Discovery of fully synthetic FKBP12-mTOR molecular glues

Robin C. E. Deutscher[‡],[a] Christian Meyners[‡],[a] Sabine C. Schäfer,[a] Maximilian L. Repity,[a] Wisely Oki Sugiarto,[a] Tim Heymann,[a] Jürgen M. Kolos,[a] Thomas M. Geiger,[a] Stefan Knapp[b] and Felix Hausch*[a,c]

[a] Institute for Organic Chemistry and Biochemistry, Technical University Darmstadt, Peter-Grünberg-Straße 4, 64287 Darmstadt (Germany)

[b] Institut für Pharmazeutische Chemie, Goethe-University Frankfurt, Biozentrum, Max-von-Laue-Str. 9, 60438 Frankfurt am Main, Germany and Structural Genomics Consortium, Goethe-University Frankfurt, Buchmann Institute for Life Sciences, Max-von-Laue-Str. 15, 60438 Frankfurt am Main; German Cancer Consortium (DKTK)/German Cancer Research Center (DKFZ), DKTK site Frankfurt-Mainz, 69120 Heidelberg, Germany

[c] Centre for Synthetic Biology, Technical University of Darmstadt, 64287 Darmstadt (Germany)

ABSTRACT: Molecular glues are a class of drug modalities with the potential to engage otherwise undruggable targets. However, the rational discovery of molecular glues for desired targets is a major challenge and most known molecular glues have therefore been discovered by serendipity. Here we present the first fully synthetic FKBP12-mTOR molecular glues, which were discovered from a FKBP-focused, target-unbiased ligand library. Our biochemical screening of >1000 in-house FKBP ligands yielded one hit that induced dimerization of FKBP12 and the FRB domain of mTOR. The crystal structure of the ternary complex revealed the binding mode of this hit, which bound to the same surface area as rapamycin, but with a radically different interaction pattern. Structure-guided optimization improved potency 500-fold and led to compounds, which initiate FKBP12-FRB complex formation and S6 kinase inhibition in cells. Our results show that molecular glues targeting flat surfaces can be discovered by focused screening and support the use of FKBP12 as a versatile accessory protein for molecular glues.

INTRODUCTION

For a long time, intracellular proteins without suitable ligand binding pockets have been considered undruggable. The emergence of molecular glues as a drug modality challenged that notion.[1] Through the help of an additional protein - an accessory protein - the available binding surface of the molecular glue-protein-complex can become large enough to bind even flat, featureless target protein surfaces with high affinity.[2; 3] If the accessory protein is an E3 ligase, the degradation of the target protein through the proteasome machinery can be enabled, providing molecular glue degraders.[4; 5] Unfortunately, both molecular glues and molecular glue degraders are still largely discovered by serendipity as strategies to identify them by a more rational strategy are rare.[6] The first and most prominent examples for the serendipitous discovery of molecular glues are the clinically used immunosuppressants rapamycin **1**, FK506 **2** and cyclosporin A **3** (Chart 1).[7–9] Being among the first of their kind, their function was discovered first, followed by the identification of the accessory protein, and finally the target protein itself. FK506 **2** and cyclosporin A **3** are now known to bind to FKBP12 (FK506 binding protein 12) and cyclophilin 18 (Cyp18), respectively, and their binary complexes bind to calcineurin, blocking access to its substrate binding site.[10–12] Rapamycin **1** binds to FKBP12 and then their complex binds to the FRB (FKBP-rapamycin **1** binding domain of mTOR) domain of mTOR (mechanistic target of rapamycin **1**), thereby inhibiting functions of the mTORC1 complex.[13] FKBP12 and Cyp18 might be preferred accessory proteins as nature used them repeatedly for molecular glues such as WDB002 **4** (Chart. 1), inducing FKBP12-CEP250 complexes[14], and sanglifehrin A, which induces Cyp18-IMPDH2 complexes.[15; 16] Additionally, there are several other FKBP12-binding natural products (e.g. Meridamycin and Antascomicin B)[17; 18] that can be considered orphan molecular glues, as their postulated ternary target proteins have not yet been identified.[19]

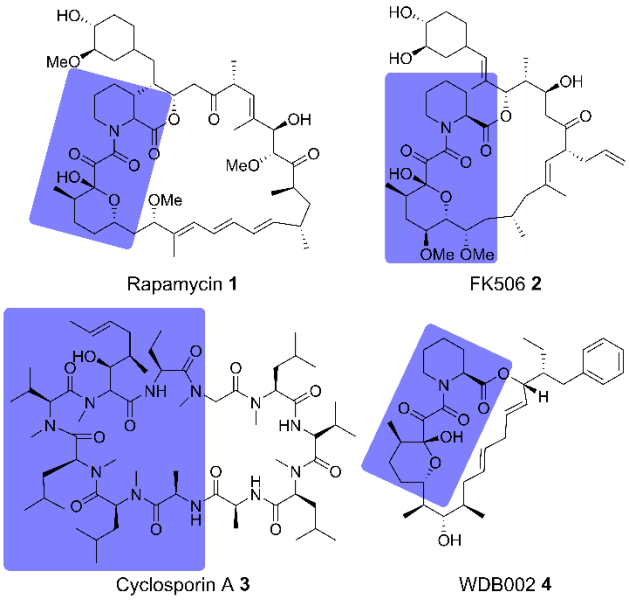
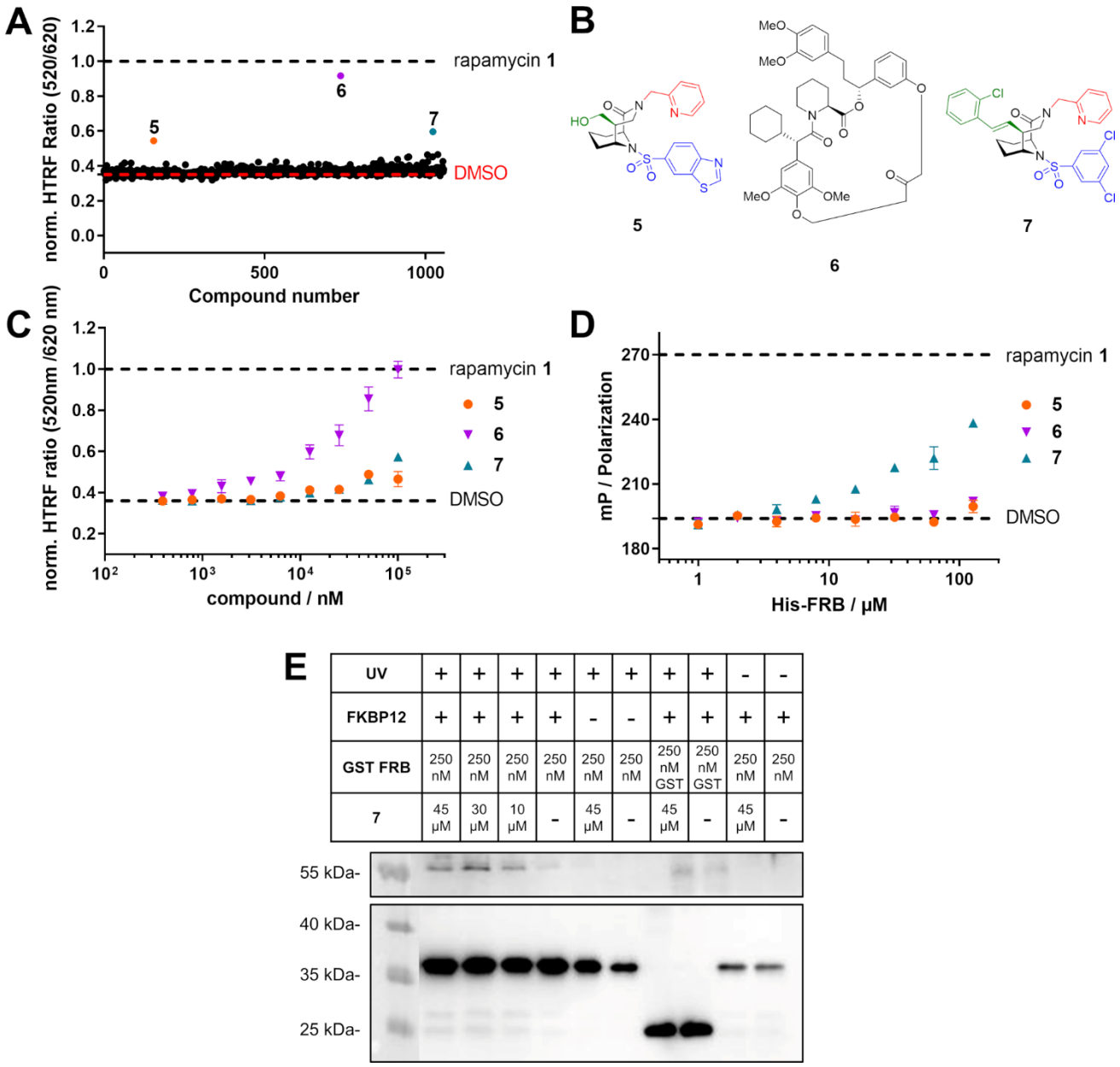


Chart 1. Natural product molecular glues rapamycin 1, FK506 2, cyclosporine A 3 and WDB002 4. FKBP12 or Cyp18 binding moieties are highlighted in slate blue.

Recently, rapamycin **1** analog libraries (rapafucins) have been developed by Liu and coworkers[20] as potential synthetic FKBP-based molecular glues, which led to inhibitors for hENT1[20], GLUT1,[21] and PAANIB-1 after phenotypic screening.[22] Based on early work by WarpDriveBio, the company Revolution Medicines developed the Cyp18-based covalent-reactive $KRAS^{G12C}$ inhibitor RMC-6291, which is currently investigated in a phase I clinical trial (NCT05462717).[23] As of today there is no universally applicable strategy to systematically identify molecular glues hits and little is known about the prospects for subsequent optimization.

RESULTS AND DISCUSSION

To explore the likelihood to discover novel molecular glues from scratch we used FKBP12 and the FKBP12/rapamycin **1**-binding domain (FRB) of mTOR as a well-established model system. We opted for a HTRF (homogeneous time-resolved fluorescence) screening assay using a His-eGFP-FKBP12 and GST-tagged FRB constructs. To enable the detection of weak initial hits, we optimized the assay conditions to allow for high compound concentrations (Fig. S1). Using this assay, we screened our internal compound library containing >1000 FKBP focused ligands (Fig. 1A), originally developed for human FKBP51 or bacterial FKBPs (Fig. S2).[24–37]



UV	+	+	+	+	+	+	+	+	-	-
FKBP12	+	+	+	+	-	-	+	+	+	+
GST FRB	250 nM	250 nM	250 nM	250 nM	250 nM	250 nM	250 nM GST	250 nM GST	250 nM	250 nM
7	45 µM	30 µM	10 µM	-	45 µM	-	45 µM	-	45 µM	-

Figure 1. Identification of compound **7** as a FKBP12-FRB molecular glue. **A** Initial HTRF screening for the compound-induced formation of the ternary FKBP12-FRB complex using 100 µM His-eGFP-FKBP12, 20 nM GST-FRB and 1 nM Terbium-labelled anti-GST antibody. **B** Structure of the three initial screening hits **5**[33], **6**[24] and **7**[38]. **C** Compounds **5**, **6**, and **7** dose-dependently increase the HTRF signal indicative of induced proximity between GFP-FKBP12 and the Terbium-labelled antibody/GST-FRB complex. **D** Compound **7**, but not **5** or **6**, increases polarization in a FRB dose dependent fluorescence polarization assay using 20 nM fluorescein-labelled FKBP12^{E140C} and 5 µM compound. **E** Western-Blot of photoreactive, diazirine labelled FKBP12^{T43C} mutants photocrosslinked with GST-FRB. UV light-induced GST-reactive bands at a size of approx. 55 kDa are indicative of the ternary complex of compound **7**, FKBP12 and FRB being formed in vitro. (**A**, **C**, **D**) Rapamycin **1** and DMSO were used as positive and negative controls, respectively.

Three hits, compounds **5**,[33] **6**[24] and **7**[38] (Fig. 1B), were found to induce the HTRF signal in a dose-dependent manner (Fig. 1C). However, only compound **7** dose-dependently induced higher fluorescence polarization, indicative of ternary complex formation, in an orthogonal fluorescence polarization (FP) assay with fluorescein-labelled FKBP12 in the presence of high concentrations of FRB (Fig. 1D). For compound **6**, we were able to attribute the strong activity in the HTRF-assay to compound-induced binding of GFP-FKBP12 directly to the anti-GST antibody (Fig. S3). The desired activity of **7** was further validated by in vitro photocrosslinking experiments using FKBP12 site-specifically labelled with a photocrosslinking moiety (Fig. 1E). With these two experiments we firmly validated the weak molecular glue activity of compound **7**.

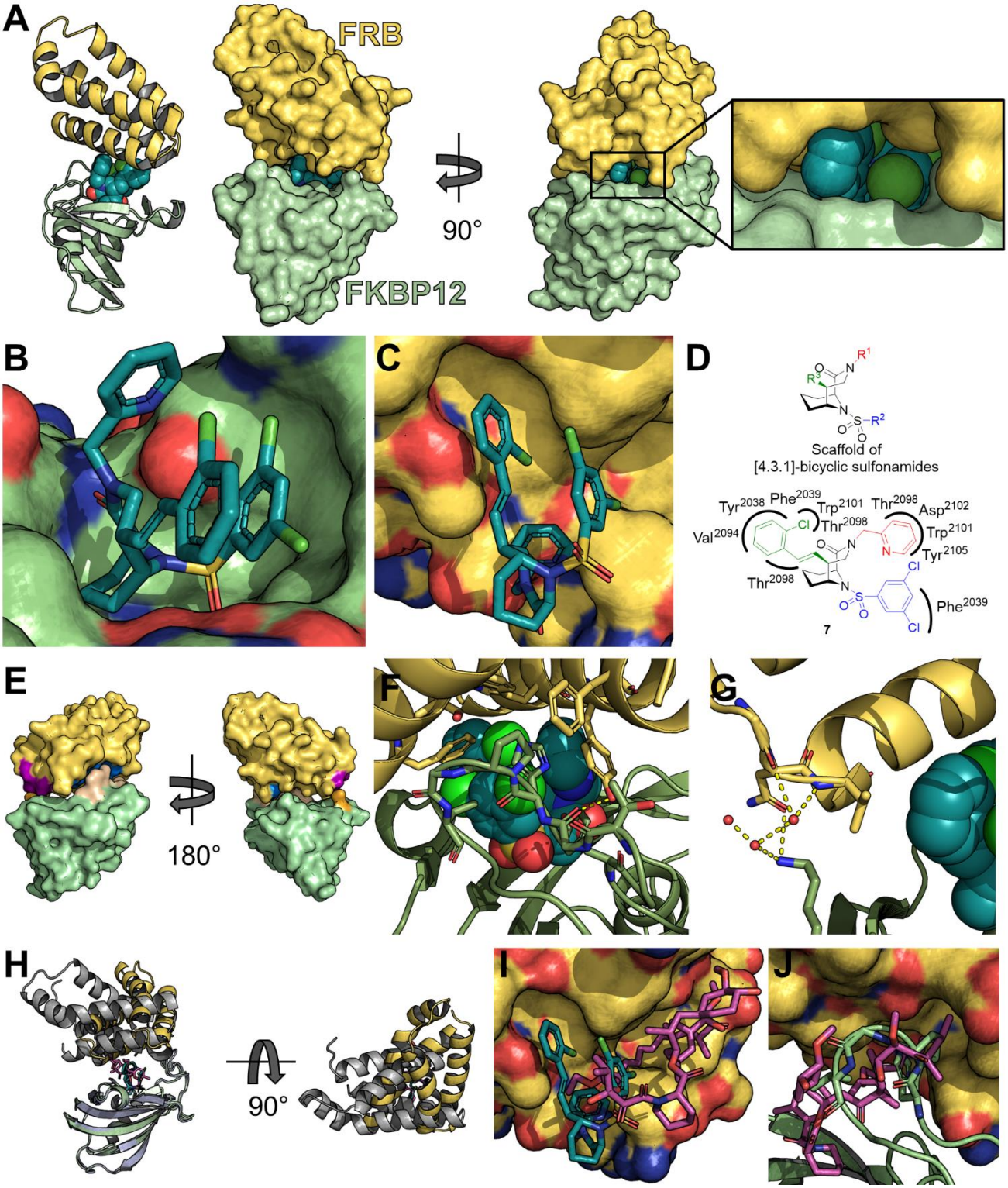


Figure 2. Cocrystal structure of the FKBP12-compound **7**-FRB ternary complex (PDB: 8PPZ). **A** Structure of overall complex of compound **7** (spheres in dark cyan), FKBP12 (surface or cartoon in light green) and FRB (surface or cartoon in yellow). **B** Binding mode of compound **7** (dark cyan sticks) towards FKBP12 (light green surface) in the ternary complex. FRB omitted for clarity. **C** Binding mode of compound **7** (dark cyan sticks) towards FRB (yellow surface) in the ternary complex. FKBP12 omitted for clarity. **D** Scaffold of [4.3.1]-bicyclic sulfonamides with R^1-position residues in red, R^2-position residues in blue and R^3-position substituents in green and two-dimensional (2D) interaction map of compound **7** with the FRB domain of mTOR. **E** FKBP12 (shown as green surface) and FRB (shown as yellow surface) with direct amino acid contacts colored in wheat and orange for FKBP12 and marine and purple for FRB (primary and secondary interaction sites, respectively). **F** Complex of FKBP12, FRB and compound **7** (dark cyan spheres) with amino acids participating in primary and secondary direct contacts shown as sticks. Hydrogen bond is indicated in yellow. **G** Complex of FKBP12, FRB and compound **7** (dark cyan spheres) with amino acids participating in secondary direct contacts between FKBP12 and FRB shown as sticks. Water and

water mediated hydrogen bond are shown as red spheres and yellow dashes. **H** Overlay of FKBP12 of the ternary complexes of compound **7**, FKBP12 and FRB (PDB: 8PPZ) with the ternary complex of rapamycin **1**, FKBP12 and FRB (PDB: 1NSG). FKBP12 molecules were omitted for clarity. Rapamycin **1** (magenta sticks) and compound **7** (dark cyan sticks) lead to different orientations of FRB (yellow for complex with compound **7**, grey in complex with rapamycin **1**). **I** Overlay of FRB of the cocrystal structures of compound **7** (dark cyan sticks, FKBP12 omitted for clarity) with the cocrystal structure of rapamycin **1** (magenta sticks, FRB in gray), highlighting the different binding mode of both complexes. **J** Overlay of FRB of the cocrystal structures of compound **7** (dark cyan sticks, FKBP12 in green cartoon and sticks) with the cocrystal structure of rapamycin **1** (magenta sticks, PDB: 1NSG).

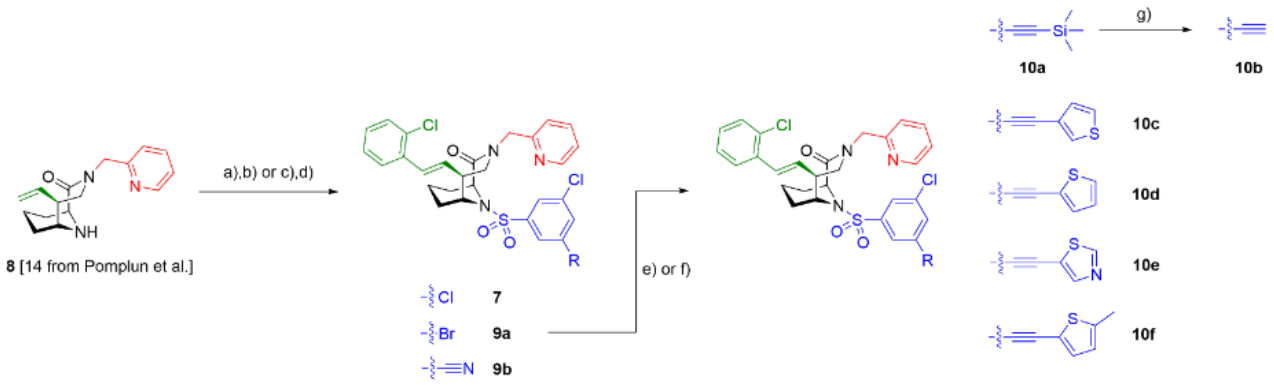
To clarify the molecular binding mode, we determined the cocrystal structure of the FKBP12-**7**-FRB ternary complex (Fig. 2A). The binding of **7** to FKBP12 was similar as observed with related ligands from the [4.3.1]-bicyclic sulfonamide class and all key interactions were conserved (e.g. hydrogen bonds to the backbone NH of Ile^{56} or to the phenol of Tyr^{82}, Fig. 2B). The interactions between **7** and FRB were largely hydrophobic (Fig. 2C&D). All three substituents of the [4.3.1]-bicyclic core engaged in contacts with the FRB domain (Fig. 2C). The R^1-pyridine formed van-der-Waals contacts with Thr^{2098}, Trp^{2101}, Asp^{2102}and Tyr^{2105}. One chlorine and the para-position of the R^2-phenyl ring formed van-der-Waals contacts with Phe^{2039}. The R^3-substituent of **7** formed most interactions with the FRB domain, incl. van-der-Waals contacts to Tyr^{2038}, Phe^{2039}, Val^{2094}, Thr^{2098} and Trp^{2101}.

The ternary complex was enhanced by several direct FKBP12- FRB contacts, located in two regions (Fig. 2E). The major contacts were formed between the 80s loop of FKBP12 (Tyr^{82} and Thr^{85}-Ile^{90}) and the side chains of Ser^{2035}, Phe^{2039}, Trp^{2101}, Tyr^{2105}, and Phe^{2108} of FRB (Fig. 2F). This included a direct hydrogen bond from the phenol group of Tyr^{2105} (FRB) to the backbone carbonyl of Gly^{86} (FKBP12). In the second region, the amine group of Lys^{44} of FKBP12 formed a hydrogen bond to the primary amide carbonyl bond of Asn^{2093} (FRB), as well as a hydrogen bond to Gly^{2092}, which was mediated by two water molecules (Fig. 2G). The side chain of Lys^{44} of FKBP12 also formed van-der-Waals contacts with Val^{2094} of the FRB domain.

The comparison with the known FKBP12-rapamycin **1**-FRB ternary complex (PDB: 1NSG)[39] revealed that the FKBP12-**7** and FKBP12-rapamycin **1** binary complexes target a similar surface region on FRB. However, the specific interactions radically differed since the orientation of the FRB was rotated by 90° between the two ternary complexes. (Fig. 2H). While the binding surface on the FRB domain partially matched comparing compound **7** and rapamycin **1**, both also formed unique interactions with parts of the FRB-domain (Fig. 2I). Interestingly, in the FKBP12-**7**-FRB complex the 80s loop of FKBP12 mimicked some of the interactions formed by the conjugated triene moiety of rapamycin **1** in the FKBP12-**1**-FRB complex (Fig. 2J).

The total binding interface, calculated with PISA[40], between the FKBP12-**7**-complex and the FRB-domain is 632 Å^2, which was similar to the interaction surface between the FKBP12-rapamycin **1** complex and FRB (698 Å^2). However, the contributions of the compounds vs FKBP12 differed substantially. While in the FKBP12-**7**-FRB complex, 194 Å^2 of the contact surface were contributed by compound **7** and 428 Å^2 by 'direct' contacts of FKBP12, in the FKBP12-**1**-FRB complex 395 Å^2 were being contributed by rapamycin **1** and 303 Å^2 by FKBP12.

Scheme 1. Synthesis of [4.3.1]-bicyclic sulfonamide analogs.

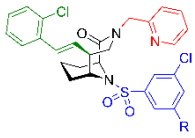
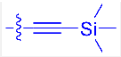
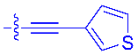
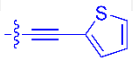
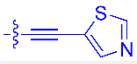
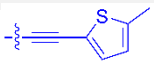


Reactions and conditions: a) sulfonyl chloride, DIPEA, MeCN, rt, compound **7**: 18, 48%, **9b**: 16 h, 49%; b) 1-bromo-2-chlorobenzene, K_2CO_3, Pd(dppf)Cl_2•CH_2Cl_2, 100°C, compound **7**: dioxane, 40 h, 57% yield, **9b**: DMF, 18 h, 25% yield; c) 1-bromo-2-chlorobenzene, K_2CO_3, Pd(dppf)Cl_2•CH_2Cl_2, dioxane:H_2O=20:1, 100°C, 19 h, 78% yield; d) 3-bromo-5-chlorosulfonyl chloride, DIPEA, MeCN, rt, 46 h, 55% yield; e) alkyne, Pd(dppf)Cl_2•CH_2Cl_2, CuI, TMEDA, 80°C, **10c**: 2,5 h, 68% yield, **10d**: 3 h, 55% yield; f) TMS-alkyne, Pd(dppf)Cl_2•CH_2Cl_2, CuI, TMEDA:DMF=1:1, 80°C, **10e**: 22 h, 53%, **10f**: 14,5 h, 75%; g) K_2CO_3, MeOH, rt, 2,5 h 94% yield.

To increase the potency of the initial hit **7**, we analyzed the structure of the ternary complex and studied the role of the chlorine pointing into a small cavity between FKBP12 and FRB domain (Fig. 2A insert, chlorine shown as green sphere). To explore this position, we substituted one of the meta chlorines with small substituents like bromine, nitrile, and acetylene (Scheme 1). This led to compounds 9a/b and 10a/b with slightly improved potencies for ternary complex induction (Tab. 1). Gratifyingly, the extension by an additional heterocycle substantially enhanced the ternary complex formation 50-500-fold. Thiophenes (10c, 10d), thiazole (10e) and methylthiophene (10f) all induced formation of

the ternary complex with <2μM potency in the FP-assay. All analogs retained high affinity to purified FKBP12 alone (Kd < 12nM) and occupied FKBP12 inside human cells with in EC50 between 40-1400nM (Tab. 1).

Table 1. Affinity data for compound binding to purified human FKBP12, determined by a fluorescence polarization assay (FP), affinity data for the binding of the FKBP12-compound complex towards FRB, determined by a fluorescence polarization assay (FP), and nanoBRET data for intracellular FKBP12 occupancy for R2-substituted compounds.

No.	human FKBP12 FP, K_i [nM][41]	FRB with FKPB12-FSM, IC50 [μM]	FKBP12 nanoBRET IC50 [nM][42]	
rapamycin **1**	0.6	0.039 ± 0.006	30.3±1.5	-
7	6.3 ± 2.2	92.9 ± 20.9	81.2 ± 16.3	
9a	5.8 ± 1.1	56.3 ± 10.3	40.6 ± 5.3	
9b	3.6 ± 1.1	54.0 ± 6.2	47.8 ± 10.7	
10a	11 ± 1.5	49.7 ± 4.7	405 ± 219	
10b	13 ± 2.1	63.1 ± 6.1	101 ± 19	
10c	4.1 ± 0.6	0.56 ± 0.03	313.7 ± 21.3	
10d	7.2 ± 1.7	0.63 ± 0.06	799 ± 183	
10e	4.7 ± 1.8	1.82 **± 0.11**	49.2 ± 4.0	
10f	4.8 ± 0.8	0.18 ± 0.02	1325 ± 195	

To test if the synthetic FKBP12-FRB molecular glues were active in cells, we first performed a nanoBRET assay using C-terminal nanoLuc-tagged FKBP12 and C-terminal Halo-Tag-tagged FRB (Fig. 3A). Like rapamycin **1**, compounds **10e** and **10f** dose-dependently induced the FKBP12-FRB complex in HEK293 cells. In the same cells, compounds **10e** and **10f** blocked the phosphorylation of p70 S6K, a key downstream target of mTOR (Fig. 3B&C). This indicated that recruiting FKBP12 to the FRB domain of mTOR was sufficient to allosterically block mTOR kinase activity while the orientation of FRB-bound FKBP12 seemed unimportant.

CONCLUSION

Our screening approach enabled us to identify a novel molecular glue targeting the flat surface of the FRB-domain of mTOR. Screening at high target protein concentrations was crucial to identify an initially very weak hit, which would have been difficult to detect by other approaches. Although our approach was unbiased regarding the binding site on FRB, the identified molecular glues target a similar region on FRB as rapamycin **1**. The surface on FRB around Tyr^{2028}/Phe^{2039}, Val^{2094}- Thr^{2098}, and Trp^{2101}-Phe^{2105}, while not *a priori* apparent, thus appeared to represent a preferred region for protein-protein contacts. Indeed, this site has been suggested to assist in the binding of mTOR targets such as S6K and PRAS40, as well as phosphatidic acid (PA).[43; 44] The preference for this region was not due to specific contact with FKBP12, since in the context with compound **7,** FKBP12 engages FRB in a completely different manner than in context with rapamycin **1**. However, for both rapamycin **1** and compound **7**, direct FKBP12-FRB contacts were crucial in order to dramatically enhance the affinity of the FKBP12-compound complex to FRB compared to FRB-binding of the compound alone.[45; 46] The substantially higher affinity of the FKBP12-rapamycin **1** complex over FKBP12-**7** for FRB is likely due to higher-quality interactions.

Aided by the crystal structure we were able to improve the affinity of our initial hit by rational design up to 500-fold, leading to compounds **10e** and **10f**, which inhibit kinase activity mTOR intracellularly at micromolar concentrations.

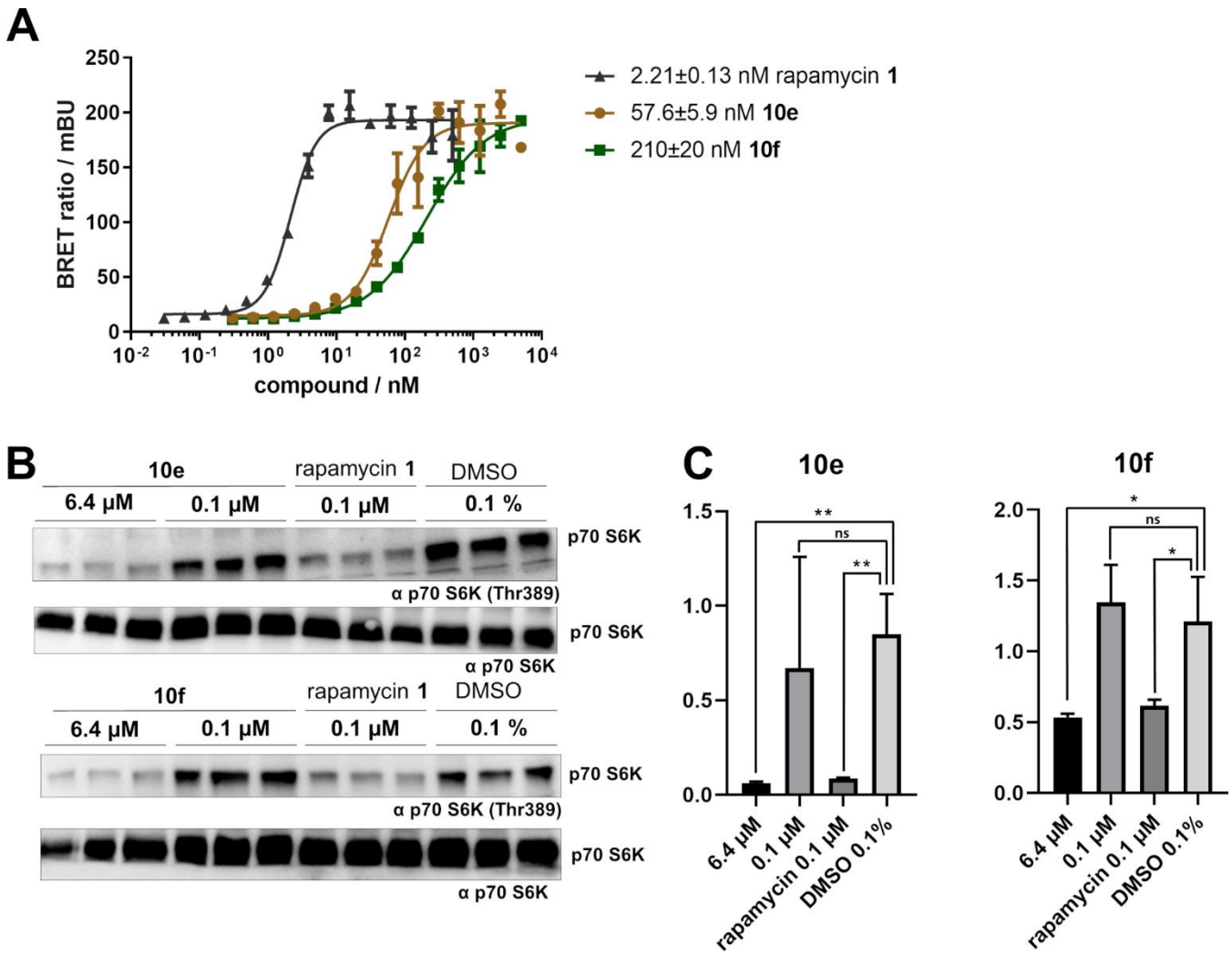


Figure 3. Cellular characterization of FKBP12-FRB molecular glues. A) Compound-induced FKBP12-FRB ternary complex formation in HEK293 cells determined by nanoBRET using C-terminal nanoLuc-tagged FKBP12 and C-terminal HaloTag-tagged FRB. B) The impact of the ternary complex resulting in inhibition of phosphorylation of p70 S6K was determined by Western Blot compared to total p70 S6K level in HEK293T cells. C) Inhibition of p70 S6K was quantified in ImageJ and Student's t-test was utilized to calculate significance of inhibition level to DMSO control (**p < 0.01; *p < 0.05; ns = not significant).

Our findings have several implications for the discovery of molecular glues. (i) Molecular glues might be less rare than initially thought as we found one hit within a relatively small focused library. (ii) Screening approaches with high compound and accessory protein concentrations were necessary to find such weak molecular glue hits. Biochemical approaches seem to be most adequate as weak activity is easier to detect. (iii) The use of a focused library targeted to the accessory proteins (FKBP12 in our case) likely facilitates the identification of molecular glues substantially since part of the recognition problem is already pre-engineered. (iv) Weak initial molecular glue hits can be used as a starting point for rational design to get more potent molecular glues. Even for weak molecular glues hits, the ternary complex structure can be obtained, which facilitated optimization substantially. (v) Shallow hydrophobic surfaces seem to be a preferred interaction site for molecular glues, in line with the binding modes of rapamycin **1**, FK506 **2** and WBD002 **4**.[47; 10–12] (vi) The choice of the accessory protein is likely a key factor. FKBP12 (like Cyp18) might be a privileged accessory protein featuring high abundance in many tissues[48; 49], absence of negative effects by binding of FKBP12 alone, availability of potent ligands as docking scaffolds, and numerous exit vectors on the latter. These features likely contributed to the prevalence of FKBPs (and cyclophilins) as accessory proteins in nature and support their use to target otherwise undruggable protein in drug discovery.

ASSOCIATED CONTENT

The supporting information is available free of charge via the Internet at http://pubs.acs.org.

Additional experimental details (Figures S1–S4), materials and methods, X-ray crystallography, ^{1}H NMR, ^{13}C NMR, and LC-MS spectra of molecular glues.

AUTHOR INFORMATION

Corresponding Author

Felix Hausch - Institute for Organic Chemistry and Biochemistry, Technical University Darmstadt, Alarich-Weiss-Straße 4, 64287 Darmstadt, Germany, Centre for Synthetic Biology, Technical University of Darmstadt, Darmstadt, Germany; orcid.org/0000-0002-3710-8838; Email: felix.hausch@tu-darmstadt.de.

Present Addresses

Jürgen M. Kolos - Immundiagnostik AG, Stubenwald-Allee 8a, 64625 Bensheim, Germany

Author Contributions

‡R.C.E.D. and C.M. contributed equally. Organic synthesis was performed by R.C.E.D. and J.M.K.. Protein production, HTRF-screening, HTRF dose response and FP-assay dose response were performed by C.M. and W.O.S.. Photocrosslinking assay was performed by M.L.R., with contributions from T.H. and ternary nanoBRET assay was performed by M.L.R. with contributions from S.K.. Crystallization was performed by C.M. FKBP12 nanoBRET was performed by T.M.G.. p70S6K assay was performed by S.C.S.. F.H. conceived and supervised the project. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Graphic entry for the Table of Contents (TOC)