Screening of Small Molecule Interactor Library by Using In-Cell NMR Spectroscopy (SMILI-NMR)

Jingjing Xie, Rajiv Thapa, Sergey Reverdatto, David S. Burz, and Alexander Shekhtman*

State University of New York at Albany, Department of Chemistry, 1400 Washington Avenue, Albany, New York 12222

Received January 19, 2009

We developed an in-cell NMR assay for screening small molecule interactor libraries (SMILI-NMR) for compounds capable of disrupting or enhancing specific interactions between two or more components of a biomolecular complex. The method relies on the formation of a well-defined biocomplex and utilizes in-cell NMR spectroscopy to identify the molecular surfaces involved in the interaction at atomic scale resolution. Changes in the interaction surface caused by a small molecule interfering with complex formation are used as a read-out of the assay. The in-cell nature of the experimental protocol insures that the small molecule is capable of penetrating the cell membrane and specifically engaging the target molecule(s). Utility of the method was demonstrated by screening a small dipeptide library against the FKBP−FRB protein complex involved in cell cycle arrest. The dipeptide identified by SMILI-NMR showed biological activity in a functional assay in yeast.

Introduction

Biomolecular complexes present attractive targets to control cellular processes in disease states by using small drug-like molecules as therapeutic agents. One proven approach to the drug discovery process entails high throughput screening (HTS)[a] of compound libraries. However, finding inhibitors or enhancers of complex formation proved to be difficult for HTS.[1,2] In vitro biochemical assays used in HTS may fail to consider physiologically relevant conformations of the complexes, while in vivo assays can be impeded by the difficulty in distinguishing small molecules that directly affect the interactions of interest from those that exert their influence through other cellular components. NMR-based in vitro screening, utilized in drug discovery programs,[3−7] has become a powerful method for identifying and analyzing low molecular weight compounds that specifically bind to biomolecular targets.

We developed a robust NMR spectroscopy-based screening procedure, screening of small molecule interactor library by using in-cell NMR (SMILI-NMR), to rapidly screen small molecule libraries for compounds capable of either strengthening or weakening protein−protein interactions within a biomolecular complex. SMILI-NMR is uniquely positioned between HTS techniques capable of screening hundreds to thousands of protein complexes a day and atomic resolution structural methods for studying protein−ligand complexes that require significantly longer periods of time.

SMILI-NMR utilizes recently developed structural interactions by using in-cell NMR (STINT-NMR) technology,[8−10] to produce biomolecular complexes inside the cell in which one of the constituent proteins is uniformly [*U*-, ^{15}N] labeled with NMR-active nuclei. By monitoring the in-cell NMR spectrum of the labeled protein, we can directly observe the formation of high-affinity ternary complexes or changes in structure induced by the binding of a small drug-like molecule that disrupts or enhances the stability of the complex. In this way, STINT-NMR serves as a direct assay for protein−drug interactions, facilitating high throughput screening. SMILI-NMR technology offers unique advantages for the screening of therapeutic agents against chosen disease-related protein targets. We can selectively screen for drug-like molecules that block the interaction surface that is critical for the normal functioning of the biocomplex. This greatly reduces the likelihood of compensatory mutations or suppressor gene products arising that may restore the function of the drug-compromised biocomplex.

We used a well-studied system of two interacting proteins, FKBP and FRB, as a model system to demonstrate the efficacy of SMILI-NMR in the screening of small molecules that can facilitate heterodimerization. The FKBP−FRB interaction constitutes one of the immunomodulatory systems in mammalian cells.[11] FKBP, fujimycin (FK-506)[12] binding protein, is a 12 kDa cytoplasmic protein that functions as a folding chaperone for proteins containing proline residues. FKBP belongs to the immunophilin family and has peptidyl−prolyl *cis*−*trans* isomerase (PPIase) activity, promoting *cis*−*trans* isomerization, the slow step in the process of protein folding.[11] FKBP is highly conserved from yeast through humans and is relatively abundant in most tissues and cell types.[13] In the down-regulation of T-cell activation, FKBP is notable as an inhibitor of cell cycle progression and as the initial intracellular target for the natural product immunosuppressive drugs fujimycin and rapamycin.[14]

In complex with rapamycin, FKBP binds to FRB, the 100 amino acid FKBP−rapamycin binding domain of the mammalian target of rapamycin (mTOR),[15,16] inhibiting the ability of this kinase to mediate mitogenic responses.[17] mTOR functions as a sensor of cellular nutrient and energy levels and redox status.[18] Misregulation of the mTOR pathway is implicated as a contributing factor to various human diseases, especially various types of cancer.[19] The key factor, rapamycin, is currently undergoing clinical trials for a variety of cancer treatments.[20,21]

* To whom correspondence should be addressed. Phone: (518) 442-4163. Fax: (518) 442-3462. E-mail: ashekhta@albany.edu.

[a] Abbreviations: HTS, high throughput screening; SMILI-NMR, screening of small molecule interactor library by using in-cell NMR; STINT-NMR, structural interactions by using in-cell NMR spectroscopy; FKBP, fujimycin binding protein; FRB, FKBP rapamycin binding protein; mTOR, mammalian target of rapamycin; PPIase, peptidyl−prolyl *cis*−*trans* isomerase; ^{1}H{^{15}N}−HSQC, ^{15}N edited heteronuclear single quantum coherence, [*U*-, ^{15}N]−, uniformly ^{15}N labeled protein; IPTG, isopropyl *β*-D-1-thiogalactopyranoside; YPD, yeast, peptone, dextrose; OD_{600}, cell optical density at 600 nm.

10.1021/jm9000743 CCC: $40.75
Published on Web 05/07/2009

Thus, screening of small molecules that function similarly to rapamycin in vivo becomes a very significant and interesting undertaking.

In this work we show that: (1) the formation of a biocomplex is required to generate a high resolution NMR spectrum to study interacting molecules; (2) small molecule binding alters the interaction surfaces involved in biocomplex formation; (3) we can rapidly screen a library of small molecules to select compounds that alter the interactions between the components of the biocomplex and observe the change in the structure of the biocomplex; (4) the functional ramifications of the binding by the small molecule identified by this selection process can be measured using a biological functional assay.

Results

1. The Formation of a Biocomplex Was Required to Obtain an NMR Spectrum of FKBP. To demonstrate the efficacy of SMILI-NMR, we first set up the in-cell model system by sequentially overexpressing [*U*-, ^{15}N]−FKBP and unlabeled FRB in *E. coli* using two compatible plasmids, pRSF−FKBP and pBAD−FRB, and recorded the in-cell ^{15}N-edited heteronuclear single quantum coherence (^{1}H{^{15}N}−HSQC) NMR spectrum (Supporting Information Figures 1 and 2). When only [*U*-, ^{15}N]-FKBP was overexpressed, the in-cell NMR spectrum showed no well-resolved peaks, implying that the single species is part of a large complex, e.g., possibly membrane-bound and therefore invisible to NMR.[22−24] When unlabeled FRB was then sequentially overexpressed in the same cells the ^{1}H{^{15}N}−HSQC NMR spectrum of FKBP became evident but only at the highest FRB concentrations, indicating the formation of a complex (Figure 1A). Similar results were obtained when [*U*-,^{15}N]−FRB and unlabeled FKBP were sequentially overexpressed in the same cells (Supporting Information Figure 3). The in-cell NMR spectrum of [*U*-,^{15}N]−FRB contained a significant number of broadened peaks suggesting that FRB interacts nonspecifically with the cell interior.

To rule out the possibility that the NMR spectra of [*U*-, ^{15}N]−FKBP−FRB and [*U*-, ^{15}N]−FRB−FKBP were due to extracellular protein,[23,25] after obtaining the in-cell NMR spectrum, the cells were centrifuged and the supernatant was examined. No NMR spectra of either [*U*-, ^{15}N]−FKBP−FRB or [*U*-, ^{15}N]−FRB−FKBP was observed above noise level (Supporting Information Figure 4), implying that there is no leakage or cell lysis occurring during the time it takes to acquire the NMR spectrum.

Banaszynski et al.[26] indicated that any interaction between free FKBP and FRB is weak ($K_d > 50$ μM). However, within the cell, overexpressed proteins can reach concentrations exceeding 100 μM, which is sufficiently high to sustain weak interactions.[10,26] In fact, both FKBP and FRB were highly overexpressed (Supporting Information Figure 2). Furthermore, macromolecular crowding may play a role in stabilizing weak interactions inside the cell.[23,24] These observations support the notion that creating a proper protein complex is necessary for high-resolution studies.

2. Small Molecule Binding Alters the Interaction Surfaces Involved in Biocomplex Formation. Once the in-cell system was established, we titrated cells containing sequentially overexpressed [*U*-,^{15}N]−FKBP and unlabeled FRB with the immunosuppressant drug rapamycin to trigger the formation of the ternary complex FKBP−rapamycin−FRB. Rapamycin is a macrolide antifungal antibiotic first isolated from *Streptomyces hygroscopicus* in 1975.[14] Rapamycin binds to FKBP with 200 pM affinity[27,28] and to FRB with 26 μM affinity.[26] While FKBP and FRB interact with each other only weakly,[26] the FKBP−rapamycin complex binds to FRB with 12 nM affinity[26] to form a well-characterized high-affinity ternary complex.[29] In this complex, rapamycin acts mostly as a linker: The crystal structure of FKBP−rapamycin−FRB revealed that there is only a limited FKBP−FRB interaction surface[29] (Figure 2A).

Adding rapamycin to the cell suspension resulted in visible changes in the ^{1}H{^{15}N}−HSQC spectrum of [*U*-,^{15}N]−FKBP (Figure 1B). These changes involved 32 out of the 107 residues in FKBP. Chemical shift changes obtained from this in-cell titration experiment were mapped onto the three-dimensional structure of FKBP (Figure 2A). Adding rapamycin to cells overexpressing [*U*-,^{15}N]−FRB and unlabeled FKBP also resulted in changes in the in-cell NMR spectrum (Supporting Information Figure 4). These results indicated the formation of a high-affinity ternary complex between FKBP−rapamycin and FRB.

Ascomycin[30] is a macrolide complex with immunosuppressant activity and is an ethyl analogue of the known immune suppressant fujimycin.[30] Ascomycin acts by binding to immunophilins, especially FKBP. The immunophilin−ascomycin complex, in turn, inhibits calcineurin, a type 2B phosphatase. Ascomycin acts as a competitive inhibitor of rapamycin, binding to FKBP with 1.4 nM affinity, but does not bind to FRB.[31,32] Furthermore, although ascomycin binding to FKBP is comparable to that of rapamycin, the ascomycin−FKBP complex binds to FRB with considerably lower affinity.[31,32]

Adding ascomycin to the cell suspension also resulted in visible changes to the in-cell NMR spectrum of [*U*-,^{15}N]−FKBP (Figure 1C). These changes involved 38 of the 107 residues in FKBP, 29 of which were similarly perturbed upon rapamycin binding (Figures 1C, 2B). However, the ascomycin binding surface on FKBP involved an additional 9 residues, resulting in an expanded surface area on FKBP relative to that of rapamycin alone (Figure 2B). STINT-NMR analysis revealed an FKBP interaction surface characteristic of a high affinity complex between FKBP and ascomycin.[32] Adding ascomycin to the cells overexpressing [*U*-,^{15}N]−FRB and unlabeled FKBP did not result in changes of the in-cell NMR spectrum (not shown).

Ascomycin was reported to attenuate heterodimerizaton in the FKBP−FRB system.[31] We used ascomycin to compete rapamycin away from FKBP in cells containing sequentially overexpressed [*U*-,^{15}N]−FKBP and unlabeled FRB. We observed the in-cell ^{1}H{^{15}N}−HSQC spectrum of [*U*-,^{15}N]−FKBP as ascomycin was titrated into a cell suspension containing 100 μM rapamycin. As the concentration of ascomycin increased, rapamycin was partially competed out as evidenced by the gradual appearance of FKBP−ascomycin peaks and the disappearance of the FKBP−rapamycin peaks. When the concentration of ascomycin reached 200 μM, the competition experiments resulted in the complete recovery of an FKBP−ascomycin spectrum (not shown).

Adding rapamycin to cells overexpressing labeled FKBP in the absence of FRB or labeled FRB in the absence of FKBP did not produce an NMR spectrum. In each case, coexpression of the second protein was required to generate an in-cell NMR spectrum. Thus, the binding of rapamycin or ascomycin to FKBP resulted in specific, high affinity biocomplexes with characteristic NMR spectra. STINT-NMR analysis of the binding revealed the biologically relevant, functional interaction surface on FKBP. In this way, STINT-NMR can be used to screen for small drug-like molecules that bind to this surface, thereby preventing FKBP from engaging in its normal activities.

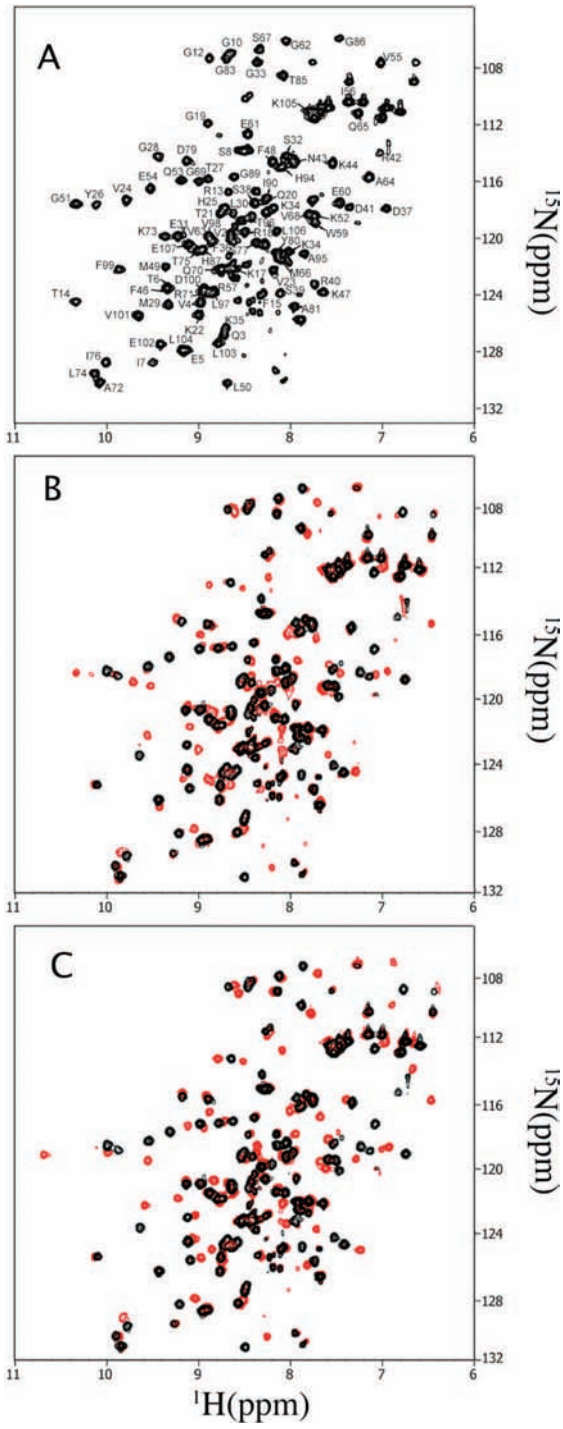


Figure 1. In-cell NMR spectra of the ternary [*U*-, ^{15}N]−FKBP−FRB−rapamycin and [*U*-, ^{15}N]−FKBP−FRB−ascomycin complexes. (A) Backbone assignment of FKBP after 4 h overexpression of [*U*-, ^{15}N]−FKBP and 4 h sequential overexpression of FRB. At typical intracellular concentrations, there is no interaction between FKBP and FRB,[26] however, within the cell, overexpressed proteins can reach concentrations levels exceeding 100 μM, which is sufficiently high to sustain weak interactions. Besides, macromolecular crowding may play a role in stabilizing weak interactions inside the cell.[23,24] We believe that the weak interactions form the biocomplex to generate an in-cell NMR spectrum of FKBP. (B) Overlay of ^{1}H{^{15}N}−HSQC spectra of *E. coli* after 4 h overexpression of [*U*-, ^{15}N]−FKBP and 4 h sequential overexpression of FRB in the absence (black) and presence (red) of 150 μM rapamycin. (C) Overlay of ^{1}H{^{15}N}−HSQC spectra of *E. coli* after 4 h overexpression of [*U*-, ^{15}N]−FKBP and 4 h sequential overexpression of FRB in the absence (black) and presence (red) of 150 μM ascomycin.

3. Screening of Dipeptide Library Identified Heterodimerizing Interactors. We chose to screen a dipeptide chemical library[33] composed of 17 × 17 dipeptides.[33,34] The library was chosen to provide a collection of compounds that are capable of interacting with the biological target of interest at a detectable level. The compounds must also provide suitable starting points for subsequent optimization into credible drug candidates.[35] Thus, the dipeptide library was considered useful as a potential source of novel lead structures because: first, dipeptides have been shown, by many groups,[33,36] to be an excellent starting point for drug design because they can easily be prepared at relatively low cost; second, a library containing only 289 compounds can provide a data set that spans a broad spectrum of physicochemical properties, and no deconvolution is required to identify the lead structures;[35] and third, because bacterial and eukaryotic cells possesses a transport system to take up dipeptides, these small biologically relevant molecules can pass through the cell membrane and interact with the target protein directly in vivo.[37,38]

We used a standard screening procedure, which we called the matrix method, in which library compounds located in one row or one column of a matrix plate were mixed and tested (Figure 3A). Individual mixtures were examined for their ability to change the in-cell NMR spectrum of the FKBP−FRB biocomplex. Samples exhibiting similar spectral changes, located at the intersection of rows and columns, were used in the second round of screening to deconvolute and validate the initial findings. In this way, we can screen a matrix of 289 (17 × 17) compounds by examining 34 (17 + 17) samples (Figure 3A).

The 34 dipeptide mixtures, A-X, D-X, E-X, F-X, G-X, H-X, I-X, K-X, L-X, N-X, Q-X, R-X, S-X, T-X, V-X, W-X, Y-X, X-A, X-D, X-E, X-F, X-G, X-H, X-I, X-K, X-L, X-N, X-Q, X-R, X-S, X-T, X-V, X-W, and X-Y, were screened at 1 mM concentration in cells overexpressing [*U*-,^{15}N]−FKBP and unlabeled FRB. We expected to see changes in the in-cell NMR spectrum of the protein complex when at least one of the compounds in the mixture binds to the interaction surfaces involved in complex formation. Most of the dipeptide mixtures showed no interaction with the target protein FKBP, i.e., the ^{1}H{^{15}N}−HSQC did not change upon adding these mixtures. But the mixture of A-X showed a totally different change from the rapamycin induced ternary complex. Unlike the changes in chemical shifts of specific amino acid observed for the rapamycin induced ternary complex, formation of the dipeptide-induced complex resulted in extreme broadening and the disappearance of some peaks in the NMR spectrum at the highest concentration used (10 mM). We also found that the mixtures of D-X, T-X, L-X, X-E, X-I, X-A, and X-T resulted in similar broadening of the spectra, which usually indicates the formation of a large complex.

Thus, the dipeptides located at the intersection of rows, A-X, D-X, T-X, and L-X and columns X-E, X-I, X-A, and X-T were screened in a second round (Figure 3A). A-A, A-T, A-E, A-I, D-A, D-T, D-E, D-I, T-A, T-T, T-E, T-I, L-A, L-T, L-E, and L-I were titrated into cells individually. At a concentration of 10 mM, A-A, A-E, D-A, D-T, D-E, T-E, and L-E showed similar broadening as the mixtures. Only A-E showed the same interaction with FKBP at 5 mM (Figure 3B). Similar changes upon adding 5 mM A-E were observed in the in-cell NMR spectrum of the [*U*-,^{15}N]−FRB and unlabeled FKBP (not shown), suggesting that A-E facilitated heterooligomerization of FKBP and FRB.

We used competition experiments with rapamycin and ascomycin to confirm that A-E binds specifically to the

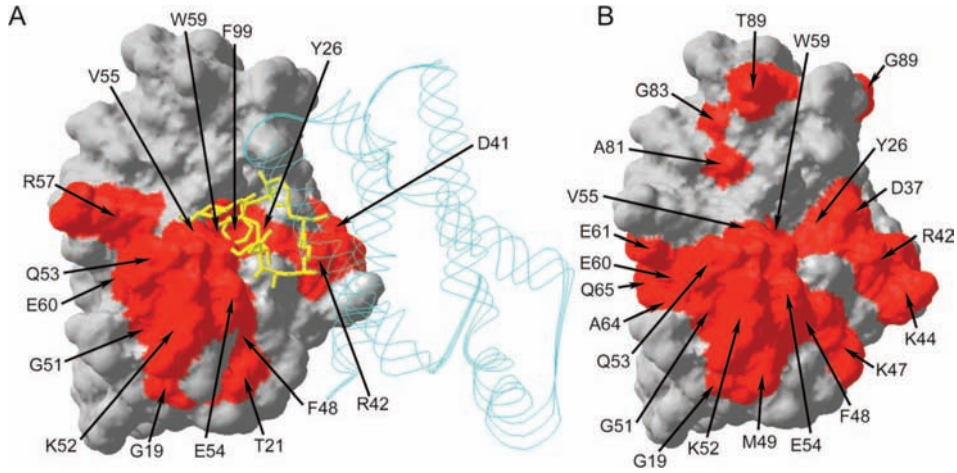
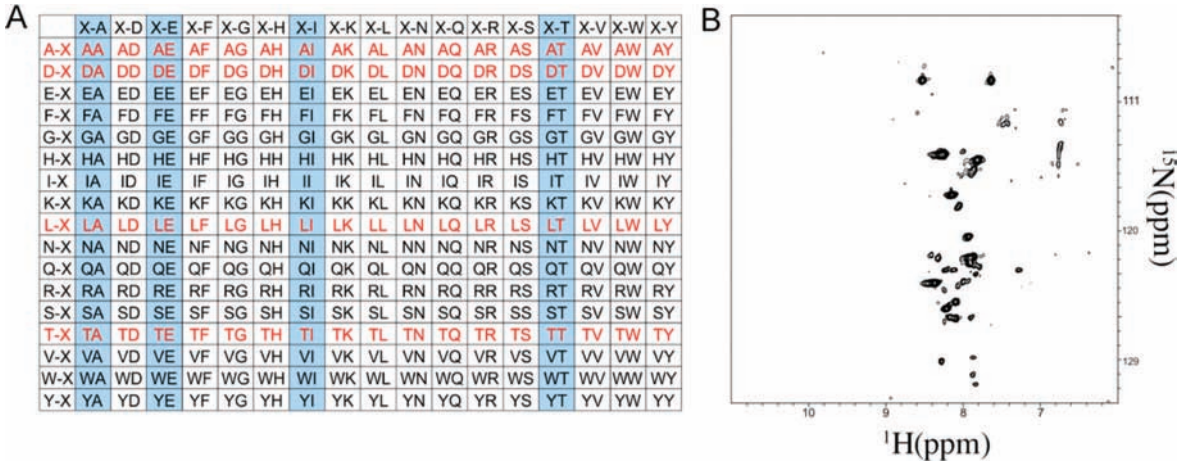


Figure 2. Interaction surfaces on FKBP. (A) Interaction surface mapped on the 3D structure of FKBP (PDB code 1FKR)[39] was based on chemical shift changes from the in-cell titration with rapamycin (yellow). The backbone structure of FRB was shown in blue. (B) Interaction surface mapped on the 3D structure of FKBP (PDB code 1FKR) was based on chemical shift changes from the in-cell titration with ascomycin.



	X-A	X-D	X-E	X-F	X-G	X-H	X-I	X-K	X-L	X-N	X-Q	X-R	X-S	X-T	X-V	X-W	X-Y
A-X	AA	AD	AE	AF	AG	AH	AI	AK	AL	AN	AQ	AR	AS	AT	AV	AW	AY
D-X	DA	DD	DE	DF	DG	DH	DI	DK	DL	DN	DQ	DR	DS	DT	DV	DW	DY
E-X	EA	ED	EE	EF	EG	EH	EI	EK	EL	EN	EQ	ER	ES	ET	EV	EW	EY
F-X	FA	FD	FE	FF	FG	FH	FI	FK	FL	FN	FQ	FR	FS	FT	FV	FW	FY
G-X	GA	GD	GE	GF	GG	GH	GI	GK	GL	GN	GQ	GR	GS	GT	GV	GW	GY
H-X	HA	HD	HE	HF	HG	HH	HI	HK	HL	HN	HQ	HR	HS	HT	HV	HW	HY
I-X	IA	ID	IE	IF	IG	IH	II	IK	IL	IN	IQ	IR	IS	IT	IV	IW	IY
K-X	KA	KD	KE	KF	KG	KH	KI	KK	KL	KN	KQ	KR	KS	KT	KV	KW	KY
L-X	LA	LD	LE	LF	LG	LH	LI	LK	LL	LN	LQ	LR	LS	LT	LV	LW	LY
N-X	NA	ND	NE	NF	NG	NH	NI	NK	NL	NN	NQ	NR	NS	NT	NV	NW	NY
Q-X	QA	QD	QE	QF	QG	QH	QI	QK	QL	QN	QQ	QR	QS	QT	QV	QW	QY
R-X	RA	RD	RE	RF	RG	RH	RI	RK	RL	RN	RQ	RR	RS	RT	RV	RW	RY
S-X	SA	SD	SE	SF	SG	SH	SI	SK	SL	SN	SQ	SR	SS	ST	SV	SW	SY
T-X	TA	TD	TE	TF	TG	TH	TI	TK	TL	TN	TQ	TR	TS	TT	TV	TW	TY
V-X	VA	VD	VE	VF	VG	VH	VI	VK	VL	VN	VQ	VR	VS	VT	VV	VW	VY
W-X	WA	WD	WE	WF	WG	WH	WI	WK	WL	WN	WQ	WR	WS	WT	WV	WW	WY
Y-X	YA	YD	YE	YF	YG	YH	YI	YK	YL	YN	YQ	YR	YS	YT	YV	YW	YY

Figure 3. Matrix method of screening chemical libraries. (A) A library containing 289 compounds is screened by examining individual mixtures located in the first row and first column of a matrix plate. Mixtures that result in similar changes in the in-cell NMR spectrum, located at the intersection of rows (red) and columns (blue), are used in the second round of screening. (B) ^{1}H{^{15}N}−HSQC spectrum of *E. coli* after 4 h overexpression of [*U*-, ^{15}N]-FKBP and 4 h sequential overexpression of FRB in the presence of 5 mM A-E.

FKBP−FRB complex. Adding 5 mM A-E and 150 μM ascomycin to cells overexpressing [*U*-,^{15}N]−FKBP and unlabeled FRB resulted in complete recovery of FKBP−ascomycin in-cell NMR spectrum, proving that ascomycin successfully competed A-E out (Figure 1C and Supporting Information Figure 5A,B). Unexpectedly, adding 5 mM A-E and 150 μM rapamycin to the cells did not result in recovery of high quality in-cell NMR spectrum characteristic of ternary FKBP−FRB−rapamycin complex (Figure 1B and Supporting Information Figure 5C,D). The in-cell NMR spectrum of [*U*-,^{15}N]−FKBP−FRB−rapamycin−A-E complex exhibited extreme broadening characteristic of the [*U*-,^{15}N]−FKBP−FRB−A-E spectrum (Figure 3B and Supporting Information Figure 5D). Because both rapamycin and ascomycin bind to a similar FKBP site,[39,40] it is unlikely that the ability of ascomycin to compete out A-E is simply due to steric occlusion. Thus, we concluded that A-E binds to a FKBP surface that is different from that of both rapamycin and ascomycin. Our studies showed that the interaction surface of ascomycin on FKBP was larger than that of rapamycin (Figure 2A,B). In addition, according to structural studies,[39,40] there were small but significant changes between the tertiary structures of FKBP−rapamycin and FKBP−ascomycin. We assumed that inability of rapamycin as compared to ascomycin to compete A-E out was due to this difference in binding mode.

4. Functional Assay in Yeast. To further confirm the effectiveness of SMILI-NMR, we examined the biological activity of A-E in yeast. Yeast and human FKBP share 54% identity and many conservative amino acid substitutions. Rapamycin is toxic to the yeast *Saccharomyces cerevisiae*, forming the FKBP−rapamycin−FRB complex, which inhibits progression through the G1 phase of the cell cycle.[41] The mutant lacking yeast FKBP (*fpr1*$^{-}$) is drug resistant and viable.[41]

The haploid yeast strains (*S. cerevisiae*) JK9-3dα [*FPR1*$^{+}$] (wild type), which produces FKBP, and JH3-3b [*fpr1*$^{-}$], which does not, were asssayed for growth.[41] Both strains were grown on YPD medium containing either 100 μM rapamycin or 5 mM A-E (Figure 4). In the absence of rapamycin or A-E, both strains grew normally. JH3-3b also grew in the presence of rapamycin and A-E due to the lack of FKBP in this strain, which precludes the formation of the toxic ternary biocomplex. JK9-3dα did not grow in the presence of rapamycin as expected[41] and grew only slightly in the presence of the dipeptide, indicating that the dipeptide induced the formation of a biocomplex similar to that induced by rapamycin, disturbing the progression of the cell cycle in this strain. The slight growth exhibited by JK9-3dα

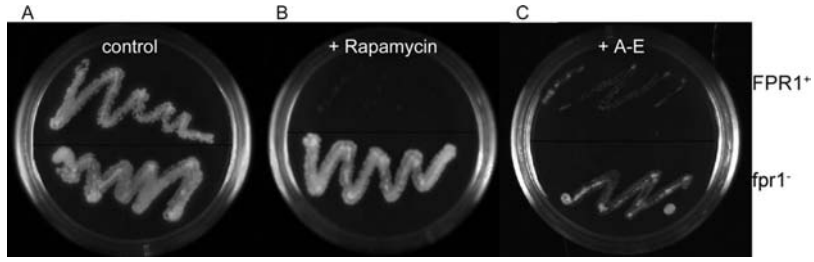


Figure 4. Yeast assay for biological activity of the dipeptide, A-E. Isogenic haploid yeast strains that express (*FPR1*$^+$) or lack (*frp1*$^-$) the FKBP proline isomerase were grown for 3 days on YPD medium. (A) Control plate. (B) 100 μM rapamycin. (C) 5 mM A-E.

likely reflects the weaker binding affinity of A-E for FKBP, relative to that of rapamycin. These results validate the finding that A-E exhibited biological activity comparable to that of rapamycin.

Discussion

SMILI-NMR presents a unique approach to small molecule screening due to the in-cell nature of the assay combined with high resolution NMR as a readout. SMILI-NMR is used to directly observe changes in biocomplex binding epitopes caused by small molecule interference at the level of individual amino acids. Our evaluation of SMILI-NMR for screening showed that in the first round of the assay, we could efficiently identify a mixture of compounds that bind to the selected biocomplex, FKBP−FRB, with millimolar affinities, from a small library of 289 dipeptides. In the second round, we deconvoluted the mixture into contributions from individual compounds, thus validating the findings. As a final step, we found that at least one of the dipeptides from the second round of screening, A-E, was active in the FKBP−FRB functional assay in yeast.[41] These results proved that SMILI-NMR can be used to screen small libraries of carefully selected compounds, such as those used for fragment-based screening.[6,42]

There are four critical issues that are important for evaluating drug screening methods: biochemical validity of the assay, assay speed, signal-to-noise ratio, and cost.[43,44] Arguably, the strongest appeal of SMILI-NMR is the biological relevance of the screening. Because observations are performed inside the cell, SMILI-NMR allows us to screen a library of compounds against biocomplexes under physiologically relevant conditions. By introducing posttranslational modifications, different conformational states of the biocomplex can be assessed.[45] Importantly, in-cell NMR is capable of detecting multiple conformations and is ideally suited to observe low affinity and transient complexes. SMILI-NMR can be especially valuable to screen against target biocomplexes of bacterial origin. The method may be applicable to proteins that are soluble in vivo but are difficult to purify; very specific nonbacterial protein-mediated interactions can be examined in a low background, and either protein can be expressed first and either or both proteins can be labeled using different strategies.[9]

The method is comparatively simple and fast. It has the advantages of STINT-NMR, which requires minimal sample preparation and eliminates the need for extensive protein purification. Assay speed is limited by the in-cell NMR acquisition time, which, depending on the protein expression level, can be up to an hour. To automate SMILI-NMR, robotic HTS accessories, such as liquid handlers and NMR tube changers, can be used together with modern NMR spectrometers. The atomic resolution nature of in-cell NMR increases the informational content of screening, allowing us to deconvolute mixtures of compounds used for screening. In the present work, we were able to successfully screen mixtures of 34 dipeptides. In general, the optimal number of mixture components will depend on the quality of the in-cell NMR spectrum of the biocomplex. By using the matrix method, we increased the testing rate of $M \times N$ samples by examining $M + N$ samples in the first round of screening. A further increase in the assay speed can be achieved by increasing the dimensions of the matrix (Figure 3A), which would entail a subsequent increase in the number of mixture components.

The sensitivity of SMILI-NMR is similar to the sensitivity of standard structure−activity relationships screening by NMR (SAR-NMR).[5] Similar to STINT-NMR,[8] SMILI-NMR is limited primarily by the amount of protein expression that can be achieved. The other limitation is the integrity of the interacting proteins. Cell lysis during storage and in-cell NMR experiments can also limit the sensitivity of SMILI-NMR. Keeping the cells below the freezing point with a cryoprotectant, such as 10% glycerol, and using an ultrasensitive NMR cryoprobe to decrease NMR data acquisition time, can overcome these limitations.

While SMILI-NMR requires a high magnetic field, preferably 700 MHz or higher, and an NMR spectrometer equipped with a cryoprobe to perform in-cell NMR experiments, the cost of the assay can be mitigated by the fact that SMILI-NMR obviates the need for multiple in vitro binding assays. The cell membrane provides a selectivity filter for drug-like molecules capable of entering the cell, which increases the chance that a selected candidate will perform well in in vivo functional assays. Also, in-cell protein overexpression results in higher local concentrations of interaction partners than in lysates, thus increasing the likelihood of selecting weaker interactors for fragment-based screening.[6,42] SMILI-NMR can discriminate binding to different surfaces on the target protein, further facilitating fragment-based screening.

Conclusions

Overall, SMILI-NMR promises to be a valuable and important addition to the repertoire of drug screening tools used in small molecule−protein complex interaction studies. It provides an important means to bridge the gap between biochemical identification of small ligands capable of interfering with target biocomplexes and functional in vivo data on the inhibition of cellular processes by using these ligands.

Experimental Section

General Procedures. All reagents and solvents were obtained from commercial suppliers and used without further purification. Restriction enzymes and Taq polymerase were from NEB. The specified chemical purity of rapamycin (A.G. Scientific, Inc.) and ascomycin (A.G. Scientific, Inc.) was better than 98%. The specified chemical purity of dipeptides (EZBiolab) was better than 95%. All other chemicals were reagent grade or better (Sigma-Aldrich). All NMR spectra were recorded on a Bruker Avance II NMR

spectrometer, operating at a ^{1}H frequency of 700 MHz, equipped with triple resonance cryoprobes and processed using TOPSPIN 2.0 (Bruker, Inc.). Chemical shifts were referenced by using tetramethylsilane as an internal standard.

Plasmid Constructions. DNA coding for full-length FKBP was amplified from pC4EN-F1 (ARIAD Pharmaceuticals, Inc.) using the oligonucleotides 5′- TTT TTT CCA TGG TGT CTA GAG GAG TGC AGG TGG AAA CC-3′ and 5′-TTT TTT GGT ACC TTA ATA ACT AGT TTC CAG TTT TAG AAG CTC CAC ATC-3′. The gene was ligated into pRSF-1b (Novagen) using the *Nco*I and *Kpn*I linker sites. The resulting plasmid, pRSF−FKBP, expresses FKBP from the T7lac promoter, which is induced by IPTG. pRSF−FKBP contains the kanamycin resistance gene (Kan^R) and *lac*I gene to ensure sufficient expression. DNA coding for FRB was amplified from pC4-RHE (ARIAD Pharmaceuticals, Inc.), using the oligonucleotides 5′-TTT TTT CCA TGG CTT CTA GAA TCC TCT TTG AGG TGC-3′ and 5′-TTT TTT GGT ACC TTA ACT AGT CTT TGA GAT TCG TCG GAA CAC ATG-3′. The gene was ligated into pBAD-HisA (Invitrogen) using the *Nco*I and *Kpn*I linker sites. The resulting plasmid, pBAD−FRB, expresses FRB from the araBAD promoter, which is induced by L-arabinose. pBAD−FRB contains the ampicilin resistance gene (Amp^R), a pBR322 replication origin and the *ara*C gene, which codes for a transcription regulator.

Labeling and Expressing FKBP and FRB: [U-,^{15}N]−FKBP and −FRB. Both pRSF−FKBP and pBAD−FRB were cotransformed into *E. coli* strain BL21(DE3) codon+ (Novagen) for overexpression. Cells were grown overnight to an OD_{600} of 1.0−1.2 at 37 °C in 1 L of Luria−Bertani medium (LB) supplemented with 100 μg/mL ampicillin, 35 μg/mL kanamycin, and 0.2% glucose. Glucose was added to suppress FRB transcription fom the araBAD promoter. The cells were washed once with minimal medium (M9) salts and resuspended to an OD_{600} of 0.5−0.6 in 2 L of M9 containing 100 μg/mL ampicillin, 35 μg/mL kanamycin, and 1.0 g/L $^{15}NH_4Cl$ and 0.2% glucose as the sole nitrogen and carbon sources, respectively. The culture was incubated at 37 °C for 10 min and [*U*-,^{15}N]−FKBP expression was induced by adding 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The induction was allowed to proceed for 4 h, during which time the OD_{600} of the culture approximately doubled. A 100 mL sample was collected, the cells were centrifuged, washed three times with 50 mL of 10 mM potassium phosphate buffer [pH 7.0] containing 10% glycerol, and stored at −80 °C for subsequent NMR analysis. The remaining culture was centrifuged, washed once with M9 salts, and a sufficient number of cells containing [*U*-,^{15}N]-labeled FKBP cells were resuspended in 2 L of LB to yield an OD_{600} of 0.5−0.6. The culture was incubated at 37 °C for 10 min, and FRB overexpression was induced by adding 0.2% of L-arabinose. Expression of FRB was allowed to proceed for 4 h, during which time the OD_{600} of the culture increased by ∼10%. Then 100 mL samples were collected, centrifuged, washed three times with 50 mL of 10 mM potassium phosphate buffer [pH7.0] containing 10% glycerol, and stored at −80 °C for subsequent NMR analysis. We found that freezing and thawing the cells produces minimal cell lysis, allowing the NMR samples to be stored for at least one month.

Labeling and Expressing FKBP and FRB: [U-,^{15}N]−FRB and −FKBP. Similar to the preparation of [*U*-,^{15}N]−FKBP and −FRB sample, cells cotransformed with pRSF−FKBP and pBAD−FRB were grown overnight at 37 °C to an OD_{600} of 1.0−1.2 in 1 L of LB supplemented with 100 μg/mL ampicillin, 35 μg/mL kanamycin, and 0.2% glucose. The cells were washed once with M9 salts and resuspended to an OD_{600} of 0.5−0.6 in 2 L of M9 containing 100 μg/mL ampicillin, 35 μg/mL kanamycin, and 1.0 g/L$^{15}NH_4Cl$ and 4 mL/L glycerol as the sole nitrogen and carbon sources, respectively. The culture was incubated at 37 °C for 10 min and [*U*-,^{15}N]−FRB expression was induced by adding 0.2% of L-arabinose. The induction was allowed to proceed for 4 h, during which time the OD_{600} of the culture approximately doubled. A 100 mL sample was collected and the cells were centrifuged, washed three times with 50 mL of 10 mM potassium phosphate buffer [pH7.0] containing 10% glycerol, and stored at −80 °C for subsequent NMR analysis. The remaining culture was centrifuged, washed once with M9 salts, and a sufficient number of cells containing [*U*-,^{15}N]−FRB were resuspended in 1 L LB to yield an OD_{600} of 0.5−0.6. The culture was incubated at 37 °C for 10 min, and expression of FKBP was induced by adding 0.5 mM IPTG. Expression of FKBP was allowed to proceed for 4 h, during which time the OD_{600} of the culture increased by ∼10%. Then 100 mL samples were collected, centrifuged, washed three times with 50 mL of 10 mM potassium phosphate buffer [pH7.0] containing 10% glycerol, and stored at −80 °C for subsequent NMR analysis.

NMR Experiments. [*U*-, ^{15}N] labeled cells were resuspended in 0.5 mL of NMR buffer (10 mM potassium phosphate, pH 7.0, 90%/10% H_2O/D_2O) and transferred to an NMR tube. To rule out the possibility that the visible NMR spectrum was due to extracellular proteins, we sedimented the cells from the NMR sample and acquired the ^{1}H{^{15}N}−HSQC spectrum of the resultant supernatant. No protein NMR signal was visible above the noise level. All NMR spectra were acquired at 25 °C. We used a watergate version of the ^{1}H{^{15}N}−HSQC experiment. ^{1}H{^{15}N}-edited HSQCs were recorded with 32 transients as 64 complex points, apodized with a squared cosine-bell window function, and zero-filled to 128 points prior to Fourier transformation. The corresponding sweep widths were 12 and 35 ppm in ^{1}H and ^{15}N dimensions, respectively. Chemical shifts of human FKBP were assigned by Rosen et al.[46] These assignments closely matched the in-cell NMR spectrum of [*U*-, ^{15}N]-FKBP. To resolve residual ambiguities in the chemical shift assignments due to spectral crowding, we collected ^{15}N-edited TOCSY and NOESY spectra with mixing times of 60 and 100 ms, respectively, on a cell lysate of the in-cell NMR sample (Supporting Information Figure 1). This procedure allowed us to assign 94% of all backbone amide proton and nitrogen resonances.

We prepared 34 dipeptide mixtures, A-X, D-X, E-X, F-X, G-X, H-X, I-X, K-X, L-X, N-X, Q-X, R-X, S-X, T-X, V-X, W-X, Y-X, X-A, X-D, X-E, X-F, X-G, X-H, X-I, X-K, X-L, X-N, X-Q, X-R, X-S, X-T, X-V, X-W, and X-Y, where X stands for any one of all 17 amino acids in a mixture. For example, A-X is a mixture of dipeptides, in which the first amino acid is alanine and the second amino acid is any one of 17 amino acids. These mixtures were added individually at 1 mM final concentrations to *E. coli* cells overexpressing [*U*-, ^{15}N]-labeled FKBP and unlabeled FRB. After the first round of screening, A-X, D-X, T-X, and L-X and X-E, X-I, X-A, and X-T showed extreme broadening, leading to the disappearance of the spectrum. Thus the dipeptides located at the intersection of rows, A-X, D-X, L-X, and T-X and columns X-A, X-E, X-I, and X-T were screened in a second round. These dipeptides (A-A, A-E, A-I, A-T, D-A, D-E, D-I, D-T, T-A, T-E, T-I, T-T, L-A, L-E, L-I, and L-T) were titrated into the cells individually.

Yeast Assay. Two isogenic haploid strains of *Saccharomyces cerevisiae* were used in this work, one that expressed normal FKBP proline isomerase and was sensitive to rapamycin, JK9-3da (*FPR1*$^+$), and another strain with disrupted FKBP gene, JH3-3b (*fpr1*$^-$), which conferred rapamycin resistance.[41] Yeast strains were grown for 65 h at 30 °C on YPD plates containing rapamycin at 0 or 100 mg/mL or 5 mM dipeptide A-E.

Acknowledgment. We thank Dr. Heitman for *Saccharomyces cerevisiae* JK9-3dα and JH3-3b strains. Support by the grant 1-06-CD-23 from the American Diabetes Association to A.S. is acknowledged.

Supporting Information Available: Overlay of the in-cell NMR and cell lysate spectra of [*U*-, ^{15}N]−FKBP−FRB; SDS-PAGE of FKBP and FRB sequential expression; overlay of the in-cell NMR spectra of the ternary FKBP−[*U*-, ^{15}N]−FRB−rapamycin and FKBP−[*U*-, ^{15}N]−FRB complexes; NMR spectrum of cell suspension supernatant; competition between the dipeptide, A-E, and rapamycin or ascomycin for the [*U*-, ^{15}N]−FKBP−FRB complex. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Fernandes, P. B. Technological advances in high-throughput screening. *Curr. Opin. Chem. Biol.* **1998**, *2*, 597–603.
(2) Silverman, L.; Campbell, R.; Broach, J. R. New assay technologies for high-throughput screening. *Curr. Opin. Chem. Biol.* **1998**, *2*, 397–403.
(3) Peng, J. W.; Lepre, C. A.; Fejzo, J.; Abdul-Manan, N.; Moore, J. M. Nuclear magnetic resonance-based approaches for lead generation in drug discovery. *Methods Enzymol.* **2001**, *338*, 202–230.
(4) Pellecchia, M.; Sem, D. S.; Wuthrich, K. NMR in drug discovery. *Nat. Rev. Drug Discovery* **2002**, *1*, 211–219.
(5) Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. Discovering high-affinity ligands for proteins: SAR by NMR. *Science* **1996**, *274*, 1531–1534.
(6) Hajduk, P. J.; Burns, D. J. Integration of NMR and high-throughput screening. *Comb. Chem. High Throughput Screening* **2002**, *5*, 613–621.
(7) Pellecchia, M.; Bertini, I.; Cowburn, D.; Dalvit, C.; Giralt, E.; Jahnke, W.; James, T. L.; Homans, S. W.; Kessler, H.; Luchinat, C.; Meyer, B.; Oschkinat, H.; Peng, J.; Schwalbe, H.; Siegal, G. Perspectives on NMR in drug discovery: a technique comes of age. *Nat. Rev. Drug Discovery* **2008**, *7*, 738–745.
(8) Burz, D. S.; Dutta, K.; Cowburn, D.; Shekhtman, A. Mapping structural interactions using in-cell NMR spectroscopy (STINT-NMR). *Nat. Methods* **2006**, *3*, 91–93.
(9) Burz, D. S.; Dutta, K.; Cowburn, D.; Shekhtman, A. In-cell NMR for protein−protein interactions (STINT-NMR). *Nat. Protoc.* **2006**, *1*, 146–152.
(10) Burz, D. S.; Shekhtman, A. Structural biology: Inside the living cell. *Nature* **2009**, *458*, 37–38.
(11) Harding, M. W.; Galat, A.; Uehling, D. E.; Schreiber, S. L. A receptor for the immunosuppressant FK-506 is a *cis*−*trans* peptidyl-prolyl isomerase. *Nature* **1989**, *341*, 758–760.
(12) Kino, T.; Hatanaka, H.; Hashimoto, M.; Nishiyama, M.; Goto, T.; Okuhara, M.; Kohsaka, M.; Aoki, H.; Imanaka, H. FK-506, a novel immunosuppressant isolated from a *Streptomyces*. I. Fermentation, isolation, and physicochemical and biological characteristics. *J. Antibiot. (Tokyo)* **1987**, *40*, 1249–1255.
(13) Siekierka, J. J.; Wiederrecht, G.; Greulich, H.; Boulton, D.; Hung, S. H.; Cryan, J.; Hodges, P. J.; Sigal, N. H. The cytosolic-binding protein for the immunosuppressant FK-506 is both a ubiquitous and highly conserved peptidyl-prolyl *cis*−*trans* isomerase. *J. Biol. Chem.* **1990**, *265*, 21011–21015.
(14) Vezina, C.; Kudelski, A.; Sehgal, S. N. Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *J. Antibiot. (Tokyo)* **1975**, *28*, 721–726.
(15) Brown, E. J.; Albers, M. W.; Shin, T. B.; Ichikawa, K.; Keith, C. T.; Lane, W. S.; Schreiber, S. L. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature* **1994**, *369*, 756–758.
(16) Sabatini, D. M.; Erdjument-Bromage, H.; Lui, M.; Tempst, P.; Snyder, S. H. RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell* **1994**, *78*, 35–43.
(17) Brown, E. J.; Beal, P. A.; Keith, C. T.; Chen, J.; Shin, T. B.; Schreiber, S. L. Control of p70 s6 kinase by kinase activity of FRAP in vivo. *Nature* **1995**, *377*, 441–446.
(18) Tokunaga, C.; Yoshino, K.; Yonezawa, K. mTOR integrates amino acid- and energy-sensing pathways. *Biochem. Biophys. Res. Commun.* **2004**, *313*, 443–446.
(19) Hidalgo, M.; Rowinsky, E. K. The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. *Oncogene* **2000**, *19*, 6680–6686.
(20) Dudkin, L.; Dilling, M. B.; Cheshire, P. J.; Harwood, F. C.; Hollingshead, M.; Arbuck, S. G.; Travis, R.; Sausville, E. A.; Houghton, P. J. Biochemical correlates of mTOR inhibition by the rapamycin ester CCI-779 and tumor growth inhibition. *Clin. Cancer Res.* **2001**, *7*, 1758–1764.
(21) Vogt, P. K. PI 3-kinase, mTOR, protein synthesis and cancer. *Trends Mol. Med.* **2001**, *7*, 482–484.
(22) Serber, Z.; Straub, W.; Corsini, L.; Nomura, A. M.; Shimba, N.; Craik, C. S.; Ortiz de Montellano, P.; Dotsch, V. Methyl groups as probes for proteins and complexes in in-cell NMR experiments. *J. Am. Chem. Soc.* **2004**, *126*, 7119–7125.
(23) Li, C.; Charlton, L. M.; Lakkavaram, A.; Seagle, C.; Wang, G.; Young, G. B.; Macdonald, J. M.; Pielak, G. J. Differential dynamical effects of macromolecular crowding on an intrinsically disordered protein and a globular protein: implications for in-cell NMR spectroscopy. *J. Am. Chem. Soc.* **2008**, *130*, 6310–6311.
(24) McNulty, B. C.; Young, G. B.; Pielak, G. J. Macromolecular crowding in the *Escherichia coli* periplasm maintains alpha-synuclein disorder. *J. Mol. Biol.* **2006**, *355*, 893–897.
(25) Cruzeiro-Silva, C.; Albernaz, F. P.; Valente, A. P.; Almeida, F. C. In-Cell NMR spectroscopy: inhibition of autologous protein expression reduces *Escherichia coli* lysis. *Cell Biochem. Biophys.* **2006**, *44*, 497–502.
(26) Banaszynski, L. A.; Liu, C. W.; Wandless, T. J. Characterization of the FKBP−rapamycin−FRB ternary complex. *J. Am. Chem. Soc.* **2005**, *127*, 4715–4721.
(27) Siekierka, J. J.; Hung, S. H.; Poe, M.; Lin, C. S.; Sigal, N. H. A cytosolic binding protein for the immunosuppressant FK-506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature* **1989**, *341*, 755–757.
(28) Bierer, B. E.; Mattila, P. S.; Standaert, R. F.; Herzenberg, L. A.; Burakoff, S. J.; Crabtree, G.; Schreiber, S. L. Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK-506 or rapamycin. *Proc. Natl. Acad. Sci. U.S.A* **1990**, *87*, 9231–9235.
(29) Choi, J.; Chen, J.; Schreiber, S. L.; Clardy, J. Structure of the FKBP12−rapamycin complex interacting with the binding domain of human FRAP. *Science* **1996**, *273*, 239–242.
(30) Arai, T.; Kouama, Y.; Suenaga, T.; Honda, H. Ascomycin, an antifungal antibiotic. *J. Antibiot. (Tokyo)* **1962**, *15*, 231–232.
(31) Kawai, M.; Lane, B. C.; Hsieh, G. C.; Mollison, K. W.; Carter, G. W.; Luly, J. R. Structure−activity profiles of macrolactam immunosuppressant FK-506 analogues. *FEBS Lett.* **1993**, *316*, 107–113.
(32) Petros, A. M.; Kawai, M.; Luly, J. R.; Fesik, S. W. Conformation of two nonimmunosuppressive FK-506 analogs when bound to FKBP by isotope-filtered NMR. *FEBS Lett.* **1992**, *308*, 309–314.
(33) Horwell, D. C.; Hughes, J.; Hunter, J. C.; Pritchard, M. C.; Richardson, R. S.; Roberts, E.; Woodruff, G. N. Rationally designed "dipeptoid" analogues of CCK. α-Methyltryptophan derivatives as highly selective and orally active gastrin and CCK-B antagonists with potent anxiolytic properties. *J. Med. Chem.* **1991**, *34*, 404–414.
(34) Horwell, D. C.; Howson, W.; Ratcliffe, G. S.; Rees, D. C. The design of a dipeptide library for screening at peptide receptor sites. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 799–802.
(35) Boden, P.; Eden, J. M.; Hodgson, J.; Horwell, D. C.; Hughes, J.; McKnight, A. T.; Lewthwaite, R. A.; Pritchard, M. C.; Raphy, J.; Meecham, K.; Ratcliffe, G. S.; Suman-Chauhan, N.; Woodruff, G. N. Use of a dipeptide chemical library in the development of nonpeptide tachykinin NK3 receptor selective antagonists. *J. Med. Chem.* **1996**, *39*, 1664–1675.
(36) Ondetti, M. A.; Rubin, B.; Cushman, D. W. Design of specific inhibitors of angiotensin-converting enzyme: new class of orally active antihypertensive agents. *Science* **1977**, *196*, 441–444.
(37) Olson, E. R.; Dunyak, D. S.; Jurss, L. M.; Poorman, R. A. Identification and characterization of dppA, an *Escherichia coli* gene encoding a periplasmic dipeptide transport protein. *J. Bacteriol.* **1991**, *173*, 234–244.
(38) Saito, H.; Inui, K. Dipeptide transporters in apical and basolateral membranes of the human intestinal cell line Caco-2. *Am. J. Physiol.* **1993**, *265*, G289−G294.
(39) Van Duyne, G. D.; Standaert, R. F.; Karplus, P. A.; Schreiber, S. L.; Clardy, J. Atomic structures of the human immunophilin FKBP-12 complexes with FK-506 and rapamycin. *J. Mol. Biol.* **1993**, *229*, 105–124.
(40) Meadows, R. P.; Nettesheim, D. G.; Xu, R. X.; Olejniczak, E. T.; Petros, A. M.; Holzman, T. F.; Severin, J.; Gubbins, E.; Smith, H.; Fesik, S. W. Three-dimensional structure of the FK-506 binding protein/ascomycin complex in solution by heteronuclear three- and four-dimensional NMR. *Biochemistry* **1993**, *32*, 754–765.
(41) Heitman, J.; Movva, N. R.; Hall, M. N. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science* **1991**, *253*, 905–909.
(42) Schuffenhauer, A.; Ruedisser, S.; Marzinzik, A. L.; Jahnke, W.; Blommers, M.; Selzer, P.; Jacoby, E. Library design for fragment based screening. *Curr. Top. Med. Chem.* **2005**, *5*, 751–762.
(43) Zhang, J.; Chung, K.; Oldenburg, A. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screening* **1999**, *4*, 27–32.
(44) Copeland, R. A. Mechanistic considerations in high-throughput screening. *Anal. Biochem.* **2003**, *320*, 1–12.
(45) Burz, D. S.; Shekhman, A. In-cell Biochemistry Using NMR Spectroscopy. *PloS One* **2008**, *3*, e2571; doi: 10.1371/journal.pone.0002571.
(46) Rosen, M. K.; Michnick, S. W.; Karplus, M.; Schreiber, S. L. Proton and nitrogen sequential assignments and secondary structure determination of the human FK-506 and rapamycin binding protein. *Biochemistry* **1991**, *30*, 4774–4789.

JM9000743