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Synthesis and Application of Light-Switchable Arylazopyrazole Rapamycin Analogs

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Abstract:

Rapamycin-induced dimerization of FKBP and FRB has been utilized as a tool for co-localizing two proteins of interest in numerous applications. Due to the tight binding interaction of rapamycin with FKBP and FRB, the ternary complex formation is essentially irreversible. Since biological processes occur in a highly dynamic fashion with cycles of protein association and dissociation to generate a cellular response, it is useful to have chemical tools that function in a similar manner. We have developed arylazopyrazole-modified rapamycin analogs which undergo a configurational change upon light exposure and we observed enhanced ternary complex formation for the *cis*-isomer over the *trans*-isomer for one of the analogs.

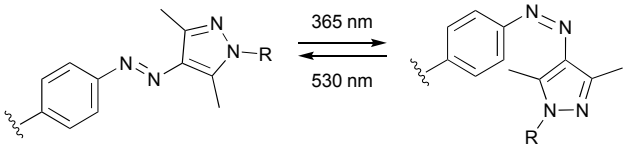
Introduction:

Chemical inducers of dimerization (CIDs) are prominent tools used by chemical biologists to place biological processes under conditional control.[1-4] The most commonly utilized CID is rapamycin, a natural product that binds to FK506 binding protein (FKBP) with a 0.2 nM K_d. This FKBP-rapamycin complex then binds to the FKBP-rapamycin binding domain of mTOR (FRB), forming a ternary complex with a K_d of 12 nM.[5] Due to the small size of FKBP and FRB (12 and 11 kDa, respectively) and their remarkably tight binding affinity with rapamycin, these two domains have been fused to numerous proteins and have been extensively applied as heterodimerization tools for small molecule switches. Processes that have been placed under rapamycin control include Golgi/endoplasmic reticulum association to study mitosis,[6] phosphoinositide control of endocytic trafficking,[7] inactivation of proteins by rerouting them to the mitochondria,[8] cell surface glycosylation,[9] and split-kinase activation.[10] In order to obtain more precise control of ternary complex formation, several caged rapamycin derivatives have been synthesized.

The Deiters group has developed a nitrobenzyl caged rapamycin analog, pRap, that required protein engineering of FKBP to generate iFKBP, a truncated protein, in order to minimize background dimerization activity in the absence of light.[11] Inoue addressed this limitation by using a biotin-streptavidin moiety attached to a nitrobenzyl protecting group to sequester the CID outside of the cell, until irradiation generated a cell-permeable rapamycin derivative that could induce dimerization.[12] In order to alleviate the need for an external avidin protein while still capitalizing on the dramatic steric demand provided through recruitment of a protein to the caging group, a second rapamycin molecule was attached to generate a symmetric caged rapamycin dimer that utilized an unmodified FKBP.[13] A different caging strategy was taken by the Woolley group, in which they replaced the C16-methoxy group with a nitrobenzyl group, such that interaction with FRB was inhibited in the absence of light.[14] These examples provide precise control of the activation of ternary complex formation; however, protein dimerization is essentially irreversible once the caging group is removed through covalent bond photolysis.

Since most biological processes occur reversibly in a highly-regulated spatiotemporal manner (for example, regulation of the MEK/ERK pathway by protein phosphatases[15, 16]), it would be beneficial to develop tools that enable chemical biologists to control cycles of protein dimerization/localization with light. To date, truly reversible control of rapamycin-induced dimerization has yet to be achieved in mammalian cells. Wandless and Crabtree demonstrated reversibility through addition of a large excess of FK506M, which acts by outcompeting rapamycin and inducing proteasomal degradation, requiring 9 hours for depletion in mice.[17] Lee utilized a similar approach of outcompeting with a 50-fold excess of FK506 and observed slightly faster loss of dimerization.[18] Interestingly, in yeast, formation of rapamycin-induced dimerization can be reversed in 20 minutes by removal of rapamycin from the cells by switching to drug-free media.[19, 20] Taken together, these approaches allow for a single on-then-off cycle of dimerization, but at a timescale that is often not amenable for dissecting dynamic biological processes.

Therefore, we aimed to develop the first rapamycin analogs that would enable multiple cycles of activation and deactivation of ternary complex formation that could be applied to existing FKBP/FRB systems. In order to render rapamycin reversibly switchable, we envisioned that addition of a diazobenzene or arylazopyrazole (AAP) light-switchable moiety to rapamycin would enable reversible control using light. Azobenzenes have been applied to a wide range of biological processes including reversible control of transcription factors, protein-protein interactions of α-helices, cell membrane receptors, catabolite-binding proteins, DNA duplex formation, RNA aptamers and several others.[21-28] However, the arylazopyrazole is a recently developed photoswitchable group with improved photostationary states and slower thermal reversion compared to traditional azobenzenes.[29] Arylazopyrazoles utilize switching wavelengths of *trans*-to-*cis* (365 nm) and *cis*-to-*trans* (530 nm) and we decided to focus on developing arylazopyrazole modified rapamycin analogs (**Figure 1**). Although non-methylated arylazopyrazoles provide longer thermal stability (1000 days with 3,5-H vs 10 days with 3,5-CH_3), we decided to use dimethylated analogs as they display near quantitative switching between the two stereoisomers.[30] Since prediction of the molecular interactions of the *cis*- vs *trans*-AAP modified-rapamycin analogs with FKBP/FRB is impossible, we made no initial assumptions regarding which isomer, if either, would allow for ternary complex formation.



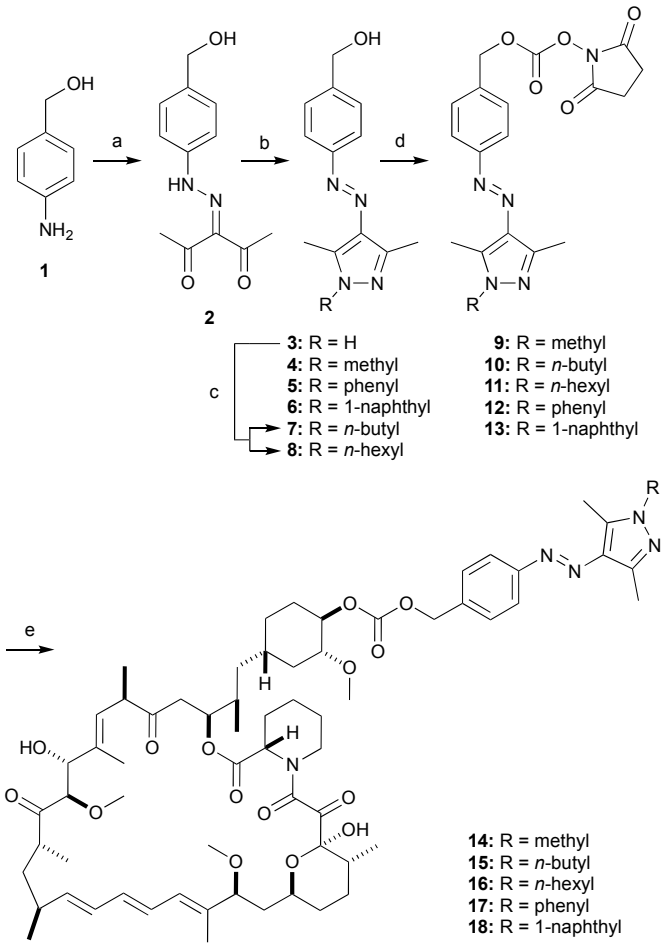
trans-AAP *cis*-AAP

Figure 1. The photoswitchable arylazopyrazole group undergoes *trans*-to-*cis* isomerization upon exposure to 365 nm light. The reverse *cis*-to-*trans* isomerization can happen thermally (slow) or upon irradiation with 530 nm light (fast).

Results and Discussion:

We synthesized five AAP-modified rapamycin analogs for subsequent biological testing. Both alkyl and aryl substituents were utilized in order to cover a broad steric range from a small methyl to a bulky naphthyl group. Modification at the *N*-1 position was selected due the synthetic versatility of using various hydrazines to generate a small panel of analogs. Although the methyl groups at positions 3 and 5 could be varied depending on the dione used in the condensation reaction, we avoided modifying these positions with bulkier substituents which could adversely affect *trans*-to-*cis* switching. The five analogs were synthesized following similar procedures

(**Scheme 1**). Synthesis commenced with diazotization of 4-aminobenzyl alcohol **1**, followed by enolate addition with acetylacetone to yield the hydrazone, **2**. Methyl, phenyl, or naphthyl-hydrazine was used to generate the corresponding arylazopyrazoles, **4** – **6**. However, when the corresponding *n*-butyl and *n*-hexyl hydrazines were used, only trace amounts of product were observed. Thus, we utilized hydrazine to generate the N-H pyrazole **3**, which was subsequently alkylated with the corresponding alkyl halide to generate the *n*-butyl and *n*-hexyl arylazopyrazoles **7** and **8**, respectively. Activation of the benzyl alcohol was achieved using DSC to generate the NHS-carbonates **9** – **13**. Addition to rapamycin was performed in the presence of base to generate the AAP-rapamycin analogs **14** – **18**. With acylation at the C-40 hydroxy position showing generally low yields in the literature (~30-50%),[11, 13] we found that the use of 9-azajulolidine improved this reaction in some cases.[31] For these analogs in particular, product polarity was similar to unreacted rapamycin starting material, resulting in purification issues and slightly lower yields.



Scheme 1. Synthesis of the methyl-, *n*-butyl-, *n*-hexyl-, phenyl-, and 1-naphthyl-AAP rapamycin analogs **14**-**18**. Reagents and conditions: (a) i. Sodium nitrite, hydrochloric acid, acetic acid; ii. acetylacetone, sodium acetate, ethanol, water; 96%. (b) $RNHNH_2$, ethanol; 17-95%. (c) RX, potassium hydroxide, ethanol; X = I, 86%; X = Br, 40%. (d) *N,N'*-Disuccinimidyl carbonate, triethylamine, acetonitrile, 63-97%. (e) Rapamycin, 4-dimethylaminopyridine or 9-azajulolidine, methylene chloride, 9-40%.

The photostationary states and thermal stabilities were determined by ^{1}H-NMR (**Table 1**). Solutions of the five AAP analogs were prepared in 20% D_2O:80% d_6-DMSO at 4 mM concentration. Unfortunately, due to the poor aqueous solubility of rapamycin, we were unable to generate more physiologically-relevant solvent conditions for these photoswitching studies. A UV transilluminator (365 nm) was used for irradiations when determining the photostationary state (PSS) of the *trans*-to-*cis* conversion. A 530 nm LED was utilized for the *cis*-to-*trans* isomerization. For *cis*-thermal stability studies, the solutions were irradiated with 365 nm light until the PSS was reached, then maintained at 37 ºC and NMR spectra were acquired every 24 hours.

Table 1. Characterization of the PSS at 365 nm and 530 nm irradiation and thermal stability of the AAP-rapamycin analogs.

	***trans*:*cis* (365 nm)**	***trans*:*cis* (530 nm)**	***cis* half-life at 37 °C**
14	6 : 94	93 : 7	1.3 days
15	13 : 87	91 : 9	1.7 days
16	16 : 84	92 : 8	2.8 days
17	11 : 89	98 : 2	1.7 days
18	10 : 90	94 : 6	3.6 days

The AAP-rapamycin analogs were tested in cells using a split luciferase reporter. The split luciferase reporter was chosen because activity can easily be quantified with high sensitivity and linearity across several orders of magnitude. Additionally, the reporter works well with low (nanomolar) concentrations of rapamycin as opposed to translocation reporters which require micromolar ligand (data not shown). This reporter utilizes the N-terminus of a split firefly luciferase fused to FRB, while the C-terminus is attached to FKBP.[32] When co-expressed in HEK293T cells, addition of rapamycin generates the FKBP-rapamycin-FRB ternary complex and brings the two luciferase halves in close proximity in order to reconstitute the active enzyme (**Figure 2a**).

HEK293T cells expressing the split luciferase reporter were treated with pre-formed *cis*- and *trans*-isomers of the five AAP-analogs, incubated for 2.5 hours, and lysed. Luciferase substrates were added and recorded luminescence values were normalized to DMSO (negative control) (**Figure 2b-c**). While most the stereoisomers of **15**-**18** did not show significant differential activity, the methyl analog **14** was >2-fold more active as the *cis*-isomer compared to the *trans* isomer at both 100 nM and 25 nM concentrations. The larger alkyl and aryl modifications resulted in a loss of isomer selectivity, and the *n*-butyl and 1-naphthyl isomers only allowed for moderate ternary complex formation for both the *cis*- and *trans*-isomers. Overall, *cis*-**14** displayed 80% and 65% activity compared to unmodified rapamycin at 100 nM and 25 nM, respectively.

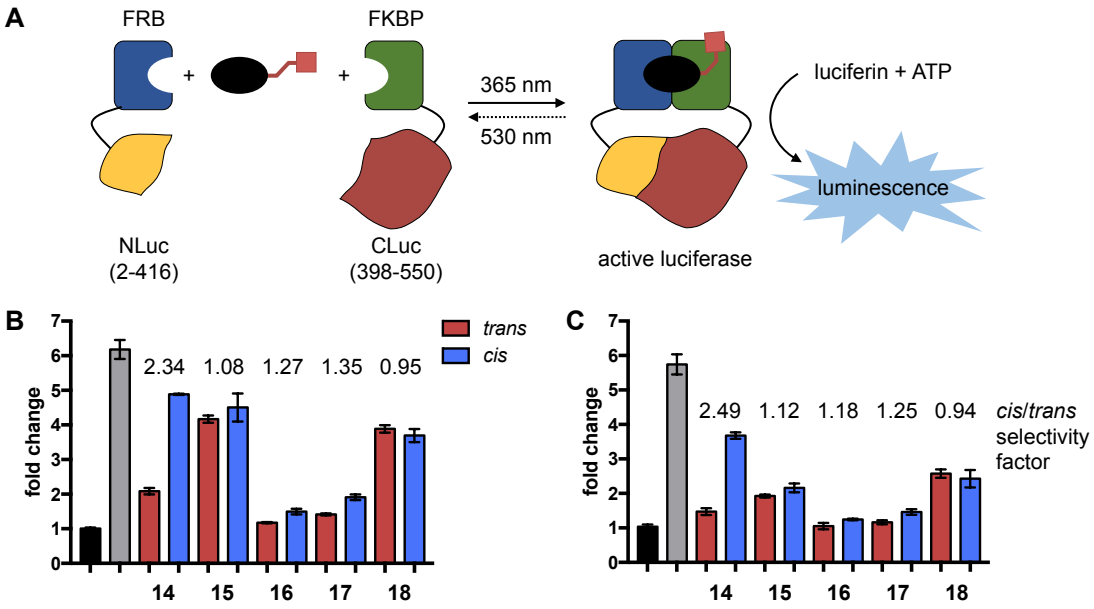


Figure 2. A split luciferase reporter was used to test the efficacy of the photoswitchable rapamycin analogs. a) A schematic of the split luciferase reporter is provided. In the presence of the *trans*-isomer, minimal ternary complex formation is expected; however, upon generation of the *cis*-isomer and ternary complex formation, an active luciferase is formed. In theory, irradiation with 530 nm light should regenerate the inactive, *trans*-isomer (indicated by a dotted arrow). b-c) HEK293T cells expressing the split luciferase reporter were treated with pre-formed *cis*- or *trans*-isomers at 100 nM (b) or 25 nM (c) for 2.5 hours, then luciferase substrates were added and luminescence was measured. Black bars represent the DMSO control and grey bars represent rapamycin. Raw values were normalized to DMSO and error bars represent standard deviations from experiments conducted in triplicate.

Based on the encouraging results of our preliminary testing with wild-type FKBP, we wanted to explore whether FKBP protein engineering would result in further improved enhancement of ternary complex formation for one isomer over the other. Based on our previous experience with iFKBP,[11, 33] we generated the corresponding CLuc-iFKBP expression construct to use in the split enzyme reporter. When cells co-expressing the CLuc-iFKBP and FRB-NLuc reporter were treated with rapamycin or the pre-formed *cis*- or *trans*-**14** for 2.5 hours, no dimerization was observed for either isomer at concentrations as high as 5 μM (**Figure S1a**). iFKBP requires higher compound concentration due to the decreased affinity for rapamycin. Since no improvement in selectivity was achieved with iFKBP, we looked into alternative approaches to modify FKBP. We analyzed the crystal structure of FKBP-rapamycin-FRB (PDB: 1FAP)[34] to find residues that were proximal to the C-40 hydroxyl of rapamycin (the position where the AAP-moiety was appended). We selected glutamine 53 to mutate to phenylalanine and to tryptophan with two different rationales in mind: (1) increase in steric bulk would reduce the background binding of the *trans*-isomer or (2) introduction of π–π stacking opportunities would preferentially stabilize one isomer over the other (**Figure S3**). Cells co-expressing either CLuc-FKBP Q53F or Q53W with FRB-NLuc were treated with rapamycin or pre-formed *cis*- or *trans*-**14** at 25 nM for 2.5 hours (**Figure S1b**). No significant reduction in *trans*-**14** background activity was observed and no significant increase in *cis*/*trans* preferential complexation was obtained. Unfortunately, neither of these approaches resulted in any improvement, thus we proceeded with additional testing of the methyl-AAP analog **14** with wild-type FKBP.

Since the methyl analog displayed the highest degree of *cis*/*trans* selectivity when added to cells as the pre-formed isomers, we next tested whether this analog would allow for activation and/or deactivation of ternary complex formation by irradiating cells following the addition of compound. We first validated that **14** could undergo multiple cycles of photoswitching without displaying fatigue or degradation (**Figure 3a**). Reversible switching was performed four times by irradiating the sample alternatingly with 365 and 530 nm light, with ^{1}H NMR analysis performed after every irradiation step. To test the turn-on ability of **14**, cells expressing the split luciferase reporter were treated with the *trans*-isomer for one hour. A fraction of the cells was irradiated using a 365 nm UV transilluminator for 0.5, 1, or 2 minutes. Incubation was performed for an additional 90 minutes (for 2.5 hours total) to allow for ternary complex formation, then cells were lysed, luciferase substrates were added, and luminescence was measured. Gratifyingly, we observed significant off-to-on activation in this experiment with minimal adverse effect of the UV exposure as seen by the irradiated rapamycin control (**Figure 3b**).

After demonstrating that the system could be turned on in cells, we wanted to explore the turn-off nature of this analog. For this, cells expressing the split luciferase reporter were treated with the pre-formed *cis*-isomer for one hour to allow for uptake and initial complex formation. Irradiations were then performed with a 530 nm LED for 10, 20, or 40 seconds to intracellularly generate the *trans*-isomer, followed by incubation for an additional 90 minutes. Interestingly, the luminescence readout showed no significant decrease in signal, indicating the inability to optically break up the FKBP-rapamycin-FRB complex (**Figure 3c**). We hypothesize that isomerization to the *trans*-isomer does not provide sufficiently disfavorable steric interactions to disrupt an already formed ternary complex that involves very high-affinity protein-ligand and protein-protein interactions with a K_D of 12 nM.[5] A split luciferase reporter system with FKBP/FRB replaced with a kinase/kinase interacting peptide has previously been utilized for screening kinase inhibitors as detected by loss of luciferase activity,[35] thus implicating that the inability to detect a decrease in luminescence here is a direct result of the FKBP-**14**-FRB complex remaining intact. Alternatively, the ternary complex that is formed with the *cis*-isomer could be oriented in such a way that *trans* isomerization is inhibited or minimized, although this is less likely to be the case. Unfortunately, since these experiments are performed at very low compound concentrations in cells, monitoring absorbance to confirm isomerization is not possible.

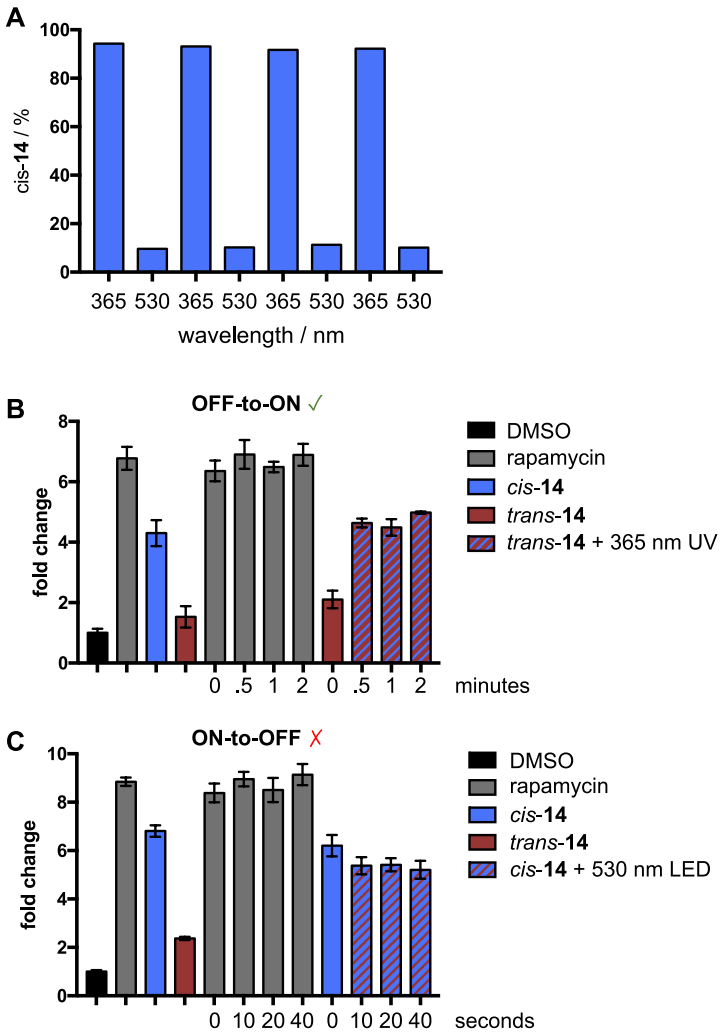


Figure 3. The photocycling stability of **14** was analyzed, and the ability to reversibly control ternary complex formation with **14** was tested in the split luciferase assay. a) Photoswitching of analog **14** in D_2O/d_6-DMSO (1:4) demonstrated robust switching with fatigue resistance following irradiations with both 365 and 530 nm light over four cycles, as measured by NMR. b) HEK293T cells expressing the split luciferase reporter were treated with the pre-formed *trans*-isomer for one hour. Irradiation to generate the *cis*-isomer was performed using a UV transilluminator and cells were incubated for an additional 90 minutes to allow for ternary complex formation. Successful off-to-on activation was achieved. c) HEK293T cells expressing the split luciferase reporter were treated with the pre-formed *cis*-isomer for one hour. A handheld 530 nm LED was used to convert to the *trans*-isomer and cells were incubated for an additional 90 minutes. Interestingly, minimal on-to-off activity was observed.

Conclusion:

In conclusion, five arylazopyrazole-modified rapamycin analogs were synthesized and their photoswitching properties were characterized. These analogs exhibit favorable switching properties with photostationary states that result in almost complete *cis* or *trans*-isomer formation depending on the irradiation wavelength. Moreover, they provided very high thermal stability with half-lives of greater than 1 day for the *cis*-isomer of all five analogs. Additionally, we demonstrated that the AAP results in fatigue resistance over eight alternating irradiations. In a split luciferase

reporter, the methyl-substituted derivative provided ~2.5 fold enhancement in the FKBP-rapalog-FRB ternary complex formation for the photoswitched *cis*-isomer over the ground-state *trans*-isomer, comparing favorably to negative (DMSO) and positive (natural rapamycin) controls. The remaining four analogs did not induce ternary complex formation with any significant preference for one isomer over the other. We explored the methyl analog as a potential on/off light-switch and found that we could successfully turn on dimerization activity in cells through irradiation with 365 nm light; however, once the protein complex was formed, we were unable to turn off dimerization with this analog. The FRB-rapamycin-FKBP complex is extremely stable, which allows complete dimerization of a wide range of fusion proteins at low rapamycin concentration. Since previous reports had to use 50-fold excess of competing ligand to reverse the ternary complex over the course of several hours,[18] reversing the complex through photoswitching of a small molecule ligand may not be achievable. In order to further develop systems that can undergo multiple cycles of dimerization activation/deactivation, we hypothesize that a weaker ternary complex may need to be engineered (e.g., through further mutational analysis of the protein-protein interface) in order to successfully achieve reversible complex formation. While protein engineering to reduce the binding affinity should, in theory, be possible for this CID system, one should keep in mind that the high binding affinity is often an attractive feature. Thus, a fine balance of weaker affinity to allow for reversibility and high affinity for persistent ternary complex formation at low small molecule dimerizer concentrations. Additionally, alternative photoswitchable groups could be chemically appended to rapamycin in an effort to improve the preferential binding, in contrast to biological optimization of the protein environment. Unlike photoswitchable protein dimerizers (e.g., LOV domains, PhyB/PIF, CRY2/CIB),[36] FKBP and FRB are significantly smaller proteins and thus are less likely to interfere with the biological question being addressed. Additionally, small molecule photoswitches often do not require pulsed (or even constant) irradiation like their optogenetic counterparts, thus minimizing the potential for phototoxicity.

Methods:

See Supplemental Information for detailed experimental protocols and supplemental figures.

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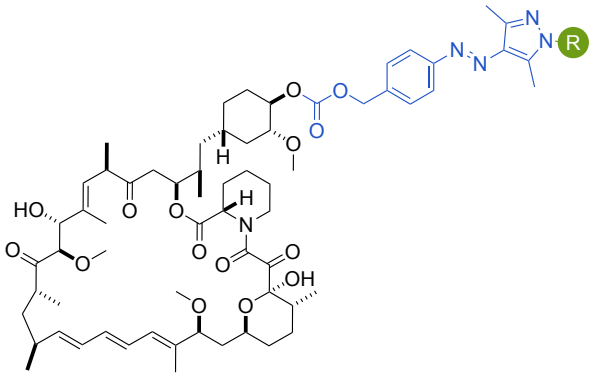
References:

1. Putyrski, M.; Schultz, C., Protein translocation as a tool: The current rapamycin story. *FEBS letters* **2012,** *586* (15), 2097-105.
2. Voss, S.; Klewer, L.; Wu, Y. W., Chemically induced dimerization: reversible and spatiotemporal control of protein function in cells. *Curr Opin Chem Biol* **2015,** *28*, 194-201.
3. Fegan, A.; White, B.; Carlson, J. C.; Wagner, C. R., Chemically controlled protein assembly: techniques and applications. *Chemical reviews* **2010,** *110* (6), 3315-36.

4. DeRose, R.; Miyamoto, T.; Inoue, T., Manipulating signaling at will: chemically-inducible dimerization (CID) techniques resolve problems in cell biology. *Pflugers Archiv : European journal of physiology* **2013,** *465* (3), 409-17.
5. Banaszynski, L. A.; Liu, C. W.; Wandless, T. J., Characterization of the FKBP.rapamycin.FRB ternary complex. *J Am Chem Soc* **2005,** *127* (13), 4715-21.
6. Pecot, M. Y.; Malhotra, V., Golgi Membranes Remain Segregated from the Endoplasmic Reticulum during Mitosis in Mammalian Cells. *Cell* **2004,** *116* (1), 99-107.
7. Zoncu, R.; Perera, R. M.; Balkin, D. M.; Pirruccello, M.; Toomre, D.; De Camilli, P., A Phosphoinositide Switch Controls the Maturation and Signaling Properties of APPL Endosomes. *Cell* **2009,** *136* (6), 1110-1121.
8. Robinson, M. S.; Sahlender, D. A.; Foster, S. D., Rapid inactivation of proteins by rapamycin-induced rerouting to mitochondria. *Developmental cell* **2010,** *18* (2), 324-31.
9. Kohler, J. J.; Bertozzi, C. R., Regulating Cell Surface Glycosylation by Small Molecule Control of Enzyme Localization. *Chemistry & Biology 10* (12), 1303-1311.
10. Diaz, J. E.; Morgan, C. W.; Minogue, C. E.; Hebert, A. S.; Coon, J. J.; Wells, J. A., A Split-Abl Kinase for Direct Activation in Cells. *Cell Chem Biol* **2017**.
11. Karginov, A. V.; Zou, Y.; Shirvanyants, D.; Kota, P.; Dokholyan, N. V.; Young, D. D.; Hahn, K. M.; Deiters, A., Light regulation of protein dimerization and kinase activity in living cells using photocaged rapamycin and engineered FKBP. *J Am Chem Soc* **2011,** *133* (3), 420-3.
12. Umeda, N.; Ueno, T.; Pohlmeyer, C.; Nagano, T.; Inoue, T., A photocleavable rapamycin conjugate for spatiotemporal control of small GTPase activity. *J Am Chem Soc* **2011,** *133* (1), 12-4.
13. Brown, K. A.; Zou, Y.; Shirvanyants, D.; Zhang, J.; Samanta, S.; Mantravadi, P. K.; Dokholyan, N. V.; Deiters, A., Light-cleavable rapamycin dimer as an optical trigger for protein dimerization. *Chemical communications (Cambridge, England)* **2015,** *51* (26), 5702-5.
14. Sadovski, O.; Jaikaran, A. S. I.; Samanta, S.; Fabian, M. R.; Dowling, R. J. O.; Sonenberg, N.; Woolley, G. A., A collection of caged compounds for probing roles of local translation in neurobiology. *Bioorg Med Chem* **2010,** *18* (22), 7746-7752.
15. Volmat, V.; Pouyssegur, J., Spatiotemporal regulation of the p42/p44 MAPK pathway. *Biology of the cell* **2001,** *93* (1-2), 71-9.
16. Caunt, C. J.; Armstrong, S. P.; Rivers, C. A.; Norman, M. R.; McArdle, C. A., Spatiotemporal regulation of ERK2 by dual specificity phosphatases. *The Journal of biological chemistry* **2008,** *283* (39), 26612-23.
17. Stankunas, K.; Bayle, J. H.; Gestwicki, J. E.; Lin, Y. M.; Wandless, T. J.; Crabtree, G. R., Conditional protein alleles using knockin mice and a chemical inducer of dimerization. *Molecular cell* **2003,** *12* (6), 1615-24.
18. Lee, K. B.; Hwang, J. M.; Choi, I. S.; Rho, J.; Choi, J. S.; Kim, G. H.; Kim, S. I.; Kim, S.; Lee, Z. W., Direct Monitoring of the Inhibition of Protein–Protein Interactions in Cells by Translocation of PKCδ Fusion Proteins. *Angewandte Chemie International Edition* **2011,** *50* (6), 1314-1317.
19. Geda, P.; Patury, S.; Ma, J.; Bharucha, N.; Dobry, C. J.; Lawson, S. K.; Gestwicki, J. E.; Kumar, A., A small molecule-directed approach to control protein localization and function. *Yeast (Chichester, England)* **2008,** *25* (8), 577-94.
20. Patury, S.; Geda, P.; Dobry, C. J.; Kumar, A.; Gestwicki, J. E., Conditional nuclear import and export of yeast proteins using a chemical inducer of dimerization. *Cell biochemistry and biophysics* **2009,** *53* (3), 127-34.
21. Mart, R. J.; Allemann, R. K., Azobenzene photocontrol of peptides and proteins. *Chemical Communications* **2016,** *52* (83), 12262-12277.
22. Zhu, M.; Zhou, H., Azobenzene-based small molecular photoswitches for protein modulation. *Organic & biomolecular chemistry* **2018,** *16* (44), 8434-8445.

23. Beharry, A. A.; Woolley, G. A., Azobenzene photoswitches for biomolecules. *Chemical Society reviews* **2011,** *40* (8), 4422-4437.
24. Broichhagen, J.; Damijonaitis, A.; Levitz, J.; Sokol, K. R.; Leippe, P.; Konrad, D.; Isacoff, E. Y.; Trauner, D., Orthogonal Optical Control of a G Protein-Coupled Receptor with a SNAP-Tethered Photochromic Ligand. *ACS Cent Sci* **2015,** *1* (7), 383-93.
25. Klippenstein, V.; Hoppmann, C.; Ye, S.; Wang, L.; Paoletti, P., Optocontrol of glutamate receptor activity by single side-chain photoisomerization. *eLife* **2017,** *6*, e25808.
26. Bose, M.; Groff, D.; Xie, J.; Brustad, E.; Schultz, P. G., The incorporation of a photoisomerizable amino acid into proteins in E. coli. *J Am Chem Soc* **2006,** *128* (2), 388-9.
27. Lotz, T. S.; Halbritter, T.; Kaiser, C.; Rudolph, M. M.; Kraus, L.; Groher, F.; Steinwand, S.; Wachtveitl, J.; Heckel, A.; Suess, B., A light-responsive RNA aptamer for an azobenzene derivative. *Nucleic acids research* **2018,** *47* (4), 2029-2040.
28. Asanuma, H.; Liang, X.; Yoshida, T.; Komiyama, M., Photocontrol of DNA Duplex Formation by Using Azobenzene-Bearing Oligonucleotides. *Chembiochem : a European journal of chemical biology* **2001,** *2* (1), 39-44.
29. Weston, C. E.; Richardson, R. D.; Haycock, P. R.; White, A. J.; Fuchter, M. J., Arylazopyrazoles: azoheteroarene photoswitches offering quantitative isomerization and long thermal half-lives. *J Am Chem Soc* **2014,** *136* (34), 11878-81.
30. Calbo, J.; Weston, C. E.; White, A. J.; Rzepa, H. S.; Contreras-Garcia, J.; Fuchter, M. J., Tuning Azoheteroarene Photoswitch Performance through Heteroaryl Design. *J Am Chem Soc* **2017,** *139* (3), 1261-1274.
31. Singh, S.; Das, G.; Singh, O. V.; Han, H., Development of More Potent 4-Dimethylaminopyridine Analogues. *Organic Letters* **2007,** *9* (3), 401-404.
32. Luker, K. E.; Smith, M. C. P.; Luker, G. D.; Gammon, S. T.; Piwnica-Worms, H.; Piwnica-Worms, D., Kinetics of regulated protein–protein interactions revealed with firefly luciferase complementation imaging in cells and living animals. *Proceedings of the National Academy of Sciences of the United States of America* **2004,** *101* (33), 12288-12293.
33. Karginov, A. V.; Ding, F.; Kota, P.; Dokholyan, N. V.; Hahn, K. M., Engineered allosteric activation of kinases in living cells. *Nature biotechnology* **2010,** *28* (7), 743-7.
34. Choi, J.; Chen, J.; Schreiber, S. L.; Clardy, J., Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. *Science (New York, N.Y.)* **1996,** *273* (5272), 239-42.
35. Jester, B. W.; Cox, K. J.; Gaj, A.; Shomin, C. D.; Porter, J. R.; Ghosh, I., A Coiled-Coil Enabled Split-Luciferase Three-Hybrid System: Applied Toward Profiling Inhibitors of Protein Kinases. *Journal of the American Chemical Society* **2010,** *132* (33), 11727-11735.
36. Spiltoir, J. I.; Tucker, C. L., Photodimerization systems for regulating protein–protein interactions with light. *Current Opinion in Structural Biology* **2019,** *57*, 1-8.

TOC



The first photoswitchable rapamycin analogs were synthesized and evaluated in split-enzyme assays using FKBP/FRB fusion, revealing configurational specificity of the system.