

# MLN0128, an ATP-Competitive mTOR Kinase Inhibitor with Potent *In Vitro* and *In Vivo* Antitumor Activity, as Potential Therapy for Bone and Soft-Tissue Sarcoma

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## Abstract

The mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase that exists in two complexes (mTORC1 and mTORC2) and integrates extracellular and intracellular signals to act as a master regulator of cell growth, survival, and metabolism. The PI3K/AKT/mTOR prosurvival pathway is often dysregulated in multiple sarcoma subtypes. First-generation allosteric inhibitors of mTORC1 (rapalogues) have been extensively tested with great preclinical promise, but have had limited clinical utility. Here, we report that MLN0128, a second-generation, ATP-competitive, pan-mTOR kinase inhibitor, acts on both mTORC1 and mTORC2 and has potent *in vitro* and *in vivo* antitumor activity in multiple sarcoma subtypes. *In vitro*, MLN0128 inhibits mTORC1/2 targets in a concentration-dependent fashion and shows striking antiproliferative effect in rhabdomyosarcoma

(RMS), Ewing sarcoma, malignant peripheral nerve sheath tumor, synovial sarcoma, osteosarcoma, and liposarcoma. Unlike rapamycin, MLN0128 inhibits phosphorylation of 4EBP1 and NDRG1 as well as prevents the reactivation of pAKT that occurs via negative feedback release with mTORC1 inhibition alone. In xenograft models, MLN0128 treatment results in suppression of tumor growth with two dosing schedules (1 mg/kg daily and 3 mg/kg b.i.d. t.i.w.). At the 3 mg/kg dosing schedule, MLN0128 treatment results in significantly better tumor growth suppression than rapamycin in RMS and Ewing sarcoma models. In addition, MLN0128 induces apoptosis in models of RMS both *in vitro* and *in vivo*. Results from our study strongly suggest that MLN0128 treatment should be explored further as potential therapy for sarcoma. *Mol Cancer Ther*; 14(2): 395–406. ©2014 AACR.

## Introduction

mTOR is a serine/threonine protein kinase that exists in two distinct protein complexes (mTORC1 and mTORC2) that regulate metabolism, homeostasis, survival, and proliferation (1–3), and is often dysregulated in multiple sarcoma subtypes (4–6). Abnormal signals both upstream and downstream of the mTOR kinase lead to aberrant activity in sarcomas, and its dysregulation has been well documented by elevated phosphorylation of multiple components of the pathway (7–10). This deregulated activity of mTOR and its surrounding axis have been shown to correlate

with poor clinical outcomes in sarcomas (11–13), as well as other tumor types (14). Upstream, overexpression or constitutive activation of platelet-derived growth factor receptor (PDGFR; ref. 15), insulin-like growth factor I receptor (IGFIR; refs. 16, 17), VEGFR (18) and fibroblast growth factor receptor (FGFR; ref. 19), has been demonstrated to play a role, while S6 kinase (S6K), and eukaryotic initiation binding factor 4E (eIF4E; ref. 11) are implicated downstream. Other critical pathways, such as the MAPK pathway, have been shown to interact with mTOR in sarcoma, and cross-talk between them is implicated in treatment-mediated resistance (20, 21). Most recently, large-scale genomic sequencing projects have revealed distinct mutations clustered in and around the PI3K/AKT/mTOR axis (22–24), which further verify mTOR as a valid antitumor target in the treatment of sarcomas.

The importance of the PI3K/AKT/mTOR axis in sarcoma has driven interest in therapies targeted to mTOR, and led to a focus on rapamycin and a group of roughly equivalent structural analogues (25) termed rapalogues. Despite significant preclinical enthusiasm, the utility of these first-generation agents has been limited because of the allosteric inhibition of only one of the two mTOR complexes, mTORC1 (26). This inhibition of mTORC1 only leaves the downstream effectors of mTORC2 unchecked, and contributes to the unwanted reactivation of AKT, which occurs via release of negative feedback in an IGFIR-dependent fashion (27). Taken together, these limitations suggest that rapalogues do not comprehensively exploit the antitumor potential of mTOR inhibition, and have driven development of second-generation agents (28).

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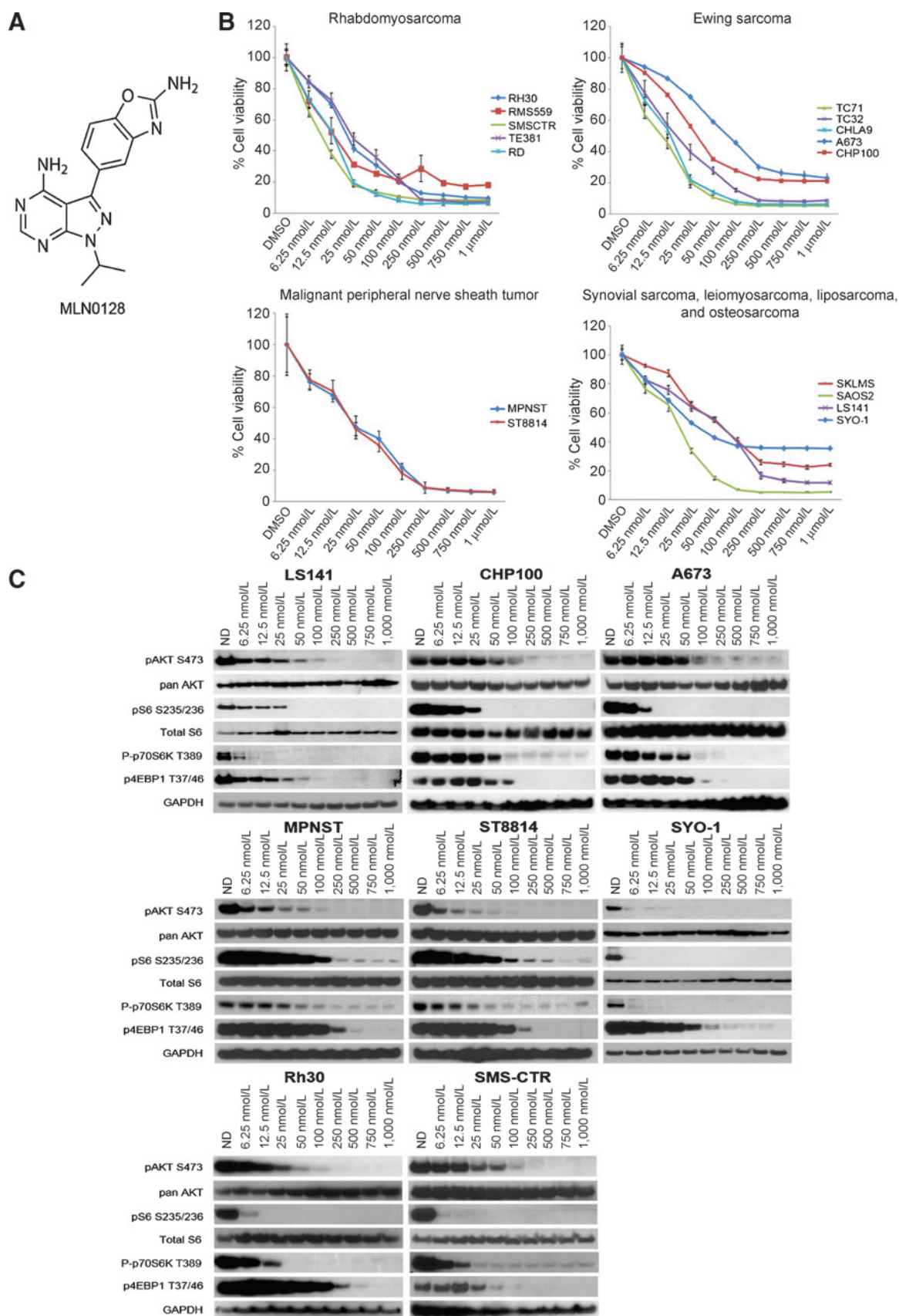
**Note:** Supplementary data for this article are available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

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doi: 10.1158/1535-7163.MCT-14-0711

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**Table 1.** Approximate IC<sub>50</sub> values for MLN0128 in 20 bone and soft-tissue sarcoma subtypes are shown

Cell line	IC <sub>50</sub>
RMS	
A204	2 nmol/L
SMS-CTR	4 nmol/L
SK-RMS -3	6 nmol/L
RD	8 nmol/L
Rh28	9 nmol/L
RMS-559	15 nmol/L
Rh30	28 nmol/L
TE-381	30 nmol/L
SK-RMS -4	70 nmol/L
Ewing sarcoma	
TC-71	6 nmol/L
CHLA9	7 nmol/L
CHP100	64 nmol/L
TC-32	17 nmol/L
A673	130 nmol/L
MPNST	
MPNST	25 nmol/L
ST8814	25 nmol/L
Leiomyosarcoma	
SKLMS	29 nmol/L
Osteosarcoma	
SaOS-2	14 nmol/L
Liposarcoma	
LS141	53 nmol/L
Synovial sarcoma	
SYO-1	82 nmol/L

MLN0128 (Millennium/Takeda Pharmaceuticals) is a selective, highly potent, and orally bioavailable adenosine triphosphate (ATP) competitor of both mTORC1 and mTORC2, which is currently in phase I and II clinical trials as a single agent in patients with advanced solid malignancies (NCT01899053, NCT01058707, NCT01351350, NCT0133183, NCT02091531), in combination with bevacizumab in patients with glioblastoma multiforme or advanced solid tumors (NCT02142803), in combination with MLN1117 (PI3K inhibitor) in patients with advanced nonhematologic malignancies (NCT01899053), in combination with ziv-afilbercept in recurrent solid tumors (NCT02159989), and breast cancer (NCT02049957). An additional dose-escalation study in relapsed or refractory multiple myeloma or Waldenstrom-Macroglobulinemia has recently been completed (NCT01118689). Preclinically, MLN0128 has been shown to have antitumor activity in prostate cancer (29), B-cell leukemia (30), breast cancer (31, 32), and renal cell carcinoma (33). In the current study, we describe the preclinical characterization of MLN0128 in bone and soft-tissue sarcomas, evaluate its *in vitro* and *in vivo* effects, and demonstrate its antitumor properties superior to those of its first-generation rapalogue predecessors.

## Materials and Methods

### Chemicals and drugs

MLN0128 was provided by Millennium/Takeda Pharmaceuticals. Rapamycin was purchased from EMD chemicals.

MLN0128 and rapamycin were dissolved in DMSO and stored at  $-20^{\circ}\text{C}$ .

### Cell culture and reagents

Cells were cultured in RPMI media with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin, maintained at  $37^{\circ}\text{C}$  in 5% CO<sub>2</sub>, and passaged for no more than 4 months. Initial stocks of all cell lines were received from their sources within the past 3 years. Malignant peripheral nerve sheath tumor (MPNST; ST8814) and rhabdomyosarcoma (RMS) cell line RMS-559 were supplied by Dr. Jonathan Fletcher (Dana Farber Cancer Institute, Boston, MA). RMS-559 (22), MPNST, and ST8814 (34) cell lines were authenticated as previously described. Ewing sarcoma (CHP100, A673) cell lines were obtained from Dr. Melinda S. Merchant (Center for Cancer Research, NCI/NIH, Bethesda, MD). Dedifferentiated liposarcoma cell lines (LS141, DDLS) were obtained from Dr. Samuel Singer [Memorial Sloan Kettering Cancer Center (MSKCC), New York, NY], and were authenticated by gene expression profiling before distribution (35). Synovial sarcoma cell lines (SYO-1 and HSSY-II) were obtained from Dr. Marc Ladanyi (MSKCC). RMS cell lines Rh28, Rh30, RD, SMS-CTR and Ewing sarcoma cell lines TE-381, TC32, TC71, and CHLA9 were obtained from Dr. Timothy Triche (University of Southern California, Los Angeles, CA). SK-RMS -3 and SK-RMS -4 were derived from patient tissues and provided by Dr. Christine Pratilas (Johns Hopkins Kimmel Comprehensive Cancer Center, Baltimore, MD). SK-RMS -3 and SK-RMS -4 were derived from patient tumors and use of patients' tumor material was conducted under an MSKCC IRB approved protocol for the use of human biospecimen (IRB 10-130) and with patient authorization for research use (IRB 06-107). Osteosarcoma cell line (SaOS2) was obtained from ATCC. RMS cell lines generously provided by Drs. Pratilas and Triche were not independently authenticated unless otherwise mentioned. Cell lines TC32, TC71, CHP100, A673, and CHLA9 were authenticated using RT-PCR, and found to have their expected characteristic chromosomal translocations. SYO-1 and HSSY cell lines were authenticated by confirming the expression of the pathognomonic SYT-SSX fusion gene by RT-PCR. All cell lines were determined to be mycoplasma free via testing in the MSKCC Monoclonal Antibody Core Facility using biochemical assay MycoAlert.

### Cell viability assays

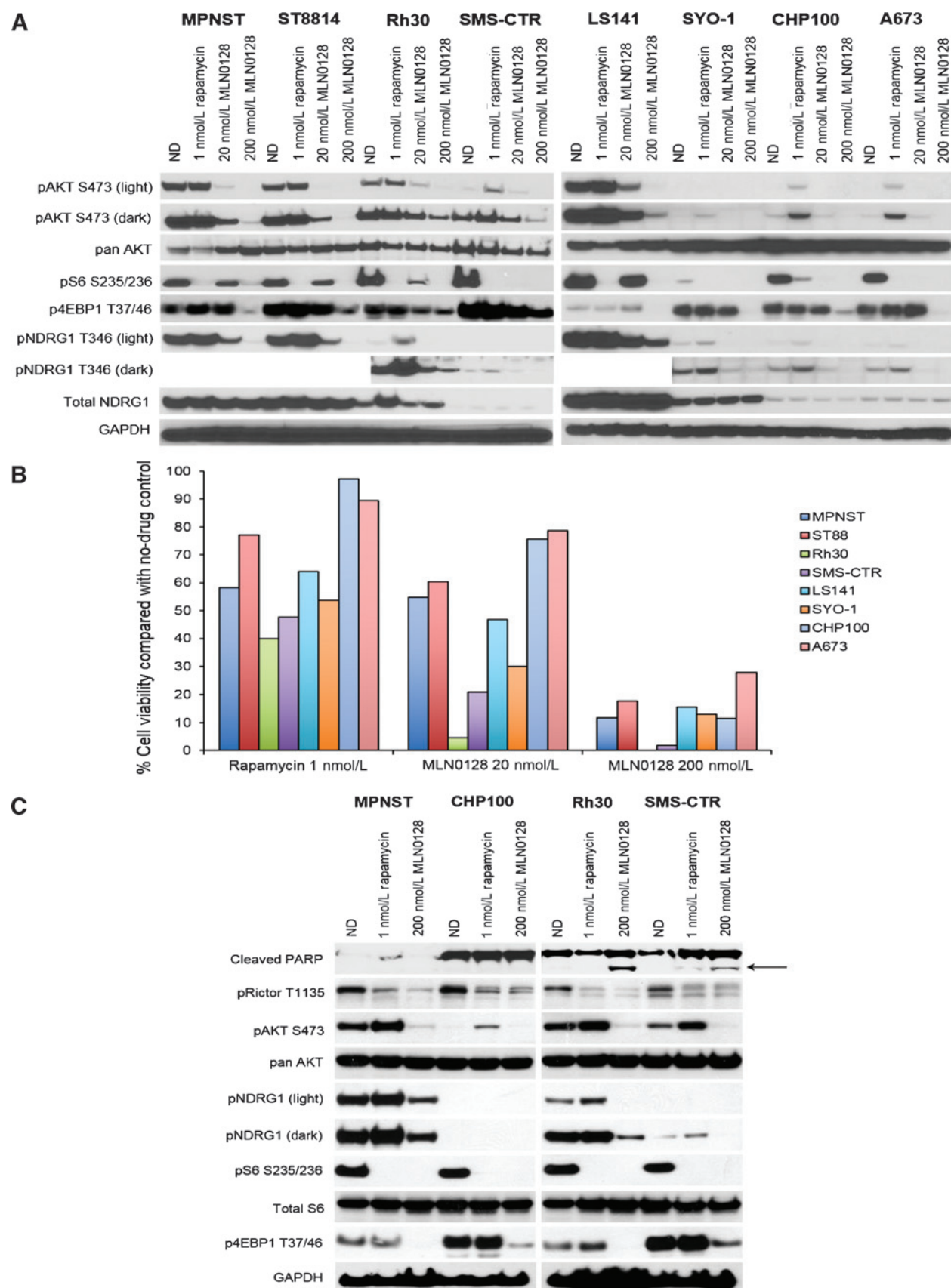
Cell viability assays were carried out using the Dojindo Molecular Technologies (CCK-8) kit as per manufacturer's instructions. Briefly, 2,000 to 5,000 cells were plated in 96-well plates, allowed to grow overnight, and then treated with the indicated drugs for 72 hours. Media were replaced with 100  $\mu\text{L}$  of media with 10% serum and 10% CCK-8 solution (Dojindo Molecular Technologies Kit). After 1 hour, the optical density was read at 450 nm using a Spectra Max 340 PC (Molecular Devices Corporation) to determine viability. Background values from negative control wells without cells were subtracted for final sample quantification. Survival is expressed as a percentage of untreated cells. IC<sub>50</sub> was extrapolated from

**Figure 1.**

MLN0128 is a potent, ATP-competitive mTOR kinase inhibitor. A, chemical structure of MLN0128. B, indicated cell lines were plated in 96-well plates and treated in six wells per condition with increasing doses of MLN0128 for 72 hours. Cell viability was measured using Dojindo Cell Counting Kit 8. Dose-response curves were generated as a percentage of the no-drug control. C, indicated cell lines were grown to 60% confluency in 60-mm plates and treated for 6 hours using indicated drugs. Twenty to 30  $\mu\text{g}$  of RIPA lysates was then loaded on SDS/PAGE and immunoblotted using indicated antibodies.



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cell viability data using CompuSyn software according to the manufacturer's instructions.

### Western immunoblotting

Cells and tissues were lysed with RIPA buffer supplemented with protease inhibitor cocktail tablets (Roche Diagnostics) and 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>. Equal amounts (20–30 µg) of protein were electrophoresed onto 4% to 12% gradient gels (Life Technologies) and transferred onto polyvinylidene difluoride or 0.45-micron nitrocellulose membranes. Membranes were blocked with 5% nonfat dried milk and probed with primary antibodies. All named antibodies were obtained from Cell Signaling Technology (see Supplementary Table S1 for a complete list of antibodies with catalog numbers). Ku70 (E-5) antibody was obtained from Santa Cruz Biotechnology (Catalog # sc-17789). Bound antibodies were detected with horseradish peroxidase secondary antibodies (GE Healthcare) and visualized by enhanced chemiluminescence reagent (GE Healthcare).

### Xenograft studies

Approximately 8-week-old athymic female mice were injected with 10 to 15 million cells in Matrigel of the cell line of interest and allowed to grow until tumors reached 100 mm<sup>3</sup> in volume before treatment with either vehicle, rapamycin, or MLN0128. Tumors were measured every 2 to 3 days using calipers and volumes calculated using the formula  $p/6 \times (\text{large diameter}) \times (\text{small diameter})$ . Animal weights were measured every 2 to 3 days as a surrogate marker for overall toxicity. Animals were sacrificed 4 hours after the final dose of treatment week 1, or 4 hours after the final dose of treatment (week 3 or 5 as indicated). Tumors were extracted from surrounding tissue and either flash-frozen in liquid nitrogen for Western immunoblotting or placed in formalin or paraformaldehyde for IHC studies. Flash-frozen tissue was ground in RIPA lysis buffer and resin as per the manufacturer's instructions using Sample Grinding Kit (GE Healthcare) and analyzed via Western immunoblotting as described above. Formalin-fixed tissues were sectioned and stained with the indicated antibodies following standard protocols in the Molecular Cytology Core Facility at MSKCC.

### Statistical analysis

All *in vitro* experiments were carried out at least three times unless otherwise indicated. *P* values were calculated using Student *t* test, with values of  $\leq 0.05$  determined to be statistically significant. SE was calculated as the SD divided by the square root of the number of samples tested.

## Results

### MLN0128 exhibits potent antiproliferative activity in multiple sarcoma cell lines and blocks mTORC1/2 targets in a concentration-dependent fashion *in vitro*

MLN0128 (Fig. 1A) was tested against a broad panel of bone and soft-tissue sarcoma cell lines following 3 days of exposure to

increasing concentrations of the drug to determine its antiproliferative activity. MLN0128 was found to inhibit proliferation of all sarcoma subtypes tested in a dose-dependent fashion at low nanomolar concentrations (Fig. 1B) with IC<sub>50</sub> values ranging from 2 to 130 nmol/L (Table 1).

At concentrations ranging from 6.25 to 1 µmol/L, the target inhibition profile of MLN0128 was examined using Western immunoblotting (Fig. 1C). Inhibition of mTORC1 activity was determined using the well-characterized mTORC1 substrates P70-S6K1 and 4EBP1 (36). Phosphorylation of S6 ribosomal protein was used as an additional marker of mTORC1 activity given its direct phosphorylation by S6 kinase 1 (S6K1). Inhibition of mTORC2 activity was assessed via phosphorylation of AKT at Ser473 (pAKTS473). In all sarcoma subtypes tested, MLN0128 potently inhibited each of these anticipated targets in a dose-dependent fashion (Fig. 1C). Phosphorylation of S6 and P70-S6K1 was inhibited significantly with nearly complete blockade at low nanomolar concentrations (Fig. 1C). Inhibition of phosphorylation of 4EBP1 and pAKTS473 was also observed at low nanomolar concentrations (approximately at 100 and 50 nmol/L respectively, Fig. 1C). AKT phosphorylation at Thr308 was, however, limited because of weak signal and detection of nonspecific proteins in many of the sarcoma cell lines studied, but when detectable, MLN0128 inhibited this PDK1 phosphorylation site (Supplementary Fig. S1A).

### MLN0128 demonstrates superior downregulation of mTORC1/2 substrates when compared with rapamycin

Rapamycin allosterically inhibits mTORC1 kinase activity via binding of the FKBP12–rapamycin (FR) complex to the FR-binding domain of mTORC1 (37), while MLN0128 provides direct, ATP-competitive kinase inhibition of both mTORC1 and mTORC2 (29). Although there is evidence for molecular interplay between mTORC2 and rapamycin (38, 39), this second complex is largely unresponsive to rapamycin and related rapalogues. Western immunoblotting was therefore used to further investigate the variances in molecular signaling between rapamycin and MLN0128. Phosphorylation of serum/glucocorticoid-regulated kinase 1 (SGK1), one of the three isoforms activated by insulin and other growth factors, is regulated by mTORC2, but not mTORC1, as evidenced by absence of activity in mTORC2 knockout fibroblasts (40). Because SGK1 was not readily detectable by Western immunoblotting (data not shown), its direct substrate, N-myc downstream-regulated gene 1 (NDRG1), whose activity is also ablated in mTORC2 knockout fibroblasts (40), was used in addition to pAKTS473 as a marker of mTORC2 activity.

Similar to rapamycin treatment, MLN0128 treatment resulted in concentration-dependent inhibition of phosphorylation of S6K1 and S6. MLN0128, however, also inhibited phosphorylation of 4EBP1 in all sarcoma subtypes suggesting more comprehensive mTORC1 targeting when compared with rapamycin (Fig. 2A). In addition to complete blockade of the mTORC1

**Figure 2.**

MLN0128 has a molecular profile distinct from that of rapamycin. A, eight cell lines representing a broad range of bone and soft-tissue sarcoma subtypes were treated with indicated concentrations of rapamycin and MLN0128 for 6 hours. Twenty to 30 µg of RIPA lysates was then loaded on SDS/PAGE and immunoblotted using indicated antibodies. B, cell lines examined in A were treated with MLN0128 for 72 hours to compare the antiproliferative effect of rapamycin and MLN0128. Cell viability was measured using Dojindo Cell Counting Kit 8 and shown as a percentage of the no-drug control. Note that error bars representing SEM have been added but are too small to be seen. C, representative cell lines from A and B were treated with indicated concentrations of rapamycin or MLN0128 for 6 hours. Twenty to 30 µg of RIPA lysates was then loaded on SDS/PAGE and immunoblotted using indicated antibodies.

targets pS6K1, pS6 and p4EBP1, MLN0128 treatment also inhibited phosphorylation of NDRG1 at Thr346 indicating distinct mTORC2 inhibition. In contrast, rapamycin had no effect on NDRG1 phosphorylation, supporting its primarily mTORC1 effects (Fig. 2A). Importantly, simultaneous inhibition of both mTOR complexes by MLN0128 prevented feedback reactivation of pAKT, whereas rapamycin led to an increase in pAKTS473 (Fig. 2A) as has been previously reported (27, 41). Taken together, these data indicate that MLN0128 provides more comprehensive inhibition of the mTOR axis, including superior mTORC1 inhibition when compared with that of rapamycin as evidenced by downregulation of p4EBP1 in addition to pS6K1 and pS6, effective targeting of the mTORC2 complex as illustrated by inhibition of pNDRG1, as well as attenuation of the release of negative feedback observed with rapamycin treatment.

To determine whether the broader molecular profile of MLN0128 also translated into superior antiproliferative effect *in vitro* when compared with rapamycin, concentrations based upon complete blockade of downstream targets were chosen for testing. Figure 2B illustrates that MLN0128 treatment resulted in superior antiproliferative effect compared with that of rapamycin ( $P < 0.04$  and  $P < 0.000006$  when comparing rapamycin at 20 and 200 nmol/L MLN0128, respectively) across the broad spectrum of cell lines tested.

#### MLN0128, but not rapamycin, induces apoptosis in models of alveolar and embryonal rhabdomyosarcoma

MLN0128 was next compared with rapamycin in its ability to induce cleavage of PARP. In models of alveolar (ARMS, Rh30) and embryonal (ERMS, SMS-CTR) RMS, MLN0128 treatment at 200 nmol/L induced cleavage of PARP at time points as early as 6 hours (Fig. 2C). Induction of PARP cleavage was also seen in two other RMS cell lines, RMS-559 as well as SK-RMS -3, only when treated with MLN0128 but not with rapamycin (Supplementary Fig. S1B). MLN0128, however, was unable to induce cleavage of PARP in an MPNST and Ewing sarcoma model (MPNST, CHP100; Fig. 2C) as well as in liposarcoma, leiomyosarcoma, or synovial sarcoma (data not shown). Treatment with rapamycin, using concentrations established in Fig. 2A, was unable to induce cleavage of PARP in any of the cell lines tested (Fig. 2C and Supplementary Fig. S1B, and data not shown).

Both MLN0128 and rapamycin were able to inhibit phosphorylation of Rictor, a component of mTORC2, at Thr1135 (Fig. 2C) as has been shown in previously published studies (39, 42).

To confirm superior *in vitro* effects of MLN0128 treatment when compared with those of rapamycin, and that increased concentrations of rapamycin would not improve the antiproliferative potency or induce apoptotic effects in the cell lines tested, we next tested equivalent concentrations of both rapamycin and MLN0128 up to 1  $\mu\text{mol/L}$ . Figure 3A indicates that rapamycin exerts its maximal antiproliferative effects at low concentrations, and continued dose escalation does not potentiate these effects. As observed previously, MLN0128 exhibited superior antitumor effect when compared with rapamycin in all cell lines tested.

To further compare the molecular signaling profiles of rapamycin and MLN0128 at higher and equivalent doses, two cell lines, MPNST and Rh30, were chosen for additional analysis. Despite using concentrations as high as 1  $\mu\text{mol/L}$ , rapamycin treatment was unable to effectively inhibit pAKTS473 or p4EBP1T37/46. Similar to earlier results (Fig. 2C), neither rapamycin nor MLN0128 treatment resulted in induction of cleaved

PARP in MPNST, however, treatment of Rh30 cells with MLN0128 but not with rapamycin resulted in dose-dependent induction of cleaved PARP as well as cleaved caspase-3 (Fig. 3B) strongly indicating induction of apoptosis.

To further confirm apoptosis in RMS models, an *in vitro* assay (Caspase Glo) was carried out to determine induction of cleaved caspase-3/7 after 6 hours of treatment with rapamycin or MLN0128 (Fig. 3C). As seen earlier (Fig. 3B), induction of cleaved caspase-3/7 activity was observed only in the RMS model, but not in the MPNST model (Rh30 =  $P < 0.0008$  and  $P < 0.0006$  and MPNST =  $P < 0.1$  and  $P < 0.09$  when comparing 200 and 1  $\mu\text{mol/L}$ , respectively). Overall, it is particularly notable that markers of apoptosis (cleavage of PARP by Western immunoblotting, induction of cleaved caspase-3/7 by Western immunoblotting, induction of cleaved caspase by *in vitro* assay) consistently occurred in RMS cell lines (Rh30 in Figs. 2C and 3B, SMS-CTR in Fig. 2C, RMS-559 and SK-RMS -3 in Supplementary Fig. S1B), but not in other sarcoma subtypes. These apoptotic markers occurred at relatively low and clinically achievable dosing levels (20 and 200 nmol/L), and were not noted when equivalent and even higher doses of up to 1  $\mu\text{mol/L}$  of rapamycin were used to treat the same cell lines. Although the exact mechanism warrants further investigation, these data are highly suggestive of MLN0128's ability to induce apoptosis in RMS models.

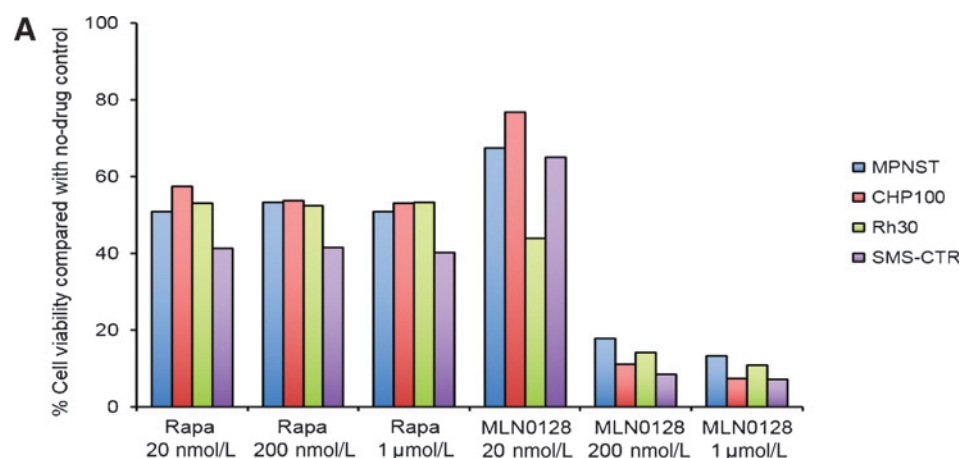
#### MLN0128 inhibits tumor growth *in vivo* and is superior to rapamycin in xenograft models of Ewing sarcoma (CHP100) and rhabdomyosarcoma (Rh30)

The efficacy of MLN0128 was next tested in multiple tumor xenograft models. A 1-mg/kg daily dosing schedule had been previously analyzed and found to be tolerable in other tumor types (32, 33). Treatment of MPNST and CHP100 xenografts with MLN0128 resulted in suppression of tumor volume (Fig. 4A and B) compared with vehicle control. Pharmacodynamic analysis of tumors harvested 4 hours following last dose showed inhibition of expected molecular targets pS6, p4EBP1, and pAKT473 (Fig. 4A and B).

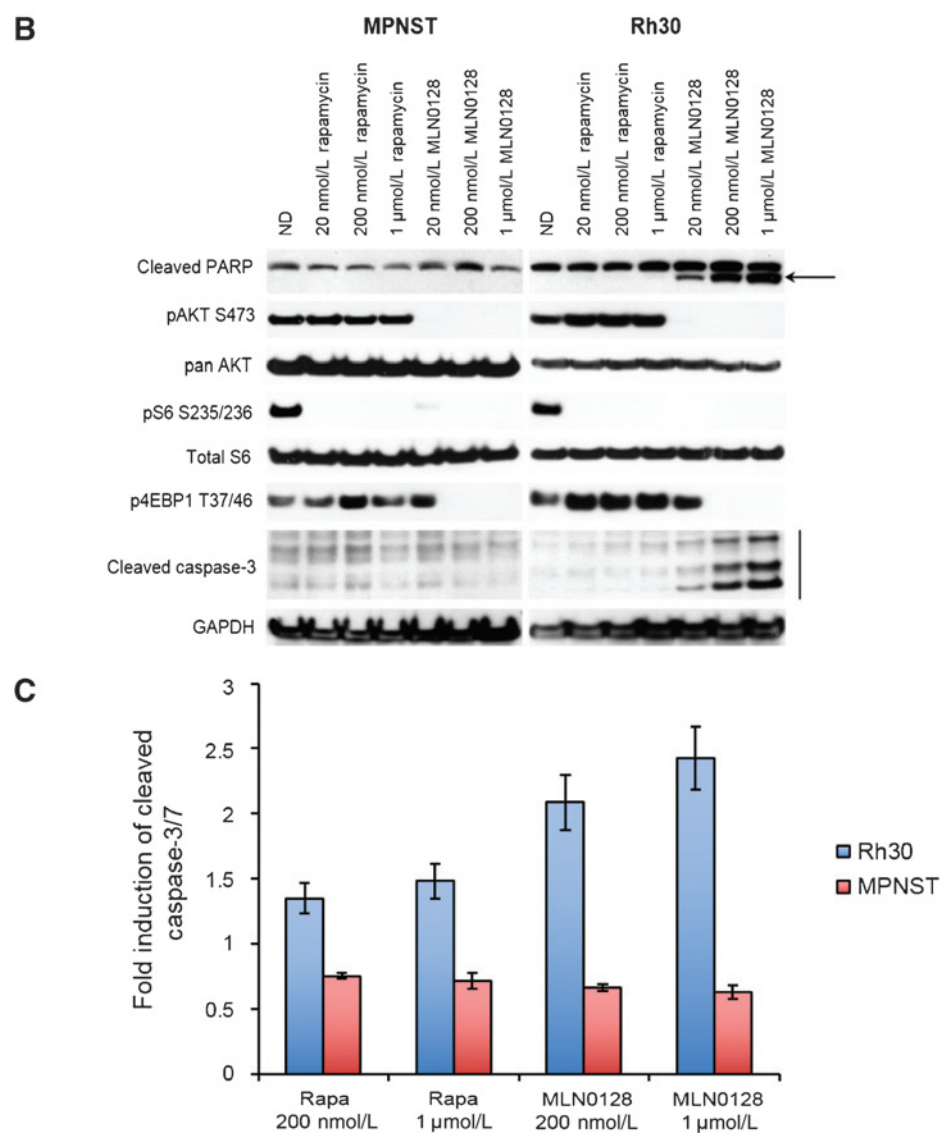
Although MLN0128 activity in these models was confirmed at a dosing schedule of 1 mg/kg orally daily, only tumor growth suppression but not tumor stability or regression was noted. In addition, molecular targets were effectively inhibited but not comprehensively ablated (Fig. 4A and B) at the tested dose. Given these observations, and the testing of higher dosing schedules including 3 mg/kg three times weekly in other tumor models (32), we next completed a toxicity study to determine which dosing schedules that provided higher overall dose intensity were likely to be tolerated (Fig. 4C). This study indicated that a high-dose, intermittent schedule of 3 mg/kg twice daily on Mondays, Wednesdays, and Fridays was tolerable, although 3 mg/kg twice daily on three consecutive days, that is, Mondays, Tuesdays, and Wednesdays was not tolerated and resulted in high toxicity.

Given the tolerability of a 3-mg/kg twice daily Mondays, Wednesdays, and Fridays dosing schedule, and our hypothesis that this higher intermittent dosing might provide more comprehensive inhibition of downstream targets and therefore tumor growth, this dosing schedule was next chosen for comparison against rapamycin. Treatment of CHP100 (Ewing sarcoma) tumor xenografts with MLN0128 resulted in significantly enhanced



**Figure 3.**

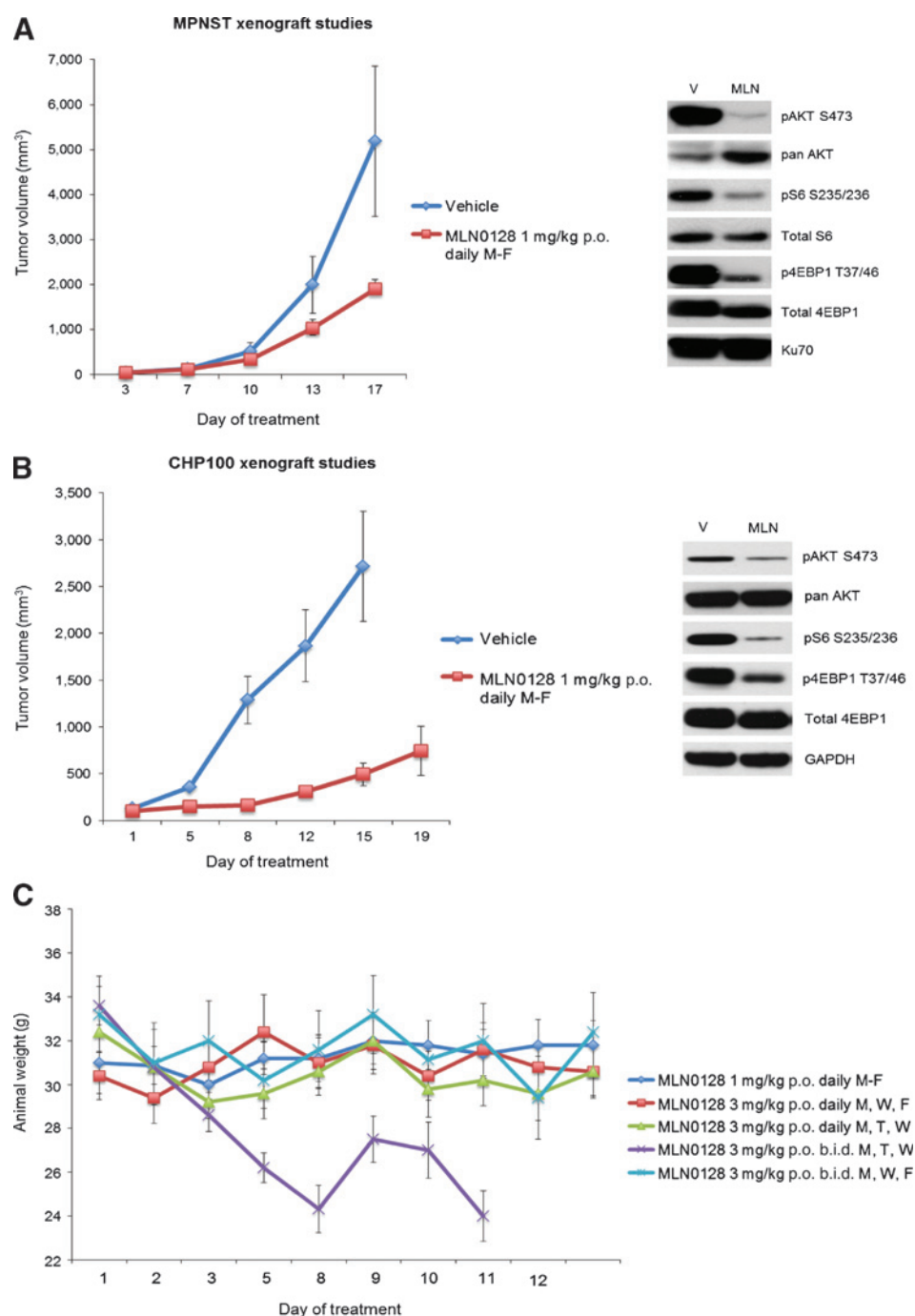
Increasing concentrations of MLN0128 but not rapamycin inhibit cell proliferation and phosphorylation of downstream targets, and induce apoptosis in RMS cells. A, representative cell lines were exposed to indicated concentrations of rapamycin and MLN0128 for 72 hours. Cell viability was measured using Dojindo Cell Counting Kit 8 and shown as a percentage of the no-drug control. Note: error bars representing SEM have been added but are too small to be seen. B, equimolar concentrations established in A were used to treat indicated cell lines for 6 hours. Twenty to 30 µg of RIPA lysates was loaded on SDS/PAGE and immunoblotted using indicated antibodies. C, indicated cell lines were treated with rapamycin or MLN0128 for 6 hours. Induction of caspase-3/7 activity was determined using CaspaseGlo (Promega) assay according to the manufacturer's instructions.



tumor suppression when compared with rapamycin (Fig. 5A) at the end of 5 weeks of treatment ( $P < 0.01$ ). As anticipated, Western immunoblotting analysis (Fig. 5A) showed inhibition of down-

stream targets such as pAKT and pS6. Similar to *in vitro* studies, only MLN0128 but not rapamycin treatment resulted in inhibition of p4EBP1 *in vivo* (Fig. 5A).

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**Figure 4.**

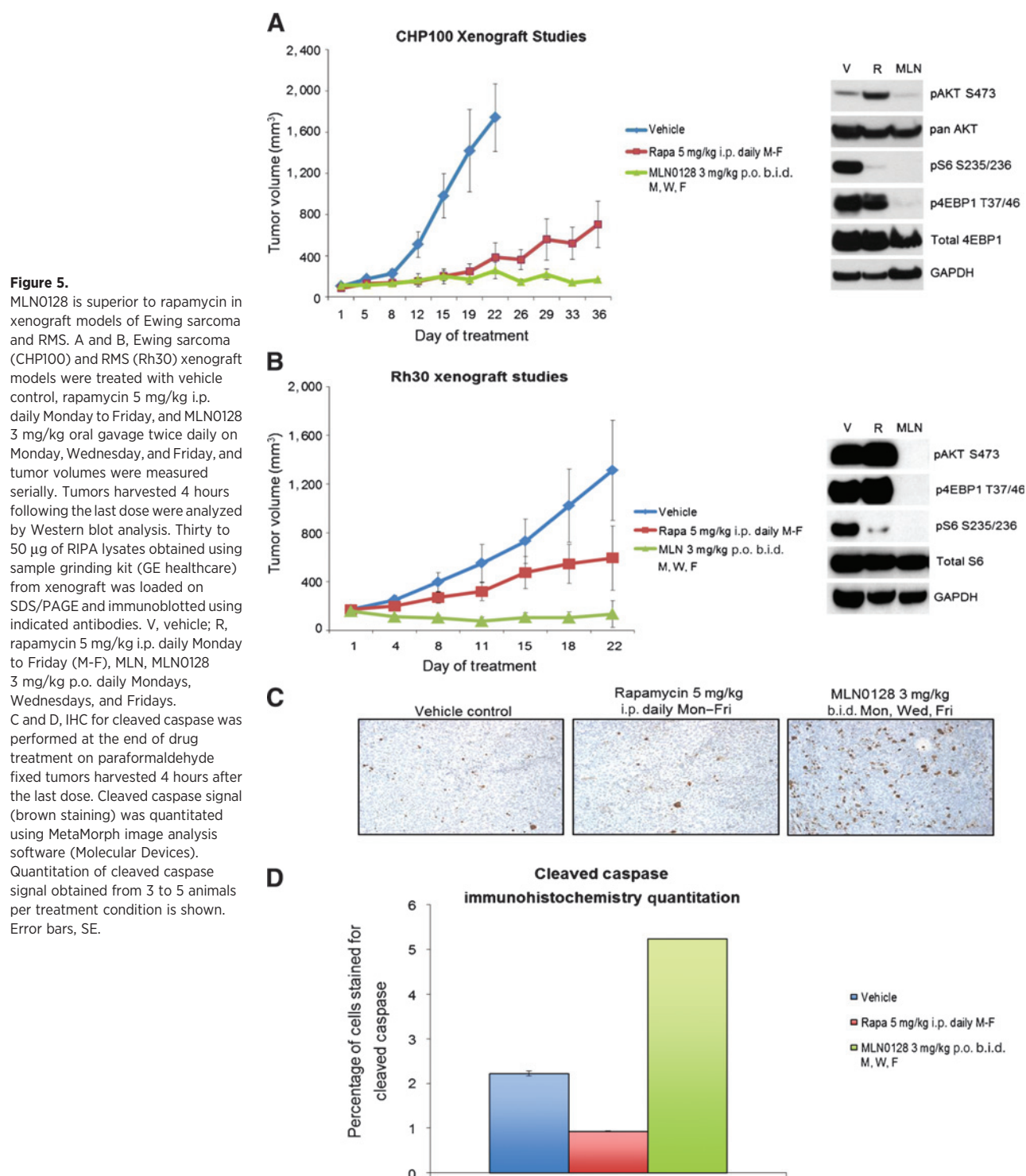
MLN0128 inhibits tumor growth in sarcoma xenograft models *in vivo*. A and B, MPNST (MPNST) and Ewing sarcoma (CHP100) xenograft models were treated with either vehicle control or MLN0128 1 mg/kg by oral gavage daily Monday through Friday and tumor volumes were measured serially. A subset of tumors was harvested 4 hours following the last dose for Western blot analysis. Of note, 30 to 50  $\mu$ g of RIPA lysates obtained using sample grinding kit (GE healthcare) from xenograft was loaded on SDS/PAGE and immunoblotted using indicated antibodies. V, vehicle; MLN, MLN0128 1 mg/kg p.o. daily Monday to Friday (M-F). C, five different dosing schedules of MLN0128 were tested in athymic mice without tumors to determine tolerability of higher dosing schedules than those tested in A and B.

Given the apoptotic potential of MLN0128 in RMS model *in vitro* (Fig. 3B and C), we next investigated MLN0128's *in vivo* antitumor effects in Rh30 xenografts. MLN0128 resulted in significant tumor suppression when compared with rapamycin (Fig. 5B,  $P < 0.001$ ) as well as resulted in significant inhibition of downstream targets such as pS6 and p4EBP1 (Fig. 5B). In concordance with the apoptosis observed *in vitro* in the Rh30 cell line, IHC analysis of Rh30 xenograft tissue (Fig. 5C and D) revealed increased cleaved caspase staining ( $P < 0.0001$ ).

## Discussion

The present study reports the *in vitro* and *in vivo* preclinical characterization of MLN0128, a potent and orally bioavailable pan-mTOR kinase inhibitor, in a broad range of sarcoma subtypes. MLN0128 exerts potent antiproliferative effect and has a molecular signaling profile distinct from that of rapamycin *in vitro* and *in vivo*. MLN0128 induces apoptosis in models of RMS, and provides superior antitumor effect when compared with rapamycin in *in vivo* RMS and Ewing sarcoma models.





Considerable preclinical and clinical data using first-generation allosteric mTORC1 inhibitors have validated mTOR as an anticancer target, and there is a robust 3-fold rationale for its specific use in the treatment of multiple sarcoma subtypes. First, hyper-phosphorylation of multiple components of the mTOR pathway suggests increased activity in numerous models likely occurring in an autocrine/paracrine fashion

(5, 7–11). Second, aberrant upstream signals from PDGFR (15), IGFIR (16, 17), VEGFR (18), FGFR (19, 43) pathways and others have been shown to contribute to dysregulation of the PI3K/AKT/mTOR axis in various sarcoma subtypes. Finally, more recently discovered discrete genetic mutations (22–24) in or clustered around the PI3K/AKT/mTOR axis, document vulnerabilities in the genetic landscapes of sarcoma

that can be exploited via mTOR inhibition. The first-generation rapamycin and its analogues have had limited clinical utility despite robust preclinical rationale, thought to be at least partly a result of release of the negative feedback from S6K1 to insulin receptor substrate 1 (IRS1), leading to an unwanted increase in the prosurvival pAKT. In addition, rapalogues do not inhibit 4EBP1 (44), which allows cap-dependent translation to proceed unchecked, and have essentially no effect on mTORC2 or its downstream effectors.

MLN0128 potently inhibits proliferation of all sarcoma cell lines tested in a dose-dependent fashion with IC<sub>50</sub> values in the low nanomolar range (2–130 nmol/L). Although differences in sensitivity between various cell lines tested are not well explained by the biomarkers used in this study (Fig. 1C), we can hypothesize that related pathway components, PTEN or v-raf murine sarcoma viral oncogene homolog B (BRAF) status, for example, A673 which carries a BRAF V600E mutation, could play a role in sensitivity to antiproliferative effects of the drug. Future investigation into such differential responses to mTOR inhibition is certainly warranted. In contrast, rapamycin does not exhibit significant dose dependency despite escalation to doses as high as 1 µmol/L, and achieves its maximal antiproliferative effect at very low concentrations. This threshold effect is consistent with the effective downregulation of P-p70-S6K at 1 nmol/L noted in the current study, as well as the early (10–20 minutes) feedback reactivation of AKT-S473 that has been documented following treatment with 1 nmol/L rapamycin (27). These antiproliferative and molecular signaling events justify the unequal concentrations used to compare MLN0128 and rapamycin in the current work, although we have additionally shown that equivalent concentrations up to 1 µmol/L of each agent do not alter outcomes. In all conditions tested *in vitro*, MLN0128 provides profound antiproliferative effect that is superior to that of rapamycin.

In all sarcoma subtypes tested, MLN0128 successfully inhibited both of the major regulators of protein synthesis downstream of mTORC1, p70-S6K1 (45), and 4EBP1 (46) in a dose-dependent fashion. In contrast, rapamycin inhibited only P-p70-S6K as observed in previously published studies (44). In other tumor types, there is emerging evidence that 4EBP1 is critical to the response to both rapalogues and mTOR kinase inhibition (47), making MLN0128's effective downregulation of particular importance. The exact role that 4EBP1 plays in mediating the antiproliferative effects of MLN0128 and other mTOR kinase inhibitors in sarcomas remains under investigation, and will be indispensable to better understand the role of mTOR inhibition in sarcoma.

MLN0128 potently inhibited phosphorylation of AKT at Ser473 in all sarcoma subtypes tested. Analysis of phosphorylation on AKT at Thr308 was limited because of weak signal and detection of nonspecific proteins in many of the sarcoma cell lines studied, but when detectable, MLN0128 did inhibit this PDK1 phosphorylation site (Supplementary Fig. S1A). These data are consistent with that of other mTOR kinase inhibitors and are likely at least in part due to decreased ability of PDK to phosphorylate AKT at Thr308 when Ser473 is fully inhibited (48, 49). Genetic knockout of SIN1 or RICTOR, however, is noted to completely suppress Ser473, but not Thr308 phosphorylation (50), suggesting that MLN0128 may have additional signaling effects not mediated

through mTORC2 that downregulate phosphorylation of AKT at Thr308.

SGK1 is phosphorylated by mTORC2, but not mTORC1, as SGK1 activity is ablated in fibroblasts possessing mTORC1 activity, but lacking the mTORC2 components RICTOR, Sin1, and mST8 (40). The physiologic substrate of SGK1, NDRG1 is similarly affected making the phosphorylation of NDRG1 a ready surrogate marker for mTORC2 activity. MLN0128 inhibited the phosphorylation of NDRG1 in a dose-dependent fashion in all cell lines tested, corroborating the anticipated mTORC2 kinase inhibition. As expected, rapamycin had no effect on NDRG1 recapitulating its lack of interaction with mTORC2.

MLN0128 was well-tolerated *in vivo* and resulted in dose-dependent growth inhibition in a broad range of sarcoma xenograft models. Pharmacodynamic analysis revealed downregulation of expected targets *in vivo*, in a fashion consistent with *in vitro* data. At a dosing schedule of 3 mg/kg b.i.d. by oral gavage three times weekly, MLN0128 provided significantly better tumor suppression when compared with rapamycin in tumor xenograft models of RMS ( $P < 0.001$ ) and Ewing sarcoma ( $P < 0.01$ ). In addition, MLN0128 was able to induce apoptosis seen as increased cleaved caspase both *in vitro* and *in vivo* model of RMS (Rh30). Further studies are warranted to determine the exact mechanism by which apoptosis is induced in this tumor subtype but not others.

In summary, MLN0128 is a potent and orally bioavailable pan-mTOR kinase inhibitor with effects on both mTORC1 and mTORC2 and their downstream effectors, and has a molecular signaling profile distinct from that of rapamycin. MLN0128 induces dose-dependent inhibition of tumor growth superior to that of rapamycin in a broad range of sarcoma models *in vitro*. In models of RMS, MLN0128 induces apoptosis. Finally, in *in vivo* models of RMS and Ewing sarcoma, MLN0128 provides superior antitumor effect when compared with that of rapamycin. MLN0128 warrants further evaluation as a potential therapy for sarcoma.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** E. Slotkin, P.P. Patwardhan, E. de Stanchina, G.K. Schwartz

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## Acknowledgments

The authors thank Dr. Rachael Brake from Millennium/Takeda Pharmaceuticals for her valuable input and providing MLN0128, and Dr. Lee Spraggon for his suggestions and critical reading of the manuscript. The authors would also like to thank Dr. Christine Pratilas for her generosity in sharing patient-derived cell lines.

## Grant Support

This work was supported by NIH grant R01CA140331-04 (to G.K. Schwartz).

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Received August 22, 2014; revised November 20, 2014; accepted December 7, 2014; published OnlineFirst December 17, 2014.

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## MLN0128, an ATP-Competitive mTOR Kinase Inhibitor with Potent *In Vitro* and *In Vivo* Antitumor Activity, as Potential Therapy for Bone and Soft-Tissue Sarcoma

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*Mol Cancer Ther* 2015;14:395-406. Published OnlineFirst December 17, 2014.

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