Design and Synthesis of Ligands for the FK506-Binding Proteins and the Serotonin Transporter

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Erklärung


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Abstract

The aim of this cumulative thesis was to develop new chemical tools to investigate proteins involved in depression. The thesis has been divided in two parts with the first major part aimed at generating the first synthetic ligands for FKBP51 and FKBP52. FKBP51 and FKBP52 are co-chaperons of steroid hormone receptor-HSP90 complexes. FKBP51 has been implicated in various mood affective disorders. The second part of my thesis is aimed at the synthesis of chemical tools to study the targets of clinically used antidepressants.

Part 1: The FK506-binding proteins 51 and 52 are co-chaperons that modulate the signal transduction of the glucocorticoid receptor. Single nucleotide polymorphisms in the gene encoding FKBP51 have been associated with a variety of psychiatric disorders. FK506 and rapamycin are two macrocyclic natural products, which unselectively bind to these proteins with nanomolar affinity. A structural alignment of FKBP51 and 52 revealed a structural divergence at the 80s loop which is a major functional determinant for the effect on steroid hormone receptors. Hence the ligand-80s loop interaction is likely to be functionally important and further offers the possibility to discriminate between FKBP homologs. Taking a simplified FK506 analog as a chemical starting point we followed two different approaches to target the 80s loop of FKBP51 and FKBP52.

In the first approach the tert-pentyl group in the synthetic lead compound was replaced with cyclohexyl derivatives that resembled the pyranose group in the natural product FK506. A detailed SAR was established which indicated that FKBP5s are tolerant towards changes in the stereochemistry of the cyclohexyl (pyranose) substituents. In the second approach we envisaged to bio-isostERICally replace the α-ketoamide moiety by a sulfonamide. For a rapid and efficient derivatization of a focused sulfonamide library I established a solid phase strategy which has led to the identification of 2 series of ligands with submicromolar affinity. Co-crystal structures of representative FKBP ligands of both series confirmed the hypothesized binding mode. The best substructures identified in both approaches were subsequently integrated into bi/polycyclic scaffolds with reduced confirmational flexibility. The sulfonamide substructures turned out to be highly active in this context.
Part 2: Tricyclic antidepressants (TCA) have an extremely broad pharmacology that is still not completely understood. To explore the mechanism of antidepressants in more detail we envisioned a photo-labeling approach to better define the TCA-binding proteome.

Towards this goal, I synthesized the imipramine analogs Azidopramine and Azidobupramine which retained the drug-like properties of the parent tricyclic antidepressants and the strong inhibitory activity of the human serotonin transporter. Both compounds were photoreactive while Azidobupramine contained an additional acetylene tag for click chemistry.

Importantly, these probes are amenable to integral transmembrane proteins which comprise all currently known antidepressant targets. These probes could enable the activity based-profiling of known antidepressant targets in endogenous tissues as well as the structural fine-mapping of the binding sites in these proteins.
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1. Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52
1.1 Introduction

1.1.1 FK506-Binding protein (FKBP) family

The immunosuppressive drugs FK506, Rapamycin and Cyclosporin A bind to a highly conserved class of protein family referred to as immunophilins, which exhibit peptidyl prolyl cis/trans isomerase (PPIase) activity\(^1\text{-}^3\). The immunophilin family consists of the FKBP family of proteins which binds to FK506 or Rapamycin and the cyclophilin family which bind cyclosporin A.

Table 1\(^3\text{-}^6\)

<table>
<thead>
<tr>
<th>No.</th>
<th>Protein</th>
<th>Cellular compartment where found</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>FKBP12</td>
<td>Cytosolic</td>
</tr>
<tr>
<td>2</td>
<td>FKBP12.6</td>
<td>Cytosolic</td>
</tr>
<tr>
<td>3</td>
<td>FKBP 13</td>
<td>Endoplasmatic reticulum</td>
</tr>
<tr>
<td>4</td>
<td>FKBP 15</td>
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<td>5</td>
<td>FKBP 22</td>
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<td>FKBP 24</td>
<td>Endoplasmatic reticulum</td>
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<td>7</td>
<td>FKBP 25</td>
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</tr>
<tr>
<td>14</td>
<td>FKBP 133</td>
<td>Nuclear</td>
</tr>
</tbody>
</table>

The mammalian FKBP family can be classified based on their molecular weight or the cellular compartments where they are found. In humans the FKBP family consists of FKBP12, FKBP12.6, FKBP 13, FKBP15, FKBP22, FKBP24, FKBP25, FKBP36, FKBP38, FKBP51, FKBP52, FKBP60, FKBP65 and FKBP133 (numbers indicate their molecular weight)\(^3\text{-}^5\text{,}^7\text{-}^8\) most of which bind to FK506 and exhibit PPIase activity. FKBP38 doesn’t have an intrinsic PPIase activity but the PPIase activity of this domain is stimulated by Ca\(^{2+}/\)CaM\(^9\text{-}^{10}\). Within the
FKBP family FKBP12, FKBP12.6, FKBP38, FKBP51 and FKBP52 are the most studied and explored members. All the available FKBP structures are largely analogous\(^3,4\).

These PPIases are distributed in three major cellular compartments (cytosolic, endoplasmic reticulum, nuclear) and control a number of cellular processes. Human and microbial FKBPs have also been shown to exhibit chaperone activity independent of their PPIase activity e.g. FKBP52, PfFKBP35 etc (Table 1).

### 1.1.2 Domain Structure of FKBPs

Sequence alignment and structural data across the human FKBP family suggest that the amino acid residues which form the PPIase active site and the FK506 binding site remain conserved. The domain to which the prototypical natural products bind is termed as the FK1 binding domain which also has the peptidyl-prolyl \textit{cis–trans} isomerase (PPIase) activity. PPIase (also known as rotamase activity) is a function that catalyzes the conversion of peptidyl-prolyl bonds from \textit{trans-} to \textit{cis-proline} or vice versa, which often is a rate-limiting step in protein folding\(^11\). The smaller FKBPs like FKBP12 and FKBP12.6 contain only one domain. Larger FKBPs can contain a second FKBP-like domain which is often devoid of the PPIase activity and is termed as the FK2 domain. Larger FKBPs like FKBP51 and FKBP52 contain a FK2 domain which has higher similarity to FKBP38. FKBP60 and FKBP65 contain up to four PPIase domains. The next accompanying domain in FKBP51 and FKBP52 is the tetratricopeptide repeat domain or the TPR domain. TPR domains often mediate binding to the Hsp90 machinery.

### 1.1.3 Immunosuppressive FKBP Ligands

The prototypical ligands of the FKBP family are FK506 (Fig. 1) and Rapamycin (2) (Fig. 1). These natural products have immunosuppressive activity and are used in the clinic for the suppression of immune responses after organ transplantation to prevent allograft rejection.

FK506 (Tacrolimus) \(^1\) was first isolated and characterized from \textit{Streptomyces tsukubaensis} which gave the nomenclature to its protein targets, the family of FK506-binding proteins (FKBP). FK506 consist of two domains, first a FKBP binding domain and second an effector domain which mediates the immunosuppressive activity. The FKBP-FK506 complex binds and allosterically inhibits the secondary target calcineurin to which the effector domain binds and thus induces its immunosuppressive effect\(^12\). FKBP12 is the major player that mediates the immunosuppressive action of FK506\(^13,14\).
Figure 1 Structures of the immunosuppressive natural product ligands. Blue: immunophilin-binding domain (FKBP for FK506 and Rapamycin); red: effector domain (calcineurin for FK506, FRB domain of mTOR for Rapamycin); green: binds to both FKBP and CaN/mTOR. The second natural binder of FKBP, Rapamycin (Sirolimus), was isolated from Streptomyces hygroscopicus. The immunosuppressive activity of this compound is exhibited via a different ternary partner, the serine-threonine protein kinase mammalian target of Rapamycin (mTOR). mTOR inhibition has been shown to block various signaling pathways that control protein translation which play a crucial role in cell cycle progression.

A series of immunosuppressive Rapamycin and FK506 analogs are presently used in the clinic or in various phases of clinical trials as these natural products have been shown to be effective in various disorders like breast cancer, melanoma and advanced renal cell carcinoma, metastatic soft-tissue sarcomas etc.

1.1.4 Non-Immunosuppressive FKBP Ligands

1.1.4.1 Neuroimmunophilin Ligands

Apart from their immunosuppressive activity Rapamycin and FK506 were also shown to have additional neuroprotective and neurotrophic effects. Studies have shown that the natural products partially mediate these effects via the calcineurin or mTOR dependent pathway. In any case the immunosuppressive effects of FK506 and Rapamycin limit the chronic use of these agents for neurological indications. Contrasting studies have shown that some neurological effects are partially independent of the calcineurin or mTOR inhibition which stimulated intense efforts across the pharmaceutical industry to identify and develop non-immunosuppressive immunophilin ligands.
The medicinal chemistry campaigns undertaken by the pharmaceutical industry resulted in identification of many FKBP ligands without immunosuppressive activity (Fig. 2 and 3). Several of these compounds were shown to be neuroprotective or neuroregenerative. Apart from these primary cellular effects these compounds were also shown to have effects in animal models of diabetic neuropathy, traumatic brain injury, Parkinson’s disease, cerebral ischemia, as well as in various models of physical neuronal injury.

![Image of FKBP ligands](image-url)

**Figure 2:** Natural product-derived non-immunosuppressive FKBP ligands. Modifications compared to FK506 or Rapamycin are shaded.

The non-immunosuppressive FKBP ligands differed from the immunosuppressive counterparts in two different ways. In the semi- or bio-synthetic analogs the substituents at the effector region were altered which resulted in complete loss of binding to calcineurin/ mTOR (e.g., FK1706, meridamycin, normeridamycin, ILS920, Way-124466, Wye-592, L685-818, shown in the figure).
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

The developed synthetic ligands mimicked the dicarbonyl pipecolyl moiety of the natural products and were devoid of the effector region substituents which bind to calcineurin or mTOR (e.g., V-10,367, JNJ460/GM284, GPl1046, GPl1485 in Fig. 3 or compound 3 shown in Fig. 4).

Clinical trials of some of the above mentioned compounds suggested that FKBP blockade seems to be well tolerated in humans as no toxic side effects have been reported at doses which might result in complete saturation of intracellular FKBP pools (in blood)²².

Nearly all of the biochemical studies reported have been carried out for FKBP12. Most of the reported synthetic FKBP ligands are based on the dicarbonyl pipecolyl/prolyl-scaffold which is derived from the natural products FK506 or Rapamycin (Fig. 1). GPl1046 was one of the first small molecule analogs of FK506 which was designed to preserve the FKBP binding part. It was originally reported to be a potent inhibitor of FKBP12 but these findings have been contradicted by a number of groups¹⁵,³³.

![Figure 3: Synthetic FKBP ligands. The dicarbonyl pipecolyl moiety derived from the central core of FK506 or Rapamycin or equivalent groups are shaded¹⁵.](image)

There are also discrepancies on the effect of GPl1046 regarding its neurotrophic/neuroprotective effects³⁴,³⁵. Other GPl1046 analogs (e.g., compound ³⁶ or JNJ460/GM284³⁷) have also been reported for their biological effects and FKBP12 inhibition. In addition JNJ460/GM284 has also been reported to have submicromolar FKBP52 inhibition³⁷. GPl1046 is believed to be a pro-drug and metabolizes in the body to release GPl1485. GPl1485 has been
shown to be devoid of PPIase activity\textsuperscript{38} and has failed to show efficacy in clinical trials for Parkinson’s disease/erectile dysfunction after nerve injury\textsuperscript{39}.

V-10,367 and Biricodar are the most potent synthetic FKBP12 ligand reported to date\textsuperscript{40,41}. V-10,367 has been tested in a number of cellular and animal models for neuroprotection or neuroregeneration. A very close analog Biricodar (VX-710) was reported to retain very high potency for FKBP12 in a PPIase assay (K\textsubscript{d}=3.7nM)\textsuperscript{41} and inhibit the P-glycoprotein (MDR1) with 0.75µM\textsuperscript{42}. Biricodar has also been investigated in several clinical trials as a chemosensitizing agent\textsuperscript{43}, however, without any beneficial clinical effects.

Apart from GPI1046 and Biricodar, Timcodar is the third FK506 analog to have entered clinical trials. The natural products FK506 and Rapamycin apart from inhibiting FKBPs are also known to be inhibitors of P-glycoprotein 1, a major drug efflux transporter\textsuperscript{44}. Similar to Biricodar, Timcodar (VX-853, \textbf{Fig. 3}) has also been shown to be a potent inhibitor of P-gp \textsuperscript{42}. P-gp inhibition could be a partial contributor to the observed neurological effects of Timcodar and analogs thereof (e.g., V13-661 and V-13670) since these compounds have been shown to lack FKBP binding but retain P-gp inhibition\textsuperscript{15}.

All though these FKBP analogs have reached the clinics their selectivity profile is not yet known. The only FKBP protein for which selective binders have been identified and reported in literature is hFKBP38. The Cycloheximide analog DM-CHX (\textbf{Fig. 4}) has been shown to selective bind FKBP38 (K\textsubscript{d}=85nM) and showed >200-fold selectivity against several other FKBP homologs\textsuperscript{30}. A co-crystal structure of a close analog (Cycloheximide N-ethylethanoate) was solved in complex with an FKBP-like protein from \textit{Burkholderia pseudomallei} revealing a totally novel FKBP-ligand interaction pattern\textsuperscript{45}. In contrast to its precursor Cycloheximide, DM-CHX is devoid of inhibition of protein translation.

\textbf{Figure 4: Synthetic FKBP ligands.} The dicarbonyl pipecolyl moiety derived from the central core of FK506 or Rapamycin or equivalent groups are shaded\textsuperscript{15}. 
1.1.4.2 Rotamase Ligands

Most of the rotamase (PPIase) ligands have been developed for the prototypical FKBP12 ligand binding pocket. The basic pharmacophore that is required for FKBP binding is depicted in Fig. 546.

![Diagram of the minimal binding domain required for compounds to bind to FKBP12](image)

**Figure 5:** The minimal binding domain that is required for compounds to bind to FKBP12.

Many of the novel FKBP ligands that have been described in literature have the above basic pharmacophore conserved in one form or the other. A detailed structure activity relationship (SAR) around each of the minimal binding domain is surveyed below.

a) Pipecolate core.

The pipecolate core that is present in FK506 and Rapamycin sits in the pipecolate binding pocket which is formed by Val55, Phe46 and the Asp37 in FKBP12 and the indole ring of Trp-59 which forms the floor of the FKBP12 binding pocket47,48. The first compounds had the pipecolate core conserved47. In GPI1046 (Fig. 3) the six membered pipecolate core was replaced by a proline. The next generation of compounds were the rigidified analogs by Agouron/Pfizer, where the relatively open binding pocket of FKBP12 had been taken into advantage49. In these compound a substituent at the axial position at C6 of the pipecolate core was introduced and further cyclized with the C1 carbonyl to yield a [3.3.1] aza amide core (6) or a polycyclic scaffold 7 (Fig. 6) These polycyclic analogs were shown to be useful cores for binding to FKBP12.
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

Figure 6: General structures of various pipecolate core analogs identified for FKBP12.

b) Pyranose group.

The pyranose moiety present in the natural products FK506 and Rapamycin have been substituted by many different analogs. Holt et al. made a series of compounds and showed that the oxygen on the pyranose group is not required for activity (Table 2, e.g., compounds 10-12). The pyranose group was further completely substituted by tert-pentyl group to give compounds with better binding affinity. This tert-pentyl group was subsequently adopted in many of the reported FKBP12 compounds.

Table 2 Pyranose group substituents.

Tatlock et al. replaced the pyranose moiety with the (R)-(−)-Carvone moiety followed by a derivatization of the C-15 position which resulted in compound 14 exhibiting remarkable affinity for FKBP12. The reason for its high affinity could be due to a hydrophobic collapse caused by the additional alkyl side chain which causes better FKBP12 binding.

c) Dicarbonyl group.

The electrophilicity of the α-ketoamide moiety present in most of the non-immunosuppressive FK506 analogs is an undesired reactive liability that could result in metabolic instability or potential toxicity. The amide carbonyl was shown to be important for activity as it is involved
in hydrogen bonding with the Tyr82 of FKBP12. Reduction of the ketone to alcohol\textsuperscript{46} or substitution by a di-fluoride \textsuperscript{15}\textsuperscript{54} did not result in a detrimental change in activity. Bioisosteric replacement of the dicarbonyl group with a sulfonamide (16) resulted in compounds having equivalent affinity compared to the ketoamides\textsuperscript{55,56}.

**Figure 7**: Compounds where the diketo moiety has been replaced by other substituents.

d) Cyclohexylethyl substituent (Top group)

The pipecolate C\textsuperscript{1} ester was also replaced by an amide (17) which completely abolished binding to FKBP12\textsuperscript{46,57}. Introduction of substituents around the phenyl group (18-20) resulted in compounds having better binding affinity.
Substitution at the carbinol centre (C10) with phenyl or cyclohexyl groups led to a 10-20 fold increase in activity compared to that of 18-20. The stereochemistry at the new chiral centre is of importance46. R enantiomer is 20-40 fold more active than S enantiomer. Compound 25 with two substituents and phenyl substitution at carbinol centre had the best activity in this series of compound synthesized46.

1.1.4.3 Microbial inhibitors.

Apart from the human FKBP1Ps, FKBP isoforms have also been identified in various parasites and microorganisms which have been suggested as potential anti-infective targets58. The most widely studied microbial FKBP homolog is the Mip (macrophage infectivity potentiator) protein which is present in human pathogens like Legionella pneumophila, the causing agent of Legionnaire’s disease, or Trypanosoma cruzi, the pathogen causing Chagas disease. L. pneumophila Mip and the Mip from T. cruzi were shown to facilitate infectivity and invasion in host tissues in a PPIase dependent and FK506-sensitive manner59,60. Oz et al. further showed that the non-immunosuppressive analog L-685,818 (Fig. 2) was active in an animal model of T. cruzi infection61 since the immunosuppressive activity of FK506 would confound the study of the role microbial FKBP5s on pathogenicity of microorganisms and parasites. The NMR structure of L. pneumophila Mip in complex with Rapamycin was solved by Ceymann et al.62 which gave an insight into the binding mode of these compounds in the microbial FKBP5s. A structure based design approach was carried out by Juli et al. where they designed a series of pipecolate-containing sulfonamides as surrogates of Rapamycin. Exemplary compound 4 (Fig. 4) inhibited the microbial FKBP5 with an IC₅₀ of 6µM compared to 0.2µM for human FKBP12. However, compound 4 was inactive in an assay for macrophage-like cell infection whereas Rapamycin as control was active63.

A NMR structure of N-ethyl-CHX (Fig. 4) with Mip from Burkholderia pseudomallei revealed a highly unexpected dynamic rearrangement of the active site that has never been observed in
any of the FKBP co-crystal structures before\textsuperscript{45}. The N-ethyl-CHX analog inhibited the PPIase activity of \textit{Burkholderia pseudomallei} Mip with a \textit{K}_i=6.5\mu M.

\textbf{1.1.5 Interaction of FKBP51 and FKBP52 with the Hsp90 machinery and its role in GR maturation and gene transcription}

The steroid hormone receptors (SHRs), especially the glucocorticoid receptor (GR), reside in the cytosol and migrate to the nucleus upon activation. Other are nuclear in both forms, i.e., in the presence or absence of the ligand (e.g. progesterone receptor)\textsuperscript{64}. Before the newly synthesized SHRs become receptive to the ligand / hormone they must undergo a heat shock protein (Hsp) assisted maturation process (\textbf{Fig. 9}). The first step includes the binding of Hsp70 and Hsp40 to the GR. The second step includes the binding of SHRs to Hsp90 which occurs in the presence of Hsp70 and Hsp organizing protein (Hop), which binds the chaperones by virtue of two separate tetratricopeptide repeat (TPR) domains\textsuperscript{65}.

A stabilization protein (p23) further stabilizes the Hsp90-SHR complex in its ATP bound form to form the intermediate complex\textsuperscript{66}. Binding of ATP reduces the affinity of Hsp90 for Hop, which results in the dissociation of Hop and Hsp70 followed by the simultaneous recruitment of other TPR proteins, Cyp40, FKBP51, FKBP52 or PP5, to form the oligomeric SHR-Hsp90 mature complex. The mature complex, depending on the co-chaperone it contains, maintains the SHR in a structural conformation that is either highly (FKBP52) or weekly (FKBP51) responsive to hormone binding\textsuperscript{67-69}. This antagonistic effect of FKBP51 and FKBP52 towards the GR has been attributed to differences in the 80s loop of the FK1 domain in these two proteins\textsuperscript{70}. The FKBPs compete for binding to the SHR-HSP90 complex to form the mature complex and, as a result, over-expression of FKBP51 will decrease the receptor regulation by FKBP52\textsuperscript{67}. After hormone binding the hormone-receptor complex translocates to the nucleus where it binds to hormone response elements and regulates gene transcription\textsuperscript{71}.
Figure 9: Schematic representations of the steroid hormone receptor maturation process and hormone binding regulated by FKBP5s.

1.1.6 Biological roles of FKBP51 and FKBP52

1.1.6.1 Stress related disorders

Chronic and acute stress coping behavior in humans is controlled by a stress hormone system, the hypothalamus-pituitary-adrenal (HPA) axis. An imbalance in this system is thought to underlie the risk and course of diseases like post-traumatic stress disorder (PTSD), major depression, anxiety disorder and bipolar depression\(^{72,73}\). The HPA axis is a complex hormone cascade mechanism comprising the hypothalamus which secretes the corticotrophin-releasing hormone (CRH) after external stress stimuli. Upon stimulation by CRH the pituitary gland triggers the synthesis and secretion of the adrenocorticotropic hormone (ACTH) which in turn acts on the adrenal cortex and results in the secretion of glucocorticoid hormones (especially cortisol) which act on various tissues (Fig. 10). A critical feature of the HPA axis is the negative feedback inhibition exerted by cortisol via the GR which keeps the stress reaction in balance. During stress related disease conditions the basal set point of the HPA axis hormones
and the reactivity of the HPA axis is altered which is interpreted as the body’s inability to adequately cope and terminate the stress response, resulting in an increased risk of disease development\textsuperscript{73}. The opposing functions of FKBP51 and FKBP52 on GR\textsuperscript{67,68}, along with human genetic studies have identified FKBP51 as a candidate associated with major depression\textsuperscript{74}. Several studies have shown a correlation between FKBP51 genetic polymorphisms and antidepressant response\textsuperscript{74-77}. FKBP51 polymorphisms have also been linked with suicidal tendency\textsuperscript{78-81}, peri-traumatic dissociation\textsuperscript{82}, psychosocial stress coping\textsuperscript{83}, and PTSD\textsuperscript{84}. FKBP51 polymorphisms have also been shown to modify the effects of early life trauma in PTSD\textsuperscript{84,85} and depression\textsuperscript{86}.

![Figure 10: Schematic representation of role of FKBP51 in the HPA axis and regulation of the HPA axis during external stress.](image)

The role of FKBP51 in stress coping behavior has very recently been firmly shown in several independent animal model studies\textsuperscript{87-90}. These findings strongly supported FKBP51 as novel therapeutic target for psychiatric disorders. Unfortunately neither FK506 nor Rapamycin can be used as tools to further dissect the role of FKBP51 and FKBP52 as they have nearly equipotent affinities for all FKBPs. Hence, selective FKBP inhibitors are required to better understand the underlying biology of these larger FKBPs with respect to psychiatric disorders.
1.1.6.2 Cancer etiology

FKBP51 and FKBP52 have been recently implicated in a variety of cancers\cite{91,92}. Both proteins have been identified to be regulated in a number of cancers. FKBP51 was found to be up-regulated in prostatic hyperplasia and in prostate cancer cells\cite{93}. In addition, the FK506-binding ability of FKBP51 has been identified as a positive regulator of androgen receptor and androgen-dependent cell growth in prostate cancer cells\cite{94,95}. The effect of FKBP51 on GR has been suggested to suppress proliferation in colorectal adenocarcinomas\cite{96}. A recent study has outlined the role of FKBP51 in melanocyte malignancy\cite{97}. Apoptosis induced by irradiation was seen in cells with silenced FKBP51, while control cells showed autophagy. This study showed that inhibition of apoptosis in control cells involved FKBP51-dependent activation of NF-κB upon irradiation\cite{97}. In a pancreatic cell line Pei et al. identified FKBP51 as a scaffolding protein to enhance the dephosphorylation of the cell growth regulator Akt by the phosphatase PHLPP\cite{98}. FKBP51 has been shown to be up- or down-regulated depending on the cell and cancer sub-type and activation or inhibition of FKBP51 can ultimately produce a beneficial effect to treat proliferating cancer cells. Thus, these opposing effects of FKBP51 have to be fine-tuned and taken into consideration before developing a FKBP51-based cancer therapy.

1.1.6.3 Other Biological Implications

Neurite Outgrowth: FKBP52 and FKBP51 have also been found to have antagonistic effects on neurite outgrowth. In N2a cells FKBP52 was shown to increase neurite outgrowth while FKBP51 was shown to shunt neurite outgrowth\cite{99}.

Immune system: FKBP51 has also been discovered to play a role in immune related disease and inflammation. In patients suffering from rheumatoid arthritis FKBP51 is found to be expressed in bone marrow cells\cite{100}. During the treatment therapy of chronic obstructive pulmonary disease in patients FKBP51 expression is seen to be increased in sputum cells\cite{101}.

1.1.7 Structural Differences between FKBP51 and FKBP52 FK1 domain

A three dimensional alignment of several crystal forms of the FK506-binding domain of FKBP51 (305R)\cite{102} and FKBP52 (1P5Q\cite{103}, to be published) revealed that the largest structural divergence between the two proteins are found at the adjacent 40s and the 80s loop (residues 71-76 and 118-122 for FKBP51, respectively) (Fig. 11). In the 40s loop of FKBP51 Asp\textsuperscript{74} and Glu\textsuperscript{75} is replaced by Lys\textsuperscript{74} and Asp\textsuperscript{75} in FKBP52. The Glu\textsuperscript{75} in FKBP51 is about 2Å closer to the PPIase active site than Asp\textsuperscript{75} in FKBP52. The tip of the 80s loop in these proteins also has structural divergences which basically comprises of Leu\textsuperscript{119} (FKBP51) and Pro\textsuperscript{119} (FKBP52).
The Leu\textsuperscript{119}-Pro\textsuperscript{120} peptide bond is always found in \textit{cis} conformation in all known FKBP51 structures while the Pro\textsuperscript{119}-Pro\textsuperscript{120} bond has been suggested to be present either in a \textit{cis} or a \textit{trans} conformations. Cellular studies have shown the residue at position 119 to be a major functional determinant for the diverging effects of FKBP51 and FKBP52 on the steroid hormone receptor\textsuperscript{70}.

\textbf{Figure 11}: Overlay and cartoon representation of the crystal structure of the FK506-binding domains of FKBP51 (305R) shaded in light brown and FKBP52 (unpublished) shaded in light blue. The amino acid residues forming the FK506-binding pocket, the 40s and the 80s loop are shown in stick representation. The structural differences between the two proteins are indicated, the amino acid residue indicated in dark brown corresponds to FKBP51 and amino-acid residue in dark blue corresponds to FKBP52.
1.1.7.1 Comparison of FKBP51 and 52 structures with PPIase inhibitors

The core interactions of FK506 are well conserved in the co-crystal structures of FKBP51 (305R) and FKBP52 (unpublished). The pipecolate core of FK506 sits atop the indole ring of Trp\(^90\), which forms the bottom of the binding pocket. The C\(^1\)-carbonyl has a tight hydrogen bond contact to the backbone amide of Ile\(^87\), which is also seen in the co-crystal structures of FKBP12. The C\(^8\)-carbonyl is involved in a hydrogen bond with Tyr\(^113\). Tyr\(^113\) in FKBP51 approaches the C\(^1\)-carbonyl at an angle of 107° with respect to the carbonyl plane and below van der Waals distance of 3.17 Å which is consistent with an attractive dipolar interaction\(^{102,104}\). In FKBP52 this attractive dipolar interaction is less pronounced with the Tyr\(^113\) approaching the C\(^1\) carbonyl at an angle of 104.5° and larger distances of 3.36Å to 3.48 Å. In both the FK506 co-crystal structures the exocyclic hydroxyl group at C\(^10\) engages in a hydrogen bond with the side chain of Asp\(^68\). The pyranose group of FK506 (1) approaches the 80s loop in both the structures and the C\(^11\) methyl group fills the hydrophobic pocket formed by Ile\(^122\), Tyr\(^113\) and Phe\(^67\) (Fig. 9a, 9c and 9d). The C\(^9\)-keto oxygen in both the structures occupies a cavity which is formed by the \(\varepsilon\)-hydrogens of Tyr\(^57\), Phe\(^67\) and Phe\(^130\).

The crystal structures of FKBP51 and FKBP52 with FK506 (1) and compound 26\(^{105}\) adopt a very similar binding topology (unpublished). In compound 26 (Fig. 12b) most of the above described interactions with FKBP51 and FKBP52 are well conserved as seen in the FK506 structures. The C\(^9\)-keto oxygen in 26 occupies a similar position to the keto group of FK506. Owing to the absence of corresponding hydroxyl group in 26 the hydrogen bond with Asp\(^68\) is no longer observed. The tert-pentyl group sits in the same pocket that is occupied by the pyranose group in the FK506 co-crystal structure. The dimethoxyphenyl ring A sits in the cradle that is created by residues Gly\(^84\) – Ile\(^87\) and Tyr\(^113\). Ring B stacks on the edge of Phe\(^77\). In FKBP51 the carboxyl group engages in electrostatically enhanced hydrogen bonds with Lys\(^109\) and Arg\(^31\) of a neighboring FKBP51 molecule in the crystal.
Figure 12: Natural and synthetic FKBP ligands (a) Structure of FK506 (1), (b) prototypic synthetic ligand of FKBP51 (26) which is devoid of immunosuppressive activity (hydrophobic contacts with FKBP51 are indicated in green, hydrogen bonds formed are dotted in pink and atom numbering of both ligands are shown in blue), (c) binding mode of FK506 (1) in complex with the FK1 domain of FKBP51(305R) \(^{102}\), (d) binding mode of FK506 (1) in complex with the FKBP52 FK1 domain (unpublished). The conserved H-bonds between O\(^1\)-1 and HN-Ile\(^{87}\) (blue), O\(^9\)-1 and HO-Tyr\(^{113}\) (red) and HO\(^{10}\)-1 and O-Asp\(^{68}\) (magenta) dotted in black. Leu\(^{119}\) and Pro\(^{120}\) at the top of the 80s loop in FKBP51 and Pro\(^{119}\) and Pro\(^{120}\) at the top of the 80s loop in FKBP52 are colored in cyan in both the structures.

Based on this observations it was hypothesized that optimization of interaction with the 80s loop of the protein has the highest probability of achieving selectivity and functional relevance within the two large FKBP5s.
1.2 Manucripts and Patents

1. The Chemical Biology of Immunophilin Ligands (Manuscript-1)

2. Evaluation of Synthetic FK506 analogs as Ligands for FKBP51 and FKBP52 (Manuscript-2)

3. Exploration of Pipecolate Sulfonamides as Binders of the FK506-Binding Proteins 51 and 52 (Manuscript-3)

4. Design of Ligand efficiency by conformation control (Manuscript-4)
1.2.1 The Chemical Biology of Immunophilin Ligands (Manuscript-1)

The immunophilin ligands Cyclosporin A, FK506 and Rapamycin are known and used in the clinic for their immunosuppressive properties. Pharmaceutical companies have over the years invested on many medicinal chemistry campaigns to develop drugs based on these immunosuppressive natural products. The immunosuppressive and the non-immunosuppressive analogs have been clinically used or investigated in various types of cancers, coronary angioplasty, dermatology, hepatitis C infections, and neuroprotection. The immunophilins have further been found to play a role in various conditions which has led to increased interest in novel immunophilin ligands. Furthermore, the immunophilin ligands have been used as sophisticated tools in chemical biology for the understanding of various cellular functions and mechanisms. The progress in the above areas in the last five years has been reviewed in the underlying manuscript. Part of this review has been implemented in the introduction section of this thesis.

My main contribution to this review where sections 3.3 FKBP51 and FKBP52, 3.4 Microbial FKBPs and their role in anti parasitic action, 3.5 Non-immunosuppressive cyclosporin analogs, 3.6 Non-peptidic cyclophilin inhibitors.

➢ The Chemical Biology of Immunophilin Ligands.

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The Chemical Biology of Immunophilin Ligands

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Abstract: The immunophilin ligands cyclosporin A, FK506 and rapamycin are best known for their immunosuppressive properties and their clinical use in transplantation medicine. These compounds or their analogs are also clinically used or investigated in various types of cancer, coronary angioplasty, dermatology, hepatitis C infections, and neuroprotection. Furthermore, the role of immunophilins in various pathologies is increasingly being recognized, supporting the preclinical drug development for novel immunophilin targets. Finally, immunophilin ligands are widely used as sophisticated tools in chemical biology. This review shows the progress on three major areas made in the last five years. An update of the immunosuppressive ligands and their clinical applications is discussed in the first part of the review, followed by a discussion about the emerging immunophilin targets and their respective ligands. The final section gives a detailed assessment of immunophilin ligand-based tools.

Keywords: FK506, rapamycin, cyclosporin, immunosuppressant, chemical dimerizers.

1. INTRODUCTION

The characterization of the molecular mechanism of action of the natural products cyclosporin A (CsA), FK506 and rapamycin in the early 1990s constitutes one of the finest examples in the discipline of chemical biology. The elucidation of chemically induced protein-protein dimerization as the underlying principle of the immunosuppressive action of these compounds continues to fascinate and inspire generations of scientists.

CsA, FK506, and rapamycin are truly remarkable molecules. First, they bind with very high affinity to their cognate immunophilin partners, called cyclophilins (CyP) for CsA and FK506-binding proteins (FKBP), for FK506 and rapamycin. The binding to immunophilins usually inhibits their common enzymatic activity, the catalysis of the isomerization around a peptidyl-prolyl bond. In some cases the active sites of immunophilins are also involved in non-catalytic protein-protein interactions that are blocked by immunophilin ligands, possibly giving rise to novel therapeutic options. Second, and most remarkably, the natural product ligands CsA, FK506 and rapamycin impart a gain-of-function on their immunophilin partners thereby endowing them with the ability to form ternary complexes with the phosphatase calcineurin (for CsA and FK506) or with the kinase mTOR (mammalian Target Of Rapamycin). The possibility to rapidly dimerize appropriately tagged proteins within living cells has inspired numerous sophisticated chemical approaches to address biological questions.

Third, the ternary interaction partners calcineurin and mTOR are key nodes in signal transduction pathways that are essential - amongst others - for immune responses. The inhibition of the latter proteins accounts for the potent immunomodulatory activities of CsA, FK506 or rapamycin. This property has transformed the practice of transplantation medicine. Finally, the structural complexity of these natural immunophilin ligands continues to inspire organic chemists to devise total syntheses for these natural products with ever-increasing sophistication.

This review focuses on recent advances in the use of immunophilin ligands in basic as well as translational life sciences. In the first part, we provide a brief overview of the immunosuppressive immunophilin ligands and natural or synthetic analogs thereof. In more detail we discuss the established or emerging clinical applications of rapamycin analogs beyond the immune system and the recent findings on the mechanism of action of rapamycin. The second part covers non-immunosuppressive immunophilin analogs, starting with their historically prominent role in neurobiology. We then extend to immunophilin homologs (beyond the prototypical FKBP12 and Cyp5A) that have been postulated to be involved in these and other novel possible clinical indications. We then provide an update of the recently published non-immunosuppressive immunophilin ligands. For a summary on the wealth of synthetic approaches to immunophilin ligands before 2005 the reader is referred to comprehensive prior reviews [1,2]. Likewise, ligands for parvalbumin, the third major family of prolylpeptidyl isomerasers, have been covered extensively elsewhere [3]. In the last two sections we present the assay systems that are available to characterize immunophilins and recently developed methods where immunophilin ligands (most of FKBP12) play a key role in chemically addressing or controlling proteins in living cells or even higher organisms.

2. IMMUNOSUPPRESSIVE IMMUNOPHILIN LIGANDS

The prototypic ligands of immunophilins are the immunosuppressants cyclosporin A (CsA), FK506 and rapamycin (Fig. 1). All three compounds are used in the clinic for immunosuppression after organ transplantation to prevent allograft rejection.

Cyclosporin A (CsA, the active ingredient of Sandimmun®, Fig. 1) was first isolated in 1976 from Tryptosomy polyporus and was the first macrolide with immunosuppressive properties to be discovered. It is a cyclic undecapeptide that binds to the class of cyclophilins (CyP) [4]. The CyP-CsA complex forms a ternary complex with the phosphatase calcineurin (CN). In this heterocomplex calcineurin is unable to dephosphorylate its substrate nuclear factor of activated T-cells (NF-AT) which is required for T-cell activation and IL-2 expression [5].

FK506 (Tacrolimus, the active ingredient of Prograf®, Fig. 1) was isolated from Streptomyces tsukubaensis and gave the name to its protein targets, the family of FK506-binding proteins (FKBP). FKBP consists of a FKBP-binding domain and an effector domain that conveys the immunosuppressive activity (Fig. 1). Like CyP-CsA, the FKBP-FK506 complex binds to and allosterically inhibits the common secondary target calcineurin and thereby induces the same immunosuppressive effect [5]. The main FKBP that mediates the immunosuppressive action of FK506 is thought to be FKBP12, with minor contributions of FKBP12.6 and FKBP51 [6, 7].

Rapamycin (Sirolimus, the active ingredient of Rapamune®, Fig. 1) was isolated from Streptomyces hygroscopicus in the mid-1970s. It also binds to FKPs but exhibits its immunosuppressive activity via a different mechanism. The FKBP-rapamycin complex differs from FK506 in its ternary partner, the PTEN-BREMEN protein kinase mammalian target of rapamycin (mTOR). The allosteric inhibition of the latter results in the blockade of various downstream signaling pathways that control translation of proteins crucial for cell cycle progression [8-10]. mTOR resides

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endogenously in two multiprotein hetero-complexes, called mTORC1 and mTORC2. The phosphorylation of the mTORC1 substrate S6K has consistently been shown to be highly rapamycin-sensitive. However, the phosphorylation of the second well established mTORC1 substrate 4EBP1 was recently shown by several groups to be partially rapamycin-resistant, probably in a cell-context dependent manner [11, 12]. This could contribute to the rapamycin-resistance that has been observed for several cancer cell lines. On the other hand, the partial inhibition of only mTORC1 might contribute to the effectiveness on the immune system and the rather benign side effect profile of rapamycin as it was shown to be much more effective on normal lymphocytes than active-site mTOR inhibitors [13]. The selective inhibition by rapamycin of only some mTORC1 phosphorylation sites was attributed to the allosteric nature of the inhibition by the FKBP12-rapamycin complex. The differentiated inhibitory activity of rapamycin – compared to mTORC1 knockdown or mTOR active site inhibitors – seems to be a more general phenomenon as it was also shown for certain phosphorylation sites in the recently identified mTORC1 substrate Gln10 [10] and for the mTORC1-controlled autophagy pathway [14].

The second mTORC2 complex, an activating kinase of Akt/FKBPs, is thought to be largely rapamycin-resistant upon acute treatment. However, mTORC2 was shown to be sensitive to long-term rapamycin treatment in susceptible cell lines. This was explained by the inhibition of the assembly of novel mTORC2 complexes from de novo synthesized mTOR protein [15, 16].

The action of rapamycin is usually discussed and investigated in the context of FKBPs as the relevant co-inhibitory partner although there is little experimental evidence for this exclusive role. Recently, rapamycin and the analog WAY-179663 (Fig. 5) were shown to bind and inhibit mTOR directly at high concentrations in an FKBP-independent manner [17]. Importantly, the FKBP-independent inhibition by rapamycin was effective on mTOR downstream targets that were previously thought to be rapamycin-resistant in an FKBP-context.

The molecular details of mTOR signaling and rapamycin action in the immune system have emerged to be complex and are still not fully understood. It is now clear that rapamycin should be rather considered an immunomodulator as it was shown to have various and sometimes even activating effects on several cell types of the adaptive as well as on the innate immune system [18-21]. For example, rapamycin was shown to enhance proinflammatory responses in myeloid dendritic cells and in macrophages and it facilitated the generation of long-lived CD8\(^+\) memory cells [22, 23]. Of particular importance might be the finding that rapamycin can actually facilitate the maturation and expansion of regulatory T cells thereby possibly enhancing allograft tolerance. This could be used for the tailored ex vivo expansion of Treg cells in cell-based immunotherapies [24]. In summary, the functional profile of rapamycin in the immune system is complex and could contribute to the effectiveness and tolerability of this molecule in organ transplantations.

Chronic rapamycin treatment has recently been shown to enhance the life span in mice [25, 26]. Rapamycin had previously been shown to induce longevity in a number of invertebrate animal models. Mechanistically, the life span prolonging activity of rapamycin was interpreted as a pharmacological mimic of cellular starvation, reminiscent of calorie restriction which is a well established protocol to promote longevity. This is consistent with the well-known role of mTOR as a coordinator of cellular energy status and nutrient availability [27]. Importantly, in addition to life span extension rapamycin might also improve the quality of life in the elderly as rapamycin was shown to improve the cognitive and behavioral deficits in several animal models of neurodegeneration like Huntington’s, Parkinson’s and Alzheimer’s disease [28-32]. Rapamycin is known to induce autophagy, via mTOR inhibition and it was shown that this may enhance the clearance of protein aggregates in animal models of Huntington’s and Alzheimer’s disease [28, 29, 31]. In animal models of Parkinson’s disease rapamycin was shown to protect from L-DOPA induced mTOR-mediated side effects or via modulation of 4E-BP1-mediated translation. Intriguingly, the allosteric inhibition of only the mTORC1 complex but not the mTORC2 complex by rapamycin was found to be crucial for the protective effects of rapamycin as the active-site competitive mTOR inhibitor Torin1 contrarily induced neuronal death [30, 32].

As mTOR acts on various downstream targets like S6K and 4E-binding protein that promote cell growth and proliferation rapamycin and its analogues were also developed as anti-cancer drugs. A major limitation of rapamycin is its poor solubility. Nevertheless, there are several ongoing clinical test phases for evaluation of rapamycin as a single agent or in combination therapy mainly in tumors with hyperactive PI3K/Akt signaling, e.g., where the expression of the tumor suppressor PTEN is reduced. Abraxis BioScience developed Nab<sup>TM</sup>-Rapamycin (ABL-309) which is bound to albumin to utilize the tumor’s attraction to albumin which delivers higher concentrations of the drug to where it is needed [33]. A clinical test phase I is still ongoing (NCT00635824).
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

To improve the aqueous solubility of rapamycin, several analogs or produgs were developed that all carry a hydrophilic group at the C-40 position of rapamycin (Fig. 2). Temsirolimus (CCI-779), the active ingredient of Torisel®, is a dihydroxysteroid of rapamycin obtained by lipase-catalyzed esterification of the C-40 hydroxyl group [34]. It displays better aqueous solubility and showed effects in clinical trials of a wide variety of cancer subtypes such as breast cancer, melanoma and advanced renal cell carcinoma. Temsirolimus was approved in 2007 for treatment of advanced kidney cancer by the FDA, Everolimus (RAD001, the active ingredient of Afinitor®, Zortract/Certican®, Probus® and Xemece®) is used for preventing allograft rejection of adult kidney transplant recipients and for patients with advanced kidney cancer after failure of either sorafenib or sunitinib and was approved by the FDA in 2009 and 2010, respectively. Everolimus also shows strong effects in a number of other tumors like neuroendocrine tumors, breast cancer, advanced hepatocellular carcinoma or advanced gastric cancer where numerous clinical test phases in stage II or III are ongoing. Everolimus has become a major economical success reaching sales of 1.9 billion dollar worldwide in 2009. Ridaforolimus (Difefolorimus, AP23573, MK-8669, Fig. 2), the C40-dimethylphosphonate derivative of rapamycin, has succeeded in clinical test phase III in January 2011 for treating patients with metastatic soft-tissue sarcomas.

Besides immunosuppression and oncology, the third major indication for rapamycin analogs is coronary angioplasty [35, 36]. Here, they are used in drug-eluting stents where they suppress the restenosis of vessel stents by proliferating smooth muscle cells that depend on the mTOR pathway. Zotarolimus (ABT-578, Fig. 2), the C40-thiazole [37], was approved in 2008 by the FDA and is used as a coronary smooth muscle cell proliferation inhibitor for drug eluting coronary stents. Biolinus (Umirolimus, Biolinus A9), the C40-thiophenethyl derivative of rapamycin is also clinically used for this indication. Mycophenol (SAR-493) is the 32-desoxyderivative of rapamycin that was reported to have improved metabolic stability [38, 39]. It is clinically investigated for use in drug-eluting coronary stents [http://elixirmedical.com/products/drug-eluting-stent-system/mycophenol].

Ascomycin (FK-520, FR-900520, Immunocyn, L-883590, Fig. 3) was isolated from Streptomyces hygroscopicus and differs from FK506 only at position 21 where the allyl group is exchanged by ethyl. Ascomycin also shows strong immunosuppressant effects [40]. The more lipophilic C33-chlorine analog pimecrolimus (SDZ/ASM 981, Elidel®, Fig. 3) is clinically used as a topical immunosuppressant for treating skin diseases [41].

Sanglifehrin A is a member of a class of macrolides isolated from the actinomycete strain Streptomyces A92-308110 which binds potently to cyclophilins. It also exhibits immunosuppressant activity, although less potently than CsA. The stereo lactam moiety of sanglifehrin A was shown to be dispensable for cyclophilin
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

binding but necessary for immunosuppressive properties [42, 43]. The Cyp-sanglifehrnin A complex is not binding or inhibiting calcineurin nor mTOR indicating a different mechanism of action compared to the before mentioned immunophilin ligands. The relevant target(s) of sanglifehrnin A remain to be established. Recently, sanglifehrnin A was shown to suppress the expression of numerous cytokines as well as the migration of dendritic cells. Importantly, this effect was insensitive to an excess of CsA or a non-immunosuppressive CsA analog, indicating that cyclophilin binding is likely not required for the immunosuppressive effect of sanglifehrnin A [44].

E-ISA247 (voceporin) is a semisynthetic analog of cyclosporin A, where the non-canonical amino acid 4-[(E)-2-butaryl]-2,4-dimethyl-L-threonine (McBmt) in position 1 was replaced by 4-[(E)-2,4-pentenyl]-2,4-dimethyl-L-threonine (E-MePmt). The affinity of E-ISA247 for CypA was found to be comparable to CsA (13-15 nM) while the Z-isomer (Z-ISA247) bound slightly weaker (60 nM). The molecular binding mode of the CypA/E-ISA247 complex was determined to be conserved. The additional terminal methylene group in E-ISA247 was designed to improve the interaction with calcineurin in the ternary complex with CypA. In turn E-ISA247 was found to have higher immunosuppressive activity than CsA. E-ISA247 is being investigated in two phase III trials for psoriasis and in two phase III trials for non-infectious uveitis [45].

Total syntheses have been developed for most of the natural product immunophilin ligands. Their structural complexity, however, still challenges the capabilities of modern organic chemistry and these molecules continue to be synthetic targets for improved synthetic methodologies [46, 47]. More recently, the gene clusters coding for the biosynthesis of several immunophilin-binding natural products have been elucidated [48-51]. This has enabled biotechnological approaches for genetically engineered analogs of these very complex natural products.

3. NON-IMMUNOSUPPRESSIVE IMMUNOPHILIN LIGANDS

3.1. Neuroimmunophilin Ligands

In the early 1990s FK506, rapamycin and CsA were shown to have additional neuroprotective and neurotrophic effects. These effects can partially be explained by the classical (i.e., immunosuppressive) activity, i.e., via calcineurin as it was recently unambiguously shown that the direct inhibition of calcineurin can protect neurons [52]. In contrast, rapamycin was recently found to block mTOR-dependent axon regeneration explaining some of the earlier reported discrepancies for the neuroprotective actions of rapamycin [53]. In any case, the suppression of immune responses that comes along with FK506, CsA or rapamycin would severely limit the chronic use of these agents for neurological indications. Therefore, the finding that the neuroprotective and neurotrophic effects were partially independent of calcineurin or mTOR inhibition in several experimental paradigms stimulated intense efforts across the pharmaceutical industry for the identification of non-immunosuppressive immunophilin ligands [2, 54, 55].

Several non-immunosuppressive FKBP ligands (Fig. 5 and 6) were shown to be neuroprotective [56] or to increase neurite outgrowth in a variety of neuronal cell systems [54, 57-67]. In addition, non-immunosuppressive immunophilin ligands were active in animal models of diabetic neuropathy [68], traumatic brain injury [69], cerebral ischemia [57, 70], Parkinson's disease [58, 59, 71], as well as various types of physical neuronal injury [54, 60-62, 68, 72]. The non-immunosuppressive FKBP ligands are either semi-synthetic analogs of FK506 or rapamycin where the effector domain was abolished (e.g., FK1706, medetamycin, nomivudimycin, IL-8920, Wye-124466, Wye-592, L-685,818), shown in Fig. 5) or small synthetic molecules that mimic the deacetyl piperidyl moiety of the natural products (e.g., V-10,367, JN3460/CM284, GPF1406, GPF1457, compound J shown in Fig. 6). FK1706 is a semi-synthetic non-immunosuppressive derivative of FK506 obtained by Wacker oxidation of the allyl-group at C21 [279]. The pharmacokinetic parameters of FK1706 were recently investigated in a small phase 1 clinical trial [74]. Pharmacokinetic measurements showed that at the doses used the intracellular FKBP pools (in blood) were likely saturated. The fact that no drug-induced discontinuations were reported over the two-week time period indicate that FKBP blockade seems to be tolerated in humans for extended periods of time.

L-685,818 is a semi-synthetic non-immunosuppressive analog of ascomycin obtained by allylic selenium oxidation [75]. The related 13-Me-18-OH FK520 is the selenium oxidation product of a biosynthetic analog of ascomycin obtained from a genetically engineered Z. harringtonii strain [60]. Its affinity to FKBP12 (0.2 nM) and to FKBP52 (80 nM) is identical to FK506. L-685,818 and 13-Me-18-OH FK520 were active in a rat sciatic nerve crush model [54, 60].

WYE-592, ILS-920 and WAY-124,466 (Fig. 5) are semi-synthetic derivatives of rapamycin that were modified at the triene.
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

Fig. (5). Natural product-derived non-immunosuppressive FKBP ligands. Modifications compared to FK506 or rapamycin are shaded.

Meridamycin and 3-normeridamycin (Fig. 5), isolated from Streptomyces strains, and the antascomycins (Fig. 5), isolated from a strain of Microsporum gypseum, are structurally similar to FK506 and rapamycin in the FKBP-binding part but differ in the effector region. Likewise, they potently bind FKBP12 but lack
immunosuppressive effects. Meridamycin and 3-normeridamycin showed activity in neuronal cell-based assays [58, 59].

Most reported synthetic FKBP ligands are based on the dicarbonyl piperidylprolyl-scaffold derived from the natural products FK506 or rapamycin (Fig. 6). V-10,367 is the most potent synthetic FKBP12 ligand reported to date \( K_{i}=0.5 \mu M \) [77]. It has been tested in a number of cellular and animal models for neuroprotection or neurorregeneration. A very close analog of V-10,367, bincorcar (VX-710), was reported to retain very high potency for FKBP12 in a PPlase assay \( K_{i}=3 \mu M \) [78] and to bind the P-glycoprotein \( (MDR1) \) with a 0.75 \( \mu M \) [79]. Bincorcar was investigated in several clinical trials as a chemosensitizing agent where it was generally well tolerated but showed only very weak efficacy in enhancing the performance of chemotherapeutics against various cancers [80]. In these trials steady state serum levels of 10 \( \mu M \) bincorcar where achieved suggesting that a prolonged blockade of FKBP12 is tolerated in humans.

FKBP1046 is a typical small molecule analog of FK506 designed to preserve the FKBP-binding part of FK506. It has been extensively reported to promote a variety of neurotrophic/neuroprotective effects, sometimes with extreme potencies, although there were also contrary reports [81, 82]. Neurotrophic/neuroprotective activities have also been reported for FKBP12 analogs (e.g., compound 1 [83] or JNJ460/13284 [62]). FKBP1046 was originally reported to be a potent FKBP12 inhibitor but this has been challenged by a number of groups [57, 82, 84-86, own unpublished results]. While FKBP1046 is inactive for at least two FKBP homologs (FKBP95 and FKBP25, own unpublished results), JNJ460/13284 was reported as a submicromolar FKBP52 inhibitor that should be metabolically more stable as the labile ester linkage of FKBP1046 was removed [62]. FKBP1046 is likely at least partially a produg, releasing FKBP12 after in vivo ester hydrolysis [55]. FKBP1046 was tested in two phase I clinical trials for Parkinson’s disease and for erectile dysfunction after nerve injury where it failed to show efficacy [88]. FKBP1046 was reported to be inactive in a PPlase assay [278], the profile for other FKBP’s or other targets is unknown. In light of these uncertainties any conclusions regarding the implication of FKBP’s in experiments with FKBP1046 should be interpreted very cautiously.

While most of the early studies focused on the prototypical FKBP12 it is now clear that at least some of the effects of FK506 analogs must be mediated by additional targets. These conclusions
were reached by studying primary neurons from FKBp52-deficient mice [89] as well as by using various FK506 analogs that do not bind to FKBp52, e.g., compounds like timocarol and V-13,661 or V-13,670 that are presumably analogs of timocarol (Fig. 6) [61, 71, 90]. The selectivity profile of these compounds for other FKBp family members is, unfortunately, unknown. Timocarol was reported to be active and equipotent to V-10,367 in two animal models for peripheral nerve diseases (protein-induced large fiber sensory neuropathy and streptozotocin-induced diabetic neuropathy) [90]. Timocarol was tested in two prospective double-blind placebo-controlled proof-of-concept study for enhancement of endogenous nerve regeneration and for regenerative sprouting of epidural nerve fibers where it was shown to be safe but ineffective for the clinical end points [91, 92]. Vertex advanced timocarol in a phase II clinical study for diabetic neuropathy in 1998 but results of this trial have not been disclosed.

In addition to FKBp1, several other FKBp homologs have been put forward as relevant targets of neuroimmunophilin ligands. For FKBp52 the cycloheximide analog DM-CHX (Fig. 7) was developed that potently bound human FKBp55 (K2-BsFinM) and showed >200-fold selectivity against several other FKBp homologs [70]. As such, DM-CHX is the most promiscuous FKBp ligand published to date. Recently, the structure of a close analog (cycloheximide N,N-diethylthiophanilide) was solved in complex with an FKBp-like protein from Borrelidella pseudomuritana revealing a totally novel FKBp-ligand interaction pattern [93]. It will be interesting to see if FKBp52 adopts a similar conformation in complex with DM-CHX. Of note, cycloheximide is best known for its potent inhibition of protein translation and as such of little use to study FKBp biology in cellular systems. It is therefore important that DM-CHX is devoid of the protein synthesis inhibition associated with the parent cycloheximide. DM-CHX was active in an animal model of focal cerebral ischemia.

A number of studies have suggested the large FKBp52 as a possible neuroimmunophilin target candidate [57, 62, 89, 94, 95]. Most studies assigned an anti-neurotrophic function to FKBp52 based on FKBp52 overexpression, FKBp52 knockdown or anti-FKBp52 antibody treatment. However, in N2a cells FKBp52 was found to increase neurite outgrowth while for the closely related FKBp51 the opposite effect was observed in these cells [95]. Mechanistically, a number of hypotheses have been suggested to explain the anti-neurotrophic effects of FKBp52, including the actions on steroid hormone receptors and on calcium channels (discussed further below). The Beattie lab showed that FKBp52 can act to reduce microtubule-associated protein tau [96]. Recombinant FKBp52 also inhibits the tau-induced polymerization of purified tubulin although this was not reproduced in a whole cell lysate system [97]. The effect of FKBp52-ligands was tested and since it was reported earlier that the FK506-binding domain of FKBp52 was dispensable for the microtubule-dissassembling activity [98] it is unclear how to integrate these findings into a mechanism for neuroimmunophilin ligands. The closely related FKBp51 was also shown to bind tau and tubulin in vitro [97, 98]. The Dickey lab showed that FKBp51 can stabilize tau possibly by reducing tau ubiquitylation/lysation. In their system FKBp51 enhanced microtubule formation in a P-kinase-dependent manner although no direct pharmacological inhibition studies were performed. Given the partially diverging findings, the physiological and pharmacological relevance of the role of the large FKBp52 in tau and microtubule dynamics needs to be further defined in animal models and in studies with inhibitors.

While it is clear that FKBp52 ligands can have neuroprotective or neurotrophic activities in appropriate settings the field has been plagued by inconsistencies and conflicting results possibly caused by differences in the neuronal systems, animal models or ligands used. Importantly, the molecular mechanisms underlying the neuroprotective/neurotrophic activities of immunophilin ligands or the relevant targets are still a matter of substantial debate [55], and a number of hypotheses have been put forward. FKBp12 is known to bind the TGFβ type 1 receptor, and FKBp ligands were shown to enhance TGFβR1 signaling [62]. Interestingly, this is one of the examples of a direct protein-FKBp interaction that utilizes the FK506-binding site but does not seem to involve peptide-prolyl isomerase activity [99]. The Ras/Raf/MEK/ERK pathway was shown to be involved in the enhancement of neurite outgrowth by FKBp ligands [67]. This can be explained by the recently described enhancement of Ras-GTPa expression in FKBp12 [100]. The aggregation of α-synuclein was shown to be enhanced by FKBp12 and other FKBp12s in an FK506-sensitive manner [87, 101, 102]. This could contribute to the effects observed in animal models of Parkinson’s disease. To identify the targets of the non-immunosuppressive rapamycin analogs WYE-592 and ILS-920 (Fig. 8) a proteomic analysis of pull-down experiments identified FKBp52 as a preferred interaction partner. Compared to the parent compound rapamycin these two derivates seem to have further acquired the additional property to bind the CACNB1 subunit of L-type voltage-gated calcium channel leading to a diminished Ca²⁺ion conductance [57].

The regulation of Ca²⁺signaling is a recurring but still insufficiently defined theme in FKBp biology. FKBp12 and 12.6 have long been known for their FK506-sensitive association with the ryanodine or IP₃ receptors which release Ca²⁺from the endoplasmic reticulum or from the sarcoplasmic reticulum in muscle cells. FK506 and rapamycin were reported to weakly inhibit the sarco-endoplasmic reticulum Ca²⁺-ATPase (SERCA) which transports Ca²⁺from the cytosol into intracellular Ca²⁺stores [103-105]. However, these results have been challenged [106, 107]. In any case, the conomum of FK506 or rapamycin that were necessary for SERCA inhibition were very high and are difficult to reconcile with the potencies usually observed for neurotrophic effects. The action of GPH146 was also reported to deplete sarco-endoplasmic Ca²⁺stores but the underlying molecular mechanism was not elucidated [107].

FKBP12 and FKBp52 were both shown to interact with numerous transient receptor potential receptor channels (TRP-C) in an FK506-sensitive manner [94, 108-110]. FKBP12 reduced the channel conductivity of TRPC1 and TRPV5 while it enhanced it for TRPC1. The effects on TRP channel conductance were sensitive to P-kinase-inactivating mutations in FKBp52 and proline mutations in the TRP channel. TRPC1 is known to mediate axon guidance and Shum et al. could show that this was modulated by FKBp52 whereas a P-kinase-inactive mutant displayed a dominant-negative phenotype [94].

![Fig. 7](image-url) Constrained polycyclic FKBp52 ligands.

The three natural products CsA, FK506 and rapamycin as well as several of their derivatives are all inhibitors of the P-glycoprotein 1, a major drug efflux transporter [111]. In fact, three non-immunosuppressive derivatives of CsA and FK506 have been developed as specific P-gp 1 inhibitors, e.g., valsepoda (FSC-833,
2.8 Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

Fig. 9, biricodar (XV-710, a close analog of XV-19:367) and timocard (XV-853, Fig. 6). The direct binding of FK506 analogs to P-gp 1 was proven by photorecroslinking experiments using a radioactively labeled, photoreactive analog of biricodar [79]. The fact that timocard and presumably close relatives thereof (e.g., XV-853661 and V-13670) lack FKBP binding but retain P-gp inhibition [90] suggests that P-gp inhibition could contribute to some of the observed antitumor neoplastic effects as has been discussed in the patent literature [2].

3.2 Novel FKBP Ligands

Compared to the activities before 2005, relatively few efforts for discovery of novel synthetic FKBP ligands have been described in the last six years. This is likely due to inconsistencies and uncertainties regarding the relevant target(s) and due to the disappointing results from the clinical trials for GPI1485 and timocard. It should be noted that while effective in animal models for neurological disorders both compounds do not target human FKBP52 and may not target FKBP51 at all.

Most medicinal chemistry efforts have targeted the prototype FKBP12. Pfizer elaborated on earlier work [112] to investigate the polycyclic scaffold 4 as a preferred FKBP binding motif [113]. They made use of the relatively open FKBP12 binding site to introduce a substituent at the axial position at C= of the tricyclic that constitutes the core of FK506 and rapamycin and is found in most FKBP ligands. The C= substituent was further cyclized to a 1,4-diaza-3-anilinocyclopropane core where the C2-carbonyl to yield a very rigid [3.3.1] azo-amide core where the C1-carbonyl is present to facilitate H-bond interaction with the 2 of FKBP12. Hudzik et al. further installed a tetrahydrodiphenylisopinocamphor moiety via azo amine chemistry. Systematic elaboration of the structure activity relationship identified the oxygen at C4 as being essential whereas ketaasides, sulfonamides, sulfaamides, ureas and α-difluoro amides where tolerated as substituents at N3. The best compounds were compounds 46 and 66b with 3.4nM and 5.4nM affinity for FKBP12, respectively. A follow-up methodology paper by the Pfizer group described the optimized library synthesis of sulfamide ligands for FKBP12 but no biological data were presented [114].

Based on computer modeling, Zhao et al. designed and synthesized a series of non-cyclic derivatives of GPI-1046 exemplified by compounds 2 and 6 (Fig. 6) [63]. They proposed that these compounds could adopt an energetically favored binding mode analogous to GPI-1046 when bound to FKBP12. Six out of eleven test compounds were effective in a cellular nutrient outgrowth assay but no binding data for FKBP12 were presented.

FKBP12 has been the seminal example for a structure-based screening approach of small organic fragments using nuclear magnetic resonance spectroscopy [115]. Following this tradition, Rohrig et al. linked two fragments previously known to bind to two adjacent sites on the FKBP12 surface with different linker structures and evaluated them using an NMR-based method previously developed by the authors [116]. The best compound 5 (Fig. 8) had an affinity for FKBP12 of 80nM determined in a fluorescence quenching assay, similar to previously described compounds [115]. NMR was also applied by Stobbs et al. to screen a small library of fragments or natural products for novel FKBP12 binders [117]. Several hits with micromolar affinity were identified and confirmed by isothermal calorimetry. Exploration of hit 6a (Fig. 8) led to compounds 6b with an estimated affinity of 0.2 μM as determined by NMR titration. Compounds 6a and 6b were active in a neurite outgrowth assay. The affinity of fragment 6a was confirmed by an independent group by isothermal calorimetry, surface plasmon resonance and a mass spectrometry-based assay [118].

3.3 FKBP51 and FKBP52

In addition to FKBP12, the two large human FKBP51 and FKBP52 have recently received considerable attention. These large FKBP homologs are co-chaperones that are best known for the regulation of steroid hormone receptors in association with the heat shock protein 90 (Hsp90) [119]. Interestingly, in spite of their high degree of homology FKBP51 and FKBP52 regulate the glucocorticoid receptor in opposing directions, with FKBP51 being an inhibitory and FKBP52 being a stimulatory factor. Knockout mice revealed an essential role of FKBP52 for a correct sex steroid hormone-dependent development [120]. The importance of the inhibitory FKBP51 on organismal steroid homeostasis is supported by primates species that naturally overexpress a hyperinhibitory version of FKBP51 [119]. Intriguingly, FKBP51 and FKBP52 might also share a mutually redundant but essential function as the double knockout of both proteins was reported to be embryonic lethal [121]. The nature of this synthetic lethality remains to be elucidated.

The expression of FKBP51 is consistently induced by stress and by numerous steroids [122-124]. Numerous human genetic studies have indicated a role of FKBP51 in the pathogenesis of stress-related psychiatric disorders [125, 126]. Mechanistically, FKBP51 is thought to reduce the responsiveness of the glucocorticoid receptor to the hormone cortisol that is secreted in response to stressful situations. Knockdown of FKBP51 in the amygdala was shown to reduce an anxiety-like behavior in the elevated plus maze test, but only after a prior stress challenge [122]. Similar results were observed with constitutive FKBP51-knockout mice [127, 128].

In addition to affective disorders FKBP51 has also emerged as a potential target for various types of cancer [129, 130]. FKBP51 has been repeatedly been shown to be upregulated in many cancer cell lines including prostate cancer. Recently, the Paschal and the Sanchez groups independently showed that FKBP51 unexpectedly

Fig. (8). FKBP ligands discovered by NMR screening approaches.
enhanced androgen receptor signaling in various prostate cancer cells [131, 132] possibly leading to a vicious forward feedback loop. Importantly, the stimulating effect of FKBP51 on the androgen receptor was found to be sensitive to FK506. Whether these findings are directly relevant to the situation in vivo or to a clinically more important androgen-independent prostate cancer subtypes remains to be established.

Romano et al. showed that silencing of FKBP51 sensitizes malignant melanoma cells to radiation in cell culture and in a xenograft model [133]. The authors showed that FKBP51 silencing abolished irradiation-induced NF-κB activation which had previously been shown to depend on FKBP51 [134]. The role of FKBP51 in cancers is, however, complex and likely cell-type dependent. In a pancreatic cell line FKBP51 silencing was shown to enhance chemo-resistance. In the search for an underlying mechanism Pii et al. identified FKBP51 as an adapter protein to enhance the dephosphorylation of Akt, a kinase frequently deregulated in cancer, by the phosphatase PP2A [135]. Whether FKBP51 functions as a tumor suppressor remains to be investigated and recent data from the Romano lab indicate that this does not seem to be the general case [277].

The effects of FKBP51 and FKBP52 on steroid hormone receptors were both demonstrated to be independent of peroxisome proliferator-activated receptor activity. However, the effects of both proteins were shown to be sensitive to FKBP ligands like FK506 or rapamycin indicating that the FK506-binding site is important for steroid hormone receptor regulation [130-138] However, the effects of FKBP ligands on steroid hormone receptors in cellular assays are confounded by the P-glycoprotein inhibition typically exerted by these compounds (i.e., active concentration) of steroid hormones is modulated by P-gp [139, 140]. The cellular characterization of FKBP51 and FKBP52 is further complicated by the antagonistic role of these close homologs on steroid hormone signaling stressing the need for subtype-selective inhibitors. Towards this goal a series of high-resolution structures of the FK506-binding domain of FKBP51 was recently published [141]. This showed the expected high structural similarity of the active site that was previously observed within the FKBP51 family. But it is also indicated divergences in the loop regions that might be a starting point for achieving subtype selectivity. So far only one small synthetic ligand, SFL (also known as APU-P7, Fig. 16c), has been described for FKBP51 in addition to FK506 and rapamycin [142].

3.4 Microbial FKBP5 and their Role in Antiparasitic Action

Several FKBP isoforms have been identified in various pathogenic microorganisms and parasites and were suggested as potential anti-infective targets [143]. One of the most widely studied microbial FKBP homolog is the Mip (macrophage infectivity potentiator) protein which is present in human pathogens like Legionella pneumophila, the causative agent of Legionnaire’s disease, or Trypanosoma cruzi, the pathogen causing Chagas disease. L.pneumophila Mip was shown to facilitate intracellular survival and infectivity in an animal model in a Ppase-dependent manner [144]. Likewise, the Mip from T. cruzi enhanced Hela cell invasion by this species in an FK506-sensitive manner [145]. However, immunosuppression by FK506 would be counterproductive for the study or treatment of pathogens and Oz et al. showed that the non-immunosuppressive analog L-685,818 (Fig. 5) but not FK506 itself was active in an animal model of T. cruzi infection [146].

Ceymann et al. solved the NMR structures of L.pneumophila Mip in complex with rapamycin [147]. Building on this structure Jaki et al. rationally designed a series of picolactone-containing subanodines [148]. The best compound 2 (Fig. 6) inhibited L.pneumophila Mip with an IC50 of 0.6μM compared to 0.2μM for human FKBP12. However, compound 2 was inactive in an assay for macrophage-like cell infection whereas rapamycin as control was active.

Recently, an ultra-high resolution structure of Mip from Burkholderia pseudomallei as well as an NMR structure in complex with the DM-CHX analog cycloheximide N-ethylthioanazole was solved. This compound inhibited the PPlase activity with a KIC of 6.5μM. Most importantly, the NMR structure revealed a highly unexpected dynamic rearrangement of the active site that is distinct from all coxyster structures obtained with FK506-derived inhibitors so far [149].

The other intensively characterized microbial immunophilin is FKBP53 from Plasmodium falciparum, the causing agent of malaria [143]. A related PfFKBP35 was found in P. vivax while T.gondii has a dual immunophilin, i.e., a protein having both a FKBP domain and a Cyp domain. FK506 and rapamycin are known to inhibit the growth of the above parasites in culture [143]. Monaghan et al. showed that non-immunosuppressive 18-hydroxy derivatives of avasimibe (e.g., L-685,818, Fig. 5) dose-dependently inhibited the growth of Plasmodium falciparum in culture [150]. In this context it should be kept in mind that while L-685,818 does not inhibit calciumin complex with human FKBP12, it does so in complex with yeast FKBP12 [151]. It is therefore possible that L-685,818 retains the ability to form trienic complexes with PfFKBP35 and PFCaN which in turn could mediate the observed growth inhibition. Kumar et al. used affinity chromatography to show that PfFKBP35 is the predominant immunophilin in P. falciparum that binds to ascomycin (the only detectable protein in their pull-down experiment) [152]. In contrast, Monaghan et al. used a series of ascomycin analogs that did not bind the PfFKBP35 active site to show that they still inhibited P. falciparum growth [150]. This might suggest that while PfFKBP35 is the most abundant FK506-binding protein in P. falciparum other less abundant proteins might be more relevant for the growth-inhibitory effects. Further pharmacological studies with genetically engineered P. falciparum strains might be necessary to clarify the role of PfFKBP35.

3.4. Non-Immunosuppressive Cyclosporin Analogues

Several viruses have been shown to rely on cyclophilins as essential host factors for their replication cycle and/or for infectivity. Viral interaction partners of cyclophilins are for example the p24 capsid protein expressed by the human immuno deficiency virus-1 and the hepatitis C virus non-structural proteins NS2, NS3A and NS5B.

Several studies have shown cyclophilin ligands to reduce HCV-specific RNA replication in vitro and in vivo. Importantly, this effect was independent of calciuminhibition by CsA leading to the development of the non-immunosuppressive analogs Deba-025, NIM-811 and SCT-035 that are currently clinically evaluated for HCV infection. To abrogate the binding to calciumin, these derivatives were designed to contain modifications in position 3 and 4 of CsA which were known to be critical for calciumin engagement (Fig. 9). The branched or more bulky residues at position 4 (e.g., the Val-4 side chain of Deba-025) are thought to sterically block the interaction with the hydrophobic pocket of CsA. In addition these modifications increase the affinity for CycP [153, 154]. Antiviral efficacy was also shown for sangefhirnin A and analogs thereof [43, 155-157].

Mechanistically, a consensus emerged for CycP as the major physiological anti-HCV target of cyclophilin ligands, with minor possible contributions of CypB, Cyp E, Cyp H and Cyp40 [43, 156]. Recently, a novel CsA-binding protein called CAH1 was identified that formed complexes with NS5B and CycP, the latter interaction being sensitive to CsA [158].

A recurring problem in antiviral therapies is the rapid emergence of drug-resistant variants due to the high mutation rate of
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Fig. (9). Non-immunosuppressive cyclosporin analogs that have been investigated in clinical trials.

the viruses. Recent studies revealed a high barrier to resistance during Delbo-025 treatment, indicating that the acquisition of independence from a host factor is a rather rare event. Importantly, no cross-resistance was observed for NIM811 or Delbo-025 in combination with the current standard antiviral therapies (pegylated interferon and ribavirin) or with new HCV-specific treatments like polymerase inhibitors or recently approved HCV protease inhibitors. Instead, additive or even synergistic antiviral activities were observed [150].

For all three CsA analogs impressive antiviral effects were observed during clinical studies which have been extensively reviewed elsewhere [160, 161]. The most prominent adverse effect seems to be hyperkalemia. Alisporivir (Delbo-025) is the most advanced candidate which has recently entered a phase III clinical trial (ClinicalTrials.gov Identifier: NCT01316094).

Cyclosporin A is a well-established inhibitor of the P-glycoprotein 1 (Pgp 1, also called MDR-1 or ABCB1), one of the major cellular efflux pumps thought to contribute to drug resistance during chemotherapy. Valspodar (PSC833, the active agent of Amduay®, Fig. 9) was designed as a non-immunosuppressive cyclosporin D analog that has improved inhibition activity for the P-glycoprotein (10nM) and specificity vs. several other human efflux transporters [162]. The oxidation of the hydroxyl group of the Lmt group in position 1 and the replacement of 2-aminoobutyric acids in position 2 by valine abolish the affinity for most human cyclophilins [43]. Valspodar was evaluated in four phase III clinical trials in combination treatment for cancer, mostly acute myeloid leukemia. These did not show a clinical benefit compared to established chemotherapeutics used alone but rather indicated additional toxicity leading to a stop of the further clinical evaluation of PSC833.

CsA as well as three non-immunosuppressive analogs were reported to inhibit the sarcoendoplasmatic reticulum Ca²⁺ ATPase (SERCA) pump, with SERCA2b being the primary target. While the potency of CsA was found to be modest (>5µM), NIM811 and Sanglifehrin A inhibited SERCA2b at 10nM and 50nM concentrations, respectively [163, 164]. The direct relevant binding sites and the physiological relevance of these activities remain to be established.

Cyclophilin A is usually an intracellular protein. However, after oxidative stress or inflammatory stimuli it can be released into the extracellular space, possibly via a vesicular pathway [163].
Extracellularly it acts as a chemoattractant by binding to the cell surface receptor CD147 (also called EMMPRIN) on leukocytes to recruit them into inflamed tissues. NIM11 completely blocked neutrophil migration in response to CypA in vitro and it significantly reduced the infiltration of neutrophils into LPS-challenged lung tissue in vivo [164]. To selectively address the extracellular pool of cyclophilins, Fischer and coworkers synthesized cell-impermeable CSA derivatives [165]. Based on earlier work [166] a highly charged oligo-glutamate tag was introduced at position 8 of [D-Ser]-CSA (compound 2, Fig. 10). In addition a TAMRA dye was added to track the cellular location of 2. As expected, this compound was incapable to penetrate cells which also abrogated the immunosuppressive properties of the conjugated [D-Ser]-CSA. However, the cell-impermeable CSA derivative still inhibited CypA-induced T cell chemotaxis. Furthermore, 2 was also active in an animal model of allergic lung inflammation after nasal application [167]. This approach could thus allow a highly focused targeting of the extracellular cyclophilins in the lung thereby substantially improving the therapeutic index of cyclophilin inhibitors for this indication.

Cyclophilin D, encoded by the Ppfd gene, is located at the inner mitochondrial membrane where it facilitates the opening of the mitochondrial pore transition pore (PTP). The latter plays a key role in cell death and has been implied in a variety of diseases. Studies with CsA, sangliphepin A or non-immunosuppressive analogs thereof in combination with PPTP transgenic mice have validated CypD as a target in animal models for heart or brain ischemia-reperfusion, collagen IV-related disorders, Alzheimer’s disease, diabetes, and bipolar disorder [73, 168, 169].

A first clinical support for the concept of CypD inhibition was provided in two small trials of patients with myocardial infarction [170] or collagen IV myopathy [171]. It should be noted that the doses of CsA used in these studies were rather low leaving some uncertainties whether immunosuppressive (i.e., calcineurin inhibition) or non-immunosuppressive (i.e., CypD inhibition) is primarily responsible for the observed effects. Recently, a phase II/III clinical trial with cyclopordin was initiated by the European brain injury consortium for traumatic brain injury (press release www.neurocrine.com).

It should be noted that the CD147-CypA system discussed above has recently been shown to also contribute to reperfusion injury after myocardial ischemia [172]. It is therefore possible that some of the effects observed with CsA analogs in animal models of myocardial ischemia might be due to the blockade of the CD147-CypA engagement.

The structures of CypA and CypD were shown to be very similar [173] indicating that the development of subtype-specific Cyp-inhibitors is likely challenging. In order to preferentially target CypD in cells, Malouite et al. introduced a triphenyloxiphenium moiety at the 3-position of CSA (compound 8 in Fig. 10) [174]. This modification abrogated calcium binding. The lipophilic cationic TPP+ group was intended to enrich the conjugate in the negatively polarized inner membrane of mitochondria where CypD is located. Subsequent cellular assays suggested a preferential inhibition of mitochondrial CypD compared to cytosolic CypA.

3.6. Non-Peptidyl Cyclophilin Inhibitors

Numerous groups explored novel cyclophilin inhibitors unrelated to the archetypical CsA scaffold. Towards this end a variety of in silico screening methods were applied based on the available crystal structures.

Guchiev et al. defined two hydrophobic pockets that harbor the CsA residues McVall11 and Abu2 in the CsA/CypA co-crystal structure. In addition, a hydrogen bond acceptor corresponding to the carbonyl of MeH419 was defined as a key interaction [175]. Based on this pharmaphore they analyzed a database containing 296,387 commercially available compounds for potential CypA binders. Among the identified in silico hits compound 2 (Fig. 11) was reported to inhibit CypA PPIase activity with an IC50 of 0.32 μM although this could not be replicated in a competitive fluorescence polarization assay (own unpublished observations) [176]. Numerous analogs of this hit were prepared with reported inhibitory activities up to 14 μM. 2 was shown to block the cell infection competence of HIV-1 virions. Liu and coworkers described quinoline derivatives as possible lead structures to inhibit cyclophilin D [177, 178]. The best compound 10 bound CypD with an affinity of 2 μM as determined by surface plasmon resonance and confirmed by tryptophan quenching and PPIase assays. This compound also inhibited Ca2+-dependent mitochondrial swelling and showed a ten-fold selectivity for CypD vs. CypA.

Liu and colleagues also employed high-throughput in silico docking to identify potential CypA ligands resulting in compounds like 11 (Fig. 11) with a reported IC50=5μM in a PPIase assay [179]. In a subsequent study these authors merged the identified sulfonamide moiety with the 2,3-difluoro-2-yquinoline scaffold identified earlier (e.g., in compound 10) resulting in compounds like 12 with a reported PPIase-inhibitory activity of 1μM [180]. In both studies the binding observed by surface
plasmon resonance were substantially weaker than the data from the PPlase assays leaving some uncertainties regarding the true affinities.

Ni et al. used a computational de novo design approach based on the CypA/sanglifehrin cocrystal. This led to a series of acyclics exemplified by compound 13, which was reported to inhibit the PPlase activity of CypA with a remarkable IC_{50} of 1.5nM [181].

Yang and coworkers designed dual inhibitors for CypA and for the HIV-1 capsid protein based on a thiourea motif [182]. Compound 14 was described to inhibit CypA PPlase activity with an IC_{50} of 0.63µM while the affinity determined in a tryptophan fluorescence quenching assay was 50nM. The compound was shown to reduce the assembly rate of the capsid protein and to display anti-Simian immunodeficiency virus activity. Control experiments confirmed that compound 14 was inactive at the HIV protease or HIV integrase. Further modification around compound 14 resulted in compound 15 which inhibits CypA PPlase activity with an IC_{50} of 0.13 µM and having equivalent antiviral activity [183].

Mori et al. recently identified compound 16 in a medium throughput screening for the PPlase Pim1. Surprisingly, this substance also inhibited CypA with an IC_{50} of 14µM while it was inactive for FKBP12 [184].

Walkinshaw and colleagues used the LIDEAUS docking algorithm to search the Maybridge chemical database consisting of 50,000 different compounds [185]. Dimedone 18 was identified as a weakly binding fragment which was confirmed by X-ray crystallography. Based on the observed binding mode analogs like compound 17 were synthesized that inhibited CypA with 28µM. The affinity was confirmed by surface plasmon resonance and tryptophan quenching experiments, and the molecular binding mode was confirmed by X-ray crystallography. Based on these results the Walkinshaw and Turner groups went on to design constrained dimedone analogs like compound 19 with an IC_{50} of 8.6µM in a PPlase assay and a K_{d} of 16µM as determined in a tryptophan quenching assay [186]. When applied to C. elegans 19 caused a phenotype characterized by reduced fecundity and growth.

Dearmond et al. used a mass spectrometry based H/D exchange technology to identify CypA ligands [187]. Among the multiple hits compound 20 was selected by the MS assay to have an affinity of 2µM. This substance was shown to inhibit the PPlase activity of CypA in a single dose assay.

Dagave and coworkers used the special properties of oxorhenium complexes for a combinatorial self-assembly approach to generate novel CypA ligands [188]. A small library of fragments was derivatized with an oxorhenium precursor and reacted with a small library of thiol-containing building blocks. The resulting...
Fig. (12). Principles and applications of chemical dimerizers.

a) Principle of chemical homodimerization. The protein of interest is fused to a dimerizable protein tag like FKBP12 (shaded). The dimerization is triggered by the addition of a symmetrical chemical dimerizer (squares) like AP1510 to induce a trimeric complex. b) Example of homodimerization used to mimic receptor tyrosine kinase activation. A receptor tyrosine kinase domain is fused to an immunophilin binding tag (shaded) and a membrane localization sequence (TM). The addition of a homodimerizer induces autophosphorylation of the kinase domains and activates downstream signaling. c) Principle of heterodimerization. One protein of interest is fused to an immunophilin binding tag (e.g., FKBP12), the other is fused to an orthogonal recognition domain (e.g., FRB for rapamycin). The dimerization is triggered by the addition of an unsymmetrical chemical dimerizer (square + triangle) like rapamycin to induce a trimeric complex. d) The yeast three hybrid system as an application of heterodimerization. An immunophilin binding domain like FKBP12 (shaded) is fused to a DNA binding domain (DBD) from a transcription factor. An immunophilin ligand (square) like rapamycin or FK506 is conjugated to a chemical compound of interest (triangle). A library of proteins (grey dotted) is fused to the activation domain of a transcription factor (AD). In vivo trimerization only those fusion proteins that are able to bind the chemical compound of interest are recruited to the DNA and induce transcription and detectable expression of a reporter gene. e) Principle of reverse dimerization. The FKBP12 (F363M) mutant (white dotted) was shown to inherently dimerize. Tagging of proteins of interest with this mutant leads to their constitutive dimerization. These homodimers can be disrupted by the addition of an immunophilin binding compound, e.g., FK506 (square). f) Principle of the ‘bump and hole’ strategy in the application of chemical dimerizers. To gain specificity for the protein of interest over endogenous proteins, the dimerizing agent is modified by a bulky group (bump) that abolishes binding to endogenous proteins (e.g., native FKBP51). The corresponding binding tag is modified by a mutation able to accommodate the bumped ligand (e.g., FKBP12F363V, shaded + dotted). g) Example of chemically controlled protein stability. A protein of interest is fused to an immunophilin mutant (e.g., FKBP12L106P, shaded) that is inherently unstable and impairs constitutive cellular degradation on the fusion protein in the absence of a ligand. Upon addition of an FKBP ligand (e.g., Shield-1, square) the FKBP domain is stabilized and the fusion protein accumulates.
complexes were stable towards purification and were tested for CypA binding. The best substances like II-8 had an IC₅₀ of 1.1μM in a tryptophan quenching assay. The authors then extended this approach in a dynamic setting where the rhodium-mediated self-assembly was carried out with mixtures of building blocks in the presence of CypA (also called Cyp18). The best compounds were reported to quench tryptophan fluorescence and to inhibit PPlase activity of CypA with IC₅₀ of 0.3μM [180].

Daum et al. discovered ary1-1-imidazolones as a preferred binding motif for CypA [190]. This motif was thought to mimic the threedimensional structure of the putative transition state of the PPlase reaction. Building on previous work they found compound 21 (Fig. 11) to inhibit the PPlase activity of CypA with an IC₅₀ of 0.5μM which was confirmed by isothermal calorimetry and competitive fluorescence polarization. Importantly, compound 21 exhibited excellent specificity vs. numerous other human Cyp paralogs, e.g., the closely related CypB. It also was specific vs. FKBP12, albeit not vs. Pim1 the enzyme it was originally developed for.

4. IMMUNOPHILIN ASSAYS

The classical assay for immunophilins is the particle-prolyl isomerase (PPlase) assay that measures the catalytic activity of these enzymes to accelerate the cis-trans isomerization of amide bonds preceding a proline [191]. However, this assay is rather labor-intensive, requires the use of water-free reagents and has to be run at low temperatures within a very short time window. Using specialized equipment this assay was recently adapted to a high-throughput screening format [192].

The second commonly used assay is a tryptophan fluorescence titration assay. Many immunophilins contain a (single) tryptophan residue close to the active site (e.g., Trp59 for FKBP12 or Trp21 for CypA). Upon binding of ligands these tryptophan residues are getting buried or displaced which reduces (for CypA) their intrinsic fluorescence [193, 194]. While readily adapted in most laboratories, this assay has a rather low sensitivity and is often confounded by the intrinsic spectroscopic activity of test substances.

Radioactive assays with excellent sensitivity, high robustness and moderate to high throughput have been established [84, 195] but the radioactively labeled [3H]-FK506 is no longer commercially available.

The method of choice currently seems to be fluorescence polarization assays and a number of fluorescent tracers have been developed, e.g., FL-SLF (Fig. 14e) [142, 176, 199, 196]. These assays have been miniaturized and adapted to medium or high throughput formats. Fluorescence chemical denaturation is a label-free assay format that has recently been applied to FKBP12 in a 96-well format [197].

Classical biophysical methods like surface plasmon resonance or isothermal calorimetry have been used to confirm the affinities of novel putative FKBP ligands. In several cases this revealed substantial discrepancies in the measured affinities, depending on different assay formats applied. Not surprisingly, immunophilins have been used as model systems in proof-of-concept experiments for novel assay technologies like mass spectrometry-based screenings [118, 198]. FKBP12 has been the seminal example for an NMR-based fragment screening [115], a technology that continues to be improved [116, 117]. Finally, cellular assays for the detection of FKBP12 ligands have been developed, usually based on the disruption of a readout that depends on the chemically induced demethylation [199].

5. IMMUNOPHILIN LIGANDS AS CHEMICAL BIOLOGICAL TOOLS

In the last two decades immunophilin ligands received increasing attention as tools for various applications in chemical biology. These applications often draw on overlapping molecular mechanisms and we try here to discretion their use as tools for chemical dimeterization, including three hybrid applications and conditional intramolecular protein localization, or as tools to chemically trigger living cells, including approaches to regulate protein or compound stability [200, 201].

5.1. Chemical Dimeterizers

The most widespread application of immunophilin ligand tools is their use as chemically induced dimeterizer (CID), allowing the fast conditional formation of protein complexes inside living cells. CID is based on the intrinsic property of the natural compounds FK506, rapamycin and cyclosporin A to bind two different protein targets at the same time. Therefore these compounds are often referred to as heterodimeterizers. FK506 and rapamycin bind to FKBP proteins and to calcineurin (for FK506) or to the FRB domain of mTOR (for rapamycin). CsA binds simultaneously cyclophilins and calcineurin. For artificial CID and rapamycin as chemical dimeterizer, pairs of proteins of interest are expressed in cells as fusion with FKBP12 and with FRB, respectively (Fig. 12c). Alternatively, fusions of FKBP12 and CsA for the application with FK506 as dimeterizer were used [202, 203]. Upon addition of the cell-permeable chemical dimeterizers, the classical trimeric complexes are formed (i.e., FKBP12-Rap-FRB in the case of rapamycin) bringing the two fused proteins into close spatial proximity.

While all three classes of natural immunophilin ligands can be employed for CID applications rapamycin-based technologies and methods using synthetic FKBP ligands have by far received most attention [204-208]. Rapamycin has the advantage to form a very tight trimeric complex (KC=2-10μM) between two very small well-folding fusion domains (FKBP12=2-4Da, FRB=11-2Da) in a highly ordered fashion [196]. The latter is due to the very low affinity of rapamycin to the FRB domain alone (KC=260μM, 10,000-fold lower than the binding of rapamycin to FKBP12 (KC=0.2μM). In practice, the Rap-FRB species that in principle could interfere with the ternary complex formation in excess of rapamycin can therefore be neglected. This is important since the concentration of rapamycin in living systems is difficult to control.

A major problem in the use of the natural immunophilin ligands as tools in cellular systems is their inherent immunosuppressive activity, mediated by the inhibition of calcineurin (in the case of FK506 and CsA) or of mTOR (in the case of rapamycin). This can severely confound the intended biological experiments. For rapamycin this problem was solved by the synthesis of rapamycin derivatives like C16-(R)-methylly rapamycin (C16-MaRap, Fig. 13) [209] or the slightly more active isomer C20-(R)-methylrapamycin [210]. The triene moiety and position C16 in particular is a preferred part in rapamycin since it can easily be derivatized with a number of nucleophiles [211]. These modifications disrupt the interaction with the wildtype FRB and abolish the binding to (and therefore the inhibition of) endogenous mTOR. To still allow for disassembly with the desired protein fusion constructs compensating mutations were introduced in the fused FRB-domain (called FRB*I baroorg K209S, T208L, and W210F). These allowed for binding of the “bumped” rapamycin analog and for ternary complex formation. Later improved versions like AP21967 and iRap (Fig. 13) were developed that were reported to display better pharmacokinetic parameters (solubility, stability, cell permeability) [212, 213] and allowed as new applications [214-217]. AP21967, originally developed by Astellas Pharmaceuticals, is now commercially available from Clontech (Clontech: Ligands for Chemically Induced Dimeterization / http://www.clontech.com/ products/detail.asp?product_id=227800&bundle=2). A conceptually similar approach was also described for a “bumped” FK506 and calcineurin mutants [218].
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

In the special case where two identical proteins are desired to be dimerized (e.g., conditional homo-dimerization of receptor tyrosine kinases constructs, Fig. 12b) the dimerizing problem was solved by the development of symmetric dimerizers (Fig. 12a). The first generation of this type of dimerizers was based on FK506 and CSK [219-221] but were soon replaced by smaller synthetic bivalent FKBP ligands like API1510 (Fig. 14a) [222]. A major drawback of these compounds is their high affinity to the abundant endogenous FKBP ligands which leads to stochastic mixtures of homo- and heterodimers between overexpressed FKBP-fusion proteins and endogenous FKBPs. This problem was again circumvented by the so-called “jump-hole” approach (Fig. 12f). Based on a FKBP12-ligand co-crystal structure the C9 carbonyl of API1510 was replaced by an ethyl group which would sterically clash with the F36 of FKBP12 [223, 224]. A compensatory F36V mutation was introduced in FKBP12 fusion proteins to accommodate the ethyl “jump” in compounds like AP20187 (Fig. 14b) [225]. The use of these bumped chemical dimerizers greatly enhanced the specificity for the dimerization of appropriately tagged proteins and significantly reduced unproductive binding to endogenous proteins. The most frequently used bumped homodimerizers for bumped FKBP12 mutants is AP20187 which is commercially available from Clontech (Clontech: Ligands for Chemically Induced Dimerization http://www.clontech.com/products/detail.asp?product_id=237800 &database=3).

It should be noted that rapamycin-based dimerizers like AP21967 still bind to endogenous FKBP12 and therefore likely also induce trimeric FKBP12/Rap-FRB-fusion protein complexes. The functional consequences of this remain to be established. For CSK the orthogonal “jump-hole” pair Meli1ICA and hyOP(699T, F113A, C115M) was generated that is devoid of binding to the endogenous cyclophilins [226, 227].

The biological applications of dimerizers are very broad. In a classical approach, CIDs are used in the (homo-) dimerization or oligomerization (if more than one FKBP-tag is added) and activation of membrane receptors like the T-cell receptor or membrane tyrosine kinases (TCR) [219], ErbB family EGF receptor [273], PDGF-GFR, insulin receptor [228], epithelial growth factor receptors / hepatocyte growth factor receptor / thrombopoietin receptor [229].

Another frequent application is the induced activation of apoptosis, which can be achieved by the dimerization of the Fas receptor or the dimerization of various caspases (Fas [221], [222] / Procaspase-8 [228], Caspase 8: [230], [231], [232] / Caspase9: [276], Procaspase2: [235]).

An in vivo application of CID-induced apoptosis is the mouse model for the study of peritoneal adhesions using macrophage Fas-induced apoptosis (MAFIA-mouse model). These mice carry a transgene for the expression of a membrane targeted FKBP12-Fas fusion protein under the control of the eGFP promoter, which directs the specific expression in macrophages. Injection of the molecular dimerizer AP20187 leads to the apoptotic depletion of macrophages and peritoneal adhesion formation in these transgenic mice [234, 235].

Another widely used application of CIDs is the chemical regulation of transcription. In analogy to yeast two-hybrid systems, transcription factors can be split in a DNA binding domain and a transactivation domain. The transcription factor can be activated by its reconstitution, which in turn is induced by the addition of a chemical dimerizer. Often used combinations are the DNA binding domain ZFHD1 (Zinc finger homedomain 1) and VP16 as transactivation activator of the Gal4 DNA binding domain together with the Gal4 activation domain, each fused to FKBP12 and FRB respectively and dimerized via rapamycin [204, 236]. In combination with AAV vector systems as vehicle this method allows tissue-specific inducible expression in mice or the potential use for gene therapy, as for example shown by the rapamycin-inducible expression of human growth hormone [237] or erythropoietin [238] in mice after intramuscular gene transfer.

A modification of the inducible transcription system is the yeast three-hybrid approach (Fig. 12d). Originally, this method was first used to identify protein binding partners of chemical compounds. In the initial application, dexamethasone was conjugated to FK506 [238]. Correspondingly, a DNA-binding domain was fused to the hormone-binding domain of the glucocorticoid receptor, which recruited the dexamethasone-FK-506 conjugate to the DNA. Screening of a cDNA library fused to a transcriptional activator domain yielded FKBP12 as an interaction partner of the dexamethasone-FK-506 conjugate. Later, the three-hybrid system
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The Chemical Biology of Immunoaphilin Ligands

(Fig. 12, Cont.)

![Chemical structures]

was adapted to mammalian cell systems, for example to screen for FRB mutants binding to rapamycin derivatives [209] or to bacterial systems, e.g., using FK506-methotrexate conjugates which isolate dihydrofolate reductase as a binder [230]. The orthogonal methotrexate-dihydrofolate reductase system represents a general alternative for heterodimerization, e.g., when combined with synthetic FKBP ligands like Tmt-SLF (Fig. 14b) [240).

Similar to the reconstitution of split transcriptional activators, other proteins like enzymes may be inactivated by splitting into two pieces and reactivated by chemically induced dimerization as for example shown for the activation of a split CRE recombinase [241] or the tobacco etch virus (TEV) protease [242].

Another field where chemically induced dimerization is widely used is the conditional transport or localization of proteins or vesicles. The most popular use in this area is the inducible recruitment of signaling proteins to the plasma membrane. For this purpose, one partner of the dimerizing system is usually expressed as a modified form or as a fusion with a membrane anchor. The other partner is then transferred to the membrane by dimerization. Examples for this application are the membrane recruitment of the guanine nucleotide exchange factor SOS [243], the ZAP70 kinase [244] or the AKT2 kinase [245]. By the use of suitable anchoring proteins, complementarily tagged proteins may also be targeted to the endoplasmic reticulum. Golgi or organelles like mitochondria [208]. For example, this allows replacing proteins from their endogenous site of action to a different cellular localization, where it is not functional, a method called “knocksideways” approach. As an example, this method was used for the redirection of the adaptor protein API from clathrin-coated vesicles to the mitochondrial membrane [207]. Similar experiments were reported where nuclear localization (NLS) or nuclear export signals were chemically recruited to regulate nuclear trafficking [246–248]. The transport of synaptic vesicles can also be influenced by the inducible dimerization of presynaptic proteins like VAMP2 / synaptobrevin and synaptophyisin [215]. The suppression of the synaptic vesicular trafficking in transgenic mice by these MISTs (molecules for inactivation of synaptic transmission) could be linked to deficits in learning and related behavior.

Last but not least, dimers can be used to control posttranslational modifications like intein splicing, sumoylation or ubiquitylation. The chemical dimerization-induced split intein fusion is able to activate trans-splicing of the fused inteins. This method was used to inducibly reconstitute an autoregulated protein kinase [249, 250]. For the induced sumoylation of proteins essentially two methods have been reported. Zhu et al. showed that a FKBP-tagged SUMO can be added to a FRB-tagged target protein by addition of rapamycin [251]. This way they studied the biological effects of sumoylation of RanGAP1. An alternative method is the UBC9 substrate dimerization-dependent sumoylation (USDDS) [252]. This approach uses the recruiting of the same ligating enzyme UBC9 to a target protein by chemical dimers.

Immunophillin ligands were also used to coordinately disrupt constitutive dimers in a method called reverse dimerization (Fig. 12e) [253]. In this method a mutated FKBP12 (mut) protein is used which has the intrinsic ability to dimerize without addition of a chemical inducer. Upon addition of a ligand selective for the FKBP mutant (e.g., AP21998, Fig. 14d) the contact points are blocked and the FKBP12(mut) dimers are disrupted. This technique was applied by Rivera et al. [254] to chemically induce the secretion of human growth hormone and insulin constructs that had been trapped in the
ER by aggregation of the fused FKBP12Δ331. The secretion of the hormones could be induced by adding compound AP21998 or AP22342 which dissolved the aggregate.

5.2. Immunphin Ligands to Control Protein Stability

A rather new field for immunphin ligands, partly overlapping with their use as molecular dimers, is the regulation of protein stability. In one method a protosomal subunit was fused to the yeast FKBP12 homolog while the target protein was tagged with the FRB subunit [255]. The rapamycin-induced recruitment of the protosome to the target protein seems to be sufficient to induce the degradation of the target protein.

Similar to the split intein method described above, a split inducible ubiquitin method can be used to target proteins for degradation [256]. In this method, called split ubiquitin for the rescue of function (SURE), the protein of interest is fused to a tag derived from the C-terminal domain of ubiquitin followed in frame by FRB and/or a degron (a signal targeting the protein to proteasomal degradation). Per default, this protein fusion construct will therefore be constitutively degraded. By addition of rapamycin this construct can dimerize with a second construct harboring the N-terminal domain of ubiquitin fused to FKBP12 which induces the reconstitution of functional ubiquitin. This in turn leads to the release of the target protein from the degron by an ubiquitin-specific protease and thereby to its rescue from degradation. This technology was recently extended by the incorporation of a mutated FKBP12 tag that functioned as the degron [257].

Sankaran et al. observed that a triple mutation of FRB (FRB K203SP, T2098L, and W2101F) reported by Libertes et al. imparted protein instability to the proteins it was fused to, which in turn got degraded [209, 210]. Importantly, the fusion protein could be protected by the addition of C20- (R)-methallyl rapamycin which bound to and stabilized the fused FRB* tag. This approach was further extended recently by the optimization of FRB mutants [258].

The Wandl三位 group developed a method to control protein stability with immunphin ligands that do not depend on dimerization anymore. This approach is based on the inherent instability of FKBP12 mutants like L106P, which act as destabilizing domains when fused to other proteins (Fig. 12g) [259]. L106P was shown to be the most efficient destabilizing mutant and is most frequently used, either as single mutation or in FKBP12 F35V/L106P double mutants. A modified SLF ligand, called Shield-1 (Fig. 14d), can be used to stabilize the FKBP12 mutants. In further studies, this method evolved by the development of improved ligands like Shield-2 [260] or by the discovery of more efficient FKBP degradation tags [261-263]. The FKBP12Δ331 mutant represents a “ligand-on” system where the target fusion protein is continuously degraded in the absence of the ligand. The latest trend seems to be the development of a “ligand-off” system where the addition of an FKBP ligand exposes unfolded portions of a mutant FKBP12 fusion construct thereby inducing the degradation of the fused protein [264].

Protein degradation can also be induced with appropriately functionalized immunphin ligands. An approach called fluorophore-assisted laser inactivation (FALI) uses a fluorescent-labeled FKBP ligand (FL-SLF) to target FKBP12Δ331-tagged proteins inside cells. Using a laser, the fluorophore-bound target proteins can be rapidly inactivated in a spatially defined manner by the local generation of reactive oxygen species [265]. An alternative method chemically induced ubiquitylation is used to target proteins for proteasomal degradation. This method is based on the recruitment of an E3-ubiquitin ligase to a protein target via PROTACs, a PROTACs. This was achieved by conjugating an FKBP ligand to a peptide E3-ubiquitin recognition sequence. As a proof-of-principle, the ligand-induced ubiquitin ligase-dependent degradation of a GFP-FKBP12Δ331 fusion protein was shown [266].

In addition to dimerization and protein (de)stabilization, the exquisite recognition properties of immunphin ligands have been employed in a number of other chemical biology applications. Recently, three groups have reported caged versions of rapamycin (Fig. 15) where rapamycin is linked to a photoactivatable moiety that interferes with cell permeability, with binding to a FKBP12 mutant or with binding to the FRB domain. Photodeprotection restores the binding of rapamycin to its target, like the inhibition of mTORC1 by rapamycin [267], the recruitment of the small GTPase RAC to the plasma membrane [268] or the activation of inactive FKBP-fused focal adhesion kinase (Fak) by binding to rapamycin [269].
The main advantage of caged rapamycin derivatives is the excellent spatial and temporal control of activation that can be achieved with light.

Marince et al. expanded on previous observations that the binding of FK506 or rapamycin to endogenous FKBP proteins shelters these natural products from oxidation and degradation by cytochrome P450. They postulated that other pharmacologically active compounds might also show an elongated activity when attached to immunophilin ligands. As a proof-of-concept the HIV-1 protease inhibitor amprenavir was conjugated to SLF (SLF-WV, Fig. 14c), which increased the half-life of the conjugate in mice [270]. Importantly, the amprenavir-SLF conjugate retained its inhibitory activity against HIV protease.

The Walkless group employed FKBP ligand conjugates to control the activity of the attached immunophilin in a cell type-dependent manner. The linker in the MTXSL conjugate (Fig. 14c) was designed to preclude simultaneous binding to FKBP5 and dihydrofolate reductase. Complexation of the conjugate with FKBP12 was shown to reduce the inhibitory activity of the attached immunophilin. In human cells MTXSL is trapped by the high concentrations of the high-affinity human FKBP5. This effect is much smaller in Plasmodium falciparum cells, consistent with the weaker affinity of PFKBP53 for MTXSL [271, 272].

Fluorescent FKBP ligands can be used for live cell imaging of proteins tagged with the FKBP domain. Recently, a group by Pirtovsag showed that 5(6)-TAMRA-EDA-Oly-SLP (Fig. 14c) is cell-penetrable, non-toxic, displays an excellent signal-to-noise ratio and is compatible with several other commonly used fluorescent protein tags [273].

6. CONCLUSION

Immunophilin ligands have had – and continue to have – an enormous impact on human health and on basic life sciences alike. Whenever biological problems can be addressed by recombinantly overexpressed fusion proteins, immunophilin fusion variants offer an additional layer of chemical control. Often the use of immunophilin ligands imparts excellent temporal control (rapid and reversible) or they bring in novel functionalities unavailable through natural protein fusions.

As the biological role of human immunophilins is becoming clearer, several members of the endogenous FKBP or Cyp families are emerging as potential therapeutic targets. So far, semisynthetic analogs of CSA, FK506 and rapamycin have been by far the most successful immunophilin ligand derivatives. It is notable that, despite the importance of the immunophilins, there is a striking paucity of published results from high throughput screening campaigns, the traditional ligand generation engine of the pharmaceutical industry. The rational design approach has been more successful, with a number of synthetic ligands described for FKBP12. Most of them retain the piperazinolike motif derived from FK506 or rapamycin. There are comparatively few reported examples of de novo generated immunophilin ligands that are truly structurally unrelated to FK506 or rapamycin. Those novel scaffolds have yet to show their broader biological applications.

Many studies that pharmacologically probe immunophilins still employ the prototypical immunosuppressants FK506, CSA or rapamycin, likely because they are commercially available. The interpretation of the effects observed with these compounds is however complicated by the concomitant inhibition of calcineurin and mTOR which likely occurs at lower concentrations than those required to block the immunophilin binding sites. As outlined in this review numerous non-immunosuppressive analogs are now known which are devoid of this confounding activity.

So far most studies focused on the prototypical FKBP12 or CypA. In the few cases where the affinity for other immunophilins have been investigated most reported immunophilin ligands tended to be rather unspecific within the FKBP or Cyp family. This is likely attributable to the very high degrees of structural homology observed in the active site of immunophilins. For the Cyp family addressing a more diverse second binding pocket adjacent to the CysA-binding site was proposed as a strategy to achieve selectivity [274].

The need for subtype-selective inhibitors is particularly prominent in the field of neuroimmunophilins where it has been impossible so far to clearly define the contributions of individual FKBP homologs to the observed neuroprotective or neurotoxic effects. Progress in this direction is eagerly awaited.

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Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52


Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52


1.2.2 Aim of Manuscripts 2 and 3

X-ray co-crystal structures of FK506 with FKBP51 (305R) and FKBP52 (in preparation) revealed that the pyranose group of FK506 (1) is in close proximity and contacts the 80s loop. SAR studies around the pyranose sub-structure have shown that that the methyl group at C\(^{11}\) of FK506 analogs is important while the pyranose ring oxygen is dispensable for binding to FKBP12 (Table 2)^50^-^52. The above SAR conclusion is consistent with the FK506-FKBP51 and FK506-FKBP52 co-crystal structures where the pyranose oxygen does not seem to be involved as a hydrogen bond acceptor while the C\(^{11}\)-methyl fills the small hydrophobic cavity\(^ {102}\). The exocyclic hydroxyl group present in FK506 at C\(^{10}\) engages in a hydrogen bond with Asp\(^{68}\) of FKBP51/52 which is absent in the co-crystal structures of the non-immunosuppressive analogs with FKBP51 and FKBP52. The crystal structures further revealed that the pocket outlined by the 80s loop is more open and there could be a potential hydrogen bond interaction partner (S\(^ {118}\)). Taking these as structural starting points we decided to follow two different approaches to target the 80s loop of FKBP51 and FKBP52 to gain affinity and selectivity. The patent applications of the compounds and the treatment for which these compounds can be useful have been filed.

- **Pipecolate-diketoamides for treatment of psychiatric disorders.**

**1.2.2.1 Evaluation of Synthetic FK506 Analogs as Ligands for FKBP51 and FKBP52 (Manuscript-2)**

In this manuscript we report the co-crystal structure of FKBP51 with a simplified α-ketoamide analog derived from FK506 and the first structure-activity relationship analysis for FKBP51 and FKBP52 based on this compound. Further, the tert-pentyl group of this ligand was systematically replaced by a cyclohexyl ring system which more closely resembles the pyranose ring in the high affinity ligands Rapamycin and FK506. The compounds in this series had various alkyl substituents at the C\(^{11}\) position. The interaction with FKBP5s was found to be surprisingly tolerant to the stereochemistry of the attached cyclohexyl substituents. The molecular basis for this tolerance was elucidated by X-ray co-crystallography.

**Figure 13:** Prototypical pyranose containing FK506 analogs
Own Contributions:
In the manuscript, my personal contributions have been the following:

1. Partial optimization of synthesis protocol (Scheme-1) and synthesis of analogs 6a, 6b, 6e, 6f in Table 1 of the manuscript.
2. Establishment of the synthesis protocol (Scheme-2) and optimization of synthesis protocol (Supp. Scheme-S1, S5 and partially S3) for the synthesis of α-keto acids and further incorporation into the corresponding α-ketoamides (Scheme-3). Synthesis and purification of all intermediates and compounds (3a*- 3j*) followed by structural characterization of all the compounds in Table 1 and 2.
3. Characterization of the final compounds in the fluorescence polarization assay together with B. Hoogeland and C. Kozany. Data analysis of the tested compounds.
Evaluation of Synthetic FK506 Analogs as Ligands for FKBP51 and FKBP52

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Abstract: The FK506-binding proteins (FKBP) 51 and 52 are co-chaperones that modulate the signal transduction of steroid hormone receptors. Both proteins have been implicated in prostate cancer. Furthermore, single nucleotide polymorphisms in the gene encoding FKBP51 have been associated with a variety of psychiatric disorders. Rapamycin and FK506 are two macrocyclic natural products that bind to these proteins indiscriminately, but with nanomolar affinity. We here report the co-crystal structure of FKBP51 with a simplified α-ketoamide analog derived from FK506 and the first structure-activity relationship analysis for FKBP51 and FKBP52 based on this compound. In particular, the tert-pentyl group of this ligand was systematically replaced by a cyclohexyl ring system which more closely resembles the pyranose ring in the high affinity ligands rapamycin and FK506. The interaction with FKBP5 was found to be surprisingly tolerant to the stereochemistry of the attached cyclohexyl substituents. The molecular basis for this tolerance was elucidated by X-ray co-crystallography.

Stereochemistry at C¹⁶ and C¹⁷ does not effect binding to the FKBP5s.
**Introduction:**

Immunosuppressant natural products like FK506 (Fig. 1a) and rapamycin bind with high affinity to immunophilins of the FKBP (FK506 binding protein) family, which often also possess peptidyl-propyl isomerase (PPIase) activity. The best-characterized member of the FKBP family is FKBP12, a 12kD protein, which consists only of the FK506-binding domain. FKBP12-FK506 and FKBP12-rapamycin complexes create binding surfaces for binding to calcineurin (CaN) and mTOR, respectively\(^1\). The inhibition of the latter proteins mediates the immunosuppressive action of the two natural products. FKBP12 has also been shown to modulate the ryanodine receptor (RyR) channels and to bind to the transforming growth factor β receptor I. FK506 inhibits these interactions consistent with a shared common binding site\(^2\).

The higher molecular weight FKBP homologs FKBP51 and FKBP52 act as co-chaperones for the heat shock protein 90 (Hsp90). In the Hsp90 heterocomplex FKBP51 and FKBP52 have been shown to modulate signal transduction by the glucocorticoid receptor in a mutually antagonistic direction\(^3\)-\(^5\). FK506 was shown to inhibit the proliferation of prostate cancer cells. This was attributed to blockade of the enhancing effect of FKBP51 on the androgen receptor in these cells\(^6\),\(^7\). Numerous human genetic studies have shown that single nucleotide polymorphisms in the gene encoding FKBP51 are associated with a variety of psychiatric disorders\(^8\). Very recently, several independent studies using knockdown or knockout mice strongly supported an important role of FKBP51 in stress-coping behaviour\(^9\)-\(^12\). These findings have rendered FKBP51 as a novel target for treatment of psychiatric disorders. However, neither FK506 nor rapamycin can be used as a tool to investigate the roles of individual FKBPs in mammalian system due to strong off-target effects and lack of selectivity. Thus, non-immunosuppressive and selective inhibitors for the large FKBPs are required.

At the end of the last millennium various sub-classes of high affinity FKBP12 ligands were described which were devoid of the immunosuppressive activity present in FK506 and rapamycin\(^13\),\(^14\). α-Ketoamide derivatives without the effector region were the most widely studied series exemplified by compound 2\(a\)\(^15\) (Fig. 1). For FKBP12 the tert-pentyl group in 2\(a\) was found to be a good surrogate for the pyranose group in FK506 and rapamycin\(^16\). While the high affinity of the natural products FK506 and rapamycin were retained for the larger FKBPs, the binding affinity of 2\(a\) for the larger FKBPs was substantially weaker\(^17\). We thus first set out for a basic characterization of the structure-activity relationship of 2\(a\). To analyze the
interactions with the 80s loop in more detail we then substituted the tert-pentyl group in 2a with cyclohexyl analogs which more closely mimic the pyranose group in the high affinity natural product ligands (Fig. 1c).

**Result and Discussion**

**Crystal structure of the 2a-FKBP51 complex**

As a structural starting point for a rational design the co-crystal structure of 2a, the only synthetic ligand known for FKBP51, was solved in complex with the FK506-binding domain of FKBP51 at 1.5 Å resolution (Fig. 1d and 1e). Upon binding of compound 2a FKBP51 adopts a very similar conformation as found in the FK506 complex\(^{18}\) (Fig. 1d). Most active site residues are virtually superimposable in the two co-crystal structures. Compared to the FK506 complex (3O5R), Phe\(^{77}\) moves into the binding pocket, while Asp\(^{68}\) and the tip of the 80s loop (Leu\(^{119}\)-Lys\(^{122}\)) move outward in the FKBP51-2a complex, the latter in part due to crystal contacts with a neighboring FKBP51 molecule.
Fig. 1 Natural and synthetic FKBP ligands: (a) Structure of FK506 (1), (b) prototypic synthetic ligand of FKBPs 2a, which is devoid of immunosuppressive activity (hydrophobic contacts with FKBP51 are indicated in green, hydrogen bonds are represented as pink dotted lines), (c) prototypic cyclohexyl-substituted ligand 3, (d, e) binding mode of 2a with FKBP51, (d) surface representation of FKBP51 in complex with 2a (green). FK506 bound to FKBP51 (3O5R) is superimposed in yellow. (e) Ribbon representation of FKBP51 showing the conserved H-bonds between O1-2a and HN-Ile87 (dark blue) and between O8-2a and HO-Tyr113 (red) as black dotted lines. Leu119 and Pro120 at the top of the 80s loop are colored in cyan. The dipolar interaction between OH-Tyr113 and C1-carbonyl is shown as a dotted line in magenta.

The core interactions of FK506 are conserved for 2a with the common pipecolate ring sitting atop the indole of Trp90, which forms the floor of the FKBP binding pocket. The C1-carbonyl of the pipecolate forms a hydrogen bond with the backbone amide of Ile87 (d= 2.92 Å), while the C8-carbonyl of the α-ketoamide engages in a hydrogen bond with the hydroxyl group of Tyr113 (d= 2.65 Å). The latter approaches the C1-carbonyl at an angle of 107° and below van-der-Waals distance (3.17Å) consistent with an attractive dipolar interaction19. The C9-keto oxygen of 2a occupies a position similar to the keto group of FK506, while the hydrogen bond with Asp68 seen in 305R is no longer conserved owing to the absence of the corresponding hydroxyl group in compound 2a. The tert-pentyl group of compound 2a sits in pocket formed by the 80s loop (Ser118-Ile122) which is occupied by the pyranose group of FK506 in the FK506-FKBP51 complex. Compared to a similar compound (SB3) in a complex with FKBP12 (1FKG16) the ethyl of the tert-pentyl group is rotated by 180° and faces the 80s loop. The dimethoxyaryl group (ring A) of 2a sits in a cradle formed by residues Gly84-Ile87 and Tyr113 and engages in van-der-Waals contacts with Glu20 from a neighboring FKBP51 molecule in the crystal. The acetyloxyaryl group (ring B) stacks on top of the edge of Phe77 and its carboxyl moiety forms electrostatically enhanced hydrogen bonds with Lys108 and Arg31 from a neighboring molecule.

Structure-activity relationship (SAR) of the pipecolate core and ester substituent

So far virtually nothing is known about the interaction of the large FKBPs with small molecule ligands. To the best of our knowledge only one and three synthetic ligands have been described for FKBP51 and FKBP52, respectively17,20,21.

As a first characterization of the recognition properties of FKBP51 and FKBP52 we engaged on a basic structure-activity relationship analysis of the prototypic ligand 2a. The analogs of 2a (Tab. 1) were synthesized by esterification or by alkylation of the C1 carboxylate of the
building blocks 4a-d as outlined in Scheme 1 or Scheme S4. The latter were prepared from the corresponding piperolate analogs by N-oxalylation, introduction of the tert-pentyl moiety followed by deprotection of the C1 carboxylate (scheme S3). The 4,5-dehydro-piperolate building block 4c was synthesized from allyl glycine in four steps (scheme S2 and S3). Building block 5a was obtained in 98% enantiomeric excess and 94% yield by a Noyori-catalyzed enantioselective reduction of the known keto precursor 13a (Scheme S1). Building blocks 5b (Scheme S1) and 5c were synthesized as described.

Method A

\[ \text{Method A} \]

\[ a) X= Y = \text{CH}_2 \]
\[ b) X = \text{CH}_2, Y = \text{none} \]
\[ c) X = Y = \text{CH} \]
\[ d) X = \text{CH}_2, Y = S \]

\[ 4a-d \]

\[ 5a-c \]

\[ a \text{ or } b \]

\[ 2a-2d, 6a-6f \]

Method B

\[ 4a \]

\[ 7a-c \]

\[ c \]

\[ 5c, 5d \]

\[ 7a \]

\[ 7b \]

\[ 7c \]

Scheme 1: General synthesis protocol of compounds 2a-2d, 6a-6h. Reagent and conditions: (a) DCC, DMAP, rt, 12h. (b) (i) DCC, DMAP, rt, 12h. (ii) 20% TFA in DCM, rt, 6h. (c) DIPEA, toluene, reflux, 40h.

In an initial SAR analysis we explored the contributions of individual substructures in 2a by first focusing on the piperolate core. Replacement by a proline (2b) or a 4,5-dehydro-piperolic acid (2c) decreased the affinity for FKBPs 4-6 fold while thiophene-3-carboxylic acid (2d) abrogated detectable binding to the large FKBPs. Since even small changes at the core diminished affinity we kept the piperolate core constant in all further derivatives. We then replaced the piperolate C1 ester by an amide (2e) which completely abolished binding to larger FKBPs. This was anticipated since the additional hydrogen bond donor would point to the hydrophobic tert-pentyl group of 2e when bound in a homologous binding mode as 2a.

We next explored the requirements of the ester “top” group. Smaller substituents like in 6a-6d resulted in analogs with 7-100 fold lower affinity for FKBP12 and no activity for larger FKBPs.
as compared to 2a. Compound 6b (also called GPI-104624) has been reported as one of the most potent and advanced inhibitors for FKBP12. Similar to the corresponding piperolate analog 6e, GPI-1046 (6b) had no binding to larger FKBP8 and micromolar affinity to FKBP12 in the fluorescence polarization assay which is consistent with the discrepancies previously observed as reviewed by Gaali et.al for GPI-1046 by others1. To eliminate the negative charge in 2a we exchanged the free acid moiety by a morpholine group (6f) which increased affinity 2-4 fold and induced a slight preference for FKBP52 vs. FKBP51. In contrast to the carboxylic acid analog 2b, the morpholine-containing proline derivative 6f retained detectable but three-fold reduced binding. Replacement of the oxyacetyl group in 6g by an amine resulted in compound having similar affinity.

Table-1

<table>
<thead>
<tr>
<th>Compd. No</th>
<th>Structure</th>
<th>Purity</th>
<th>FKBP12 IC50(µM)</th>
<th>FKBP51 IC50(µM)</th>
<th>FKBP52 IC50(µM)</th>
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<tr>
<td>2a</td>
<td><img src="image" alt="Structure" /></td>
<td>&gt; 99%</td>
<td>0.17 ± 0.05</td>
<td>8.36 ± 0.98</td>
<td>10.5 ± 1.5</td>
</tr>
<tr>
<td>2b</td>
<td><img src="image" alt="Structure" /></td>
<td>&gt; 99%</td>
<td>0.80 ± 0.05</td>
<td>51.5 ± 31.9</td>
<td>41.6 ± 15.8</td>
</tr>
<tr>
<td>2c</td>
<td><img src="image" alt="Structure" /></td>
<td>&gt; 99%</td>
<td>0.55 ± 0.06</td>
<td>32.73 ± 12.3</td>
<td>49.2 ± 24.6</td>
</tr>
<tr>
<td>2d</td>
<td><img src="image" alt="Structure" /></td>
<td>&gt; 98%</td>
<td>1.29 ± 0.14</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>2e</td>
<td><img src="image" alt="Structure" /></td>
<td>&gt; 99%</td>
<td>3.38 ± 0.54</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

6a

> 99%  17.1 ± 2.7  >100  >100

6b

> 99%  1.24 ± 0.33  >100  >100

6c

> 99%  2.11 ± 0.20  >150  >150

6d

> 99%  2.45 ± 0.44  >100  >100

6e

> 99%  0.10 ± 0.02  4.15 ± 1.45  2.8 ± 1.10

6f

> 99%  1.05 ± 0.09  15.34 ± 1.94  5.55 ± 1.16

6g

≥98%  0.10 ± 0.05  3.8 ± 1.05  1.07 ± 0.84

6h

> 99%  0.15 ± 0.02  19.3 ± 6.6  11.6 ± 1.6

6i

>98%  0.017 ± 0.020  8.52 ± 2.81  7.37 ± 3.28

6j

> 99%  >100  >100  >100
Table-1 Purity of the compounds was confirmed by using HPLC. Binding affinities to FKBP12, FKBP51 (FK1 domain) and FKBP52 (FK1 domain) were determined by a fluorescence polarization assay\textsuperscript{17}.

Finally, we replaced the tert-pentyl group with 3,4,5-trimethoxyphenyl in 6h (scheme S6) which led to a two-fold decrease in affinity for FKBP51 while having equivalent binding for FKBP12 and FKBP52. Additionally, two FK506 analogs that had been evaluated in the clinic were tested for their binding to the larger FKBPs\textsuperscript{1}. Biricodar (VX-710, 6i) potently bound to FKBP12 while displaying moderate affinity for the larger FKBPs. In contrast, the related Timcodar (VX-853, 6j) which lacks the pipecolate core had no binding affinity for any FKBPs, consistent with the SAR data observed above.

**Exploration of pyranose/tert-pentyl analogs**

A three-dimensional alignment of FKBP12 and the FK506-binding domains of FKBP51 and FKBP52 revealed that the core residues of the binding pockets are highly conserved. The largest differences were found in the adjacent 40s and 80s loops (residues 71-76 and 118-122 for FKBP51, respectively). The 80s loop of FKBP51 further contains Leu\textsuperscript{119} which is replaced by Pro\textsuperscript{119} in FKBP52. Cellular studies have shown the residue at position 119 to be a major functional determinant for the effect on steroid hormone receptors\textsuperscript{25}. Optimization of interactions with this part of the protein thus could impart selectivity and functional efficacy towards steroid hormone receptor for the large FKBPs. We therefore decided to investigate the interaction with this part of the protein in more detail.

The X-ray structure of FK506 with FKBP12(1FKJ)\textsuperscript{26}, with the FK1 domains of FKBP51(PDB code 305R)\textsuperscript{18} and FKBP52 (manuscript in preparation) revealed that the pyranose group in FK506 (1) contacts the 80s loop. SAR studies around the pyranose group have shown that the methyl group at C\textsuperscript{11} of FK506 analogs is important while the pyranose ring oxygen is dispensable for binding to FKBP12\textsuperscript{27-29}. This is consistent with the FK506-FKBP51 co-crystal structure where the C\textsuperscript{11}-methyl fills a small hydrophobic cavity, while the pyranose ring oxygen of FK506 does not seem to act as a hydrogen bond acceptor\textsuperscript{18}. The pyranose of FK506 further contains an exocyclic hydroxyl group at C\textsuperscript{10} that engages in a hydrogen bond with Asp\textsuperscript{68} of FKBP51. This could contribute to the higher affinity observed for the natural product. The 2a-FKBP51 co-crystal structure shows that the tert-pentyl group in 2a occupies the same subpocket below the 80s loop as the pyranose ring in FK506. We therefore decided to replace
the tert-pentyl group in 2a with cyclohexyl derivatives that more closely resembled the pyranose in the high-affinity ligand FK506 (1). The first series of compounds investigated had a methyl substituent (3a) at C\(^{11}\) as in FK506. The FK506-FKBP51 crystal structure (305R) further revealed that the 80s subpocket in the large FKBPs is more open and has a potential hydrogen bond interaction partner (S\(^{118}\)) in its vicinity. We therefore also prepared cyclohexyl analogs with larger or hydrophilic C\(^{11}\) substituents.

Scheme 2: General synthesis protocol of diketo acids 11a-e. Reagent and conditions: (a) L-threonine, MgSO\(_4\), HCHO, THF, 5 days. (b) MOMCl, DIPEA, DCM 12h. (c) TMS acetylene, n-BuLi, -78°C, 2h. (d) N-bromosuccinimide, AgNO\(_3\), acetone, 2h. (e) KMnO\(_4\), pH 7 (MgSO\(_4\), NaHCO\(_3\)), MeOH: H\(_2\)O: 1:1, 0°C to room temperature, 1h. (f) 1M LiOH, MeOH, 6h.

A four step synthesis scheme for the α-keto acids 11a-b and 11d-e was set up starting from the corresponding racemic cyclohexanones 8a or 8b (Scheme 2). Alternatively, for 11c the enantiopure MOM-protected 2-hydroxymethyl cyclohexanone 8c was used. The latter was obtained in two steps from cyclohexanone by an organocatalyzed formylation\(^{30,31}\). TMS acetylene was reacted with 8a-c to obtain the cis and trans diastereomers 9a-9f (stereochemistry assigned by NMR\(^{33}\)) in nearly equal amounts which could be separated using column chromatography. N-bromosuccinimide was used to cleave the TMS group and introduce the bromide at the terminal alkynes (10a-e)\(^{34}\) followed by oxidation of the activated alkynes by KMnO\(_4\) to yield the corresponding α-keto esters\(^{35-38}\). These were further hydrolysed to give the α-keto acids 11a-b and 11d-e, as racemic mixtures, and enantiopure 11c.
The α-keto acids (11a-e) were coupled with the piperolic acid building blocks 12a-d as outlined in Scheme 3 to give compounds 3a-3g and 3i-3j as mixture of diastereomers and 3h as a single pure diastereomers. The affinities for FKBPs were either tested as mixture of diastereomers (3a-3g, 3i-3j) or after diastereomeric separation using preparative HPLC (Table-2).

**Scheme 3**: General synthesis protocol of compounds 3a-3j. *Reagent and conditions : (a) (S)-1-Boc-piperidine-2-carboxylic acid, K₂CO₃, KI, 60°C, 12 h. (b) 20% TFA in DCM, rt, 2 h. (c) (S)-1-Fmoc-piperidine-2-carboxylic acid, DCC, DMAP, rt, 12 h. (d) 20% 4-methylpiperidine in DCM, rt, 4 h. (e) 11a-e, HATU, DIPEA, rt, 16 h. (f) (i) 11a-e, HATU, DIPEA, rt, 16 h. (ii) 20% TFA in DCM, rt, 6 h.

Introduction of the FK506-like cyclohexyl moiety in 3a increased affinity for FKBPs two-fold compared to 2a indicating that the cyclohexyl moiety might indeed better interact with the 80s loop than the tert-pentyl group. We next explored the influence of the ester “top” group in the context of the cyclohexyl substituent. Removing the acetyloxaryl ring (ring B) as in 3c reduced the affinity for FKBPs by 6 fold. This is in contrast to the results observed for the C₁₁-ethyl analog 3d and the corresponding tert-pentyl containing substance 6d. Further shortening of the linker connecting the dimethoxyaryl moiety (ring A) as in 3b substantially decreased affinity for all FKBPs. This indicates that the linker length is critical for optimal positioning of the dimethoxyaryl moiety, at least in the cyclohexyl series. Similar to the tert-pentyl series, replacement of the carboxylate by a morpholine in compounds 3e and 3g increased affinity for FKBPs and induced a slight preference for FKBP52 compared to FKBP51.

We next investigated the role of the C₁₁ substituent on the cyclohexyl moiety. The C₁₁-methyl (3a*), C₁₁-ethyl (3f*) and C₁₁-hydroxymethyl derivative (3h) had similar binding for the larger FKBPs while the affinity for FKBP12 was reduced. Importantly, however, we also found that the diastereomeric mixtures 3i* and 3j* had almost equivalent binding to FKBPs as their FK506-like counterparts 3a* and 3f*. This was somewhat surprising since in the “unnatural”
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52 diastereomers $3i^*$ and $3j^*$ the Asp$^{68}$-HO$^{10}$ hydrogen bond and hydrophobic 80s loop contacts of the C$^{11}$-substituent are not possible at the same time. To further investigate the influence of stereochemistry and the substitution pattern at the cyclohexyl ring in more detail we separated the individual diastereomers $3a$-$1$, $3a$-$2$, $3i$-$1$, $3i$-$2$, $3f$-$1$ and $3f$-$2$.

Again, these diastereomers had almost equivalent binding to the proteins. These observation led us to conclude that the stereochemistry around the pyranose group in FK506/rapamycin like ligands is not as important for activity as previously thought and that the 80s loop is flexible enough to accommodate the small stereo chemical changes in the active site.

**Table-2**

<table>
<thead>
<tr>
<th>Compd. No.</th>
<th>R1</th>
<th>FKBP12 IC$_{50}$(µM)</th>
<th>FKBP51FK1</th>
<th>FKBP52FK1</th>
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<tr>
<td>$3a^*$</td>
<td><img src="image1" alt="Image" /></td>
<td>0.055 ± 0.004</td>
<td>4.20 ± 0.11</td>
<td>2.13 ± 0.21</td>
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<tr>
<td>$3b^*$</td>
<td><img src="image2" alt="Image" /></td>
<td>2.2 ± 0.5</td>
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<td>&gt;100</td>
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<tr>
<td>$3c^*$</td>
<td><img src="image3" alt="Image" /></td>
<td>0.31 ± 0.04</td>
<td>29.39 ± 8.5</td>
<td>11.7 ± 6.4</td>
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<tr>
<td>$3d^*$</td>
<td><img src="image4" alt="Image" /></td>
<td>2.78 ± 0.02</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>$3e^*$</td>
<td><img src="image5" alt="Image" /></td>
<td>0.057 ± 0.004</td>
<td>2.02 ± 0.14</td>
<td>0.89 ± 0.06</td>
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Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_1$ (μM)</th>
<th>$K_B$ (μM)</th>
<th>$K_i$ (μM)</th>
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<tr>
<td>3f*</td>
<td>0.32 ± 0.025</td>
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<td>9.5 ± 1.3</td>
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<tr>
<td>3f-1</td>
<td>0.128 ± 0.03</td>
<td>5.8 ± 0.6</td>
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<tr>
<td>3f-2</td>
<td>0.343 ± 0.09</td>
<td>3.9 ± 0.6</td>
<td>3.5 ± 0.6</td>
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<tr>
<td>3g*</td>
<td>0.47 ± 0.06</td>
<td>9.66 ± 0.83</td>
<td>3.72 ± 1.02</td>
</tr>
<tr>
<td>3h</td>
<td>0.507 ± 0.08</td>
<td>8.5 ± 0.6</td>
<td>6.2 ± 0.5</td>
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<tr>
<td>3i*</td>
<td>0.055 ± 0.004</td>
<td>4.13 ± 0.20</td>
<td>2.64 ± 0.19</td>
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<tr>
<td>3a-1</td>
<td>0.056 ± 0.004</td>
<td>4.27 ± 0.19</td>
<td>2.44 ± 0.17</td>
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<td>3a-2</td>
<td>0.048 ± 0.006</td>
<td>4.54 ± 0.24</td>
<td>2.88 ± 0.21</td>
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<tr>
<td>3i-1</td>
<td>0.049 ± 0.005</td>
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<td>2.20 ± 0.13</td>
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<tr>
<td>3i-2</td>
<td>0.063 ± 0.003</td>
<td>4.96 ± 0.25</td>
<td>2.64 ± 0.25</td>
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</table>
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

Table-2: *Mixture of diastereomers. Binding affinity to FKBP12, FKBP51 (FK1 domain) and FKBP52 (FK1 domain) determined by fluorescence polarization assay17.

Crystal structures of 3f-1 and 3f-2

To understand the unexpected binding of the non-canonical diastereomers we solved the co-crystal structure of both 3f-1 and 3f-2 with the FK506-binding domain of FKBP51 (Fig. 2). Depending on whether the complexes were crystallized or the compounds were added to pre-formed crystals, different crystal forms were obtained. In both co-crystal lattices the ligands engaged Glu20, Arg31 and Lys108 of a neighboring FKBP51 molecule, similar to the crystal contacts observed for 2a (see above).

Upon binding of compound 3f-1 or 3f-2 FKBP51 adopts the same structure as found in FKBP51 complexed with 1 and 2a. Likewise, the binding modes for the piperolate, the ester “top” group and the α-keto amide of 3f-1 or 3f-2 were almost perfectly superimposable to those found for 2a in complex with FKBP51. In particular the hydrogen bond network and the dipolar interaction comprising Ile87-NH, C1=O, Tyr113-OH and C8=O is conserved. In 3f-1 a hydrogen bond of C10-OH with Asp68 (d= 2.75 Å) is formed similar to the one observed for the pyranose group of FK506 (PDB code 3O5R). However, the cyclohexyl group in 3f-1 is slightly lifted out of the binding pocket and slightly rotated likely to relieve a steric clash of the larger C11 substituent. For the C11 substituent two orientations seem to be possible which occupy similar positions like the ethyl group of the tert-pentyl moiety in 2a (Fig. 2a). In the case of 3f-2 the cyclohexyl moiety is rotated by 180° which allows the C11 ethyl substituent to occupy almost an identical position as for 3f-1 indicating that this hydrophobic interaction might be rather important (Fig. 2b). In this conformation the hydrogen bond with Asp68 is no longer possible but the C10-OH now forms water-mediated hydrogen bonds to Tyr113 and Ser118. This water network might provide the binding energy to compensate for the loss of the C10-OH···Asp68 H-bond.
**Fig. 2** X-ray crystal structure of **3f-1** and **3f-2** in the FK506-binding domain of FKBP51. The hydrogen bonds between O¹ and HN-Ile⁸⁷ (shadowed blue) and between O⁸ and HO-Tyr¹¹³ (shadowed red) are represented as dotted black lines. The dipolar interaction between OH-Tyr¹¹³ and C¹ carbonyl is indicated by a dotted pink line. Leu¹¹⁹ and Pro¹²⁰ of the 80s-loop are indicated in cyan. (a) Binding mode of **3f-1** in the active site of FKBP51. The additional hydrogen bond between HO⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻˓ newArray.png
Experimental section:

Chemistry: Chromatographic separations were performed either by manual flash chromatography or by automated flash chromatography using an Interchim Puriflash 430 with an UV detector. Organic phases were dried over MgSO₄, and the solvents were removed under reduced pressure. Merck F-254 (thickness 0.25mm) commercial plates were used for analytical TLC to follow the progress of reactions. Silica gel 60 (Merck 70-230 mesh) was used for manual column chromatography. Unless otherwise specified, ¹H NMR spectra, ¹³C NMR spectra, 2D HSQC, HMBC and COSY of all intermediates were obtained from the Department of Chemistry and Pharmacy, LMU, on a Bruker AC 300, a Bruker XL 400, or a Bruker AMX 600 at room temperature. Chemical shifts for ¹H or ¹³C are given in ppm (δ) relative to tetramethylsilane (TMS) as internal standard. Mass spectra (m/z) were recorded on a Thermo Finnigan LCQ DECA XP Plus mass spectrometer at the Max Planck Institute of Psychiatry, while the high resolution mass spectrometry was carried out at MPI for Biochemistry (Microchemistry Core facility) on Varian Mat711 mass spectrometer. The purity of the compounds was verified by reversed phase HPLC.

HPLC conditions for product analysis: Column: Jupiter 4 µm Proteo 90 A, 250 x 4.6 mm, Phenomenex, Torrance, USA, Wavelength: 224nm, 280nm Flow rate: 1ml/min, Buffer A: 0.1% TFA in 5% MeCN/Water, Buffer B: 0.1% TFA in 95% MeCN/water. Gradient A: After 1 min elution with 100% buffer A, linear gradient of 0-100% buffer B for 30 min.

LCMS conditions for product analysis: Column: YMC Pack Pro C8, 100 x 4.6 mm, 3µm Wavelength: 224nm, 280nm Flow rate: 1ml/min, Buffer A: 0.1% HCOOH in 5% MeCN/water, Buffer B: 0.1% HCOOH in 95% MeCN/water. Gradient A: 1min 100% buffer A, then linear gradient of 0-100% buffer B for 11 min.

Preparative HPLC for diasteromer seperation: Compound was dissolved in 40% buffer B and the purification was carried out with a injection loop volume of 2ml, Column: Jupiter 10µm Proteo 90 A, 250 x 21.7 mm, 10micron Phenomenex, Torrance, USA, Wavelength: 224nm, Flow rate: 25ml/min, Buffer A: 0.1% TFA in 5% MeOH/Water, Buffer B: 0.1% TFA in 95% MeOH/water..

Synthesis of (S)-methyl 1-(3,3-dimethyl-2-oxopentanoyl)piperidine-2-carboxylate (4a)
The compound was prepared as described previously\textsuperscript{16}.
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Synthesis of (S)-methyl 1-(3,3-dimethyl-2-oxopentanoyl)pyrrolidine-2-carboxylate (4b)
Prepared from the methyl ester of L-Proline in an analogous manner to 4a.

General Method A.
A solution of alcohol 5a-c, carboxylic acid 4a-d and DMAP in DCM at room temperature was treated with DCC. After stirring for 12 h the mixture was diluted with EtOAc and filtered through a plug of celite. The filtrate was concentrated and the crude material flash chromatographed to afford the product.

General Method B.
A solution of bromide 7a-c and carboxylic acid 4a or 4b was treated with DIPEA in toluene at reflux for 40 h. Afterwards, the mixture was diluted with EtOAc (30ml) and filtered through a plug of celite. The filtrate was concentrated and the crude material flash chromatographed to afford the product.

Synthesis of 2-(3-((R)-3-(3,4-dimethoxyphenyl)-1-((S)-1-(3,3-dimethyl-2-oxopentanoyl)piperidine-2-carbonyloxy)propyl)phenoxy)acetic acid 2a
The compound was prepared as described previously. 1

Synthesis of 2-(3-((R)-3-(3,4-dimethoxyphenyl)-1-((S)-1-(3,3-dimethyl-2-oxopentanoyl)pyrrolidine-2-carbonyloxy)propyl)phenoxy)acetic acid 2b
General method A was used for coupling of alcohol 5a (75.9mg, 0.188 mmol) and acid 4b (50 mg, 0.207 mmol) using DMAP (2.5 mg, 0.0207 mmol) and DCC (43mg, 0.207 mmol). The crude product was chromatographed using Hexane: EtOAc 3: 1 to afford ester 2.

TLC (Hexane: EtOAc 3:1): Rf = 0.19.
HPLC (Gradient A) retention time= 30.1-30.4min

Ester 2 was treated with 20% TFA in DCM at room temperature. The mixture was stirred for 6h. TFA and DCM were evaporated under reduced pressure to yield the free acid 2b (22.6mg, 0.039mmol, 21%) over two steps.

TLC (Hexane: EtOAc: MeOH: AcOH: 6:3: 0.5: 0.5): Rf = 0.25.
HPLC (Gradient A) retention time= 24.8-25.2min

1H NMR (600 MHz, CDCl3) δ= 0.79 (t, 3H, J= 7.2Hz), 1.14 (s, 3H), 1.16 (s, 3H), 1.59-1.70 (s, 2H), 1.89-2.05 (m, 4H), 2.15-2.24 (m, 2H), 2.48-2.60 (m, 2H), 3.44-3.70 (m, 2H), 3.80 (s, 3H),
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3.82 (s, 3H), 4.55 (s, 2H), 4.58-4.61 (m, 1H), 5.66-5.72 (m, 1H), 6.62-6.66 (m, 2H), 6.74 (d, 1H, J= 8.4Hz), 6.78-6.81 (m, 1H), 6.85-6.89 (m, 2H), 7.18-7.21 (m, 1H).

$^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$= 9.05, 23.20, 23.76, 24.99, 29.89, 31.36, 32.33, 38.26, 47.05, 47.46, 56.04, 58.78, 65.74, 76.39, 111.52, 112.03, 112.56, 114.58, 119.63, 120.39, 129.86, 133.76, 141.91, 147.48, 149.02, 158.05, 163.74, 165.53, 170.83, 207.13.

MS (ESI) m/z: found Rt 11.52 min. (Method LCMS), 592.32 [M + Na]$^+$. HRMS 570.3268 [M + H]$^+$, calculated 570.3225 [M + H]$^+$.

Synthesis of 2-(3-((R)-3-(3,4-dimethoxyphenyl)-1-((S)-1-(3,3-dimethyl-2-oxopentanoyl)-1,2,3,6-tetrahydropyridine-2-carbonyloxy)propyl)phenoxy)acetic acid 2c

General method A was used for coupling of alcohol 5a (71.7mg, 0.178 mmol) and acid 4c (50 mg, 0.19 mmol) using DMAP (2.4 mg, 0.0196 mmol) and DCC (39.2 mg, 0.19 mmol). The crude product was chromatographed using Hexane: EtOAc 3: 1 to afford ester 3 (115mg, 0.180 mmol, 91%)

TLC (Hexane: EtOAc 1: 1): Rf = 0.8.

HPLC (Gradient A) retention time= 30.2-30.6min

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$= 0.77-0.91 (m, 3H), 1.12 (d, 2H, J= 4.2Hz), 1.21 (s, 3H), 1.22 (s, 3H), 1.47 (s, 9H), 1.96-2.08 (m, 1H), 2.15-2.28 (m, 1H), 2.44-2.62 (m, 3H), 2.66-2.83 (m, 1H), 3.72-4.00 (m, 8H), 4.34-4.47 (m, 1H), 4.52 (s, 2H), 5.48-5.59 (m, 1H), 5.69-5.86 (m, 2H), 6.64-6.67 (m, 2H), 6.75-6.87 (m, 3H), 6.92 (d, 1H, J= 7.8Hz), 7.21-7.27 (m, 1H).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$= 9.00, 23.54, 23.69, 26.21, 28.24, 31.36, 32.71, 38.21, 43.15, 46.9, 54.6, 56.03, 56.14, 65.95, 76.87, 82.52, 111.57, 111.99, 113.24, 114.50, 119.94, 120.40, 122.61, 124.07, 129.90, 133.65, 141.50, 147.56, 149.11, 158.32, 167.42, 168.07, 169.61, 207.48.


Ester 3 (100 mg, 0.157 mmol) was treated with 20% TFA in DCM at room temperature. The mixture was stirred for 6h. TFA and DCM were evaporated under reduced pressure to yield the free acid 2c (61mg, 0.104mmol, 67%).

TLC (Hexane: EtOAc: AcOH: 5:5: 0.5): Rf = 0.35.

HPLC (Gradient Acetate) retention time= 25.4-25.9min

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$= 0.89 (t, 3H, J= 7.5 Hz), 1.22 (s, 3H), 1.23 (s, 3H), 1.67-1.75 (m, 3H), 1.99-2.29 (m, 2H), 2.51-2.64 (m, 3H), 3.73 (t, 1H, J= 18.8 Hz), 3.85 (s, 3H), 3.86 (s,
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$^3$H), 3.97 (d, 1H, J= 18.7 Hz), 4.37-4.38 (m, 1H), 4.68 (s, 2H), 5.50-5.89 (m, 3H), 6.66 (s, 1H), 6.69 (s, 1H), 6.77-6.81 (m, 1H), 6.83- 6.94 (m, 3H), 7.17-7.24 (m, 1H).

$^{13}$C NMR (75 MHz, CDCl$_3$) δ= 9.06, 23.56, 23.66, 27.38, 29.37, 31.44, 32.73, 38.31, 43.26, 54.75, 56.10, 56.17, 65.36, 76.88, 111.64, 112.06, 112.82, 114.92, 120.48, 122.57, 123.98, 125.53, 130.07, 133.61, 141.88, 147.63, 149.14, 158.07, 167.34, 169.54, 172.76, 207.48.


**Synthesis of 2-(3-((R)-3-(3,4-dimethoxyphenyl)-1-((R)-4-(3,3-dimethyl-2-oxopentanoyl)thiomorphone-3-carbonyloxy)propyl)phenoxy)acetic acid 2d**

General method A was used for coupling of alcohol 5a (67mg, 0.166 mmol) and acid 4d (50 mg, 0.183 mmol) using DMAP (2.2 mg, 0.018 mmol) and DCC (38 mg, 0.183 mmol). The crude product was chromatographed using Hexane: EtOAc 3: 1 to afford ester 4 (104mg, 0.158 mmol, 87%)

TLC (Hexane: EtOAc 1: 1): Rf = 0.80.

HPLC (Gradient A) retention time= 30.2-30.6min

$^1$H NMR (600 MHz, CDCl$_3$) δ= 0.83-0.86 (m, 3H), 1.19 (s, 3H), 1.21 (s, 3H), 1.44 (s, 9H), 1.56-1.63 (m, 1H), 1.68-1.76 (m, 1H), 2.01-2.08 ((m, 1H), 2.20-2.26 (m, 1H), 2.33-2.38 (m, 1H), 2.51-2.65 (m, 2H), 2.71-2.81 (m, 1H), 2.91-3.03 (m, 1H), 3.08-3.27 (m, 1H), 3.51-3.58 (m, 2H), 3.81 (s, 3H), 3.82 (s, 3H), 4.48 (s, 2H), 5.55-5.56 (m, 1H), 5.74-5.84 (m, 1H), 6.65-6.68 (m, 2H), 6.74-6.76 (m, 1H), 6.79 (d, 1H, J= 8Hz), 6.86 (s, 1H), 6.92 (d, 1H, J= 8.2Hz), 7.20-7.25 (m, 1H).

$^{13}$C NMR (150 MHz, CDCl$_3$) δ= 8.92, 23.05, 23.84, 27.37, 28.23, 28.72, 31.36, 32.64, 38.23, 45.60, 47.00, 51.72, 56.02, 56.12, 65.96, 77.37, 82.56, 111.55, 112.03, 113.46, 114.37, 119.98, 120.41, 129.94, 133.71, 141.32, 147.54, 149.07, 158.33, 167.87, 168.05, 171.27, 207.64.


**Ester 4** (100 mg, 0.152 mmol) was treated with 20% TFA in DCM at room temperature. The mixture was stirred for 6h. TFA and DCM were evaporated under reduced pressure to yield the free acid 2d (58.1mg, 0.096mmol, 64%).

TLC (Hexane: EtOAc: AcOH: 5:5: 0.5): Rf = 0.42.

HPLC (Gradient A) retention time= 25.2-25.7min
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$^{1}$H NMR (600 MHz, CDCl$_3$) $\delta$ = 0.84-0.87 (m, 3H), 1.17 (s, 3H), 1.20 (s, 3H), 1.55-1.76 (m, 2H), 2.05-2.10 (m, 1H), 2.21-2.28 (m, 1H), 2.34-2.41 (m, 1H), 2.56-2.69 (m, 2H), 2.74-2.84 (m, 1H), 2.92-3.04 (m, 1H), 3.10-3.24 (m, 1H), 3.50-3.59 (m, 2H), 3.82 (s, 3H), 3.84 (s, 3H), 4.64 (s, 2H), 5.56-5.57 (m, 1H), 5.76-5.83 (m, 1H), 6.65-6.69 (m, 2H), 6.75-6.76 (m, 1H), 6.80-6.86 (m, 2H), 6.91 (d, 1H, J= 7.8 Hz), 7.21-7.25 (m, 1H).

$^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$ = 8.91, 23.04, 23.81, 27.38, 28.76, 31.42, 32.65, 38.30, 45.67, 47.06, 51.96, 56.08, 56.14, 65.30, 80.28, 111.61, 112.11, 112.44, 114.87, 130.11, 133.65, 141.72, 147.56, 149.08, 158.02, 167.78, 168.02, 173.02, 207.55.


**Synthesis of 2-(3-(3,4-dimethoxyphenyl)-1-((S)-1-(3,3-dimethyl-2-oxopentanoyl)piperidine-2-carboxamido)propyl)phenoxy)acetic acid (mixture of diasteromers) 2e**

General method A was used for coupling of amine 19 (71.5mg, 0.178 mmol) and acid 4a (50 mg, 0.195 mmol) using DMAP (2.3 mg, 0.019 mmol) and DCC (40 mg, 0.195 mmol). The crude product was chromatographed using Hexane: EtOAc 1: 1 to afford ester 5 (110mg, 0.172 mmol, 96%)

**TLC (Hexane: EtOAc 1: 1):** Rf = 0.7.

**HPLC (Gradient A) retention time= 28.2-28.8min**

$^{1}$H NMR (300 MHz, CDCl$_3$) $\delta$ = 0.82-0.92 (m, 3H), 1.18-1.26 (m, 6H), 1.46 (s, 9H), 1.57-1.75 (m, 5H), 2.23-2.70 (m, 3H), 3.16-3.38 (m, 1H), 3.83 (s, 3H), 3.84 (s, 3H), 4.43-4.45 (m, 2H), 4.87-4.96 (m, 1H), 5.06 (t, 1H, J= 6Hz), 6.63-6.96 (m, 6H), 7.19-7.28 (m, 1H).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ = 8.94, 9.03, 20.60, 21.21, 22.92, 22.96, 23.33, 23.39, 23.44, 23.72, 25.26, 25.36, 28.37, 32.66, 32.89, 32.91, 38.45, 38.78, 44.59, 44.64, 46.77, 46.80, 51.59, 53.43, 53.46, 56.02, 56.11, 60.55, 65.91, 82.46, 111.55, 112.07, 113.32, 113.58, 119.72, 119.77, 120.36, 120.38, 130.01, 130.10, 133.93, 134.00, 143.95, 147.48, 149.06, 149.08, 158.42, 158.46, 166.64, 166.74, 168.08, 169.00, 169.17, 171.27, 207.98, 208.01, 210.02, 210.31.


**Ester 5 (110 mg, 0.172 mmol) was treated with 20% TFA in DCM at room temperature.** The mixture was stirred for 6h. TFA and DCM were evaporated under reduced pressure to yield the free acid 2e (40mg, 0.068mmol, 40%).

**TLC (Hexane: EtOAc: AcOH 5: 5: 0.5):** Rf = 0.19.
HPLC (Gradient A) retention time= 26.2-26.8 min

$^1$H NMR (600 MHz, CDCl$_3$) δ= 0.86-0.94 (m, 3H), 1.08-1.16 (m, 6H), 1.42 (s, 9H), 1.49-1.65 (m, 5H), 2.20-2.60 (m, 3H), 3.05-3.40 (m, 1H), 3.79 (s, 3H), 3.80 (s, 3H), 4.46-4.49 (m, 2H), 4.86-4.96 (m, 1H), 5.07 (t, 1H, J= 6Hz), 6.63-6.95 (m, 6H), 7.20-7.28 (m, 1H).

$^{13}$C NMR (150 MHz, CDCl$_3$) δ= 8.94, 9.03, 20.60, 21.21, 22.92, 22.96, 23.33, 23.39, 23.44, 23.72, 25.26, 25.36, 32.66, 32.89, 32.91, 38.45, 38.78, 44.59, 44.64, 46.77, 46.80, 51.59, 53.43, 53.46, 56.02, 56.11, 60.55, 65.91, 111.55, 112.07, 113.32, 113.58, 119.72, 119.77, 120.36, 120.38, 130.01, 130.10, 133.93, 134.00, 143.95, 147.48, 149.06, 149.08, 158.42, 158.46, 166.64, 166.74, 168.08, 169.00, 169.17, 171.27, 207.98, 208.01, 210.02, 210.31.

MS (ESI) m/z: found Rt 11.01 min. (Method LCMS), 583.28 [M + H]$^+$, 605.60 [M + Na]$^+$.


**Synthesis of (S)-methyl 1-(3,3-dimethyl-2-oxopentanoyl)piperidine-2-carboxylate 6a**

The compound was prepared as described previously$^{16}$. 

TLC (Hexane : EtOAc 8: 2): Rf = 0.40, Yield- 640mg (73%).

HPLC (Gradient A) retention time= 22.8-23.1 min

$^1$H NMR (400 MHz, CDCl$_3$) δ= 0.83 (dt, 3H, J= 1.2, 7.2Hz), 1.14 (s, 3H), 1.17 (s, 3H), 1.26-1.50 (m, 2H), 1.58-1.74 (m, 5H), 2.24 (d, 1H, J= 14Hz), 3.12-3.19 (m, 1H), 3.33 (d, 1H, J= 13.8Hz), 3.70 (s, 3H), 5.20 (d, 1H, J= 5.6Hz).

$^{13}$C NMR (100 MHz, CDCl$_3$) δ= 8.69, 21.11, 22.85, 23.54, 24.82, 26.27, 32.45, 43.89, 46.70, 51.06, 52.35, 167.49, 170.77, 207.77.

MS (ESI) m/z: found Rt 12.94 min. (Method LCMS), 270.30 [M + H]$^+$, 292.24 [M + Na]$^+$.


**Synthesis of (S)-3-(pyridin-3-yl)propyl 1-(3,3-dimethyl-2-oxopentanoyl)pyrrolidine-2-carboxylate 6b**

General method A was used for coupling of alcohol 5d (34mg, 0.249 mmol) and acid 4b (60 mg, 0.248 mmol) using DMAP (3.3 mg, 0.027 mmol) and DCC (65.8 mg, 0.298 mmol). The crude product was chromatographed using DCM: MeOH 9.3: 0.7 to afford 6b (52mg, 0.144 mmol, 57%).

TLC (DCM: MeOH 9:7:0.3): Rf = 0.39.

HPLC (Gradient A) retention time= 14.87-15.26 min
$^1$H NMR (400 MHz, CDCl$_3$) $\delta=$ 0.85 (t, 3H, $J = 7.6$ Hz), 1.20 (s, 3H), 1.24 (s, 3H), 1.64-1.71 (m, 2H), 1.91-2.04 (m, 5H), 2.19-2.30 (m, 1H), 2.66-2.73 (m, 2H), 3.40-3.54 (m, 2H), 4.08-4.20 (m, 2H), 4.49-4.52 (m, 1H), 7.19-7.23 (m, 1H), 7.50-7.53 (m, 1H), 8.45 (s, 2H).

$^{13}$C NMR (100 MHz, CDCl$_3$) $\delta=$ 8.92, 23.10, 23.70, 24.87, 29.17, 29.92, 32.34, 33.93, 46.94, 47.17, 58.33, 64.18, 123.37, 135.98, 147.54, 149.83, 165.14, 171.41, 206.98


**Synthesis of (S)-3-(pyridin-3-yl)propyl(3,3-dimethyl-2-oxopentanoyl)piperidine-2-carboxylate 6c**

General method B was used for reacting the bromide 7a (37.8mg, 0.19mmol) and acid 4a (48.5 mg, 0.19 mmol) using DIPEA (24.7 mg, 0.23 mmol). The crude product was chromatographed using DCM: MeOH 9.3: 0.7 to afford 6c (45mg, 0.12 mmol, 64%)

TLC (DCM: MeOH 9.7:0.3): Rf = 0.30,

HPLC (Gradient A) retention time- 16.9-17.4 min

$^1$H NMR (300 MHz, CDCl$_3$) $\delta=$ 0.82 (t, 3H, $J = 7.5$ Hz), 1.16 (d, 6H, $J = 9.3$ Hz), 1.36 (s, 2H), 1.59-1.70 (m, 4H), 1.87-1.95 (m, 2H), 2.23 (d, 2H, $J = 14.16$ Hz), 2.65 (t, 2H, $J = 7.9$ Hz), 3.15-3.31 (m, 2H), 4.12 (t, 2H, $J = 8.0$ Hz), 5.21 (d, 1H, $J = 5.3$ Hz), 7.46 (d, 2H, $J = 7.8$ Hz), 8.40 (s, 2H).

MS (ESI) m/z: found Rt 4.80 min. (Method LCMS), 375.39 [M + H]$^+$, HRMS 375.2642 [M + H]$^+$, calculated 375.2606 [M + H]$^+$.

**Synthesis of (S)-2-(3,4-dimethoxyphenoxy)ethyl 1-(3,3-dimethyl-2-oxopentanoyl)piperidine-2-carboxylate 6d**

General method B was used for reacting the bromide 7b (50mg, 0.19mmol) and acid 4a (50mg, 0.196mmol) using DIPEA (24.7mg, 0.23mmol). The crude product was chromatographed using Hexane: EtOAc 4:1 to afford 6d (70mg, 0.16mmol, 84%).

TLC (Hexane: EtOAc 4:1): Rf = 0.45.

HPLC (Gradient A) retention time= 25.8-26.4 min

$^1$H NMR (300 MHz, CDCl$_3$) $\delta=$ 0.86 (t, 3H, $J = 7.5$Hz), 1.16 (s, 3H), 1.20 (s, 3H), 1.58-1.79 (m, 7H), 2.33 (d, 1H, $J = 14.1$ Hz), 3.18-3.28 (m, 1H), 3.38 (d, 1H, $J = 13.2$ Hz), 3.82 (s, 3H), 3.84 (s, 3H), 4.13 (t, 2H, $J = 4.8$ Hz), 4.44-4.56 (m, 2H), 5.30 (d, 1H, $J = 5.1$Hz), 6.37 (dd, 1H, $J = 2.7$, 8.7 Hz), 6.50 (d, 1H, $J = 4.05$ Hz), 6.76 (d, 1H, $J = 8.7$ Hz).
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$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta =$ 8.94, 21.37, 23.07, 23.87, 25.11, 26.62, 32.07, 44.19, 46.94, 51.42, 56.08, 56.64, 63.90, 66.50, 101.31, 104.22, 112.03, 144.16, 150.15, 153.16, 167.74, 170.59, 207.98.


Synthesis of (S)-(R)-3-(3,4-dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propyl-1-(3,3-dimethyl-2-oxopentanoyl)piperidine-2-carboxylate 6e

General method A was used for coupling of alcohol 5b (95mg, 0.235 mmol) and acid 4a (60 mg, 0.235 mmol) using DMAP (3.1 mg, 0.026 mmol) and DCC (62.1 mg, 0.282 mmol). The crude product was chromatographed using DCM: MeOH 9.3: 0.7 to afford 6e (51mg, 0.079 mmol, 34%)

TLC (DCM: MeOH 9.7:0.3): Rf = 0.62.

HPLC (Gradient A) retention time= 18.87-19.32 min

$^1$H NMR (400 MHz, CDCl$_3$) $\delta =$ 0.87 (t, 3H, J= 7.6 Hz), 1.19 (s, 3H), 1.21 (s, 3H), 1.56-1.76 (m, 7H), 1.99-2.08 (m, 1H), 2.19-2.37 (m, 2H), 2.47-2.59 (m, 6H), 2.80 (t, 2H, J= 5.2Hz), 3.14 (dt., 1H, J= 3.2, 13.2 Hz), 3.34 (d, 1H, J= 13.2 Hz), 3.73 (t, 4H, J= 4.4 Hz). 3.84 (s, 3H), 3.85 (s, 3H), 4.11, (t, 2H, J= 5.6 Hz), 5.30 (d, 1H, J= 5.2 Hz), 5.75 (q, 1H, J= 2.4, 5.6 Hz), 6.65-6.68 (m, 2H), 6.75-6.78 (m, 1H), 6.81-6.84 (m, 1H), 6.87-6.92 (m, 2H), 7.22-7.26 (m, 1H).

$^{13}$C NMR (100 MHz, CDCl$_3$) $\delta =$ 8.74, 21.18, 23.09, 23.49, 24.94, 26.41, 31.24, 32.45, 38.01, 44.12, 46.66, 51.23, 54.04, 55.80, 55.89, 57.63, 65.68, 66.81, 77.02, 111.25, 111.69, 113.01, 114.20, 119.04, 120.11, 129.64, 133.43, 141.31, 147.29, 148.83, 158.82, 167.19, 169.65, 207.76.


Synthesis of (S)-(R)-3-(3,4-dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propyl-1-(3,3-dimethyl-2-oxopentanoyl)pyrrolidine-2-carboxylate 6f

General method A was used for coupling of alcohol 5b (50mg, 0.124 mmol) and acid 4b (30 mg, 0.124 mmol) using DMAP (1.6 mg, 0.013 mmol) and DCC (33 mg, 0.149 mmol). The crude product was chromatographed using DCM: MeOH 9.3: 0.7 to afford 6f (45mg, 0.072 mmol, 59%).
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

TLC (DCM: MeOH 9.7:0.3): Rf = 0.38.

HPLC (Gradient A) retention time= 18.79-19.19 min

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$= 0.81-0.86 (m, 3H), 1.19-1.22 (m, 6H), 1.56-1.75 (m, 3H), 1.89-2.01 (m, 4H), 2.17-2.25 (m, 1H), 2.45-2.61 (m, 6H), 2.79 (s, 2H), 3.45-3.54 (m, 2H), 3.72 (t, 4H, J= 4.8 Hz), 3.83 (s, 3H), 3.85 (s, 3H), 4.11 (t, 2H, J= 5.6 Hz), 4.56-4.66 (m, 1H), 5.65-5.77 (m, 1H), 6.64-6.66 (m, 1H), 6.67 (s, 1H), 6.75-6.77 (m, 1H), 6.79-6.84 (m, 1H), 6.88-6.90 (m, 2H), 7.20-7.25 (m, 1H).

$^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ = 8.92, 23.23, 23.59, 24.93, 31.07, 32.20, 33.93, 38.04, 46.86, 47.14, 49.06, 54.04, 55.81, 57.63, 65.67, 66.85, 76.19, 111.21, 111.79, 112.70, 114.08, 118.75, 120.11, 129.50, 131.63, 141.48, 147.21, 148.78, 158.78, 165.04, 170.62, 207.01.

MS (ESI) m/z: found Rt 10.59 min. (Method LCMS), 625.47 [M + H]$^+$,


(S)-(R)-1-(3-aminophenyl)-3-(3,4-dimethoxyphenyl)propyl 1-(3,3-dimethyl-2-oxopentanoyl)piperidine-2-carboxylate 6g

Obtained from Cayman Chemicals, Compound name: SLF, Cat no. 1000974.

Synthesis of 2-(3-((R)-3-(3,4-dimethoxyphenyl)-1-((S)-1-(2-oxo-2-(3,4,5-trimethoxyphenyl)acetyl)piperidine-2-carboxyloxy)propyl)phenoxy)acetic acid 6h

To a stirred solution of the 12c (51mg, 0.1mmol) in DCM under argon was added sequentially N,N-Diisopropyl-ethylamine (DIPEA) (15.7mg, 0.125mmol) to which was added 22 (26mg, 0.1mmol). The reaction mixture was stirred for 1h at room temperature. Saturated NH$_4$Cl solution was added to the reaction and the solution was stirred for 10 min. The organic phase was separated and the aqueous phase was extracted with ethyl acetate (3x 100ml). The combined organic phases were washed with brine (10ml), dried over MgSO$_4$ and the residual solid was purified by column chromatography using Hexane : EtOAc 4:1 to afford ester 6 (35.8mg, 0.048mmol, 49%).

TLC (Hexane: EtOAc 4:1): Rf = 0.45.

HPLC (Gradient A) retention time= 18.92-19.16min

$^1$H NMR (600 MHz, CDCl$_3$) $\delta$ = 1.48 (s, 9H), 1.58-1.66 (m, 1H), 1.78-1.85 (m, 3H), 2.06-2.12 (m, 1H), 2.24-2.30 (m, 2H), 2.44 (d, 1H, J= 12.6 Hz), 2.52-2.57 (m, 1H), 2.59-2.64 (m, 1H), 3.27 (t, 1H, J= 12.6 Hz), 3.48 (d, 1H, J= 12.6Hz), 3.82 (s, 6H), 3.85 (s, 6H), 3.92 (s, 3H), 4.52 (s, 2H), 5.41 (d, 1H, J= 4.8Hz), 5.73 (t, 1H, J= 6.6Hz), 6.63-6.70 (m, 3H), 6.76-6.81 (m, 2H), 6.90 (s, 1H), 6.95 (d, 1H, J= 7.8 Hz), 7.09 (s, 1H), 7.28-7.35 (m, 1H).
Ester 6 (35.8 mg, 0.048 mmol) was treated with 20% TFA in DCM at room temperature. The mixture was stirred for 6 h. TFA and DCM were evaporated under reduced pressure to yield the free acid 6h (30.9 mg, 0.045 mmol, 96%).

TLC (Hexane: EtOAc: AcOH 5:5: 0.5): Rf = 0.37.

HPLC (Gradient A) retention time = 23.8–24.6 min

$^1$H NMR (600 MHz, CDCl$_3$) δ = 1.50–1.56 (m, 1H), 1.61–1.66 (m, 1H), 1.83–1.91 (m, 2H), 1.98–2.10 (m, 2H), 2.22–2.28 (m, 1H), 2.42 (d, 1H, J = 13.8 Hz), 2.56–2.61 (m, 1H), 2.64–2.69 (m, 1H), 3.25–3.30 (m, 1H), 3.45 (d, 1H, J = 12 Hz), 3.71 (s, 6H), 3.84 (s, 3H), 3.85 (s, 3H), 3.88 (s, 3H), 4.58 (s, 2H), 5.40 (d, 1H, J = 5.4 Hz), 5.56–5.66 (m, 1H), 6.66–6.67 (m, 2H), 6.75–6.78 (m, 1H), 6.82 (d, 1H, J = 7.8 Hz), 6.87–6.88 (m, 2H), 7.16 (s, 2H), 7.22 (t, 1H, J = 7.8 Hz).

$^{13}$C NMR 150 MHz, CDCl$_3$ δ = 24.84, 28.41, 28.44, 31.65, 38.45, 44.56, 52.17, 56.06, 56.14, 56.39, 61.14, 77.40, 107.09, 111.43, 111.58, 111.89, 114.92, 119.85, 120.41, 127.87, 129.85, 133.48, 142.25, 144.19, 147.64, 149.15, 153.63, 158.17, 158.36, 170.23, 177.39, 190.70

MS (ESI) m/z: found Rt 13.29 min. (Method LCMS), 702.22 [M + Na]$^+$.


Synthesis of (S)-1,7-di(pyridin-3-yl)heptan-4-yl 1-(3,3-dimethyl-2-oxopentanoyl)piperidine-2-carboxylate (Biricodar) 6i

The compound was prepared as described previously.$^{23,39}$

Timcodar 6j

Obtained from the group of Dr. Edwin Sanchez.

Synthesis of (S)-2-((MOM)methyl)cyclohexanone 8c

The (S)-2-(hydroxymethyl)cyclohexanone was prepared as already described.$^{31}$ To this compound (1.5 g, 11.7 mmol) in DCM was added $N,N$-Diisopropyl-ethylamine (3.7 g, 29.3 mmol). The mixture was stirred at 0°C for 5 min before MOM-Cl (2 g, 25.7 mmol) was added. After stirring for 3 h the mixture was concentrated under reduced pressure and subjected to purification by column chromatography using Hexane: EtOAc 8:2 to afford 8c (1.8 g, 10.5 mmol, 90%) as a colorless liquid.

TLC (Hexane: EtOAc 8:2): Rf = 0.45
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ = 1.36-1.49 (m, 1H), 1.54-1.71 (m, 2H), 1.77-1.89 (m, 1H), 1.95-2.06 (m, 1H), 2.13-2.38 (m, 3H), 2.50-2.60 (m, 1H), 3.29 (s, 3H), 3.41-3.47 (m, 1H), 3.75-3.80 (m, 1H), 4.58 (d, 2H, $J = 2.1$ Hz).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta = 24.63, 27.62, 31.16, 41.99, 50.67, 55.08, 66.72, 96.62, 211.13$


**General procedure for the synthesis of 2-alkyl-1-((trimethylsilyl)ethynyl)cyclohexanol 9**

The THF solution of lithium reagent was generated by treating trimethylsilylacetylene (3ml, 21.4 mmol) with $n$-BuLi (2M in hexane, 11.6ml) at -78°C. The solution was stirred for 0.5h at that temperature. To this a solution of 2-alkylcyclohexanone (8a-8c) (17.8 mmol) in THF (5ml) was added and stirred for an additional 2h. Then the solution was quenched by addition of a saturated aqueous NH$_4$Cl solution. The organic phase was separated and the aqueous phase was extracted with ethyl acetate (3x 100ml). The combined organic phases were washed with brine (30ml) and dried over MgSO$_4$. The solution was concentrated and then flash chromatographed using Hexane: EtOAc 9:1 to afford each of the two diastereomers 9a-9f.

**(1S,2R)-2-Methyl-1-((trimethylsilyl)ethynyl)cyclohexanol and (1R,2S)-2-Methyl-1-((trimethylsilyl)ethynyl)cyclohexanol 9a**

Compound 9a (1.2g, 33%) was obtained from 8a (2g) as a colorless liquid.

TLC (Hexane: EtOAc 9:1): Rf = 0.36

$^1$H NMR (300 MHz, CDCl$_3$) $\delta = 0.17$(s, 9H), 1.06(d, 3H, $J = 6.9$ Hz), 1.23-1.33 (m, 1H), 1.48-1.71 (m, 7p), 1.95-2.02 (m, 1P).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta = 0.01, 16.00, 21.06, 25.02, 29.15, 39.15, 40.48, 69.77, 86.95, 110.61$


**(1S,2R)-2-Ethyl-1-((trimethylsilyl)ethynyl)cyclohexanol and (1R,2S)-2-Ethyl-1-((trimethylsilyl)ethynyl)cyclohexanol 9b**

Compound 9b (1.65g, 46%) was obtained from 8b (2.2g) as a colorless liquid.

TLC (Hexane: EtOAc 9:1): Rf = 0.40

$^1$H NMR (300 MHz, CDCl$_3$) $\delta = 0.17$(s,9H), 0.86-0.96 (m, 6H), 1.12-2.42 (m, 22H).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta = 0.024, 11.68, 12.30, 21.23, 22.88, 24.80, 25.46, 28.00, 33.28, 39.47, 41.94, 47.47, 52.31, 70.32, 87.09, 110.85$

(1S,2S)-2-((methoxymethoxy)methyl)-1-(((trimethylsilyl)ethynyl)cyclohexanol 9c:
Compound 9c (2.1g, 46%) was obtained from 8c (3g) as a colorless liquid.
TLC (Hexane: EtOAc 9:1): Rf = 0.45
$^1$H NMR (300 MHz, CDCl$_3$) δ= 0.17(s, 9H), 1.31-2.04 (m, 9H), 3.40(s, 3H), 3.58 (dd, 1H, J = 2.4, 9.3 Hz), 4.26 (dd, 1H, J = 3.6, 9.6 Hz), 4.64 (dd, 2H, J = 6.6, 10.8 Hz).
$^{13}$C NMR (75 MHz, CDCl$_3$) δ = 0.00, 20.70, 24.67, 25.57, 39.18, 45.07, 55.41, 69.77, 70.75, 87.06, 96.54, 110.30.

(1R,2R)-2-Methyl-1-(((trimethylsilyl)ethynyl)cyclohexanol and (1S,2S)-2-Methyl-1-((trimethylsilyl)ethynyl)cyclohexanol 9d:
Compound 9d (1.1g, 31%) was obtained from 8a (2g) as a colorless liquid.
TLC (Hexane: EtOAc 9:1): Rf = 0.32
$^1$H NMR (300 MHz, CDCl$_3$) δ = 0.18 (s, 9H), 1.04 (d, 3H, J = 6.6 Hz), 1.17-1.32 (m, 2H), 1.41-1.73 (m, 6H), 1.96-2.02 (m, 1H).
$^{13}$C NMR (75 MHz, CDCl$_3$) δ = 0.02, 16.03, 24.29, 25.50, 32.29, 40.63, 42.53, 73.42, 90.63, 106.80.

(1R,2R)-2-Ethyl-1-(((trimethylsilyl)ethynyl)cyclohexanol and (1S,2S)-2-Ethyl-1-((trimethylsilyl)ethynyl)cyclohexanol 9e:
Compound 9e (1.3g, 37%) was obtained from 8b (2.2g) as a colorless liquid.
TLC (Hexane: EtOAc 9:1): Rf = 0.37
$^1$H NMR (300 MHz, CDCl$_3$) δ = 0.19(s, 9H), 0.94 (t, 3H, J= 7.2Hz), 1.04-1.30 (m, 4H), 1.43-1.74 (m, 4H), 1.80-2.01 (m, 3H).
$^{13}$C NMR (75 MHz, CDCl$_3$) δ = 0.03, 11.93, 21.73, 24.15, 25.49, 28.36, 41.11, 49.56, 73.04, 90.46, 107.33.

(1R,2S)-2-((methoxymethoxy)methyl)-1-(((trimethylsilyl)ethynyl)cyclohexanol 9f:
Compound 9f (1.9g, 41%) was obtained from 8c (3g) as a colorless liquid.
TLC (Hexane: EtOAc 9:1): Rf = 0.40
1H NMR (300 MHz, CDCl₃) δ= 0.18 (s, 9H), 1.03-1.29 (m, 2H), 1.46-1.55 (m, 3H), 1.67-1.71 (m, 2H), 1.80-1.90 (m, 1H), 2.02-2.06 (m, 1H), 3.41 (s, 3H), 3.48 (dd, 1H, J= 4.2, 9.6 Hz) 3.87 (T, 1H, J= 9.9 Hz), 4.64 (s, 2H).

13C NMR (75 MHz, CDCl₃) δ= 0.04, 23.16, 24.94, 26.29, 39.48, 46.06, 55.59, 71.68, 72.87, 90.49, 96.49, 106.78.


**General procedure for the synthesis of 2-alkyl-1-(bromoethynyl)cyclohexanol 10**

To solution of 9a-e (1.3 mmol), N-bromosuccinimide (1.5 mmol) and AgNO₃ (0.5 mmol) in acetone (10 ml) was added and the resulting solution was stirred in darkness for 2h at room temperature. Acetone was evaporated under reduced pressure and the solids were removed by filtration through a celite pad (washing with ether). The combined organic phase were concentrated and subjected to purification by column chromatography using Hexane: EtOAc 9:1 to yield 10a-e as yellow liquids.

(1S,2R)-1-(Bromoethynyl)-2-methylcyclohexanol and (1R,2S)-1-(Bromoethynyl)-2-methyl cyclohexanol 10a

Compound 10a (256 mg, 91%) was obtained from 9a (273mg) as a yellow liquid.

TLC (Hexane: EtOAc 9:1): Rf = 0.30

1H NMR (300 MHz, CDCl₃) δ= 1.06 (d, 3H, J = 6.9 Hz), 1.29-1.73 (m, 8H), 1.97-2.04 (m, 1H).

13C NMR (75 MHz, CDCl₃) δ= 16.05, 20.94, 24.95, 29.09, 39.17, 40.52, 43.04, 70.79, 84.60.


(1S,2R)-1-(Bromoethynyl)-2-ethylcyclohexanol and (1R,2S)-1-(Bromoethynyl)-2-ethylcyclohexanol 10b

Compound 10b (264 mg, 88%) was obtained from 9b (292 mg) as a yellow liquid.

TLC (Hexane: EtOAc 9:1): Rf = 0.36

1H NMR (300 MHz, CDCl₃) δ= 0.96 (d, 3H, J = 7.2 Hz), 1.13-1.30 (m, 3H), 1.36-1.45 (m, 1H), 1.50-1.74 (m, 5H), 1.83-2.02 (m, 2H).

13C NMR (75 MHz, CDCl₃) δ= 12.17, 21.10, 23.03, 24.81, 25.35, 39.55, 43.17, 47.78, 71.39, 84.74.

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**(1S,2S)-1-(Bromoethynyl)-2-((methoxymethoxy)methyl)cyclohexanol 10c**

Compound **10c** (327 mg, 90%) was obtained from **9c** (350 mg) as a yellow liquid.

TLC (Hexane: EtOAc 9:1): Rf = 0.39

$^1$H NMR (300 MHz, CDCl$_3$) δ= 1.33-2.07 (m, 9H), 3.41 (s, 3H), 3.59 (dd, 1H, J = 2.4, 9.6 Hz), 4.24 (dd, 1H, J = 3.3, 9.6 Hz), 4.65 (dd, 2H, J = 6.6, 13.2 Hz).

$^{13}$C NMR (75 MHz, CDCl$_3$) δ= 20.61, 24.67, 25.51, 39.17, 43.23, 45.09, 55.39, 70.66, 70.87, 84.39, 96.51.


**(1R,2R)-1-(Bromoethynyl)-2-methylcyclohexanol and (1S,2S)-1-(Bromoethynyl)-2-methylcyclohexanol 10d**

Compound **10d** (254 mg, 90%) was obtained from **9d** (270 mg) as a yellow liquid.

TLC (Hexane: EtOAc 9:1): Rf = 0.28

$^1$H NMR (300 MHz, CDCl$_3$) δ= 1.05 (d, 3H, J= 6.6 Hz), 1.20-1.29 (m, 2H), 1.48-1.75 (m, 6H), 2.00-2.05 (m, 1H).

$^{13}$C NMR (75 MHz, CDCl$_3$) δ= 16.66, 24.18, 25.43, 32.16, 40.63, 42.73, 45.35, 74.40, 81.18.


**(1R,2R)-1-(Bromoethynyl)-2-ethylcyclohexanol and (1S,2S)-1-(Bromoethynyl)-2-ethylcyclohexanol 10e**

Compound **10e** (267 mg, 89%) was obtained from **9e** (292 mg) as a yellow liquid.

TLC (Hexane: EtOAc 9:1): Rf = 0.32

$^1$H NMR (300 MHz, CDCl$_3$) δ= 0.97 (d, 3H, J = 7.2 Hz), 1.09-1.25 (m, 3H), 1.36-1.76 (m, 6H), 1.87-2.03 (m, 2H).

$^{13}$C NMR (75 MHz, CDCl$_3$) δ= 12.57, 23.15, 24.97, 25.68, 25.55, 40.51, 45.60, 48.11, 75.20, 81.16.


**General procedure for the synthesis of α-ketoesters of 11a-e**

To a solution of **10a-e** (1.08 mmol) in MeOH (5 ml) was added a solution of NaHCO$_3$ (45.5 mg, 0.54 mmol) and MgSO$_4$ (261 mg, 2.16 mmol) in water (5 ml) at 0°C. The mixture was stirred for 10 min vigorously before KMnO$_4$ (513 mg, 3.25 mmol) was added. The mixture was allowed to warm to room temperature and stirred at this temperature for 1 h. The solids were removed by filtration through celite pad and washed with ethyl acetate. The organic phase was separated.
and the aqueous phase was extracted with ethyl acetate (3x 100ml). The combined organic phases were washed with brine (30ml) and dried over MgSO₄. The solution was concentrated and then flash chromatographed using Hexane: EtOAc 9:1 to afford the corresponding α-ketoesters of 11a-e.

**Methyl 2-((1S,2R)-1-hydroxy-2-methylcyclohexyl)-2-oxoacetate and Methyl 2-((1R,2S)-1-hydroxy-2-methylcyclohexyl)-2-oxoacetate**

The corresponding α-ketoester of 11a (117 mg, 65%) was obtained from 10a (235 mg) as an oily liquid.

TLC (Hexane: EtOAc 9:1): Rf = 0.61

^1^H NMR (300 MHz, CDCl₃) δ = 0.81 (d, 3H, J = 6.6 Hz ), 1.25-1.90 (m, 8H), 2.03- 2.16 (m, 1H), 3.90 (s, 3H).

^1^3^C NMR (75 MHz, CDCl₃) δ = 16.00, 20.16, 25.35, 29.27, 34.99, 36.25, 52.74, 80.88, 163.61, 200.66.

**Methyl 2-((1S,2R)-2-ethyl-1-hydroxycyclohexyl)-2-oxoacetate and Methyl 2-((1R,2S)-2-ethyl-1-hydroxycyclohexyl)-2-oxoacetate**

The corresponding α-ketoester of 11b (160 mg, 69%) was obtained from 10b (250 mg) as an oily liquid and was further hydrolyzed without further purification.

TLC (Hexane: EtOAc 9:1): Rf = 0.64

**Methyl 2-((1S,2S)-1-hydroxy-2-((methoxymethoxy)methyl)cyclohexyl)-2-oxoacetate**

The corresponding α-ketoester of 11c (170 mg, 60%) was obtained from 10c (300 mg) as an oily liquid.

TLC (Hexane: EtOAc 9:1): Rf = 0.60

^1^H NMR (300 MHz, CDCl₃) δ= 1.44-2.40 (m, 8H), 2.59-2.70 (m,1H), 3.27(s, 3H), 3.38- 3.42 (m, 2H), 3.87 (s, 3H), 4.42 (dd, 2H, J = 6.6, 18.6 Hz).

^1^3^C NMR (75 MHz, CDCl₃) δ= 20.74, 23.65, 24.81, 35.62, 42.23, 52.54, 55.50, 68.34, 78.81, 96.24, 161.37, 197.77.

MS (ESI) m/z 282.73[M + Na]^+, calculated 282.71 [M + Na]^+.

**Methyl 2-((1R,2R)-1-hydroxy-2-methylcyclohexyl)-2-oxoacetate and Methyl 2-((1S,2S)-1-hydroxy-2-methylcyclohexyl)-2-oxoacetate**
The corresponding α-ketoester of 11d (85 mg, 47%) was obtained from 10d (235 mg) as an oily liquid.

TLC (Hexane: EtOAc 9:1): Rf = 0.58

$^1$H NMR (300 MHz, CDCl$_3$) δ= 0.76 (d, 3H, J = 3.6 Hz), 1.29-1.83 (m, 8H), 1.99- 2.11 (m, 1H), 3.86 (s, 3H).

$^{13}$C NMR (75 MHz, CDCl$_3$) δ= 15.96, 20.11, 25.31, 29.22, 34.95, 36.21, 52.68, 80.84, 163.62, 200.69.

**Methyl 2-((1R,2R)-2-ethyl-1-hydroxycyclohexyl)-2-oxoacetate and Methyl 2-((1S,2S)-2-ethyl-1-hydroxycyclohexyl)-2-oxoacetate**

The corresponding α-ketoester of 11e (127 mg, 55%) was obtained from 10e (250 mg) as an oily liquid and was further hydrolyzed without further purification.

TLC (Hexane: EtOAc 9:1): Rf = 0.61

**General procedure for the synthesis of 2-(1-hydroxy-2-alkycyclohexyl)-2-oxoacetic acid (11a-e)**

To the above synthesized α-ketoesters was added 1M LiOH in MeOH: H$_2$O (1:1) and the reaction stirred for 6h at room temperature. The reaction was acidified to pH=2 by addition of 1M HCl. The aqueous layer was extracted with ethyl acetate (3x 20ml). The combined organic phases were washed with brine (30ml) and dried over MgSO$_4$. The solution was concentrated under reduced pressure to furnish the free acid 11a-e as an oily liquid.

**2-((1S,2R)-1-Hydroxy-2-methylcyclohexyl)-2-oxoacetic acid and 2-((1R,2S)-1-Hydroxy-2-methylcyclohexyl)-2-oxoacetic acid 11a**

Compound 11a (105 mg, overall yield for 2 steps 52%) was obtained from 10a (235 mg) as a oily liquid.

TLC (Hexane: EtOAc: TFA 9:1:0.1): Rf = 0.28

$^1$H NMR (400 MHz, CDCl$_3$) δ= 0.78 (d, 3H, J = 6.8 Hz), 1.33-1.95 (m, 8H), 2.15 - 2.24 (m, 1H).

$^{13}$C NMR (100 MHz, CDCl$_3$) δ= 16.32, 20.32, 25.46, 29.41, 35.12, 36.57, 81.55, 162.81, 200.53.

**2-((1S,2R)-2-Ethyl-1-hydroxycyclohexyl)-2-oxoacetic acid and 2-((1R,2S)-2-Ethyl-1-hydroxycyclohexyl)-2-oxoacetic acid 11b**
Compound **11b** (141 mg, overall yield for 2 steps 65%) was obtained from **10b** (250 mg) as a oily liquid.

TLC (Hexane: EtOAc: TFA 9:1:0.1): Rf = 0.26

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ = 0.83 (t, 3H, J = 7.6 Hz), 1.13-1.36 (m, 4H), 1.57-1.62 (m, 2H), 1.73-1.96 (m, 5H).

$^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ = 11.82, 20.39, 23.95, 25.08, 25.46, 35.32, 43.14, 82.20, 164.12, 201.23.

**2-((1S,2S)-1-Hydroxy-2-((methoxymethoxy)methyl)cyclohexyl)-2-oxoacetic acid 11c**

Compound **11c** (170 mg, overall yield for 2 steps 59%) was obtained from **10c** (300 mg) as an oily liquid.

TLC (Hexane: EtOAc: TFA 9:1:0.1): Rf = 0.26

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ = 1.44-1.67 (m, 7H), 2.70-2.80 (m, 1H), 3.26-3.41 (m, 6H), 4.41-4.47 (m, 2H).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ = 20.59, 23.34, 24.53, 35.27, 42.79, 55.74, 68.03, 78.38, 96.12, 160.39, 196.63.

**2-((1S,2S)-2-Methoxycyclohexyl)-2-oxoacetic acid and 2-((1S,2S)-1-Hydroxy-2-methylo cyclohexyl)-2-oxoacetic acid 11d**

Compound **11d** (68 mg, overall yield for 2 steps 34%) was obtained from **10d** (235 mg) as an oily liquid.

TLC (Hexane: EtOAc: TFA 9:1:0.1): Rf = 0.24

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ = 0.76 (d, 3H, J = 6.6 Hz), 1.29-1.51 (m, 3H), 1.55-1.62 (m, 2H), 1.67-1.72 (m, 2H), 1.83-1.93 (m, 1H), 2.06-2.18 (m, 1H).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ = 16.00, 20.22, 25.37, 29.31, 34.88, 36.21, 80.82, 164.55, 201.85

**2-((1R,2R)-2-Ethyl-1-hydroxycyclohexyl)-2-oxoacetic acid and 2-((1S,2S)-2-Ethyl-1-hydroxy cyclohexyl)-2-oxoacetic acid 11e**

Compound **11e** (101 mg, overall yield for 2 steps 51%) was obtained from **10e** (250 mg) as an oily liquid.

TLC (Hexane: EtOAc: TFA 9:1:0.1): Rf = 0.23

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ = 0.83 (t, 3H, J = 4.2 Hz), 1.12-1.33 (m, 4H), 1.56-1.63 (m, 2H), 1.72-1.97 (m, 5H).
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$^3$C NMR (75 MHz, CDCl$_3$) δ= 11.79, 20.38, 23.94, 25.08, 25.47, 35.31, 43.15, 82.21, 164.10, 201.14

**Synthesis of tert-butyl 2-(3-(3-(3,4-dimethoxyphenyl)propanoyl)phenoxy)acetate 13a**

A solution of corresponding phenol$^{15}$ (5g, 15.46 mmol) and K$_2$CO$_3$ (4.8g, 34.9mmol) in acetone (30mL) was treated with tert-butyl bromoacetate (3.7g, 19.21mmol) and stirred at room temperature for 20h. After this time the reaction mixture was filtered, concentrated and flash chromatographed to afford compound 5 (6.6g, 16.5mmol, 94%).

TLC (Hexane:EtOAc 8:2): RF = 0.50.

$^1$H NMR (400 MHz, CDCl$_3$) δ= 1.45 (s, 9H), 2.97 (t, 2H, J = 8 Hz), 3.22 (t, 2H, J = 8 Hz), 3.82 (s, 3H), 3.83 (s, 3H), 4.52 (s, 2H), 6.73-6.78 (m, 3H), 7.09 (dd, 1H, J = 0.8, 2.4 Hz), 7.33 (t, 1H, J = 8 Hz), 7.43 (m, 1H), 7.53 (dd, 1H, J = 1.2, 7.6).

$^{13}$C NMR (100 MHz, CDCl$_3$) δ= 27.99, 29.77, 40.70, 55.80, 65.63, 82.55, 111.35, 111.81, 113.06, 119.99, 120.12, 121.39, 129.66, 133.77, 138.22, 147.38, 148.88, 158.12, 167.57, 198.77. MS (ESI) m/z 439.13 [M + K]$^+$, calculated 439.15 [M + K]$^+$. 

**Synthesis of 3-(3,4-Dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propan-1-one 13b**

A solution of the corresponding phenol (15.6 g, 59.5 mmol) in dry DMF (300 ml) under an atmosphere of nitrogen was treated with K$_2$CO$_3$ (33.2 g, 240 mmol) and 4-(2-chloroethyl)morpholine hydrochloride (11.1 g, 59.6 mmol). The mixture was heated with stirring at 90°C for 2 hours until TLC indicated complete conversion. The mixture was cooled to room temperature and poured into ice-cold water (3.2 l). The precipitate of the title compound was collected by filtration, washed with water (3 x 200 ml) and dried in vacuo to yield 13b (17.7 g, 44.3 mmol, 74%) The product was used for the next step without further purification.

$^1$H NMR (300 MHz, CDCl$_3$) δ= 2.49-2.54 (m, 4H), 2.75 (t, J = 5.7 Hz, 2H), 2.94 (t, J = 7.7 Hz, 2H), 3.20 (t, J = 7.7 Hz, 2H), 3.65-3.69 (m, 4H), 3.79 (s, 3H), 3.81 (s, 3H), 4.08 (t, J = 5.6 Hz, 2H), 6.69-6.74 (m, 3H), 7.04 (dd, J = 0.8 Hz, J = 2.6 Hz, 1H), 7.29 (t, J = 7.9 Hz, 1H), 7.41-7.49, (m, 2H).

**Synthesis of (R)-tert-butyl2-(3-(3-(3,4-dimethoxyphenyl)-1-hydroxypropyl)phenoxy)acetate 5a**

A solution of the ketone 13a (6.5g, 16.48mmol) in isopropanol (50 ml) was charged into the hydrogenation reactor (High-pressure laboratory autoclave Model IV from Roth) along with
K$_2$CO$_3$ (2.3g, 16.48mmol). The reactor was flushed twice with argon. Afterwards Noyuri catalyst (ABCR AB131600) was added. The reactor was flushed with argon, sealed and hydrogen gas was flushed into the reactor twice. The reaction was then stirred at room temperature with hydrogen gas at 15 bar pressure for 6 days. Afterwards the reaction was filtered through celite pad and washed continuously with diethyl ether. The organic solvent was dried under vacuum to yield compound 6 (6.2g, 15.42mmol, 94%, 99% ee using chiracel OD-H column Hexane: isopropanol isocratic gradient).

TLC (Hexane:EtOAc 8:2): Rf = 0.3

$^1$H NMR (300 MHz, CDCl$_3$) δ= 1.47 (s, 9H), 2.03 (m, 2H), 2.642 (m, 2H), 3.83 (s, 3H), 3.84 (s, 3H), 4.49 (s, 2H), 4.66-4.61 (m, 1H), 6.70-6.80 (m, 4H), 6.95-6.91 (m, 2H), 7.24(t, 1H, $J = 7.8$ Hz).

$^{13}$C NMR (75 MHz, CDCl$_3$) δ= 28.01, 31.56, 40.62, 55.79, 55.90, 65.62, 73.55, 82.32, 111.31, 111.80, 112.18, 113.53, 119.08, 120.19, 129.48, 134.40, 146.56, 147.16, 148.82, 158.07, 168.01.


**Synthesis of (R)-3-(3,4-Dimethoxyphenyl)-1-(3-(2-morphinoethoxy)phenyl)propan-1-ol 5b**

Dry THF (35 ml) was added under an atmosphere of nitrogen to the 13b (25 g, 62.6 mmol). The mixture was cooled to -20°C and a 1.8 M solution of (+)-B-chlorodiisopinocampheylborane [(+)-DIP chloride] in hexane (53 ml, 95.4 mmol) which had been diluted with dry THF (70 ml) was slowly added dropwise. The temperature was kept below -10°C and the mixture stirred for 3 hours. It was placed in a refrigerator overnight and another 0.2 equivalents of (+)-DIP chloride was added. After another day at -20°C the solvent was removed under reduced pressure, the residue treated with ether (170 ml) and the mixture cooled to 0°C. Diethanolamine (60 ml) was added and it was stirred for a while. The formed precipitate was removed by filtration and washed with ether. The combined filtrates were concentrated under reduced pressure, and the title compound was obtained from the residue by column chromatography as oil (silica gel; CH$_2$Cl$_2$ / MeOH gradient 100:0 to 94:6) to give 5b (17.2 g, mmol, 68 %).

$^1$H NMR (300MHz, CDCl$_3$) δ= 1.84-2.08 (m, 2H), 2.27 (bs, 1H), 2.45-2.52 (m, 4H), 2.52-2.67 (m, 2H), 2.70 (t, $J = 5.8$ Hz, 2H), 3.61-3.67 (m, 4H), 3.78 (2s, 6H), 4.02 (t, $J = 5.8$ Hz, 2H), 4.54-4.60 (m, 1H), 6.62-6.76 (m, 4H), 6.82-6.87 (m, 2H), 7.14-7.21 (m, 1H).

**Synthesis of (S)-1-(((9H-fluoren-9-yl)methoxy)carbonyl)piperidine-2-carboxylic acid 28**

A solution of the L-pipecolic acid 27 (3.6g, 38.7 mmol) in 40ml of 10% sodium carbonate was dissolved in a round bottom flask and stirred for 5 min at room temperature. To this solution was added Fmoc oxy-succinimide (8.5g, 34.8 mmol) dissolved in 35 ml dioxane and the reaction was stirred overnight. After 24h water was added and the aqueous layer was extracted with ethyl acetate. The aqueous layer was acidified (pH=2) by addition of concentrated HCl. The acidic layer was extracted with ethyl acetate (3x 40ml). The organic phase was washed with 1N HCl followed by brine, dried over MgSO₄ and concentrated under reduced pressure to yield an oily colorless liquid. The oily liquid was dissolved in ether and cooled to yield a fluffy white solid which was washed with hexane and dried to yield compound 7 (8.2g, 38.7mmol, 83%).

TLC (Hexane:EtOAc: TFA 1:1: 0.2): RF = 0.60

HPLC (Gradient A) retention time= 24.6-24.8 min

^1^H NMR (300 MHz, CDCl₃) δ= 1.28-1.53 (m, 2H), 1.69-1.82 (m, 3H), 2.19-2.37 (m, 1H), 3.15 (t, 1H, J= 13.2Hz), 4.05-4.33 (m, 2H), 4.37-4.49 (m, 2H), 4.76-5.05(m, 1H), 7.28-7.41 (m, 4H), 7.55-7.62 (m, 2H), 1.77 (s, 2H).

^1^3^C NMR (75 MHz, CDCl₃) δ= 20.72, 24.70, 26.55, 41.94, 47.25, 54.19, 67.86, 119.97, 125.08, 127.07, 127.68, 141.33, 143.89, 156.65, 177.36

MS (ESI) m/z 352.66 [M + H]^+, calculated 352.40 [M + H]^+.

**Synthesis of (S)-1-tert-butyl 2-(3,4-dimethoxyphenethyl) piperidine-1,2-dicarboxylate (precursor of 12a)**

To a solution of 7c (385 mg, 1.57 mmol) in acetone (10ml) was added (S)-1Boc-piperidine-2-carboxylic acid (300mg, 1.30 mmol), K₂CO₃ (217 mg, 1.57 mmol), and KI (catalytic amount). The reaction mixture was stirred at 60°C for 12h. The mixture was filtered and the solid residue washed with ethyl acetate (3 X 30 ml). The combined organic phases were washed with brine (30ml) and dried over MgSO₄. The solution was concentrated and then the residue was purified by chromatography using Hexane: EtOAc 8:2 to afford **precursor of 12a** (440mg, 1.12 mmol, 85%).

TLC (Hexane:EtOAc 8:2): RF = 0.46

^1^H NMR (300 MHz, CDCl₃) δ= 1.46 (s,1H), 1.58-1.59 (m, 3H), 2.17 (d, 1H, j= 13.2 Hz), 2.91 (t, 2H, J= 6.9 Hz), 3.87 (s, 3H), 3.89 (s, 3H), 3.91-4.17 (m, 2H), 4.35 (t, 2H, J= 6.9 Hz), 4.71-4.88(m, 1H), 6.75-6.83(m, 3H).
13C NMR (75 MHz, CDCl3) δ= 28.36, 34.75, 55.80, 55.94, 65.53, 79.89, 111.35, 112.11, 120.90, 147.78, 148.95.


**Synthesis of 4-(2-bromoethoxy)-1,2-dimethoxybenzene (7b)**

A solution of 3,4-dimethoxyphenol (500mg, 3.24 mmol) in acetone (5ml) was added to K₂CO₃ (538 mg, 3.89 mmol) and the reaction was stirred for 10min. Dibromoethane (2.4g, 12.97 mmol) was added to the reaction mixture before being heated to reflux for 12h. Afterwards the mixture was cooled to room temperature and 1M NaOH solution was added. The organic phase was separated and the aqueous phase was extracted with ethyl acetate (3x 30ml). The combined organic phases were washed with brine (30ml), dried over MgSO₄ and the residual product was purified by chromatography using Hexane:EtOAc 7:3 to afford 7b (510mg, 1.95 mmol, 60%).

TLC (Hexane:EtOAc 7:3): RF = 0.41

1H NMR (300 MHz, CDCl3) δ= 3.64 (t, 2H, J= 6.3 Hz), 3.85 (s,3H), 3.87 (s,3H), 4.26 (t, 2H, J= 6.3 Hz), 6.42 (dd, 1H, J= 2.7, 8.7 Hz), 6.57 (d, 1H, J= 2.7Hz), 6.79 (d, 1H, J= 8.7Hz).

13C NMR (75 MHz, CDCl3) δ= 20.30, 55.89, 56.42, 65.59, 101.37, 104.27, 111.75, 144.09, 149.97, 152.05

MS (ESI) m/z 261.18 [M + H]+, calculated 261.26 [M + H]+.

**Synthesis of (S)-1-tert-butyl2-(2-(3,4-dimethoxyphenoxy)ethyl)piperidine-1,2-dicarboxylate (precursor of 12b)**

To a solution of 7b (200mg, 0.76 mmol) in acetone (10ml) was added (S)-1Boc-piperidine-2-carboxylic acid (150 mg, 0.65 mmol), K₂CO₃ (108 mg, 0.78 mmol), and KI (catalytic amount). The reaction mixture was stirred at 60°C for 12h. The mixture was filtered and the solid residue was washed with ethyl acetate (3 X 30 ml). The combined organic phases were washed with brine (30ml) and dried over MgSO₄. The solution was concentrated and then the residual crude product was purified by chromatography using Hexane:EtOAc 7:3 to afford precursor of 12b (200mg, 0.50 mmol, 77%).

TLC (Hexane:EtOAc 7:3): RF = 0.39

1H NMR (600 MHz, CDCl3) δ= 1.42 (d, 9H, J= 19.2 Hz), 1.56-1.65 (m, 4H), 2.17-2.23 (m, 1H), 2.86- 3.01 (m, 1H), 3.82 (s,3H), 3.83 (s,3H), 3.88-4.02 (m, 1H), 4.11 (t, 2H, J= 4.8 Hz), 4.45 (t, 2H, J= 4.8 Hz), 4.75 (s, 0.5H), 4.91 (s, 0.5H), 6.37 (dd, 1H, J= 3, 9 Hz), 6.51 (d, 1H, J= 1.2 Hz), 6.76 (d, 1H, J= 8.4 Hz).
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$^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$ = 26.79, 28.37, 41.03, 42.08, 53.79, 54.86, 55.81, 56.39, 63.17, 66.49, 101.08, 103.94, 111.68, 143.82, 149.84, 153.02, 171.89.

MS (ESI) m/z 432.20[M + Na$^+$], 448.20[M + K$^+$], calculated 432.20[M + Na$^+$], 448.17[M + K$^+$].

Synthesis of (S)-1-(9H-fluoren-9-yl)methyl 2-((R)-1-(3-(2-tert-butoxy-2-oxoethoxy)phenyl)-3-(3,4-dimethoxyphenyl)propyl) piperidine-1,2-dicarboxylate: (precursor of 12c)

A solution of alcohol 5a (2g, 4.97 mmol), carboxylic acid 28 (1.9g, 5.47 mmol), and DMAP (0.06 g, 0.54mmol) in 30mL DCM at room temperature was treated with DCC (1.1g, 5.47 mmol). The mixture was stirred for 12h. Afterwards the organic solvent was dried and the solid was dissolved in diethyl ether (50mL) and filtered through a plug of celite. The filtrate was concentrated and then flash chromatographed using Hexane: EtOAc 2:1 to afford precursor of 12c (3.5g, 4.78 mmol, 96%).

TLC (Hexane :EtOAc 2:1): Rf = 0.4.

$^1$H NMR (600 MHz, CDCl$_3$) $\delta$ = 1.46 (s, 10H), 1.68-1.76 (m, 3H), 1.98-2.04 (m, 1H), 2.14-2.22 (m, 1H), 2.29-2.33 (m, 1H), 2.41-2.58 (m, 2H), 2.96-3.15 (m, 1H), 3.81 (s, 3H), 3.83 (s, 3H), 4.07-4.15 (m, 2H), 4.26-4.49 (m, 5H), 5.02 (d, 1H, J= 5.4 Hz), 5.73-5.77 (m, 1H), 6.57-6.63 (m, 2H), 6.72-6.81 (m, 2H), 6.88 (s, 1H), 6.93-6.95 (m, 1H), 7.16-7.24 (m, 2H), 7.28-7.48 (m, 2H), 7.57-7.80 (m, 1H), 7.69-7.71 (m, 1H), 7.75-7.77 (m, 1H).

$^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$ = 15.35, 17.89, 20.80, 24.76, 26.81, 28.00, 31.10, 38.03, 42.02, 47.20, 55.77, 55.89, 63.75, 65.72, 67.77, 76.18, 82.35, 104.18, 111.26, 111.56, 111.65, 113.23, 113.90, 119.64, 119.88, 119.89, 119.93, 125.06, 127.02, 127.62, 127.66, 129.65, 133.46, 141.26, 141.67, 143.85, 144.08, 147.25, 148.81, 156.38, 158.01, 167.88, 170.86.

MS (ESI) m/z 758.60[M + Na$^+$], 774.53[M + K$^+$], calculated 758.33[M + Na$^+$], 774.30 [M + K$^+$].

Synthesis of (S)-1-(9H-fluoren-9-yl)methyl 2-((R)-1-(3-(3,4-dimethoxyphenyl)propyl) piperidine-1,2-dicarboxylate (precursor of 12d)

A solution of alcohol 5b (171 mg, 0.427mmol), carboxylic acid 28 (150mg, 0.427 mmol), and DMAP (5.7 mg, 0.047mmol) in 10mL DCM at room temperature was treated with DCC (113 mg, 0.51 mmol). The mixture was stirred for 12h after which the organic solvent was dried. The solid was dissolved in diethyl ether (50mL) and filtered through a plug of celite. The filtrate was concentrated and then flash chromatographed using DCM: MeOH 9.7:0.3 to afford precursor of 12d (280 mg, 0.38 mmol, 89%).
TLC (EtOAc: 1): Rf = 0.56.

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ = 1.31-1.51 (2H), 1.67-1.82 (m, 3H), 1.89-1.99 (m, 1H), 2.14-2.39 (m, 2H), 2.47-2.64 (m, 6H), 2.70-2.83 (m, 2H), 2.96-3.21 (m, 1H), 3.74 (s, 4H), 3.83 (s, 3H), 3.85 (s, 3H), 4.00-4.04 (m, 1H), 4.09-4.17 (m, 4H), 4.26-4.49 (m, 3H), 5.74-5.80 (m, 1H), 6.60-6.67 (m, 2H), 6.74-6.94 (m, 4H).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ = 21.04, 24.95, 25.63, 31.22, 38.09, 41.98, 47.22, 54.00, 55.81, 55.92, 57.59, 60.38, 65.57, 66.75, 67.78, 76.28, 111.33, 111.73, 113.04, 113.93, 118.95, 119.95, 120.10, 125.06, 127.06, 127.67, 129.64, 133.51, 141.27, 141.52, 141.71, 143.87, 144.08, 147.33, 148.87, 156.38, 158.77, 170.94.

MS (ESI) m/z 735.57 [M + H]$^+$, calculated 735.40 [M + H]$^+$.

**Synthesis of (S)-3,4-dimethoxyphenethyl piperidine-2-carboxylate 12a**

Precursor of 12a (390 mg, 0.991 mmol) was treated with 20% TFA in DCM at room temperature. The mixture was stirred for 2h. TFA and DCM were evaporated under reduced pressure to yield 12a (280mg, 0.95mmol, 96.2%).

TLC (Hexane: EtOAc: TEA 7:2.8: 0.2): Rf = 0.24.

$^1$H NMR (300 MHz, CDCl$_3$) $\delta$ = 1.57 (s, 1H), 1.84 (s, 4H), 2.18 (d, 1H, J= 12.9 Hz), 3.54 (s, 1H), 3.86 (s, 3H), 3.87 (s, 3H), 3.89-3.96 (m, 1H), 4.37 (d, 3H, J= 7.8 Hz), 6.73 (d, 2H, J= 6.9 Hz), 6.81 (d, 1H, J= 9.3 Hz).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ = 21.04, 24.95, 25.63, 31.22, 38.09, 41.98, 47.22, 54.00, 55.81, 55.92, 57.59, 60.38, 65.57, 66.75, 67.78, 76.28, 111.33, 111.73, 113.04, 113.93, 118.95, 119.95, 120.10, 125.06, 127.06, 127.67, 129.64, 133.51, 141.27, 141.52, 141.71, 143.87, 144.08, 147.33, 148.87, 156.38, 158.77, 170.94.

MS (ESI) m/z 294.17 [M + H]$^+$, calculated 294.41 [M + H]$^+$.

**Synthesis of (S)-2-(3,4-dimethoxyphenoxy)ethyl piperidine-2-carboxylate 12b**

Precursor of 12b (200 mg, 0.488 mmol) was treated with 20% TFA in DCM at room temperature. The mixture was stirred for 2h. TFA and DCM were evaporated under reduced pressure to yield 12b (150mg, 0.48mmol, 99%).

TLC (Hexane: EtOAc: TEA 7:2.8: 0.2): Rf = 0.16.

$^1$H NMR (600 MHz, CDCl$_3$) $\delta$ = 1.54-1.61 (m, 1H), 1.82-1.97 (m, 4H), 2.24-2.28 (m, 1H), 2.99-3.04 (m, 1H), 3.55 (d, 1H, J= 12.6 Hz), 3.82 (s, 3H), 3.83 (s, 3H), 3.92 (dd, 1H, J= 3.6, 11.4 Hz), 4.11 (t, 2H, J= 4.2 Hz), 4.45-4.54(m, 2H), 6.35 (dd, 1H, J= 3, 9 Hz), 6.50 (d, 1H, J= 3 Hz), 6.76 (d, 1H, J= 9 Hz).

$^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$ = 21.50, 21.74, 25.60, 44.14, 55.81, 56.39, 56.83, 64.71, 65.85, 100.97, 103.94, 111.74, 143.98, 149.91, 152.71, 168.48.
MS (ESI) m/z 310.36 [M + H]^+, calculated 310.26 [M + H]^+.

**Synthesis of (S)-(R)-1-(3-(2-tert-butoxy-2-oxoethoxy)phenyl)-3-(3,4-dimethoxyphenyl)-propyl piperidine-2-carboxylate 12c**

Precursor of 12c (3.5 g, 4.8 mmol) was treated with 20% 4-methylpiperidine in DCM at room temperature. The mixture was stirred for 4h. 4-methylpiperidine and DCM were evaporated under reduced pressure. Saturated NH₄Cl solution was added to the filtrate and the solution was stirred for 10 min. The organic phase was separated and the aqueous phase was extracted with ethyl acetate (3x 100ml). The combined organic phases were washed with brine (30ml), dried over MgSO₄ and the residual solid was purified by chromatography using Hexane: EtOAc : TEA 7:2.8: 0.2 to yield 12c (2.2g, 4.3mmol, 91%).

TLC (Hexane: EtOAc: TEA 7:2.8: 0.2): Rf = 0.33.

$^1$H NMR (600 MHz, CDCl₃) δ= 1.42 (s, 9H), 1.43-1.50 (m, 2H), 1.52-1.57 (m, 1H), 1.61-1.68 (m, 1H), 1.69-1.74 (m, 1H), 1.99-2.04 (m, 2H), 2.15-2.21 (m, 1H), 2.44-2.55 (m, 2H), 2.62-2.66 (m, 1H), 3.05-3.08 (m, 1H), 3.42 (dd, 1H, J= 3, 10.2 Hz), 3.78 (s, 3H), 3.79 (s, 3H), 4.45 (s, 2H), 5.65-5.71 (m, 1H), 6.58-6.62 (m, 2H), 6.70-6.74 (m, 2H), 6.81-6.89 (m, 2H), 7.18 (t, 1H, J= 8.4Hz).

$^{13}$C NMR (150 MHz, CDCl₃) δ= 23.53, 24.78, 27.97, 28.41, 31.19, 37.83, 45.08, 55.80, 55.85, 58.08, 65.63, 75.83, 82.29, 112.27, 111.63, 113.07, 113.79, 119.72, 120.09, 129.56, 133.44, 141.60, 146.26, 148.81, 157.97, 167.84, 171.63.

MS (ESI) m/z 514.53[M + H]^+, calculated 514.27 [M + H]^+.

**Synthesis of (S)-(R)-3-(3,4-dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propyl piperidine-2-carboxylate 12d**

Precursor of 12d (250 mg, 0.34 mmol) was treated with 20% 4-methylpiperidine in DCM at room temperature. The mixture was stirred for 4h. 4-methylpiperidine and DCM were evaporated under reduced pressure. Saturated NH₄Cl solution was added to the filtrate and the solution was stirred for 10 min. The organic phase was separated and the aqueous phase was extracted with ethyl acetate (3x 100ml). The combined organic phases were washed with brine (30ml), dried over MgSO₄ and the residual solid was purified by chromatography using Hexane: EtOAc : TEA 7:2.8: 0.2 to yield 12d (160 mg, 0.31mmol, 84%).

TLC (Hexane: EtOAc: TEA 7:2.8: 0.2): Rf = 0.3.

$^1$H NMR (300 MHz, CDCl₃) δ= 1.48-1.60 (m, 1H), 1.67-1.89 (m, 2H), 1.89-2.10 (m, 3H), 2.17-2.31 (m, 2H), 2.44-2.62 (m, 1H), 2.85 (s, 2H), 3.06 (s, 3H), 3.08 (s, 3H), 3.27-3.42 (m, 1H),
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3.77-3.85 (m, 5H), 4.18-4.35 (m, 2H), 5.70 (t, 1H, J= 7.2 Hz), 6.62-6.66 (m, 2H), 6.72-6.80 (m, 2H), 6.86 (d, 1H, J= 7.8Hz), 6.95 (s, 1H), 7.19 (t, 1H, J= 8.1 Hz).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ = 21.59, 22.00, 26.01, 31.15, 37.82, 44.12, 45.89, 55.91, 55.95, 56.67, 57.17, 64.55, 65.60, 77.65, 111.35, 111.83, 112.36, 111.72, 119.27, 120.18, 129.57, 133 .31, 140.98, 147.29, 148.84, 158.45, 167.88.

MS (ESI) m/z 513.29 [M + H]$^+$, calculated 513.32 [M + H]$^+$.

**General procedure for coupling of 12a-d with 11a-e to yield 3a-3j**

To a stirred solution of the free amines (12a-d) in acetonitrile under argon was added sequentially N,N-Diisopropyl-ethylamine (DIPEA), HATU and the di-ketoacids (11a-e). The reaction mixture was stirred for 16h at room temperature. Saturated NH$_4$Cl solution was added to the reaction and the solution was stirred for 10 min. The organic phase was separated and the aqueous phase was extracted with ethyl acetate (3x 100ml). The combined organic phases were washed with brine (10ml), dried over MgSO$_4$ and the residual solid was purified by column chromatography.

**Synthesis of 2-(3-((R)-3-(3,4-dimethoxyphenyl)-1-((S)-1-(2-((1S,2R)-1-hydroxy-2-methyl cyclohexyl)-2-oxoacetyl)piperidine-2-carbonyloxy)propyl)phenoxy)acetic acid (3a*)**

To 12c (211mg, 0.412 mmol) was added DIPEA (160 mg, 1.24 mmol), HATU (234 mg, 0.618 mmol), 11a (92 mg, 0.494 mmol) and the reaction was treated as described above. The residual solid obtained was purified by column chromatography using Hexane: EtOAc 6:4 to yield 3a* ester (46mg, 0.067 mmol, 20%).

TLC (Hexane: EtOAc 6:4): Rf = 0.41.

HPLC (Gradient A) retention time= 32.1-32.6 min

$^1$H NMR (600 MHz, CDCl$_3$) $\delta$= 0.84 (t, 3H, J= 5.4 Hz), 1.30-1.41 (m, 6H), 1.47 (m, 9H), 1.61-1.85(m, 7H), 2.00-2.13 (m, 2H), 2.20-2.28(m, 1H), 2.36 (d, 1H, J= 13.8Hz), 2.47-2.61 (m, 2H), 3.10-3.17 (m, 1H), 3.49 (d, 1H, J= 13.2 HZ), 3.85 (s, 6H), 4.53 (s, 2H), 5.29 (s, 1H), 5.74-5.79 (m, 1H), 6.66-6.67 (m, 2H), 6.76-6.78 (m, 1H), 6.81-6.84 (m, 1H), 6.88-6.93 (m, 1H), 6.95-7.00 (m, 1H), 7.25-7.28 (m, 1H).

$^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$= 16.14, 20.33, 20.92, 25.33, 26.24, 28.02, 29.97, 36.89, 38.05, 44.31, 51.53, 55.80, 65.70, 76.69, 81.21, 82.35, 111.27, 111.68, 113.18, 114.25, 119.85, 120.13, 129.73, 133.47, 141.38, 147.34, 148.83, 158.07, 166.55, 167.87, 169.27, 205.06.

MS (ESI) m/z 682.07 [M + H]$^+$, calculated 682.85 [M + H]$^+$. 

**3a**<sup>*</sup> ester (46 mg, 0.067 mmol) was treated with 20% TFA in DCM at room temperature. The mixture was allowed to stir for 6 h. TFA and DCM was evaporated under reduced pressure to yield the free acid **3a**<sup>*</sup> (32 mg, 0.051 mmol, 77%).

TLC (Hexane: EtOAc: TFA 6:3.9: 0.1): Rf = 0.33.

HPLC (Gradient A) retention time = 24.6-25.1 min

<sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ = 0.82-0.88 (m, 3H), 1.36-1.92 (m, 13H), 2.03-2.13 (m, 2H), 2.23-2.38 (m, 2H), 2.50-2.67 (m, 2H), 3.24-3.31 (m, 1H), 3.48-3.55 (m, 1H), 3.85 (s, 3H), 3.86 (s, 3H), 4.67 (s, 2H), 5.25-5.27 (m, 2H), 5.74-5.77 (m, 1H), 6.56-6.70 (m, 2H), 6.77-6.80 (m, 1H), 6.82-6.87 (m, 1H), 6.89-6.96 (m, 2H), 7.26-7.29 (m, 1H).

<sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>) δ = 16.3, 20.16, 20.87, 24.79, 25.29, 26.55, 29.30, 31.35, 35.59, 37.60, 39.45, 44.28, 51.92, 55.87, 55.92, 65.07, 76.86, 82.24, 111.37, 111.70, 115.71, 116.21, 119.71, 120.20, 129.90, 133.21, 141.51, 147.41, 148.89, 157.74, 167.39, 169.20, 171.63, 205.23.

MS (ESI) m/z: found Rt 13.88 min. (Method LCMS), 648.45 [M + Na]<sup>+</sup>, HRMS 626.2902 [M + H]<sup>+</sup>, calculated 626.2887 [M + H]<sup>+</sup>.

The diasteromeric mixture was further separated using preparative HPLC Gradient 62-77% B for 35 min to yield diasteromer 3a-1 (6 mg) and 3a-2 (9 mg).

**3a-1**

HPLC (Gradient A) retention time = 24.6-24.8 min

<sup>1</sup>H NMR (600 MHz, CDCl<sub>3</sub>) δ = 0.82 (d, 3H, J = 5.4 Hz), 1.38-1.43 (m, 2H), 1.44-1.48 (m, 2H), 1.53-1.58 (m, 2H), 1.64-1.70 (m, 3H), 1.74-1.81 (m, 2H), 2.04-2.12 (m, 2H), 2.22-2.28 (m, 1H), 2.52-2.67 (m, 2H), 2.98 (d, 1H, J = 5.4 Hz), 3.08 (s, 1H), 3.12 (s, 1H), 3.25 (dt, 1H, J = 2.4, 13.2 Hz), 3.53 (d, 1H, J = 13.2 Hz), 3.64-3.67 (m, 1H), 3.72 (s, 1H), 3.85 (s, 3H), 3.86 (s, 3H), 4.63 (s, 2H), 5.24 (d, 1H, J = 4.8 Hz), 5.74-5.80 (m, 1H), 6.66-6.69 (m, 2H), 6.77-6.79 (m, 1H), 6.83-6.94 (m, 3H), 7.26-7.28 (m, 1H).

<sup>13</sup>C NMR (150 MHz, CDCl<sub>3</sub>) δ = 16.15, 20.18, 21.06, 24.79, 25.27, 26.52, 29.68, 31.41, 35.57, 36.61, 37.64, 44.18, 51.88, 55.86, 55.92, 63.81, 81.38, 111.35, 111.68, 115.65, 115.66, 119.54, 120.16, 129.85, 133.19, 141.53, 147.45, 148.93, 157.92, 167.57, 169.26, 169.26, 205.46.

MS (ESI) m/z: found Rt 13.87 min. (Method LCMS), 648.40 [M + Na]<sup>+</sup>, calculated 648.45 [M + Na]<sup>+</sup>.

**3a-2**

HPLC (Gradient A) retention time = 24.9-25.1 min
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$^1$H NMR (600 MHz, CDCl$_3$) $\delta$ = 0.84 (d, 3H, J= 6.6 Hz), 1.38-1.85 (m, 10H), 2.06 (s, 2H), 2.20-2.31 (m, 1H), 2.49-2.65 (m, 2H), 2.97 (d, 1H, J= 6.6 Hz), 3.05 (s, 1H), 3.12 (s, 1H), 3.25 (t, 1H, J= 12.6 Hz), 3.48 (d, 1H, J= 10.8 Hz), 3.65 (s, 1H), 3.72 (s, 2H), 3.84 (s, 3H), 3.85 (s, 3H), 4.81 (s, 2H), 5.26 (s, 1H), 5.74 (s, 1H), 6.66-6.68 (m, 2H), 6.77-6.94 (m, 4H), 7.21-7.24 (m, 1H).

$^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$ = 16.15, 20.21, 20.94, 24.82, 25.31, 26.40, 29.68, 31.35, 35.31, 36.72, 37.15, 42.16, 43.25, 44.25, 44.54, 46.53, 48.81, 51.75, 55.86, 55.92, 56.79, 63.84, 81.66, 111.34, 111.70, 115.51, 119.59, 120.17, 129.82, 133.32, 141.58, 147.41, 148.91, 157.91, 167.37, 169.34, 205.95.


Synthesis of 3,4-dimethoxyphenethyl 1-((2-((1S,2R)-1-hydroxy-2-methylcyclohexyl)-2-oxoacetyl)piperidine-2-carboxylate ($3b^*$)

To 12a (33mg, 0.112 mmol) was added DIPEA (43.4 mg, 0.336 mmol), HATU (40.5mg, 0.168 mmol) and 11a (25 mg, 0.134 mmol). The reaction was treated as described above. The residual solid obtained was purified by column chromatography using Hexane: EtOAc 6:4 to yield $3b^*$ (25mg, 0.054 mmol, 49%).

TLC (Hexane: EtOAc 6:4): Rf = 0.57.

HPLC (Gradient A) retention time= 25.8-26.2 min

$^1$H NMR (600 MHz, CDCl$_3$) $\delta$ = 0.83 (m, 3H), 1.39-1.77 (m, 14H), 2.07-2.12 (m, 2H), 2.26 (d, 1H, J= 14.4 Hz), 2.89-2.94 (m, 2H), 3.09-3.17 (m, 1H), 3.46 (t, 1H, J= 11.4 Hz), 3.86 (s, 3H), 3.87 (s, 3H), 4.31-4.43 (m, 2H), 5.24 (s, 1H), 6.72-6.81 (m, 3H).

$^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$ = 16.12, 20.35, 20.80, 24.95, 25.37, 26.30, 29.50, 34.56, 35.67, 36.81, 44.04, 51.46, 55.86, 55.91, 65.99, 81.23, 111.29, 111.99, 120.93, 129.85, 147.78, 148.95, 166.83, 169.99, 204.98.


Synthesis of 2-(3,4-dimethoxyphenoxy)ethyl 1-((2-((1S,2R)-1-hydroxy-2-methylcyclohexyl)-2-oxoacetyl)piperidine-2-carboxylate ($3c^*$)

To 12b (34.6 mg, 0.112 mmol) was added DIPEA (43.4 mg, 0.336 mmol), HATU (40.5mg, 0.168 mmol) and 11a (25 mg, 0.134 mmol). The reaction was treated as described above. The residual solid obtained was purified by column chromatography using Hexane : EtOAc 1:1 to yield $3c^*$ (33mg, 0.069 mmol, 62%).
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TLC (Hexane: EtOAc 1:1): Rf = 0.28.
HPLC (Gradient A) retention time= 25.8-26.2 min

\[^1\text{H}\text{ NMR}\ (600 \text{ MHz}, \text{CDCl}_3) \delta = 0.821 \text{ (dd, 3H, } J= 6.6 \text{ Hz}), 1.41-1.79 \text{ (m, 13H), 2.04-2.13 \text{ (m, 1H), 2.35 \text{ (d, 1H, } J= 13.8 \text{ Hz), 3.22-3.34 \text{ (m, 1H), 3.51 \text{ (t, 1H, } J= 12 \text{ Hz), 3.83 \text{ (s, 3H), 3.85 \text{ (s, 3H), 4.13-4.15 \text{ (m, 2H), 4.43-4.56 \text{ (m, 2H), 5.30 \text{ (d, 1H, } J= 5.4 \text{ Hz), 6.37-6.40 \text{ (m, 1H), 6.52 \text{ (t, 1H, } J= 3 \text{ Hz), 6.76 \text{ (d, 1H, } J= 9 \text{ Hz).}}}

\[^{13}\text{C NMR}\ (150 \text{ MHz, CDCl}_3) \delta = 16.13, 20.33, 21.06, 24.84, 26.40, 29.47, 34.60, 35.64, 36.82, 44.09, 51.49, 55.85, 56.40, 63.77, 66.35, 81.18, 101.09, 104.07, 111.71, 143.92, 149.88, 152.83, 166.98, 170.03, 205.03.


2-(3,4-dimethoxyphenoxy)ethyl 1-(2-((1S,2R)-2-ethyl-1-hydroxycyclohexyl)-2-oxoacetyl)piperidine-2-carboxylate (3d*)
To 12b (64.4 mg, 0.208 mmol) was added DIPEA (81 mg, 0.624 mmol), HATU (75 mg, 0.312 mmol) and 11b (50 mg, 0.250 mmol). The reaction was treated as described above. The residual solid obtained was purified by column chromatography using Hexane: EtOAc 1:1 to yield 3d* (25 mg, 0.051 mmol, 25%).
TLC (Hexane: EtOAc 6:4): Rf = 0.50.
HPLC (Gradient A) retention time= 25.5-25.9 min

\[^1\text{H}\text{ NMR}\ (600 \text{ MHz}, \text{CDCl}_3) \delta = 0.85-0.88 \text{ (m, 3H), 1.41-1.88 \text{ (m, 18H), 2.35 \text{ (d, 1H, } J= 12 \text{ Hz), 3.22-3.29 \text{ (m, 1H), 3.49-3.53 \text{ (m, 1H), 3.83 \text{ (s, 3H), 3.85 \text{ (s, 3H), 4.13-4.16 \text{ (m, 2H), 4.35-4.57 \text{ (m, 2H), 5.31 \text{ (s, 1H), 6.37-6.40 \text{ (m, 1H), 6.51-6.53 \text{ (m, 1H), 6.77 \text{ (d, 1H, } J= 9 \text{ Hz).}}}

\[^{13}\text{C NMR}\ (150 \text{ MHz, CDCl}_3) \delta = 11.78, 20.66, 20.90, 23.67, 24.94, 25.46, 26.42, 35.91, 43.72, 44.06, 55.85, 56.40, 63.70, 66.36, 82.34, 101.10, 104.08, 111.72, 143.97, 149.88, 152.85, 152.90, 166.82, 170.01, 205.09.

MS (ESI) m/z: found Rt 13.69 min. (Method LCMS), 492.21 [M + H]^+, calculated 492.24 [M + H]^+.

**Synthesis of (S)-((R)-3-(3,4-dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propyl) 1-(2-((1S,2R)-1-hydroxy-2-methylocyclohexyl)-2-oxoacetyl)piperidine-2-carboxylate (3e*)**
To 12d (60 mg, 0.117 mmol) was added DIPEA (60 mg, 0.468 mmol), HATU (66 mg, 0.175 mmol) and 11a (26 mg, 0.14 mmol) and the reaction was treated as described above. The
residual solid obtained was purified by column chromatography using Hexane : EtOAc 1:1 to yield 3e* (41mg, 0.060mmol, 52%).

TLC (DCM: MeOH 9.3:0.7): Rf = 0.50.

HPLC (Gradient A) retention time= 21.1-21.7 min

$^1$H NMR (400 MHz, CDCl$_3$) $\delta$ = 0.79-0.86 (m, 3H), 1.40-1.74 (m, 13H), 1.98-2.38 (m, 7H), 2.48-2.61 (m, 2H), 2.83-2.87 (m, 1H), 2.96-3.05 (m, 2H), 3.09-3.18 (m, 1H), 3.47-3.51 (m, 1H), 3.77-3.85 (m, 1H), 4.17-4.24 (m, 2H), 5.29 (t, 1H, J= 4.4 Hz), 5.74-5.77 (m, 1H), 6.65-6.68 (m, 2H), 6.75-6.78 (m, 2H), 6.81-6.84 (m, 1H), 6.86-6.96 (m, 2H), 7.22-7.27 (m, 1H).

$^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ = 16.12, 16.14, 20.25, 20.29, 20.90, 21.11, 24.88, 24.92, 25.32, 25.35, 26.23, 26.32, 29.34, 29.41, 31.17, 31.25, 34.85, 35.31, 36.89, 36.90, 38.01, 38.24, 44.24, 44.29, 51.49, 51.64, 53.67, 53.74, 55.81, 55.90, 57.31, 57.36, 64.82, 64.82, 65.83, 66.03, 77.22, 81.16, 81.23, 111.23, 111.26, 111.65, 111.69, 112.73, 113.06, 114.08, 114.38, 119.23, 119.40, 120.11, 120.13, 129.73, 129.78, 133.30, 133.46, 141.41, 141.59, 147.33, 148.82, 148.85, 158.36, 158.42, 166.59, 166.73, 169.31, 169.40, 205.13, 205.47.


**Synthesis of 2-((R)-3-(3,4-dimethoxyphenyl)-1-((S)-1-2-((1S,2R)-2-ethyl-1-hydroxy-cyclohexyl)-2-oxoacetyl)piperidine-2-carbonyloxy)propyl)phenoxy)acetic acid (3f*)**

To 12c (214mg, 0.416 mmol) was added DIPEA (161 mg, 1.25 mmol), HATU (236 mg, 0.624 mmol) and 11b (100 mg, 0.499 mmol) and the reaction was treated as described above. The residual solid obtained was purified by column chromatography using Hexane : EtOAc 6:4 to yield 3f* ester (62mg, 0.089 mmol, 21%).

TLC (Hexane: EtOAc 6:4): Rf = 0.71.

HPLC (Gradient A) retention time= 32.6-32.9 min

$^1$H NMR (600 MHz, CDCl$_3$) $\delta$ = 0.87 (t, 3H, J= 7.2Hz), 1.28-1.37 (m, 4H), 1.48 (s, 9H), 1.65-1.88 (m, 1H), 2.00-2.09 (m, 1H), 2.20-2.27 (m, 1H), 2.37 (d, 1H, J=13.8Hz), 2.47-2.62 (m, 2H), 3.05-3.20 (m, 1H), 3.49-3.53 (m, 1H), 3.85 (s, 3H), 3.86 (s, 3H), 4.52 (d, 2H, J=4.8 Hz), 5.31 (d, 1H, J= 5.4 Hz), 5.75-5.79 (m, 1H), 6.65-6.67 (m, 2H), 6.76-6.78 (m, 1H), 6.81-6.83 (m, 1H), 6.87-7.00 (m, 2H), 7.25-7.28 (m, 1H).

$^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$ = 11.78, 20.65, 23.65, 23.66, 24.92, 25.27, 26.26, 28.02, 29.66, 31.17, 35.27, 38.04, 44.28, 51.65, 55.80, 55.90, 65.70, 76.69, 81.99, 82.36, 111.27, 111.68, 113.44, 114.22, 119.87, 120.13, 129.72, 133.48, 141.37, 147.27, 148.83, 156.05, 158.06, 166.58, 167.83, 169.26, 205.17.
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MS (ESI) m/z 696.84 [M + H]⁺, calculated 696.72 [M + H]⁺.

3f* ester (62 mg, 0.089 mmol) was treated with 20% TFA in DCM at room temperature. The mixture was allowed to stir for 6h. TFA and DCM was evaporated under reduced pressure to yield the free acid 3f* (40mg, 0.062mmol, 80%).

TLC (Hexane: EtOAc: TFA 1:1: 0.2): Rf = 0.45.

HPLC (Gradient A) retention time= 25.3-25.9 min

MS (ESI) m/z: found Rt 15.93 min. (Method LCMS), 662.63 [M + Na]⁺.


The diasteromeric mixture was further separated using preparative HPLC Gradient 65-70% B for 15min to yield diasteromer 3f-1 (5mg) and 3f-2 (7mg).

3f-1
HPLC (Gradient A) retention time= 25.3-25.5min

3f-2
HPLC (Gradient A) retention time= 25.7-25.9min

Synthesis of (S)-(R)-3-(3,4-dimethoxyphenyl)-1-(3-(2-morphinoethoxy)phenyl)propyl)-1-(2-((1S,2R)-2-ethyl-1-hydroxycyclohexyl)-2-oxoacetyl)piperidine-2-carboxylate (3g*)

To 12d (60 mg, 0.117 mmol) was added DIPEA (55 mg, 0.425 mmol), HATU (60mg, 0.158 mmol) and 11b (28 mg, 0.139 mmol) and the reaction was treated as described above. The residual solid obtained was purified by column chromatography using Hexane: EtOAc 1:1 to yield 3g* (20mg, 0.028 mmol, 25%).

TLC (DCM: MeOH 9.7: 0.3): Rf = 0.48.

HPLC (Gradient A) retention time= 21.7-22.3 min

¹H NMR (300 MHz, CDCl₃) δ= 0.77-0.86 (m, 3H), 1.41-1.74 (m, 15H), 1.96-2.36 (m, 7H), 2.45-2.61 (m, 2H), 2.87-2.89 (m, 1H), 3.00-3.10 (m, 2H), 3.12-3.19 (m, 1H), 3.47-3.51 (m, 1H), 3.77-3.86 (m, 11H), 4.19-4.25 (m, 2H), 5.29 (t, 1H, J= 4.8 Hz), 5.74-5.77 (m, 1H), 6.65-6.69 (m, 2H), 6.76-6.78 (m, 2H), 6.81-6.84 (m, 1H), 6.84-6.98 (m, 2H), 7.23-7.28 (m, 1H).
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$=11.83, 12.40, 20.51, 20.63, 21.55, 22.68, 23.63, 24.93, 25.25, 25.45, 29.36, 30.57, 31.19, 31.29, 31.92, 34.27, 35.56, 38.61, 43.69, 43.77, 44.31, 51.50, 51.62, 53.65, 55.87, 55.95, 57.31, 61.77, 65.56, 70.36, 70.61, 77.22, 82.04, 111.38, 111.78, 113.11, 114.10, 114.42, 120.20, 129.83, 130.91, 133.39, 133.53, 141.61, 147.42, 148.94, 158.32, 158.45, 166.65, 166.69, 169.34, 169.43, 205.13, 205.41.

MS (ESI) m/z: found Rt 8.95 min. (Method LCMS), 695.45 [M + H]$^+$.


**Synthesis of 2-(3-((R)-3,4-dimethoxyphenyl)-1-((S)-2-((1S,2S)-1-hydroxy-2-(hydroxymethyl)cyclohexyl)-2-oxoacetyl)piperidine-2-carbonyloxy)propyl)phenoxy)acetic acid (3h)**

To 12c (208mg, 0.404 mmol) was added DIPEA (158 mg, 1.22 mmol), HATU (230 mg, 0.608 mmol) and 11c (120 mg, 0.487 mmol) and the reaction was treated as described above. The residual solid obtained was purified by column chromatography using Hexane: EtOAc 1:1 to yield 3h ester (60mg, 0.080 mmol, 20%).

TLC (Hexane: EtOAc 1:1): Rf = 0.48.

$^1$H NMR (600 MHz, CDCl$_3$) $\delta$= 1.26-1.37 (m, 4H), 1.46 (s, 9H), 1.51-1.63 (m, 3H), 1.64-1.79 (m, 5H), 1.96-2.08 (m, 1H), 2.15-2.26 (m, 2H), 2.33 (d, 1H, J= 14.4 Hz), 2.45-2.62 (m, 2H), 3.13-3.21 (m, 1H), 3.25 (s, 1H), 3.27-3.29 (m, 1H), 3.30 (s, 1H), 3.48-3.53 (m, 1H), 3.55-3.58 (m, 1H), 3.64-3.67 (m, 1H), 3.83-3.84 (m, 6H), 4.44-4.48 (m, 1H), 4.50 (s, 2H), 4.51-4.59 (m, 1H), 5.28 (t, 1H, J= 5.4 Hz), 5.72-5.78 (m, 1H), 6.64-6.67 (m, 2H), 6.75-6.77 (m, 1H), 6.79-6.82 (m, 1H), 6.88-7.00 (m, 2H), 7.23-7.26 (m, 1H).

$^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$= 14.09, 20.38, 20.40, 22.67, 24.67, 24.72, 24.97, 24.99, 25.12, 25.17, 26.32, 26.46, 29.35, 29.67, 29.69, 31.13, 31.91, 31.92, 35.50, 36.16, 37.98, 38.03, 41.50, 41.64, 44.13, 44.20, 51.60, 51.67, 55.29, 55.35, 55.80, 55.89, 65.69, 69.84, 69.89, 76.56, 76.58, 81.26, 81.28, 82.36, 82.38, 96.39, 96.49, 111.26, 111.67, 113.27, 114.06, 114.13, 119.77, 119.89, 120.13, 129.62, 129.69, 133.40, 133.48, 141.35, 141.54, 147.26, 147.29, 148.82, 148.83, 159.01, 158.04, 165.87, 166.07, 166.81, 167.09, 167.89, 167.90, 169.47, 169.50, 205.48, 206.00


3h ester (60 mg, 0.080 mmol) was treated with 20% TFA in DCM at room temperature. The mixture was allowed to stir for 6h. TFA and DCM was evaporated under reduced pressure to yield the free acid 3h (16mg, 0.024mmol, 31%).

TLC (Hexane: EtOAc: TFA 6: 4: 0.1): Rf = 0.41.
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

HPLC (Gradient A) retention time = 25.7-26.1 min

$^1$H NMR (600 MHz, CDCl$_3$) δ = 1.32-1.88 (m, 13H), 2.02-2.10 (m, 1H), 2.16-2.28 (m, 2H), 2.37-2.40 (m, 1H), 2.51-2.65 (m, 2H), 3.20-3.26 (m, 1H), 3.51-3.59 (m, 1H), 3.60-3.67 (m, 1H), 3.73-3.80 (m, 1H), 3.85 (s, 6H), 4.63-4.70 (m, 2H), 5.32 (d, 1H, J = 5.4 Hz), 5.73-5.79 (m, 1H), 6.66-6.70 (m, 2H), 6.77-6.80 (m, 1H), 6.84-6.89 (m, 2H), 6.92-6.96 (m, 1H), 7.25-7.29 (m, 1H).

$^{13}$C NMR (150 MHz, CDCl$_3$) δ = 20.67, 20.80, 21.19, 23.60, 24.92, 25.47, 26.60, 29.68, 31.29, 34.48, 44.50, 44.56, 51.92, 55.91, 65.06, 69.07, 76.67, 81.87, 111.35, 111.72, 115.07, 115.16, 119.99, 120.18, 129.84, 133.26, 141.63, 147.40, 148.85, 157.77, 166.31, 169.38, 169.47, 204.17.


**Synthesis of 2-((3-((R)-3-(3,4-dimethoxyphenyl)-1-((S)-1-((1R,2R)-1-hydroxy-2-methyl cyclohexyl)-2-oxoacetyl)piperidine-2-carboxyloxy)propyl)phenoxy)acetic acid (3i*)**

To 12c (210 mg, 0.412 mmol) was added DIPEA (160 mg, 1.24 mmol), HATU (234 mg, 0.618 mmol) and 11d (92 mg, 0.494 mmol) and the reaction was treated as described above. The residual solid obtained was purified by column chromatography using Hexane: EtOAc 6:4 to yield 3i* ester (38 mg, 0.055 mmol, 14%).

3i* ester (38 mg, 0.055 mmol) was treated with 20% TFA in DCM at room temperature. The mixture was allowed to stir for 6h. TFA and DCM was evaporated under reduced pressure to yield the free acid *3i (27 mg, 0.043 mmol, 77%).

HPLC (Gradient A) retention time = 27.3-27.7 min


The diasteromeric mixture was further separated using preparative HPLC Gradient 61-71% B for 20min to yield diasteromer 3i-1 (5 mg) and 3i-2 (8 mg).

**3i-1**

HPLC (Gradient A) retention time = 27.2-27.4 min.

$^1$H NMR (600 MHz, CDCl$_3$) δ = 0.82 (d, 3H, J = 6.6 Hz), 1.36-1.51 (m, 3H), 1.54-1.59 (m, 2H), 1.65-1.72 (m, 3H), 1.77-1.82 (m, 1H), 1.92 (d, 1H, J = 12.6 Hz), 2.03-2.13 (m, 2H), 2.23-2.30 (m, 1H), 2.37 (d, 1H, J = 14.4 Hz), 2.52-2.68 (m, 2H), 3.54 (d, 1H, J = 12.6 Hz), 3.64-3.65 (m, 2H), 4.63-4.70 (m, 2H), 5.32 (d, 1H, J = 5.4 Hz), 5.73-5.79 (m, 1H), 6.66-6.70 (m, 2H), 6.77-6.80 (m, 1H), 6.84-6.89 (m, 2H), 6.92-6.96 (m, 1H), 7.25-7.29 (m, 1H).
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

1H), 3.72 (s, 3H), 3.85 (s, 3H), 3.86 (s, 3H), 4.67 (s, 2H), 5.25 (d, 1H, J= 5.4 Hz), 5.75-5.77(m, 1H), 6.67-6.70 (m, 2H), 6.77-6.80 (m, 1H), 6.83 (s, 1H), 6.89-6.94 (m, 2H), 7.27-7.29 (m, 1H).


3i-2
HPLC (Gradient A) retention time= 27.3-27.6min.

1H NMR (600 MHz, CDCl3) δ= 0.85 (d, 3H,J= 6.6Hz), 1.37-1.86 (m, 12H), 2.02-2.14 (m, 2H), 2.23-2.29 (m, 1H), 2.36 (d, 1H, J= 14.2 Hz), 2.52-2.66 (m, 2H), 3.65 (d, 1H, J= 5.4 Hz), 3.73 (s, 3H), 3.85 (s, 3H), 3.86 (s, 3H), 4.68 (s, 2H), 5.27 (d, 1H, J= 5.4 Hz), 5.74-5.79 (m, 1H), 6.65-6.70 (m, 2H), 6.79 (d, 1H, J= 7.8 Hz), 6.81-6.95 (m, 3H), 7.24-7.29 (m, 1H).


**Synthesis of 2-(3-(3,4-dimethoxyphenyl)-1-((S)-1-((1R,2R)-2-ethyl-1-hydroxy cyclohexyl)-2-oxoacetyl)piperidine-2-carbonyloxy)propyl)phenoxy)acetic acid (3j)**

To 12c (21.4mg, 0.041mmol) was added DIPEA (15.1 mg, 0.117 mmol), HATU (23.7 mg, 0.063 mmol) and 11e (10.0 mg, 0.05 mmol) and the reaction was treated as described above. The residual solid obtained was purified by column chromatography using Hexane:EtOAc 6:4 to yield 3j ester (6.6 mg, 0.009 mmol, 22%).

TLC (Hexane:EtOAc 6: 4): Rf = 0.46.

HPLC (Gradient A) retention time= 31.5-31.9 min


3j ester (6.6 mg, 0.009 mmol) was treated with 20% TFA in DCM at room temperature. The mixture was allowed to stir for 6h. TFA and DCM was evaporated under reduced pressure to yield the free acid 3j* (5.7 mg, 0.007 mmol, 91%).

TLC (Hexane:EtOAc: TFA 1:1: 0.1): Rf = 0.35.

HPLC (Gradient A) retention time= 25.6-26.1 min


**Supporting Information.** Reaction schemes of intermediates. This material is available free of charge via the Internet at [http://pubs.acs.org](http://pubs.acs.org).
**Crystallography**

Crystals and Co-crystals of the FKBP51 Fk1 domain construct comprising residues 16-140 and containing mutation A19T were obtained as previously described\(^\text{18}\). Diffraction data were collected at the European Synchrotron Radiation Facility (ESRF) in Grenoble, France. The data were processed with MOSFLM\(^\text{40}\) and XDS\(^\text{41}\), SCALA\(^\text{42}\) and TRUNCATE\(^\text{43}\). The crystal structures were solved by molecular replacement employing the program MOLREP\(^\text{44}\). The dictionaries for the ligand compounds were generated with the PRODRG server\(^\text{45}\). The structures were refined with REFMAC\(^\text{46}\). Manual model building was performed with COOT\(^\text{47}\). Molecular-graphics figures were generated using PyMOL (http://www.pymol.org).

**Acknowledgement:**

We thank Dr. Gerd Rühter and the Lead Discovery Center (Dortmund) for providing building block 5b and 5c and Drs. B. Gold and E.R. Sanchez for providing a sample of Timcodar. We are indebted to Claudia Dubler (LMU, Munich, Germany) and Elisabeth Weyher (MPI of Biochemistry, Martinsried, Germany) for NMR spectroscopy and HRMS measurements respectively. We thank Prof. Florian Holsboer for continuous and generous financial support. Support by the Joint Structural Biology Group at the ESRF beamlines is gratefully acknowledged.

**Reference:**


(23) Stivanello, M.; Leoni, L.; Bortolaso, R. Synthesis of 1,5-bis(triphenylphosphonium)pentan-3-ol dichloride and its application to the preparation of 1,7-di(pyridin-3-yl)heptan-4-ol. Organic Process Research & Development 2002, 6, 807-810.


Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52


Supporting Information

Evaluation of Synthetic FK506 Analogs as Ligands for FKBP51 and FKBP52

Ranganath Gopalakrishnan¹, Christian Kozany¹, Steffen Gaali¹, Christoph Kress¹, Bastiaan Hoogeland¹, Andreas Bracher², Felix Hausch¹*.

Supporting Information Table of Contents:

Scheme S1 Enantioselective synthesis of Compound 5a and 5b

Scheme S2 Synthesis of pipecolic acid analogs.

Scheme S3

Scheme S4 Synthesis scheme of compound 6d

Scheme S5

Scheme S6 Synthesis scheme of compound 6h

Table-S1 Data collection and Refinement Statistics (crystallographic data)
Scheme S1 Enantioselective synthesis of Compound 5a and 5b

Scheme S2 Synthesis of pipecolic acid analogs

Scheme S3
Scheme S4 Synthesis scheme of compound 6d

Scheme S5

Scheme S6 Synthesis scheme of compound 6h
### Table S1: Data collection and Refinement Statistics

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### Refinement

| reflection range | 20 – 1.5 | 20 - 1.48 | 20 – 1.07 | 20 - 1.1 | 20 - 1.8 |
| reflections (test set) | 37697 (1995) | 21603 (1135) | 59549 (3141) | 51373 (2699) | 14559 (769) |
| Rcryst | 0.1658 | 0.1760 | 0.1548 | 0.1477 | 0.2200 |
| Rfree | 0.1995 | 0.2082 | 0.1724 | 0.1725 | 0.2609 |
| number of atoms | 2610 | 1299 | 1364 | 1357 | 1205 |
| r.m.s.d. bonds (Å) | 0.014 | 0.015 | 0.011 | 0.013 | 0.013 |
| r.m.s.d. angles (°) | 1.579 | 1.604 | 1.600 | 1.613 | 1.472 |

### Ramachandran plot

| % most favored region*** | 98.94 | 98.71 | 97.3 | 98.9 | 99.21 |
| % additionally allowed*** | 0 | 0.92 | 1.8 | 0 | 0 |

* Values in parenthesis for outer shell.
** As defined in Scala.
*** As defined in Coot.
1.2.2.1.1 Discussion (Manuscript-2)

In this study an extensive SAR analysis of α-ketoamide containing piperacolates as binders for FKBP51 and FKBP52 was carried out. This class of compounds has been extensively studied and validated for their binding to FKBP12. Here a direct comparison of the binding affinities of these compounds between FKBP12 and larger FKBPs has been studied.

Starting from the X-ray crystal structure of the lead compound (2a), a structure-based design approach was followed to study the contributions of each substructure on the binding affinity for FKBP51 and FKBP52. Firstly, the piperacolate core which is present in 2a and the natural product was substituted with other core structures (2b, 2c and 2d). The piperacolate core was found to be essential for binding to the larger FKBPs. Next the effect of top group modifications on the binding affinity was studied. Larger top group substituents (2a, 6e) were found to have better binding affinity as compared to the smaller top groups (6a, 6b and 6c). The morpholine top group was found to have the best binding affinity as compared to all other substituents. To further optimize the ligand for their binding affinity, the piperacolic core and the larger top groups were kept constant for the rest of the studies.

Next the interaction with the 80s loop was investigated in detail for gaining affinity and selectivity as both the proteins have a structural divergence in this loop. The tert-pentyl group in the lead compound (2a) was proposed to be substituted with a substituent that closely mimics the pyranose group in the natural products FK506 and Rapamycin. The cyclohexyl group was chosen as the pyranose oxygen in FK506 and Rapamycin is dispensable and has been shown to have no interaction with the protein surface102. This substitution resulted in compound 3a* having 2-5 fold better affinity to the larger FKBPs as compared to 2a. Next an extensive SAR around the cyclohexyl group was carried out to systematically understand the importance of the C10- and C11-substituents. First the role of C11-substituent on the cyclohexyl moiety was investigated. A series of cyclohexyl containing compounds were synthesized with varying chain length at the C11-methyl substituent (3a*, 3f*, 3h*). The binding affinity of this series of compounds was similar for the larger FKBPs thereby concluding that chain length or substitution of carbon with oxygen doesn’t affect the binding affinity.

Finally, analogs were designed to investigate the effect of stereochemistry at the C11-methyl and C10-OH on the binding affinities. This series comprised of the diastereomeric mixture 3a*, 3f*, 3i* and 3j*. All these compounds surprisingly had equivalent binding affinities. This observation was unexpected. The natural diastereomers 3a*, 3f* (stereochemistry at C10 and C11
similar to FK506 and Rapamycin) were expected to bind to the protein with a similar binding mode as the natural compound. The unnatural diasteromers 3i* and 3j* on the other hand were hypothesized to bind weekly as hydrogen bond between OH\textsuperscript{10} - Asp\textsuperscript{68} and the hydrophobic 80s loop contacts of the C\textsuperscript{11}-methyl/ethyl substituents was not possible simultaneously. To further dissect the above observations with respect to the pure diasteromers, the individual diasteromers 3a\text{-}1, 3a\text{-}2, 3i\text{-}1, 3i\text{-}2, 3f\text{-}1 and 3f\text{-}2 of the diasteromer mixture 3a*, 3i* and 3f* were separated. All these compounds were found to have equivalent binding to the FKBPs. From the above set of compounds we could thus conclude that the stereochemistry at the C\textsuperscript{10} and C\textsuperscript{11} was not important. This observations concluded from the binding studies were further supported by X-ray co-crystal structures (3f\text{-}1, 3f\text{-}2).

The X-ray co-crystal structure showed that the binding mode of both the compounds in the active site of FKBP51 was similar. In case with 3f\text{-}1 all three hydrogen bonds observed in the co-crystal structure of FK506 are conserved. In 3f\text{-}2 the cyclohexyl ring is flipped and the hydrogen bond between HO\textsuperscript{10}, 3f\text{-}1 and O-Asp\textsuperscript{68} is not present. But the retention of the binding affinity can be argued owing to the fact there is presence of an additional water mediated hydrogen bond with Tyr\textsuperscript{113} and Ser\textsuperscript{118} of FKBP51 and C\textsuperscript{10} - OH of 3f\text{-}2.

This elaborate study thus helps us to conclude that the stereochemistry at the hydroxyl and the methyl substituent on the pyranose ring of FK506 and Rapamycin are not important to gain affinity towards the protein subtypes. Overlay of different FKBP51 FK1 domain co-crystal has shown the 80s loop to be flexible. We thus infer that it is this loop flexibility that might make the FKBPs tolerant towards subtle changes in the stereochemistry around the cyclohexyl group. The rationale for binding of all the diasteromers with multiple binding modes and conserved 80s loop interaction can thus be explained.
**1.2.2.2 Exploration of Pipecolate Sulfonamides as Binders of the FK506-Binding Proteins 51 and 52 (Manuscript 3)**

The electrophilicity of the α-keto amide moiety present in most of the non-immunosuppressive FK506 analogs (as well as in the compounds of manuscript 2) is an undesired reactive liability that could result in metabolic instability or potential toxicity. In the second approach a bioisosteric replacement of the α-keto amide moiety of Rapamycin and FK506 with a sulfonamide was envisaged with the retention of the conserved hydrogen bonds. For a rapid and efficient derivatization of a focused sulfonamide library we envisaged a solid phase synthesis strategy which led to ligands with submicromolar affinity for FKBP51 or with 4-fold selectivity versus FKBP52. The molecular binding mode for one sulfonamide analog was confirmed by X-ray crystallography.

![Figure 14: Prototypic sulfonamide containing analogs.](image)

**Own Contributions:**

In the attached manuscript my personal contributions have been the following:

1. Establishment of the solid support synthesis protocol (Scheme-1) and the solution phase protocol (Scheme-2). Synthesis, purification and structural characterization of all compounds except compound 38 and 39.

2. Characterization of the final compounds in the fluorescence polarization assay, supported by B. Hoogeland and C. Kozany. Data analysis of the tested compounds.
Exploration of Pipecolate Sulfonamides as Binders of the FK506-Binding Proteins 51 and 52

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Correspondence: Email: hausch@mpipsykl.mpg.de, phone: +49(89)30622640, fax: +49(89)30622610

Abstract: FK506-binding proteins (FKBP) 51 and 52 are co-chaperones that modulate the signal transduction of steroid hormone receptors. Single nucleotide polymorphisms in the gene encoding FKBP51 have been associated with a variety of psychiatric disorders. Rapamycin and FK506 are two macrocyclic natural products, which tightly bind to all these proteins. A bioisosteric replacement of the α-ketoamide moiety of rapamycin and FK506 with a sulfonamide was envisaged with the retention of the conserved hydrogen bonds. A focused solid support-based synthesis protocol was developed, which led to ligands with submicromolar affinity for FKBP51 and FKBP52. The molecular binding mode for one sulfonamide analog was confirmed by X-ray crystallography.

![Chemical structures and binding affinities for FKBP51 and FKBP52]
Introduction:

Members of the FKBP (FK506-binding protein) family display peptidyl prolyl isomerase (PPIase) activity and bind to the immunosuppressive natural products FK506 and rapamycin. The prototypical FKBP12 is the most widely studied member of this family. In complex with FKBP12, FK506 and rapamycin also interact with and inhibit calcineurin (CaN) and mTOR, respectively, thereby mediating their immunosuppressive action. Prior studies led to analogs devoid of immunosuppressive activity\(^1-3\), as exemplified by compound \(\mathbf{2}\) (Fig. 1)\(^4\). The high molecular weight multi-domain homologs of FKBP12, FKBP51, and FKBP52 act as co-chaperones for the heat shock protein 90 (Hsp90) and modulate the signal transduction of the glucocorticoid receptor in a mutually antagonistic direction\(^5-7\). Human genetic studies have shown single nucleotide polymorphisms in the gene encoding FKBP51 to be associated with various stress-related psychiatric disorders\(^8\). Recent characterization of FKBP51 knockout mice has further validated these findings\(^9-12\). To further dissect the role of larger FKBPs and to better understand the underlying biology, selective inhibitors targeting FKBP51 are required. Neither FK506 nor rapamycin can be used as tools as they have nearly equipotent affinities for all FKBPs.

Extensive medicinal chemistry campaigns on analogs of FK506 and rapamycin have shown that the two conserved hydrogen bonds shown in Fig. 1 are required for binding to FKBPs. The electrophilicity of the \(\alpha\)-ketoamide moiety present in most of the non-immunosuppressive FK506 analogs is an undesired reactive liability that could result in metabolic instability or potential toxicity. For FKBP12 it has been shown that the \(\alpha\)-ketoamide can be bioisostERICALLY replaced by a sulfonamide moiety to yield compounds that retain binding to FKBP12\(^2,13-15\).
Fig. 1 Natural and synthetic ligands that bind to large FKBPs. (a) Structure of FK506 (1). (b) The prototypic synthetic ligand 2, which is devoid of immunosuppressive activity. Key hydrogen bonds with FKBP51 are indicated by dotted lines; the position of the 80s loop interacting with the tert-pentyl moiety is indicated in cyan.

However, these compounds have not been tested for their binding profile with the larger FKBPs. Until very recently, compound 2 has been the only synthetic ligand tested for its binding affinity for FKBP51. In quest for finding improved inhibitors of FKBP51 or FKBP52 we envisaged a solid phase synthesis methodology for the synthesis of pipecolate sulfonamide compounds to gain insight into the structure activity relationship (SAR) of this series for the larger FKB isoforms.

Results and Discussion:

Chemistry:

Strategy: A three-dimensional alignment of the FK506-binding domains of FKBP51(3O5E)\(^{16}\), FKBP52 (to be published) and FKBP12 (2PPN)\(^{17}\) revealed the largest structural divergences close to the binding pocket at the 80s loop (Ser\(^{118}\)-Ile\(^{122}\) of FKBP51/52). The 80s loop of FKBP51 contains Leu\(^{119}\) which is replaced by Pro\(^{119}\) in FKBP52 possibly contributing for the structural difference in this region. Importantly, the residue at position 119 was shown to be a major functional determinant for the effect on steroid hormone receptor\(^{18}\). Hence an optimization of interactions with this part of the protein has a higher probability of achieving selectivity and functional relevance within the FKBp family. The X-ray structures of FK506 with the FK1 domain of FKBP51 (3O5R) and FKBP52 (unpublished) confirmed that the pyranose group in FK506 (1) contacts the 80s loop. Sulfonamide substituents as replacements of the pyranose group have been shown to have contact with the 80s loop in FKBP12\(^{2,13,14,19}\). Compound 2, until very recently the only known synthetic FKBP51 ligand, was chosen as a starting point for the synthesis of sulfonamide analogs. For a rapid derivatization of compounds targeting the 80s loop we envisaged a solid phase strategy for synthesis of a focused sulfonamide library.

Solid phase synthesis of a focused sulfonamide library: The precursor 3 was synthesized as described\(^{20}\). This was further coupled with the pipecolic acid 4 followed by liberation of the acid to give the building block 5. The latter was anchored on a 2-chloro trityl resin.
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

**Scheme-1. Synthesis of the pipecolate framework**

Reagents and conditions: (a) DCC, DMAP, 0 °C to r.t., 20h (for 3a); HATU, DIPEA, r.t., 2h (for 3a) (b) 20% TFA in CH₂Cl₂, r.t., 6h. (c) DIPEA, CH₂Cl₂, r.t., 16h; and DIPEA, methanol, r.t., 4h. (d) 20% 4-methyl-piperidine, CH₂Cl₂, r.t., 1h. (e) DIPEA, CH₂Cl₂, r.t., 16h. (f) 1% TFA in CH₂Cl₂, r.t., 1h.

The immobilized building block was deprotected to give 6 and was further reacted with a library of commercial sulfonyl chlorides 7. Cleavage form the solid support under mild acidic conditions yielded compounds \(8-37, 49-79\) and \(46, 47\). This solid support protocol was used for the synthesis of a small focused library followed by primary screening as well as for the resynthesis of hits for further characterization. The best sulfonamide analogs of this series were further attached to pipecolate core where the free acid moiety in \(8-37, 49-79\) was exchanged by a morpholine group in \(40-45\) (scheme 2).
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Scheme-2. Synthesis of the various top groups containing sulfonamide

Reagent and conditions : (a) DIPEA, RSO$_2$Cl, CH$_2$Cl$_2$, r.t., 16h.

**Biology:**

The sulfonyl chloride building blocks were designed to initially probe a variety of aliphatic and aromatic sulfonyl moieties as well as substituents around the aromatic rings. Out of 36 compounds in the medium-throughput screening, 28 compounds inhibited tracer binding to FKBP12 by more than 15% while 8 compounds displayed inhibition of more than 85% at 5 μM (supplementary information and Table-1). 7 compounds inhibited the tracer binding to the FK1 domain of FKBP52 by more than 15% at 5 μM, whereas 5 hits were identified for the FK1 domain of FKBP51. The initial screening assay results indicated that sulfonamides can be surrogates of the α-ketoamides in the context of this scaffold, but that efficient binding critically depended on the nature of the sulfonamide substituent, at least for the larger FKBPs. In general, the inhibitory activity was much higher for FKBP12 than for the larger FKBPs. This could be related to the core structure 6 which was designed and optimized for FKBP12 as well as to the more concave 80s loop of FKBP12.

The most promising compounds 8-12 from the primary screening were selected, resynthesized in larger scale and characterized in more detail. The binding affinities of all sulfonamide hits were weaker for all tested FKBPs compared to the reference compound 2. However, in general, the affinity for FKBP12 was compromised in a stronger way than those for the larger FKBPs.
Compounds 9 and 10 turned out to have the highest binding affinity for FKBP51 and 52 and were further evaluated in two series (Table-2).

![Chemical structure](image)

Table-1 Binding affinities for the primary hits towards FKBP paralogs.

<table>
<thead>
<tr>
<th>Compd. No.</th>
<th>R</th>
<th>Purity %</th>
<th>FKBP12</th>
<th>FKBP51FK1</th>
<th>FKBP52FK1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC&lt;sub&gt;50&lt;/sub&gt;(µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>&gt;99</td>
<td>0.114 ± 0.015</td>
<td>6.3 ± 0.9</td>
<td>9.37 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>&gt;99</td>
<td>1.8 ± 0.1</td>
<td>62.8 ± 10.7</td>
<td>&gt;100</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>&gt;98</td>
<td>1.2 ± 0.2</td>
<td>30.7 ± 15.7</td>
<td>32.8 ± 14.5</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>&gt;99</td>
<td>1.1 ± 0.1</td>
<td>11.6 ± 1.1</td>
<td>32.5 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>&gt;96</td>
<td>10.1 ± 1.0</td>
<td>&gt;100</td>
<td>&gt;100</td>
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</tr>
<tr>
<td>12</td>
<td>&gt;99</td>
<td>4.6 ± 0.15</td>
<td>67.1 ± 12.1</td>
<td>&gt;100</td>
<td></td>
</tr>
</tbody>
</table>

Binding affinity to FKBP12, FKBP51 (FK1 domain) and FKBP52 (FK1 domain) determined by a fluorescence polarization assay<sup>21</sup>.

**Exploration of the SAR:** The first series of derivatives probed the influence of the substituent at the meta position. The compounds in this series included m-CN 13, m-NO<sub>2</sub> 14, m-NH<sub>2</sub> 15, m-(2-methylpyrimidin-4-yl) 16, m-(pyrimidin-4-yl) 17, m-F 18, and m-Br 19 aromatic sulfonamides. All these compounds had binding affinity between 0.3-10 µM for FKBP12. The meta-substituted halogen derivatives had better binding affinity to the larger FKBP1s, among these 19 being the best. Compound 15 was synthesized by reduction of the nitro group in 14.
The m-CN 13, m-NO₂ 14 and m-NH₂ 15 analogs had slightly reduced binding affinities to the FKBPs compared to the m-Cl aromatic sulfonamide 9, while compound 16 and 17 were inactive for the larger FKBPs.

We next set out to explore multi-substituted aromatic sulfonamide groups. The di-chloro substituted aromatic sulfonamide 20 had slightly better binding affinity than the mono meta-chloro substituted aromatic sulfonamide 9 (Table-2). In contrast, an additional chloro-substituent in the para-position 21 was found to substantially reduce binding to the FKBPs. A similar result was found for the meta, para-dimethoxy substituted sulfonamide 22 but, interestingly, compound 23 having an m-Cl, p-OMe substitution had better binding affinity. This series of compounds indicated the following SAR m-di-Cl > m-Cl > m,p-di-Cl >> p-Cl >> o-Cl for the aryl sulfonamide substituents.

To further explore the acceptable nature of the groups at the meta positions the derivatives m-difluoro 24, 3,5-bis(trifluoromethyl) 25, 3-bromo-5-(trifluoromethyl) 26, and 3,5-bis(carboxy-methyl) 27 were synthesized. Compound 24 had reduced affinity compared to the mono-fluoro substituted analog 18, while the three other compounds were inactive for FKBP51 or FKBP52. This series led us to conclude that a halogen is a preferred substituent at the meta-position for the larger FKBPs (Table-2).

![Chemical structure](image)

**Table-2** Meta substituted analogs synthesized for SAR extrapolation.

<table>
<thead>
<tr>
<th>Compd. No.</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>Purity %</th>
<th>FKBP12</th>
<th>FKBP51FK1</th>
<th>FKBP52FK1</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IC₅₀(µM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>CN</td>
<td>H</td>
<td>H</td>
<td>&gt;98</td>
<td>3.8 ± 0.3</td>
<td>28.5 ± 9.6</td>
<td>69.9 ± 65.4</td>
</tr>
<tr>
<td>14</td>
<td>NO₂</td>
<td>H</td>
<td>H</td>
<td>&gt;98</td>
<td>1.9 ± 0.13</td>
<td>47.2 ± 7.1</td>
<td>&gt;100</td>
</tr>
<tr>
<td>15</td>
<td>NH₂</td>
<td>H</td>
<td>H</td>
<td>&gt;99</td>
<td>1.9 ± 0.2</td>
<td>45.4 ± 13.1</td>
<td>&gt;100</td>
</tr>
<tr>
<td>16</td>
<td>NH</td>
<td>H</td>
<td>H</td>
<td>&gt;99</td>
<td>8.1 ± 1.2</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
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<tr>
<td>17</td>
<td>NH</td>
<td>H</td>
<td>H</td>
<td>&gt;99</td>
<td>9.1 ± 0.7</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>18</td>
<td>F</td>
<td>H</td>
<td>H</td>
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<td>1.09 ± 0.07</td>
<td>54.05 ± 7.0</td>
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<td>Br</td>
<td>H</td>
<td>H</td>
<td>&gt;99</td>
<td>0.32 ± 0.03</td>
<td>15.78 ± 1.25</td>
<td>15.69 ± 6.84</td>
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<tr>
<td>20</td>
<td>Cl</td>
<td>H</td>
<td>Cl</td>
<td>&gt;99</td>
<td>0.80 ± 0.08</td>
<td>22.6 ± 8.2</td>
<td>14.3 ± 1.8</td>
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<tr>
<td>21</td>
<td>Cl</td>
<td>Cl</td>
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<td>&gt;99</td>
<td>6.1 ± 5.7</td>
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<td>22</td>
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<td>OMe</td>
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<td>&gt;100</td>
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<tr>
<td>23</td>
<td>Cl</td>
<td>OMe</td>
<td>H</td>
<td>&gt;99</td>
<td>0.60 ± 0.07</td>
<td>29.6 ± 1.9</td>
<td>40.3 ± 5.1</td>
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<tr>
<td>24</td>
<td>F</td>
<td>H</td>
<td>F</td>
<td>&gt;98</td>
<td>1.00 ± 0.06</td>
<td>88.2 ± 11.6</td>
<td>Not measured</td>
</tr>
<tr>
<td>25</td>
<td>CF$_3$</td>
<td>H</td>
<td>CF$_3$</td>
<td>&gt;99</td>
<td>5.1 ± 0.4</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>26</td>
<td>CF$_3$</td>
<td>H</td>
<td>Br</td>
<td>&gt;99</td>
<td>2.4 ± 0.2</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>27</td>
<td>COO</td>
<td>Me</td>
<td>H</td>
<td>COOMe</td>
<td>&gt;98</td>
<td>4.5 ± 0.5</td>
<td>&gt;100</td>
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<tr>
<td>28</td>
<td>Cl</td>
<td>OH</td>
<td>Cl</td>
<td>&gt;99</td>
<td>0.67 ± 0.04</td>
<td>6.2 ± 0.5</td>
<td>20.3 ± 1.9</td>
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<tr>
<td>29</td>
<td>Cl</td>
<td>OMe</td>
<td>Cl</td>
<td>&gt;99</td>
<td>0.23 ± 0.02</td>
<td>16.4 ± 1.7</td>
<td>17.7 ± 1.6</td>
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<tr>
<td>30</td>
<td>Cl</td>
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<td>Cl</td>
<td>&gt;98</td>
<td>1.18 ± 0.04</td>
<td>16.1 ± 0.96</td>
<td>20.5 ± 2.7</td>
</tr>
<tr>
<td>31</td>
<td>OMe</td>
<td>OMe</td>
<td>COOH</td>
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<td>No binding</td>
<td>No binding</td>
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<tr>
<td>32</td>
<td>NO$_2$</td>
<td></td>
<td></td>
<td>&gt;98</td>
<td>1.5 ± 0.14</td>
<td>27.2 ± 3.1</td>
<td>43.9 ± 10.1</td>
</tr>
<tr>
<td>33</td>
<td>NH$_2$</td>
<td></td>
<td></td>
<td>&gt;98</td>
<td>2.57 ± 0.55</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>34</td>
<td>H</td>
<td></td>
<td></td>
<td>&gt;98</td>
<td>1.2 ± 0.09</td>
<td>18.4 ± 1.4</td>
<td>26.5 ± 4.9</td>
</tr>
</tbody>
</table>
The purity of the compounds was confirmed using HPLC. The binding affinity of the compounds to FKBP12, FKBP51 (FK1 domain) and FKBP52 (FK1 domain) was determined by a fluorescence polarization assay. The second series of sulfonamide derivatives was designed to probe the substitution pattern at the fused thiazole ring of compound 10. A substitution by methyl at C-2 resulted in compound 34 which had equivalent binding to 10. Conversion to the corresponding benzothiazol-2(3H)-one 35 resulted in nanomolar affinity for FKBP12, low micromolar binding for FKBP51 and high micromolar affinity to FKBP52. The reasons for this striking selective preference for FKBP12 are currently unknown. However, the sulfur in the meta-position seems to be extremely important since substitution by a methylene as in 36 or oxygen 37 resulted in a dramatic loss of affinity for all FKBP5s (Table-2).

**X-Ray Crystal Structure.** The X-ray crystal structure of the FK506-binding domain of FKBP51 complexed with ligand 20 was solved to 1.0 Å resolution. In this complex, FKBP51 adopts the same folding topology as found in FKBP51 complexed with 1 and 2. Compared to the latter structures Asp 68 moves into the binding pocket, while Tyr 113 and Ser 118 move out. The ligand adopts a similar binding mode compared to that of 1 or 2 with the common pипецолate ring being nearly superimposable (Fig. 2b). The pипецолyl ring of each ligand sits atop the indole of Trp 90, which forms the floor of the FKBP binding pocket. Similar to FK506 the C1-carbonyl of the pипецолate forms a hydrogen bond with the backbone amide of Ile 87. One oxygen of the sulfonamide (S=Oa) engages the ε-hydrogen of Phe 130 and the hydroxyl group of Tyr 113. This latter contact is substantially longer (3.37Å) compared to the corresponding hydrogen bonds formed between Tyr 113 and the C8-carbonyl groups of α-ketoamides like FK506, 2 or analogs thereof. The p-оxygen of Tyr 113 engages in a rather short dipolar contact with the C1-carbonyl of 20 (3.06Å). Similar although less intense dipolar interactions have also been observed in FKBP51-complexes with FK506 and 2. FKBP51 and 20 engage in a number of aromatic CH ––Ο-acceptor interactions, e.g., the oxygen of the sulfonamide (S=Ob) and the...
ε-hydrogens of Tyr\textsuperscript{57}, Phe\textsuperscript{67} and Phe\textsuperscript{130}. These interactions correspond to the contacts formed by the C\textsuperscript{9}-keto group of FK506 with the same residues of FKBP51 thereby confirming that the sulfonamide is a bioisosteric mimic of the α-ketoamide moiety. As expected, the dichloro aryl ring sits below the 80s loop and packs on Ile\textsuperscript{122}. The two ortho-hydrogens of the sulfonylephenyl ring form close contacts (2.95Å) with the p-oxygen of Tyr\textsuperscript{113} and with carboxylate of Asp\textsuperscript{68}, respectively. One of the aromatic chlorines might form a van-der-Waals contact with Lys\textsuperscript{121}, while the other chlorine engages Ser\textsuperscript{118}. For the latter, two conformations seem possible, one compatible with a hydrogen bond to the aromatic chloride, the other with a linear C-Cl-O geometry consistent with a halogen bond\textsuperscript{22,23}. The dimethoxyphenyl and acetoxyphenyl rings were poorly resolved in the electron density map indicating strong disorder. In the most populated conformer the top group is rotated by 120° compared to compound 2, most likely stabilized by π-π stacking interactions between the acetoxyphenyl ring B and the dichloro aryl substituent of the sulfonamide. The dimethoxyphenyl ring A stacks on the edge of Phe\textsuperscript{77} and points into a solvent channel. Its ortho-hydrogen forms an aromatic hydrogen bond (d=2.97Å) to the backbone carbonyl of Gln\textsuperscript{85}.

Fig. 2 X-ray crystal structure of 20 in complex with the FK1 domain of FKBP51. (a) Chemical structure of 20. Hydrophobic contacts with FKBP51 are indicated in green, hydrogen bonds are shown as dotted lines in pink, aromatic hydrogen bonds are indicated in blue and the unresolved groups are in grey. (b) 20 bound to the FK1 domain of FKBP51. The three hydrogen bonds between O\textsuperscript{1}-20 and HN-Ile\textsuperscript{87}, between O\textsuperscript{8}-20 and HO-Tyr\textsuperscript{113}, and between Cl\textsuperscript{11} and O-S\textsuperscript{118} are shown as dotted red lines. The dipolar interaction between the C\textsuperscript{1}-carbonyl and HO-Tyr\textsuperscript{113} and the halogen bond between Cl\textsuperscript{11} and O-S\textsuperscript{118} are shown in black. Aromatic hydrogen bonds between ring A and Gln\textsuperscript{85}, C\textsuperscript{10}.H and OH-Tyr\textsuperscript{113}, C\textsuperscript{14}.H and OH-Asp\textsuperscript{68} are shown in blue. Leu\textsuperscript{119} and Pro\textsuperscript{120} at the top of the 80s loop are colored in cyan and the conserved water below the 80s loop is shown in yellow.
**SAR extension**

The X-ray co-crystal structure of the sulfonamide 20 revealed a water molecule engaged in a hydrogen bond with the amide of Lys121 that is situated close to the para-position of the sulfonamide aromatic ring (Fig. 2b). Water molecules below Pro120 of the 80s loop have been observed in several FKBP51 crystal structures16 (unpublished observations). We therefore explored whether this conserved water molecule could be engaged by substituents in the para-position of the sulfonamide aromatic ring. Introduction of a p-OH substituent in compound 28 did not affect the affinity for FKBP51 while improving the selectivity vs. FKBP52 three-fold (Table 2). A similar trend was observed for the p-NHAc substituted analog 30, but not in the p-OMe substituted analog 29. The tri-substituted analog 33 had similar affinities compared to the corresponding mono-substituted derivative 15, whereas for 32 the affinities were slightly increased compared to 14. The tri-substituted analog 31 was inactive, similar to the disubstituted analog 22.

**Modification of the top group**

The charged carboxylic acid attached to ring B in the above series is likely to reduce the cell permeability of these compounds. To remove this undesired property the free acid moiety was replaced by various groups as shown in Scheme 2 to yield compounds 38-48. Simplified substituents at C-1 as in compounds 38 and 39 resulted in complete loss of activity for the large FKBPs. The next series of compounds included the substitution of the free acid moiety with a morpholine group. Surprisingly, in the morpholine-series phenyl sulfonamides substituted with meta-dichloro 40, with meta-dichloro, p-OMe 41 and the benzothiazole analog 42 were inactive for all FKBPs including FKBP12 (Table 3). This could be attributed in part to a detection limit imposed by the lower solubility of these compounds. In striking contrast, the meta-dichloro, para-hydroxy substituted analog 42 displayed submicromolar affinities for all tested FKBPs. This potency and the almost equal affinity for the large FKBPs vs. FKBP12 is remarkable, especially when compared to the very close analogs 22 (carboxyl group instead of morpholine), 40, 43 (para-hydrogen or para-methoxy instead of para-hydroxy) and 45 (para NH-acetyl). A similar unexpected activity was observed for the morpholine-containing benzothiazol-2(3H)-one analog 44 which was much more active than thiazole-containing analog 41 or the carboxyl-derivative 35. The affinity of 44 rivaled those of the natural product
FK506 (Table-2). The molecular underpinnings for the extraordinary activities of 42 and 44 remain to be established.

Last but not the least we replaced the pipecolate C\(^1\) ester by an amide (46, 47), which completely abolished the binding to larger FKBPs. Compound 47 retained substantial binding to FKBP12 in line with the preference of this substituent for FKBP12 observed with 44. The loss of binding affinity of 46 and 47 can be attributed to the additional hydrogen bond donor that would point in the direction of the aromatic ring when bound in a homologous binding mode as 20. Finally, in 48 the top group was replaced by a symmetric top group as present in Biricodar\(^3\) which resulted in equivalent affinity as 28.

Table-3. FKBP binding affinity of sulfonamides with different pipecolate ester substituents

<table>
<thead>
<tr>
<th>Compd. No.</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>Purity %</th>
<th>FKBP12</th>
<th>FKBP51FK1</th>
<th>FKBP52FK1</th>
</tr>
</thead>
<tbody>
<tr>
<td>38</td>
<td>Cl</td>
<td>H</td>
<td>Cl</td>
<td>&gt;98</td>
<td>&gt;100</td>
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</tr>
<tr>
<td>39</td>
<td>H</td>
<td>H</td>
<td>Cl</td>
<td>&gt;99</td>
<td>0.20 ± 0.10</td>
<td>66.27 ± 37.9</td>
<td>&gt;100</td>
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<tr>
<td>40</td>
<td>Cl</td>
<td>H</td>
<td>Cl</td>
<td>&gt;99</td>
<td>&gt;50 (\mu)M (^a)</td>
<td>&gt;50 (\mu)M (^a)</td>
<td>&gt;50 (\mu)M (^a)</td>
</tr>
<tr>
<td>41</td>
<td>H</td>
<td>H</td>
<td>Cl</td>
<td>&gt;98</td>
<td>&gt;50 (\mu)M (^a)</td>
<td>&gt;50 (\mu)M (^a)</td>
<td>&gt;50 (\mu)M (^a)</td>
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<td>Cl</td>
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<td>Cl</td>
<td>&gt;99</td>
<td>0.115 ± 0.014</td>
<td>0.456 ± 0.05</td>
<td>0.71 ± 0.10</td>
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<tr>
<td>43</td>
<td>Cl</td>
<td>OMe</td>
<td>Cl</td>
<td>&gt;99</td>
<td>&gt;50 (\mu)M (^a)</td>
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<tr>
<td>44</td>
<td>H</td>
<td>H</td>
<td>Cl</td>
<td>&gt;99</td>
<td>0.003 ± 0.0005</td>
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<td>45</td>
<td>Cl</td>
<td>N-Ac</td>
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<td>&gt;99</td>
<td>0.45 ± 0.03</td>
<td>12.3 ± 18.9</td>
<td>8.3 ± 6.8</td>
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Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

<table>
<thead>
<tr>
<th></th>
<th></th>
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<tr>
<td>47</td>
<td>H</td>
<td></td>
<td></td>
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<td>1.4 ± 0.21</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<td>&gt;99</td>
<td>0.87 ± 0.07</td>
<td>9.69 ± 0.76</td>
<td>15.13 ± 0.23</td>
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*a* Low solubility impaired binding measurements.

**Conclusion:** Using a bioisosteric replacement strategy we converted the α-ketoamide motif derived from FK506 and rapamycin into a sulfonamide motif with conservation of the hydrogen bond pattern as confirmed by the co-crystal structure of 20. By using a solid phase synthesis protocol we were able to generate, screen and optimize a focused library of these compounds. This led to the identification of aromatic sulfonamides with soft substituents in the meta-position as preferred binders of the larger FKBPs. In combination with the morpholine-modified top group this resulted in a 15-20 fold enhancement in affinity for FKBP51 or FKBP52 compared to the starting compound 2. The most advanced compound 42 is the best synthetic ligand known for the large FKBPs. Compound 44 has exceptionally high affinity for FKBP12 rivaling those of the natural products FK506 and rapamycin.

**Experimental section:**

**Chemistry:** All solvents were purchased from Roth, reagents were bought from Aldrich-Fluka and the sulfonyl chlorides were obtained from Maybridge, Sigma Aldrich, ABCR or AKos, unless otherwise stated.

Chromatographic separations were performed either by manual flash chromatography or by automated flash chromatography using an Interchim-Puriflash 430 with a UV detector. Extracts were dried over MgSO₄ and the solvents were removed under reduced pressure. Merck F-254 commercial plates were used for analytical TLC to follow the course of reaction and visualized by UV light at either 254 or 365 nm. Silica gel 60 (Merck 70-230 mesh) was used for column chromatography. NMR spectra of all compounds were obtained from the Department of Chemistry and Pharmacy, LMU, on a Bruker AC 300, a Bruker XL 400, or a Bruker AMX 600 at room temperature in deuterio-CDCl₃ with tetramethylsilane (TMS) as internal standard, unless otherwise stated. Mass spectra (m/z) were recorded on a Thermo Finnigan LCQ DECA XP Plus mass spectrometer at the Max Planck Institute of Psychiatry, while the high resolution mass spectrometry was carried out at the MPI for Biochemistry (Microchemistry Core Facility) on a Varian Mat711 mass spectrometer.
HPLC analysis was carried out using a Jupiter 4 µm Proteo column (250 x 4.6 mm, 5µm particle size), **Wavelength**: 224nm, 280nm; **Flow rate**: 1ml/min; **Buffer A**: 0.1% TFA in 5% MeCN/water; **Buffer B**: 0.1% TFA in 95% MeCN/water; **Gradient A**: After 1min elution with 100% buffer A, linear gradient of 0-100% buffer B for 30 min.

**Method LCMS**: YMC Pro C-8 (100 x 4.6 mm, 3µm particle size) column, **Wavelength**: 224nm, 280nm; **Flow rate**: 1ml/min; **Buffer A**: 0.1% HCOOH in 5% MeCN/water; **Buffer B**: 0.1% HCOOH in 95% MeCN/water; **Gradient B**: 1min 100% buffer A, then linear gradient of 0-100% buffer B for 11 min.

Final compounds were purified using a preparative HPLC Jupiter 10µm Proteo (250 x 21.7 mm, 10µm particle size) column. Compounds were dissolved in 40% buffer B and the purification was carried out with an injection loop volume of 2ml. **Wavelength**: 224nm; **Flow rate**: 25ml/min; **Buffer A**: 0.1% TFA in 5% MeOH/Water; **Buffer B**: 0.1% TFA in 95% MeOH/water; **Gradient C**: 40% B, then a linear gradient of 60-70% B for 15 min.

**Synthesis of 2-(3-(((1R)-1-(1-(((9H-fluoren-9-yl)methoxy)carbonyl)piperidine-2-carbonyloxy)-3-(3,4-dimethoxyphenyl)propyl)phenoxy)acetic acid 5a.**

The reaction was performed as previously described to yield the pipecolic ester 1.

To the **pipecolic ester 1** (3g, 4mmol) a solution of 20% TFA in DCM (20mL) was added at 0°C. The mixture was allowed to warm to room temperature and stirred for 6h after which time it was diluted with DCM and evaporated under reduced pressure to remove solvents and TFA. The crude material was then subjected to column chromatography using hexane: EtOAc: TFA 7:2:2.8: 0.2 to afford product 5a (2.7g, 4mmol, 100%).

TLC (Hexane: EtOAc: TFA 7:2.8:0.2): Rf = 0.38.

HPLC (Gradient A) retention time= 32.21-32.62 min

$^1$H NMR (600 MHz, CDCl$_3$) δ= 1.39-1.49 (m, 2H), 1.74 (dd, 3H, J= 11.7, 45.6 Hz), 2.02-2.11 (m, 1H), 2.15-2.31 (m, 2H), 2.53-2.70 (m, 2H), 2.75-2.83 (m, 1H), 3.19 (t, 1H, J= 12.1 Hz), 3.85 (s, 6H), 3.99-4.12 (m, 1H), 4.22 (t, 1H, J= 6.8Hz), 4.33-4.45 (m, 1H), 4.65 (dd, 1H, J= 16.6 35.3 Hz), 4.83 (s, 0.5H), 4.99 (d, 0.5H, J= 4 Hz), 5.67 (dd, 1H, J= 4.8, 8.3 Hz), 6.54-6.71 (m, 2H), 6.72-6.82 (m, 2H), 6.82-6.97 (m, 2H), 7.16-7.31 (m, 4H), 7.31-7.41 (m, 2H), 7.51-7.55 (m, 1H), 7.67-7.77 (m, 2H).

$^{13}$C NMR (150 MHz, CDCl$_3$) δ= 21.07, 25.02, 27.27, 31.80, 38.43, 42.24, 47.39, 54.95, 56.23, 56.30, 65.56, 68.68, 76.94, 110.38, 111.76, 112.07, 115.98, 120.07, 120.33, 120.60, 125.32, 127.45, 128.11, 130.17, 133.71, 141.60, 142.52, 143.91, 144.09, 147.70, 149.21, 157.22, 158.17, 170.94, 172.24.
Synthesis of 2-(3-((1R)-1-(1-(((9H-fluoren-9-yl)methoxy)carbonyl)piperidine-2-carbonyloxy)-3-(3,4-dimethoxyphenyl)propyl)phenoxy)acetic acid 5b.

The reaction was done as previously described to yield the pipecolic ester 2.

To the pipecolic ester 2 (0.52 g, 0.71 mmol) a solution of 20% TFA in DCM (20mL) was added at 0°C. The mixture was allowed to warm to room temperature and stirred for 2.5 h after which time it was diluted with DCM and evaporated under reduced pressure to remove solvents and TFA. The crude material was then subjected to column chromatography using Hexane: EtOAc: TFA 6:4: 0.1 to yield product 5b (410g, 0.60 mmol, 84%).

TLC (Hexane: EtOAc: TFA 6:4:0.1): Rf = 0.38.


Coupling of free acid 5a to the trityl resin.

2-Chloro tritylresin (6.1g, 7.9mmol, Novabiochem) resin was swollen in DCM for 1h and added to a mixture of 2-(3-((1R)-1-(1-(((9H-fluoren-9-yl)methoxy)carbonyl)piperidine-2-carbonyloxy)-3-(3,4-dimethoxyphenyl)propyl)phenoxy) acetic acid 5a (2.7 g, 3.97mmol ) and i-Pr2EtN (2g, 15.89mmol) in 20 ml dry DCM. The reaction was monitored by removing aliquots of the reactant mixture (10 μL) over a period of 1- 24h which were filtered and dried. The analytes were then re-dissolved in buffer A subjected to HPLC analysis and checked for the disappearance of compound 5a. The slurry was agitated for 16h and then filtered. The resin was washed with DCM (3 x 50ml); DCM: MeOH: i-Pr2EtN 17:2:1 (3 x 50ml); DCM (3 x 20ml); DMF (3 x 25ml); MeOH (3 x 50ml); CHCl3 (3 x 50ml) and diethyl ether (3 x 50ml). The resin was dried under high vacuum overnight to yield a free flowing resin immobilized with compound 5a. The loading of the free acid was calculated using Fmoc deprotection and measurement of the Fmoc absorbance.

Coupling of free acid 5b to the trityl resin.

Loading reaction was carried out as described previously.

Final Library Synthetic route:

Fmoc-protected immobilized pipecolate 5a was pre-weighed (approximately 50 mg, 0.019 mmol) and transferred to each of 36 wells of a 96-well parallel synthesis reactor platform obtained from FlexChem® peptide synthesis system. The resin was swollen for 1h in 1mL
DCM followed by addition of 2mL of 20% 4-methyl piperidine in DCM and the reactor was stirred for 1h for the Fmoc deprotection to give 6a. The wells were washed with DCM (1X 3mL) by vacuum assisted filtration. The resins were dried and the sulfonyl chlorides (0.095 mmol) obtained commercially from Maybridge were weighed (15- 40mg) and added to the wells as a solution in DIPEA (30 mg, 0.237 mmol) in 0.25mL DCM for the first coupling and 15 mg (0.119 mmol) in 0.25mL DCM for the second coupling with 0.057 mmol (5- 12mg) sulfonyl chloride. The reaction time was 4h for first coupling and 20h for the second coupling. The wells were subsequently washed with DCM and ethanol to completely remove excess of unreacted sulfonyl chlorides. The compounds were finally cleaved in presence of 1mL of 1% TFA solution in DCM for 20 min. Each of the solutions were collected by vacuum filtration and dried by air blowing to give approximately 12mg of the crude products. The purity of the above crude products was analyzed by HPLC using gradient A and 36 of these compounds were further purified by preparative HPLC using the gradient B. The remaining compound 50 was purified using ion exchange column chromatography to get rid of the traces of unreacted educt. The purified compounds were characterized using mass spectroscopy and dried under high vacuum to yield approximately 1-3 mg of the final desired sulfonamides.

Medium Scale synthesis:

**Deprotection of Fmoc resin 6a:**
The coupled resin 5a was weighed (210 mg, 0.08mmol) and added to syringes, swollen in DCM (4 mL) for 1h, and the Fmoc protecting group was removed using 20% 4-methyl piperidine/DCM (4ml) for 1h. After filtration, the resin was washed with DCM (3 x 5ml) and used for the next coupling step.

**Synthesis of sulfonamides:**
To the above resin i-Pr₂EtN (40mg, 0.317mmol) in dry DCM (3 mL) was added and stirred for 20min. To this solution the sulfonyl chloride (0.237mmol) in 500 µL of DCM was added and the reaction was stirred for 4h at room temperature. After the first coupling step the resins were filtered, washed with DCM (3 x 10ml) and then subjected to second coupling with i-Pr₂EtN (30mg, 0.237mmol), sulfonyl chloride (0.158 mmol) in DCM (3 mL) and stirred for 16h at room temperature. The resins were washed with DCM (3 x 5ml) and dried to give the derivatized resins. These were re-swollen in DCM reacted with 1% TFA/DCM (3ml) for 1h and then washed with 1% TFA/DCM (3 x 3ml) and further washed with DCM (3 x 5ml). The combined filtrates were concentrated *in vacuo* to yield the compounds 8-37. (crude weight ~
TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.18, yield= 13.5mg (30%).
HPLC (Gradient A) retention time= 25.1-25.3 min

1H NMR (600 MHz, CDCl3) δ= 1.29 (t, 1H, J = 7.2 Hz), 1.55 (dd, 1H, J = 3.6, 9 Hz), 1.67 (d, 1H, J = 12.6), 1.72 (d, 1H, J = 13.8 Hz), 1.85-1.91 (m, 1H), 1.93-1.99 (m, 1H), 2.13-2.19 (m, 1H), 2.24 (d, 1H, J = 13.2 Hz), 2.45-2.50 (m, 2H), 2.55-2.59 (m, 1H), 3.11-3.16 (m, 2H), 3.75 (d, 1H, J = 9.6 Hz), 3.82 (s, 3H), 3.83 (s, 3H), 4.91 (d, 1H, J = 4.8 Hz), 5.53 (q, 1H, J = 3, 4.8 Hz), 6.61 (s, 1H), 6.63 (d, 1H, J = 7.8 Hz), 6.74 (d, 1H, J = 7.8 Hz), 6.80 (s, 1H), 6.92 (d, 1H, J = 7.2 Hz), 7.01(d, 1H, J = 7.8 Hz), 7.32-7.35 (m, 2H), 7.59 (d, 2H, J = 8.4 Hz), 8.25 (d, 2H, J = 8.4 Hz), 8.91 (d, 2H, J = 4.8 Hz).

13C NMR (150 MHz, CDCl3) δ= 20.02, 24.74, 28.17, 31.42, 38.30, 42.45, 45.66, 55.33, 55.82, 65.24, 76.43, 111.28, 111.60, 112.94, 114.37, 118.85, 120.02, 120.09, 127.61, 128.51, 130.01, 133.25, 140.05, 141.31, 142.19, 147.34, 157.41, 157.77, 162.90, 169.73.

Synthesis of 2-(3-((R)-1-(S)-1-(3-chlorophenylsulfonyl)piperidine-2-carboxyloxy)-3-(3,4-dimethoxy phenyl) propyl-phenoxy)acetic acid 9
TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.50, yield= 25.4 mg (57%).
HPLC (Gradient A) retention time= 26.1-26.5 min

1H NMR (400 MHz, DMSO) δ= 1.12-1.18 (m, 4H), 1.59 (t, 2H, J = 13.6 Hz), 1.98-2.13 (m, 2H), 2.40 (t, 2H, J = 6.8 Hz), 3.07 (t, 1H, J = 12 Hz), 3.61 (d, 1H, J = 12 Hz), 3.69 (s, 3H), 3.70 (s, 3H), 4.55 (s, 2H), 4.68 (d, 1H, J = 4Hz), 5.50 (t, 1H, J = 4.8Hz), 6.56 (d, 1H, J = 7.6 Hz), 6.60 (s, 1H), 6.72 (d, 1H, J = 8 Hz), 6.76-6.80 (m, 3H), 7.19-7.26 (m, 2H), 7.46 (d, 2H, J = 7.2 Hz), 7.61 (s, 1H).

13C NMR (100 MHz, DMSO) δ= 20.05, 24.51, 27.74, 31.05, 37.92, 42.79, 55.32, 55.76, 64.99, 76.13, 111.95, 112.23, 112.85, 114.11, 119.22, 120.33, 125.36, 126.62, 129.77, 130.88, 132.66, 133.48, 134.48, 141.75, 141.95, 147.34, 148.92, 158.18, 169.61, 172.41.
MS (ESI) m/z: found Rt 12.34 min. (Method LCMS), 654.17, 656.16 [M + Na]+.
Synthesis of 2-(3-((R)-1-((S)-1-(benzo[d]thiazol-5-ylsulfonil)piperidine-2-carbonyloxy)-3-(3,4-dimethoxyphenyl)propyl)phenoxy)acetic acid 10

TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.20, yield= 25.4mg (59%).

HPLC (Gradient A) retention time= 24.6-25.0 min

$^1$H NMR (600 MHz, CDCl$_3$) δ = 1.08-1.15 (m,1H), 1.38-1.45 (m,1H), 1.60 (t, 2H, J = 12 Hz), 1.70-1.76 (m,1H), 1.94-2.00 (m,1H), 2.11- 2.18 (m,1H), 2.43- 2.54 (m,1H), 3.23 (dt, 1H, J = 3, 6 Hz), 3.76-3.78 (m,1H), 3.85(s, 6H), 4.65 (s, 2H), 4.84 (d, 1H, J = 4.2 Hz), 5.55 (t, 1H, J = 7.2 Hz), 6.63 (s, 1H), 6.64 (d, 1H, J = 1.8 Hz), 6.78 (d, 2H, J = 5.4 Hz), 6.81 (dd, 1H, J = 2.4, 6 Hz), 6.85 (d, 2H, J = 8.5 Hz), 7.22 (t, 1H, J = 7.8 Hz), 7.8 (dd, 1H, J = 1.8, 8.4 Hz), 8.13 (d, 1H, J = 8.4 Hz), 8.46 (d, 1H, J = 1.8 Hz), 9.18 (s, 1H).

$^{13}$C NMR (150 MHz, CDCl$_3$) δ= 19.91, 24.61, 27.69, 31.22, 37.87, 42.81, 55.27, 55.91, 55.92, 65.00, 76.54, 111.34, 111.73, 112.91, 114.16, 119.88, 120.14, 121.92, 123.95, 124.83, 129.78, 133.20, 133.83, 137.56, 141.50, 147.39, 148.87, 154.92, 157.56, 158.03, 169.95, 171.84


Synthesis of 2-(3-((R)-3-(3,4-dimethoxyphenyl)I-((S)-1-(furan-3-ylsulfonil)piperidine-2-carbonyloxy) propyl)phenoxy)acetic acid 11

TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.37, yield= 25mg (56%).

HPLC (Gradient A) retention time= 24.4-24.8 min

$^1$HNMR (300 MHz, CDCl$_3$) δ= 1.10-1.18 (m,1H), 1.43-1.52 (m,1H), 1.61-1.81(m,2H), 2.00-2.12(m,1H), 2.19-2.30 (m, 2H), 2.37 (s,2H), 2.52-2.65 (m,2H), 3.14-3.28 (m,2H), 3.87(s,3H), 3.88 (s,3H), 4.68 (s, 2H), 4.82 (d, 1H, J = 4.5 Hz), 5.70 (dd. 1H, J = 2.1,5.7 Hz) , 6.58 (q,1H, J = 0.9, 1.2Hz), 6.70 (dd, 2H, J = 2.1, 4.5 Hz), 6.79-6.87 (m,2H), 6.91-6.96 (m, 2H), 7.25 (d, 1H, J = 2.1 Hz), 7.44 (t, 1H, J = 2.4 Hz), 7.91 (q, 1H, J = 0.9 Hz).

$^{13}$C NMR (75 MHz, CDCl$_3$) δ= 21.45, 24.62, 27.72, 31.25, 37.92, 42.57, 55.18, 55.93, 55.94, 64.92, 76.40, 108.52, 111.40, 111.80, 112.88, 114.30, 119.97, 120.20, 127.37, 128.21, 133.29, 141.86, 144.42, 145.50, 147.42, 148.92, 157.66, 170.10


Synthesis of 2-(3-((R)-1-((S)-1-(benzo[b]thiophen-2-ylsulfonil)piperidine-2-carbonyloxy)-3-(3,4-dimethoxyphenylpropyl)phenoxy)acetic acid 12
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TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.62, yield= 23.8 mg (55%).

HPLC (Gradient A) retention time= 21.8-22.2 min

$^1$HNMR (300 MHz, CDCl$_3$) δ= 1.12- 1.21 (m,1H), 1.44-1.52(m,1H), 1.60-1.64 (m,2H), 1.70-1.81 (m,1H), 1.96-2.07 (m,1H), 2.14-2.26(m,2H), 2.47-2.65(m,2H), 3.36 (dt, 1H, J = 3 Hz), 3.86 (s,3H), 3.87 (s, 3H), 4.66 (s,2H), 4.87 (d, 1H, J = 4.2 Hz), 5.65 (dd, 1H, J = 2.4, 6.6 Hz), 6.66-6.69 (m,2H), 6.77-6.85 (m,2H), 6.91(d, 2H, J = 7.2), 7.24 (t, 1H, J = 8.1 Hz), 7.40-7.49(m, 2H), 7.79-7.84 (m,3H).

$^{13}$C NMR (75 MHz, CDCl$_3$) δ= 19.87, 24.47, 27.45, 31.28, 37.98, 40.44, 55.47, 55.93, 55.94, 64.90, 76.44, 111.39, 111.84, 112.73, 114.36, 119.95, 120.22, 122.66, 125.36, 125.58, 127.08, 128.91, 129.76, 133.36, 137.63, 141.35, 141.61, 141.82, 147.39, 148.90, 157.59, 169.84.


Synthesis of 2-(3-((R)-1-((S)-1-(3-cyanophenylsulfonyl)piperidine-2-carbonyloxy)-3-(3,4-dimethoxyphenyl)propyl)phenoxy)acetic acid 13

TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.58, yield= 29.5mg (61%).

$^1$HNMR (600 MHz, CDCl$_3$) δ= 1.02- 1.10 (m,1H), 1.40-1.47 (m,1H), 1.60-1.64 (m,2H), 1.72-1.78 (m,1H), 1.99-2.06 (m,1H), 2.16-2.22 (m,2H), 2.49-2.59 (m,2H), 3.15(dt,1H, J = 3 Hz), 3.7 (d, 1H, J = 9.6 Hz), 3.86 (s,3H), 3.87 (s, 3H), 4.67 (d, 1H, J = 4.8 Hz), 5.60 (dd, 1H, J = 6 Hz), 6.67-6.69 (m,2H), 6.81 (d, 1H, J = 7.8 Hz), 6.83-6.84 (m, 1H), 6.90 (d,1H, J = 7.8 Hz), 7.26 (dd, 1H, J = 7.8 Hz), 7.56 (t, 1H, J = 7.8 Hz), 7.78 (t, 1H, J = 1.2 Hz, 7.8 Hz), 7.99-7.99 (m, 1H), 8.07 (t, 1H, J = 1.8Hz).

$^{13}$C NMR (150 MHz, CDCl$_3$) δ= 19.83, 24.63, 27.80, 31.25, 37.82, 42.88, 55.39, 55.93, 55.97, 64.78, 76.70, 111.37, 111.82, 112.95, 113.38, 114.23, 117.30, 120.00, 120.16, 129.87, 130.70, 131.05, 133.16, 135.56, 141.58, 141.77, 147.43, 148.88, 157.53, 169.67, 171.85.


Synthesis of 2-(3-((R)-3-(3,4-dimethoxyphenyl)1-((S)-1-(3-nitrophenylsulfonyl)piperidine -2-carbonyloxy propyl)phenoxy)acetic acid 14

TLC (Hexane: EtOAc: TFA 5.5:4.5:0.2): Rf = 0.44, yield= 24.0 mg (48%).

HPLC (Gradient A) retention time= 24.4-24.8 min

$^1$HNMR (600 MHz, CDCl$_3$) δ= 1.00- 1.01 (m,1H), 1.43-1.49 (m,1H), 1.60-1.63 (m,2H), 1.74-1.80 (m,1H), 1.98-2.04 (m,1H), 2.15-2.23 (m,2H), 2.46-2.57 (m,2H), 3.15 (dt,1H, J = 2.4 Hz,
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12.6 Hz), 3.74 (td, 1H, J = 2.4, 9.6 Hz), 3.85 (s, 3H), 3.86 (s, 3H), 4.65 (s, 2H), 4.85 (d, 1H, J = 4.2 Hz), 5.57 (dd, 1H, J = 6.6 Hz), 6.65-6.67 (m, 2H), 6.79-6.82 (m, 3H), 6.87 (d, 1H, J = 7.8 Hz), 7.24 (dd, 1H, J = 7.8 Hz), 7.62 (t, 1H, J = 7.8 Hz), 8.06-8.08 (m, 1H), 8.34-8.36 (m, 1H), 8.59 (t, 1H, J = 1.8 Hz).

13C NMR (150 MHz, CDCl₃) δ= 19.81, 24.65, 27.86, 31.21, 37.76, 42.88, 55.47, 55.92, 55.93, 64.76, 76.73, 111.35, 111.77, 112.98, 114.14, 120.12, 122.34, 126.91, 129.85, 130.13, 132.62, 133.17, 141.53, 142.09, 147.40, 148.07, 157.55, 169.65, 172.65


**Synthesis of 2-(3-((R)-1-((S)-1-(3-aminophenylsulfonyl)piperidine-2-carbonyloxy)-3-(3,4-dimethoxyphenyl)propyl)phenoxy)acetic acid 15**

TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.28, yield= 35 mg (66%).

HPLC (Gradient A) retention time= 21.32-21.52 min

¹HNMR (600 MHz, CDCl₃) δ= 1.21-1.29 (m, 1H), 1.46-1.52 (m, 1H), 1.60-1.66 (m, 1H), 1.69-1.74 (m, 1H), 1.79-1.86 (m, 1H), 1.89-1.96 (m, 1H), 2.07-2.13 (m, 1H), 2.42-2.56 (m, 2H), 3.10-3.25 (m, 1H), 3.74-3.79 (m, 1H), 3.84 (s, 6H), 4.66 (s, 2H), 4.70-4.74 (m, 1H), 5.53 (s, 1H), 6.60-6.68 (m, 3H), 6.74-6.80 (m, 2H), 6.82-6.86 (m, 2H), 7.13-7.20 (m, 2H), 7.36-7.54 (m, 2H).

¹³C NMR (150 MHz, CDCl₃) δ= 20.06, 24.80, 28.03, 31.36, 37.99, 42.76, 55.20, 55.89, 55.92, 65.02, 76.38, 111.33, 111.68, 112.54, 114.44, 118.69, 119.84, 120.12, 123.19, 124.25, 129.94, 130.21, 133.19, 139.69, 140.92, 141.93, 147.39, 148.87, 157.64, 169.72, 172.07

MS (ESI) m/z: found RT 10.87 min. (Method LCMS), 613.12 [M + H]⁺, 635.17 [M + Na]⁺.


**Synthesis of 2-(3-((R)-3-(3,4-dimethoxyphenyl)-1-((S)-1-(3-(2-methylpyrimidin-4-yl)phenylsulfonyl)piperidine-2-carbonyloxy)propyl)phenoxy)acetic acid 16**

TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.22, yield= 48.3 mg (90%).

HPLC (Gradient A) retention time= 23.1-23.5 min

¹HNMR (600 MHz, CDCl₃) δ= 1.14-1.21 (m, 1H), 1.51-1.58(m,1H), 1.61-1.63 (m,1H), 1.71-1.75 (m,1H), 1.83-1.90 (m,1H), 1.96-2.02 (m,1H), 2.12-2.18 (m,1H), 2.32 (d, 1H, J = 13.8 Hz), 2.45-2.49 (m,1H), 2.52-2.57(m,1H),3.66 (d, 1H, J = 12 Hz), 3.79 (s,3H), 3.80 (s, 3H), 4.59 (d,1H, J = 16.8 Hz), 4.67 (d, 2H, J = 16.8 Hz), 4.98 (d, 1H, J = 5.4 Hz), 5.51 (dd, 1H, J = 5.4 Hz), 6.66-6.64 (m,2H), 6.75 (m, 2H, J= 8.4 Hz), 6.81-6.86 (m, 2H), 7.26 (m, 1H), 7.66 (t, 1H, J= 8.4 Hz), 8.06-8.08 (m, 1H), 8.34-8.36 (m, 1H).
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= 7.8 Hz), 8.01(d, 1H, J = 6 Hz), 8.01- 8.08 (m, 1H), 8.41-8.43(m,1H), 8.73 (t, 1H, J = 1.8 Hz), 9.07 (d, 1H, J = 6.6 Hz).

$^{13}$C NMR (150 MHz, CDCl$_3$) δ = 20.19, 22.94, 24.75, 28.08, 31.23, 37.79, 43.03, 55.21, 55.87, 55.88, 65.36, 77.06, 111.41, 111.61, 112.78, 115.21, 115.33, 118.94, 120.22, 127.02, 129.86, 130.19, 131.83, 132.08, 133.02, 134.97, 141.62, 141.84, 147.39, 148.85, 151.43, 157.99, 164.28, 167.69, 170.58, 171.54


**Synthesis of 2-(3-((R)-3-(3,4-dimethoxyphenyl)-1-((S)-1-(3-(pyrimidin-4-yl)phenylsulfonyl)piperidine-2-carbonyloxy)propyl)phenoxy)acetic acid 17**

TLC (Hexane: EtOAc: TFA 5:4.8:0.2): Rf = 0.43, yield= 41.9 mg (80%).

HPLC (Gradient A) retention time= 24.2–24.6 min

$^1$HNMR (600 MHz, CDCl$_3$) δ = 1.10- 1.17 (m,1H), 1.37-1.44 (m,1H), 1.58-1.61 (m,2H), 1.66-1.72 (m,1H), 1.94-2.00 (m,1H), 2.13-2.19 (m,2H), 2.42-2.47 (m,1H), 2.50- 2.55 (m,1H), 3.25(dt,1H, J = 3 Hz, 12.6 Hz),3.83 (d, 1H, J = 6 Hz), 3.85 (s,3H), 3.85 (s, 3H), 4.67 (d, 2H, J =6 Hz), 4.83 (d, 1H, J = 4.2 Hz), 5.60 (dd, 1H, J =5.4 Hz),6.62-6.64 (m,2H), 6.77 (d, 1H, J =8.4 Hz), 6.80-6.82 (m, 1H), 6.86-6.88 (m,2H), 7.22 (t, 1H, J =8.4 Hz), 7.28 (t, 1H, J = 4.8 Hz), 7.59 (t, 1H, J = 7.8 Hz), 7.92-7.94 (m, 1H), 8.55 (dd, 1H, J = 1.2 Hz, 7.8Hz), 8.85 (d, 3H, J = 4.8 Hz).

$^{13}$C NMR (150 MHz, CDCl$_3$) δ = 19.99, 24.53, 27.41, 31.28, 38.02, 42.93, 55.35, 55.88, 55.92, 64.85, 111.31, 111.74, 112.30, 114.60, 119.88, 119.95, 120.17, 127.01, 129.17, 129.45, 129.71, 132.08, 133.42, 137.71, 141.12, 141.77, 147.27, 148.79, 157.41, 157.63, 162.78, 170.08, 172.04.


**Synthesis of 2-(3-((R)-3-(3,4-dimethoxyphenyl)-1-((S)-1-(3-fluorophenylsulfonyl)piperidine-2-carbonyloxy)propyl)phenoxy)acetic acid 18**

TLC (Hexane: EtOAc: TFA 5.5:4.5:0.2): Rf = 0.44, yield= 23.5 mg (49%).

HPLC (Gradient A) retention time= 24.70-24.85 min

$^1$HNMR (300 MHz, CDCl$_3$) δ = 1.24-1.33 (m,1H), 1.41-1.53(m,1H), 1.63-1.85 (m, 3H), 1.94-2.06 (m,1H), 2.12-2.27 (m, 2H), 2.45-2.61 (m, 2H), 3.18 (t, 1H, J= 12.7 Hz), 3.74 (d, 1H, J=
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

12.6 Hz), 3.85 (s, 6H), 4.71 (s, 2H), 4.80 (d, 1H, J= 4.8Hz), 5.63 (t, 1H, J= 7.8 Hz), 6.65-6.67 (m, 2H), 6.75 (d, 1H, J= 8.7 Hz), 6.89- 6.93 (m, 3H), 7.13- 7.34 (m, 3H), 7.39-7.41 (m, 2H).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$= 20.05, 24.62, 27.89, 31.24, 37.95, 42.77, 55.37, 55.88, 55.93, 65.32, 76.34, 111.38, 111.78, 112.56, 114.18, 114.50, 119.43, 119.71, 120.18, 122.83, 129.86, 130.49, 133.29, 135.46, 141.77, 147.39, 148.89, 157.75, 160.53, 163.86, 165.31, 169.73.

MS (ESI) m/z: found Rt 13.19 min. (Method LCMS), 638.19 [M + Na]$^+$.


Synthesis of 2-(3-((R)-1-((S)-1-(3-bromophenylsulfonyl)piperidine-2-carbonyloxy)-3-(3,4-dimethoxyphenyl)propyl)phenoxy)acetic acid 19

TLC (Hexane: EtOAc: TFA 5.5:4.5:0.2): Rf = 0.36, yield= 29.87mg (57%).

HPLC (Gradient A) retention time= 25.89-26.02 min

$^{1}$HNMR (300 MHz, CDCl$_3$) $\delta$ = 1.24-1.32 (m, 1H), 1.39-1.52 (m,1H), 1.62-1.85 (m, 3H), 1.94-2.05(m, 1H), 2.11–2.27(m, 2H), 2.44-2.62 (m, 2H), 3.16 (dt, 1H, J= 2.7; 12.6 Hz), 3.71 (d, 1H, J= 9.9 Hz), 4.70 (s, 2H), 4.80 (d, 1H, J= 4.5 Hz), 5.65 (t, 1H, J= 8.1 Hz), 6.65-6.68 (m, 2H), 6.79 (d, 1H, J= 8.7 Hz), 6.88-6.93 (m, 3H), 7.11 (t, 1H, J= 8.1 Hz), 7.31 (t, 1H, J= 8.25 Hz), 7.52 (dd, 1H, J= 0.9; 7.8 Hz), 7.59 (dd, 1H, J= 0.9, 7.9 Hz), 7.87 (t, 1H, J= 1.8Hz).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$= 20.06, 24.59, 27.87, 31.23, 37.96, 42.79, 55.48, 55.89, 55.94, 64.86, 76.43, 111.41, 111.80, 112.60, 114.53, 120.09, 120.21, 122.73, 125.64, 129.89, 130.33, 133.32, 135.46, 141.60, 141.75, 147.37, 148.86, 157.75, 169.81, 172.68.

MS (ESI) m/z: found Rt 13.33 min. (Method LCMS), 698.31, 700.12 [M + Na]$^+$.

HRMS 676.1716, 678.1715 [M + H]$^+$, calculated 676.1718 [M + H]$^+$.

Synthesis of 2-(3-((R)-1-((S)-1-(3,5-dichlorophenylsulfonyl)piperidine-2-carbonyloxy)-3-(3,4-dimethoxyphenyl)propyl)phenoxy)acetic acid 20

TLC (Hexane: EtOAc: TFA 5.5:4.5:0.2): Rf = 0.58, yield= 22.6mg (44%).

HPLC (Gradient A) retention time= 26.8-27.3 min

$^{1}$HNMR (600 MHz, CDCl$_3$) $\delta$= 1.03- 1.10 (m,1H), 1.40-1.47 (m,1H), 1.60-1.63 (m,2H), 1.71-1.77 (m,1H), 1.99-2.06 (m,1H), 2.16-2.23 (m,2H), 2.49-2.59 (m,2H), 3.20 (dt,1H, J = 2.4 Hz, 12.6 Hz), 3.71 (td, 1H, J = 1.2, 12.6 Hz), 3.85 (s,3H), 3.86 (s, 3H), 4.66 (s,2H), 4.78 (d, 1H, J = 4.8 Hz), 5.63 (dd, 1H, J = 6 Hz), 6.66-6.68 (m,2H), 6.79 (d, 1H, J = 7.8 Hz), 6.83-6.84 (m, 1H), 6.85 (t,1H, J = 2.4 Hz), 6.90 (d, 1H, J = 7.8 Hz), 7.26 (dd, 1H, J = 7.8 Hz), 7.49 (t, 1H, J = 1.8 Hz), 7.66 (d, 1H, J = 1.8 Hz).
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

$^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$= 19.74, 24.61, 27.74, 31.28, 37.84, 42.89, 55.32, 55.92, 64.78, 76.65, 111.33, 111.75, 112.95, 114.17, 120.00, 120.17, 125.45, 129.87, 132.42, 133.21, 135.73, 141.63, 142.89, 147.40, 148.86, 157.53, 169.57, 172.53.


**Synthesis of 2-(3-((R)-1-(2,3-dichlorophenylsulfonyl)piperidine-2-carbonyloxy)-3-(3,4-dimethoxyphenyl)propyl)phenoxy)acetic acid 21**

TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.50, yield = 18.7 mg (43%)

HPLC (Gradient A) retention time= 27.1-27.5 min

$^1$HNMR (600 MHz, CDCl$_3$) $\delta$= 1.02- 1.08 (m,1H), 1.41-1.46 (m,1H), 1.60-1.64 (m,2H), 1.70-1.76 (m,1H), 2.00-2.06 (m,1H), 2.15-2.24 (m,2H), 2.50-2.60 (m,2H), 3.21 (dt,1H, $J = 2.4$ Hz, 12.6 Hz), 3.70 (td, 1H, $J = 1.2$, 12.6 Hz), 3.85 (s,3H), 3.86 (s, 3H), 4.65 (s,2H), 4.77 (d, 1H, $J = 4.8$ Hz), 5.63 (dd, 1H, $J = 6$ Hz), 6.65-6.67(m,2H), 6.73 (d, 1H, $J = 7.8$ Hz), 6.80-6.82 (m, 1H), 6.85 (t,1H, $J = 2.4$ Hz), 6.92 (s,1H), 7.25 (dd, 1H, $J = 7.8$ Hz), 7.49 (t, 1H, $J = 1.8$ Hz), 7.67 (d, 1H, $J = 1.8$ Hz).


**Synthesis of 2-(3-((R)-3-(3,4-dimethoxyphenyl)-1-((S)-1-(3,4-dimethoxyphenylsulfonyl)piperidine-2-carbonyloxy)propyl)phenoxy)acetic acid 22**

TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.38, yield = 32.89 mg (62%).

HPLC (Gradient A) retention time= 23.1-23.5 min

$^1$HNMR (600 MHz, CDCl$_3$) $\delta$= 1.08- 1.15 (m,1H), 1.32-1.39(m,1H), 1.53-1.60(m,2H), 1.64-1.70(m,1H), 1.99-2.06 (m,1H), 2.14-2.23(m,2H), 2.48-2.59(m,2H), 3.19 (dt, 1H, $J = 3$, 10.2 Hz), 3.63 (d,1H, $J = 11.4$Hz), 3.83 (s,3H), 3.84 (s,3H), 3.87 (s,3H), 3.87 (s, 3H), 4.65 (s,2H), 4.78 (d, 1H, $J = 4.8$ Hz), 5.64 (dd, 1H, $J = 1.2$, 6.0 Hz), 6.66 (d,2H, $J = 7.2$ Hz), 6.73 (s, 1H, $J = 8.4$Hz), 6.81- 6.83(m, 2H), 6.88-6.91 (m, 2Hz), 7.22-7.24(m,2H), 7.41 (dd,1H, $J = 0.6$, 8.4Hz).

$^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$= 20.04, 24.43, 27.53, 31.25, 37.98,42.63, 55.16, 55.88,55.89, 56.06, 56.17, 64.89, 76.48, 109.76, 110.41, 111.32, 111.74, 112.74, 114.40, 119.85, 120.16, 121.03, 129.75, 131.88, 133.30, 141.79, 147.34, 148.84, 148.93, 152.41, 157.58

Synthesis of 2-(3-((R)-1-((S)-1-(3-chloro-4-methoxyphenylsulfonyl)piperidine-2-carbonyloxy)-3-(4-dimethoxyphenyl)propyl)phenoxy)acetic acid

TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.45, yield= 11.7mg (25%).

HPLC (Gradient A) retention time= 24.98-25.12 min

$^1$HNMR (300 MHz, CDCl$_3$) $\delta$ = 1.25-1.34 (m, 1H), 1.41-1.54 (m, 1H), 1.63-1.85 (m, 3H), 1.95-2.06 (m, 1H), 2.13-2.27 (m, 2H), 2.46-2.64 (m, 2H), 3.15 (dt, 1H, J= 2.7, 12.6 Hz), 3.69 (d, 1H, J= 12.1 Hz), 3.86 (s, 6H), 3.89 (s, 3H), 4.72 (s, 2H), 4.81 (d, 1H, J= 4.5 Hz), 5.66 (t, 1H, J= 6.6 Hz), 6.65-6.72 (m, 3H), 6.78 (d, 1H, J= 8.7 Hz), 6.91-6.96 (m, 3H), 7.32 (t, 1H, J= 8.3 Hz), 7.47 (dd, 1H, J= 2.4, 9.3 Hz), 7.72 (d, 1H, J= 2.4 Hz)

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ = 20.09, 24.60, 27.93, 31.25, 38.11, 42.63, 55.31, 55.90, 55.94, 56.37, 64.87, 76.21, 111.33, 111.38, 111.79, 112.22, 114.68, 120.14, 120.19, 122.95, 127.55, 129.16, 129.85, 132.20, 133.30, 142.00, 147.40, 148.89, 157.74, 158.07, 169.87, 172.04.

MS (ESI) m/z: found Rt 13.48 min. (Method LCMS), 684.27 [M + Na]$^+$.


Synthesis of 2-(3-((R)-1-((S)-1-(3,5-difluorophenylsulfonyl)piperidine-2-carbonyloxy)-3-(4-dimethoxyphenyl)propyl)phenoxy)acetic acid

TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.38, yield= 23.5mg (57%).

HPLC (Gradient A) retention time= 25.22-25.39 min

$^1$HNMR (300 MHz, CDCl$_3$) $\delta$ = 1.21-1.35 (m, 1H), 1.42-1.55 (m, 1H), 1.65-1.84 (m, 3H), 1.96-2.08 (m, 1H), 2.14-2.30 (m, 2H), 2.45-2.63 (m, 2H), 3.17 (t, 1H, J= 12 Hz), 3.73 (d, 1H, J= 11.9 Hz), 3.85 (s, 6H), 4.78 (s, 2H), 4.72 (s, 1H), 5.66 (t, 1H, J= 6 Hz), 6.65-6.68 (m, 2H), 6.79 (d, 1H, J= 4.4 Hz), 6.87-6.93 (m, 4H), 7.20 (d, 2H, J= 1.8 Hz), 7.31 (t, 1H, J= 7.5 Hz).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ = 20.09, 24.66, 27.88, 31.23, 37.83, 42.99, 53.42, 55.39, 55.94, 55.93, 77.23, 107.66, 107.98, 110.43, 110.79, 111.39, 111.76, 112.74, 114.49, 120.09, 120.18, 129.93, 133.26, 141.51, 143.17, 147.40, 148.88, 157.69, 160.92, 164.13, 169.63.

MS (ESI) m/z: found Rt 13.46min. (Method LCMS), 656.19 [M + Na]$^+$.


Synthesis of 2-(3-((R)-1-((S)-1-(3,5-bis(trifluoromethyl)phenylsulfonyl)piperidine-2-carbonyloxy)-3-(4-dimethoxyphenyl)propyl)phenoxy)acetic acid

TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.47, yield= 30.1mg (51%).
HPLC (Gradient A) retention time = 27.5-27.8 min

\(^1\)HNMR (600 MHz, CDCl\(_3\)) \(\delta = 0.99-1.01 \text{ (m,1H)}, 1.40-1.47 \text{ (m,1H)}, 1.59-1.61 \text{ (m,2H)}, 1.73-1.79 \text{ (m,1H)}, 1.99-2.05 \text{ (m,1H)}, 2.14-2.23 \text{ (m,2H)}, 2.46-2.56 \text{ (m,2H)}, 3.09 \text{ (dt, 1H, } J = 2.4 \text{ Hz)}, 3.69 \text{ (dd, 1H, } J = 1.8 \text{ Hz)}, 3.84 \text{ (s,3H)}, 3.85 \text{ (s, 3H)}, 4.64 \text{ (s,2H)}, 4.83 \text{ (d, 1H, } J = 4.2 \text{ Hz)}, 5.56 \text{ (t, 1H, } J = 6.6 \text{ Hz)}, 6.63-6.66 \text{ (m,2H)}, 6.78 \text{ (d,1H, } J = 7.8 \text{ Hz)}, 6.80-6.82 \text{ (m,2H)}, 6.88 \text{ (d, 2H, } J = 7.8 \text{ Hz)}, 7.24 \text{ (t, 1H, } J = 7.8 \text{ Hz)}, 8.031 \text{ (s, 1H)}, 8.20 \text{ (s, 1H)}.

\(^{13}\)C NMR (150 MHz, CDCl\(_3\)) \(\delta = 19.74, 24.58, 27.88, 31.21, 37.71, 42.83, 55.59, 55.87, 55.88, 64.73, 76.92, 111.31, 111.71, 113.02, 114.13, 119.98, 120.09, 121.62, 123.44, 125.27, 127.29, 127.51, 129.87, 131.12, 141.57, 142.63, 147.39, 148.83, 157.48, 169.47, 172.22

MS (ESI) \(m/z\) : found Rt 14.65 min. (Method LCMS), 756.20 [M + Na].


**Synthesis of 2-(3-((R)-1-((S)-1-((3,5-bis(methoxycarbonyl)phenylsulfonyl)piperidine-2-carbonyloxy)-3-(3,4-dimethoxyphenyl)propyl)phenoxy)acetic acid 26**

TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.50, yield = 28.5mg (47%).

HPLC (Gradient A) retention time = 27.5-27.8 min

\(^1\)HNMR (600 MHz, CDCl\(_3\)) \(\delta = 0.97-1.04 \text{ (m,1H)}, 1.40-1.46 \text{ (m,1H)}, 1.59-1.62 \text{ (m,2H)}, 1.71-1.77 \text{ (m,1H)}, 1.99-2.05 \text{ (m,1H)}, 2.15-2.22 \text{ (m,2H)}, 2.47-2.57 \text{ (m,2H)}, 3.15 \text{ (dt, 1H, } J =2.4, 12.6 \text{ Hz)}, 3.68 \text{ (dd, 1H, } J = 2.6Hz), 3.84 \text{ (s,3H)}, 3.85 \text{ (s, 3H)}, 4.65 \text{ (s,2H)}, 4.80 \text{ (d, 1H, } J = 4.8 \text{ Hz)}, 5.59 \text{ (t, 1H, } J = 7.2 \text{ Hz)}, 6.64-6.67 \text{ (m,2H)}, 6.78 \text{ (d, 1H, } J = 8.4Hz), 6.81-6.84 \text{ (m, 2H), 6.90(d,1H, } J = 7.2Hz), 7.25 \text{ (t, 1H, } J = 7.8Hz), 7.90 \text{ (s, 1H)}, 7.95 \text{ (s,1H)}, 8.08 \text{ (s,1H)}

\(^{13}\)C NMR (150 MHz, CDCl\(_3\)) \(\delta = 19.74, 24.59, 27.80, 31.24, 37.77, 42.86, 55.45, 55.90, 64.75, 76.77, 111.32, 111.74, 112.98, 114.15, 119.99, 120.14, 122.80, 123.32, 129.87, 132.12, 132.15, 133.17, 133.25, 141.60, 142.89, 147.39, 148.84, 157.49, 169.49, 172.39.

MS (ESI) \(m/z\) : found Rt 14.60 min. (Method LCMS), 766.16, 768.08 [M + Na].

HRMS 766.1568, 768.1556 [M + Na]^+, calculated 744.1511.

**Synthesis of 2-(3-((R)-1-((S)-1-((3,5-bis(methoxycarbonyl)phenylsulfonyl)piperidine-2-carbonyloxy)-3-(3,4-dimethoxyphenyl)propyl)phenoxy)acetic acid 27**

TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.52, yield = 22mg (41%).

HPLC (Gradient A) retention time = 24.82-25.02 min

\(^1\)HNMR (400 MHz, CDCl\(_3\)) \(\delta = 1.21-1.31 \text{ (m, 1H)}, 1.37-1.47 \text{ (m, 1H)}, 1.61-1.80 \text{ (m, 3H)}, 1.91-2.00 \text{ (m, 1H)}, 2.08-2.17 \text{ (m, 1H)}, 2.28 \text{ (d, 1H, } J = 6.8 \text{ Hz)}, 2.40-2.55 \text{ (m, 2H)}, 3.15 \text{ (dt, 1H, } J = 2.8, 12.8 \text{ Hz)}, 3.78 \text{ (d, 1H, } J = 12.6 \text{ Hz)}, 3.82 \text{ (s, 3H)}, 3.83 \text{ (s, 3H)}, 3.93 \text{ (s, 6H)}, 4.70 \text{ (s, 2H)}, 5.26 \text{ (t, 1H, } J = 7.8 \text{ Hz)}, 5.80 \text{ (s, 1H)}, 6.66 \text{ (s, 1H)}, 7.20 \text{ (s, 1H)}, 7.90 \text{ (s, 1H)}, 8.05 \text{ (s, 1H)}.

\(^{13}\)C NMR (150 MHz, CDCl\(_3\)) \(\delta = 19.74, 24.59, 27.80, 31.24, 37.77, 42.86, 55.45, 55.90, 64.75, 76.77, 111.32, 111.74, 112.98, 114.15, 119.99, 120.14, 122.80, 123.32, 129.87, 132.12, 132.15, 133.17, 133.25, 141.60, 142.89, 147.39, 148.84, 157.49, 169.49, 172.39.

MS (ESI) \(m/z\) : found Rt 14.60 min. (Method LCMS), 766.16, 768.08 [M + Na].

HRMS 766.1568, 768.1556 [M + Na]^+, calculated 744.1511.
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13C NMR (100 MHz, CDCl₃) δ = 20.21, 24.65, 27.77, 31.13, 37.76, 43.04, 52.85, 55.61, 55.84, 55.89, 64.91, 76.47, 111.27, 111.68, 112.70, 114.57, 120.08, 120.10, 129.79, 131.53, 131.95, 133.25, 134.01, 141.30, 141.73, 147.31, 148.80, 157.58, 164.86, 169.61, 172.02.

MS (ESI) m/z: found Rt 11.80 min. (Method LCMS), 736.16 [M + Na]+.


Synthesis of 2-(3-((R)-1-((S)-1-(3,5-dichloro-4-hydroxyphenylsulfonyl)piperidine-2-carbonyl-oxy)-3-(3,4-dimethoxyphenyl)propyl)phenoxy)acetic acid 28

TLC (Hexane: EtOAc: TFA 4.8 :5:0.2): Rf = 0.50, yield = 12.3mg (23%).

HPLC (Gradient A) retention time = 26.8-27.1 min

1H NMR (300 MHz, CDCl₃) δ = 1.00-1.10 (m, 1H), 1.38-1.53 (m, 1H), 1.60-1.68 (m, 2H), 1.74-1.87 (m, 1H), 2.01-2.12 (m, 1H), 2.16-2.26 (m, 2H), 2.53-2.62 (m, 2H), 3.21 (t, 1H, J = 12Hz), 3.72 (d, 1H, J = 12Hz), 3.87 (s, 6H), 4.65 (s, 2H), 4.77 (d, J = 2.7Hz), 5.63 (t, J = 6Hz), 6.69-6.71(m, 2H), 6.80-6.93(m, 4H), 7.28 (s, 1H), 7.78 (s, 1H), 7.99 (s, 1H).

13C NMR (75 MHz, CDCl₃) δ = 19.66, 24.66, 27.85, 31.34, 37.88, 43.01, 55.4, 55.95, 64.82, 77.21, 111.44, 111.83, 113.02, 114.23, 119.84, 120.21, 122.20, 127.86, 128.97, 129.89, 139.94, 141.75, 145.83, 147.22, 148.89, 153.52, 157.56, 169.51, 172.35.

MS (ESI) m/z: found Rt 13.66 min. (Method LCMS), 704.68 [M + Na]+.


Synthesis of 2-(3-((R)-1-((S)-1-(3,5-dichloro-4-methoxyphenylsulfonyl)piperidine-2-carbonyl-oxy)-3-(3,4-dimethoxyphenyl)propyl)phenoxy)acetic acid 29

TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.41, yield = 18.3mg (39%).

HPLC (Gradient A) retention time = 27.25-27.47 min.

1H NMR (600 MHz, CDCl₃) δ = 1.02-1.09 (m, 1H), 1.39-1.46(m,1H), 1.59-1.62(m,2H), 1.70-1.76(m,1H), 1.99-2.05(m,1H), 2.15-2.23(m,2H), 2.49-2.59(m,2H), 3.18 (dt, 1H, J = 2, 12 Hz, ), 3.68 (dd, 1H, J = 3, 12.6 Hz), 3.84 (s,3H), 3.85 (s, 3H), 3.92 (s, 3H), 4.66 (s,2H), 4.76 (d, 1H, J = 4.8 Hz), 5.61 (t, 1H, J = 6.6 Hz), 6.64-6.67 (m,2H), 6.78 (d, 1H, J = 8.4Hz), 6.81-6.84(m, 2H), 6.89(d,1H, J = 7.8Hz), 7.25 (t, 1H, J = 7.8Hz), 7.71(s, 2H).

13C NMR (150 MHz, CDCl₃) δ = 19.78, 24.58, 27.70, 31.28, 37.83, 42.84, 55.31, 55.90, 60.95, 64.85, 76.64, 111.34, 111.76, 112.90, 114.23, 120.01, 120.17, 127.73, 129.85, 130.06, 133.19, 136.95, 141.66, 147.37, 148.84, 155.65, 157.48, 169.67.
MS (ESI) m/z: found RT 11.84 min. (Method LCMS), 718.37, 720.26 [M + Na]+.

Synthesis of 2-(3-((R)-1-((S)-1-(4-acetamido-3,5-dichlorophenyl)sulfonyl)piperidin-2-carboxyloxy)-3-(3,4-dimethoxyphenyl)propyl)phenoxy)acetic acid 30

TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.48, yield= 10.98 mg (21%).
HPLC (Gradient B) retention time= 22.45-22.67min

1H NMR (300 MHz, CDCl3) δ = 1.27 (s, 3H), 1.63-1.81 (m, 1H), 1.95-2.05 (m, 2H), 2.12-2.33 (m, 5H), 2.51 (t, 2H, J= 6.9 Hz), 3.06 (t, 1H, J= 10.8 Hz), 3.74 (d, 1H, J= 10.2 Hz), 3.85 (s, 3H), 3.86 (s, 3H), 4.67 (s, 3H), 4.80 (d, 1H, J= 4.8 Hz), 5.63 (t, 1H, J= 6 Hz), 6.65-6.68 (m, 2H), 6.74(s, 1H), 6.80 (d, 1H, J= 8.4 Hz), 6.85-6.92 (m, 2H), 7.29 (t, 1H, J= 7.9Hz), 7.68 (s, 2H).

13C NMR (75 MHz, CDCl3) δ = 20.14, 24.80, 28.02, 29.69, 31.24, 37.65, 43.11, 55.61, 55.86, 55.96, 64.97, 76.72, 111.41, 111.77, 112.99, 114.45, 120.25, 120.30, 127.00, 133.20, 134.24, 135.52, 140.49, 141.28, 147.38, 148.84, 157.66, 169.49, 169.60, 171.46.

MS (ESI) m/z: found Rt 12.25 min. (Method LCMS), 723.02, 725.02 [M + 1]+.

Synthesis of 5-((S)-2-(((R)-1-((3-carboxymethoxy)phenyl)-3-(3,4-dimethoxyphenyl)propoxy)carbonyl)piperidine-1-ylsulfonyl)-2,3-dimethoxybenzoic acid 31

TLC (DCM: MeOH 9.7:0.3): Rf = 0.38, yield= 7.23mg (45%).
HPLC (Gradient A) retention time- 22.79-22.95 min

1H NMR (400 MHz, DMSO) δ= 1.58-1.73 (m, 3H), 1.94-2.05 (m, 2H), 2.15-2.30 (m, 4H), 2.71-2.97 (m, 3H), 3.67-3.71 (m, 9H), 3.80 (s, 3H), 4.22 (t, 1H, J= 7.6 Hz), 4.58 (s, 2H), 5.35 (s, 1H), 6.68-6.72 (m, 1H), 6.75 (s, 1H), 6.81-6.89 (m, 4H), 6.95-6.99 (m, 1H), 7.06-7.11 (m, 1H), 7.16-7.20 (m, 1H).

13C NMR (100 MHz, DMSO) δ= 21.20, 25.10, 31.08, 31.61, 36.50, 51.04, 52.19, 55.33, 55.92, 56.03, 56.06, 61.24, 64.84, 108.62, 111.06, 112.33, 112.40, 112.62, 114.33, 120.46, 120.71, 129.88, 133.63, 135.79, 137.97, 145.07, 147.45, 147.74, 148.33, 148.59, 149.09, 151.85, 158.28, 170.61.

MS (ESI) m/z: found Rt 10.48 min. (Method LCMS), 702.10 [M + H]+.
**Synthesis of** 2-((R)-(3,4-dimethoxyphenyl))-1-(S)-(7-nitro-2,3-dihydrobenzofuran-5-ylsulfonyl)piperidine-2-carboxyloxy)propylphenoxy)acetic acid 32

TLC (DCM: MeOH 9.7:0.3): Rf = 0.43, yield= 22mg (42%).

HPLC (Gradient A) retention time= 24.08-24.24 min

1H NMR (400 MHz, CDCl$_3$) $\delta$ = 1.24-1.32 (m, 1H), 1.47-1.50 (m, 1H), 1.67-1.77 (m, 2H), 1.82-1.91 (m, 1H), 1.92-2.01 (m, 1H), 2.09-2.19 (m, 1H), 2.28 (d, 1H, J= 12.4Hz), 2.41-2.60 (m, 2H), 2.92-3.04 (m, 1H), 3.11-3.20 (m, 2H), 3.74 (d, 1H, J= 12Hz), 3.83 (s, 3H), 3.84 (s, 3H), 4.70 (s, 2H), 4.78-4.87 (m, 3H), 4.78-4.87 (m, 3H), 5.55-5.59 (m, 1H), 6.61-6.64 (m, 2H), 6.76 (d, 1H, J= 8Hz), 6.80-6.88 (m, 3H), 7.27 (t, 1H, J= 8Hz), 7.52 (d, 1H, J= 2Hz), 8.24 (d, 1H, J= 2Hz).

13C NMR (100 MHz, CDCl$_3$) $\delta$ = 20.17, 24.75, 28.16, 28.29, 31.28, 38.25, 42.17, 55.52, 55.85, 55.90, 64.78, 74.88, 76.31, 111.30, 111.62, 111.97, 114.46, 119.62, 120.12, 124.34, 128.80, 129.83, 131.69, 132.14, 133.13, 133.79, 141.85, 147.37, 148.85, 157.74, 157.90, 169.71, 171.79

MS (ESI) m/z: found Rt 11.54 min. (Method LCMS), 707.14 [M + Na]$^+$.


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**Synthesis of** 2-((R)-1-((S)-1-(7-amino-2,3-dihydrobenzofuran-5-ylsulfonyl)piperidine-2-carboxyloxy)-3-(3,4-dimethoxyphenyl)propylphenoxy)acetic acid 33

TLC (DCM: MeOH 9.7:0.3): Rf = 0.35, yield= 6.7mg (14%).

HPLC (Gradient A) retention time= 21.68-21.89 min

1H NMR (600 MHz, CDCl$_3$) $\delta$ = 1.24-1.32 (m, 1H), 1.47-1.50 (m, 1H), 1.67-1.77 (m, 2H), 1.82-1.91 (m, 1H), 1.92-2.01 (m, 1H), 2.09-2.19 (m, 1H), 2.28 (d, 1H, J= 12.4Hz), 2.41-2.60 (m, 2H), 2.92-3.04 (m, 1H), 3.11-3.20 (m, 2H), 3.74 (d, 1H, J= 12Hz), 3.83 (s, 3H), 3.84 (s, 3H), 4.70 (s, 2H), 4.78-4.87 (m, 3H), 4.78-4.87 (m, 3H), 5.55-5.59 (m, 1H), 6.61-6.64 (m, 2H), 6.76 (d, 1H, J= 8Hz), 6.84 (s, 1H), 6.90-6.98 (m, 3H), 7.27 (t, 1H, J= 8Hz), 8.24 (d, 1H, J= 2Hz).

13C NMR (150 MHz, CDCl$_3$) $\delta$ = 20.17, 24.75, 28.16, 28.29, 31.28, 38.25, 42.17, 55.52, 55.85, 55.90, 64.78, 74.88, 76.31, 111.30, 111.62, 111.97, 114.46, 119.62, 120.12, 124.34, 128.80, 129.83, 131.69, 132.14, 133.13, 137.16, 141.85, 147.37, 148.85, 157.74, 157.90, 169.71, 171.79

MS (ESI) m/z: found Rt 12.22 min. (Method LCMS), 655.08 [M + H]$^+$, 677.25 [M + Na]$^+$.

Synthesis of 2-(3-((R)-3-(3,4-dimethoxyphenyl)-1-((S)-1-(2-methylbenzo[d]thiazol-6-ylsulfonyl)piperidine-2-carbonyloxy)propyl)phenoxy)acetic acid 34

TLC (DCM: MeOH 9.7:0.3): Rf = 0.26, yield= 22mg (43%).

HPLC (Gradient A) retention time= 24.22-24.38 min

1H NMR (400 MHz, CDCl3) δ = 1.24-1.32 (m, 1H), 1.50-1.61 (m, 1H), 1.65-1.73 (m, 2H), 1.85-1.95 (m, 2H), 2.03-2.13 (m, 1H), 2.18 (d, 1H, J=13.6 Hz), 2.38-2.54 (d, 2H), 2.89 (s, 3H), 3.17-3.25 (m, 1H), 3.81 (s, 3H), 3.82 (s, 3H), 4.72 (d, 1H, J= 8.4 Hz), 4.82 (d, 1H, J= 4.4 Hz), 5.44-5.47 (m, 1H), 6.58-6.61 (m, 2H), 6.68 (s, 1H), 6.74 (d, 1H, J= 8 Hz), 6.98-7.02 (m, 1H).

13C NMR (100 MHz, CDCl3) δ = 19.76, 19.96, 24.74, 28.21, 31.33, 38.54, 42.44, 55.07, 55.82, 55.88, 65.29, 75.86, 111.25, 111.55, 112.34, 114.90, 119.22, 120.06, 120.93, 121.81, 125.10, 130.15, 133.14, 134.98, 135.77, 142.02, 147.34, 148.83, 154.03, 157.70, 169.43, 171.12, 172.52.

MS (ESI) m/z: found Rt 11.66 min. (Method LCMS), 668.16 [M + H]+, 691.11 [M + Na]+.


Synthesis of 2-(3-((R)-3-(3,4-dimethoxyphenyl)-1-((S)-1-(2-oxo-2,3-dihydrobenzo[d]thiazol-6-ylsulfonyl)piperidine-2-carbonyloxy)propyl)phenoxy)acetic acid 35

TLC (DCM: MeOH 9.7:0.3): Rf = 0.37, yield= 24.20mg (46.8%).

HPLC (Gradient A) retention time= 22.74-22.94 min

1H NMR (600 MHz, CDCl3) δ = 1.38-1.45 (m, 1H), 1.59-1.66 (m, 1H), 1.73-1.75 (m, 2H), 1.89-1.99 (m, 2H), 2.09-2.16 (m, 2H), 2.41-2.46 (m, 1H), 2.52-2.57 (m, 1H), 3.23 (t, 1H, J= 10.2 Hz), 3.78 (d, 1H, J= 7.8Hz), 3.82 (s, 3H), 3.83 (s, 3H), 4.72-4.82 (m, 3H), 5.41-5.43 (m, 1H), 6.18 (d, 1H, J= 8.4Hz), 6.58-6.65 (m, 3H), 6.76 (d, 1H, j= 8.4 Hz), 6.92 (d, 1H, J= 6.6Hz), 7.01 (d, 1H, J= 8.4Hz), 7.09 (d, 1H, J= 8.4Hz), 7.41 (t, 1H, J= 7.8Hz), 7.66 (d, 1H, J= 1.8Hz).

13C NMR (150 MHz, CDCl3) δ = 19.52, 24.83, 28.47, 31.44, 38.63, 42.16, 54.77, 55.85, 55.92, 64.28, 75.54, 111.34, 111.60, 111.69, 113.56, 118.85, 120.12, 121.10, 124.32, 125.93, 130.27, 133.04, 133.23, 134.88, 142.51, 147.43, 148.89, 157.44, 169.46, 172.87, 173.00.

MS (ESI) m/z: found Rt 11.08 min. (Method LCMS), 693.26 [M + Na]+.


Synthesis of 2-(3-((R)-3-(3,4-dimethoxyphenyl)-1-((S)-1-(2-oxoindolin-5-ylsulfonyl)piperidine-2-carbonyloxy)propyl)phenoxy)acetic acid 36

TLC (Hexane: EtOAc: TFA 6:4:0.1): Rf = 0.25, yield= 6.0mg (12.5%).

HPLC (Gradient A) retention time= 21.21-21.40min
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

\[ \text{HNMR (600 MHz, CDCl}_3 \text{)} \delta = 1.55-1.64 \text{ (m, 1H), 1.69-1.76 (m, 2H), 1.91-2.00 (m, 2H), 2.10-2.16 (m, 2H), 2.43-2.48 (m, 1H), 2.53-2.58 (m, 1H), 3.16-3.21 (m, 1H), 3.39 (d, 1H, J= 22.8 Hz), 3.52 (d, 1H, J= 22.8 Hz), 3.73 (s, 1H), 3.82 (s, 3H), 3.83 (s, 3H), 3.87 (s, 1H), 4.71-4.81 (m, 3H), 5.43-5.45 (m, 1H), 6.25 (d, 1H, J= 8.4 Hz), 6.60-6.62 (m, 2H), 6.72-6.77 (m, 2H), 6.84-6.89 (m, 1H), 7.04-7.10 (m, 2H), 7.38 (t, 1H, J= 8.4 Hz), 7.45 (s, 1H).}

\[ \text{C NMR (150 MHz, CDCl}_3 \text{)} \delta = 19.63, 24.79, 28.43, 31.39, 36.16, 38.61, 42.13, 54.81, 55.83, 55.90, 63.93, 75.75, 110.47, 111.35, 111.61, 113.16, 113.50, 118.85, 120.14, 123.25, 125.28, 128.33, 130.10, 133.09, 142.60, 145.55, 147.37, 148.85, 157.49, 169.65, 171.81, 179.46.

\[ \text{MS (ESI) m/z: found Rt 11.03 min. (Method LCMS), 675.18 [M + Na]^+ \text{, calculated 653.2591 [M + H]^+}.}]

\[ \text{Synthesis of 2-(3,4-dimethoxyphenyl)-1-((R)-3,4-dimethoxyphenyl)ethyl 1-(3,5-dichlorophenylsulfonyl)piperidine-2-carboxylate 37} \]

TLC (Hexane: EtOAc: TFA 6:4:0.1): Rf = 0.42, yield= 6.4mg (24.6%).

HPLC (Gradient A) retention time= 21.68-21.85 min

\[ \text{HNMR (600 MHz, CDCl}_3 \text{)} \delta = 1.57-1.64 \text{ (m, 1H), 1.701.75 (m, 2H), 1.91-1.99 (m, 2H), 2.10-2.16 (m, 2H), 2.43-2.48 (m, 1H), 2.53-2.59 (m, 1H), 3.19-3.24 (m, 1H), 3.36 (d, 1H, J= 22.2 Hz), 3.48 (d, 1H, J= 22.2 Hz), 3.74 (s, 2H), 3.83 (s, 3H), 3.84 (s, 3H), 4.75 (d, 2H, J= 19.0 Hz), 4.80-4.81 (m, 1H), 5.44-5.46 (m, 1H), 6.20 (d, 1H, J= 8.4 Hz), 6.59-6.63 (m, 2H), 6.72-6.78 (m, 2H), 6.89 (d, 1H, J= 8.4 Hz), 7.05 (d, 1H, J= 7.8Hz), 7.10 (d, 1H, J= 7.8 Hz), 7.39 (t, 1H, J= 8.4 Hz), 7.44 (s, 1H).}

MS (ESI) m/z: found Rt 14.66 min. (Method LCMS), 677.66 [M + Na]^+ \text{, calculated 677.54 [M + Na]^+}.]

\[ \text{Synthesis of 2-(3,4-dimethoxyphenoxy)ethyl 1-(3,5-dichlorophenylsulfonyl)piperidine-2-carboxylate 38} \]

To 71a ((50mg, 0.162mmol) in DCM DIPEA (62.7mg, 0.485mmol) and 3,5-dichlorobenzene sulfonyl chloride (39.7mg,0.162mmol) were added. The reaction was stirred at room temperature overnight and was purified by flash chromatography using cyclohexane : EtOAc 3:1 to yield 38.

TLC (Cyclohexane: EtOAc 3:1): Rf = 0.57, yield= 17mg (20%).

HPLC (Gradient A) retention time= 22.74-22.94 min

\[ \text{HNMR (600 MHz, CDCl}_3 \text{)} \delta = 1.47-1.63 \text{ (m, 2H), 1.65-1.71 (m, 2H), 1.73-1.85 (m, 1H), 2.16-2.21 (m, 1H), 3.16-3.24 (m, 1H), 3.72-3.77 (m, 1H), 3.84(s, 3H), 3.85 (s, 3H), 3.97-4.03 (m,
1H), 4.03-4.08 (m, 1H), 4.25-4.30 (m, 1H), 4.35-4.40 (m, 1H), 4.75-4.80 (m, 1H), 6.34 (dd, 1H, J= 2.83, 8.73 Hz), 6.48 (d, 1H, J= 2.8Hz), 6.76 (d, 1H, J= 8.7Hz), 7.49 (t, 1H, J= 1.86, 1.86 Hz), 7.64(d, 2H, J= 1.85 Hz).

$^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$ = 19.88, 24.73, 27.92, 42.88, 55.31, 55.85, 56.41, 63.48, 66.13, 101.06, 103.99, 111.76, 125.55, 132.29, 135.64, 142.67, 143.99, 149.92, 152.82, 170.23

MS (ESI) m/z: found Rt 13.97 min. (Method LCMS), 518.55, 520.57 [M + H]$^+$, 540.45, 542.23 [M + Na]$^+$.


**Synthesis of 2-(3,4-dimethoxyphenoxy)ethyl 1-(benzo[d]thiazol-6-ylsulfonyl)piperidine-2-carboxylate 39**

To 71a (50mg, 0.162mmol) in DCM DIPEA (62.7mg,0.485mmol) and benzo[d]thiazole-6-sulfonyl chloride(76mg, 0.323mmol) were added. The reaction was stirred at room temperature overnight and was purified by flash chromatography using cyclohexane: EtOAc 1:1 to yield 39

TLC (Cyclohexane: EtOAc 1:1): Rf = 0.3, yield= 39mg (48%).

HPLC (Gradient A) retention time= 22.74-22.94 min

$^1$HNMR (300 MHz, CDCl$_3$) $\delta$= 1.3-1.62 (m, 2H), 1.62-1.74(m,2H), 1.74-1.88 (m, 1H), 2.15-2.25 (m, 1H), 3.21-3.34 (m, 1H), 3.74-3.83 (m, 1H), 3.85 (s, 3H), 3.86 (s, 3H), 3.89-4.05(m,2H), 4.09-4.38(m, 2H), 4.85-4.91 (m, 1H), 6.31-6.37(m, 1H), 6.47-6.51(m, 1H), 6.75-6.81(m, 1H), 7.90-7.96 (m, 1H), 8.19-8.24(m, 1H), 8.47-8.51(m, 1H), 9.18-9.22(m,1H).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$= 19.98, 24.75, 27.94, 42.80, 55.21, 55.89, 56.44, 63.47, 66.16, 101.09, 104.07, 111.80, 122.00, 123.98, 124.98, 133.95, 137.34, 144.01, 149.04, 152.84, 155.20, 157.63, 170.56.

MS (ESI) m/z: found Rt 12.15 min. (Method LCMS), 507.56 [M + H]$^+$, 529.38 [M + Na]$^+$.


**General Procedure for the synthesis of Compound 40-45.**

To 71b (19mg, 0.037 mmol) DIPEA (9.2 mg, 0.055 mmol) and the corresponding sulfonyl chloride (0.055 mmol) were added The reaction was stirred at room temperature overnight and was purified by prep HPLC using gradient C to yield compound 40-45.

**Synthesis of (S)-((R)-3-(3,4-dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propyl)1-(3,5-dichlorophenylsulfonyl)piperidine-2-carboxylate 40**

TLC (DCM: MeOH 9.7:0.3): Rf = 0.48, yield= 16.42 mg (62%).
HPLC (Gradient A) retention time= 20.8-21.2 min

1H NMR (600 MHz, CDCl₃) δ= 1.28-1.31 (m, 1H), 1.38-1.47 (m, 1H), 1.64-1.79 (m, 1H), 1.98-2.04 (m, 1H), 2.15-2.21 (m, 1H), 2.27 (d, 1H, J= 13.8 Hz), 2.48-2.53 (m, 1H), 2.56-2.60 (m, 1H), 3.09-3.18 (m, 3H), 3.56 (t, 2H, J= 4.8 Hz), 3.66 (dd, 1H, J= 2.4, 13.2 Hz), 3.76 (d, 2H, J= 12 Hz), 3.84 (s, 3H), 3.85 (s, 3H), 3.95 (t, 2H, J= 12.6 Hz), 4.02 (d, 1H, J= 2.4 Hz), 4.04 (d, 1H, J= 2.4 Hz), 4.39 (t, 2H, J= 4.6 Hz), 4.79 (d, 1H, J= 4.8 Hz), 6.65-6.67 (m, 2H), 6.77 (d, 1H, J= 7.8 Hz), 6.80-6.82 (m, 1H), 6.85-6.86 (m, 1H), 6.91 (d, 1H, J= 7.8 Hz), 7.29 (t, 1H, J= 8.1 Hz), 7.46 (t, 1H, J= 1.8 Hz), 7.55 (s, 1H), 7.55 (s, 1H).

13C NMR (150 MHz, CDCl₃) δ= 20.15, 24.71, 27.93, 31.28, 37.86, 43.01, 53.93, 55.64, 55.86, 55.95, 57.55, 65.55, 66.45, 77.21, 111.34, 111.73, 112.87, 114.24, 119.07, 120.13, 125.53, 129.82, 132.35, 133.33, 135.64, 141.19, 142.98, 147.42, 148.92, 158.7, 169.61

MS (ESI) m/z: found Rt 8.76 min. (Method LCMS), 721.65, 723.37 [M + H]+.


**Synthesis of (S)-((R)-3-(3,4-dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propyl) I-(benzo[d]thiazol-6-ylsulfonyl)piperidine-2-carboxylate 41**

TLC (DCM: MeOH 9.7:0.3): Rf = 0.27, yield= 31.72mg (91%).

HPLC (Gradient A) retention time= 20.78-20.98 min

1H NMR (300 MHz, CDCl₃) δ= 1.61-1.74 (m, 3H), 1.77-1.87 (m, 3H), 1.94-2.06 (m, 1H), 2.13-2.31 (m, 1H), 2.47-2.66 (m, 2H), 3.05-3.23 (m, 3H), 3.55-3.57 (m, 2H), 3.66-3.81 (m, 3H), 3.85 (s, 6H), 4.00 (s, 4H), 4.46 (s, 2H), 2.90 (d, 1H, J= 4.5 Hz), 5.58-5.63 (m, 1H), 6.65-6.68 (m, 2H), 6.88-6.96 (m, 3H), 7.24-7.29 (m, 1H), 7.32-7.37 (m, 1H), 7.64-7.68 (m, 1H), 7.89-7.96 (m, 1H), 8.35 (s, 1H), 9.20 (s, 1H).

13C NMR (75 MHz, CDCl₃) δ= 24.56, 27.80, 31.30, 33.33, 42.74, 46.50, 52.74, 55.42, 55.85, 55.93, 56.47, 62.47, 63.91, 76.23, 111.37, 111.73, 112.28, 114.31, 119.97, 120.16, 121.93, 123.74, 124.85, 129.02, 130.08, 133.25, 137.21, 142.29, 147.42, 148.93, 155.00, 157.39, 157.99, 169.98.

MS (ESI) m/z: found Rt 8.45 min. (Method LCMS), 710.51 [M + H]+.

HRMS 710.2517 [M + H]+, calculated 710.2492 [M + H]+.

**Synthesis of (S)-((R)-3-(3,4-dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propyl) I-(3,5-dichloro-4-hydroxyphenyl)sulfonyl)piperidine-2-carboxylate 42**

TLC (DCM: MeOH 9.7:0.3): Rf = 0.45, yield= 15.96mg (58%).

HPLC (Gradient A) retention time= 19.28-19.61 min
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

$^1$HNMR (300 MHz, CDCl$_3$) $\delta$ = 1.54-2.06 (m, 6H), 2.14-2.30 (m, 2H), 2.44-2.64 (m, 2H), 2.99-3.27 (m, 3H), 3.51 (s, 2H), 3.74-3.80 (m, 3H), 3.86 (s, 3H), 3.87 (s, 3H), 4.06 (s, 4H), 4.43 (s, 2H), 4.72 (d, 1H, J= 4.2Hz), 5.53-5.59 (m ,1H), 6.65-6.69 (m, 3H), 6.78-6.85 (m, 2H), 6.92 (d, 1H, J=7.8 Hz), 7.29-7.33 (m, 1H), 7.49 (s, 2H).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ = 20.15, 24.94, 28.07, 31.45, 37.87, 42.86, 53.05, 55.25, 55.87, 55.95, 56.81, 62.50, 63.96, 76.25, 111.39, 111.71, 112.71, 114.02, 119.57, 120.15, 121.53, 127.30, 130.22, 132.31, 133.18, 141.75, 147.47, 148.95, 151.71, 157.01, 169.52.

MS (ESI) m/z: found RT 8.76 min. (Method LCMS), 737.04, 739.13 [M + H]$^+$. HRMS 737.2532, 739.2506 [M + H]$^+$, calculated 737.2501, 739.2542 [M + H]$^+$.

**Synthesis of (S)-((R)-3-(3,4-dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propyl) 1-(3,5-dichloro-4-methoxyphenylsulfonyl)piperidine-2-carboxylate 43**

TLC (DCM: MeOH 9.7:0.3): Rf = 0.42, yield= 21mg (75%).

HPLC (Gradient A) retention time= 23.31-23.63 min

$^1$HNMR (600 MHz, CDCl$_3$) $\delta$ = 1.27-1.31 (m, 1H), 1.41-1.46 (m, 1H), 1.65 (d, 1H, J= 13.8Hz), 1.71-1.80 (m, 2H), 1.99-2.05 (m, 1H), 2.17-2.23 (m, 1H), 2.28 (d, 1H, J= 14.4 Hz), 2.49-2.54 (m, 1H), 2.56-2.61 (m, 1H), 3.09 (t, 2H, J= 11.4Hz), 3.15(dt, 1H, J= 3, 12.6Hz), 3.54 (t, 2H, J= 5.2Hz), 3.66 (d, 1H, J= 10.8Hz), 3.71-3.74 (m, 2H), 3.84 (s, 6H), 3.91 (s, 3H), 3.96-4.03 (m, 4H), 4.40 (d, 2H, J=5.0Hz), 4.79 (d, 1H, J= 4.8Hz), 5.68 (q, 1H, J=3, 5.4 Hz), 6.65-6.67 (m, 2H), 6.77 (d, 1H, J= 7.8Hz), 6.81-6.83 (m, 1H), 6.85(t, 1H, J= 1.8Hz), 6.90(d, 1H, J= 7.2Hz), 7.29 (t, 1H, J= 7.8 Hz), 7.61 (s, 2H).

$^{13}$C NMR (150 MHz, CDCl$_3$) $\delta$ = 20.05, 24.56, 27.73, 31.26, 37.95, 42.93, 52.87, 55.57, 55.82, 55.91, 56.58, 60.94, 62.32, 63.90, 76.44, 111.32, 111.70, 112.33, 141.12, 120.03, 120.15, 127.73, 129.98, 130.07, 133.24, 137.01, 141.83, 147.38, 148.88, 155.61, 157.27, 159.80.


**Synthesis of (S)-((R)-3-(3,4-dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propyl) 1-(2-oxo-2,3-dihydrobenzo[d]thiazol-6-ylsulfonyl)piperidine-2-carboxylate 44**

TLC (DCM: MeOH 9.7:0.3): Rf = 0.39, yield= 6.4mg (24.6%).

HPLC (Gradient A) retention time= 18.92-19.16 min

$^1$HNMR (300 MHz, CDCl$_3$) $\delta$ = 1.71-1.81 (m, 2H), 1.84-2.01 (m, 2H), 2.06-2.22 (m, 2H), 2.39-2.62 (m, 2H), 3.01-3.12 (m, 2H), 3.26-3.37 (m, 2H), 3.54 (s, 2H), 3.80-3.91 (m, 12H), 4.06 (s, 4H), 4.26-4.50 (m, 2H), 4.74 (d, 1H, J= 4.8Hz), 5.36-5.41 (m, 1H), 6.55-6.64 (m, 4H), 6.77 (d,
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

1H, J = 7.5Hz), 6.89 (d, 1H, J = 6.9 Hz), 6.99 (d, 1H, J = 7.5 Hz), 7.19 (d, 1H, J = 8.4 Hz), 7.38 (t, J = 7.6 Hz), 7.62 (d, 1H, J = 1.8Hz).

13C NMR (75 MHz, CDCl3) δ = 24.98, 28.29, 31.43, 38.45, 42.48, 53.47, 54.79, 55.87, 55.94, 57.28, 62.02, 63.93, 75.83, 111.03, 111.37, 111.69, 112.73, 113.79, 119.18, 120.17, 121.54, 124.28, 125.73, 130.46, 133.10, 133.39, 138.88, 142.22, 147.47, 148.93, 156.99, 169.62.

MS (ESI) m/z: found Rt 9.47 min. (Method LCMS), 726.29 [M + H]^+.

HRMS 726.3111 [M + H]^+, calculated 726.3091 [M + H]^+.

Synthesis of (S)-(R)-3-(3,4-dimethoxyphenyl)-1-(3-(2-morpholinoethoxy)phenyl)propyl)-4-acetamido-3,5-dichlorophenylsulfonylpiperidine-2-carboxylate 45

TLC (DCM: MeOH 9.7:0.3): Rf = 0.12, yield= 15.4mg (70.8%).

HPLC (Gradient A) retention time= 19.64-19.81min

1H NMR (600 MHz, CDCl3) δ = 1.27-1.29 (m, 1H), 1.49-1.51 (m, 1H), 1.67 (d, 1H, J=12.6 Hz), 1.73 (d, 1H, J= 13.2 Hz), 1.78-1.84 (m, 1H), 1.95-2.00 (m, 1H), 2.13-2.19 (m, 1H), 2.26 (s, 4H), 2.48-2.55 (m, 2H), 3.08-3.14 (m, 3H), 3.54 (s, 2H), 3.65-3.74 (m, 3H), 3.81 (s, 3H), 3.84 (s, 3H), 3.92-3.98 (m, 2H), 4.00-4.04 (m, 2H), 4.36 (m, 2H), 4.78 (d, 1H, J= 4.8Hz), 5.58 (t, 1H, J= 7.2 Hz), 6.62-6.64 (m, 2H), 6.76-6.81 (m, 3H), 6.90 (d, 1H, J= 7.8 Hz), 7.28 (t, 1H, J= 7.8 Hz), 7.61 (s, 2H).

13C NMR (150 MHz, CDCl3) δ = 19.95, 24.60, 27.88, 29.66, 31.16, 37.54, 42.98, 53.07, 55.76, 55.93, 56.80, 61.99, 63.80, 63.82, 76.65, 111.38, 111.68, 114.17, 116.07, 120.06, 120.30, 126.96, 126.97, 130.13, 133.21, 135.30, 141.68, 147.29, 148.76, 157.19, 159.79, 160.05, 160.32, 160.58, 169.76.

MS (ESI) m/z: found Rt 10.30 min. (Method LCMS), 778.25, 780.31 [M + H]^+, 800.23, 802.20 [M + Na]^+.


Deprotection of Fmoc (resin 6b)
The coupled resin 5b was weighed (190 mg, 0.05mmol) and added to syringes, swollen in DCM (4 mL) for 1h, and the Fmoc protecting group was removed using 20% 4-methyl piperidine/DCM (4ml) for 1h. After filtration, the resin was washed with DCM (3 x 5ml) and used for the next coupling step.
**Synthesis of sulfonamides**

To the above resin $i$-Pr$_2$EtN (25mg, 0.20mmol) in dry DCM (3 mL) was added and stirred for 20min. To this solution the sulfonyl chloride (0.15mmol) in 500 µL of DCM was added and the reaction was stirred for 4h at room temperature. After the first coupling step the resins were filtered, washed with DCM (3 x 10ml) and then subjected to second coupling with $i$-Pr$_2$EtN (30mg, 0.237mmol), sulfonyl chloride (0.158 mmol) in DCM (3 mL) and stirred for 16h at room temperature. The resins were washed with DCM (3 x 5ml) and dried to give the derivatized resins. These were re-swollen in DCM reacted with 1% TFA/DCM (3ml) for 1h and then washed with 1% TFA/DCM (3 x 3ml) and further washed with DCM (3 x 5ml). The combined filtrates were concentrated *in vacuo* to yield the compounds $46-47$. (crude weight ~50mg). The crude compounds were further purified by preparative HPLC using Gradient C. The purified peaks were further dried by lyophilization.

**Synthesis of 2-(3-((R)-1-((S)-1-(3,5-dichloro-4-hydroxyphenylsulfonyl)piperidine-2-carboxamido)-3-(3,4-dimethoxyphenyl)propyl)phenoxy)acetic acid 46**

TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.31, yield= 8 mg (17.4 %).

HPLC (Gradient A) retention time= 22.6-22.9 min

$^1$HNMR (600 MHz, CDCl$_3$) $\delta$= 1.30-1.36 (m, 1H), 1.46 (d, 1H, J= 13.2 Hz), 1.51-1.55 (m, 1H), 2.01-2.14 (m, 3H), 2.26 (d, 1H, J= 13.2 Hz), 2.49-2.63 (m, 2H), 2.85-2.94 (m, 1H), 3.79-3.83 (m, 2H), 3.85 (s, 3H), 3.86 (s, 3H), 4.49 (d, 1H, J= 4.8 Hz), 4.67 (s, 2H), 4.92-4.97 (m, 1H), 6.68-6.70 (m, 2H), 6.77-6.80 (m, 2H), 6.82-6.84 (m, 1H), 6.92 (d, 1H, J= 7.8 Hz), 7.28 (t, 1H, J= 7.8 Hz), 7.75 (s, 2H).


**Synthesis of 2-(3-((R)-3-(3,4-dimethoxyphenyl)-1-((S)-1-(2-oxo-2,3-dihydrobenzo[d]thiazol-6-ylsulfanyl)piperidine-2-carboxamido)propyl)phenoxy)acetic acid 47**

TLC (Hexane: EtOAc: TFA 6:3.8:0.2): Rf = 0.32, yield= 8.2 mg (17.5 %).

HPLC (Gradient A) retention time= 21.14-21.35 min

$^1$HNMR (600 MHz, CDCl$_3$) $\delta$= 1.38-1.42 (m, 1H), 1.57-1.66 (m, 5H), 1.93-2.17 (m, 3H), 2.44-2.56 (m, 2H), 3.71-3.76 (m, 1H), 3.83-3.84 (m, 6H), 4.55-4.60 (m, 1H), 4.72-4.73 (m, 2H), 4.77-4.84 (m, 1H), 6.46 (t, 1H, J= 8.4 Hz), 6.64-6.73 (m, 3H), 6.77 (d, 1H, J= 7.8 Hz), 6.81-6.83 (m, 1H), 6.93-6.96 (m, 1H), 7.29-7.36 (m, 2H), 7.78-7.79 (m, 1H).

Synthesis of (S)-1,7-di(pyridine-3-yl)heptane-4-yl 1-(3,5-dichloro-4-hydroxyphenylsulfonyl)piperidine-2-carboxylate 48

TLC (DCM: MeOH 9.5:0.5): Rf = 0.49, yield = 10.2 mg (47.7%).
HPLC (Gradient A) retention time = 28.9-29.2 min
MS (ESI) m/z: found Rt 7.01 min. (Method LCMS), 606.18, 608.20 [M + H]⁺
Calculated 606.18, 608.22 [M + H]⁺.

X-ray crystallography
Crystals and co-crystals of the FKBP51 Fk1 domain construct comprising residues 16-140 and containing mutation A19T were obtained as previously described16. Diffraction data were collected at beamline X06DA of the Swiss Light Source (SLS) synchrotron in Villigen, Switzerland. The data were processed with MOSFLM24 and XDS25, SCALA26 and TRUNCATE27. The crystal structure was isomorphous with the apo structure (PDB code 3O5R). The dictionaries for the ligand compounds were generated with the PRODRG server28. The structures were refined with REFMAC29. Manual model building was performed with COOT30. Molecular graphic figures were generated using PyMOL (http://www.pymol.org).

Supporting Information. Purity and activity data for compounds 47-77 from the high throughput synthesis and assay. This material is available free of charge via the Internet at http://pubs.acs.org.

Acknowledgments
We thank Dr. Gerd Rühter and the Lead Discovery Center (Dortmund) for providing the precursor for the synthesis of morpholine analogs and compound 46. We thank Prof. Florian Holsboer and the CIPSM for financial support. We are indebted to Mrs. E. Weyher and Dr. S. Uebel (MPI of Biochemistry) and to Mrs. C. Dubler (Ludwig-Maximilians-Universität Munich) for HRMS and NMR measurements. This research project has been supported by the European Commission under the 7th Framework Program: Research Infrastructures. Grant Agreement Number 226716.
References


Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52


(20) Gopalakrishnan, R.; Hausch, F. Evaluation of Synthetic FK506 Analogs as Ligands for FKBP51 and FKBP52. *To be Submitted*.


Supporting Information

Exploration of Pipecolate Sulfonamides as Binders of the FK506-Binding Proteins 51 and 52

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³Technical University Munich, Lichtenbergstr. 4, 85747 Garching, Germany
Supporting Information Table of Contents:

1. Medium throughput small scale synthesis
2. Library Testing
3. Table-S1 Summary of the sulfonamide library synthesized by the solid support protocol for screening along with the mass, % purity and % inhibition.
4. Table-S2 Data collection and Refinement Statistics (crystallographic data)
1. **Medium throughput small scale synthesis:**

Preweighed samples of Fmoc protected immobilized piperolate solid support were distributed to each of 36 wells of a 96 well parallel synthesis reactor platform obtained from FlexChem® peptide synthesis system. The Fmoc deprotection was carried out individually in each of the wells followed by coupling with sulfonyl chlorides obtained commercially from Maybridge. The unreacted excess sulfonyl chlorides were washed followed by the cleavage of the piperolate sulphonamides from the resin under mild acidic condition.

Out of the 36 compounds 35 compounds were purified by preparative HPLC. Analytical HPLC showed the compounds had at least > 90% purity with most compounds were > 95% pure (except 50, 65 and 73). The correct identity was confirmed by mass spectroscopy, as summarized in Table-S1. Compound 50 was purified using ion exchange chromatography to only 81% purity.

2. **Library Testing:**

The 36 purified and chemically validated compounds were tested for their binding to FKBP12 and to the FK1 domains of FKBP51 and FKBP52 in a fluorescence polarization assay. The binding of the compounds to the proteins was analyzed by calculating the % inhibition at a concentration of 5 µM (Table 1).

Compounds having better than 15% inhibition were considered as hits. This threshold yielded 5 compounds (8-12) having activity for all the three proteins.
3. Table-S1:

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<th>Compound No. (a)</th>
<th>Substituent Structure</th>
<th>Mol Wt Calc</th>
<th>Mol Wt found</th>
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<td>630.27 [M + H]+, 652.27 [M + Na]+, 668.20</td>
<td>97</td>
<td>O</td>
<td>601.18</td>
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Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

<table>
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<tr>
<th></th>
<th>Structure</th>
<th>Mass (Da)</th>
<th>% Purity</th>
<th>% Inhibition</th>
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<td>691.2</td>
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<td>95</td>
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Table S1: Summary of the sulfonamide library synthesized by the solid support protocol for screening along with the mass, % purity and % inhibition. (a) Negative mode; (b) two peaks in HPLC spectrum. The major peak (86%) while the other is a minor peak corresponding to 12% of the AUC.
4. Table-S2 Data collection and Refinement Statistics

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<thead>
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<th>Dataset</th>
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<td>Ligand</td>
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</tr>
<tr>
<td>beamline</td>
<td>SLS, X06DA</td>
</tr>
<tr>
<td>wavelength (Å)</td>
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</tr>
<tr>
<td>space group</td>
<td>$P_2_1_2_1_2_1$</td>
</tr>
<tr>
<td>cell dimensions, a, b, c (Å); α, β, γ (°)</td>
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<tr>
<td>resolution limits (Å)*</td>
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<td>Rmerge ***</td>
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</tr>
<tr>
<td>I/sigma ***</td>
<td>18.4 (3.6)</td>
</tr>
<tr>
<td>multiplicity *</td>
<td>3.8 (3.6)</td>
</tr>
<tr>
<td>completeness (%) *</td>
<td>99.5 (99.0)</td>
</tr>
</tbody>
</table>

**Refinement**

| resolution range                 | 20 – 1.0      |
| reflections (test set)           | 66559 (3503)  |
| Rcryst                           | 0.1375        |
| Rfree                            | 0.1508        |
| number of atoms                  | 1332          |
| r.m.s.d. bonds (Å)               | 0.012         |
| r.m.s.d. angles (°)              | 1.724         |
| Ramachandran plot                |               |
| % most favored region***         | 97.67         |
| % additionally allowed***        | 1.16          |

* Values in parenthesis for outer shell.
** As defined in Scala.
*** As defined in Coot.
S. Reference.

1.2.2.2.1 Discussion (Manuscript 3)

Bioisosteric replacement of equivalent moieties is an established approach to design analogues to eradicate the metabolic instability or potential toxicity there by helping to improve stability and optimize activity.

In this strategy the α-ketoamide was substituted with sulfonamides to generate new scaffolds with a conserved hydrogen bond pattern. A solid phase synthesis strategy was established to generate a focused library of sulfonamide analogs. Firstly, a focused library was designed taking into consideration various substitution patterns. This library consisted of 36 compounds which were synthesized and further tested in the medium throughput screening platform (fluorescence polarization assay) at a single dose concentration. The best hits (8-12) identified were resynthesized and screened for their binding to FKBPs. This pilot study led to the primary identification of two active aromatic sulfonamides series (9, 10).

These lead structures were further optimized and a detailed systemic SAR analysis was carried out. In the first series various substituents at the meta-positions were incorporated to study their effect on the binding to the larger FKBPs (13-33). Halogen substitution at the meta position (18-21) was found to be favorable as compared to larger substituents (13-17, 24-27, 31-33). The Di-meta Cl substituted analog (20) was used as a representative compound of this series, and the postulated binding mode was confirmed by solving the co-crystal structure with the FK1 domain of FKBP51. Compound 20 bound to FKBP51 with retention of the conserved hydrogen bonds present in the crystal structure of α-ketoamides. Additional dipolar interactions and aromatic hydrogen bonds were also present. This co-crystal structure was used as a template to gain insights for further modifications to gain selectivity and affinity (28-30). Compound 28 having a p-OH substituent in addition to the Di-m-Cl substituent was found to have three folds selectivity for FKBP51 while, substitution with p-OMe rescued the affinity of 29 for FKBP52.

The second series consisted of fused ring substituents (34-37). Compound 35 was found to have an unexpected high binding affinity for FKBP12. Substitution of sulfur in the fused ring by carbon or oxygen (36, 37) resulted in complete loss of activity for the larger FKBPs. This gives an indication that sulfur at this position is important to retain binding to the FKBPs.

The best hit analogs from the two series were further combined with various top group substituents. The larger top group modifications were found to have better binding affinity (40-45, 46) as compared to small modifications (38, 39). Reference sulfonamide compounds having a similar scaffold which have been identified and published to have high binding affinities for FKBP12/ MIP were also resynthesized to evaluate their effect on larger
FKBPs\textsuperscript{55,63}. Surprisingly these compounds were not active for FKBP12 in the fluorescence polarization assay as claimed in the literature. The compounds did not show any measurable binding to the larger FKBPs (Table 3) in line with the SAR established before. The synthesis of these compounds have been incorporated in patent No. EP-11195970.6).

\[ \text{Table: 3 Purity of the re-synthesized reference compounds and their binding affinity determined in a fluorescence polarization assay.} \]

<table>
<thead>
<tr>
<th>Compd. No.</th>
<th>(R_1)</th>
<th>(R)</th>
<th>Reference</th>
<th>FKBP12</th>
<th>FKBP51FK1</th>
<th>FKBP52FK1</th>
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<tr>
<td>A</td>
<td>Et</td>
<td></td>
<td></td>
<td>63.5 ± 14.4</td>
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<td>B</td>
<td>Et</td>
<td></td>
<td></td>
<td>54.7 ± 12.7</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
<td>9.8 ± 7.4</td>
<td>&gt;100</td>
<td>&gt;100</td>
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<tr>
<td>D</td>
<td></td>
<td></td>
<td></td>
<td>9.2 ± 3.7</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

The best hit analogs (compound 42 and 44) resulted in 15-60 fold enhancement of binding affinity for the FKBPs as compared to the lead compound 2. The molecular underpinning of the unexpected high binding affinity of compound 42 and 44 is yet to be investigated. This study proved the hypothesis that sulfonamides adopt a similar binding mode as the \(\alpha\)-ketoamides in the binding site of FKBP and can be used as efficient surrogates for \(\alpha\)-ketoamides. Further, this campaign resulted in identifying and dissecting specific substituents that are important for gaining binding affinity for the FKBPs. This study has resulted in finding two potent binders, (1) compound 42 is so far the best synthetic ligand known for the larger FKBPs, (2) compound 44 is one of the most potent ligand known with equivalent potency for FKBP12 compared to the natural products FK506 and Rapamycin.
The identified sulfonamide lower parts have further being investigated and adapted in rigid bicyclic and polycyclic scaffolds. Combination of the sulfonamide substituents discovered in this work together with bicyclic scaffold has given substantial increase in the potency of this series of ligands. (Wang, Y manuscript *in prep*.). The polycyclic series of compound is discussed in detail in the next section.
1.2.3 Design of Ligand Efficiency by Conformation Control (Manuscript 4)

Yansong Wang¹, Christian Kozany¹, Ranganath Gopalakrishnan¹, Christoph Kress¹, Bastiaan Hoogeland¹, Andreas Bracher², Felix Hausch¹*.

FK506 and Rapamycin have high binding affinity to the FKBPs. This might be in part due to the rigid backbone that is present in these macrocyclic compounds. The synthetic compounds analyzed in manuscript 2 and 3 have high conformational flexibility. From the co-crystal structure elucidated above we postulated the incorporation of an axial substituent at the C⁶-position of the pipecolate ring which could be further cyclized with the C¹-carbonyl to limit the flexibility and to yield polycyclic aza-amide compounds.

One of the polycycle compounds known for FKBP12 was resynthesized and the postulated binding mode was further confirmed by solving the co-crystal structure of this polycyclic compound with FKBP51.

The most promising lower substructures identified in manuscript 2 and 3 were attached to this rigid polycycle core. This series of compounds contained four additional compounds.

![Figure 15: Prototypic compounds of the proposed series.](image)

This work will be part of the manuscript submitted to The Journal of American Chemical Society.

This work comprises a small part of the above manuscript and hence the complete manuscript will not be attached and only my contribution will be discussed in the following pages.
1.2.3.1.1 Background

The diketoamides and the sulfonamides ligands that have been described above have a high degree of conformational flexibility due to presence of rotatable bonds as compared to the natural products FK506 and Rapamycin. This might be a reason for the weak binding affinities of these analogs compared to the natural products. In order to identify more efficient scaffolds we adopted the polycyclic aza amide core which has been previously described for FKBP12\textsuperscript{106,107}. To further understand if this concept can be extended to the larger FKBPs compound 29 was resynthesized and was found to bind to FKBP51 and 52 with equal potency compared to the lead compound (compound 2a ketoamide manuscript).

The postulated binding mode of 29 was further confirmed by solving the co-crystal structure with the FK1 domain of FKBP51 to 1.05\AA. Compound 29 bound to FKBP51 similar to compounds that were described in the above two manuscripts 2 and 3. Apart from these conserved interactions ring B and ring C stack atop each other via [pi] - [pi] interactions (Fig. 16). The preorganization of ring B might lock ring C into a conformation which is favourable for binding. The stacking of these two rings represents a productive ligand hydrophobic collapse\textsuperscript{108}.

![Figure 16: X-ray crystal structure of 29 in complex with the FK1 domain of FKBP51. The two hydrogen bonds between O\textsuperscript{1} 29 and HN-Ile\textsuperscript{87}, and between O\textsuperscript{16} 29 and HO-Tyr\textsuperscript{113}, are shown as dotted red lines. The dipolar interaction between the C\textsubscript{1}-carbonyl and HO-Tyr\textsuperscript{113} is shown in green. Van-der-Waals interactions of the ligand with Y\textsuperscript{57}, D\textsuperscript{68}, F\textsuperscript{77}, Y\textsuperscript{113} and S\textsuperscript{118} are shown in black. Leu\textsuperscript{119} and Pro\textsuperscript{120} at the top of the 80s loop are colored in cyan.](image-url)
1.2.3.1.2 Result and Discussion

Taking these observations as an initial lead we further synthesized this polycycle with the cyclohexyl ring $30^*$ (Scheme-1) that mimic the pyranose of FK506 or Rapamycin and which we had identified previously as preferred substructures compared to the trimethoxyphenyl moieties in a monocyclic scaffold [manuscript 2]. In contrast, in the polycyclic context a dramatic decrease of the binding affinity was observed when ring C was changed to cyclohexyl α-keto amide $30^*$. The lower affinity of $30^*$ might be due to the loss of favourable [π] - [π] interactions leading to a loss of the preorganized conformation. Next, the best substituents from the sulfonamide series were attached to the polycyclic rigid core (Scheme-1). Compound $31$ and $32$ had low to no binding affinity for the larger FKBPs though the binding to FKBP12 was still conserved to some extent. Compound $33$ was the only compound in this series that had detectable affinity for the larger FKBPs.

Scheme-1

* Reagents and conditions : (a) HATU, DIPEA, rt, 16h, (b) DIPEA, CH$_2$Cl$_2$, r.t., 1.5h. *Mixture of Diasteromers.
<table>
<thead>
<tr>
<th>Compd. No.</th>
<th>R</th>
<th>FKBP12</th>
<th>FKBP51FK1</th>
<th>FKBP52FK1</th>
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</thead>
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<tr>
<td>+30*</td>
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<tr>
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<tr>
<td>+32</td>
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<td>&gt;100</td>
<td>&gt;200</td>
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<tr>
<td>+33</td>
<td>![Chemical Structure]</td>
<td>0.70±0.92</td>
<td>13.9±9.7</td>
<td>22.7±19.6</td>
</tr>
</tbody>
</table>

Table 4: Binding affinity to FKBP12, FKBP51 (FK1 domain) and FKBP52 (FK1 domain) determined by a fluorescence polarization assay. * Diastereomeric mixture.
1.2.3.1.3 Experimental section

Chemistry: All solvents were purchased from Roth, reagents were bought from Aldrich-Fluka and the sulfonyl chlorides were obtained from Maybridge, Sigma Aldrich, or ABCR unless otherwise stated. Chromatographic separations were performed either by manual flash chromatography or by automated flash chromatography using an Interchim-Puriflash 430 with an UV detector. Extracts were dried over MgSO₄ and the solvents were removed under reduced pressure. Merck F-254 commercial plates were used for analytical TLC to follow the course of reaction and visualized by UV light at either 254 or 365 nm. Silica gel 60 (Merck 70-230 mesh) was used for column chromatography. NMR spectra of all compounds were obtained from the Department of Chemistry and Pharmacy, LMU, on a Bruker AC 300, a Bruker XL 400, or a Bruker AMX 600 at room temperature in deuterio-CDCl₃ with tetramethylsilane (TMS) as internal standard, unless otherwise stated. Mass spectra (m/z) were recorded on a Thermo Finnigan LCQ DECA XP Plus mass spectrometer at the Max Planck Institute of Psychiatry.

HPLC analysis was carried out using a Jupiter 4 µm Proteo column (250 x 4.6 mm, 5µm particle size). Wavelength: 224nm, 280nm; Flow rate: 1ml/min; Buffer A: 0.1% TFA in 5% MeCN/water; Buffer B: 0.1% TFA in 95% MeCN/water; Gradient A: After 1min elution with 100% buffer A, linear gradient of 0-100% buffer B for 30 min.

Method LCMS: YMC Pro C-8 (100 x 4.6 mm, 3µm particle size) column, Wavelength: 224nm, 280nm; Flow rate: 1ml/min; Buffer A: 0.1% HCOOH in 5% MeCN/water; Buffer B: 0.1% HCOOH in 95% MeCN/water; Gradient B: 1min 100% buffer A, then linear gradient of 0-100% buffer B for 11 min.

Synthesis of Polycycle core (27).
The polycycle core (27) was synthesized either by Christoph Kress in the group of Dr. Felix Hausch or by the Lead Discovery Center (Taros) as previously describedⁱ⁰⁶.

Synthesis of compound 30*.
To 7 (108mg, 0.358 mmol) DIPEA (139 mg, 1.07 mmol), HATU (129 mg, 0.537 mmol), 28 (80 mg, 0.430 mmol) were added. The reaction was stirred at room temperature for 16 h and the product was purified by column chromatography (Hexane: EtOAc 2:8) to yield compound 30*.
TLC (EtOAc: TEA 9.9:0.1): Rf = 0.66, Yield= 25mg (16%).
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

HPLC (Gradient A) retention time = 19.2-19.6 min.

\(^{1}\)HNMR (600 MHz, CDCl\(_3\)) \(\delta = 0.71-0.74 \text{ (m, 3H)}; 1.12-1.42 \text{ (m, 7H)}; 1.58-1.64 \text{ (m, 3H)}; 1.80-1.91 \text{ (m, 4H)}; 2.01-2.16 \text{ (m, 2H)}; 3.76-3.88 \text{ (m, 6H)}; 4.57-4.68 \text{ (m, 1.5H)}; 4.84-4.94 \text{ (m, 1H)}; 5.07 \text{ (s, 0.5H)}; 6.23-6.26 \text{ (m, 1H)}; 6.32 \text{ (s, 0.5H)}; 6.36-6.38 \text{ (m, 0.5H)}.

\(^{13}\)C NMR (150 MHz, CDCl\(_3\)) \(\delta = 15.63, 16.02, 20.25, 20.46, 25.34, 27.22, 29.27, 29.30, 30.08, 31.79, 36.74, 36.82, 40.55, 46.86, 51.01, 52.79, 55.30, 55.47, 58.67, 58.83, 80.75, 81.24, 97.25, 97.40, 104.99, 105.25, 115.79, 116.55, 138.29, 139.04, 157.20, 157.31, 159.80, 159.93, 163.09, 163.99, 168.66, 168.92, 204.10, 204.75.

MS (ESI): \(m/z = 471.33 \text{ [M + H]}^+, \) calculated: 471.56[M + H]^+.

**Synthesis of compound 31.**

To 7 (75mg, 0.247 mmol) DIPEA (45 mg, 0.346 mmol) and 3,5-dichloro-4-hydroxybenzene-1-sulfonyl chloride (81mg, 0.346 mmol) were added. The reaction was stirred at room temperature for 1.5 h and the product was purified by column chromatography (Hexane: EtOAc 1:1) to yield compound 4 as white solid.

TLC (Hexane: EtOAc 3:7): Rf = 0.51, Yield - 100mg (77%).

HPLC (Gradient A) retention time = 27.2-27.5 min.

\(^{1}\)HNMR (300 MHz, CDCl\(_3\)) \(\delta = 1.80-2.37 \text{ (m, 8H)}; 2.56 \text{ (dt, 1H, } J = 3.3, 12.6 \text{ Hz, )}; 3.84 \text{ (s,3H)}; 3.80 \text{ (s, 3H)}; 4.48 \text{ (s, 1H)}; 4.55 \text{ (t,1H, } J = 2.1 \text{ Hz)}; 4.76-4.82 \text{ (m, 1H)}; 4.89-4.91 \text{ (m, 1H)}; 6.06 \text{ (d, 1H, } , J = 2.4 \text{ Hz )}; 6.31 \text{ (d, 1H, } J = 2.4 \text{ Hz)}; 7.24 \text{ (s, 1H)}; 7.25 \text{ (s,1H)}; 7.31 \text{ (t, 1H, } J = 1.8Hz).

\(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta = 17.56, 28.02, 29.50, 31.53, 40.44, 51.66, 54.84, 55.17, 55.50, 59.05, 97.08, 105.60, 116.06, 125.07, 132.23, 135.60, 138.15, 143.56, 157.05, 159.45, 168.06.

MS (ESI): \(m/z = 511.87, 513.47 \text{ [M + H]}^+, \) calculated: 511.48, 513.13[M + H]^+.

**Synthesis of compound 32.**

To 7 (50mg, 0.165 mmol) DIPEA (29.9 mg, 0.232 mmol) and 3,5-dichloro-4-hydroxybenzene-1-sulfonyl chloride (60.5mg, 0.232 mmol) were added The reaction was stirred at room temperature for 2 h and the product was purified by crystallization from methanol to yield 5 as white solid.

TLC (DCM: MeOH 9.6: 0.4): Rf = 0.44, Yield= 19.28mg (23%).

HPLC (Gradient A) retention time = 23.3-23.6 min
Medicinal chemistry approach to identify new ligands for FKBP51 and FKBP52

$^1$HNMR (400 MHz, CDCl$_3$ : CD$_3$OD 8 : 2) $\delta$= 1.91-2.07 (m,6H), 2.27-2.31 (m,2H), 2.50-2.57 (m, 1H), 3.76 (s,3H), 3.79 (s, 3H), 4.44 (s, 1H), 4.51-4.53 (m,1H), 4.72-4.78 (m, 1H), 4.78-4.81 (m, 1H), 6.02 (d, 1H, , $J$ = 2.4 Hz ), 6.25 (d, 1H, $J$ = 2.4 Hz), 7.24 (s, 1H), 7.25 (s,1H).

$^{13}$C NMR (100 MHz, CDCl$_3$: CD$_3$OD 8:2) $\delta$= 17.55, 27.90, 29.47, 31.34, 40.52, , 51.21, 54.53, 55.10, 55.44, 59.10, 97.93, 105.19, 116.00, 121.73, 127.00, 132.60, 137.97, 151.88, 156.95, 159.33, 168.57.


**Synthesis of compound 33.**

To 7 (50mg, 0.165 mmol), DIPEA (29.9 mg, 0.232 mmol) and benzo[d]thiazole-6-sulfonyl chloride (60.5mg, 0.232 mmol) were added The reaction was stirred at room temperature for 1.5 h and the product was purified by column chromatography (Hexane: EtOAc 2:8) to yield 6 as white solid.

TLC (Hexane: EtOAc 1:9): Rf = 0.21, Yield= 79.2mg (95%).

HPLC (Gradient A) retention time = 22.1-22.3 min.

$^1$HNMR (300 MHz, CDCl$_3$) $\delta$= 1.79-2.15 (m,8H), 2.46 (dt, 1H, $J = 3, 12.6$ Hz ), 3.74 (s,3H), 3.84 (s, 3H), 4.46 (s, 1H), 4.60-4.70 (m,2H), 5.00 (d, 1H, $J = 1.8$ Hz), 5.70 (d, 1H, $J = 2.4$ Hz), 6.27 (d, 1H, , $J = 2.4$ Hz ), 7.51 (dd, 1H, $J = 1.8, 8.7$ Hz), 7.95 (m, 2H), 9.15 (s,1H).

$^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$= 17.62, 28.10, 29.21, 31.87, 40.11, 51.83, 54.71, 55.19, 55.51, 58.86, 96.98, 104.98, 116.52, 121.40, 123.96, 124.33, 133.77, 138.10, 138.17, 155.00, 157.20, 157.40, 159.22, 168.38.

2. Synthesis of novel photoactivable Tricyclic antidepressant analogs
2.1 Introduction

2.1.1 Depression

The most common psychiatric disorders prevalent in the society are major depression and bipolar disorder. The most prominent symptoms of these disorders which effect the everyday life of the patient include long lasting depressive mood, guilt feeling, anxiety, recurrent thoughts of death and suicide and are collectively referred to as “depressive syndrome”\textsuperscript{109}. The disease treatment and the associated direct and indirect costs have a huge economic burden on the society. This explains the huge market share the antidepressants have in the CNS drug category, which has continuously increased in the past decades.

Both non-genetic and genetic factors play an equal role in the development of depression. The non-genetic social factors that are the causative reasons include physical and emotional stress, affective trauma, viral infection or neurodevelopmental abnormalities. The genetic contribution for the risk of depression has been estimated to be roughly about 30-40\%. However few individual genes that contribute to this risk have been identified\textsuperscript{110}, and a single gene abnormality fails to explain the multifaceted symptoms of depression\textsuperscript{111}.

The central topic of depression research in the last 60 years that has been driving the pharmaceutical industry to search for cure is the monoamine hypothesis. The mechanism of action of all classes of antidepressants can be accounted for by the monoamine theory. As compared to other disease conditions and disorders the knowledge of the brain and its neural circuits are limited. In addition there is a lack of objective diagnostic test which means that the diagnosis of this disorder is highly variable with no clear demarcation between a normal, mild and highly depressed individual. The antidepressants available in the market are effective in only 50\% of the treated patients which gives an indication that apart from the monoamine theory other pathways can also play a role in the etiology of depression. The known animal models for mood disorders also fail to explain the reason why chronic treatment of the antidepressants is required in humans before the clinical effects starts to be seen\textsuperscript{112}. Most of these animal models are based on the monoamine theory and hence it is unclear whether new novel antidepressants working by a novel mechanism can be identified using these models.

2.1.2 Monoamine Hypothesis

The monoamine hypothesis proposes that disruptions in the serotonergic and the noradrenergic systems can result in depression or depression-like symptoms. The therapeutic strategy is to
restore the monoamine levels in such patients. Those classical classes of drugs are present which elicit their effect in accordance with the monoamine hypothesis. The oldest antidepressants are the monoamine oxidase inhibitors (MAO-I) while the second class of drugs are the tricyclic antidepressants. However, new evidences have suggested that the monoamine hypothesis fails to explain the whole mechanism of action of antidepressants. The cytokine hypothesis of depression\textsuperscript{113-117}, the hypothalamic pituitary thyroid hypothesis of depression\textsuperscript{118-120}, the role of brain derived neurotrophic factor\textsuperscript{121-123} and cAMP response element binding protein\textsuperscript{124-126} have been recently shown to partially explain the mechanism of antidepressants.

2.1.3 Monoamine transporter inhibitors as antidepressants

The primary mechanism of action of most or all antidepressant drugs available in market is that they are modulators of monoaminergic neurotransmission. As stated above these drugs can be divided into three broad class (i) monoamine oxidase inhibitors (MAOIs), (ii) monoamine transporter inhibitors (iii) monoamine receptor ligands.

MAOIs were the first compounds that were available for therapy in the 1960s. The early MAOIs like iproniazide, tranylcypromine or phenelzine irreversibly inhibit MAO-A which is the main catabolic enzyme for the monoamine transmitter’s noradrenaline (NA), serotonin (5-HT), and dopamine (DA) (Fig. 17). The end result is the generalized increased in monoamine levels throughout the CNS\textsuperscript{127,128}. The newer analogs of this class of compounds, e.g., Moclobemide, are reversible MAO inhibitors. However, MAOIs are very powerful drugs, and are the last line of defense as its use is limited due to hepatotoxicity and prominent and lethal adverse effect (“the cheese effect”) which leads to hypertensive crisis due to high tyramine ingestion\textsuperscript{128}.

The tricyclic antidepressants (e.g., imipramine) elicited their antidepressant effect by inhibiting the membrane transporters and the reuptake of 5-HT or NE, thereby causing an increase in the synaptic concentrations of monoamines. This class of drugs is very efficient and potent although they are characterized by a wide profile of side effects arising due to a variable degree of antagonism at the muscarinic, adrenergic and histaminergic receptors (Fig. 17). The second generation of TCAs was comparatively more selective to NE uptake (desipramine, nortriptyline, maprotiline) with no significant betterment of the side effect. The subsequent development of the selective serotonin reuptake inhibitors (SSRIs) fluoxetine and its introduction in the clinic has provided the clinicians with a safer treatment alternative and a lesser side effect profile.
Figure 17 Mechanism of action of various classes of antidepressants on a prototypic monoamine transporter (serotonin).

However, the SSRIs have been found to be ineffective in treating certain class of patients which has led to the development of the dual selective drugs SNRIs (venlafaxine and duloxetine)\textsuperscript{129}. 

2.1.4 SAR studies of TCA class of drugs.

Tricyclic antidepressants or various other classes of antidepressants preferentially bind to the transporters SERT and NET. The binding mode of these compounds to the transporters is not fully clarified as the structural insights of these transporters have been controversial. Structure activity relationships and homology modeling has been able to provide clues about the distinct binding mode and binding site of these classes of drugs. Also, mutagenesis studies have provided information regarding residues that are important and required for inhibitor binding\textsuperscript{130-142}. The first tricyclic antidepressant drug, imipramine (34), was launched by Geigy in 1951. The core structure of this drug was obtained by modification of the antipsychotic drug
chlorpromazine. Further modification on the TCA ring gave rise to Clomipramine (36), Cyanopramine and Amitriptyline (37). It has been shown that modifications on the TCA ring at position 3 resulted in analogs with retention of activity\(^ {143,144} \). These drugs have the same dimethylamino group as in chlorpromazine but have different tricyclic core. Imipramine (34), Clomipramine (36), and Cyanopramine have a dibenzoazepine core while in Amitriptyline (37) the nitrogen is replaced by carbon to give the dibenzocycloheptene core and an ethylidene moiety at position 5.

**Table 4**

<table>
<thead>
<tr>
<th>No.</th>
<th>Name of the Drug</th>
<th>Structure</th>
<th>Company</th>
<th>Approved</th>
<th>( \text{Ki (nM)}^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>Imipramine</td>
<td><img src="image" alt="Imipramine structure" /></td>
<td>Geigy</td>
<td>1951</td>
<td>1.4</td>
</tr>
<tr>
<td>35</td>
<td>Desipramine</td>
<td><img src="image" alt="Desipramine structure" /></td>
<td>Geigy</td>
<td>1964</td>
<td>17.6</td>
</tr>
<tr>
<td>36</td>
<td>Clomipramine</td>
<td><img src="image" alt="Clomipramine structure" /></td>
<td>Geigy</td>
<td>1970</td>
<td>0.28</td>
</tr>
<tr>
<td>37</td>
<td>Amitriptyline</td>
<td><img src="image" alt="Amitriptyline structure" /></td>
<td>Hoffmann-La Roche</td>
<td>1961</td>
<td>4.3</td>
</tr>
<tr>
<td>38</td>
<td>Nortriptyline</td>
<td><img src="image" alt="Nortriptyline structure" /></td>
<td>Geigy</td>
<td>1963</td>
<td>18</td>
</tr>
<tr>
<td>39</td>
<td>Lofepramine</td>
<td><img src="image" alt="Lofepramine structure" /></td>
<td>LEO AB</td>
<td>1980</td>
<td>70</td>
</tr>
</tbody>
</table>

\(^a\) Values taken from\(^ {144} \).

All four compounds have equipotent affinity to SERT and NET which proved that these minor structural changes are not of crucial importance\(^ {143} \).
The corresponding N-desmethyl metabolites of the above compounds also have been shown to have therapeutic activity. The N-desmethyl metabolites Desipramine (35), and nortriptyline (38) have a slight preference on NET as compared to their corresponding precursors which have preference for SERT\textsuperscript{143}. This gives an indication that the dimethylamino substituent is important for higher SERT affinity while the N-desmethyl version gives higher NET affinity. Lofepramine (39)\textsuperscript{145}, a close analog of imipramine (34) where one of the methyl in the dimethylamino center is replaced with a longer side chain, has 14 times more preference to NET as compared to SERT\textsuperscript{146}. The above series of compounds shows that any subtle changes in the dimethylamino group of imipramine gives rise to compounds with varying selectivity towards NET and SERT.
2.2 Aim of the work (Manuscript-5)

Tricyclic antidepressants (TCA) tightly bind to a variety of proteins and exert their pharmacological action via a wide mechanism of action. The broad pharmacological background and molecular mechanisms of these drugs is still poorly understood. The unspecific binding of these drugs to wide protein class is thought to be the contributing factor for its clinical efficacy and side effects. Hence a photo-labeling approach was conceptualized to explore and define the TCA-binding proteome which will help in understanding the mechanism of action of antidepressants in detail.

To identify and fish the binding proteome, the first step is the development and functional validation of a chemically modified antidepressant analog with retention of the drug-like properties of the parent drug (Imipramine, in this case). The final tool compound should be amenable to affinity purification and identification of photo-crosslinked antidepressant binding proteins in lysates and endogenous tissue (Fig. 18). Ideally, it should also be applicable to integral transmembrane proteins which comprise all currently known antidepressant targets. Such a tool could also enable the structural fine-mapping of the binding sites in these proteins.

Figure 18. Proposed series of photoreactive TCA analogs.
Photoactivable Tricyclic Antidepressants as Trifunctional Probes for the Serotonin transporter

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Abstract:

Monoamine transporter inhibitors have been the front line therapy for depression for decades. The exact binding mode of these drugs to their canonical target, the serotonin transporter, is matter of debate. Moreover, these antidepressants are characterized by an extremely complex polypharmacology and the role of additional targets for their clinical efficacy is been unclear. Here we present the development of multifunctional analogs of the tricyclic antidepressant imipramine. These tools inhibit monoamine uptake by the serotonin transporter with nanomolar potency, allow for photocrosslinking and contain an acetylene tag for affinity enrichment by click chemistry. These chemical tools will be useful for the fine-mapping of the binding mode of tricyclic antidepressants or for the identification of alternative targets by mass spectroscopy.
Synthesis of novel photoactivable Tricyclic antidepressant analogs

Depression is one of the most common disorders and it is expected to be the leading cause of disability in 2030 [WHO: The global burden of disease (2004 update)]. The established targets for most antidepressants are transporters of monoamine neurotransmitters\(^1\). While there has been substantial progress in the structural biology of this protein class, the exact antidepressant binding site has remained controversial, in part due to the poor biochemical tractability of these integral membrane proteins\(^2\)\(^-\)\(^5\). In addition to the monoamine transporters additional protein targets have been repeatedly been discussed to contribute to the efficacy of the currently available antidepressants and the ultimate mechanism of action of these drugs has remained elusive. A better understanding of antidepressants as been complicated by their extremely broad polypharmacology as well as by a lack of adequate chemical tools\(^6\)\(^,\)\(^7\). In fact, some antidepressants are among the most “dirty” drugs currently in clinical use.

To study the targets of antidepressants in endogenous systems in an unbiased manner, we set out to develop antidepressants analogs that would allow for covalent labelling of antidepressant binding proteins. Towards this end, we started with the prototypic tricyclic antidepressant Imipramine (1) as a chemical starting point. Clinically effective analogs like Clomipramine (3) and Cyanopramine (4)\(^8\)\(^-\)\(^11\) suggested that substituents in the 3-position of the tricyclic ring system would not compromise the antidepressant activity of this class of drugs [Fig. 1]. We thus set out to introduce an azido group in the 3-position of Imipramine to graft the known photoreactivity of aromatic azides into the tricyclic ring system.

![Figure 1](image-url) Tricyclic antidepressants drugs used in the clinic.

The Imipramine analog Azidopramine 9 was synthesized from the commercially available azepine analog 1-(3-amino-10,11-dihydro-5H-dibenzo[b,f]azepin-5-yl)ethanone 6 in four steps. The primary amine of 6 was first converted to the corresponding azide 7. Basic deprotection of the acetyl group provided the free secondary amine 8 which was alkylated with 9 to yield the tricyclic antidepressant analog Azidopramine (10).
Scheme 1: Synthesis of Azidopramine (10): (a) NaNO$_2$, 10% HCl. (b)NaN$_3$, H$_2$O, rt, 1h. (c) KOH, MeOH, 60°C, 15h. (d) NaH, ClICH$_2$CH$_2$CH$_2$N(CH$_3$)$_2$(9), 0-60°C, toluene.

The photoreactivity of the aromatic azide 10 was then tested by subjecting the compound to UV light exposure (254 nm). TLC analysis (Fig. 2) showed a time dependent conversion under these conditions. This confirmed that the introduction of the azide group in Azidopramine resulted indeed in a photoreactive AD analog.

Figure 2 Photoreactivity of Azidopramine (10) when subjected to UV light (254 nm) analyzed by thin layer chromatography (TLC), mobile phase DCM: MeOH 9.2:0.8

To verify that Azidopramine retained its biological activity we then tested it in an uptake assay for its primary target, the serotonin transporter (SERT). Gratifyingly, Azidopramine inhibited the uptake of SERT with an IC$_{50}$ of 28nM, i.e., with equal potency compared to the parent compound Imipramine. This shows that addition of the azide group at position 3 was indeed tolerated (Table-1).

For Azidopramine (10) to be useful as a tool to study the mechanism of action of antidepressants, a convenient way to detect Azidopramine in biological samples would be extremely useful. We thus set out to explore the possibilities to introduce additional chemical tags into Azidopramine (10) to make it amenable to sensitive biochemical detection. Two approaches can be envisioned, (i) to convert Azidopramine into a radiotracer, (ii) to introduce additional bioorthogonal reactive chemical tags that allow a specific and sensitive labeling of Azidopramine photocrosslinking adducts.

We first envisaged the synthesis of the demethylated analog Desazidopramine 13. The coupling of the necessary Boc-protected building block 11 to the azepine 8 turned out to be more demanding than the corresponding 3-chloropropyl-dimethyl amine (9) used for the synthesis of
Azidopramine. Stronger activation with a tosyl group and optimization of the reaction conditions eventually furnished compound 13 in 64% yield which was deprotected to the desired Desazidopramine 14 (Scheme 2).

Scheme 2: Synthesis protocol of Desazidopramine (14) and Azidobupramine (15): (a) (Boc)₂O, DMAP, ACN, rt, 2h. (b) TsCl, Et₃N, DCM, 0°C. (c) NaHMDS, toluene, -78°C to 70°C, 6h. (d) 20%TFA, DCM, rt, 4h. (e) 1-bromo-3-butyne (15), K₂CO₃, KI, 60°C, 12h, acetone.

Demethylation in Desazidopramine substantially reduced activity in the uptake assay for the serotonin transporter (SERT) compared to Azidopramine (Table 1). The loss in activity was similar to the clinically used antidepressant Desipramine, in line with the known preference of the serotonin transporter for di-N-methylated TCA analogs. Desazidopramine can be used, however, as the precursor for the synthesis of tritiated Azidopramine by radioactive methylation.

To allow for a non-radioactive detection of Azidopramine, we alkylated 14 with 1-bromo-3-butyne (15) to introduce a “click tag” to generate Azidobupramine 16 (Scheme-2). This second generation antidepressant analog carried an additional terminal alkyne group for selective derivatization with labeled azides by copper-catalyzed cycloaddition. The introduction of propyne group was not envisaged in place of the butyne because the resulting compound will have a structure similar to MAOIs, e.g. pargyline. This could lead to an additional MAO inhibition thereby possibly confounding the interpretation of biological effects.

Azidubupramine (16) was further tested in the radioactive uptake assay for SERT. Its SERT-inhibiting activity was found to be weaker than Azidopramine (10), but a stronger compared to Des-azidopramine (14) and substantially stronger than the clinically used Lofepramine (5), one of the few TCA analogs with a longer N-alkyl substituent.
Table-1 SERT uptake assay: [H$^3$]-5HT Uptake assay for using HEK293 cells stably over expressing human SERT.

<table>
<thead>
<tr>
<th>Compd. No.</th>
<th>Compd. Name</th>
<th>Structure</th>
<th>IC$_{50}$(nM) hSERT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Imipramine</td>
<td><img src="image" alt="Structure" /></td>
<td>22 ± 6</td>
</tr>
<tr>
<td>2</td>
<td>Desipramine</td>
<td><img src="image" alt="Structure" /></td>
<td>1400 ± 400</td>
</tr>
<tr>
<td>5</td>
<td>Lofepramine</td>
<td><img src="image" alt="Structure" /></td>
<td>2747 ± 1573</td>
</tr>
<tr>
<td>10</td>
<td>Azidopramine</td>
<td><img src="image" alt="Structure" /></td>
<td>28 ± 15</td>
</tr>
<tr>
<td>14</td>
<td>Des-Azidopramine</td>
<td><img src="image" alt="Structure" /></td>
<td>1400 ± 400</td>
</tr>
<tr>
<td>16</td>
<td>Azidobupramine</td>
<td><img src="image" alt="Structure" /></td>
<td>560 ± 25</td>
</tr>
</tbody>
</table>

In conclusion, this study provides functionalized antidepressant analogs as chemical tools for molecular psychiatry. Azidopramine (10) allows for the covalent derivatization of membrane-localized antidepressant targets like the serotonin transporters. [H$^3$]-labelled Azidopramine or the tri-functional analog Azidobupramine (16) can both be used to fine-map the antidepressants binding site of SERT. Azidobupramine further allows for an affinity enrichment to enable a mass spectrometry analysis of the antidepressant binding proteome. Importantly, Azidobupramine (16) can interrogate antidepressant binding sites in their native environment in an unbiased manner. It is small enough that it could even be used in intact animals, e.g., in
animal model of depression. This should be useful to expand our understanding of the mechanism of action of clinically used antidepressants.

Methods:
Chemistry. Chromatographic separations were performed either by manual flash chromatography or by automated flash chromatography using an Interchim Puriflash 430 with an UV detector. Organic phases were dried over MgSO₄, and the solvents were removed under reduced pressure. Merck F-254 (thickness 0.25mm) commercial plates were used for analytical TLC to follow the progress of reactions. Silica gel 60 (Merck 70-230 mesh) was used for manual column chromatography. Unless otherwise specified, ¹H NMR spectra, ¹³C NMR spectra, 2D HSQC, HMBC and COSY of all intermediates were obtained from the Department of Chemistry and Pharmacy, LMU, on a Bruker AC 300, a Bruker XL 400, or a Bruker AMX 600 at room temperature. Chemical shifts for ¹H, ¹³C are given in ppm (δ) relative to tetramethylsilane (TMS) as internal standard. Mass spectra (m/z) were recorded on a Thermo Finnigan LCQ DECA XP Plus mass spectrometer at the Max Planck Institute of Psychiatry, while the high resolution mass spectrometry was carried out at MPI for Biochemistry. (Microchemistry Core facility) on Varian Mat711 mass spectrometer. The purity of the compounds was verified by reversed phase HPLC (Jupiter 4 µm Proteo 90 A, 250*4.6 mm, Phenomenex, Torrance, USA) using gradient A (acetonitrile:water gradient:0.1% TFA of 0 – 100% in 45 min) unless otherwise specified. Solvents were purchased from Roth, reagents were obtained from Aldrich-Fluka unless otherwise noted.

HPLC conditions for product analysis; Column: Jupiter 4 µm Proteo 90 A, 250 x 4.6 mm, Phenomenex, Torrance, USA, Wavelength: 224nm, 280nm Flow rate: 1ml/min, Buffer A: 0.1% TFA in 5% MeCN/Water, Buffer B: 0.1% TFA in 95% MeCN/water. Gradient A After 1min elution with 100% buffer A,: linear gradient of 0-100% buffer B for 30 min.

Synthesis of 1-(3-azido-10,11-dihydro-5H-dibenzo[b,f]azepin-5-yl)ethanone 7
To a stirred solution of 1-(3-amino-10,11-dihydro-5H-dibenzo[b,f]azepin-5-yl)ethanone 6 (Wako Chemicals, 100 mg, 0.396 mmol) in 10% aqueous hydrochloric acid (2ml), a solution of sodium nitrite (27.3 mg, 0.396 mmol) in water was added at 0-5°C with vigorous stirring. The mixture was kept below 5°C for 30 min, and then a solution of sodium azide (28.3 mg, 0.436 mmol) in water (5ml) was added dropwise while the reaction was kept at the same temperature. After being stirred for 1h, the mixture was warmed to room temperature and extracted with EtOAc and water. The organic layer was washed with brine, dried with MgSO₄ and
Synthesis of novel photoactivable Tricyclic antidepressant analogs

concentrated under reduced pressure to give 110mg (0.396 mmol, 100%) of compound 7 as yellow oily liquid.

TLC (Hexane: EtOAc 11:9): Rf = 0.58

1H NMR (400 MHz, CDCl3) δ= 2.03 (s, 3H, CH3), 2.77-2.86 (m, 2H, CH2CH2), 3.28-3.48 (m, 2H, CH2CH2), 6.85 (d, 1H, J = 8.4 Hz), 6.96 (s, 1H), 7.07 (s, 1H), 7.13 (d, 1H, J = 8.4 Hz), 7.29 (m, 4H).

13C NMR (100.5 MHz, CDCl3) δ= 22.64, 30.01, 30.59, 118.42, 119.19, 127.46, 127.60, 128.75, 129.88, 131.87, 137.41, 138.13, 140.94, 142.24, 143.69, 170.56.


Synthesis of 3-azido-10,11-dihydro-5H-dibenzo[b,f]azepine 8

To 110 mg of compound 7 (0.395 mmol) was added potassium hydroxide (72mg, 1.38 mmol) dissolved in 10ml of methanol and the reaction mixture was refluxed for 6 h under an argon atmosphere. Afterwards, methanol was evaporated and the mixture was extracted with CH2Cl2. The oily liquid was dissolved in minimum amount of EtOAc and then recrystallised from hexane in the cold to yield needle shaped crystals of 8 (85 mg, 0.359 mmol, 85%).

TLC (Hexane: EtOAc 9:1): Rf = 0.6.

1H NMR (300 MHz, CDCl3) δ= 3.07 (s, 4H), 6.39 (d, 1H, J = 2.1 Hz), 6.49 (dd, 1H, J = 2.1, 6 Hz), 6.75 (d, 1H, J = 0.9 Hz), 6.84 (dt, 1H, J = 1.2, 7.5 Hz), 7.03 (d, 1H, J = 8.1Hz), 7.06-7.15 (m, 2H).

13C NMR (75 MHz, CDCl3) δ= 34.55, 34.81, 108.06, 109.83, 118.15, 120.12, 125.39, 126.95, 129.02, 130.64, 132.06, 138.55, 141.87, 143.53.


Synthesis of 3-dimethylamino-1-propyl chloride 9

Sodium hydroxide and 3-dimethylamino-1-propyl chloride hydrochloride (TCI Europe) were dissolved separately in water (10 mL). These two solutions were mixed and the pH was adjusted to ~14. After extraction with dichloromethane (3x30 mL), the extracts were dried over anhydrous sodium sulfate and the solvent was removed to afford 50 mg (53%) of the free base. High vacuum was not used as the amine obtained is volatile.

Synthesis of 3-(3-azido-10,11-dihydro-5H-dibenzo[b,f]azepin-5-yl)-N,N-dimethyl propan-1-amine 10 (Azidopramine)

A solution of compound 8 (50 mg, 0.212 mmol) was prepared in dry toluene (sure seal, Fluka, 10ml) at 0°C under argon. To the solution was added a suspension of NaH (6.09 mg, 0.254
mmol) in toluene (3ml) and the reaction was stirred for 30min. Freshly prepared solution of 3-dimethylamino-1-propylchloride 9 (33.5mg, 0.275 mmol) (generated from its hydrochloride salt) as described above was added dropwise and the reaction was allowed to warm to room temperature. The reaction was heated to 60°C and stirred overnight. TLC analysis showed complete disappearance of the starting educt 8. The reaction mixture was poured into water and extracted with EtOAc. The organic layer was washed with brine, dried over MgSO₄ and concentrated by rotary evaporation. Column chromatography in dichlorormethane: MeOH 95:05 of the crude reaction mixture was performed to give compound 10 (Azidopramine, 45 mg, 0.14mmol, 66%).

TLC (DCM: MeOH 9:1): Rf = 0.38.

HPLC (gradient A) Rt: 21.2min, Purity= 99 %.

1H NMR (300 MHz, CDCl₃) δ= 1.70-1.80 (m, 2H), 2.19 (s, 6H), 2.26-2.37 (m, 2H), 3.15 (s, 4H), 3.76 (t, 2H, J = 6.9 Hz) 6.61 (dd, 1H J = 2.4, 5.7 Hz), 6.73 (d, 1H, J = 2.1 Hz), 6.984 (dt, 1H, J = 1.5, 5.4, 6.9 Hz), 7.06 (d, 1H, J = 8.1Hz), 7.11- 7.20 (m, 3H).

13C NMR (75 MHz, CDCl₃) δ= 25.94, 31.65, 32.22, 45.42, 48.84, 57.49, 110.46, 112.45, 120.56, 123.22, 126.55, 129.43, 129.96, 131.33, 135.17, 137.87, 147.91, 149.20.

MS (ESI) : m/z = 322.27 [M + H]+.


**Synthesis of 3-(tert-butoxycarbonyl(methyl)amino)propyl 4-methylbenzenesulfonate 12**

To 3-(methylamino)propan-1-ol 11 (250 mg, 6.28 mmol) in acetonitrile was added BOC anhydride (680 mg, 12.56 mmol) and a catalytic amount of DMAP. The reaction was stirred for 2 hours until the disappearance of alcohol 11. The crude mixture was subjected to column chromatography (Hexane: EtOAc 55:45) and dried under reduced pressure to obtain (460 mg, 2.43 mmol, 87%) of the desired product.

TLC (Hexane: EtOAc 1:1): Rf = 0.46.

1H NMR (300 MHz, CDCl₃) δ= 1.45 (s, 9H), 1.66-167 (m, 2H), 2.62 (s, 3H), 3.37 (s, 2H), 3.52 (s, 2H).

13C NMR (75 MHz, CDCl₃) δ= 28.35, 29.63, 34.13, 44.21, 58.08, 79.96, 157.19.

To the above compound (90 mg, 0.475 mmol) in dichloromethane was added p-toluene sulfonylchloride (136 mg, 0.713 mmol) and triethylamine (96 mg, 0.951 mmol) and the mixture was stirred at 0°C for 4 h. The reaction mixture was then quenched with water and extracted using diethyl ether. The ethereal layer was washed with brine and dried over MgSO₄.
to yield the crude product which was further subjected to column chromatography using Hexane: EtOAc 13: 7 to yield 135 mg (0.393 mmol, 84%) of 12 as white oily liquid.

TLC (Hexane: EtOAc 1:1): Rf = 0.46.

\(^1\)H NMR (300 MHz, CDCl\(_3\)) δ = 1.41 (s, 9H), 1.81- 1.90 (m, 2H), 2.44 (s, 3H), 2.78 (s, 3H), 3.23 (t, 2H, J = 6.9 Hz), 4.02 (t, 2H, J = 6.3 Hz), 7.34 (d, 2H, J = 7.8 Hz), 7.77 (d, 2H, J = 8.4 Hz).

\(^13\)C NMR (75 MHz, CDCl\(_3\)) δ = 21.63, 27.53, 28.35, 34.59, 45.28, 68.2, 79.62, 125.94, 127.87, 129.10, 129.88, 132.86, 144.87, 155.60.


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**Synthesis of tert-butyl 3-(3-azido-10,11-dihydro-5H-dibenzo[b,f]azepin-5-yl)propyl(methyl) carbamate 13**

To a solution of 3-azido-10,11-dihydro-5H-dibenzo[b,f]azepine 8 (130 mg, 0.550 mmol) in 5ml dry toluene was added 0.660mL of a 1M solution of sodium bis (trimethylsilyl) amide in hexane (0.660 mmol) under an argon atmosphere at -78°C and the mixture was stirred for 0.5h. Freshly prepared tosyl analog 12 (227 mg, 0.660 mmol) was added dropwise to the above mixture and the reaction flask was allowed to warm to room temperature. The reaction was further stirred at 70°C overnight and the completion of the reaction was monitored using thin layer chromatography. The crude product was poured into water and extracted with EtOAc. The organic layer was washed with brine and dried over MgSO\(_4\) and concentrated to dryness. Column chromatography of the crude reaction mixture was performed in (Hexane: EtOAc 19:1) as eluent to give compound 13 (150 mg, 0.368 mmol, 67%).

TLC (Hexane: EtOAc 19:1): Rf = 0.23

\(^1\)H NMR (400 MHz, CDCl\(_3\)) δ = 1.39 (s, 9H), 1.72- 1.79 (m, 2H), 2.44 (s, 3H), 2.72 (s, 3H), 3.09 – 3.16 (m, 4H), 3.23 (t, 2H, J = 6.8 Hz), 3.69 (t, 2H, J = 6.8 Hz), 6.59 (d, 1H, J = 2.4, 5.6 Hz), 6.68 (d, 1H, J = 2 Hz), 6.96 (dt, 1H, J = 1.2, 7.2 Hz), 7.05 (t, 2H, J = 8 Hz), 7.10-7.16 (m, 2H).

\(^13\)C NMR (100 MHz, CDCl\(_3\)) δ = 26.26, 28.39, 31.58, 32.14, 34.23, 46.71, 48.09, 79.31, 110.27, 112.52, 120.36, 123.33, 126.54, 129.49, 129.91, 131.37, 135.17, 137.89, 147.67, 149.12, 155.69.

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**Synthesis of 3-(3-azido-10,11-dihydro-5H-dibenzo[b,f]azepin-5-yl)-N-methylpropan-1-amine 14 (Desazidopramine)**
Compound 13 (150mg, 0.368mmol) was deprotected in the presence of 20% trifluoroacetic acid solution in DCM for 3.5h at room temperature to yield 14. TFA was evaporated under reduced pressure and the crude mixture was subjected to a small wash out silica gel column using hexane: EtOAc: TEA 3.8:6.0:0.2 to give pure Desazidopramine 14 (85mg, 0.276mmol, 75%).

TLC (Hexane: EtOAc: TEA 3.8:6.0:0.2): Rf = 0.29.

HPLC (gradient A) Rt: 19.2 min, Purity= 98%

\[ ^1H\text{ NMR (600 MHz, CDCl}_3\text{)} \delta = 1.88-1.93 (m, 2H), 2.42 (s, 3H), 2.85 (t, 2H, J = 7.2), 3.07 – 3.12 (m, 4H), 3.75 (t, 2H, J = 6.6 Hz), 6.62 (dd, 1H, J = 2.4, 6 Hz), 6.64 (d, 1H, J = 1.8 Hz), 6.967(dt, 1H, J = 1.2, 6 Hz), 7.03 (q, 2H, J = 4.8, 7.2 Hz), 7.10-7.15 (m, 2H). \]

\[ ^{13}C\text{ NMR (150 MHz, CDCl}_3\text{)} \delta = 24.69, 31.48, 31.96, 33.26, 47.49, 47.62, 110.15, 112.95, 120.12, 123.71, 126.70, 129.68, 129.98, 131.50, 135.06, 138.08, 147.15, 148.68. \]

MS (ESI) m/z 308.12 [M + H]+.


**Synthesis of N-(3-(3-azido-10,11-dihydro-5H-dibenzo[b,f]azepin-5-yl)propyl)-N-methylbut-3-yn-1-amine 16 (Azidobupramine)**

To a solution of 14 (80 mg, 0.260 mmol) in acetone (10ml) was added potassium carbonate (180mg, 1.30 mmol) and a catalytical amount of potassium iodide. The mixture was stirred for 30 min and then further reacted with the 4-bromobut-1-yn (15) (41mg, 0.312 mmol) and refluxed at 60°C overnight. Acetone was evaporated followed by an aqueous work up and extraction with CH2Cl2. The crude mixture was subjected to column chromatography in DCM: MeOH mixture to give the desired product 16 (47 mg, 0.130 mmol, 50%).

TLC (DCM: MeOH 9.2:0.8): Rf = 0.38.

HPLC (gradient A) Rt: 19.8 min, Purity= 85%

\[ ^1H\text{ NMR (300 MHz, CDCl}_3\text{)} \delta = 1.69-1.79 (m, 2H), 1.94-1.96 (t, 1H , J = 2.4 Hz), 2.21 (s, 3H), 2.27-2.33 (m, 2H), 2.45 (t, 2H, J = 7.5 Hz), 2.56 (t, 2H, J = 7.2 Hz), 3.15 (s, 4H), 3.78 (t, 2H, J = 6.9 Hz), 6.62 (dd, 1H, J= 2.4 Hz), 6.74 (d, 1H, J= 2.4 Hz), 6.90-7.20 (m, 5H). \]

\[ ^{13}C\text{ NMR (75 MHz, CDCl}_3\text{)} \delta = 15.73, 24.45, 30.55, 31.33, 40.86, 47.62, 53.77, 54.90, 67.86, 81.68, 109.36, 111.29, 119.45, 122.09, 125.41, 128.30, 128.81, 130.21, 134.06, 136.75, 146.80, 148.10 \]

MS (ESI): m/z = 360.16 [M + H]+.

Radioactive Serotonin uptake assay in HEK293 hSERT

Serotonin (5-HT) uptake was performed in HEK293 cells over expressing the human serotonin transporter SERT (hSERT). The cell line was kindly provided by the Blakely lab. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% foetal calf serum (FCS), 100U/ml penicillin, 100 µg/ml Streptomycin and for the selection 250 µg/ml geneticine G418.

For the uptake assay the protocol from Deecher et al. with some modifications was followed. 15,000 cells/well were plated in 96 well plates (Corning 3610) containing complete medium. Before the plating of the cells, wells were precoated with poly D-lysine for 2 hours. After 48 hours incubation of the cells in a cell incubator (37ºC, 5% CO₂), the medium was removed and substituted with 150µl of assay buffer (25mM HEPES, 120mM NaCl, 5mM KCl, 2.5mM CaCl₂, 1.2mM MgSO₄, 2mg/ml glucose, pH 7.4) added with 1µM pargyline. Cells were incubated with different compound concentrations in presence of 15nM [H³]-5HT (Hydoxytryptamine creatinine sulphate, 5-[1,2-³H(N)-serotonin, NET498, PerkinElmer). Compounds were diluted in DMSO with a final concentration of 0.5% in the assay.

After 30 minutes incubation with [H³]-5HT the free radioligand was removed with two washing steps using 200µl of assay buffer. Cells were than lysed with 25µl NaOH 0.25N and shaked for 5 min. Finally 75µl of scintillation cocktail was added and plate was incubated and shaken for 30 minutes. Radioactivity was counted using a Wallac Microbeta counter (PerkinElmer). The uptake assays were performed in triplicates in the plate format. The curves were analysed using SigmaPlot11. Data was fitted to a four parameter logistic curve to deduce the IC₅₀ values.

Acknowledgments

We thank Dr. Blakely for providing the HEK293 cells over expressing the human serotonin transporter SERT (hSERT). We are indebted to Mrs. E. Weyher and Dr. S. Uebel (MPI of Biochemistry) and to Mrs. C. Dubler (Ludwig-Maximilians-Universität Munich) for HRMS and NMR measurements.

Author contributions

R.G and F.H. designed the molecules. R.G. synthesized the molecules. S.C. and R.G. tested compounds. R.G. and F.H. wrote the manuscript.
References:


2.3 Discussion

In this manuscript photoactivable tricyclic antidepressants analogs were developed which can be used as tools to study the targets and mechanism of clinically used antidepressants.

These tool compounds were designed starting from clinically used antidepressants. In this strategy, in the first step the chloride or the cyano group at position 3 of Clomipramine or Cyanopramine was substituted by an azide group to generate Azidopramine (9). The corresponding aryl azide moiety was further shown to be activated by UV light, thereby generating a highly photoreactive analog, which can in principle covalently cross-link to binding partners. Azidopramine (9) was further tested in the established uptake assay and was shown to retain the high \textit{in vitro} affinity for the hSERT. This indicated that the azido substitution is well tolerated at this position. Subsequently, Azidobupramine (14) was generated as a second generation antidepressant analog, by substituting one methyl of the dimethyl amino group with a terminal alkyne to introduce a click handle. The introduction of Boc-protected 11 to azepine 8 required substantial optimization. The purification of product 13 from the educt was difficult and hence a modified synthesis protocol had to be established. Herein, the activated tosyl analog 12 was reacted with building block 8 under various conditions (Table-5).

<table>
<thead>
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<th>Base</th>
<th>Condition</th>
<th>Comment</th>
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<tr>
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<td>NaH</td>
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<td>No reaction, 8 repurifed and 12 degraded.</td>
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<tr>
<td>12</td>
<td>n-BuLi</td>
<td>-78°C, toluene</td>
<td>No reaction, 8 repurifed and 12 degraded.</td>
</tr>
<tr>
<td>12</td>
<td>NaHMDS</td>
<td>70 °C, THF</td>
<td>No reaction, 8 repurifed and 12 degraded.</td>
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<tr>
<td>12</td>
<td>NaHMDS</td>
<td>-78 to70 °C, toluene</td>
<td>Product obtained</td>
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</tbody>
</table>

Azidobupramine (16) had moderate binding ability \textit{in vitro} for the hSERT. Nevertheless, the terminal alkyne in 16 provides a handle for clicking the transporter ligand complex with biotin for enrichment and protein identification or with a fluorophore for imaging in cells. In addition this compound can also be used in fine mapping of the binding site of antidepressants in their transporters. Finally, the precursor Des-azidopramine (14) can also be used to synthesize [$^3$H]Azidopramine which can be used as a photoreactive radiotracer.
3. **Summary**

The present study employs different medicinal chemistry approaches to rationally design compounds that can be used as tools to dissect and understand the functions of specific protein targets implicated in depression.

In the first part of the thesis three different approaches were pursued to identify compounds binding to FKBP51 and FKBP52. The compounds synthesized in all three approaches were designed to target the 80s loop. The 80s loop had been shown to have structural differences resulting in divergent functions. In the first approach the tert-pentyl group of the lead compound was substituted by a cyclohexyl group which mimics the pyranose moiety in natural products FK506 and Rapamycin. Here the effect of the stereochemistry at C\textsuperscript{10} and C\textsuperscript{11} on binding affinity was studied in detail, followed by X-ray co-crystal analysis. The study revealed that the diverging 80s loop is flexible enough to accommodate various stereochemical motifs with different binding modes. In the second approach a focused library of sulfonamides was synthesized. The sulfonamides were hypothesized to be bio-isosters for the α-ketoamide motif. Medium throughput library synthesis and screening identified two potential lead compounds. These lead series was further optimized to draw a conclusive SAR. The study led to the identification of two highly potent compounds for FKBP12, FKBP51 and FKBP52. In the third methodology the best lower parts from the above two methodologies was further amalgamated with bicyclic/polycyclic rigid scaffold. The bicyclic scaffolds turned out to be more efficient than the polycyclic counterparts. This study for the first time has generated and identified putative ligands that bind to FKBP51 and FKBP52 with submicromolar affinity. Our data suggest that further medicinal chemistry and rational optimization of these leads can lead to potent selective ligands for FKBP51 and FKBP52.

In the second part of the thesis we aimed to synthesize compounds which can help to understand the function and mechanism of classical antidepressants. The chemical tools thus synthesized had an azide and an alkyne group. The azide group was incorporated for photocrosslinking while the alkyne group would be used for affinity purification followed by identification or for imaging. The designed tool compounds were shown to retain the biological property of the clinical antidepressants in an uptake assay. Further these analogs were shown to be photoreactive. The initial data suggested that these compounds can be effectively used as tools for chemo-proteomic approaches as well as for fine mapping of the binding sites in interacting proteins.
### 4. Materials

#### 4.1 Solvents, reagents and salts

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<th>Compound name</th>
<th>CAS No.</th>
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<th>Product code</th>
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## 4.2 Chemicals

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<th>Product code</th>
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<td>Maybridge</td>
<td>CC62303</td>
<td>97%</td>
</tr>
<tr>
<td>1-methyl-1H-indole-7-sulfonyl chloride</td>
<td></td>
<td>Maybridge</td>
<td>CC66903</td>
<td>97%</td>
</tr>
<tr>
<td>4-fluorobenzenesulfonyl chloride</td>
<td></td>
<td>Maybridge</td>
<td>DSHS00791</td>
<td></td>
</tr>
<tr>
<td>1-2-dimethyl-1H-imidazole-4-sulfonyl chloride</td>
<td></td>
<td>Maybridge</td>
<td>KM10104</td>
<td>95%</td>
</tr>
<tr>
<td>3-(5-methyl-1,3,4-oxadiazol-2-yl)benzenesulfonyl chloride</td>
<td></td>
<td>Maybridge</td>
<td>MO00158</td>
<td>Tech</td>
</tr>
<tr>
<td>(4-chlorophenyl)methanesulfonyl chloride</td>
<td></td>
<td>Maybridge</td>
<td>MO00927</td>
<td>90%</td>
</tr>
<tr>
<td>4-chloro-3-(trifluoromethyl)benzenesulfonyl chloride</td>
<td></td>
<td>Maybridge</td>
<td>MO07002</td>
<td>97%</td>
</tr>
<tr>
<td>Compound name</td>
<td>CAS No.</td>
<td>Company</td>
<td>Product code</td>
<td>Purity</td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>-----------------</td>
<td>--------------</td>
<td>--------------</td>
<td>--------</td>
</tr>
<tr>
<td>4-phenoxybenzenesulfonyl chloride</td>
<td></td>
<td>Maybridge</td>
<td>MO07030</td>
<td>97%</td>
</tr>
<tr>
<td>2-propanesulfonyl chloride</td>
<td></td>
<td>Maybridge</td>
<td>MO08485</td>
<td></td>
</tr>
<tr>
<td>1-ethanesulfonyl chloride</td>
<td></td>
<td>Maybridge</td>
<td>MO08486</td>
<td></td>
</tr>
<tr>
<td>4-chlorobenzene-1-sulfonyl chloride</td>
<td></td>
<td>Maybridge</td>
<td>SB00912</td>
<td>97%</td>
</tr>
<tr>
<td>4-(trifluoromethyl)benzene-1-sulfonyl chloride</td>
<td></td>
<td>Maybridge</td>
<td>TL00175</td>
<td>97%</td>
</tr>
<tr>
<td>3,4-dichlorobenzene-1-sulfonyl chloride</td>
<td></td>
<td>Maybridge</td>
<td>TL00303</td>
<td>90%</td>
</tr>
<tr>
<td>4-((tert-butyl)benzene-1-sulfonyl chloride</td>
<td></td>
<td>Maybridge</td>
<td>TL00417</td>
<td></td>
</tr>
<tr>
<td>4-methoxybenzene-1-sulfonyl chloride</td>
<td></td>
<td>Maybridge</td>
<td>TL00513</td>
<td>97%</td>
</tr>
<tr>
<td>3,4 dimethoxybenzosulfonyl chloride</td>
<td></td>
<td>Aldrich</td>
<td>452467-1G</td>
<td>98%</td>
</tr>
<tr>
<td>3,4 dimethoxyphenylethyl bromide</td>
<td>40173-90-8</td>
<td>Aldrich</td>
<td>653675-5G</td>
<td>97%</td>
</tr>
<tr>
<td>4-Nitrobenzenesulfonyl chloride</td>
<td>1694-92-4</td>
<td>Aldrich</td>
<td>N1,150-7</td>
<td>97%</td>
</tr>
<tr>
<td>Ethyl pipecolinate</td>
<td>15862-72-3</td>
<td>Aldrich</td>
<td>198803-5G</td>
<td>98%</td>
</tr>
<tr>
<td>3-Nitrobenzenesulfonyl chloride</td>
<td>121-51-7</td>
<td>Aldrich</td>
<td>254665-5G</td>
<td>97%</td>
</tr>
<tr>
<td>3,5-Dichlorobenzesulfonyl chloride</td>
<td>705-21-5</td>
<td>Aldrich</td>
<td>546933-5G</td>
<td>97%</td>
</tr>
<tr>
<td>3-Cyanobenzenesulfonyl chloride</td>
<td>56542-67-7</td>
<td>Aldrich</td>
<td>638358-1G</td>
<td>97%</td>
</tr>
<tr>
<td>3-(3,4-Dimethoxyphenyl)-1-propanol</td>
<td>3929-47-3</td>
<td>Aldrich</td>
<td>197688-5G</td>
<td>99%</td>
</tr>
<tr>
<td>Boc-Pip-OH</td>
<td>26250-84-0</td>
<td>Aldrich</td>
<td>516368-5G</td>
<td>98%</td>
</tr>
<tr>
<td>2,2-Dimethylbutyric acid</td>
<td>595-37-9</td>
<td>Aldrich</td>
<td>D15,260-9</td>
<td>96%</td>
</tr>
<tr>
<td>Cyclohexanone</td>
<td>108-94-1</td>
<td>Aldrich</td>
<td>398241-500ml</td>
<td>99%</td>
</tr>
<tr>
<td>Phenylmethanesulfonyl chloride</td>
<td>1939-99-7</td>
<td>Aldrich</td>
<td>159719-5G</td>
<td>98%</td>
</tr>
<tr>
<td>Compound name</td>
<td>CAS No.</td>
<td>Company</td>
<td>Product code</td>
<td>Purity</td>
</tr>
<tr>
<td>------------------------------------------------------------------------------</td>
<td>---------</td>
<td>-------------</td>
<td>---------------</td>
<td>--------------</td>
</tr>
<tr>
<td>Benzoic acid A.C.S. reagent</td>
<td>65-85-0</td>
<td>Aldrich</td>
<td>242381-25G</td>
<td>≥99.5%</td>
</tr>
<tr>
<td>3-Pyridinepropanol</td>
<td>2859-67-8</td>
<td>Aldrich</td>
<td>P7120-7</td>
<td>98%</td>
</tr>
<tr>
<td>3’-Hydroxyacetophenone</td>
<td>121-71-1</td>
<td>Aldrich</td>
<td>328103-25G</td>
<td>≥99%</td>
</tr>
<tr>
<td>(S)-(--) N-Boc carbonyl-2-piperidinecarboxylic acid</td>
<td>26250-84-0</td>
<td>Aldrich</td>
<td>516368-5G</td>
<td>98% ee</td>
</tr>
<tr>
<td>Pipercolinic acid</td>
<td>535-75-1</td>
<td>Aldrich</td>
<td>P4,585-0</td>
<td>98%</td>
</tr>
<tr>
<td>Cyclohexene</td>
<td>110-83-8</td>
<td>Fluka</td>
<td>29230-100ml</td>
<td>≥99.5%</td>
</tr>
<tr>
<td>tert-Butyl bromoacetate</td>
<td>5292-43-3</td>
<td>Fluka</td>
<td>17035-50ml</td>
<td>≥97%</td>
</tr>
<tr>
<td>2-Methylcyclohexanone</td>
<td>583-60-8</td>
<td>Fluka</td>
<td>66380</td>
<td>≥98%</td>
</tr>
<tr>
<td>(S)-Pyrrolidine-2-carboxylic acid</td>
<td>147-85-3</td>
<td>Fluka</td>
<td>81710-10G</td>
<td>≥99%</td>
</tr>
<tr>
<td>L-Pipercolinic acid</td>
<td>3105-95-1</td>
<td>Alfa Aesar</td>
<td>L15373</td>
<td>99%</td>
</tr>
<tr>
<td>3-bromo,5-(trifluoromethyl) benzenesulfonyl chloride</td>
<td>351003-46-8</td>
<td>ABCR</td>
<td>AB180851</td>
<td>97%</td>
</tr>
<tr>
<td>2- Ethyl cyclohexanone</td>
<td>4423-94-3</td>
<td>ABCR</td>
<td>AB126350</td>
<td>99%</td>
</tr>
<tr>
<td>3-5 Bis(trifluoromethyl) benzenesulfonyl chloride</td>
<td>39234-86-1</td>
<td>ABCR</td>
<td>AB103447</td>
<td>97%</td>
</tr>
<tr>
<td>4-Nitrobenzenesulfonyl chloride</td>
<td>98-74-8</td>
<td>ABCR</td>
<td>AB118187</td>
<td>98%</td>
</tr>
<tr>
<td>3-Bromobenzenesulfonyl chloride</td>
<td>2905-24-0</td>
<td>ABCR</td>
<td>AB114107</td>
<td>97%</td>
</tr>
<tr>
<td>3,5-Dichloro-4-hydroxybenzenesulfonyl chloride</td>
<td>13432-81-0</td>
<td>ABCR</td>
<td>AB181058</td>
<td>97%</td>
</tr>
<tr>
<td>3-Chloro-4methoxybenzenesulfonyl chloride</td>
<td>22952-43-8</td>
<td>ABCR</td>
<td>AB267265</td>
<td>95%</td>
</tr>
<tr>
<td>3-Fluorobenzene-10sulfonyl chloride</td>
<td>701-27-9</td>
<td>ABCR</td>
<td>AB226807</td>
<td>97%</td>
</tr>
<tr>
<td>3,5-Difluorobenzenesulfonyl chloride</td>
<td>210532-25-5</td>
<td>ABCR</td>
<td>AB173895</td>
<td>97%</td>
</tr>
<tr>
<td>Trimethylsilyl acetylene</td>
<td>1066-54-2</td>
<td>ABCR</td>
<td>AB102117</td>
<td>98%</td>
</tr>
<tr>
<td>3-Dimethylamino-1-propanol</td>
<td>3179-63-3</td>
<td>ABCR</td>
<td>AB116149</td>
<td>99%</td>
</tr>
<tr>
<td>3-(Methylamino)-1-propanol</td>
<td>TCI</td>
<td>M1484</td>
<td>&gt;97%</td>
<td></td>
</tr>
<tr>
<td>Compound name</td>
<td>CAS No.</td>
<td>Company</td>
<td>Product code</td>
<td>Purity</td>
</tr>
<tr>
<td>------------------------------------------------------------------------------</td>
<td>---------</td>
<td>-----------------</td>
<td>--------------</td>
<td>--------</td>
</tr>
<tr>
<td>N-Methyl-3-chloropropylamine Hydrochloride</td>
<td></td>
<td>TCI</td>
<td>M1048</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>2-Chlorotrityl chloride resin (100-200 mesh)</td>
<td></td>
<td>Novabiochem</td>
<td>01-64-0114</td>
<td></td>
</tr>
<tr>
<td>5-Acetyl-10,11-dihydro-5H-dibenz[b,f]azepin-3-amine</td>
<td></td>
<td>Wako</td>
<td>326-38523</td>
<td></td>
</tr>
<tr>
<td>4-hydroxy-3,5-diisopropylbenzenesulfonyl chloride</td>
<td></td>
<td>ChemCollect</td>
<td>SV000244</td>
<td></td>
</tr>
<tr>
<td>4-hydroxy-3, methoxy-benzenesulfonyl chloride</td>
<td></td>
<td>ChemCollect</td>
<td>SV000258</td>
<td></td>
</tr>
<tr>
<td>5-(chlorosulfonyl)-2,3-dimethoxybenzoic acid</td>
<td></td>
<td>AKOS</td>
<td>AKOS000131666</td>
<td></td>
</tr>
<tr>
<td>5-(Chlorosulfonyl)-isophthalic acid dimethyl ester</td>
<td></td>
<td>AKOS</td>
<td>AKOS001074083</td>
<td></td>
</tr>
<tr>
<td>4-Aacetamido-3,5-dichlorobenzene-1-sulfonyl chloride</td>
<td></td>
<td>AKOS</td>
<td>AKOS000153961</td>
<td></td>
</tr>
<tr>
<td>2-Methylbenzo[d]thiazole-6-sulfonyl chloride</td>
<td></td>
<td>AKOS</td>
<td>AKOS000301981</td>
<td></td>
</tr>
<tr>
<td>2-Oxo-2,3-dihydrobenzo[d]thiazole-6-sulfonyl chloride</td>
<td></td>
<td>AKOS</td>
<td>AKOS000302227</td>
<td></td>
</tr>
<tr>
<td>7-nitro-2,3-dihydro-1-benzofuran-5-sulfonyl chloride</td>
<td></td>
<td>AKOS</td>
<td>AKOS005072576</td>
<td></td>
</tr>
<tr>
<td>2,6-dimethylmorpholine-4-sulfonyl chloride</td>
<td></td>
<td>AKOS</td>
<td>AKOS000321499</td>
<td></td>
</tr>
<tr>
<td>4-bromo-1-butyne</td>
<td>38771-21-0</td>
<td>Aldrich</td>
<td>675725-5G</td>
<td>98%</td>
</tr>
</tbody>
</table>
5. **Personal Future Outlook.**

Interdisciplinary research is very important and crucial for the advancement of available medical therapies. Medicinal Chemistry and Chemical Biology are two disciplines which exemplify the symbiosis between chemistry and biology which can bring about a better understanding of disease and help in paving new drug therapies for the future. Drug research and development is a resource and money thirsty campaign normally taken by big pharmaceutical companies. Academic involvement in such projects is limited and rare.

During my PhD study, I have been lucky to be involved in an academic oriented drug discovery program either as a main driver or as a collaborator. Close collaboration and interactions with clinicians, researchers, pharmacist and leaders in the field has helped me to troubleshoot the problems and grow in various aspects of drug development during my PhD studies. This experience has helped me to learn and grow in the field of lead identification and optimization and has given me a wide and through experience in medicinal chemistry, high throughput screening and target identification platforms.

Rational drug design, structure based drug design campaigns and chemical biology technologies clearly suggest that chemical probes will be a routine armory on the table of biologists. With parallel improvements in designing chemical probes (synthetic chemistry, molecular modeling) and technology platforms (mass spectrometers, DNA sequencing), I believe that personalized medicine therapies is just around the corner.

Spending 4.5 years in a closely knit interdisciplinary environment (research and clinical setting), I believe my experience at the Max Planck Institute of Psychiatry will help me in my future endeavors. In the near future I look forward to make progress and contributions to tackle unanswered biological questions by designing versatile and potent chemical probes which can be applied in clinical and biological settings.
6. Curriculum Vitae

Ranganath Gopalakrishnan

Personal Information
Date of Birth: 5 January 1984
Place of Birth: Ahmedabad, Gujarat
Gender: Male
Family status: Unmarried
Nationality: Indian

Academic Qualification
2007-to present Ph.D. student at Hausch Lab,
Max Planck Institute of Psychiatry Munich, Germany
2005-2007 Master of Science (M. S.) Pharm Medicinal Chemistry,
National Institute of Pharmaceutical Edu. & Res. (NIPER), India
2001-2005 Bachelor of Pharmacy (B. Pharm),
K.B. Institute of Pharmaceutical Edu. & Res. Gujarat Univ. India

Patents Filed.

Talks
1. Ligands for FKBP51 and FKBP52. MPI Psychiatry Ringberg symposium 2011.

Publications and Manuscripts


**Poster presentations**


3. **Are the antidepressants as selective as originally thought?**, Institute Symposium 2011, Kirmeier T, Ganal V. Gopalakrishnan R, Werner AM, Maccarrone G, Schmidt MV, Henes K, Wotjak CT, Müller M, Turck CW, Hausch F, Holsboer F, Rein T.

7. References


(33) Gopalakrishnan, R.; Hausch, F. Evaluation of Synthetic FK506 Analogs as Ligands for FKBP51 and FKBP52. *To be Submitted*.


(146) Andersen, J.; Kristensen, A. S.; Bang-Andersen, B.; Stromgaard, K. Recent advances in the understanding of the interaction of antidepressant drugs with serotonin and norepinephrine transporters. *Chem Commun (Camb)* **2009**, *3677*-3692.