Aurora A Kinase Inhibition Selectively Synergizes with Histone Deacetylase Inhibitor through Cytokinesis Failure in T-cell Lymphoma

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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-cell lymphoma (BCL) and TCL, 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 used 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 vivo results.

**Results:** In vitro, alisertib exhibited concentration-dependent cytotoxicity in BCL and TCL cell lines. Alisertib was synergistic with romidepsin in a T-cell–specific fashion that 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. *Clin Cancer Res;* 21(18); 4097–109. ©2015 AACR.

Introduction

The T-cell lymphomas (TCL) are a heterogeneous subset of non-Hodgkin’s lymphoma that exhibit a poor prognosis. Present treatment options for patients with relapsed/refractory (RR) PTCL (peripheral T-cell lymphoma) (peripheral T-cell lymphomas) 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 FDA has approved pralatrexate, two histone deacetylase (HDAC) inhibitors and the CD30-targeted immunonconjugate brentuximab vedotin (Bv) for patients with RR PTCL and anaplastic large T-cell lymphoma (ALCL; ref. 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). Although 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 and colleagues (6) found that overexpression 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. 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 that leads to abnormal mitosis, initiating an accumulation of cells in G2 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 3 weeks exhibited 100% inhibition of tumor growth (10). Although 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 BCL and TCL, 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 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 the ATCC. SU-DHL6, SU-DHL2, Jeko-1, JVM-2, Z-138, Rec-1 are characterized BCL lines purchased from the 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 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. DMSO was obtained from Sigma-Aldrich. 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.

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 IC10–IC30. For all cytotoxicity experiments, Cell-Titer-Glo Reagent (Promega Corp.), 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 × 105 cells/mL and incubated for 72 hours with alisertib and romidepsin, alone or in combination at concentrations approximating the IC10–IC30. A minimum of 1 × 105 events were acquired for each sample. To quantitate apoptosis, cells were stained with Alex Fluor 488/Annexin V and propidium iodide (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 × 105 cells/mL and incubated with alisertib and romidepsin, alone or in combination with concentrations approximating the IC10–IC30. After 24 hours of incubation, cells were harvested and washed twice with 1 mL of cold PBS. Cells were then fixed with 70% histology grade ethanol for 2 hours. After the incubation period, cells were resuspended 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) and partially synchronized with 1 mmol/L nocodazole for 12 hours. Cells were then released from nocodazole
and subjected to different drug treatments. Before live-cell imaging, cells were incubated with Hoechst 33342 (1 mg/mL) for 30 minutes. Time-lapse microscopic images were acquired every 10 minutes in a 37°C/C02, 5% CO2 chamber for 48 hours using an inverted microscope (IX81; Olympus) with a 10x 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 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 7-week-old SCID mice were injected with 5 x 10⁶ cells/mL of HH, into the right flank subcutaneously with 50 μL of B.D. Matrigel (BD Biosciences). When the tumor volume reached an average of 50 to 100 mm3 mice were randomly divided into four cohorts of 10 mice each (control 0.01% DMSO, alisertib 20 mg/kg, romidepsin 1 mg/kg, alisertib 20 mg/kg + romidepsin 1 mg/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 minutes, 1, 2, and 6 hours in 2 mice per cohort (two time points were taken from each mouse) after day 16 of treatment. Tumor tissue was collected and harvested following 1 and 6 hours after treatment. Romidepsin and alisertib were quantified in serum and tissue by extraction using Acetonitrile followed by liquid chromatography tandem mass spectrometry (LC/MS-MS). Nine volumes of chilled acidified Acetonitrile (0.1% formic acid) were added to 100 μL of serum or 400 μL of aqueous tissue homogenate obtained by disrupting 100 mg of wet tissue using a tissue tearer homogenizer. The mixture was incubated at 4°C for 15 minutes followed by centrifugation at 13,000 × g for 10 minutes 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 50 pg/mL and 250 ng/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). A 5 μL was injected into a Phenomenex Kinetex C18 column (50 × 2.1 mm, 1.7u, 100A) column preceded by a C18 guard column that was kept at 40°C. The flow rate was maintained at 200 μL/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

Figure 2.

Alisertib in combination with romidepsin is synergistic in TCL but not in BCL. The IC10 of romidepsin (R) was used in combination with 50, 100, 1,000 nmol/L (IC10) of alisertib (A) to evaluate drug:drug interactions for up to 72 hours of treatment. A and B, following 72 hours of simultaneous drug exposure, synergy was not observed in a representative panel of BCL cell lines. EOB values were in the range of −20.03 to 5.83, signifying an antagonistic drug:drug interaction. C and D, following 72 hours of simultaneous drug exposure, synergy was observed in a panel of TCL cell lines with EOB values ranging from −9.05 to 21.01.
Alisertib Synergizes with Romidepsin in TCL

Figure 3.
Combined treatment of alisertib and romidepsin induces polyploidy in TCL cell lines following 24 hours of treatment. Cells were stained with PI following 24 hours of treatment with an IC10–20 of romidepsin (R) and 50 or 100 nmol/L of alisertib as a single agent or in combination. A, H9, TCL cell line demonstrates the percentage of cells in G1, S, G2–M, or polyploid; B, the HH TCL cell line demonstrates the percentage of cells in G1, S, G2–M, or polyploid; C, C5MJ, ATLL HTLV-1 cell line demonstrates the percentage of cells in G1, S, G2–M, or polyploid; D, DND41, T-ALL cell line demonstrates the percentage of cells in G1, S, G2–M, or polyploid.

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A
A  Control 0 min

F  2 nmol/L romidepsin 24 h

I  100 nmol/L alisertib 24 h

L  100 nmol/L alisertib + 2 nmol/L romidepsin 24 h

B  Control 10 min

G  2 nmol/L romidepsin 48 h

J  100 nmol/L alisertib 48 h

M  100 nmol/L alisertib + 2 nmol/L romidepsin 48 h

C  Control 20 min

H  2 nmol/L romidepsin 72 h

K  100 nmol/L alisertib 72 h

N  100 nmol/L alisertib + 2 nmol/L romidepsin 72 h

D  Control 30 min

E  Control 40 min

B

Control
2 nmol/L romidepsin (R)
50 nmol/L alisertib (A)
100 nmol/L alisertib (A)
2 nmol/L R + 50 nmol/L A
2 nmol/L R + 100 nmol/L A

CENP-A
HDAC3
β-Actin
Figure 4. Combined treatment of alisertib and romidepsin induces cytokinesis failure. A, live-cell imaging was used following 24 hours of treatment with 2 nmol/L of romidepsin and 100 nmol/L of alisertib both as a single agent and in combination in the H9, TCL cell line. Cells were imaged from 0 to 72 hours. A to E, images from control H9 sample demonstrate an H9 cell going through the stages of mitosis (prophase, metaphase, anaphase, telophase, and cytokinesis). Each stage is depicted every 10 minutes, for a total of 40 minutes. F, following 24 hours of 2 nmol/L romidepsin treatment H9 cells begin to display apoptotic effects depicted by the fragmented cells. G and 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 nmol/L alisertib treatment, H9 cells demonstrate a multinucleated form depicted by the arrow. J and 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 (100 nmol/L alisertib + 2 nmol/L romidepsin), the H9 cell demonstrated cytokinesis failure. M and N, cytokinesis failure was observed following 48 and 72 hours of treatment, respectively; B, evaluation of CENP-A protein levels following 72 hours of treatment with 2 nmol/L of romidepsin (R), 50 nmol/L of alisertib (A), 100 nmol/L of alisertib (A) as a single agent of in combination. Evaluation of HDAC3 protein levels following 72 hours of treatment with 2 nmol/L of romidepsin, 50 nmol/L of alisertib, 100 nmol/L of alisertib as a single agent of in combination.
approximately 40 minutes as depicted by images in Fig. 4A. 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 with control, whereas alisertib demonstrated a dose-dependent increase in the percentage of cells in 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, C5M, and DND41) following 24 hours of treatment. In all cell lines evaluated, romidepsin induced a marked increase in the percentage of cells in G2–M arrest following 50 and 100 nmol/L of drug (in the range of 13%–48%) when compared with control. The combination of alisertib and romidepsin induced a marked increase in polyploidy (in the range 10% to 42%), while inducing a significant decrease of cells in G1 relative to the control. The combination of alisertib and romidepsin at the IC10 and IC20, respectively, was the most potent in inducing polyploidy (up to 42%; Fig. 3A–D). Following 48 hours of treatment, virtually all cells were polyploid with little distinction among the other stages of cell cycle (data not shown).

Live-cell imaging of TCL confirms failure of cytokinesis with alisertib and romidepsin in TCL

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 nmol/L romidepsin, 100 nmol/L alisertib, 100 nmol/L alisertib + 2 nmol/L romidepsin) after synchronization with nocodazole in the H9 cell line. The control cells underwent normal mitosis in approximately 40 minutes as depicted by images in Fig. 4A–E. Following treatment with romidepsin, increased apoptosis was observed within 24 hours of treatment (Fig. 4F). Following treatment with alisertib, an accumulation of cells in G2–M was observed in about four hours (Fig. 4I). These results remained consistent following 48 to 72 hours of treatment with either romidepsin (Fig. 4G and H) or alisertib (Fig. 4I and 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 (Fig. 4L–N). Supplementary Fig. S2 demonstrates the live-cell imaging time-lapse video from 0 to 72 hours for all treatment conditions (control [Supplementary Fig. S2A], romidepsin [Supplementary Fig. S2B], alisertib [Supplementary Fig. S2C], combination [Supplementary Fig. S2D], respectively). Cytokinesis failure was confirmed after a corresponding increase in CENP-A protein levels following 72 hours of treatment with 100 nmol/L of alisertib, which was augmented with the combination treatment (Fig. 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 or 100 nmol/L of alisertib and 2 nmol/L of romidepsin following both single agent and combination treatment (Fig. 4B). HDAC3 is known to deacetylate AAK preventing AAK from proteolytic degradation (23). A slight decrease in HDAC3 levels following 100 nmol/L 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 IC10 of romidepsin and IC10 of alisertib both as a single agent or in combination (Fig. 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 nmol/L of romidepsin and 50 nmol/L 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 proapoptotic protein PUMA as well as a decrease in antiapoptotic protein Bcl-xL (Fig. 5B and 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 with the single-agent treatment and the control cohorts (\(P<0.05\)) over time. Figure 6B establishes that the survival of the combination cohort was also statistically significant (\(P<0.05\)) when compared with the single-agent treatment and control arms (by day 58). PK analyses were used to determine the concentration of romidepsin and alisertib on day 16, 30 minutes, 1, 2, and 6 hours after treatment (one sample collected/timepoint/treatment cohort). Figure 6C (top histogram) demonstrates that the single-agent romidepsin concentration in plasma samples was approximately 60 nmol/L at 30 minutes after treatment, and then decreased to 3 and 1 nmol/L at 2 and 6 hours after treatment, respectively. At 6 hours after treatment, the single-agent romidepsin plasma concentration approximated the IC50, which is obviously less than the IC10 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 laboratory with romidepsin in NOG mice (16). The alisertib concentration in plasma was in the range of 770 to 4,600 nmol/L in both the single agent and combination plasma samples and the concentration time pattern of the single agent was similar to that described earlier (Fig. 6C, bottom histogram; refs. 10, 25). 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 MTD. A 50 mg twice a day dose of alisertib produces a concentration of approximately 192 μmol/L. 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 and 6 hours of treatment. The intratumor concentration of romidepsin was approximately 2 and 1 nmol/L at 1 and 6 hour after 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 0.3 nmol/L per gram of tumor, with an average tumor size of 200 mg. The single-agent alisertib tumor concentration was 100 and 150 nmol/L at 1 and 6 hours after treatment, respectively. These concentrations are approximate the IC35–40 at 72 hours in our in vitro analyses. Even though, the romidepsin concentration was at the detection limit of 0.3 nmol/L per gram of tumor, the
alisertib concentration in the combination tumor samples increased from 100 to 400 nmol/L (1 hour after treatment) and 150 to 300 nmol/L (6 hours after treatment) when compared with the single-agent alisertib tumor samples. These data support the synergistic cytotoxicity of alisertib and romidepsin.

**Discussion**

AAK is a serine threonine kinase that autophosphorylates 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 and colleagues (26) have demonstrated that AAK phosphorylates histone 2b and histone 3 while maintaining its maximal kinase activity during M-phase. 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. Although AAK
Survival curve

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<td>Alisertib</td>
<td>Combination</td>
<td>0.053398</td>
<td>0.0338372</td>
</tr>
<tr>
<td>Romidepsin</td>
<td>Combination</td>
<td>0.326020</td>
<td>0.0196656</td>
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<tr>
<td>Control</td>
<td>Alisertib</td>
<td>0.040920</td>
<td>0.0651008</td>
</tr>
<tr>
<td>Control</td>
<td>Romidepsin</td>
<td>0.585513</td>
<td>0.8670352</td>
</tr>
</tbody>
</table>

Linear mixed model

<table>
<thead>
<tr>
<th>Comparison type</th>
<th>Linear mixed model</th>
<th>Regression coefficient</th>
<th>Std. Error</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compared with control intercepts</td>
<td>Intercept</td>
<td>4.56</td>
<td>0.29</td>
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<tr>
<td>Days of treatment</td>
<td>0.13</td>
<td>0.008</td>
<td>&lt;0.0001</td>
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<tr>
<td>Romidepsin</td>
<td>-0.07</td>
<td>0.41</td>
<td>0.87</td>
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<tr>
<td>Alisertib</td>
<td>-0.12</td>
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<td>0.77</td>
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<tr>
<td>Romidepsin + Alisertib</td>
<td>-0.47</td>
<td>0.41</td>
<td>0.25</td>
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</table>

Slope over time

<table>
<thead>
<tr>
<th>Pairwise comparisons of the change over time between the groups</th>
<th>Mean difference in change over time between groups</th>
<th>Std. Error</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Romidepsin</td>
<td>Controls</td>
<td>-0.032</td>
<td>0.009</td>
</tr>
<tr>
<td>Alisertib</td>
<td>Controls</td>
<td>-0.036</td>
<td>0.009</td>
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<tr>
<td>Romidepsin + alisertib</td>
<td>Controls</td>
<td>-0.050</td>
<td>0.009</td>
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<tr>
<td>Alisertib</td>
<td>Romidepsin</td>
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<td>0.0066</td>
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<td>Romidepsin + alisertib</td>
<td>Romidepsin</td>
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<td>0.0057</td>
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<tr>
<td>Romidepsin + alisertib</td>
<td>Alisertib</td>
<td>-0.0148</td>
<td>0.0059</td>
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</tbody>
</table>

Plasma romidepsin concentration (nmol/L)

![Graph showing plasma romidepsin concentration over time](image)

Plasma alisertib concentration (nmol/L)

![Graph showing plasma alisertib concentration over time](image)
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 and colleagues (27) have demonstrated that HDAC inhibitors induce degradation of AAK and Aurora B kinase. In addition to the cytokinesis failure demonstrated here, the data from Park and colleagues 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 three 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 Korttner and colleagues (28), suggesting that vorinostat in combination with a pan-auro kinase inhibitor is synergistic in BCL due to a down-regulation of c-MYC. The C-MYC protein 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 to see synergy in BCL. 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 with 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 and colleagues (24) demonstrated that prolonged exposure to low concentrations HDAC inhibitors leads to relocation and alteration within the pericentromeric heterochromatin. Corroborating these findings, Ishii and colleagues (29) 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 (30). Using a unique live suspension cell imaging system, which we developed, we demonstrated that the combination of alisertib and romidepsin induces cytokinesis failure following 15 hours of treatment. This result is supportive of the Taddei and colleagues finding that longer exposure to low concentrations of HDAC inhibitors alters the pericentromeric heterochromatin, leading to improved therapeutic effects (24, 31). These findings are supported by the downregulation of HDAC3 and an increase in CENP-A. CENP-A is a chromatin-associated protein that contains a histone H3–related fold domain that 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 with single agents in log transformed tumor burden 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 laboratory and Amiri-Kordestani and colleagues (34) demonstrated that romidepsin is a multidrug-resistant (MDR) substrate and can induce the MDR, increasing the extrusion of the drug. Although the basis for the increase in intratumor alisertib following combination treatment is unlikely correlated with the inhibition of the MDR because 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 with 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. Although 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).

Figure 6. Alisertib in combination with romidepsin is synergistic in a xenograft model of cutaneous TCL. The 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 days 1 to 21 (N = 10). Combination mice 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 with the control cohort over time (top table) and single agents over time (bottom table). B, Kaplan–Meier survival curve demonstrates that combination mice surpassed survival of all other cohorts and demonstrates statistical significance when compared with control and single agents (P < 0.05). C, PK analysis on plasma samples was performed following 30 minutes, 1, 2, and 6 hours of treatment. The top histogram depicts romidepsin (R) concentration for all timepoints evaluated whereas bottom histogram depicts the alisertib (A) concentration. D, PK analysis on tumor samples was performed on samples following 1 and 6 hours of treatment. Romidepsin intratumor concentration is depicted in the top histogram whereas alisertib intratumor concentration is depicted in the bottom histogram.
Cha and colleagues have demonstrated that panobinostat induced deacetylation of AAK and ABK (presumably through acetylation) through the ubiquitin–proteasome pathway (UPP) by directly targeting HDAC3 and HDAC6. Interestingly, when Cha and colleagues 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 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 I study of alisertib and romidepsin (NCT01482962 and NCT01897012). Future preclinical studies will be focused on the biochemical effects of these drugs and determine whether there are other mitotic agents that can more efficiently modulate mitotic proteins.

**Disclosure of Potential Conflicts of Interest**

O.A. O’Connor reports receiving a commercial research grant from Takeda/Millennium Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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**Development of methodology:** K.M. Zullo, Y. Guo, L. Cooke, X. Jirau-Serrano, R. Nandakumar, S. Cremers, D. Mahadevan, O.A. O’Connor

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** K.M. Zullo, Y. Guo, L. Cooke, X. Jirau-Serrano, R. Nandakumar, S. Cremers

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** K.M. Zullo, L. Cooke, X. Jirau-Serrano, M. Mangone, L. Scotto, J.E. Amengual, Y. Mao, R. Nandakumar, S. Cremers, J. Duong, O.A. O’Connor

**Writing, review, and/or revision of the manuscript:** K.M. Zullo, Y. Guo, L. Cooke, X. Jirau-Serrano, M. Mangone, D. Mahadevan, O.A. O’Connor

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** K.M. Zullo, Y. Guo, L. Cooke, X. Jirau-Serrano, M. Mangone, D. Mahadevan, O.A. O’Connor

**Study supervision:** L. Cooke, J.E. Amengual, D. Mahadevan, O.A. O’Connor

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**References**


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