplate Reader (Biotek Instruments, Inc) were used as previously described (11-14, 16). Synergistic interactions were measured using excess over bliss (EOB) as previously described (16-18).

**Flow Cytometry.**

Cells were seeded at a density of 3 x 10^5 cells/mL and incubated for 72 hours with alisertib and romidepsin, alone or in combination at concentrations approximating the IC_{10}-IC_{30}. A minimum of 1 x 10^5 events were acquired for each sample. To quantitate apoptosis, cells were stained with Alex Fluor 488/Annexin V and propidium iodine (PI) (Invitrogen #V13240) according to the manufacturer's instruction. Flow cytometry was performed on a FACS Calibur System and the data were analyzed with Flowjo 8.8.6.
software. Cells were considered in early apoptosis if annexin V positive but PI negative, late apoptotic if annexin V and PI positive, and dead if only PI positive.

**Cell Cycle Analysis.**

Cells were seeded at a density of $3 \times 10^5$ cells/mL and incubated with alisertib and romidepsin, alone or in combination with concentrations approximating the IC$_{10}$-IC$_{30}$. After 24 hours of incubation, cells were harvested and washed twice with 1 mL of cold phosphate buffered saline (PBS). Cells were then fixed with 70% histology grade ethanol for two hours. After the incubation period, cells were re-suspended in PBS and washed once. After cells were suspended in 1 mL of Triton 0.1x containing RNase A (Ambion #2286) and propidium iodide (Invitrogen #P3566) in a 1:50 dilution and kept at room temperature for 30 minutes. The fluorescence signal was acquired by FACS Calibur System and analyzed using Flowjo 8.8.6.
**Live Cell imaging**

Cells were plated onto 35-mm glass-bottom dishes (MatTek Corporation, Ashland MA) and partially synchronized with 1 mM nocodazole for 12 hours. Cells were then released from nocodazole and subjected to different drug treatments. Prior to live cell imaging, cells were incubated with Hoechst 33342 (1 mg/ml) for 30 min. Time-lapse microscopic images were acquired every 10 min in a 37°C, 5% CO2 chamber for 48 hrs using an inverted microscope (IX81; Olympus) with a 10 ×, NA = 0.6 dry objective lens (Olympus) and a monochrome CCD camera (Sensicam QE; Cooke), and processed using a Slidebook 5.5 software (Olympus).

**Western Blot Analysis.**

Cells were incubated with the IC10–IC30 of each drug alone (alisertib, romidepsin) and in combination (alisertib plus romidepsin) under normal growth conditions for 72 hours. Proteins from total cell lysates were resolved on 4% to 20% tris-glycine gel (Invitrogen #EC6028BOX) and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked in tris-buffered saline (TBS) containing 0.2% Tween and 5% skim milk powder. Membranes were then incubated overnight with specific primary antibodies. Antibodies were detected as previously described (12). All monoclonal and polyclonal antibodies used were from cell signaling.
In Vivo Tumor Model

In-vivo experiments were performed as previously described (11, 12, 14). Five to seven-week old SCID mice were injected with $5 \times 10^6$ cells/mL of HH, into the right flank subcutaneously with 50 μL of B.D. Matrigel (BD Biosciences, San Jose, CA). When the tumor volume reached an average of 50-100 mm$^3$ mice were randomly divided into four cohorts of 10 mice each (control 0.01% DMSO, alisertib 20mg/kg, romidepsin 1mg/kg, alisertib 20mg/kg + romidepsin 1mg/kg). Tumor volumes were assessed as previously described (11). Control mice were given 0.09% saline I.P. on days 1, 9, 16. Romidepsin was diluted in 0.09% saline given I.P. on days 1, 9, 16. Alisertib was diluted in saline and administered daily for 21 days by oral gavage. In the combination cohort, romidepsin and alisertib were administered at the same dose and frequency as the single agent cohorts. Control and drug treated mice received diluted DMSO concentrations of 0.01%.

Quantification of Alisertib and Romidepsin in Mouse Plasma and Tumor Tissue

To define and compare the pharmacokinetic (PK) profile of the two agents and their combination in blood, serial blood collections were performed at 30 mins., 1, 2, and 6 hours in two mice per cohort (two timepoints were taken from each mouse) after Day 16 of treatment. Tumor tissue was collected and harvested following 1 and 6 hours post-treatment. Romidepsin and alisertib were quantified in serum and tissue by extraction using Acetonitrile followed by liquid chromatography tandem mass spectrometry (LC-MSMS). Nine volumes of chilled acidified Acetonitrile (0.1% formic acid) were added to 100μl of serum or 400μl of aqueous tissue homogenate obtained
by disrupting 100mg of wet tissue using a tissue tearer homogenizer. The mixture was incubated at 4°C for 15 minutes followed by centrifugation at 13,000g for 10min at 4°C. The supernatant was transferred to an LCMS vial and evaporated under nitrogen stream. The extracted compounds were resuspended in sample buffer (40% methanol) for further analysis. A calibration curve was prepared by spiking untreated mouse serum spanning a range between 50pg/ml to 250ng/ml and extracted same as the samples.

The method was developed on a platform comprising an Eksigent UPLC 100 integrated to API 4000 tandem mass spectrometer controlled by Analyst 1.6 software (all from AB Sciex, Framingham, MA). Five μl was injected into a Phenomenex Kinetex C18 column (50x2.1mm, 1.7u, 100A) column preceded by a C18 guard column which was kept at 40°C. The flow rate was maintained at 200μl/min. The initial flow conditions were 60% solvent A (H2O containing 0.1% Formic acid) and 40% solvent B (Methanol with 0.1% FA). After a 1min loading time, Solvent B was raised to 95% linearly over 7 min and held for 30sec and back to initial conditions by 8 min and held constant until 10min for column equilibration and to avoid carry over. Romidepsin and Alisertib eluted at 4.37 and 6.09 min respectively. Both compounds were detected in positive electrospray ionization (ESI+) and multiple reactions monitoring (MRM) mode with optimized mass spectrometer parameters for both compounds. Romidepsin and Alisertib were detected at mass to charge transitions of 541.2>423.9 and 519.0>328.1. Concentrations of romidepsin and alisertib in all samples were calculated by comparing integrated peak areas against calibration standards. Single point standard addition method was used to assess the matrix effect of tumor tissue samples. Concentrations in tumor samples were corrected for any matrix effect.
Statistical Analysis

The Kaplan-Meier survival functions were calculated for each cohort using log-rank test to compare the median survival times among cohorts. Linear mixed model with random intercept was used to analyze log-transformed tumor burden in the *in-vivo* experiment. Predictors in the model include time, treatment group, and interactions between time and group. Statistical significance is denoted by $p$-value < 0.05.

RESULTS

**Concentration: Effect Relationship of Alisertib Across a Panel of B- and T-Cell Lymphoma Cell Lines.** Previously published reports have demonstrated an IC$_{50}$ of approximately 100 nM for alisertib in select BCL (19). Figure 1 presents the concentration effect relationships of alisertib across a broad panel of BCL (OCI-LY10, OCI-LY7, SU-DHL6, SU-DHL2, Jeko-1, JVM-2, Rec-1, and Z-138) and TCL (H9, HH, DND41, CCL119, J. Cam 1.6, SUP-T1, Tib 152, C5MJ) cell lines (Figure 1A-B). Alisertib exhibited a concentration and strong time dependent cytotoxic effect with the lowest IC$_{50}$ values achieved at 72 hours in the range of 10-100 nM and 60-1000 nM for B- and T-cell lymphoma cell lines, respectively (Figure 1 C). In addition, continuous exposure to alisertib was more cytotoxic than a 1 hour and 3 hour pulse in two TCL cell lines (data not shown). The exquisite impact of time is consistent with the cell cycle dependent effects of a M-phase specific drug, and suggests that area under the curve of exposure is critical in mediating cytotoxicity.
Alisertib Is Not Synergistic With Pralatrexate Or Ixazomib In B- And T-Cell Lymphoma Cell Lines. Single agent concentration: effect relationship curves were generated for pralatrexate and the proteasome inhibitor ixazomib (MLN-2238) in a panel of B- and T-cell lines. The 72 hour IC\textsubscript{50} values across a panel of B- and T-cell lymphomas for pralatrexate and ixazomib were in a range from 1 nM to 5 nM and 2 nM to 40 nM (ixazomib). Using the IC\textsubscript{10}-IC\textsubscript{20} of pralatrexate (0.75 nM and 1.5 nM) or ixazomib (10 nM and 15 nM) and the IC\textsubscript{10}-IC\textsubscript{30} of alisertib (50 nM, 100 nM, 1000 nM), we evaluated the drug: drug interactions in a schedule (drug A before B, drug B before drug A, and simultaneous exposure of drug A and drug B) and time dependent manner. The combinations of alisertib and pralatrexate in both BCL and TCL generated additive effects on cytotoxicity defined by excess over bliss (EOB) values less than or equal to 13 (Supplementary Figure 1 A-B). On the other hand when alisertib was combined with ixazomib, an antagonistic effect was observed across of the T-and B- cell lymphoma cell lines, with the EOB values in the range of -5 to 5 (Supplementary Figure 1 C-D). These data establish that in these models the combination of alisertib with pralatrexate or ixazomib does not produce a synergistic interaction in either B- or T-cell lymphoma cell lines.

Alisertib Is Markedly Synergistic With Romidepsin In In Vitro T-Cell Lymphoma Models But Not In In–Vitro B-Cell Lymphoma Models. Using the IC\textsubscript{10}-IC\textsubscript{30} of alisertib we evaluated the synergistic interaction using the IC\textsubscript{10}–IC\textsubscript{20} (approximately 1 nM - 2.7 nM) of romidepsin across a panel of in vitro T-and B-cell lines following 24, 48, and 72 hours of simultaneous drug exposure. The IC\textsubscript{10} - IC\textsubscript{20} of romidepsin was determined for all cell lines based on the single agent concentration: effect relationship curves. When
alisertib was combined with romidepsin in BCL, there was an additive to antagonistic interaction across all BCL cell lines evaluated (4 DLBCL and 4 MCL). Figures 2A and 2B depict two representative diffuse large B-cell lymphoma (DLBCL) cell lines (1 ABC, 1 GCB) indicating the additive to antagonistic interaction between alisertib and romidepsin following 72 hours of treatment.

Interestingly, there was a highly synergistic interaction of alisertib and romidepsin in TCL following 72 hours of drug exposure. Notably, the greatest synergistic interaction was observed in C5MJ, an alisertib resistant ATLL HTLV-1 Tax+ cell line (Figures 2C and 2D). The EOB values for all 8 TCL cell lines evaluated were greatest after 72 hours of drug exposure.

**Alisertib In Combination With Romidepsin Induces Polyploidy In T-Cell Lymphoma Cell Lines.** Romidepsin treatment has been shown to cause a G1 arrest through the up regulation of p21cip1/waf1 leading to the activation of the G1/S cell cycle checkpoint, while alisertib treatment inhibits AAK, leading to a defect in spindle formation and a G2/M arrest (20-22). We evaluated cell cycle arrest upon treatment of alisertib and romidepsin as single agents and in combination in 4 TCL cell lines (H9, HH, C5MJ, and DND41) following 24 hours of treatment. In all cell lines evaluated romidepsin induced a modest increase (≤ 3.2%) in the percentage of cells in G1 compared to control, while alisertib demonstrated a dose dependent increase in the percentage of cells in G2/M arrest following 50 nM and 100 nM of drug (in the range of 13-48%) when compared to control. The combination of alisertib and romidepsin induced a marked increase in polyploidy (in the range 10-42%), while inducing a significant decrease of cells in G1 relative to the control. The combination of alisertib...
and romidepsin at the IC$_{10}$ and IC$_{20}$ respectively, was the most potent in inducing polyploidy (up to 42%) (Figure 3 A-D). Following 48 hours of treatment virtually all cells were polyploid, with little distinction amongst the other stages of cell cycle (data not shown).

**Live Cell Imaging Of TCL Confirms Failure Of Cytokinesis With Alisertib And Romidepsin In TCL.** In order to explore the mechanism of action in more detail, we developed a live cell imaging method for suspension cells. Figure 4A depicts single cell images of all treatment conditions (control, 2 nM romidepsin, 100 nM alisertib, 100 nM alisertib + 2 nM romidepsin) after synchronization with nocodazole in the H9 cell line. The control cells underwent normal mitosis in approximately 40 minutes as depicted by images A-E. Following treatment with romidepsin increased apoptosis was observed within 24 hours of treatment (image F). Following treatment with alisertib, an accumulation of cells in G2/M was observed in about four hours (image I). These results remained consistent following 48-72 hours of treatment with either romidepsin (image G-H) or alisertib (images J-K) and support the cell cycle analysis data presented above. Interestingly, when alisertib was combined with romidepsin a spindle defect was observed inducing cytokinesis failure as soon as 15 hours following treatment (images L-N). Supplementary Figure 2 demonstrates the live cell imaging time-lapse video from 0 to 72 hours for all treatment conditions (control (A), romidepsin (B), alisertib (C), combination (D), respectively). Cytokinesis failure was confirmed after a corresponding increase in CENP-A protein levels following 72 hours of treatment with 100 nM of alisertib, which was augmented with the combination treatment (Figure 4B). CENP-A is a chromatin associated protein that is histone h3 variant and plays a role in the final
stages of cytokinesis. HDAC3 protein levels were evaluated following 72 hours of treatment with 50 nM or 100 nM of alisertib and 2 nM of romidepsin following both single agent and combination treatment (Figure 4B). HDAC 3 is known to deacetylate AAK preventing AAK from proteolytic degradation (23). A slight decrease in HDAC3 levels following 100 nM of alisertib and the combination treatment was appreciated in H9 cell line. This finding raises the prospect that the combination down regulates HDAC3, which has been shown to contribute to cytokinesis defects (24).

**Alisertib In Combination With Romidepsin Induces Apoptosis In TCL Cell Lines.** Apoptosis as measured by positive annexin V staining was analyzed in a TCL cell line following 72 hours of treatment with the IC	extsubscript{10} of romidepsin and IC	extsubscript{10-30} of alisertib both as a single agent or in combination (Figure 5A). Clearly there was an increase in apoptosis as a function of the alisertib concentration, with approximately 13% and 52% of apoptotic cells being observed following treatment with 2.7 nM of romidepsin and 50 nM of alisertib and 2.7 nM of romidepsin and 100 nM of alisertib, respectively. The induction in apoptosis corresponded with an increase in Caspase 3 activation and PARP cleavage, as well as an increase in the pro-apoptotic protein PUMA as well as a decrease in anti-apoptotic protein Bcl-xL (Figure 5B-C).

**Alisertib In Combination With Romidepsin Is Synergistic In an In-Vivo Xenograft Model of TCL.** The *in-vivo* activity of alisertib and romidepsin as single agents or in combination were evaluated in an *in vivo* xenograft model of TCL using the HH cell line. Figure 6A demonstrates that the combination cohort was statistically superior compared
to the single agent treatment and the control cohort (p< 0.05.) over time. Figure 6B establishes that the survival of the combination cohort was also statistically significant (p<0.05) when compared to the single agent treatment and control arms (by day 58). Pharmacokinetic (PK) analyses were used to determine the concentration of romidepsin and alisertib on day 16, 30 minutes, 1, 2 and 6 hours post-treatment (one sample collected/timepoint/treatment cohort). Figure 6C (top histogram) demonstrates that the single agent romidepsin concentration in plasma samples was approximately 60 nM at 30 minutes post treatment, and then decreased to 3 nM and 1 nM at 2 hours and 6 hours post-treatment, respectively. At 6 hours post treatment, the single agent romidepsin plasma concentration approximated the IC50, which is obviously less than the IC10-20 concentration used in-vitro. It is important to note that the PK profile of romidepsin was nearly identical in both single agent and combination cohorts. These data are consistent with previous PK analyses done in our lab with romidepsin in NOG mice (16). The alisertib concentration in plasma was in the range of 770 nM to 4600 nM in both the single agent and combination plasma samples and the concentration time pattern of the single agent was similar to that described earlier (10, 25) (Figure 6C bottom histogram). These concentrations approximated the IC70-80 in our in vitro studies. Interestingly, the plasma concentration of alisertib is still lower than that achieved in patients at the maximum tolerated dose (MTD). A 50 mg twice a day dose of alisertib produces a concentration of approximately 192 µM. Figure 6D presents the PK data evaluating the concentration of romidepsin (top histogram) and alisertib (bottom histogram) in tumor tissue on day 16 following 1 hour and 6 hours of treatment. The intratumor concentration of romidepsin was approximately 2 and 1 nM at 1 hour and 6
hour post-treatment respectively. These data are concordant with the single agent romidepsin concentration in plasma samples as well as our *in vitro* combination data and our previous PK analyses (16). Interestingly, the concentration of romidepsin at 1 and 6 hours was equivalent in plasma and tumor samples supporting rapid distribution of drug *in vivo*. In contrast, the combination tumor samples revealed the romidepsin concentration was at the lower detection limit of 0.3nmol/l per gram of tumor, with an average tumor size of 200mg. The single agent alisertib tumor concentration was 100 and 150 nM at 1 hour and 6 hours post-treatment, respectively. These concentrations are approximate the IC$_{35-40}$ at 72 hours in our *in vitro* analyses. Even though, the romidepsin concentration was at the detection limit of 0.3nmol/l per gram of tumor, the alisertib concentration in the combination tumor samples increased from 100 nM to 400 nM (1 hour post treatment) and 150 nM to 300 nM (6 hour post treatment) when compared to the single agent alisertib tumor samples. These data support the synergistic cytotoxicity of alisertib and romidepsin.

**DISCUSSION**

AAK is a serine threonine kinase that auto-phosphorylates at threonine 288. AAK plays a major role in the regulation of mitosis including targeting G2-M transition and DNA content. Although this role is well known the exact mechanism of action of how AAK regulates mitosis is still not clear. Marumoto *et al* have demonstrated that AAK phosphorylates histone 2b and histone 3 while maintaining its maximal kinase activity during M-phase (26). The activation of AAK is associated with activity of cyclin B-associated kinase which may suggest that AAK interacts with cyclin B1 to facilitate entry into mitosis. In addition, AAK inactivation occurs through DNA damage induced at the
end of G2. However, if AAK is overexpressed the G2 checkpoint will be abrogated and cellular proliferation will occur. While AAK plays a major role in G2-M, it is well known that HDAC’s play a role in inducing G1-S. HDAC inhibitors have been shown to alter kinetochore assembly through hyperacetylation of pericentromeric histones (23). Park et al have demonstrated that HDAC inhibitors induce degradation of AAK and Aurora B kinase (27). In addition to the cytokinesis failure demonstrated here, the data from Park et al. suggest that HDAC inhibitor mediated degradation of AAK could further complement the activity of the combination seen with an AAK inhibitor. The finding that the AAK inhibitor, alisertib, and an HDAC inhibitor were selectively active in the T-cell lineage over B-cell was unexpected. One potential explanation relates to the practical observation that HDAC inhibitors are highly active drugs in TCL with minimal activity in BCL. With 3 different HDAC inhibitors approved for clinical use all in essentially the same disease, there is irrefutable evidence that TCL are sensitive to this class of drugs. Another explanation could be related to data from Kretzner et al, suggesting that vorinostat in combination with a pan-aurora kinase inhibitor is synergistic in BCL due to a down regulation of c-MYC. C-MYC is a likely downstream target of aurora B kinase. Therefore it could be hypothesized that aurora B kinase would need to be inhibited leading to down regulation of c-MYC in order to see synergy in BCL (28). Despite this, in these model systems, the combination of alisertib and romidepsin consistently demonstrated substantially more activity in cell lines derived from T-cell malignancies compared to those derived from B-cell malignancies. What remains elusive however is that despite nearly 2 decades of preclinical and clinical research into these drugs, there
are still no good predictive biomarkers of activity in these diseases, likely owing to their pleiotropic properties.

What has been established is that HDAC inhibitors do cause chromosome segregation defects through pericentromeric heterochromatin. Taddei et al demonstrated that prolonged exposure to low concentrations HDAC inhibitors leads to relocation and alteration within the pericentromeric heterochromatin. Corroborating these findings, Ishtii et al demonstrated that HDAC3 uniquely localizes to the mitotic spindle during mitotic progression. When HDAC3 was knocked-down in HELA cells, the cells were unable to maintain a proper chromosome alignment due to defects within the mitotic spindles and kinetochore assembly (24, 29, 30). Using a unique live suspension cell imaging system we developed for these experiments, we demonstrated that the combination of alisertib and romidepsin induces cytokinesis failure following 15 hours of treatment. This result is supportive of the Taddei et al finding that longer exposure to low concentrations of HDAC inhibitors alter the pericentromeric heterochromatin, leading to improved therapeutic effects (24, 31). These findings are supported by the down regulation of HDAC3 and an increase in CENP-A. CENP-A is a chromatin associated protein that contains a histone H3 related fold domain which is regulated through AAK phosphorylation. CENP-A is required for the recruitment of centromeric proteins, proper kinetochore assembly, and chromosome segregation. After DNA replication and cytokinesis, CENP-A accumulates in the nucleosome region of replicated centromeres to maintain homeostasis between CENP-A protein levels and the epigenetic mechanism of chromatin assembly of the newly replicated centromeres (32, 33). If there is a defect in the completion of cytokinesis, then CENP-A will not
accumulate in the nucleosome region of the newly replicated centromeres and will accumulate in the cytosol as free CENP-A protein. Our data strongly support this as the primary mechanism of action for these two drugs, as cytokinesis failure results in an increase of CENP-A protein levels following treatment with alisertib and romidepsin.

Our in-vivo experiment demonstrates that combination treatment is statistically beneficial when compared to single agents in area under the curve and survival analysis. Interestingly, we found that the intratumor concentration of romidepsin following combination treatment was very low. There are a few reasons for this. First, the concentration of romidepsin was found to be at the lower detection limit of romidepsin is 0.3 nmol/l per gram of tumor with an average tumor size of 200 mg. It is possible that due to low concentration of intratumor romidepsin, the detection of the intratumor romidepsin following combination treatments was too low to quantify. Second, previous work by our lab and Bates et al demonstrated that romidepsin is a multidrug resistant (MDR) substrate and can induce the MDR, increasing the efflux of the drug (34). Although the basis for the increase in intratumor alisertib following combination treatment is unlikely correlated to the inhibition of the MDR since romidepsin is an inducer of the MDR, the data may suggest that romidepsin can selectively induce an influx pump pathway. This induction may lead to an increase in intratumor alisertib concentration following combination treatment when compared to the intratumor single agent alisertib which is observed in our PK analysis. It is important to note that even with low concentrations of romidepsin there was broad marked synergy seen in the combination cohort.
While our data demonstrate a selective synergy with an HDAC inhibitor, it establishes an additive or worse interaction with pralatrexate and ixazomib. The antagonism with pralatrexate is not that unexpected, given that exposure to a drug that induces G1/S arrest would preempt cells from entering mitosis, and thus nullify the impact of an M-phase specific AAK inhibitor. The antagonism observed when alisertib was combined with ixazomib was a little surprising, given that proteasome inhibitors have been shown to synergize with so many different classes of drugs, and are also known to induce mitotic catastrophe (11, 12, 35-37). Cha et al has demonstrated that panobinostat induced degradation of AAK and ABK (presumably through acetylation) through the ubiquitin-proteasome pathway (UPP) by directly targeting HDAC3 and HDAC6. Interestingly, when Cha et al treated HELA cells with the proteasome inhibitor MG132 there was marked suppression of panobinostat induced AAK and ABK depletion. The authors suggested that inhibition of the UPP led to accumulation of AAK, which could be a mechanism of resistance to MG132. Alternatively, it was been well established that proteasome inhibitors increase CDK inhibitors like p21 and p27, inducing a G1/S arrest. It is possible that some combination of these events could account for the observed lack of synergism.

Presently, there is a randomized phase III clinical trial with alisertib in TCL, versus dealers choice (pralatrexate, romidepsin or gemcitabine), and a Phase 1 study of alisertib and romidepsin (NCT01482962 and NCT01897012). Future preclinical studies will be focused on the biochemical effects of these drugs and determine if there are other mitotic agents that can more efficiently modulate mitotic proteins.
Acknowledgements: We are grateful to the Columbia University Lymphoma Research Fund for support. This work was supported in part by a grant from Takeda Millenium. We wish to thank University of Tennessee/West Cancer Center for research support for this project. This publication was supported by the National Center for Advancing Translational Sciences, National Institutes of Health, through Grant Number UL1TR000040.
References


Figure Legends

Figure 1: Alisertib Is Cytotoxic in T- And B-Cell Lymphoma. Cytotoxicity curves were generated for a panel of T-and B-cell lymphoma cell lines following 24, 48, 72 hours of treatment. (A) Concentration: effect of alisertib following 72 hours of treatment in four representative T-cell lymphoma cell lines (C5MJ, DND41, H9, HH). (B) Concentration: effect of alisertib following 72 hours of treatment in four representative B-cell lymphoma cell lines (LY10, SU-DHL6, LY7, Jeko-1). (C) IC₅₀ values were generated for alisertib following 24, 48, 72 hours of treatment in a panel of T- and B- cell lymphoma cell lines.

Figure 2: Alisertib In Combination With Romidepsin Is Synergistic In T-Cell Lymphoma But Not In B-Cell Lymphoma. The IC₁₀-₂₀ of romidepsin (R) was used in combination with 50 nM, 100 nM, 1000 nM (IC₁₀-₃₀) of alisertib (A) to evaluate drug: drug interactions for up to 72 hours of treatment. (A-B) Following 72 hours of simultaneous drug exposure, synergy was not observed in a representative panel of B-cell lymphoma cell lines. EOB values were in the range of -20.03 to 5.83, signifying an antagonistic drug:drug interaction. (C-D) Following 72 hours of simultaneous drug exposure, synergy was observed in a panel of T-cell lymphoma cell lines with excess over bliss (EOB) values ranging from -9.05 to 21.01.

Figure 3: Combined Treatment Of Alisertib And Romidepsin Induces Polyploidy In TCL Cell Lines Following 24hrs Of Treatment. Cells were stained with propidium iodide following 24 hours of treatment with an IC₁₀-₂₀ of romidepsin (R) and 50
nM or 100 nM of alisertib as a single agent or in combination. (A) H9, TCL cell line, demonstrates percentage of cells in G1, S, G2/M, or polyploid (B) HH TCL cell line demonstrates percentage of cells in G1, S, G2/M, or polyploid (C) C5MJ, ATLL HTLV-1 cell line, demonstrates percentage of cells in G1, S, G2/M, or polyploid (D) DND41, T-ALL cell line, demonstrates percentage of cells in G1, S, G2/M, or polyploid.

Figure 4: Combined Treatment of Alisertib And Romidepsin Induces Cytokinesis Failure.

(A) Live cell imaging was used following 24 hours of treatment with 2 nM of romidepsin and 100 nM of alisertib both as a single agent and in combination in H9, TCL cell line. Cells were imaged from 0 hours- 72 hours. (a-e) images from control H9 sample demonstrate an H9 cell going through the stages of mitosis (Prophase, Metaphase, Anaphase, Telophase, Cytokinesis). Each stage is depicted every 10 minutes, for a total of 40 minutes. (f) Following 24 hours of 2 nM romidepsin treatment H9 cells begin to display apoptotic effects depicted by the fragmented cells. (g-h) Following 48 and 72 hours of romidepsin treatment (respectively) there was an increase in fragmented cells, however no mitosis defect was observed. (I) Following 24 hours of 100 nM alisertib treatment, H9 cells demonstrate a multinucleated form depicted by the arrow. (j-k) Following 48 and 72 hours of alisertib treatment (respectively) the cell was shown to be arrested, no longer entering mitosis. (L) Following 24 hours of combination treatment (100nM alisertib + 2 nM romidepsin) the H9 cell demonstrated cytokinesis failure. (m-n) Cytokinesis failure was observed following 48 hours and 72 hours of treatment, respectively. (B) Evaluation of CENP-A protein levels.
following 72 hours of treatment with 2 nM of romidepsin (R), 50 nM of alisertib (A), 100 nM of alisertib (A) as a single agent of in combination. Evaluation of HDAC3 protein levels following 72 hours of treatment with 2 nM of romidepsin, 50 nM of alisertib, 100 nM of alisertib as a single agent of in combination.

Figure 5: Alisertib In Combination With Romidepsin Induces Apoptosis Following 72 Hours Of Treatment. (A) Following 72 hours of 2.7 nM romidepsin (R), 50 nM alisertib (A), 100 nM alisertib (A), and combination 50 nM A+R, 100 nM A+R, we analyzed apoptosis by staining for annexin V/Propidium iodide (P.I) as measure by facs. (B) Western blot analysis was used to confirm apoptosis in H9, TCL cell line. Following 72 hours of R, 50 nM A, 100 nM A, 50 nM A+R, 100 nM A+R. (C) Western blot analysis was used to confirm apoptosis in HH, TCL cell line. Following 72 hours of R, 50 nM A, 100 nM A, 50 nM A+R, 100 nM A+R.

Figure 6: Alisertib In Combination With Romidepsin Is Synergistic In A Xenograft Model Of Cutaneous T-Cell Lymphoma. HH, TCL cell line was injected into SCID mice. Control mice were injected I.P. with 0.01% DMSO (N=10). Romidepsin 1 mg/kg was given I.P. day 1,9,16(N=10). Alisertib 20 mg/kg was given orally once a day from day1-21(N=10). Combination mice were followed the same treatment schedule as single agents(N=10).Cycle 1 ended on day 21, and Cycle 2 began on day 23 and ended on day 42. (A) Combination mice showed a statistically significant(P<0.05) log-transformed tumor burden when compared to the control cohort over time (top table) and single agents over time (bottom table). (B) Kaplan-Meier survival curve demonstrates combination mice surpassed survival of all other cohorts and demonstrates statistical significance when compared to
control and single agents (P<0.05). (C) PK analysis on plasma samples was performed following 30 mins, 1 hour, 2 hours, and 6 hours of treatment. The top histogram depicts romidepsin (R) concentration for all timepoints evaluated while bottom histogram depicts the alisertib (A) concentration. (D) PK analysis on tumor samples was performed on samples following 1 hour and 6 hours of treatment. Romidepsin intratumor concentration is depicted in the top histogram while alisertib intratumor concentration is depicted in the bottom histogram.
Figure 1

A. T-Cell Lymphoma

![Graph showing cell survival and drug concentration for T-Cell Lymphoma subtypes.]

B. B-Cell Lymphoma

![Graph showing cell survival and drug concentration for B-Cell Lymphoma subtypes.]

C. IC$_{50}$ Values For Alisertib (nM) In A Panel Of B And T-Cell Lymphoma

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Impact of Time

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Figure 3

A. H9 (TCL)

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B. HH (TCL)

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C. C5MJ (ATLL HTLV-1 TAX+)

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D. DND41 (T-ALL)

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Figure 4

A.

- A. Control 0 min
- F. 2 nM Romidepsin 24h
- I. 100 nM Alisertib 24h
- L. 100 nM Alisertib + 2 nM Romidepsin 24h
- B. Control 10 min
- G. 2 nM Romidepsin 48h
- J. 100 nM Alisertib 48h
- M. 100 nM Alisertib + 2 nM Romidepsin 48h
- C. Control 20 min
- H. 2 nM Romidepsin 72h
- K. 100 nM Alisertib 72h
- N. 100 nM Alisertib + 2 nM Romidepsin 72h
- D. Control 30 min
- E. Control 40 min

B.

- Control
- 2 nM Romidepsin (R)
- 50 nM Alisertib (A)
- 100 nM Alisertib
- 2 nM R + 50 nM A
- 2 nM R + 100 nM A

- CENP-A
- HDAC 3
- β-actin
Figure 5

A. Alisertib In Combination with Romidepsin, 72hr DND41

B. H9 (CTCL)  
- Romidepsin (R)  
- 50 nM A  
- 100 nM A  
- 50 nM A + R  
- 100 nM A + R

C. HH (CTCL)  
- Romidepsin (R)  
- 50 nM A  
- 100 nM A  
- 50 nM A + R  
- 100 nM A + R

% Apoptotic Cells

Drug Concentration (nM)
Figure 6

A. Mean Tumor Burden (mm³) over Days of Treatment

B. Kaplan-Meier survival estimates

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C. Plasma Romidepsin Concentration (nM)

D. Plasma Alisertib Concentration (nM)
Clinical Cancer Research

Aurora A Kinase Inhibition Selectively Synergizes With Histone Deactylase Inhibitor Through Cytokinesis Failure In T-Cell Lymphoma


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