CHEMBIOCHEM

DOI: 10.1002/cbic.200800806

Fluorescent Probes to Characterise FK506-Binding Proteins

Christian Kozany,[a] Andreas März,[a] Christoph Kress,[a, b] and Felix Hausch*[a]

FK506-binding proteins (FKBPs) convey the immunosuppressive action of FK506 and rapamycin and mediate the neuroprotective properties of these compounds, and participate in the regulation of calcium channels. In addition, the larger homologues FKBP51 and FKBP52 act as cochaperones for Hsp90 and regulate the transactivational activity of steroid hormone receptors. To further characterize these FKBPs, we have synthesized fluorescein-coupled rapamycin analogues. In fluorescence polarization assays one of these compounds retained high affinity to all tested proteins (K_d: 0.1–20 nM) and could be used for active-site titrations. To adapt the fluorescence polarization assay for high-throughput purposes, a simplified rapamycin derivative was synthesized and labelled with fluorescein. This probe showed moderate affinity for the FK1 domains of FKBP51 (177 nM) and FKBP52 (469 nM) and allowed a highly robust, optimized, miniaturized assay ($Z' > 0.7$) sufficient for high-throughput screening of large compound libraries.

Introduction

FK506-binding proteins (FKBPs) are widely expressed immunophilins that bind the natural products FK506 and rapamycin and mediate their immunosuppressive effect. Most FKBPs display a peptidyl–prolyl isomerase activity; this indicates a role for these molecules in protein folding. In vivo, several FKBPs have been shown to regulate calcium channels.[1,2] In addition, the multidomain homologues FKBP51 and FKBP52 act as cochaperones for the heat shock protein 90 (Hsp90) and regulate the binding and transactivational activity of steroid hormone receptors in a mutually opposite direction.[3,4] In eukaryotic cells, FKBP52 has been shown to enhance the transactivation of the glucocorticoid receptor (GR) and the androgen receptor (AR).[3,5] In contrast the hormone responsiveness of the GR can be attenuated by FKBP51.[6,7] Interestingly, the expression of inhibitory FKBP51 itself is strongly induced by steroids; this gives rise to an intracellular, ultrashort negative-feedback loop that could desensitize steroid hormone receptors after initial stimulation by steroids.[8]

In addition to their well-established interaction with steroid hormone receptors, other less well-characterized functions have been ascribed for FKBP51 and -52. Several reports have implicated FKBP51 in the activation of the NF-κB pathway.[9] FKBP52 has been described to regulate transcription factors,[10] participate in copper transport,[11] inhibit viral second-strand synthesis[12] and block calcium channels in a FK506-sensitive manner.[2] Based on neutralizing antibody and siRNA knockdown experiments, FKBP52 has further been suggested to promote neurite outgrowth.[13,14] While numerous studies have reported a neuroprotective or neurotrophic action of FK506-like ligands, the exact protein target(s) that mediate this effect have not been identified so far, possibly due to the lack or uncertainty of specificity of these compounds within the FKBP family.

The physiological significance of FKBP52 has been demonstrated in knockout mice, which showed profound reproductive abnormalities, likely mediated by impaired progesterone and androgen signalling.[5,15] The physiological role of FKBP51 could be shown in the Bolivian squirrel monkey in which the low responsiveness of the GR was partially attributed to the high levels of the inhibitory FKBP51.[6,16] More recently, several reports have provided strong genetic support for the role of FKBP51 in human stress response[17] and in a variety of psychiatric disorders.[18] In light of aberrant corticosteroid signalling as a recurrent observation in mood disorders, these combined findings suggest FKBP51 as a mechanistically novel and drugable target for depression.

FKBP51 and FKBP52 are highly homologous, multidomain proteins with >60% amino acid sequence identity. This is reflected in a high degree of structural similarity observed in the X-ray crystal structures of these proteins.[19,20] Both proteins contain a FK1 domain that displays peptidyl–prolyl isomerase activity and low nanomolar affinity for FK506 and rapamycin.[21–23] This domain was also shown to be the major determinant for the regulation of steroid hormone signalling;[3,24] this suggests FKBP cochaperones as a novel target for the modulation of steroid hormone receptors. However, due to the lack of selectivity of FK506 or rapamycin, these compounds cannot be used to dissect the opposing role of FKBP51 and FKBP52 (or other FKBPs) in mammalian systems where these proteins tend to be coexpressed. For a better understanding of FKBP biology specific inhibitors are needed.

[a] Dr. C. Kozany, A. März, C. Kress, Dr. F. Hausch
Chemical Genomics Research Group, Max Planck Institute for Psychiatry
Kraepelinstrasse 2, 80804 Munich (Germany)
Fax: (+49) 89-30622610
E-mail: hausch@mpipsykl.mpg.de

[b] C. Kress
Present address: 4SC AG, Am Klopferspitz 19a
82152 Planegg-Martinsried (Germany)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.200800806.

Fluorescent labelled tracers for the founding family member FKBP12 have already been described. However, these tracers have not been tested for their applicability with other FK506 binding proteins.[25] Therefore, we have developed a fluorescence polarization assay based on fluorescein-labelled rapamycin analogues using FKBP51 and FKBP52 as desired protein targets, with the possibility to be transferred to other FKBPs. These reagents allow a comprehensive biochemical characterization of FKBP homologues that will enable the development of such pharmacological tools.

Results and Discussion

Purification of active FKBP51 and FKBP52

Full-length human FKBP51 and FKBP52 were expressed in *E. coli* as double-tagged constructs with an amino terminal hexahistidinyl tag and a carboxy terminal FLAG-tag. We also expressed the isolated FK1 domains of FKBP51 and FKBP52 since this domain contains the FK506 binding site and has a major regulatory influence on steroid hormone receptors. In addition, FKBP12, -12.6, -13 and -25 were expressed as controls with an amino terminal His-tag. All proteins were purified by Ni affinity chromatography and were sufficiently pure for further biochemical experiments. Full-length FKBP51 still showed substantial impurities after Ni affinity chromatography; this indicates its susceptibility to degradation, and it therefore required an additional round of purification by FLAG-affinity chromatography.

To demonstrate the correct folding of purified FKBP51 and -52, their enzymatic activity as peptidyl–prolyl isomerases was measured by a coupled enzymatic assay.[22,26,27] Both proteins were able to increase the velocity of the *cis–trans* isomerization of a X-Pro peptide compared to the uncatalyzed background reaction (Figure 1). Addition of rapamycin—an inhibitor of the peptidyl–prolyl isomerase (PPIase) activity of these proteins—completely blocked their enzymatic action. Likewise, the isolated FK1 domains of FKBP51 and -52 showed PPIase activity (data not shown). The enzymatic activity and the ability to bind the specific inhibitor rapamycin demonstrate the proper folding of the purified proteins.

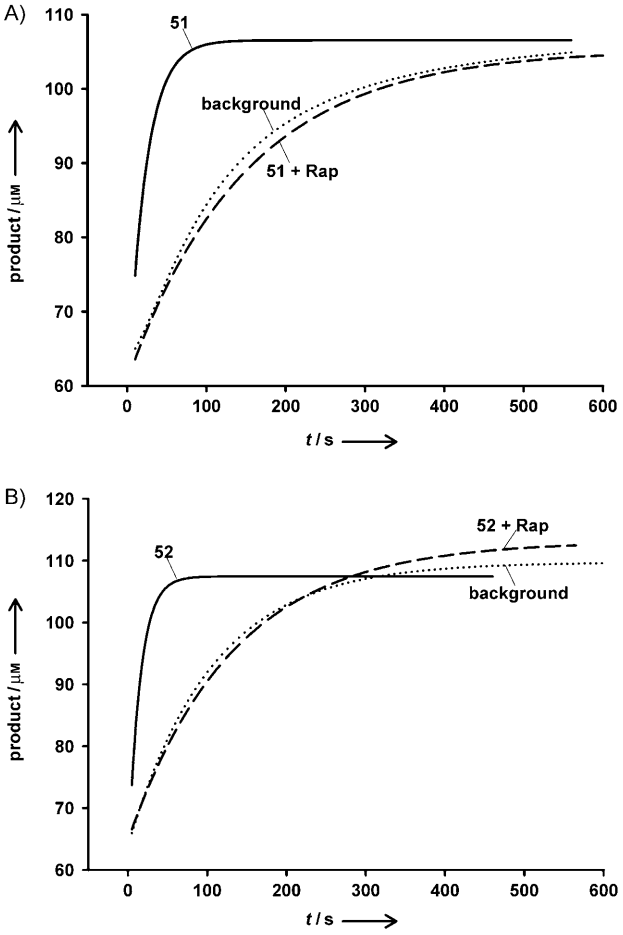


Figure 1. Purified full length FKBP51 and FKBP52 were used to measure PPIase activity by a coupled enzymatic assay. A) 300 nM FKBP51 (——) increases the velocity of *cis–trans* isomerization in a Suc-ALPF-*p*NA peptide significantly compared to the background (•••••). Addition of an excess of rapamycin (Rap; -----: 500 nM) blocked the FKBP catalysed peptidyl–prolyl isomerization. The release of *p*NA upon isomerization was followed at 390 nm; B) 300 nM FKBP52 (——) has peptidyl–prolyl isomerase activity compared to background (•••••) and can be inhibited by 500 nM rapamycin (-----).

Synthesis of tight-binding fluorescein-labelled rapamycin analogues

The PPIase assay used to measure the enzymatic activity of the FKBPs has some major technical limitations. Due to the high background rate of the uncatalyzed isomerization, the time window of the reaction is very short (< 120 s) even if performed at low temperatures (< 10 °C). Furthermore, water-free reagents are required to enrich the initial percentage of substrate in the *cis*-prolyl conformation. This makes this assay incompatible for high-throughput applications. Alternatively, the specific binding of a labelled tracer could be detected by fluorescence polarization.

To establish an assay applicable for the identification of new small molecule inhibitors, we chose rapamycin (**1a**, Scheme 1) as a starting point for the development of fluorescent tracers for FKBP51 and -52. Crystal structures of FKBP12 cocrystallised with rapamycin suggest that the C40-hydroxyl group of rapamycin acts as an attachment point that should not interfere with binding to the FKBP pocket.[28] Furthermore, this position has been used successfully for immobilization of rapamycin in an FKBP-binding competent form.[14]

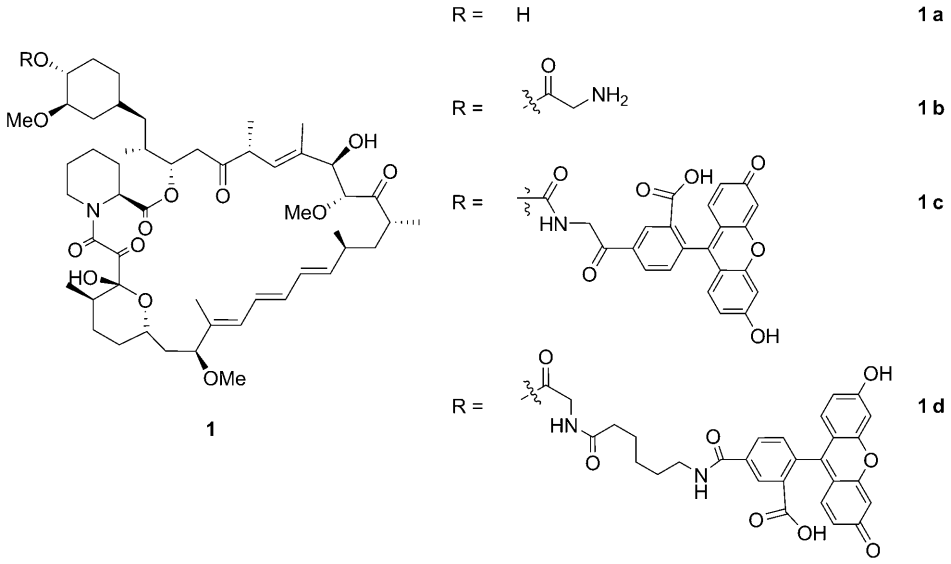
Direct fluorescein labelling of rapamycin at C40-OH has been shown to reduce binding to FKBP12 substantially ($K_d \geq$ 10 μM).[29] To alleviate possible steric hindrance, we introduced linkers of different length. First, rapamycin was coupled to fluorescein via a glycine linker (Scheme 1). When tested in a fluorescence polarization assay, tight binding of compound **1c** could be demonstrated for FKBP12 (K_d ~1.7 nM). Binding in the low nanomolar range could also be observed for FKBP52 (K_d ~3.5 nM) albeit with a reduced overall change in fluorescence anisotropy. However, only weak, unsaturatable binding and small anisotropy changes could be achieved for FKBP51 (data not shown).

We therefore introduced an additional hexanoic acid spacer between rapamycin and the fluorophore. Fortunately, compound **1d** tightly bound the isolated FK1 domain of FKBP51 at subnanomolar concentrations (K_d: (0.11 ± 0.03) nM) and showed a substantially improved fluorescence anisotropy (Figure 2A). Tight binding was also observed for FKBP12, -12.6, -13, -25 and for full-length FKBP51 and -52 (Figure 2C and Table 1).

Compound **1d** was used for a competition experiment with the natural product, rapamycin. Due to the high affinity of **1d** (fluorescence intensity at the K_d was below the detection limit of the reader) a large excess of competing rapamycin (or FK506; data not shown) was necessary to displace **1d** (Figure 2B). Nonlinear regression for a three-component system was applied to account for the saturating conditions (see the Supporting Information, appendix 3).[30] This yielded a K_i of (3.15 ± 0.27) nM of rapamycin for FKBP51FK1, which is about one order of magnitude tighter than previously reported with PPIase assays.[31,32]

As proof-of-concept, we determined K_i values for rapamycin and FK506 with a set of FKBPs by fluorescence polarization competition assays with **1d** (Table 1). Rapamycin and FK506 display subnanomolar affinity to FKBP12, as described previously.[33] Also, the K_i values for FKBP12.6 were in good agreement with published data.[23,32,34] For FKBP13 we found a K_i value for rapamycin in the low nanomolar range (7.2 ± 0.7) nM, which is similar to that published previously, (1.5 ± 0.3) nM.[23,32] For FKBP25 an IC_{50} value of 0.9 nM was reported,[23,32,35] which was obtained with PPIase assays, while we measured a K_i of (5.9 ± 1.8) nM.

The high affinity of rapamycin for FKBP51FK1 (i.e., the purified FK1 domain of FKBP51) described above, could be confirmed with full-length FKBP51, (3.7 ± 0.9) nM, but for FK506 we observed a five- to seven-times lower affinity (104 ± 14) nM compared to published data (14.6 ± 1.8) nM.[23] The published binding constants for rapamycin and FK506 to FKBP52 demonstrate a significant variability (FKBP52/Rap: 1.4–45 nM, FKBP52/FK506: 0.72–269 nM).[14,21,36] In our hands, the K_i values of rapamycin and FK506 for FKBP52 are in the published range. Altogether the observed K_i values were generally comparable to published data, which can vary significantly between different publications, depending on the methodological approach.



Scheme 1. Fluorescein labelling of rapamycin.

Table 1. K_d values determined for different FK506-binding proteins with compounds **1d** and **2b**, K_i values for rapamycin, FK506 and **2a**.

FKBP	Compound **1d** K_d [nM]	Compound **2b** K_d [nM]	Rapamycin K_i [nM]	FK506 K_i [nM]	Compound **2a** K_i [nM]
-12	0.42 ± 0.27[a]	5 ± 0.4[a]	0.6 ± 0.17	0.2 ± 0.01	12 ± 3
-12.6	0.78 ± 0.43	4 ± 0.5[e]	0.4 ± 0.06	4.4 ± 1	24 ± 3
-13	1.7 ± 0.3	90 ± 15	7.2 ± 0.7	166 ± 30	318 ± 101
-25	21 ± 2	n.b.	5.9 ± 1.8	n.d.	n.d.
-51	1.9 ± 0.5[b]	449 ± 69	3.7 ± 0.9	104 ± 14	3100 ± 1100[f]
-52	1.4 ± 0.10[b]	281 ± 30	4.2 ± 0.7	23 ± 3	2600 ± 900[f]
-51K1	0.11 ± 0.03[c,d]	177 ± 15[f]	3.2 ± 0.3[d]	79 ± 12	2650 ± 155[f]
-52K1	1.6 ± 0.6[a]	469 ± 86[f]	18 ± 4	28 ± 5	2050 ± 250[f]

The K_d values of compounds **1d** and **2b** were measured by fluorescence polarization binding assays. Typically 5 nM compound **1d** or 10 nM of **2b** were used. The K_i values of rapamycin and FK506 were determined by fluorescence polarization competition assays by using 500 pM compound **1d**. The K_i values for compound **2a** were determined by fluorescence polarization competition assays by using 500 pM of **2b**. The measurements were performed with a GENios Pro plate reader (Tecan) in duplicate. Variations are indicated; [a] 3 nM tracer; [b] 4 nM tracer; [c] 100 pM tracer; [d] cuvette-based format with spectrofluorometer (F-7000 FL; Hitachi); [e] 5 nM tracer; [f] 20 nM tracer; n.b.: no binding; n.d.: not determined.

Active-site titration of FKBPs by fluorescence polarization

The high affinity ligand **1d** was further used for rapid active-site titrations of the purified FKBPs. Fluorescence polarisation (FP) binding curves were generated by titrating the FKBP proteins in the presence of increasing amounts of labelled ligand **1d** (Figure 3A). For all experiments we confirmed that the intrinsic fluorescence of ligand **1d** was linear within the used concentration range and did not change upon binding to the proteins. For each binding curve the EC_{50} values were extracted and plotted against the corresponding ligand concentration (Figure 3A, insert). As expected, a linear rela-

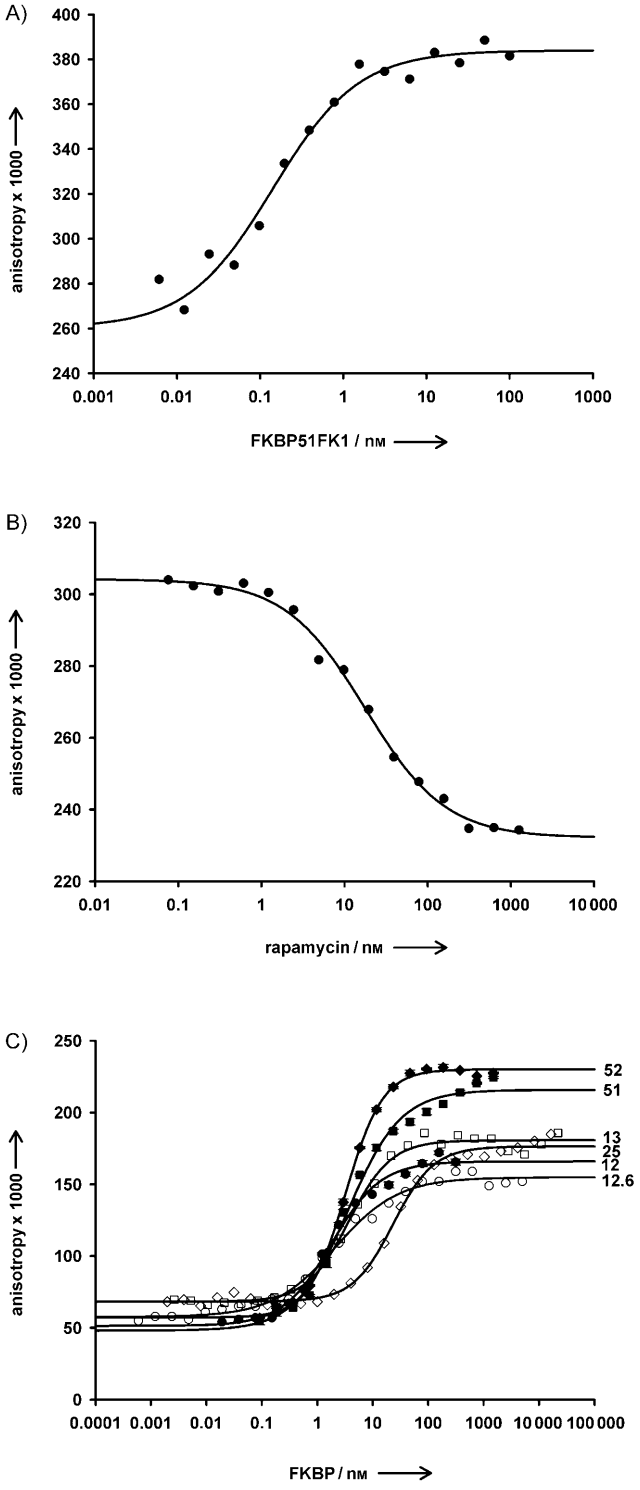


Figure 2. Compound **1 d** bound FKBPs: A) 100 pM compound **1 d** and the purified FK1 domain of FKBP51 (FKBP51FK1) were used to generate a binding curve. The measurement was performed in a cuvette-based spectrofluorometer as a single measurement (Hitachi; F7000 FL). B) Rapamycin could compete with 500 pM compound **1 d** for binding to 500 pM FKBP51FK1. The measurement was performed as in Figure 2 A. C) The binding of compound **1 d** was obtained for different FKBPs: -12 (●), -12.6 (○), -13 (□), -25 (◇), -51 (■), -52 (◆). The binding curves were generated with 3 to 5 nM compound **1 d** in duplicate, except FKBP12.6, -13 and -25, which were single experiments. The measurement was performed by using a GENios Pro plate reader (Tecan).

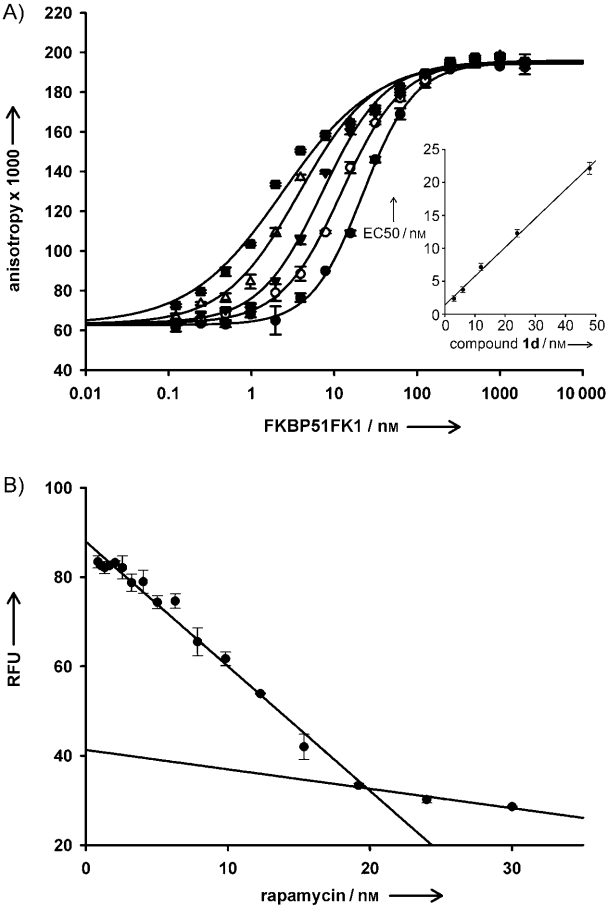


Figure 3. Active-site titration of FK506 binding proteins. A) The active-site titration for the purified FK1 domain of FKBP51 (FKBP51FK1) was performed by generation of binding curves at different concentrations of tracer **1 d** (3 nM: ■, 6 nM: △, 12 nM: ▼, 24 nM: ○, 48 nM: ●) measured by fluorescence polarization by using FKBP concentrations estimated with UV measurements. The measured EC_{50} values show a linear dependence to the tracer concentration with a slope of 0.44 (insert), which allows the active-site quantification. B) The active-site concentration was verified by tryptophan quenching. The fluorescence of Trp90 of 13 μM of FKBP51FK1 (determined by UV measurements) can be quenched by increasing amounts of rapamycin.

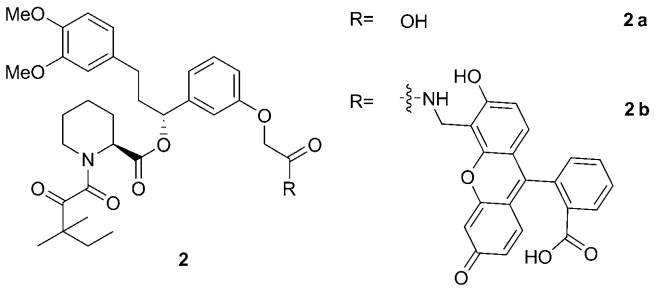
tionship was obtained, and the slope was indicative of the active concentration of the FKBPs relative to the total protein content determined by UV absorption (see the Supporting Information, appendix 1). For all FKBPs, values between 0.3–0.5 were obtained; this indicates a slight underestimation of the protein concentrations by UV determination.

To confirm the results obtained by fluorescence polarization we independently performed tryptophan quenching experiments for the isolated FK1 domains. The FK506-binding sites of FKBP51 and -52 both contain a highly conserved solvent-exposed Trp residue at their core, which is buried upon binding of a hydrophobic ligand.[19,37] Quenching of the fluorescence of this residue (Trp90 for FKBP51 and FKBP52) can be used for active-site titration of FKBPs.[23,38] An exemplary tryptophan fluorescence quenching experiment is shown for FKBP51FK1 titrated with increasing concentrations of rapamycin (Figure 3 B). The tryptophan quenching experiments are in excellent agreement with the results obtained by fluorescence polarization

and validate the latter method as a rapid procedure for the quantification of active protein content.

Fluorescence polarization competition assays with synthetic FKBP ligands

The very high affinity of ligand **1d** precluded fluorescence polarization measurement under subsaturating conditions. For the detection of weakly binding inhibitors, for example, in high-throughput screening assays, a less potent FKBP ligand would be desirable. We, therefore, synthesized the prototypic ligand **2a**, which has been described for FKBP12 and FKBP52[39,40] (Scheme 2). When tested in a PPIase assay, this



Scheme 2. Fluorescein labelling of a FK506 analogue.

compound inhibited the isomerase activity of FKBP51 and -52 with an IC_{50} of 1.2 and 0.9 μM, respectively (data not shown).

Next, we coupled compound **2a** to a fluorescein derivative and tested the fluorescent analogue **2b** for binding to full-length FKBP51 and FKBP52 by fluorescence polarization. Binding constants of (448.7 ± 68.3) nM (FKBP51) and (280.7 ± 30.5) nM (FKBP52) were observed; these indicate a slight improvement of affinity by the introduction of fluorescein. Similar affinities were observed for the isolated FK1 domains and demonstrate that these protein constructs retain full activity (Figure 4B). Low nanomolar binding affinities were also observed for FKBP12, -12.6 and -13 (Table 1); this indicates that tracer **2b** is suitable for various members of the FKBP family.

The binding capacity of the fluorescein-labelled analogue **2b** and its enhanced affinity in comparison with the unlabelled compound **2a**, were verified by PPIase assays by using the FK1 domains of either FKBP51 or -52 (Figure 4A). For FKBP51FK1 the IC_{50} was about two-times higher for the unlabelled compound **2a** (876 nM) relative to **2b** (390 nM). For FKBP52FK1 IC_{50} values of 899 nM (**2a**) and 349 nM (**2b**) were measured (data not shown).

To test the capability of the assay to identify inhibitors of FKBP proteins, the binding of **2b** was competed with the non-fluorescent analogue **2a** (Figure 4C). The K_i values of (2.65 ± 0.16) μM and (2.05 ± 0.25) μM were obtained for the FK1 domains of FKBP51 and FKBP52, respectively. This assay thus allows the rapid determination of the affinity of novel FKBP inhibitors, which is demonstrated by the determination of K_i values of compound **2a** for different FKBPs (Table 1). For full-length FKBP51 and -52 we determined comparable K_i values as for the FK1 domains. For FKBP12, -12.6 and -13 K_i values of (12 ± 3), (24 ± 3) and (318 ± 101) nM were determined.

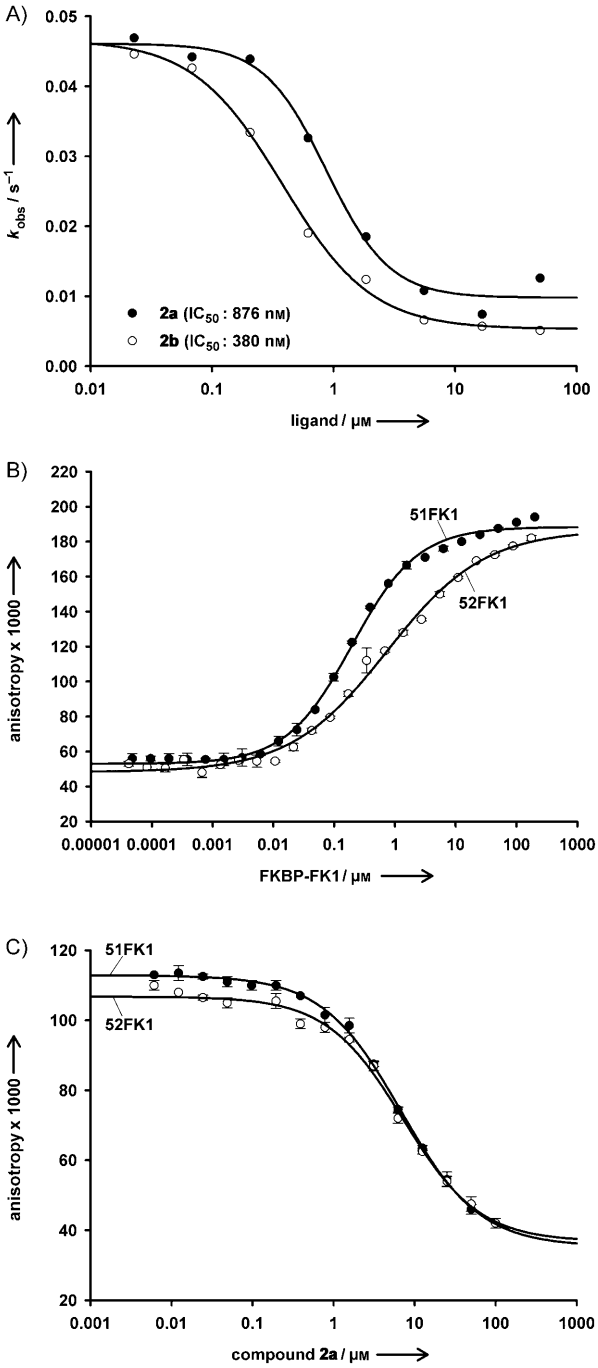


Figure 4. Compound **2b** bound to the FK1 domains of FKBP51 and -52. A) Enhanced binding of **2b** to the FK1 domains was determined by a PPIase assays with 100 nM FKBP51FK1 and titration of **2a** and **2b**. B) The binding of **2b** (20 nM) to the FK1 domains of FKBP51 and FKBP52 was measured by fluorescence polarization. C) The binding of **2b** (20 nM) was competed with compound **2a**. The competition was measured by fluorescence polarization.

Optimization of the fluorescence polarization assay

To improve the robustness of the fluorescence polarization assay according to high-throughput demands, several assay parameters (pH conditions, DMSO, NaCl, incubation time) were investigated. For the final conditions HEPES (20 mM, pH 8), Triton-X100 (0.01%), DMSO (< 1.5%) and **2b** (20 nM) in a total

volume of 60 μL were used; the binding of **2b** to the FK1 domains was optimal and stable for incubation times of up to 24 h.

The assay performance was then tested in a high-throughput screening setting. In a competition assay the FK1 domains of FKBP51 and FKBP52 were treated with rapamycin (5 μM; 1472 wells, 100% inhibition) or DMSO (1472 wells, no inhibition). For both proteins stable signals were obtained (Figure 5) with calculated *Z′* factors of 0.76 for FKBP51FK1 (Figure 5A) and 0.74 for FKBP52FK1 (Figure 5B); this demonstrates the suitability of this assay for high-throughput screening applications.

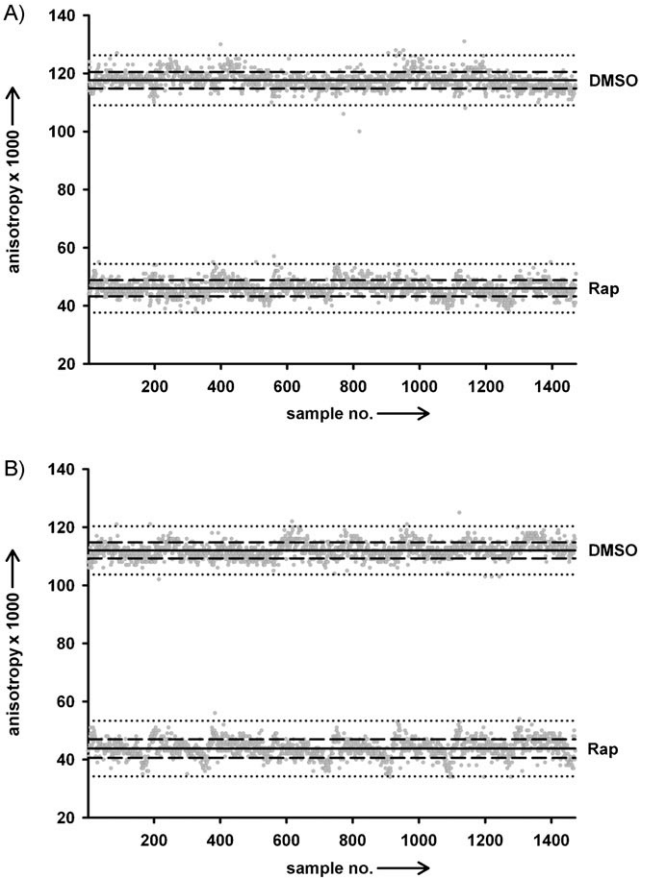


Figure 5. Compound **2b** can be used for high-throughput applications. For competition experiments with compound **2b** the *Z′* factor was determined by using a BiomekFX robot (Beckman–Coulter). Rapamycin (Rap) was used as the inhibition control, and DMSO was used for the no-inhibition control. The figure shows the mean of the controls (——), the single standard deviation (-----) and the 3× standard deviation (•••••). A) *Z′* factor determination for FKBP51FK1; B) *Z′* factor determination for FKBP52FK1.

Conclusions

In humans there are at least 17 known FKBPs that are involved in diverse cellular pathways.[41] In contrast to FKBP12, which has been studied extensively, much less is known about the physiological roles of other FKBP members, in part due to the lack of suitable chemical probes.

In this study we describe fluorescent substrates applicable to higher homologues of the FKBP family. In particular, we have generated a fluorescent tracer that retained the high affinity of rapamycin. This compound can be used for the characterization of high affinity FKBP ligands and for active-site titrations. To our knowledge, the latter represents the first application of a FP assay for the quantification of active protein content—an often tedious prerequisite for subsequent quantitative biochemical experiments. The methodology described here should be applicable to most proteins for which FP tracers are available.

We also present a moderate affinity tracer that allows the screening of small molecule libraries and the characterisation of weakly binding hits. While the primary focus of this study was the characterization of the Hsp90-associated cochaperones FKBP51 and -52, the described tracers should also be applicable to FKBP12, -12.6, -13 and -25, for example, for the generation of specificity profiles.

In the present study we have characterized full-length FKBP51 and -52, as well as the isolated FK1 domains thereof. For all experiments performed, the latter fully replicated the biochemical characteristics of their full-length counterparts. FK1 domains, therefore, are valid surrogates for a structural analysis of ligand complexes of FKBP51 and FKBP52 (unpublished results).

In summary, we have developed tracers and assays that substantially facilitate the biochemical characterisation of the larger FKBPs and their ligand interactions. These will aid the identification of more specific probes for the FKBP family.

Experimental Section

Expression and purification of FKBP constructs: Plasmids harbouring the cDNA of FKBP12 and expression plasmids for full-length FKBP51 and FKBP52 with additional carboxy terminal FLAG-tag were kindly provided by Dr. Theo Rein (Max Planck Institute for Psychiatry, Munich, Germany). Plasmids containing the cDNA sequences of FKBP12.6, -13 and -25 were kindly provided by Dr. Gunter Fischer (Max Planck Institute for Enzymology, Halle, Germany). FKBP12 was amplified with the sense primer: 5′-AAA GAA TTC ATG GGA GTG CAG GTG GAA ACC-3′, and the antisense primer: 5′-CCC GTC GAC TCA TTC CAG TTT TAG AAG CTC C-3′. Cloning into plasmid pProExHta (Invitrogen, Carlsbad, USA) was performed with the restriction enzymes EcoRI (NEB, Ipswich, USA) and SalI (NEB). For the amplification of the coding sequence of FKBP51FK1 the sense primer: 5′-CAT GCC ATG GCA ATG ACT ACT GAT G-3′, and the antisense primer: 5′-GCA GTC GAC TCA CTC TCC TTT GAA ATC AAG GAG C-3′, were used. For FKBP52FK1 the sense primer: 5′-GCG CCA TGG GGA TGA CAG CCG AGG AG-3′, and the antisense primer: 5′-GTC GAC TCA TTC TCC CTT AAA CTC AAA CAA CTC-3′ were utilized. FKBP51FK1 was cloned into pProExHta by using the restriction enzymes NcoI (NEB) and XbaI (NEB), FKBP52FK1 was cloned with NcoI and SalI. FKBP12.6 was amplified by using the sense primer: 5′-CCG GAA TTC ATG GGC GTG GAG ATC GAG-3′, and the antisense primer: 5′-CTC GAG TCA CTC TAA GTT GAG CAG CTC-3′. For FKBP13, the sense primer: 5′-CCG GAA TTC AAA AGG AAG CTG CAG ATC GG-3′, and the antisense primer: 5′-CTC GAG TTA CAG CTC AGT TCG TCG CTC-3′, were used to amplify a truncated fragment of FKBP13 (amino acids 27–142) without a leader peptide. Full-length FKBP25 was amplified by using the sense primer:

5′-CCG GAA TTC ATG GCG GCG GCC GTT CC-3′, and the antisense primer: 5′-CTC GAG TCA ATC AAT ATC CAC TAA TTC-3′. The PCR products of FKBP12.6, -13 and -25 were cloned into pProExHta by using the restriction enzymes EcoRI and XhoI (NEB).

Induction of protein expression in *E. coli* BL21(DE3)pLysS was performed by addition of IPTG (0.6 mM; Eppendorf, Hamburg, Germany). FKBP12, FKBP12.6, FKBP13, FKBP25, FKBP52, FKBP51FK1 and FKBP52FK1 were purified by a single Ni-NTA (Qiagen, Hilden, Germany) affinity column. The standard protocol was modified by using HEPES (50 mM, pH 8), NaCl (20 mM), glycerol (10%) and imidazol (30 mM) as washing buffer. The elution buffer was HEPES (50 mM, pH 8), NaCl (20 mM), glycerol (10%) and imidazol (300 mM). For full-length FKBP51 a tandem purification strategy was employed by using Ni-NTA chromatography as the first step and an additional FLAG affinity chromatography as the second step. The eluate from the Ni-NTA column was passed over FLAG M2 affinity gel (Sigma–Aldrich, St. Louis, USA) and eluted with FLAG-peptide (100 $\mu g\,mL^{-1}$; Sigma–Aldrich). Proteins were analysed by SDS-PAGE and stained with Coomassie brilliant blue (Carl Roth GmbH, Karlsruhe, Germany). The protein yield was quantified by UV quantification or Bradford assay (BioRad, Hercules, USA).

Analysis of the *cis–trans* peptidyl–prolyl isomerase activity: The PPIase activity was determined by using a previously published assay and Suc-ALPF-*p*NA (Sigma–Aldrich) as peptide substrate.[22,26,27] The buffer used contained HEPES (50 mM, pH 8) and NaCl (100 mM). The peptide substrate (4 mM) was dissolved in a solution of LiCl (470 mM) in dry trifluoroethanol and stored under argon.

All solutions and buffers used were precooled to 4 °C. For the determination of the PPIase activity, a 100× concentrated protein solution (10 μL, 30 μM according to Bradford determination) was added to the assay buffer (865 μL). After addition of chymotrypsin (100 μL, 60 $mg\,mL^{-1}$; Carl Roth GmbH, Karlsruhe, Germany) the reaction was started by addition of the peptide substrate (25 μL, 4 mM). The used final concentrations were 300 nM protein, 6 $mg\,mL^{-1}$ chymotrypsin and 100 μM peptide substrate. The increase in absorption was recorded at 390 nm and 4 °C.

To analyze the inhibition of the PPIase activity, 200× concentrated inhibitor in DMSO (5 μL) was added. As negative control the corresponding amount of DMSO was used. After addition of the protein, the samples were incubated in protein low binding cups (Eppendorf, Hamburg, Germany) for 1 h at room temperature to allow the binding equilibrium to be established. After incubation, the samples were transferred to cuvettes and the reaction was started by addition of chymotrypsin and the peptide substrate.

The amount of released *p*-nitroanilide (*p*NA) is directly proportional to the *trans* isomer of the peptide substrate, starting from a *cis–trans* mixture. The measured absorption units were correlated to released *p*NA by the molar extinction coefficient of 13300 $cm^{-1}\,M^{-1}$ at 390 nm. The curves were analyzed by using SigmaPlot9 and fitted with a three parameter fit (single) for an exponential rise to a maximum.

Synthesis of the fluorescent rapamycin derivative 1d: C40-Glycyl-rapamycin **1b** was synthesized as described.[29] Compound **1b** (25 mg, 25.7 μmol) in DMF (300 μL) was added to 6-[fluorescein-5(6)-carboxamido]hexanoic acid-*N*-hydroxysuccinimide ester (15 mg, 25.6 μmol; Fluka, Seelze, Germany). This was followed by addition of triethylammonium bicarbonate buffer (81 μL, 1 M, pH 8.5; Fluka). After being shaken for 30 min at room temperature the reaction mixture was quenched with $NaHCO_3$ and extracted with CH_2Cl_2. The organic phase was concentrated and purified by silica gel flash chromatography by using a solvent mixture of $CHCl_3$/MeOH/hexane 6:1:3→5:2:3→6.2:2 followed by reversed-phase HPLC (Jupiter 4 μ Proteo 90 A, 250×4.6 mm, Phenomenex, Torrance, USA) by using an acetonitrile:water gradient of 55–79% in 36 min (1 $mL\,min^{-1}$, t_R = 24.8 min). The pure product was dried in vacuo to yield 2 mg (1.38 μmol, 5.4%) of a yellow solid. TLC ($CHCl_3$/MeOH/hexane 6:2:2): R_f = 0.55; HRMS: calcd for $C_{80}H_{104}N_3O_{21}$ [$M+H^+$]: 1442.7156; found: 1442.7126; UV/Vis (85% CH_3CN, 5% MeOH, 10% Tris-HCl 5 mM, pH 8.8): ε_{264} = 53565 $cm^{-1}\,M^{-1}$; ε_{278} = 58240 $cm^{-1}\,M^{-1}$; ε_{288} = 44583 $cm^{-1}\,M^{-1}$; ε_{500} = 31065 $cm^{-1}\,M^{-1}$.

Synthesis of the synthetic fluorescent FKBP tracer 2b: Compound **2a** (48 mg, 82.2 μmol)[39] was preactivated for 10 min in a HBTU (150 μL, 0.5 M) solution in DMF (75 μmol) and DIPEA (75 μL). 4′-(Aminomethyl)fluorescein hydrochloride (15 mg, 37.7 μmol; Invitrogen) was added and the reaction mixture was stirred for 3 h. Purification was performed by preparative reversed-phase HPLC (Jupiter Proteo 4 μ 90 A, 250×21.6 mm, Phenomenex) by using a acetonitrile/water TFA (0.1%) gradient of 60–70% in 36 min (25 $mL\,min^{-1}$, t_R = 25–28 min). The pure product was dried in vacuo to yield 17.7 mg (19 μmol, 50%) of a yellow solid.

TLC ($CHCl_3$:MeOH 19:1): R_f = 0.38; HRMS: calcd for $C_{53}H_{55}N_2O_{13}$ [$M+H^+$]: 927.3699; found: 927.3707; UV/Vis (80% MeOH, 20% Tris-HCl, 5 mM, pH 8.8): ε_{280} = 12300 $cm^{-1}\,M^{-1}$; ε_{498} = 50600 $cm^{-1}\,M^{-1}$.

^{1}H NMR (400 MHz, CD_3OD, major rotamer): δ = 7.98 (d, J = 7.6 Hz, 1H), 7.72 (td, J = 1.2, 7.4 Hz, 1H), 7.67 (ddd, J = 1.1, 4.3, 7.4 Hz, 1H), 7.20–7.10 (m, 2H), 6.94–6.82 (m, 3H), 6.81–6.76 (m, 2H), 6.75–6.69 (m, J = 4.3 Hz, 1H), 6.62 (ddd, J = 2.0, 3.7, 8.1 Hz, 1H), 6.58–6.48 (m, 4H), 5.64 (dd, J = 5.1, 8.6 Hz, 1H), 5.14 (d, J = 4.8 Hz, 1H), 4.77 (s, 2H), 4.56 (d, J = 2.8 Hz, 2H), 3.75 (s, J = 1.3 Hz, 3H), 3.75 (s, J = 0.7 Hz, 3H), 3.37–3.33 (m, J = 5.0 Hz, 1H), 3.18–3.07 (m, J = 3.3, 8.9, 9.4 Hz, 1H), 2.58–2.43 (m, J = 8.4, 14.8 Hz, 2H), 2.26 (d, J = 14.0 Hz, 1H), 2.21–2.08 (m, 1H), 2.01–1.89 (m, 1H), 1.74–1.53 (m, 5H), 1.49–1.36 (m, 1H), 1.34–1.23 (m, 2H), 1.16 (d, J = 1.7 Hz, 3H), 1.15 (d, J = 1.4 Hz, 3H), 0.85 (td, J = 1.1, 7.5 Hz, 3H).

^{13}C NMR (101 MHz, CD_3OD): δ = 207.64, 207.55, 169.92, 169.72, 169.69, 169.58, 169.52, 167.67, 167.65, 167.00, 157.81, 152.60, 150.89, 148.90, 147.31, 141.80, 141.62, 135.01, 133.79, 133.77, 133.76, 129.69, 129.46, 129.44, 128.64, 128.00, 126.86, 126.81, 124.61, 124.11, 120.31, 119.78, 119.65, 114.08, 114.05, 112.90, 112.75, 112.72, 112.52, 112.15, 111.78, 111.09, 111.07, 110.51, 109.99, 102.47, 76.75, 76.52, 66.88, 56.83, 55.12, 55.03, 51.37, 48.42, 48.28, 48.21, 48.07, 48.00, 47.86, 47.79, 47.64, 47.57, 47.43, 47.36, 47.15, 46.93, 46.63, 46.26, 46.19, 44.24, 38.70, 37.52, 37.40, 32.17, 32.11, 31.67, 30.74, 25.85, 24.40, 22.58, 22.40, 22.35, 22.19, 22.17, 20.60, 7.70, 7.66.

Fluorescence polarization assay for the binding of labelled ligands to FKBPs: For fluorescence polarization assays the fluorescent ligands **1d** or **2b** were dissolved in HEPES (20 mM, pH 8), Triton-X100 (0.01%), at double the concentration required for the final sample.[42] The target protein was also diluted in this assay buffer at double the highest concentration required for the final sample. This protein stock was used for a 1:1 serial dilution. Each protein dilution (30 μL) was mixed with the dilution of the fluorescent ligand (30 μL) and transferred to a black 384-well assay plate (No.: 3575; Corning Life Sciences B.V., Schiphol-Rijk, Netherlands). After incubation at room temperature for 30 min the fluorescence anisotropy was measured (GENios Pro, Tecan, Männedorf, Switzerland) by using an excitation filters of 485/20 nm and emission filters of 535/25 nm. For FKBP12, -51, -52, -51FK1 and -52FK1 the binding assays were performed in duplicates in the plate format.

The controls with FKBP12.6, -13 and -25 and measurements in the cuvette format were performed as single measurements.

To achieve increased sensitivity, the binding of compound **1d** (final concentration 100 pM) to FKBP51FK1 was measured in a cuvette format. For this, compound **1d** (500 μL, 200 pM) in assay buffer and a FKBP51FK1 1:1 dilution series (500 μL, starting with 200 nM) were mixed and transferred to a quartz cuvette after 30 min incubation at room temperature. The measurement was performed by using a spectrofluorometer (F-7000 FL; Hitachi, Krefeld, Germany) with excitation at 485/10 nm and emission at 520/10 nm.

For the active-site titrations, five binding curves were generated by using ligand **1d** (final concentrations: 3, 6, 12, 24 and 48 nM). The EC_{50} values (based on protein concentrations determined by UV absorption) and the corresponding concentrations of **1d** were fitted for the equation provided in the Supporting Information (appendix 1).

The binding curves were analysed by using SigmaPlot9. Data were fitted to a four parameter logistic curve to deduce EC_{50} values. For the analysis of K_d values, data were fitted to the equation provided in the Supporting Information (appendix 2).

Fluorescence polarization assay for the competition of labelled ligands with inhibitors for binding to FKBP target proteins: The fluorescent ligand **2b** was diluted in assay buffer to a concentration of 40 nM (double the final concentration of 20 nM). The competitive ligand was dissolved in DMSO to reach a 100-times concentrated stock solution. This was used for a 1:1 serial dilution in DMSO. Every sample of this serial dilution was diluted by a factor of 50 in assay buffer supplemented with ligand **2b** (40 nM) to achieve a 2× concentrated mixture of ligand **2b** and the corresponding inhibitor. To each of these competitive ligand mixtures (30 μL), double the protein concentration (30 μL, 400 nM FKBP51FK1, 1600 nM FKBP52FK1), diluted in assay buffer was added. The samples were transferred to black 384-well assay plates (No.: 3575; Corning Life Sciences) and treated as described above.

The competition curves were analyzed by using SigmaPlot9. Data were fitted to a four parameter logistic curve to deduce the IC_{50} values. For the analysis of K_i values, data were fitted to the equation provided in the Supporting Information (appendix 3).

Active-site titration by tryptophan quenching: To determine the active-site concentration by tryptophan quenching, FKBP51FK1 (final 13 μM, determined by UV absorption) and FKBP52FK1 (final 6 μM, determined by UV absorption) were titrated with increasing concentrations of rapamycin (Cfm Oskar Tropitzsch e.K., Marktredwitz, Germany).[23,38] The protein targets and inhibitors were diluted in Tris (50 mM, pH 7), NaCl (50 mM) at double the concentration required for the final sample and mixed in a 1:1 ratio. After incubation for 30 min the quenching of tryptophan was measured at 25 °C (Luminescence Spectrometer, LS50B, Perkin–Elmer, Wellesley, USA). Excitation was achieved at (280 ± 5) nm, emission was measured at (308 ± 5) nm and (335 ± 5) nm for FKBP51FK1 and FKBP52FK1, respectively (respective values correspond to the measured absorption and emission maxima). Experiments were performed in duplicate and analysis of the data was performed by using SigmaPlot9.[43]

Determination of the *Z′* factor: FKBP51FK1 (48 μL, 250 nM) or FKBP52FK1 (48 μL, 1 μM) in HEPES (20 mM, pH 8), Triton-X100 (0.01%) was dispensed in eight 384-well black assay plates (No: 3575, Corning Life Sciences B.V.) by using a Multidrop (Combi, Thermo Fisher Scientific, Waltham, USA).

The fluorescent ligand **2b** was diluted to a concentration of 100 nM in assay buffer. One 384-well plate (No.: AB1056, Abgene, Epsom, UK) was prefilled with DMSO (1 μL, 100%) as no inhibition control (rows A–H, columns 1–23) and rapamycin (1 μL, 625 μM) in DMSO as 100% inhibition control (rows I–P, columns 1–23). The ligand solution (24 μL, **2b** in assay buffer) was added to this 384-well plate by using an automated laboratory device (Biomek FX^P, Beckman Coulter, Fullerton, USA) to achieve a 1:25 dilution (4% DMSO and 25 μM rapamycin, 96 nM ligand **2b**). An aliquot (12 μL) from this plate was transferred to the 48 μL of protein solution by the robot to reach the final concentrations of 200 nM FKBP51FK1 or 800 nM FKBP52FK1, 19.2 nM ligand **2b**, 5 μM rapamycin and/or 0.8% DMSO. For each protein 1472 wells of 100% inhibition control and 1472 wells of no inhibition control were used to calculate the *Z′* factor.

The *Z′* factor was calculated according to Equation (1):[44]

$$Z' = 1 - \left\{ \frac{[3 \times (\sigma_P + \sigma_N)]}{\Delta\mu} \right\} \quad (1)$$

σ_P: standard deviation of the positive control (rapamycin)
σ_N: standard deviation of the negative control (−rapamycin)
$\Delta\mu$: delta of the mean values of the positive and negative controls.

Acknowledgements

We thank Irene Friedle and Bastiaan Hoogeland for excellent technical assistance. We are indebted to Claudia Dubler (LMU, Munich, Germany) and Elisabeth Weyher (MPI of Biochemistry, Martinsried, Germany) for NMR spectroscopy and MS measurements. We thank Mr. Uwe Binninger (Uwe Binninger Analytik), who provided the Spectrofluorometer. We thank Professor Florian Holsboer for generous financial support.

Keywords: active-site titration · assay development · FKBP · fluorescent probes · high-throughput screening

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Received: December 3, 2008
Published online on May 5, 2009

