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6. AUTHOR(S)

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

mTOR (mammalian target of rapamycin) is a protein kinase that forms two distinct complexes mTORC1 and mTORC2. Aberrant upregulation of mTORC1 signaling has the potential to cause disease, including Tuberous Sclerosis Complex (TSC). We aim to develop novel mTORC1 inhibitors with improved potency and safety over current mTORC1 inhibitors in the market, such as Rapamycin and Everolimus (a rapamycin analog). After initial high throughput screening, we have obtained a class of molecules that inhibit mTORC1 in vitro with a different mode of action than Rapamycin. We are currently optimizing this class of molecules towards activity towards mTORC1 inhibition in cells and in mouse models.

15. SUBJECT TERMS

NONE LISTED

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1. INTRODUCTION:

Tuberous sclerosis complex (TSC) is a disease that is caused by aberrant upregulation of the mTORC1 (mammalian target of rapamycin complex 1) signaling. mTORC1 signaling inhibitors such as Rapamycin and Everolimus (a rapamycin analog), are currently approved for treatment of renal tumors, partial onset seizures and brain tumors (SEGAs) in TSC patients, as well as TSC-associated lymphagioleiomyomatosis (LAM) in LAM patients. However, they are not very well tolerated and more than 60% of TSC patients do not respond to these treatments when it comes to the treatment of seizures. This lack of efficacy of Rapamycin and Everolimus is attributed to failure of these drugs to be fully effective and selective towards mTORC1 signaling. They are deemed to lack efficacy as they are ineffective towards 4EBP1 activity, a major target of mTORC1 that plays a role in protein synthesis and they are less selective as they target mTORC2, the other complex formed by the protein kinase mTOR. We have identified a novel way of targeting mTORC1 via a mechanism of action different from rapamycin and rapamycin analogs. After initial high throughput screening, we have identified small molecules that have the potential to be more efficacious and selective in inhibiting mTORC1. Via work funded by this grant we aim to further optimize these compounds to initially obtain potency and selectivity in cell line experiments and further obtain compounds that have activity against mTORC1 in wild type and TSC deficient mice and exhibit tumor reducing capabilities in TSC xenograft mouse models.

2. KEYWORDS:

mTORC1 inhibitors, drug development, TSC, mTOR

3. ACCOMPLISHMENTS:

What were the major goals of the project?

The project stems from our previous work on searching for compounds that have the ability to inhibit mTORC1 in a more efficacious and selective manner than rapamycin and rapamycin derived analogs. Previously, via high throughput screening, utilizing an assay to identify molecules that inhibit mTORC1 via a new mode of action, we identified and started to characterize 4 different molecules. These molecules were found to inhibit mTORC1 with IC50s in the low micromolar range in vitro.
Work performed in this grant aims at further optimizing these molecules. The following are the milestones listed in the approved SOW:

Milestone 1: At least one compound that inhibits phosphorylation of 4EBP1 in cultured cells (4 months)

We have yet to obtain any activity of our molecules in cells, as such the promising effects we see in vitro with our molecules do yet translate to a cellular response.

Milestone 2: Perform compound optimization and obtain a molecule that inhibits 4EBP1 phosphorylation by mTORC1 at an IC50 of less than 500nM in cells (10 months).

We are currently in the midst of a hit optimization campaign with our most promising candidate, conducting multiple rounds of custom synthesis optimization. We have obtained a class of analogs that have low micromolar activity in vitro, however this has not yet translated to activity in cells.

Milestone 3: One compound that reduces mTORC1 signaling by at least 50% in mice (18 months)
We have not yet started experiments to achieve this milestone.

Milestone 4: One compound that reduces tumor size in TSC mouse xenograft experiments (24 months)

We have not yet started experiments to achieve this milestone.

What was accomplished under these goals?

During the first phase of this grant, we aimed at progressing further the 4 compounds obtained from high throughput screening. These compounds selectively inhibit mTORC1 with an IC50 of around 10uM in our in vitro TR-FRET assay and an IC50 of less than 30uM in our in vitro kinase assay. To optimize the compounds, we collaborated with our medicinal chemist Dr. Werner Neidhart. From a chemical point of view, we decided to omit compound 2 as from the structural groups it was deemed to be highly reactive compound. We decided to continue with compounds 1, 3 and 4 as shown in the grant proposals' project narrative. We purchased over 30 derivatives per compound from publicly available databases and determined the IC50 of mTORC1 inhibition in iterative rounds using our in vitro TR-FRET assay.

For compound 1 a few of the derivatives purchased showed very similar activity to that of the parent compound itself, as such the results of this optimization could not provide a rational to guide further optimization of the structure activity relationship of this class of compounds.

For compound 3 we found that almost none of publicly available compounds improved the potency. Moreover, upon closer inspection we noticed that even very close analogs of compound 3 did not yield any activity. We decided to test the parent compound with resynthesized material from a different provider and found it to be inactive. Thus, we conclude that an impurity in the original batch of compound 3 was responsible for the initial activity observed. We tried to dissect the possible chemical entities in the original batch using analytical models but were unable to determine the exact species necessary for the activity.

Compound 4 is the compound that we are currently following and intend to optimize towards generating a lead compound. Like the other compounds we performed initially three rounds of hit optimizations using publicly available compounds and more recently custom synthesis. We did obtain analogs with improved potency from our initial hit as well compounds with no activity or comparable activity to the parent compound (Figure 1). This helped us determine a structure activity relationship. However, as seen also in Figure 1 the potency is still in the low micromolar range and we would need to improve it to the nanomolar range in our initial invitro TR-FRET assay in order for these compounds to be active in cell assays.

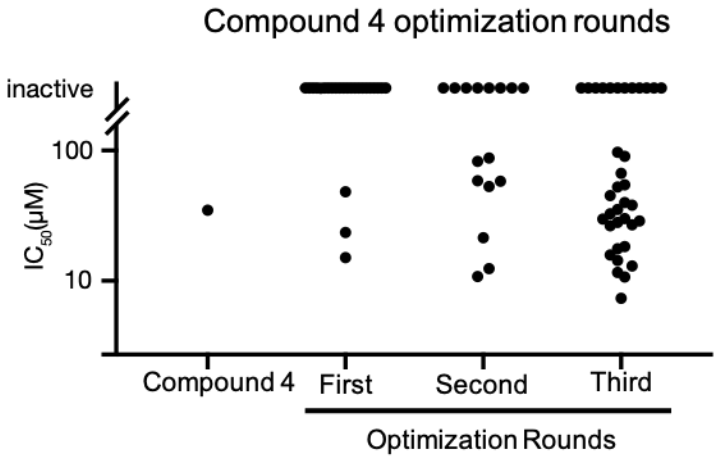

Figure 1: IC50 (uM) of compound 4 and compounds from the 3 optimization rounds. Each dot represents a compound.

Even though, via three rounds of iterations, we have managed to increase the affinity of our compounds to inhibit mTORC1 via roughly two-fold we still do not see any activity when testing analogs of compound 4 in cells. To test our compounds in cells we further refined our experimental pipeline and approach. We decided to use Alphalisa and HTRF detection systems from PerkinElmer in order to measure the effects of our compounds in various cell lines. These methods are very sensitive and quantifiable. They could be scaled up to do multiple compounds and multiple compound dilution sets at the same time. We measured the effects our compounds in blocking mTORC1 phosphorylation in cells, specifically 4EBP1 T37/46 phosphorylation and S6K T389 phosphorylation. We validated our assays using commercially available mTORC1 inhibitors such as Rapamycin and Torin 1 but did not see any activity of the analogs of compound 4 that were active in our in vitro TR-FRET assays. Two of the derivatives tested are shown in Figure 2 (Compound 4.1 and Compound 4.2).

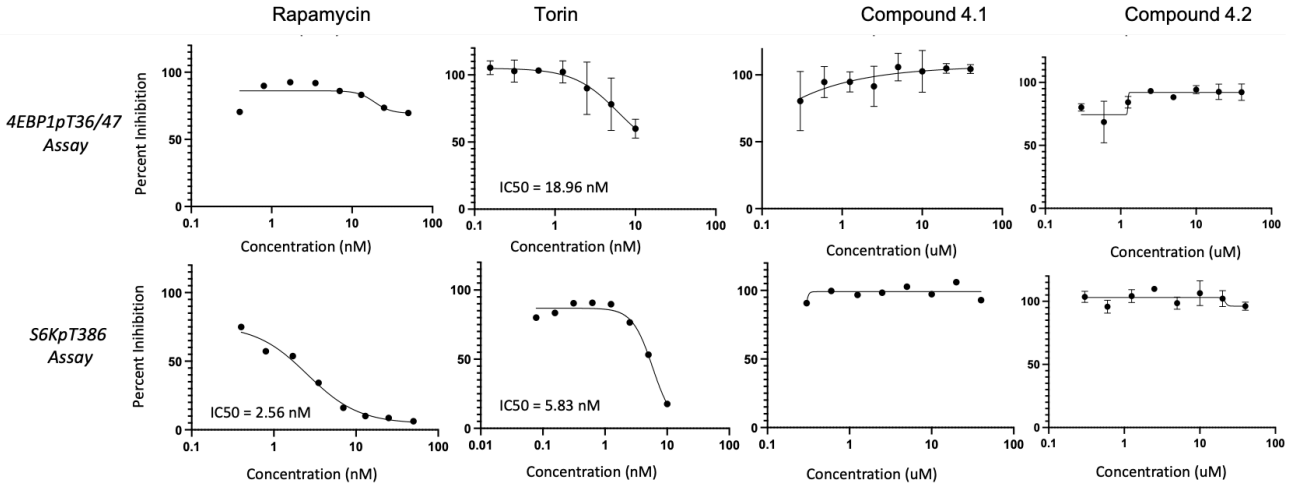

Figure 2: In-cell assay using MCF7 cell line and HTRF technology with antibodies against mTORC1 phosphorylations 4EBP1 pT36/47 and S6K pT386. Rapamycin, Torin1 and two analogues of Compound 4 that is currently undergoing optimization were tested. Cells were treated with the shown inhibitors for 2 hours and lysed. The lysates were used in 4EBP1 pT36/47 and S6K pT386 HTRF kits from PerkinElmer according to manufacturer instructions.

As these in-cell assays are validated with known mTOR inhibitors we deem them ready to use with the next batch of chemically active compounds to determine cellular potency towards mTORC1 inhibition. They will be used with compounds that show an IC50 of mTORC1 inhibition in the high nanomolar range in our in vitro TR-FRET assays in order to achieve the first two milestones and to be well on the way of achieving milestone 3.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

As noted previously, we could not accomplish the first two milestones in the proposed time as the compounds do not have yet activity in cells. During this time, we further chemically optimized one class of the compounds and refined our assays that will be used to test if the optimized compounds act to inhibit mTORC1 in a more efficacious and specific manner than Rapamycin and Everolimus (a rapamycin analog currently in the clinic). We have developed Alphalisa and HTRF (PerkinElmer) based assays to test the effects of our compounds upon a short treatment on mTORC1 targets (efficacity) and effects of our compounds upon a 24–48-hour treatment on mTORC2 targets (selectivity). Furthermore, we have developed an in-cell assay that measures viability to assess the phenotypical effects of our compounds under development. We are continuing with our chemical compound optimization plan with the aim to deliver compounds with increased potency towards mTORC1 inhibition, as measured by our in vitro TR-FRET assay. All the in-cell assays were optimized and tested using known mTORC1 inhibitors such as Rapamycin, Everolimus and Torin 1. Upon obtaining more potent compounds in vitro we will subject them to our cascade of cellular assays to determine if they are efficient and specific towards mTORC1 inhibition also in vivo. We plan on using normal tumor cell lines as well as TSC2 KO cell lines to determine the effects of our compounds in cases where mTOR signaling is "normal" as well as elevated due to the lack of the TSC2 protein, as in the case of Tuberous Sclerosis Complex disease. We are confident that we are making headway in our chemical optimization and believe than in 1 or 2 further iterations would be able to obtain more potent compounds that should begin to show activity in our cellular setup. We intend to further optimize the compounds for in cell activity to reach IC50 of mTORC1 inhibition of less than 500nM, as proposed in the second milestone.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

The in-cell assays developed during this initial period will prove very valuable for testing in vivo properties of our compounds in the coming months. As they are readily scalable and quantitative, they could also be utilized by other members of the lab in different projects to assess how different treatment do affect mTORC1 and mTORC2 signaling as opposed to western blotting (using antibodies or in-cell westerns).

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Nothing to report

Changes in approach and reasons for change

Nothing to report

Actual or anticipated problems or delays and actions or plans to resolve them

There are no changes per se in the approach taken however, we now anticipate more time will be needed until we see in vivo activity of our compounds. The compounds might need to undergo one or two more rounds of chemical optimization.
The main aim of this project is to develop compounds that have selective activity towards mTORC1 in mice. We started with 4 different hits from our high throughput screening and, as noted in milestone 1, we anticipated that one of our compound classes would have shown in cell activity within 4 to 6 months of the start of the grant. Upon trying hit optimization with the initial hits, we determined that only one class showed a promising structure activity relationship in order to advance towards the hit-to-lead-optimization stage. We are now in the process of chemically optimizing this class. The mTORC1 inhibitory activity seen in vitro so far has not been observed in cells. We aim to perform further rounds of optimization and test these compounds in the cell line experiments already devised. We expect to achieve in-cell activity within two rounds of optimization.

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals

Nothing to report

Significant changes in use of biohazards and/or select agents

Nothing to report

6. PRODUCTS

- **Publications, conference papers, and presentations**

  Nothing to report

  Journal publications

  Nothing to report

  Books or other non-periodical, one-time publications.

  Nothing to report

  Other publications, conference papers and presentations.

  Nothing to report

- **Website(s) or other Internet site(s)**

  Nothing to report

- **Technologies or techniques**

  Nothing to report

- **Inventions, patent applications, and/or licenses**

  Nothing to report

- **Other Products**

  Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: *Prof. Michael N. Hall*
Project Role: *PI*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *0.5*

Contribution to Project: *Prof Hall has supervised the project.*
Funding Support: *Enumeration as a Professor at University of Basel*

Name: *Dr. Dritan Liko*
Project Role: *Principal Scientist*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *12*

Contribution to Project: *Dr. Liko has designed and carried out the in vitro TR-FRET testing, coordinated to obtain custom synthesis compounds and performed the cellular research described.*
Funding Support:

Name: *Dr. Werner Neidhart*
Project Role: *Medicinal Chemist*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *0.8*

Contribution to Project: *Dr. Neidhart has contributed in the evaluation and design of the new chemical entities during the chemical optimization of our hits.*
Funding Support:

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report

What other organizations were involved as partners?

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Nothing to report

QUAD CHARTS:

Nothing to report

9. APPENDICES:

Nothing to report