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TORC1 and class I HDAC inhibitors synergize to suppress mature B cell neoplasms

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ABSTRACT

Enhanced proliferative signaling and loss of cell cycle regulation are essential for cancer progression. Increased mitogenic signaling through activation of the mTOR pathway, coupled with deregulation of the Cyclin D/retinoblastoma (Rb) pathway is a common feature of lymphoid malignancies, including plasmacytoma (PCT), multiple myeloma (MM), Burkitt's lymphoma (BL), and mantle cell lymphoma (MCL). Here we evaluate the synergy of pharmacologically affecting both of these critical pathways using the mTOR inhibitor sirolimus and the histone deacetylase inhibitor entinostat. A dose-matrix screening approach found this combination to be highly active and synergistic in a panel of genetically diverse human MM cell lines. Synergy and activity was observed in mouse PCT and human BL and MCL cell lines tested *in vitro*, as well as in freshly isolated primary MM patient samples tested *ex vivo*. This combination had minimal effects on healthy donor cells and retained activity when tested in a co-culture system simulating the protective interaction of cancer cells with the tumor microenvironment. Combining sirolimus with entinostat enhanced cell cycle arrest and apoptosis. At the molecular level, entinostat increased the expression of cell cycle negative regulators including CDKN1A (p21) and CDKN2A (p16), while the combination decreased critical growth and survival effectors including Cyclin D, BCL-XL, BIRC5, and activated MAPK.

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Abbreviations: PCT, plasmacytoma; MM, multiple myeloma; BL, Burkitt's lymphoma; MCL, Mantle cell lymphoma; CS-BLI, tumor cell-specific *in vitro* bioluminescence imaging; BMSC, bone marrow stromal cells; PBMC, peripheral blood mononuclear cells; EOSHA, excess over single highest agent; CI, combination index; mTORi, mechanistic target of rapamycin inhibitor; HDACi, histone deacetylase inhibitor.

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1. Introduction

Cancer is a multigenic disease often arising from a complex interplay of environmental factors with primary disease alleles, modifier loci encoding efficiency alleles, and somatic events which themselves may be affected by genetic background. As such, it is not particularly surprising that single agents with high target specificity ultimately fail to control cancer growth and metastases. Drug combinations designed to target multiple pathways are more likely to control this complex disease process, and may help ameliorate drug resistance mechanisms so common with single agent use (Dancey and Chen, 2006; Stewart, 2009; Zimmermann et al., 2007).

Two central pathways frequently dysregulated in cancer are the PI3K/Akt/mTOR/p53 (mTOR) and Cyclin/CDK/CDKI/Rb (CDK) pathways. mTOR and CDK pathway dysregulation is common in B cell neoplasias, including MCL (de Boer et al., 1995; Rizzatti et al., 2005), MM (Dilworth et al., 2000; Harvey and Lonial, 2007; Kuehl and Bergsagel, 2012; Peterson et al., 2009; Zhan et al., 2006), BL (Schmitz et al., 2012) and PCT where genetic predisposition is determined in part by alleles of *Mtor* and *Cdkn2a* (Bliskovsky et al., 2003; Zhang et al., 1998, 2001).

mTOR pathway dysregulation can mechanistically involve mutations, activation by growth factor receptor pathways, PTEN loss, and amplification of AKT and DEPTOR (Guertin and Sabatini, 2007; Harvey and Lonial, 2007; Laplante and Sabatini, 2009; Meric-Bernstam and Gonzalez-Angulo, 2009; Peterson et al., 2009; Zhang et al., 2011; Zoncu et al., 2011). mTOR is a serine-threonine kinase that forms two complexes, mTORC1 (mTOR, RAPTOR, PRAS40, mLST8, DEPTOR) and mTORC2 (mTOR, RICTOR, PROTOR, mLST8, SIN1, DEPTOR), which phosphorylate a number of downstream targets (most notably S6K1, 4EBP1,2, AKT, SGK1) to effect regulatory roles in transcription and translation, cell proliferation and survival, and immune response, metabolism, and autophagy (Guertin and Sabatini, 2007; Laplante and Sabatini, 2009; Meric-Bernstam and Gonzalez-Angulo, 2009; Zhang et al., 2011; Zoncu et al., 2011). Rapamycin (sirolimus) is a relatively specific inhibitor of mTORC1, but can also affect mTORC2 following prolonged exposure (Sarbassov et al., 2006). Clinical investigations using rapamycin or its analogs as single agents have shown only modest long-term benefit despite initial antitumor activity in some patients (Dancey, 2010).

Similarly, CDK pathway dysregulation often involves tumor suppressor gene (Rb, cyclin-dependent kinase inhibitors (CDKI) including p16, p21) loss or mutation, and Cyclin/cyclin dependent kinase (CDK) amplification (Fernandez et al., 2005; Malumbres and Barbacid, 2009). The benzamide entinostat (MS-275) is predominantly a selective Class I HDAC inhibitor with many activities, one of which is the reactivation of tumor suppressor gene (CDKI) pathways, which can ultimately affect CDK activity and lead to apoptosis (Bantscheff et al., 2011). Entinostat has strong activity against HDAC1, weak activity for HDACs2 and 3, and no activity against HDAC8 (Bantscheff et al., 2011; Witt et al., 2009); it has relatively strong activity against HDAC9, which is a Class IIA histone deacetylase (Khan et al., 2008). Recent studies have shown that entinostat has low affinity for binding to HDAC1/2-Sin3 complexes, and higher associations with HDAC3-NCOR1 and HDAC1/2-CoREST complexes (Bantscheff et al., 2011). Use of HDAC inhibitors (vorinostat and entinostat) in MM cell lines has shown decreased phospho-Rb, decreased cyclin D1 and E2f1 expression, enhanced p53 activity, and increased p21 and p27 expression (Lee et al., 2010; Mitsiades et al., 2004, 2003). Combining HDAC inhibitors with other targeted agents, radiation, or chemotherapeutics has shown efficacy in clinical trials for MM (Badros et al., 2009), and breast cancer (Huang et al., 2011), despite relatively modest benefit as single agents (Federico and Bagella, 2011; Gojo et al., 2007; Gore et al., 2008; Hess-Stumpp et al., 2007; Kummar et al., 2007).

In this study, we have investigated the synergistic effects of combining mTOR and HDAC inhibition to limit the growth of a variety of mature B cell neoplasms *in vitro* and *in vivo*. Further, we have extended these findings into an examination of activity in primary patient cells, in a model system simulating the pro-tumor effects of the microenvironment upon drug treatment, and in long-term animal studies.

2. Methods

2.1. Cell lines, primary cells and culture conditions

Human MM cell lines L363, U266, EJM, RPMI-8226, FR-4, JK-6L, OCI-MY5, LP-1, MM-1R, KMS-11, KMS-26, KMS-20, and KMS-28BM were cultured and authenticated as previously described (Gabrea et al., 2008). MOPC265 (IL6 dependent, p16(+)), and MOPC460(IL6 dependent, p16+ve, p53 partial deletion) cells were derived from pristane-induced PCTs from BALB/c mice. RMDPC 107-403 cells (p16(−), deleted) were cloned from a myc-ras retroviral-induced PCT from DBA/2 mice. MM cell lines were cultured in RPMI-1640 (2 mM L-glutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin: Invitrogen). Mouse cell lines MOPC265, MOPC460 and 107403 were cultured in RPMI-1640 (+50 μM β-mercaptoethanol and 10 ng/mlIL6), in a humidified incubator (5%CO_2 at 37 °C). MCL lines SP53 and JEKO were kindly provided by Lou Staudt (Center for Cancer Research (CCR), National Cancer Institute (NCI), Bethesda, MD) and Burkitt's lymphoma lines CB-32, Namalwa, BJAB, and DG-75 were kindly provided by Giovanna Tosato (CCR, NCI, Bethesda, MD).

Bone marrow aspirate was collected from patients with newly diagnosed multiple myeloma enrolled in clinical trials at the NCI/NIH. All patients reviewed and signed informed consent forms prior to admission. Bone marrow mononuclear cells and primary MM cells were isolated using Ficoll-Hypaque density gradient sedimentation from bone marrow aspirates according to manufacturer's protocol. $CD138^+$ cells were further separated from bone marrow samples by antibody-mediated positive selection using anti-CD138 magnetic-activated cell separation microbeads (Miltenyi Biotech, Cambridge, MA). The percentage of $CD138^+$ cells in the positive fraction was quantified by flow cytometric analysis using FlowJo software and found to be greater than 98%.