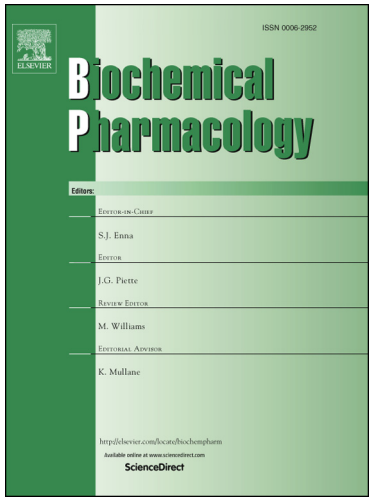

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Title:

Small molecules bind human mTOR protein and inhibit mTORC1 specifically

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Abstract (250 words)

Inhibition of mTOR activity (mechanistic target of rapamycin) is an anti-cancer therapeutic strategy. mTOR participates in two functional complexes, mTORC1 and mTORC2. Since mTORC1 is specifically activated in multiple tumors, novel molecules that inhibit mTORC1 could be therapeutically important. To identify potentially novel modulators of mTOR pathways, we screened 1600 small molecule human drugs for mTOR protein binding, using novel biolayer interferometry technology. We identified several small molecules that bound to mTOR protein in a dose-dependent manner, on multiple chemical scaffolds. As mTOR participates in two major complexes, mTORC1 and mTORC2, the functional specificities of the binders were measured by S6Kinase and Akt phosphorylation assays. Three novel 'mTOR general' binders were identified, carvedilol, testosterone propionate, and hydroxyprogesterone, which inhibited both mTORC1 and mTORC2. By contrast, the piperazine drug cinnarizine dose-dependently inhibited mTORC1 but not mTORC2, suggesting it as a novel mTORC1-specific inhibitor. Some of cinnarizine's chemical analogs also inhibited mTORC1 specifically, whereas others did not. Thus we report the existence of a novel target for some related piperazines including cinnarizine and hydroxyzine, i.e. specific inhibition of mTORC1 activity. Since mTOR inhibition is a general anti-cancer strategy, and mTORC1 is specifically activated in some tumors, we suggest the piperazine scaffold, including cinnarizine and hydroxyzine, could be proposed for rational therapy in tumors in which mTORC1 is specifically activated. Related piperazines have shown toxicity to cancer cells in vitro as single agents and in combination chemotherapy. Thus piperazine-based mTOR inhibitors could become a novel chemotherapeutic strategy.

Keywords

mTOR, mTORC1, mTORC2, piperazine, cinnarizine, hydroxyzine, cancer

1. Introduction

The mechanistic target of rapamycin (mTOR) is a 289 kDa serine/threonine kinase protein that controls various energetic functions at both the cellular and organism level (1). In 1975 on the island of Rapa Nui, an antifungal compound named rapamycin was isolated from *Streptomyces hygroscopicus* (2). Clinically rapamycin is used in organ transplants to suppress the immune system and minimize organ rejection (3). mTOR is dysregulated in multiple diseases ranging from cancer (4) to neurodegenerative disorders (5) and genetic disease (6). Specifically it is mTORC1's hyperactivation that affects multiple cancer types (7). Rapamycin and other mTOR inhibitors have been used in experimental anti-cancer therapy (8, 9). In addition, several mTOR inhibiting rapalogs have been approved for the treatment of human cancers in the clinic. Everolimus is one such rapalog marketed by Novartis and approved for use in humans as an immunosuppressant, for organ transplants, and for the treatment of specific types of lung, endocrine, and gastrointestinal cancers (10). While another rapalog, temsirolimus from Pfizer, has been approved for the treatment of renal cancer for over ten years (10).

There are two distinct mTOR complexes, mTORC1 and mTORC2, which regulate different cellular metabolic processes (11). mTORC1 functions as a sensor of nutrients and triggers the anabolic processes of protein and lipid synthesis, as well as

cellular growth (12). Raptor is the crucial mTORC1 binding partner. mTORC1 phosphorylates two downstream targets: ribosomal protein S6 Kinase beta 1 (p70 S6K) and eukaryotic translation initiation factor 4E-bidning protein 1 (4E-BP1) (13). mTORC2 regulates cell survival, cytoskeletal organization, and cell proliferation (14). In mTORC2 Rictor is the mTORC2 binding partner that phosphorylates and regulates protein kinase B also known as Akt (15). Although mTOR inhibition has been used as an experimental therapeutic strategy for cancer treatment (16, 17), and there are at least three general mTOR inhibitors used, including rapamycin, Torin, and INK128 (18).

Since specific mTORC1 activation occurs in aggressive renal, pancreatic and breast cancer (19, 20), then if mTORC1-specific binders could be identified, they might be effective therapies. To identify druglike molecules that were previously not known to engage the mTOR pathway, we took a 'repurposing' approach, screening 1600 drug-like small molecules that had been through clinical evaluation in humans, to identify mTOR binders and modulators of function.

2. Materials and Methods

2.1 Materials

The mouse liver cell line (FL83B) and human embryonic kidney cell line (HEK293T) were purchased from American Type Culture Collection (Manassas, VA) maintained at 37 °C and 5% CO_2.

The library of 1600 compounds was purchased from Microsource Discovery Systems Inc. (Gaylordsville, CT). All chemicals (DMSO, insulin, penicillin/streptomycin….) were

purchased from Sigma-Aldrich, unless indicated otherwise. Rapamycin was purchased from EMD Millipore (Billerica, MA). The PathScan® Phospho-p70 S6 Kinase (Thr389) Sandwich ELISA Kit, the PathScan® Total p70 S6 Kinase Sandwich ELISA Kit, and the PathScan® Phospho-Akt1 (Ser473) Sandwich ELISA Kit were from Cell Signaling Technologies (Danvers, MA). The avi-tagged mTOR and avi-tagged GFP plasmids were from GeneCopoeia (Rockville, MD). The SSA Super StreptAvidin Biosensors and kinetic buffers were from ForteBio (Fremont, CA). DMEM, FBS, and PSB for cell culture and assays were purchased from Corning (Corning, NY).

2.2 Real time kinetics of compound-mTOR interaction using BLI

The biotinylated, expressed in human cells (HEK 293T) mTOR or GFP were affinity purified using the SSA biosensors and Octet 384 RED instrument, and then tested against 10μM of each of the 1600 compounds in real time BLI experiment in triplicate. The GFP served as a negative control protein. For the measurements of affinity of the best 56 selected compounds, the dilution series of each of them were prepared at 24-point titration ranging from 19pM to 100μM.

2.3 pS6K/totalS6K, pAkt ELISA functional assays.

FL83B cells were seeded, 200,000 cells/well onto a 24 well plates and allowed to grow for 48 hours; the media was changed to DMEM12 without FBS, and cells were incubated for another 18 to 20 hours. Cells were treated for two hours with tested compounds at indicated concentrations; the media was aspirated, and plates were washed once with ice cold PBS. Cells were lysed with 200μl of lysis buffer; lysates were

transferred to a 96-well plate for ELISA. ELISA was performed according to manufacturer instructions.

2.4 Data Analysis and Statistics

Graphpad Prism 8.0 and Microsoft Excel were used for the statistical analysis. The three parameter sigmoidal dose response model was used for fitting analysis, Significance was assigned as follows: * P<0.05, ** P<0.01, *** P<0.001.

3. Results

3.1 Overall strategy of workflow to identify functional mTOR binders.

Our overall strategy is presented in Fig. 1. First, 1600 drug-like compounds were screened for binding to mTOR protein by biolayer interferometry (BLI), to identify 56 nominal mTOR binders. Next, we carried out dose-dependent testing for mTOR protein binding, and 16 of the 56 were confirmed. Finally we carried out downstream assays of mTORC1 (S6 Kinase phosphorylation) and mTORC2 (Akt phosphorylation) to determine the extent to which these mTOR protein binders positively or negatively affected mTORC1 and mTORC2 function.

3.2 Several drug molecules used in humans bind mTOR.

1600 small molecules were tested for their ability to bind mTOR protein at 10uM and as a control, to bind a 'decoy' non-specific protein, in this case Green Fluorescent protein (GFP). Some molecules are inherently non-specific binder, permissively binding to all

proteins, so we wanted to determine their *relative* preference to bind mTOR over other background proteins by this method. Some examples of this specificity test are shown (Fig 2A). For example, we used Rapamycin, that is known to be specific for mTOR as a positive control in the BLI test. The signal for Rapamycin association was quite strong, specific, and there was very low rapamycin-GFP binding. Sulfadiazine on the other hand is an example of a molecule that bound neither mTOR nor GFP (Fig. 2A). A Response Ratio (RR) was first used to determine and then rank the specificity of the top 56 mTOR binders.

3.3 16 drugs used in humans dose-dependently bind mTOR.

All 56 mTOR binders that passed through the primary screen were re-tested in 24-point concentration curves. The primary screen was done at a single concentration, 10μM to reduce the list of compounds to work with quickly, and 56 were relatively specific. However a secondary test of specificity tested the relative binding to mTOR and GFP at a wide range of concentrations to confirm specificity and determine an apparent binding constant (Km).

Of these 56, 16 compounds showed dose dependent binding to the mTOR with affinity ranging from 10nM to 10μM (Fig. 2B). Carvedilol is one example that bound to both mTOR and GFP at approximately the same affinity. The binding constants were determined by OctetRED384's BLI technology. Four mTOR binders included: testosterone propionate (Kd=10μM), carvedilol (Kd=55μM), hydroxyprogesterone (Kd=5μM), and cinnarizine (Kd=400nM).

3.4 Testing mTOR protein binders for functional activity through mTORC1 and mTORC2 functional assays.

We tested to what extent mTOR binders had ability to inhibit mTOR activity by assaying mTORC1 and mTORC2 activity, using phospho-S6Kinase and phospho-Akt assays, respectively, at a single concentration, 10μM (Fig. 3). Multiple drugs, for example hydroxyprogesterone, carvedilol and testosterone propionate, significantly inhibited the activity of both S6Kinase and phospho-akt at 10μM, suggesting that we had identified three new mTOR-general inhibitors. Also, in this group, cinnarizine clearly inhibited S6 kinase activity, but not phospho-Akt, and thus seemed it could be a novel, type of molecule, i.e. mTORC1-specific. The mTORC1-specificity was also supported by Western blots of pS6Kinase/total S6Kinase and phospho-Akt/total Akt activity (Fig. 4). This was then further tested in the next data figure.

3.5 Carvedilol produces a dose-dependent decrease in both mTORC1 and mTORC2, whereas Cinnarizine is mTORC1 specific.

As above we noticed that cinnarizine, unlike the mTORC-general drugs carvedilol, hydroxyprogesterone and testosterone propionate, did not appear to inhibit mTORC2, the dose-dependence of these two representatives was tested (Fig. 5). Whereas it was clear that carvedilol dose-dependently inhibited mTORC1 and mTORC2 activity, cinnarizine only inhibited mTORC1 at all doses.

3.6 Multiple piperazines are specific mTORC1 inhibitors.

We formed the hypothesis that cinnarizine might be the first member of mTOR-binding, mTORC1-specific inhibitors, and thus tested multiple chemical piperazine analogs of cinnarizine at 10μM, including hydroxyzine, meclizine, and flunarizine (Fig. 6). We observed that the property of mTORC1-specificity was conserved in this group of related molecules.

3.7 Small chemical changes in piperazines determine mTOR binding and functional activity.

We identified dose-dependence of a piperazine, hydroxyzine, with respect to mTORC1-specificity (Fig. 7A). Small differences in chemical structure were responsible for large changes in activity. For example, the addition of a benzene ring onto hydroxyzine to make buclizine, another antihistamine piperazine (https://www.drugbank.ca/drugs/DB00354), completely eliminated its ability to inhibit mTORC1 and mTORC2 (Fig. 7B). Another structurally related piperazine, meclizine, also shows the same trend of specific mTORC1 inhibition (Data not shown).

4. Discussion

4.1 mTORC1-specific inhibitors could be of use as tools and therapeutically.

mTORC1-specific inhibitors have been used as basic science tools to test the mechanistic basis of addiction (21) and have been considered as medicines for specific tumors (22). mTORC1 is hyper-activated in renal, pancreatic and breast tumors (19,

20), so if an mTORC1-specific inhibitor could be found it might be of medical benefit. Also, the availability of specific inhibitors of these important functions could lead to biological insights. Thus, the identification of specific inhibitors of mTORC1 has been pursued for basic science and medical reasons (23). Rapamycin was the first identified mTORC1 inhibitor (24), but it also partially stimulates mTORC2 via insulin receptor feedback loop (25). This is consistent with our results when 2-hour rapamycin treatment stimulated Akt activation (Figure 3, 4, 5, 6). However, long-term rapamycin treatment actually inhibits the mTORC2. This can be explained by sequestration of the mTOR from all of its complexes by rapamycin (15). mTOR is involved in several signaling pathways and exists in two protein complexes, and further research is necessary to fully understand how compounds inhibit or stimulate this protein.

4.2 Cinnarizine and hydroxyzine are the first two members of a piperazine category of mTORC1-specific inhibitors.

We identified in cinnarizine the first member of a new class of mTORC1-inhibitory drugs, all of which are anti-histamine piperazines. Testing multiple piperazines identified several members with the same mTOC1 specific profile: hydroxyzine, meclizine, flunarizine, cinnarizine. Investigation of dose dependence of cinnarizine and hydroxyzine showed that cinnarizine binds mTOR protein with an affinity of 400nM, and inhibits mTORC1 at a similar EC50 500nM. Hydroxyzine also inhibits mTORC1 at an EC50 of 500nM, although its binding affinity was never calculated because it came through an analog search. mTOR-general binders and inhibitors, and mTORC1 specific inhibitors are summarized in Fig. 8.

4.3. mTORC1-inhibiting piperazines demonstrate efficacy against tumor models alone and in combination. Cinnarizine, hydroxyzine, flunarizine and meclizine have all been tested in vitro and/or in vivo tumor models and mediate dose dependent killing. Flunarizine and cinnarizine as single agents mediate dose-dependent killing against lymphoma and multiple myeloma cells in vitro (26). In combination therapy, flunarizine potentiates vincristine's toxicity to melanoma (27), flunarizine potentiates melphalan toxicity vs. sarcoma (28), and cinnarizine and flunarizine are radiation sensitizers in murine tumors (29). The anti-tumor activity of these piperazines could be mediated through their mTOR -binding and -inhibiting activity.

4.4 Cinnarizine and hydrozyzine are relatively safe, non-toxic drugs in use for chronic diseases.

Cinnarizine and hydroxyzine have been prescribed for over 60 years. Cinnarizine was synthesized and marketed by Janssen Pharmaceutica in 1955 (https://www.drugbank.ca/drugs/DB00568) and hydroxyzine by Pfizer in 1956. Thus, both have extensive pharmacological and safety profiles. Compared to rapamycin, an immunosuppressant that cannot be given for an extended period of time, cinnarizine and hydroxyzine are antihistamine anti-nausea anti-anxiety drugs with the potential for long-term treatment. The complete mechanism of action hydroxyzine and cinnarizine is not fully understood, and beyond the scope of this paper. One mechanism via binding to the cellular H1 receptor have being proposed previously (https://www.drugbank.ca/drugs/DB00568, https://www.drugbank.ca/drugs/DB00557),

but specific inhibition of mTORC1 might be an additional mechanisms for their therapeutic effects. Further research is needed to fully understand how cinnarizine, hydroxyzine and other piperazine derivative drugs are inhibiting mTORC1. The three generations of rapamycin analogs, rapalogs (30), show limited effectiveness in treating cancer or neurodegenerative diseases, perhaps due to the general inhibition of both mTOR complexes which creates upstream and downstream feedback loops and complications (25). Since cinnarizine and hydroxyzine appear to specifically inhibit only mTORC1 they have a potential for repurposing as a first novel, specific, safe pharmacological mTORC1 inhibitors and to replace rapamycin and the rapalogs on the market.

mTORC1 is specifically activated in multiple tumor types. Through specific mTOR binding and functional assays we identified carvedilol, alpha-hydroxyprogesterone, and testosterone propionate as mTOR binders and mTORC-general inhibitors at high micromolar concentrations. By contrast, cinnarizine, hydroxyzine, flunarizine and meclizine were demonstrated to specifically inhibit mTORC1, the first time a piperazine drug with such specificity has been demonstrated. Several of these piperazines exhibit antitumor activity alone and in combination as has been shown previously (31, 32, 33), and their anti-tumor mechanism may proceed through mTOR inhibition. Thus the novel activity of these piperazines to specifically inhibit mTORC1 could have implications for novel antitumor therapeutic strategies (10).

6. Author Contributions

HTS for mTOR binding – Tomilov and Allen; Dose response binding of hits, Western blots, functional testing of mTOR inhibitors by pS6K/pAkt ELISAs - Allen; overseeing entire project – Cortopassi.

7. Conflict of interest

Authors do not have any conflict of interest associated with publishing this work.

8. Acknowledgements

Presented work was supported by unrestricted funds and Foundation awards.

Figure legends

Figure 1. High-throughput screening workflow used to identify mTOR binding and mTOR inhibiting compounds from library of 1600. OctetRed384 BLI technology was employed for small molecule binding to mTOR protein assays, while pS6K and pAkt ELISA assays were utilized for functional testing of candidate compounds in mammalian cell lines.

Figure 2. 1600 small molecules were screened in triplicate for direct binding to mTOR protein using OctetRed384 and BLI technology. **A.** Compounds were tested at a single concentration of 10μM in triplicate and ranked according to consistency. For example

while rapamycin and cinnarizine specifically bind mTOR better than GFP, sulfadiazine does not. **B.** Increasing dose curves, ranging from 10nM to 10μM, identify KDs of mTOR binding compounds vs non-specific binders.

Figure 3. Fold change refers to phospho-protein normalized to total protein, pS6K/Total S6K and pAkt/Total Akt, as determined by ELISA. At 10μM, hydroxyprogesterone, carvedilol, testosterone propionate statistically significantly inhibit both mTORC1 (pS6K-gray bars) and mTORC2 (pAkt-black bars), whereas cinnarizine only significantly inhibits mTORC1 (pS6K-gray bars).

Figure 4. Expression of the mTORC1 downstream target pS6K/total S6K, and mTORC2 downstream target pAkt/Total Akt in response to the mTOR binders. All targets normalized to α-Tubulin prior to normalization to total S6K or Akt. The data presented here is from one representative experiment and serves to verify mTORC1 and mTORC2 inhibiting compounds were confirmed by western blot.

Figure 5. Fold change refers to phospho-protein normalized to total, pS6K/Total S6K and pAkt/Total Akt, as determined by ELISA. **A.** Carvedilol dose dependently inhibits both mTORC1(pS6K-gray bars) and mTORC2 (pAkt-black bars), **B.** Cinnarizine is a dose-dependent inhibitor of only mTORC1 at these doses.

Figure 6. Fold change refers to phospho-protein normalized to total, pS6K/Total S6K and pAkt/Total Akt, as determined by ELISA. Multiple structurally related piperazine

compounds inhibit mTORC1 (pS6K-gray bars) specifically, but do not inhibit mTORC2 (pAkt-black bars) at 10μM concentration and 2hrs of drug treatment.

Figure 7. Fold change refers to phospho-protein normalized to total, pS6K/Total S6K and pAkt/Total Akt, as determined by ELISA. **A.** Cinnarizine analog hydroxyzine dose-dependently inhibits mTORC1 specifically, but not mTORC1**. B.** A small modification of the cinnarizine or hydroxyzine structure to make buclizine, eliminates buclizine's ability to inhibit mTORC1 specificity.

Figure 8. Summary. Multiple general mTOR -general binders and inhibitors have been identified by this study that decrease both mTORC1 and mTORC2 activity, including carvedilol, testosterone propionate, hydroxyprogesterone caproate. Furthermore, mTORC1-specific binders and inhibitors have been identified, including cinnarizine and hydroxyzine.

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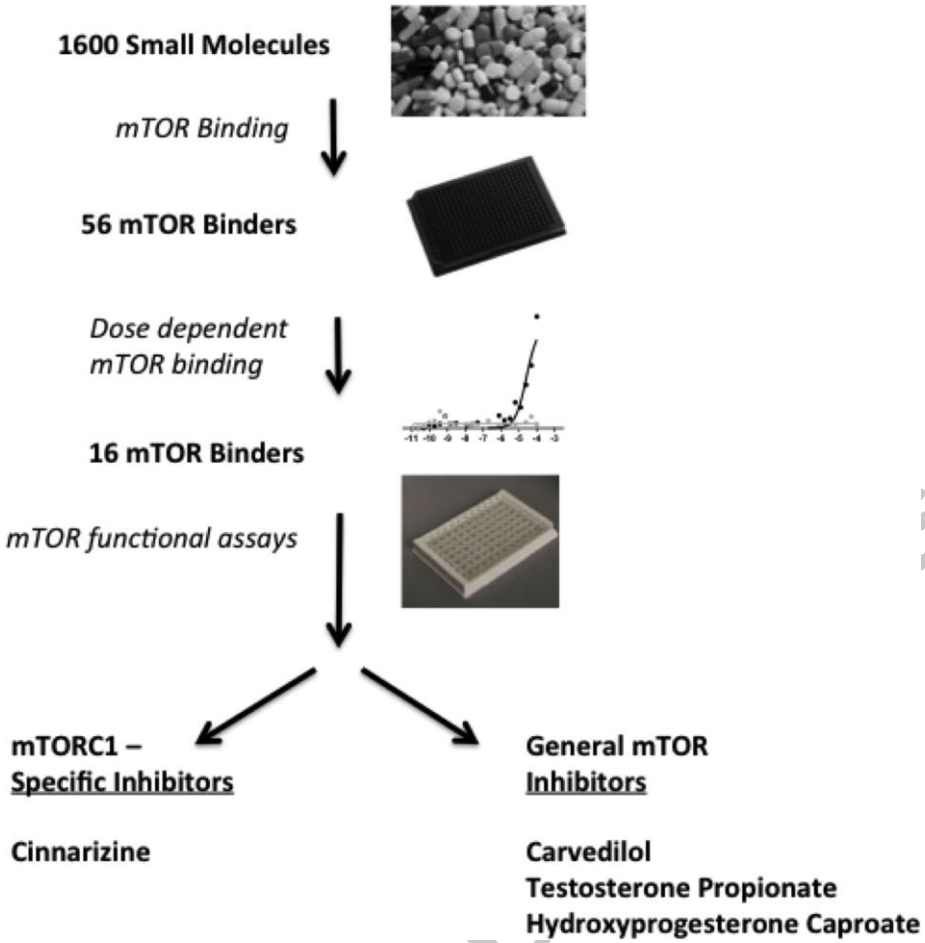

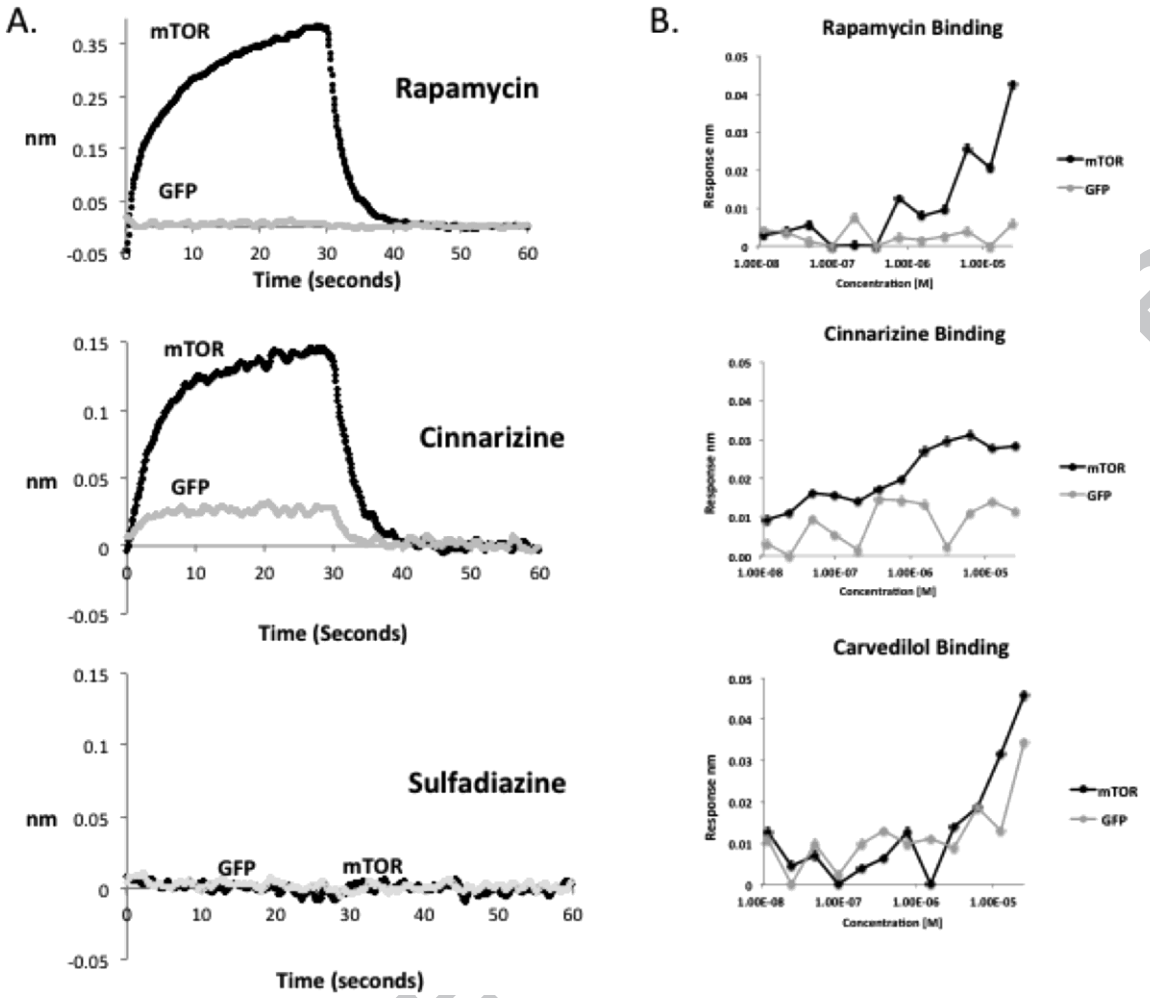

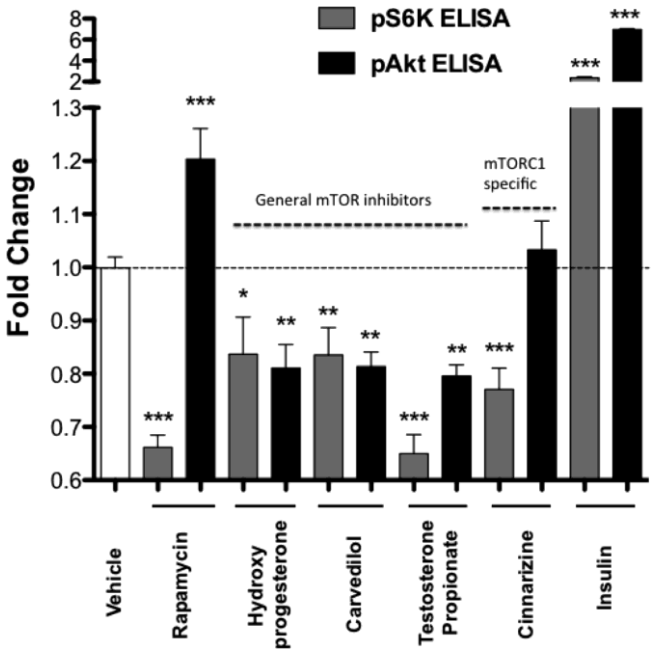

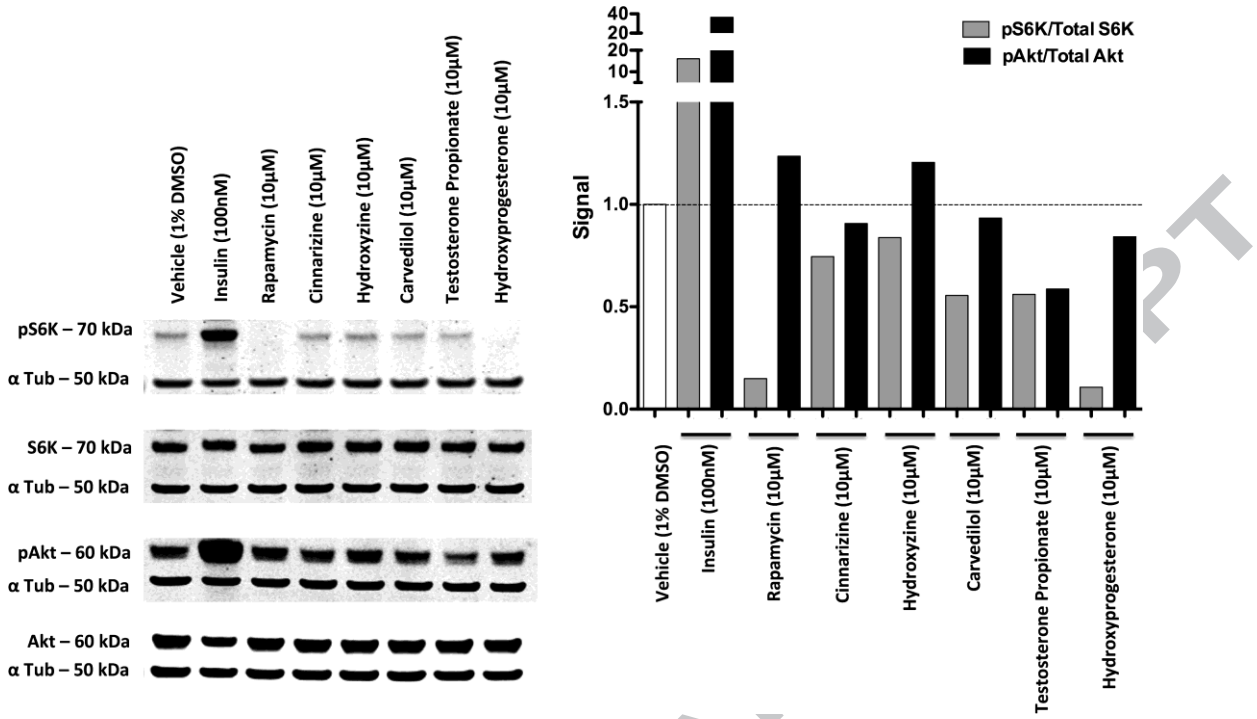

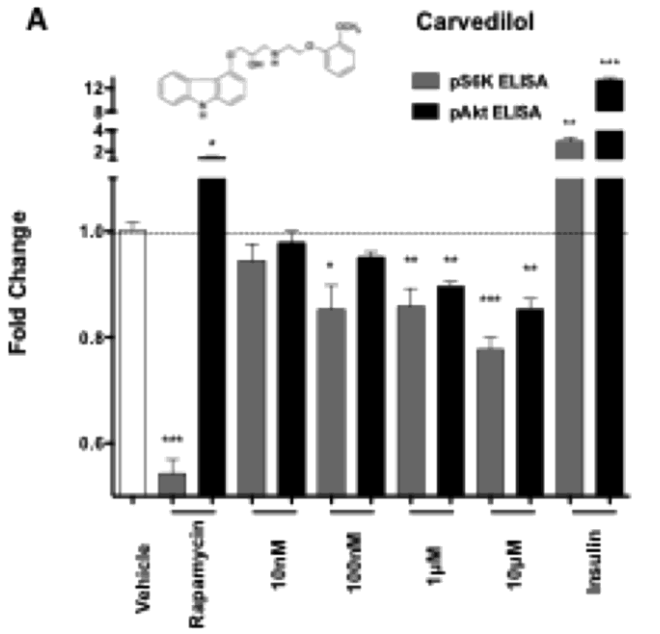
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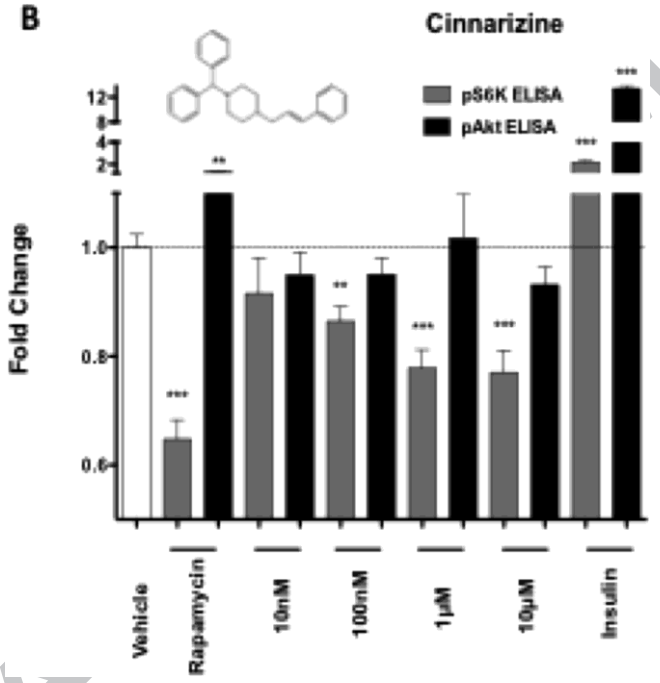
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1μM
10μM
Insulin