11-2017

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Notes/Citation Information
Published in *Molecular Cancer Therapeutics*, v. 16, issue 11, p. 2432-2441.

Digital Object Identifier (DOI)
https://doi.org/10.1158/1535-7163.MCT-17-0058

mTOR kinase inhibition effectively decreases progression of a subset of neuroendocrine tumors that progress on rapalog therapy and delays cardiac impairment

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Running title: mTOR kinase inhibitors' efficacy in rapalog resistant pNETs

Keywords: mTOR, rapalogs, neuroendocrine, tumors, carcinoids, pancreatic

Financial Support: This work was supported by grants to H.E.Thomas from the NANETS (YIA award), Just-in-Time funding from the Cincinnati Cancer Center, the department of Internal Medicine junior faculty pilot project award and division of Hematology and Oncology Translational Science Awards. The UC Genomics, Epigenomics and Sequencing Core is partially funded by an NIEHS CEG administrative core grant P30ES006096.

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Total number of figures: 6

Abstract (250 words)

Inhibition of mTOR signaling using the rapalog everolimus is an FDA-approved targeted therapy for patients with lung and gastroenteropancreatic neuroendocrine tumors (NETs). However, patients eventually progress on treatment, highlighting the need for additional therapies. We focused on pancreatic NETs (pNETs) and reasoned that treatment of these tumors upon progression on rapalog therapy, with an mTOR kinase inhibitor (mTORKi) such as CC-223 could overcome a number of resistance mechanisms in tumors and delay cardiac carcinoid disease. We performed preclinical studies using human pNET cells in vitro and injected them subcutaneously or orthotopically to determine tumor progression and cardiac function in mice treated with either rapamycin alone or switched to CC-223 upon progression. Detailed signaling and RNA sequencing analyses were performed on tumors that were sensitive or progressed on mTOR treatment. Approximately 57% of mice bearing pNET tumors which progressed on rapalog therapy showed a significant decrease in tumor volume upon a switch to CC-223. Moreover, mice treated with an mTORKi exhibited decreased cardiac dilation and thickening of heart valves than those treated with placebo or rapamycin alone. In conclusion, in the majority of pNETs that progress on rapalogs, it is possible to reduce disease progression using an mTORKi, such as CC-223. Moreover, CC-223 had an additional transient cardiac benefit on valvular fibrosis compared to placebo- or rapalog-treated mice. These results provide the preclinical rationale to further develop mTORKi clinically upon progression on rapalog therapy and to further test their long term cardioprotective benefit in those NET patients prone to carcinoid syndrome.

Introduction

The incidence of pancreatic neuroendocrine tumors (pNETs) is increasing (1). These tumors vary in their clinical presentation, depending on whether they secrete hormones and have metastasized to the liver (2). At the genetic level, mutations in key players of the mammalian Target Of Rapamycin (mTOR) pathway have been identified in pNET patient tumors by whole-exome sequencing (3, 4). Down-regulation of tumor suppressors of this pathway, including phosphatase and tensin homolog (*PTEN*) and tuberous sclerosis complex 2 (*TSC2*) leads to mTOR activation in a number of human pNETs (5). In addition, nutrients, amino acids and glucose, as well as hormones, insulin and IGF1, mediate mTOR activation. The clinical relevance of targeting mTOR signaling in these tumors is highlighted by the FDA approval of the allosteric mTOR inhibitor everolimus (RAD001), for the treatment of pNETs. The rapalog, RAD001 by targeting mTOR complex 1 (mTORC1) has both anti-proliferative and anti-angiogenic properties in tumors (6). In the phase 3 RAD001 in Advanced Neuroendocrine Tumors (RADIANT-3) clinical trial, treatment of pNET patients with RAD001 increased median progression-free survival by ~6.4 months compared to placebo, leading to its FDA approval for the treatment of pNET patients with progressive, unresectable, locally advanced or metastatic disease (7). More recently, the follow-up phase 3 RADIANT-4 trial data reported that RAD001 significantly improved the median progression-free survival in patients with progressive gastrointestinal (GI) or lung NETs by ~7.1 months (8). Nonetheless, the response to treatment is not durable, most likely due to incomplete and unsustained inhibition of mTORC1 signaling by rapalogs, and/or activation of mTOR complex 2 (mTORC2), underscoring the need for alternative therapies and drug combinations (9).

Consistent with our studies in cell culture (10), analysis of patient biopsies treated with RAD001 suggested that activation of Protein Kinase B (PKB referred to as Akt), due to loss of the mTOR/40S ribosomal protein S6 kinase 1 (S6K1) negative-feedback-loop, could contribute to tumor progression (11). Irrespective of treatment response, S6K1 mediated phosphorylation (p-) of ribosomal protein S6, an indicator of mTORC1 activation, was significantly decreased in matched neuroendocrine tumor patient biopsies after 2 weeks of RAD001/octreotide treatment (12), suggestive of effective target inhibition. However, RAD001 affects substrate specificity not kinase activity. Indeed, RAD001 can abolish S6K1 signaling, while having little impact on other mTORC1 substrates such as translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and unc-51 like kinase 1 (ULK1) (10, 13). Thus, incomplete inhibition of mTORC1 substrates and activation of survival effector Akt have the potential to lead to drug resistance. This

resistance could potentially be overcome by phosphatidylinositol 3-kinase (PI3K) and/or ATP-site competitive mTOR inhibitors, which have an added therapeutic advantage in solid tumors as they inhibit the catalytic activity of both mTOR complexes (14-16). However, there is a lack of systematic evidence demonstrating in vivo whether a switch to an mTOR kinase inhibitor (mTORKi) will be beneficial upon progression on rapalog therapy in pNETs.

Preclinical mouse models for pNETs have been valuable as predictors of clinical responses to drug combinations and targeted therapies (17-20). More recently, nude mice injected with BON cells, which were derived from a lymph node metastasis of a human pancreatic tumor (21), not only form primary tumors and metastases to the liver and abdominal lymph nodes (20), but also exhibit carcinoid syndrome. This includes cardiac carcinoid disease that phenotypically recapitulates the human disease (22). The cardiac impairment and fibrosis that develops in some pNET patients ultimately leads to congestive heart failure (23). Although increased serotonin levels secreted by the tumor have been postulated to play a role in cardiac carcinoid disease, the detailed molecular mechanism remains unclear (24).

Based on these premises and using in vivo mouse models, we investigated whether mTOR kinase inhibition provided benefit over rapalog treatment in pNETs that progress on rapalog therapy. Moreover, we determined whether the benefit from inhibiting mTOR signaling would extend beyond inhibiting tumor progression to the heart, as it has been reported to be adversely impacted by the growth of the primary tumor and its metastases.

Materials and Methods

Cell Culture: BON cells were a kind gift from Dr. Courtney Townsend (UTMB Texas) received in July, 2013. QGP1 cells were purchased in February, 2015 from the Japanese Collection of Research Bioresources Cell Bank. Both cell lines were expanded and frozen into multiple low passage aliquots. BON and QGP1 cells were passaged using DMEM: F12K media or RPMI respectively, routinely tested for mycoplasma contamination and not maintained in culture for more than 1.5 months after thawing of a frozen vial. Expression of NET markers, including chromogranin A and synaptophysin were confirmed by western blotting for both cell lines. All media contained 10% fetal bovine serum and penicillin/streptomycin antibiotics. Firefly luciferase virus obtained from GenTarget (LVP-326) was used to infect BON cells followed by selection of stable pool of cells using blasticidin (Life Technologies). TAK-228, rapamycin and RAD001 were obtained from LC laboratories and AZD2014 from Selleckchem.

Author Manuscript Published OnlineFirst on September 1, 2017; DOI: 10.1158/1535-7163.MCT-17-0058
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Mouse experiments: All in vivo studies were performed following procedures approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Cincinnati (UC). 5-week old nude male mice (J:NU stock 007850) were obtained from Jackson Laboratories and housed at a barrier facility in our institution with ad libitum access to food and water. Mice were injected with $2x10^6$ cells subcutaneously or $1x10^6$ cells in 20 µl orthotopically into the pancreas (25). Subcutaneous tumor measurements were done using digital calipers and the volume was calculated using the formula ($\pi/6$ x length x width^2). Rapamycin was administered in drinking water at (10 mg/kg body weight (BW) daily). The water was refreshed every other day. Progression on rapamycin was defined according to a modified Response Evaluation Criteria in Solid Tumor (mRECIST) by an increase of at least 20% in diameter in the majority of the mice. Mice were taken off rapamycin for 24h prior to the switch to CC-223 to allow time for the rapamycin to be cleared to avoid dosage with the combination of both drugs. CC-223, a generous gift from Celgene was gavaged twice daily at 15 mg/Kg BW. The percent change in tumor volume from baseline was calculated based on the formula: [100*(tumor volume at time of sacrifice – tumor volume at time of switch)/ tumor volume at time of switch]. Mice were starved for 3h prior to the time of euthanasia at which point all tumors and tissues collected were snap-frozen using liquid nitrogen and/or fixed in formalin. Serum serotonin levels were measured using an ELISA assay (IBL-America) following the manufacturer protocol.

Imaging: Mice were injected with potassium luciferin (Gold Biotechnology) and 10 min later were anesthetized using isoflurane. Bioluminescence imaging was performed using a 2D Carestream (Kodak) MultiSpectral FX (2D).

Echocardiographic studies: Mice were anesthetized with isoflurane and cardiac function and structure was measured via high frequency echocardiography with M-mode imaging (26), and valvular function with B-mode and color Doppler imaging (27) as previously described using a Vevo2100 ultrasound system. Post-processing of the images was done using the VevoStrain software (Visualsonic, Vevo 2100, v1.1.1 B1455) by an investigator blinded to the treatment groups.

IHC/Modified Masson Trichrome staining and scoring of valve thickness: The heart was trimmed longitudinally through both ventricles after formalin fixation. The tissue was then sectioned (at a thickness of 5µM) until the valves were visible. At which point, 20 µM were skipped and another section was saved until the tissue was sectioned throughout in order to get

an even representation throughout the whole valve. These sections were then stained with a modified Masson trichrome staining kit (ScytTek Laboratories) following the manufacturer's protocol. The stained slides were scored for cardiac valvular and endocardial histopathologic lesions by a veterinary pathologist blinded to the treatment groups.

Western blot analyses: Tumors were extracted as previously described except for the lysis buffer contained 1% NP-40 (10). Quantitative western blot analyses were performed using a Li-Cor Odyssey imaging system. All total and phosphorylated antibodies were obtained from Cell Signaling Technology except for chromogranin A which was from ThermoFisher Scientific.

RNA sequencing: RNA-seq of tumors was performed at the UC Genomics, Epigenomics and Sequencing Core following a standardized method. Briefly, RNA was isolated from each ~50 mg of each tumor sample using the mirVana miRNA Isolation Kit (ThermoFisher) according to the manufacturer protocol. Target RNA enrichment was performed from ~750 ng of RNA by isolation of polyA RNA using NEBNext Poly(A) mRNA Magnetic Isolation Module (New England BioLabs). PrepX PolyA script was run for automated polyA RNA isolation. RNA-seq library preparation was done using NEBNext Ultra Directional RNA Library Prep Kit (New England BioLabs) with dUTP for cDNA synthesis to maintain strand specificity, indexing via 11 cycles of PCR enrichment and finally cleaning up of the amplified library by AMPure XP beads for QC analysis. The quality and yield of the purified library was analyzed with a Bioanalyzer (Agilent) and a DNA high sensitivity chip; then the concentration was quantified by qPCR using the Kapa Library Quantification kit (Kapabiosystem). Individually indexed and compatible libraries were proportionally pooled (~$25x10^6$ million reads per sample) for clustering with a cBot system (Illumina). Libraries at the final concentration of 15 pM were clustered onto a single read flow cell using Illumina TruSeq SR Cluster kit v3, and sequenced to 50 bp using TruSeq SBS kit on an Illumina HiSeq system. The laboratory for Statistical Genomics and Systems Biology at UC performed the bioinformatics analyses by aligning the sequence reads to the genome using a standard Illumina sequence analysis pipeline. A list of significantly different genes between the mTOR inhibitors treatment groups was generated by a multivariate regression analysis with a significant p-value ≤0.006. Comparative networks and functional analyses were performed using the NetWalker software (28). The GEO accession number for the RNA-seq data is GSE102246.

Statistical Analyses: Statistical analyses were performed using the GraphPad Prism 5 Software. Unless otherwise specified in the figure legends, data are presented as means ±

standard error of the mean (SEM). Statistical significance was determined by a one-way analysis of variance (ANOVA) followed with a Bonferroni multiple group comparison test when applicable unless otherwise stated in the figure legend.

Results

Rapalog treatment using rapamycin or its analog RAD001, has been reported to delay tumor growth of BON cells subcutaneously injected into mice (12). To follow tumor progression in the mouse, we generated BON cells (29) that stably express luciferase (BON-luci) for in vivo bioluminescence imaging (BLI). In addition, to recapitulate the tumor microenvironment for a pNET, the BON-luci cells were injected into the pancreas. To our knowledge, we are the first to test the preclinical efficacy of mTOR inhibitors on BON cells or human pNET cell lines orthotopically rather than subcutaneously (12, 30) or intrasplenically (22). Mice were randomized into 2 groups of relatively equal tumor volume based on BLI measurements at ~5 weeks post-implantation of the cells, and treated either with placebo or rapamycin. Rapamycin treatment decreased tumor burden significantly compared to placebo (**Fig 1A, B**) after 25 days, albeit the tumors were still progressing. As previously reported for subcutaneously injected BON tumors, orthotopic tumors of rapamycin-treated mice had a significant decrease in p-S6, no inhibition of 4E-BP1 and activation of p-Akt compared to placebo-treated mouse tumors (**Fig 1C**).

Given this incomplete suppression of mTOR signaling in rapamycin-treated BON tumors, we reasoned that treatment of BON tumors upon progression on rapalog therapy with an mTORKi would overcome a number of resistant mechanisms such as those mediated by activation of Akt or 4E-BP1; owing to their more potent inhibition of these and other mTOR substrates. Indeed, treatment of BON cells in vitro with clinically relevant mTORKi(s) including CC-223, MLN0128 (also known as TAK-228, sapanisertib or INK128) and AZD2014 (vistusertib) decreased phosphorylation of a number or mTOR substrates, including p-4E-BP1, p-Akt and p-ULK1 in a dose-dependent manner compared to vehicle or rapalogs (**Fig 2A, S1**) (31-33). These mTORKis were equally effective in inhibiting mTORC1/2 signaling in QGP1 cells, another human pNET cell line (**Fig 2B, S1**). To test our hypothesis in vivo, we injected mice with BON-luci cells, subcutaneously, to allow for quantitative volumetric monitoring of tumor growth. Once tumor volumes averaged ~180 mm^3, mice were randomized into 2 groups of equal tumor volume for treatment with either placebo or rapamycin (**Fig 3A**). Rapamycin treatment resulted in a significant decrease in tumor volume compared to placebo treatment, although tumor progression continued similar to our findings with the orthotopically injected tumors. Once the

tumors progressed on rapalog therapy according to a modified RECIST criteria, these mice were divided into 2 cohorts of similar tumor distribution and either maintained on rapamycin or switched to CC-223. Placebo-treated mice were euthanized on day (D) 80 as not to exceed the tumor volume limited by IACUC. Tumor progression in mice switched to CC-223 was decreased compared to rapamycin (**Fig 3B**). More importantly, a waterfall plot of the percent change in tumor volume from the time of switch to CC-223 of each mouse revealed that while some mice responded to the switch to CC-223 others continued to progress (**Fig 3C**). Mice in the CC-223-treatment group were further separated into two subgroups termed “CC-223-responders” based on the clinical criteria of either stable disease or a better response rate to treatment; while mice with a change from baseline greater than 20% were grouped as “CC-223 non-responders” (**Fig S2**).

To gain insight into the mechanism by which CC-223 treatment led to inhibition of tumor progression in some mice and not others, we performed signaling analyses on tumors from all treatment groups to determine if the extent of inhibition of mTOR signaling would correlate with the level of tumor response. Overall, mTOR substrate inhibition of rapamycin-treated tumors compared to placebo-treated tumors was equivalent between the subcutaneously injected tumors and orthotopic ones (**Fig 4 and 1C**). CC-223 treatment induced a more pronounced inhibition of p-4E-BP1, p-Akt S473 and p-ULK1 S758 than rapamycin-treated tumors (**Fig 4**). Moreover, although there was a modest increase in phosphorylation of Extracellular signal-Regulated Kinase (ERK) at T202/Y204 in CC-223-treated tumors when compared to placebo, there was no apparent correlation between MAPK/ERK signaling and tumor response (**Fig 4**).

Since inhibition of these selected targets did not explain the differences in response to CC-223, we performed RNA sequencing (RNA-seq) analyses as an unbiased means to assess changes in gene expression. Our major goal was to identify the differences in tumor mRNAs between the CC-223- and non-CC-223 responders compared to the rapamycin alone treatment arm (**Fig 5A**). A total of 477 genes were significantly differentially expressed between CC-223 and rapamycin-treated tumors and clustered into 3 groups highlighted in the heat map (**Fig 5A**). Interestingly, RNA-seq analyses revealed that in comparison to rapamycin and the CC-223 responders, the expression of ribosomal proteins and other genes involved in translation, were increased in a number of CC-223 non-responders (cluster 1) (**Fig 5A and 5B**), consistent with the accelerated growth of these tumors. Indeed, western blot analyses revealed a modest increase in levels of ribosomal proteins (RP) L5 and S19 and eIF4E in a number of tumors of CC-223 non-responders compared to CC-223 responders (**Fig 5C**). Tumors are dependent on protein synthesis to meet high demands of growth and proliferation. It is not surprising that

inhibition of mTOR signaling, a pathway implicated in regulating translation at multiple signaling and transcriptional nodes would alter the translational landscape within tumors (32). Nonetheless, it is interesting that transcription of certain proteins involved in translation was upregulated in a number of CC-223 non-responders tumors under mTOR inhibition. Since translation is a major energy consuming process in cells (34), we determined the activation state of AMP-activated protein kinase (AMPK) a major energy sensing kinase (35). Overall, the level of p-AMPK in mTORKi-treated tumors appeared to inversely correlate with the tumor response rate and upregulation of some of the translational machinery.

Next, we set out to determine if mTOR inhibition had additional benefits beyond the direct effects on tumor progression; focusing on cardiac carcinoid disease which has been linked to decreased survival in pNET patients (36). Interestingly, the RNA-seq data revealed that transcript levels of L-DOPA decarboxylase (DDC), an enzyme involved in the synthesis of serotonin, was significantly decreased in tumors switched to CC-223 compared to rapamycin-treated tumors (**Fig S3A**). Consistent with the transcriptional downregulation of DDC, protein levels of DDC were also decreased in CC-223-treated tumors irrespective of the response rate (**Fig 5C**). To address the relative impact of mTOR inhibitors on cardiac impairment, we implanted BON-luci cells into the pancreas; as this better recapitulates human tumor growth at the primary site with occurrence of metastases to the liver and other organs upon disease progression. Two weeks after orthotopic injection, the mice were randomized into 2 groups of comparable tumor burden, based on BLI measurements, to be treated with either placebo or rapamycin for 22 days (**Fig 6A**). On day 24 (D24), rapamycin-treated mice were divided into 2 groups of relatively equal tumor burden, again based on BLI, such that one group remained on rapamycin while the other was switched to CC-223 for ~26 days (**Fig 6A, S3B**). Cardiac function and structure were measured by high frequency echocardiography with M-mode imaging (26), and valvular function with B-mode and color Doppler imaging (27) on D34 (D10 post-switch to CC-223) (**Fig 6B**) and ~2 days prior to sacrifice on D48 (D24 post-switch to CC-223) (**Fig 6C**). Both CC-223 and rapamycin-treated mice had a significant improvement in left ventricular dilation (diastolic volume) compared to placebo within ~10 days of the switch to CC-223 (**Fig 6B**) but this conferred cardiac protection was not statistically significant within ~24 days of the switch to CC-223 (**Fig 6C**). Systolic cardiac function (as measured via ejection fraction) was not significantly different among the groups at either time point. However, diastolic function (as measured by E/E') was slightly better in mice switched to CC-223 treatment than those remaining on rapamycin at D34 (D10 post-switch) (**Fig 6B),** but was less apparent by D48 (D24 post-switch to CC-223) (**Fig 6C**).

Importantly, histopathological blind scoring of trichrome-stained consecutive heart sections showed that only mice that were switched to CC-223 treatment had less histopathological lesions of their heart valves (especially the mitral and tricuspid ones) as compared to either placebo- or rapamycin-treated mice (**Fig 6D, 6E** and **Table S1**). Although rapamycin-treated mice or those switched to CC-223 had a significant decrease in tumor burden compared to placebo (**Fig S3C**), there was no difference in tumor sizes between the 2 mTOR inhibitors that could account for the difference in valvular pathology. A smaller number of histopathological valve lesions in the hearts of mice switched to CC-223 was associated with decreased expression of DDC in the tumor (**Fig S3D**). Although it was possible that DDC levels might influence serotonin synthesis, the expression of the rate-limiting serotonin synthesis enzyme tryptophan hydroxylase 1 (TPH1) was unchanged (**Fig S3D**), and serum serotonin levels were not significantly different between any of the treatment groups (**Fig S3E**). Together, these results indicate that mTOR inhibitors can potentially delay progression of cardiac impairment and that the sustained efficacy of mTORKi(s) may be more prolonged than that of rapalogs.

Discussion

Since RAD001 is FDA-approved for the treatment of pNETs, and a number of mTORKi(s) are currently in phase 1-2 clinical trials, it is translationally relevant to determine if an mTORKi would serve as next line therapy to RAD001 clinically. Our data suggest that switching to an mTORKi, here CC-223, in a subset of mice that progress on rapalog therapy, provides potential clinical benefits. Recently published data from a clinical trial using the dual mTOR/ phosphatidylinositol 3-kinase (PI3K) inhibitor BEZ235 to treat pNET patients who had progressed on an mTOR inhibitor reported at least stabilization of disease in greater than 50% of the patients albeit with numerous challenges (37). The latter consisted of multiple adverse effects, treatment interruptions and poor pharmacokinetics (PK) of BEZ235. A number of issues have dampened the initial excitement in the clinical development of PI3K and/or mTOR kinase inhibitors; ranging from higher toxicity profiles compared to rapalogs, to clinical testing of inhibitors with suboptimal formulations, PK and pharmacodynamics (PD) properties. Nevertheless, a number of mTORKi(s) with improved PK/PD are being actively pursued in the clinic. One of these, TAK-228 is currently in a phase II clinical trial for pNETs. Moreover, a third generation of mTOR inhibitors has been developed which could be best described as a hybrid of the first 2 generations of inhibitors (38) with recently reported improved efficacy compared to rapalogs (39). In summary, based on our preclinical data, treatment of pNETs with a rapalog

until progression then switching to an mTORKi was associated with tumor responses in the majority of the cases.

Activation of the MAPK/ERK pathway may signal the beginning of acquired resistance to mTOR inhibitors and has been reported in biopsied patient-tumors after RAD001 treatment (40). We found that p-ERK modestly increased in tumors of mice switched to CC-223 compared to control tumors, irrespective of tumor response. This is consistent with previous reports showing activation of MAPK following treatment of BON cells with BEZ235 (30). Future studies will be needed to determine whether MAPK/ERK activation could be clinically exploited upon development of resistance to mTORKi.

A number of additional resistance mechanisms to rapalogs have been reported to occur both in tumors extrinsically as well as in a tumor cell intrinsic manner (41). Incomplete inhibition of p-4E-BP1 is one potential mechanism of resistance to rapalogs that can be overcome by mTORKi(s). In the context of mTORKi treatment, the hypophosphorylated 4E-BP1 sequesters eIF4E, thus preventing the assembly of a productive eIF4F translation initiation complex (42). Availability of eIF4E is rate limiting for translation, and its overexpression is sufficient to induce both transformation and tumor formation (43, 44). Interestingly, a number of CC-223 non-responders had increased levels of total eIF4E and ribosomal proteins compared to CC-223-responders in addition to the decreased AMPK activity, consistent with upregulation of the translational machinery and anabolic processes, which actively promote tumor growth (**Fig 6F**).

As a secondary endpoint, our study also tested the potential cardiac benefits provided by mTOR inhibitors, as the heart is one of the organs damaged by growth of NET tumors. Earlier literature reported more than half (66 to ~77%) of NET patients developed cardiac carcinoid disease (24, 36, 45). However more recent data suggest that this percentage is lower, potentially due to earlier diagnosis and treatment with somatostatin analogs (46). Once valvular carcinoid disease develops, the affected valve can be replaced with either a mechanical or tissue valve of bovine or porcine origin. The disadvantage of a tissue valve is that it also may become damaged due to the underlying carcinoid disease. Our results suggest that mTOR inhibitors decrease progression of cardiac impairment due to valve thickening. Although serum serotonin mean levels were lower in both rapamycin- and CC-223- treated mice than in placebo-treated mice at the time of sacrifice; the differences were not significant. This suggests that either (1) the levels of serotonin had increased by that time point above a minimum threshold required to induce valvular thickening and fibrosis, (2) mTOR inhibition decreased the expression of serotonin receptors on valve cells and/or (3) other players are implicated including bradykinin or chromogranin A (45). Further investigations beyond the scope of these studies are

needed to decipher the mechanism of mTOR inhibitors on serotonin synthesis and secretion by tumor cells and uptake by valve cells. Altogether, it might be more suitable to treat pNET patients who are at a greater risk of developing cardiac carcinoid disease with mTOR inhibitors in lieu of other FDA-approved targeted therapies such as sunitinib which has been associated with cardiotoxicity (47). Teasing out the impact of everolimus on carcinoid syndrome is complicated as many patients treated with everolimus usually receive octreotide or other somatostatin analogues in conjunction to manage their carcinoid syndrome.

Altogether, our results reveal potential applicability to other GI and lung NETs given the efficacy of everolimus based on the RADIANT4 trial. Our findings warrant deeper future studies to test the efficacy of mTORKi(s) and/or to determine selection biomarkers for pNET patients who would more likely benefit from a switch to an mTORKi upon progression on rapalog therapy.

Acknowledgments

Dr. Courtney Townsend for kindly providing BON cells. Celgene for providing CC-223 and Dr. Ellen Filvaroff for discussions. Drs. Sara Kozma and George Thomas for valuable discussions and critical reading of the paper. Members of the laboratories of Drs. Carol Mercer, Atsuo Sasaki, Sara Kozma and David Plas for project discussions. Drs. Olowokure Olugbenga for clinical insights and valuable comments. Joanne Tetens-Woodring, Carissa Lester and all LAMS staff for animal care and advice. The UC Genomics and Epigenomics Core, especially Drs. Xiang Zhang and Jenny Chen. Tamara Hagen for laboratory management assistance.

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Figure legends

Figure 1. Mice-bearing orthotopically injected BON tumors had a significant decrease in tumor burden when treated with rapamycin compared to placebo. **(A)** Representative BLI images of mice treated with placebo or rapamycin at the indicated times. All images were obtained with 1 min exposure. **(B)** Mean tumor weight (±SEM) at the time of sacrifice of mice treated with placebo (n=9) and rapamycin (n=11) for 25 days. *p<0.05 using a t-test. **(C)** Western blot analyses of tumor lysates with each lane representing a mouse tumor. Actin was used as a loading control.

Figure 2. mTORKis result in more potent inhibition of mTOR signaling compared to rapalogs in a dose dependent manner in the human pNET cell lines **(A)** BON and **(B)** QGP1 cells. Cells were treated with vehicle, RAD001 (20nM), rapamycin (50nM) or mTORKis (100, 200, 400 and 800nM) for 2h prior to harvesting them for signaling analysis by western blotting.

Figure 3. A switch to CC-223 resulted in significant decrease in tumor burden in a subset of BON tumors that progressed on rapalog therapy. **(A)** Schematic diagram of timeline (day (D)) for subcutaneous injection of cells and drug treatments. **(B)** Changes in tumor volume (mean ± SEM) over time as a result of treatment with placebo (n=8), rapamycin (n=15) and CC-223 (n=15). There were 2 deaths during the course of the experiment: 1 rapamycin-treated mouse died due to a technical gavage error and 1 CC-223-treated mouse which initially developed diarrhea on rapamycin treatment and had to be euthanized for persistent symptoms. **(C)** Percent change in tumor volume from baseline (D78) until the time of euthanasia (D94) of mice treated with rapamycin or CC-223.

Figure 4. Compared to placebo- or rapamycin-treated tumors, CC-223-treated mice had a more complete inhibition of mTOR targets irrespective of tumor response. Western blot analyses of tumor lysates. Each lane was loaded with lysates from individual mouse tumors. The green and red bars indicate respectively tumors that either responded to treatment or did not respond based on mRECIST criteria. Total actin was used as a loading control.

Figure 5. Distinct significant changes occurred at the genetic and signaling levels of CC-223 responders and non-responders compared to rapamycin-treated tumors. **(A)** Hierarchical clustering of genes in rapamycin-treated tumors and tumors of CC-223 responders (green line), or non-responders (red line). Each row represents an individual tumor. **(B)** A network diagram of translation proteins which were significantly upregulated in tumors from CC-223 non-responders mice compared to tumors from rapamycin-treated mice obtained using the NetWalker software. Blue and black lines indicate reactome and protein-protein interactions respectively. **(C)** Western blot analyses of tumor lysates (same as Figure 4).

Figure 6. Mice switched to CC-223 treatment developed less histopathologic lesions of their heart valves and fibrosis than in rapamycin or placebo treatment. **(A)** Schematic diagram of timeline for orthotopic injection of cells and drug treatments. **(B and C)** Mean diastolic volume and E/E' (±SEM) on ~D10 and ~D24 respectively from the switch to CC-223. *p< 0.001. **(D)** Representative images of the modified Masson trichrome staining of the heart. The arrowheads point to the tricuspid valve leaflets with the blue color staining collagen. **(E)** Percent thickened valves based on scoring of cardiac valvular histopathologic lesions by a pathologist blinded to the treatment groups (detailed in Table S1). **(F)** Model for potential development of resistance that could occur in pNETs leading to increased tumor growth, valvular fibrosis and cardiac impairment.

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Figure 1.

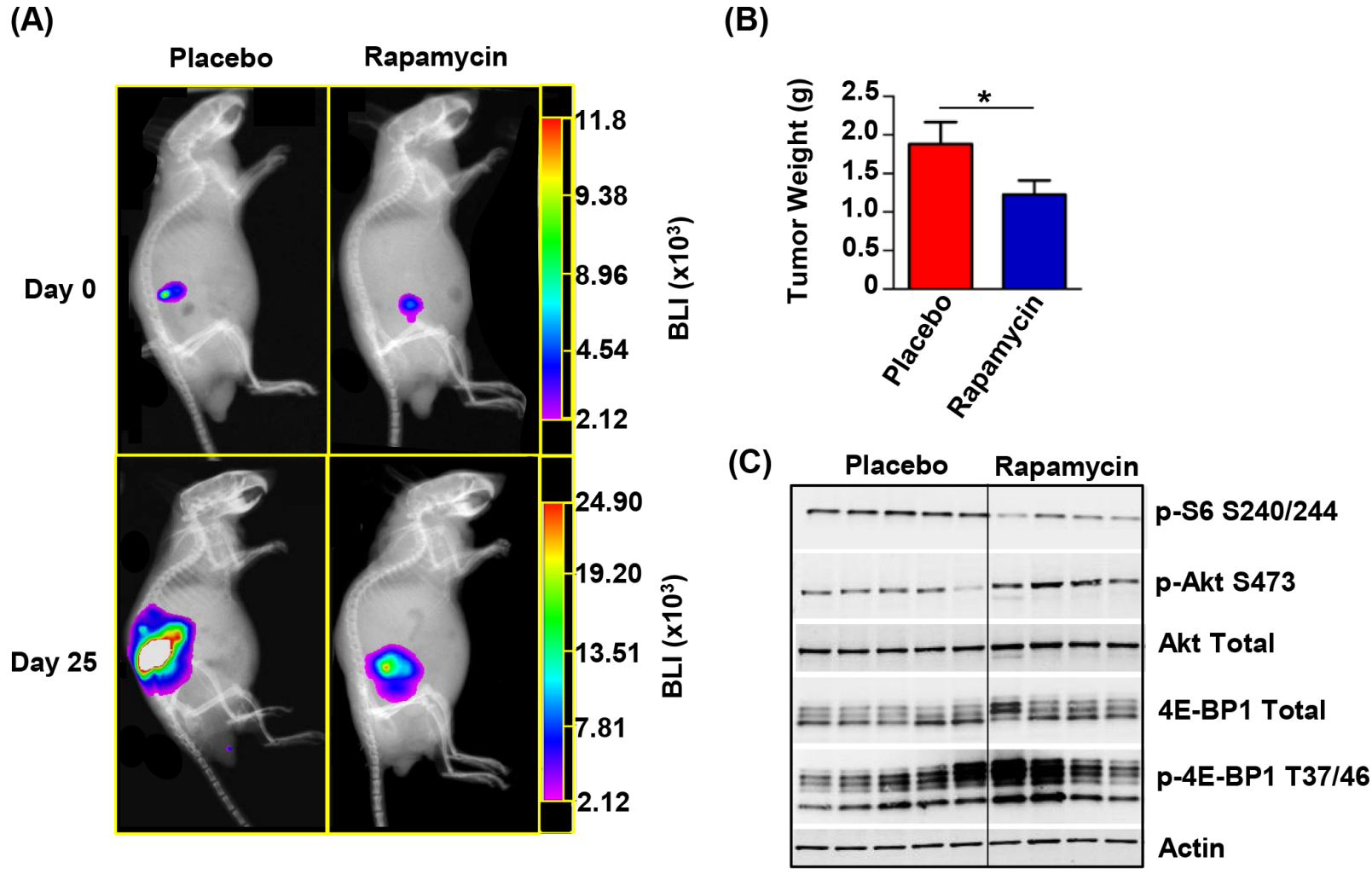

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Figure 2.

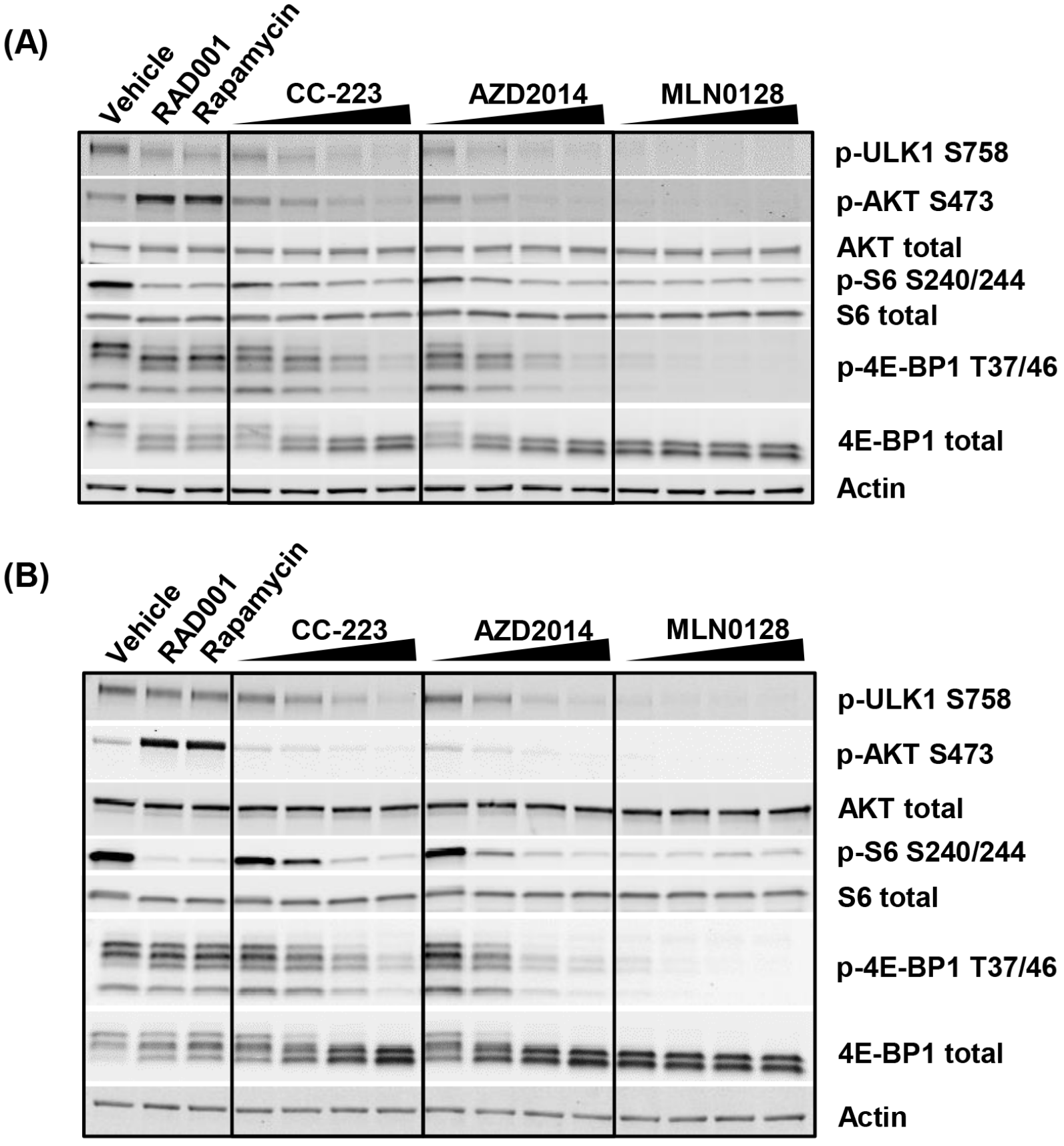

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Figure 3.

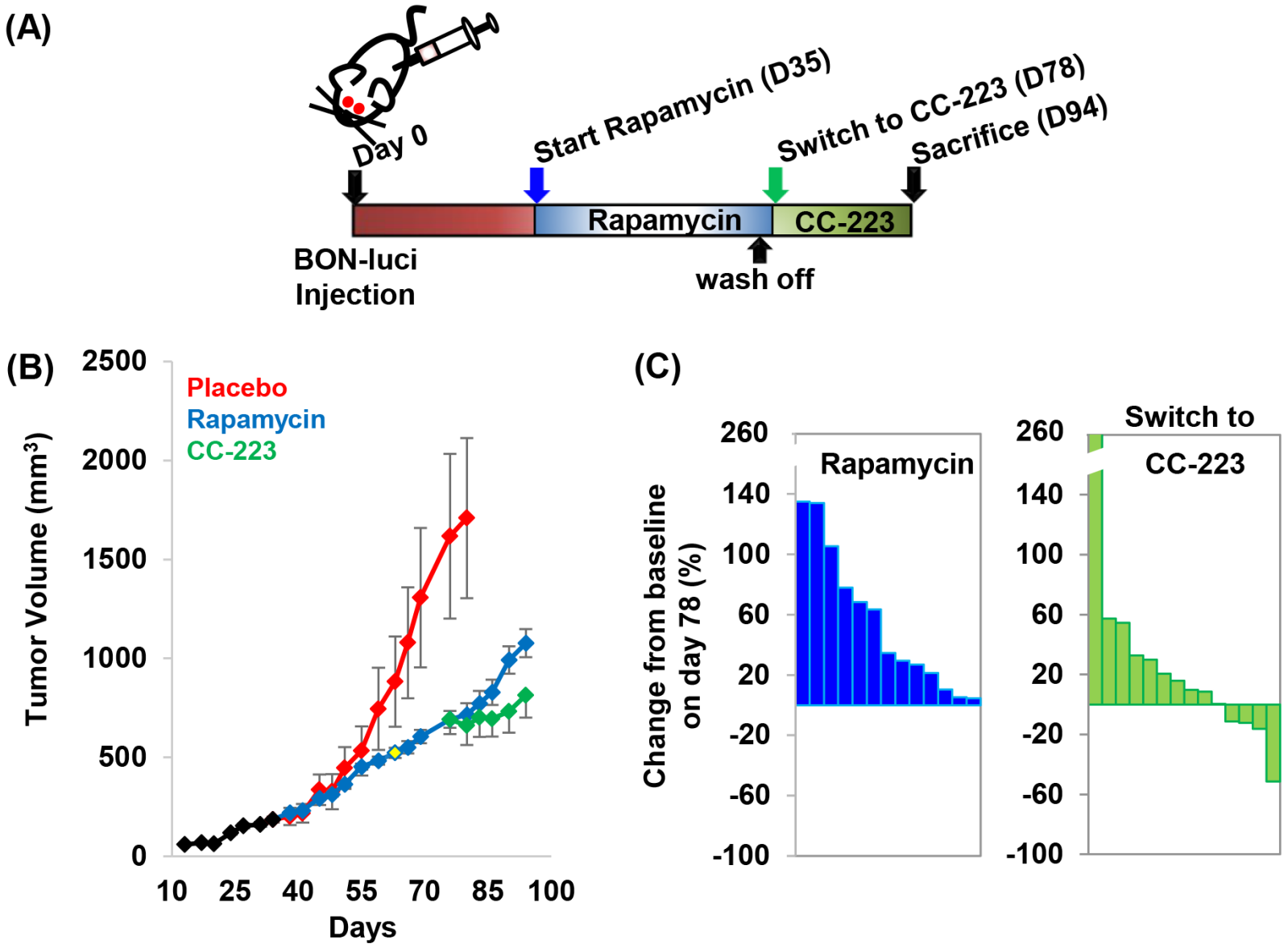

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Figure 4.

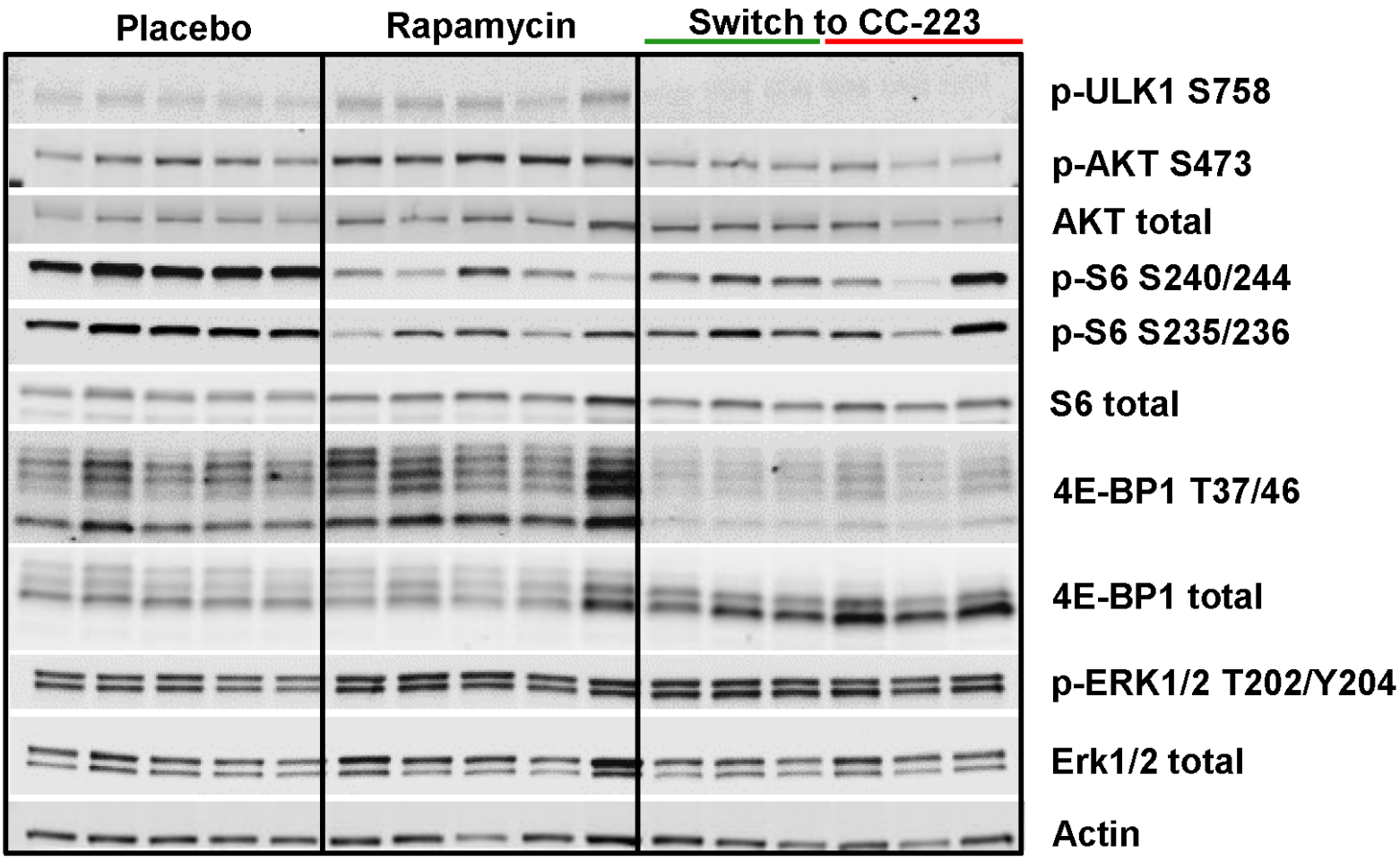

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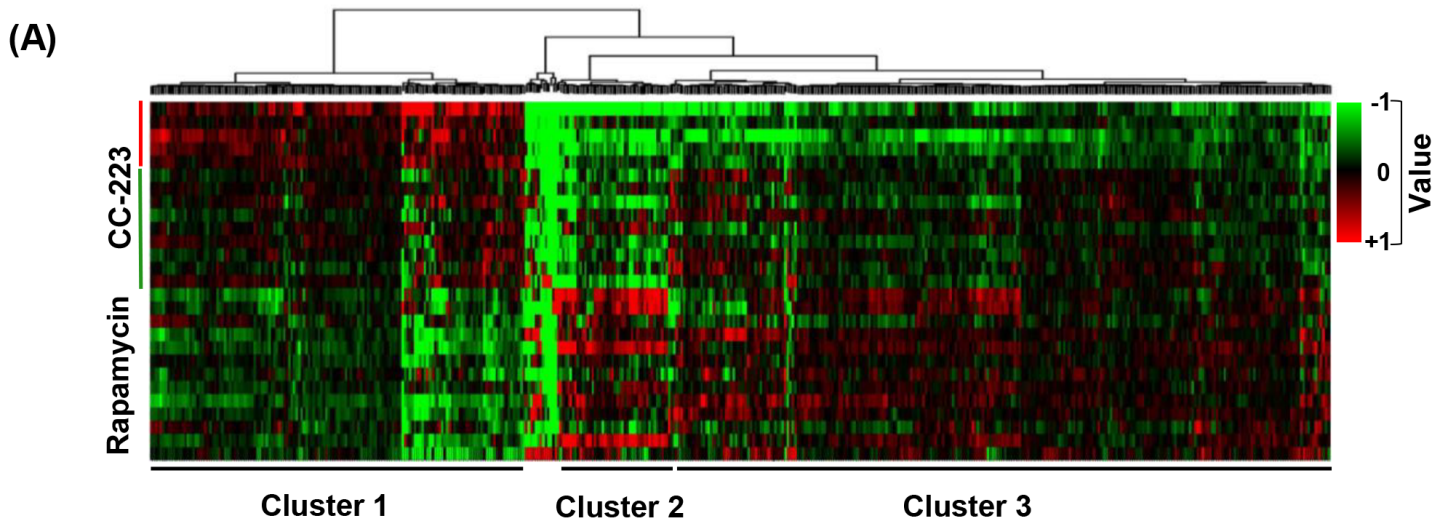
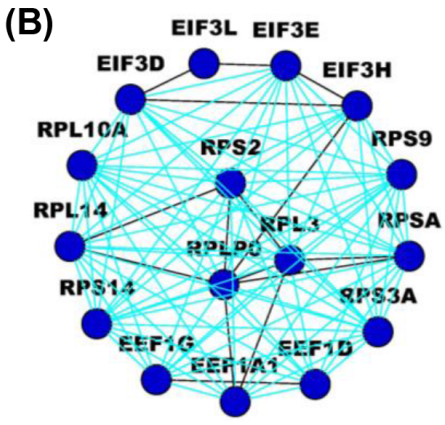
Figure 5.





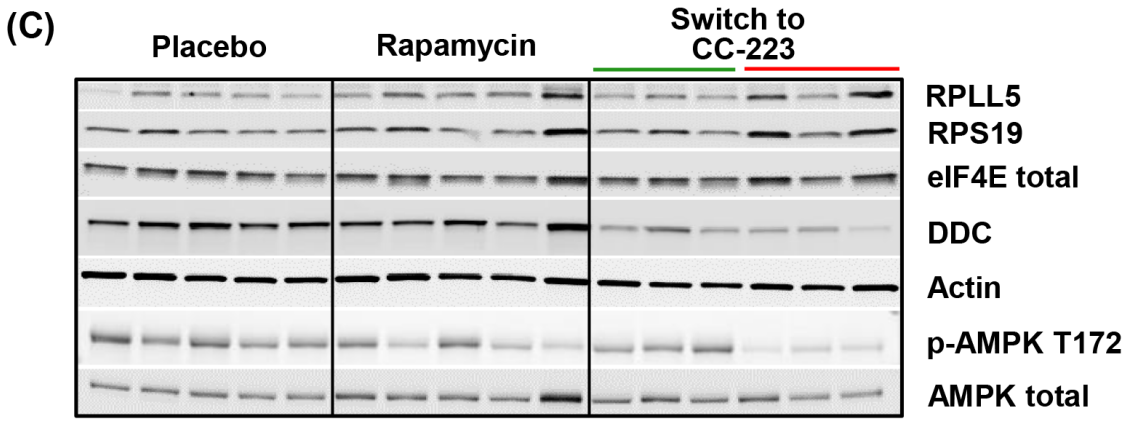


Figure 6.
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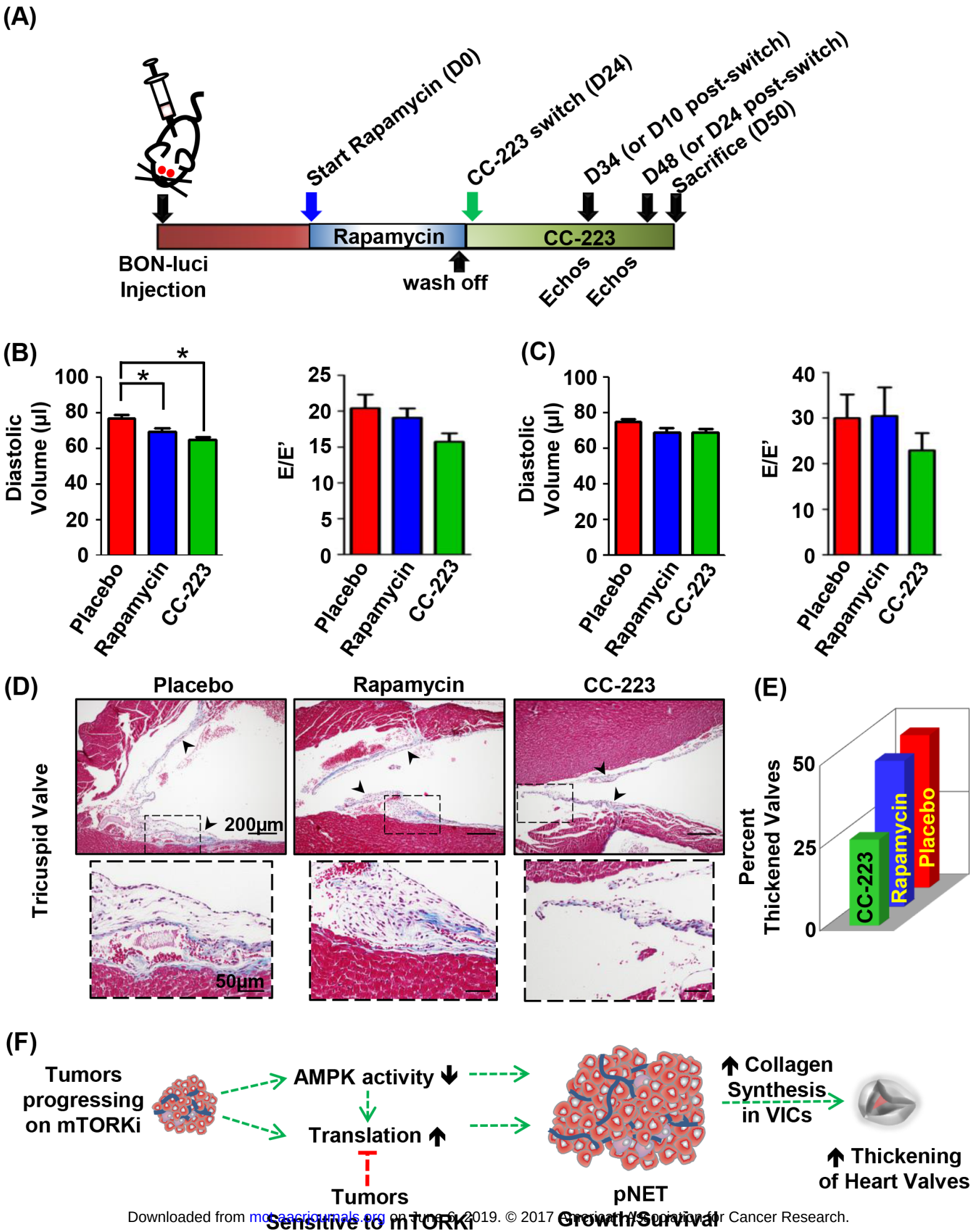

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mTOR kinase inhibition effectively decreases progression of a subset of neuroendocrine tumors that progress on rapalog therapy and delays cardiac impairment

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Mol Cancer Ther Published OnlineFirst September 1, 2017.