FKS06-binding proteins (FKBPs) are key intracellular co-receptors for the natural products FKS06 and Rapamycin\(^{[1]}\) as well as for recently discovered molecular glues.\(^{[2]}\) Moreover, several members of the FKBPs family have been suggested to play key roles in diseases such as pulmonary arterial hypertension, hereditary haemorrhagic telangiectasia, wound healing, depression, obesity, chronic pain, as well as bacterial and parasitic infections.\(^{[3]}\) Most of the pharmacologically relevant human FKBPs like FKB12, 12.6, 51 and 52 are localized in the cytosol, where they participate in regulatory protein-protein interaction exemplified by the FKB12/12.6-ryanodine receptor\(^{[4]}\) or FKB12-ALK\(^{[5]}\) or FKB12/12.6-Hsp90-steroid receptor complexes.\(^{[6]}\) Several high-affinity FKBPs ligands have been developed.\(^{[7,8]}\) However, little is known to what extent these ligands occupy FKBPs or on the degree of occupancy needed to evoke biological effects. A recent study on irreversible ligands for FKBP35 of \textit{Plasmodium falciparum} showed that intracellular activities of FKBPs ligands may be substantially weaker than biochemical affinities and do not necessarily correlate with the latter.\(^{[9]}\) Here, we report competitive NanoBRET assays\(^{[10]}\) for human FKB12, FKB12.6, FKB51 and FKB52 for rapid profiling of target engagement of FKBPs ligands in living cells.

Our work started with the identification of cell-permeable fluorescent tracers suitable as NanoBRET acceptors. While this work was in progress, a NanoBRET assay for FKB12 and FKB35 from \textit{Plasmodium falciparum} was published,\(^{[10]}\) which used a Rapamycin-derived tracer. Since we had observed solubility issues at higher tracer concentrations with a Rapamycin-derived fluorescence tracer,\(^{[10]}\) and (unpublished results) we here opted for a bicyclic [4.3.1] sulphonamide scaffold, for which preliminary cell permeability data were available. Further benefits of the chosen bicyclic [4.3.1] sulphonamides\(^{[11,12]}\) as starting scaffold are the lack of immunosuppressive properties, their pan-selectivity within the FKBPs family, which is desirable for broadly applicable tracers, and the presence of three well-defined exit vectors to allow for the introduction of fluorophores. Specifically, the alkyne 1\(a\)-c were chosen as starting points and conjugated to an azide-containing BODIPY590, via copper-catalysed cycloaddition (Figure 1). The resulting fluorescent conjugates 2\(a\)-c were tested in a fluorescence polarization assay\(^{[16]}\) and bound to purified FKBPs with moderate (FKBPS1/52) to high affinity (FKB12/12.6, Figure S1). Tracer 2\(a\) bound substantially weaker to FKBPs, especially towards FKBPS1 and FKBPS2 with \(K_D\) values of 228 \pm 39 nM and 669 \pm 99 nM, respectively, compared to the known TAMRA-containing tracer 16 g, which has affinities towards...
FKBPs between 0.57 nM and 13.8 nM. The affinities of 2b and 2c however were in line with affinities of the closest known analogues.

To explore the intracellular interaction of the tracers with the most prominent FKBPs, we generated a set of mammalian expression constructs harbouring FKBPs12, FKBPs12.6, FKBPs51 or FKBPs52 N- or C-terminally fused to a Nano-luciferase. All constructs were active and produced luminescence signals after transient transfection (Figure S2). However, the luminescence signals were more robust for the C-terminal fusion constructs, which were thus pursued for further NanoBRET experiments. When transiently transfected in HEK293T cells, all C-terminal constructs generated dose-dependent BRET signals after titration with the tracers 2a–c (Figures S3–6 and Table S1). After optimizing for cell density, transfection levels and incubation time, tracer 2a or 2b were found to provide the best results for FKBPs12 and 12.6, while tracer 2c was more suitable for FKBPs51 and FKBPs52. Pilot experiments showed that the identified construct tracer combinations were sensitive to the prototypical FKBPs ligands FK506 and Rapamycin and suitable to quantify intracellular binding in a competitive NanoBRET setup (Figure 2).

The experiments with transiently expressed NanoBRET constructs showed substantial batch-to-batch variation. Therefore, we generated HEK293T cell lines stably expressing FKBPs12/12.6/51 or 52 C-terminally fused to Nano-luciferase. The best selected cell lines displayed a robust, dose-dependent increase in BRET ratios upon incubation with their preferred tracers (Figure 3). Half-maximal stimulation (EC₅₀) was observed at concentrations in agreement with the biochemical affinities of the tracers, except for FKBPs2-NLuc, which consistently engaged tracer 2c with exceptionally high potency. The signal-to-noise ratio was excellent for FKBPs12, 12.6 and 52 and acceptable for FKBPs51.

With robust NanoBRET assays for the major cytosolic FKBPs in hand, we set out to profile representative FKBPs ligands for intracellular FKBPs occupancy. Matrix titrations with one of the tracers, except for FKBPs2-NLuc, which was necessary due to the lower assay window. Comparisons of the target engagement of Rapamycin towards the luciferase-labelled FKBPs with previously published results clearly showed that this ligand bound competitively inside cells to FKBPs12, 12.6, 51 and 52 (Figure 4, structures of the compounds used are shown in Figure 6).

The matrix titrations also defined plausible tracer concentrations suitable for further competition experiments. With these conditions in hand, we profiled representative FKBPs ligands, including the FKBPs-selective ligands SAFit1 and SAFit2 as well as the natural products FK506 and Rapamycin (Figure 5A and Figure S7). Generally, the IC₅₀ values for FKBPs51 were higher, likely due to the higher tracer concentration, which was necessary due to the lower assay window. Comparison of the target engagement of Rapamycin towards the luciferase-labelled FKBPs with previously published results clearly showed that in spite of the different assay conditions (e.g., tracer affinity and concentration) the obtained IC₅₀ values (20 nM) were remarkably similar. In light of the results by Atack et al., we conclude that the Rapamycin-based tracer as well as our bicyclic [4.3.1] sulfonamide-based tracers are useful.

From a comparative analysis of the data, several conclusions can be extracted. First, the natural products FK506 and Rapamycin, as well as the non-immunosuppressive smaller bicyclics 3 (FK[4.3.1]-16h) and 4 (FK[4.3.1]-16j) are highly effective at occupying FKBPs inside cells. Second, 4 (FK[4.3.1]-16j) is always more potent than 3 (FK[4.3.1]-16h), possibly reflecting the higher affinity of the former. Third, SAFit1 and SAFit2 are highly selective for FKBPs12 and FKBPs52 (a key unique property of SAFit-type ligands) and moderately selective over FKBPs52. Fourth, SAFit2 retains potency for FKBPs2.6 and is equipotent to Rapamycin and 4 (FK[4.3.1]-16j) for FKBPs51, in line with the biochemical affinity

![Figure 2. Competitive NanoBRET assay for Rapamycin and FK506 with HEK293T cells transiently expressing FKBPs12-NLuc (A) or FKBPs12.6-NLuc (B). Experiments were performed in three independent cellular assays for all samples.](image-url)

![Figure 3. Tracer titrations for stable FKBPs-NLuc cell lines. HEK293T cells stably expressing the indicated FKBPs-NLuc constructs were treated with tracers 2b or 2c at serially diluted concentrations for 2 h at 37 °C. BRET measurements were performed by detecting emission light intensity at 450 nm and 660 nm. Mean BRET ratios and standard deviations were calculated from three independent cellular assays. EC₅₀ values were determined by a four-parameter fit. Nd represents the affinity of the tracer to the indicated purified FKBPs determined by fluorescence polarization.](image-url)
SAFit1 was substantially less potent than the closely related SAFit2, which may reflect reduced cellular permeability or active efflux of the former. Indeed, the cell permeability ($A_{\text{B}}$) of SAFit1 was 10 times lower in a standard Caco2 assay (Eurofins) compared to SAFit2. We previously observed poor cell permeability and strong efflux ratio ($B_{\text{A}}/A_{\text{B}} > 10$) for SAFit1 in Caco2 assay (not shown). To test if P-gp-mediated active efflux contributed to the reduced BRET activity of SAFit1 assay we performed a NanoBRET experiment in the presence or absence of the P-gp inhibitor Elacridar (Figure S9). However, Elacridar did not affect the NanoBRET activities of SAFit1, SAFit2 or FK506 (the latter had been described as a P-gp substrate\cite{18}), suggesting that our NanoBRET assay is not affected by P-gp. Taken together, these results suggest that 3 (FK(4.3.1)-16h) and 4 (FK(4.3.1)-16j) (and SAFit2 for FKBP51) are valid non-immunosuppressive tools to probe the role of FKBPs inside living cells.

To further explore the scope of the established NanoBRET assays, we applied them to a recently discovered class of macrocyclic SAFit analogues (Figure 6) that are highly selective for CK2.
for FKBP51 over FKBP12.\textsuperscript{[19]} This experiment confirmed the intracellular potencies of SAFit1 and SAFit2 for FKBP12 and FKBP51 (Figure 5B and Figure S8). Importantly, the macrocyclic SAFit analogues all occupied intracellular FKBP51, with potencies reflecting their relative biochemical affinities, whereas none of them were active for FKBP12. This is consistent with preliminary findings from NanoBRET assays using transiently expressed FKBP-NLuc constructs.\textsuperscript{[19]}

Taken together, we developed a set of NanoBRET assays for the most prominent human FKBPs. High-quality tracers that have high target affinity, high cell permeability and low nonspecific binding were key to obtain high signal-to-noise ratios. Stable cell lines proved critical to reduce inter-assay variability. With these assays, we profiled some of the most widely used FKBP ligands reported in the literature. We demonstrated the high intracellular activity of the natural products FK506 and Rapamycin as well as of ligands with the bicyclic [4.3.1] scaffold. We also confirmed the biochemical selectivity of SAFit-like ligands inside cells. However, compared to the natural products or [4.3.1] bicycles, compounds of the SAFit class, especially SAFit1, had reduced intracellular potency, pointing to the need to regularly control for intracellular activity. The here developed assays will be important for the profiling of FKBP ligands and a valuable tool to guide drug discovery directed at FKBPs.

**Experimental Section**

**Competitive NanoBRET assay**

The NanoBRET assays described here are based on previously published work\textsuperscript{[10]} and modified as described below.

The fluorescent ligands \(2\ b\) or \(2\ c\) were dissolved in Opti-MEM I Reduced Serum Media at the eightfold concentration required for the final sample. For the target engagement matrix different final tracer concentrations were chosen. HEK293T cells expressing the FKBP-NanoLuc fusion protein were detached from the culture dish and resuspended in Opti-MEM I Reduced Serum Media. The cell number was adjusted to \(4.6 \times 10^5\) cells/mL using transiently transfected cells or to \(1.81 \times 10^6\) cells/mL using the stable FKBP-NanoLuc cell line. A cell-tracer mixture was prepared mixing one part of the tracer stock solution with three parts of the cell suspension (e.g. \(500\ \mu\)L tracer stock solution + \(1500\ \mu\)L cell suspension). Test ligands were dissolved in DMSO at thousand fold the concentration required for the final sample. This ligand stock was used to prepare a 1:2 serial dilution in DMSO. Each dilution was then diluted with Opti-MEM I Reduced Serum Media to generate a ligand dilution series with double the concentration required for the final sample. To a white non-binding 384-well assay plate (No.: 3574; Corning Life Sciences B.V., Schiphol-Rijk, Netherlands) \(20\ \mu\)L of cell-tracer mixture and \(20\ \mu\)L of test compound solution were added and the plate was incubated at \(37^\circ\)C for two hours. Afterwards, the plate was equilibrated at room temperature for 15 minutes. For BRET detection the Intracellular NanoGlo® Substrate/Inhibitor kit (No.: N2160; Promega) was used diluting the NanoBRET™ NanoGlo® Substrate 1: 664 and the extracellular NanoLuc® inhibitor 1 : 2000 in Opti-MEM I Reduced Serum Media. 20 \(\mu\)L of the detection solution was added per well and the plate was incubated for three minutes at room temperature. The donor emission was measured at 450 nM and the acceptor emission at 660 nM using a ClarioStar plate reader (BMG Labtech, Ortenberg, Germany) or a Tecan Spark (Cailsheim, Germany). The BRET ratio was calculated as shown in the supplementary information. \(K_{\text{app}}\) and \(K_{D,\text{app}}\) values were determined with the following formula.

\[
I_{C_{50}} = K_{\text{app}}(1 + \frac{c(\text{tracer})}{K_{D,\text{app}}})
\]
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Conflict of Interest

The authors declare no conflict of interest.

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