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Selective Targeting of Cells via Bi-Specific Molecules that Exploit Co-Expression of Two Intracellular Proteins

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Abstract

In drug discovery, small molecules must often discriminate between healthy and diseased cells. This feat is usually accomplished by binding to a protein that is preferentially expressed in the target cell or on its surface. However, in many cases, the expression of an individual protein may not generate sufficient cyto-selectivity. Here, we demonstrate that bi-specific molecules can better discriminate between similar cell types by exploiting their simultaneous affinity for two proteins. Inspired by the natural product, FK506, we designed molecules that have affinity for both FKBP12 and HIV protease. Using cell-based reporters and live virus assays, we observed that these compounds preferentially accumulated in cells that express both targets, mimicking an infected lymphocyte. Treatment with FKBP12 inhibitors reversed this partitioning, while over-expression of FKBP12 protein further promoted it. The partitioning into the target cell type could be tuned by controlling the properties of the linker and the affinities for the two proteins. These results show that bi-specific molecules create significantly better potential for cyto-selectivity, which might be especially important in the development of safe and effective anti-virals and anti-cancer compounds.

Graphical Abstract

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Supporting Information Available: This material includes synthetic schemes, compound characterization, protein expression details and additional experimental methods. This material is available free of charge *via* the Internet.

Introduction

Many drugs, especially those used to treat viral infections and cancer, require selectivity for a specific cell type amongst a “sea” of similar, healthy cells. Towards that end, bi-specific antibodies are often advantageous because they simultaneously recognize two cell surface proteins, which increases their avidity and reduces interactions with bystander cells.^{1, 2} Here, we wondered whether a similar approach might be used to drive the cyto-selectivity of a small molecule. One potential advantage of this idea would be that small molecules can access intracellular proteins, expanding the choices for discriminating between cell types. To this end, we were inspired by the natural product, FK506. This molecule is naturally bi-specific; it binds FK506-binding protein (FKBP12) with one chemical “face” and calcineurin with the other. FKBP has high affinity ($K_D \sim 0.6 \text{ nM}$)³ for FK506 and this drug-protein pair recruits calcineurin into a remarkably stable, ternary complex ($K_{app} \sim 6$ to 30 nM).^{3, 4} This unusual binding mode may also impart cyto-selectivity because FK506 is principally sequestered into lymphocytes and red blood cells after oral administration,^{5, 6} perhaps because these cell types express relatively high concentrations of the two target proteins. Synthetic molecules based on FK506 have a long history of important uses in chemical biology and drug discovery,^{7–15} yet the specific role of bi-valency in cyto-selectivity has not yet been explored.

To better understand this natural mechanism and explore ways of possibly engineering bi-specific small molecules, we chose a model system based on the human immunodeficiency virus (HIV) protease. Although they are clinically effective, HIV protease inhibitors are poorly cell penetrant and rapidly metabolized.¹⁶ We hypothesized that an “FK506-like” molecule capable of simultaneously binding FKBP12 and HIV protease might be selectively retained in cells that express both targets. To test this idea, we synthesized bi-specific molecules composed of an FKBP12 ligand and an HIV protease inhibitor attached by a modular linker. In this collection, we systematically varied the affinity of the molecules for FKBP12 and HIV protease, creating a suite of tools to ask how avidity correlates with relative cellular partitioning and retention. We found that bi-specific molecules preferentially accumulate in cells expressing both FKBP12 and HIV protease. Further, we found that the partitioning values *in vitro* and in cells were determined by the molecule’s relative affinities for the target proteins and the abundance of the targets. Indeed, the amount of molecule in the cell could be “tuned” by artificially increasing or decreasing the availability of FKBP12. These results show that bi-specific molecules, like their antibody counterparts, can be preferentially directed towards cells expressing two targets.

RESULTS AND DISCUSSION

Generation of bi-specific compounds with variable linkers

Based on extensive prior work in the general area of bi-functional molecules,^{12–15, 17} we envisioned the synthesis of compounds **1a-f** and **2a-f** composed of three major components (Fig. 1a). On one end would be a ligand for FKBP12, either FK506 itself or a Synthetic Ligand for FKBP (SLF). SLF is composed of only one half of FK506 and it is known to have a significantly weaker affinity for FKBP12.¹⁸ Thus, using FK506 or SLF as one “end” of the molecule would be expected to provide either tight or weak binding to FKBP12,

respectively. To avoid the immunosuppressive effects of using FK506, we took advantage of observations that modification of the extra-cyclic alkene destroys its affinity for calcineurin, but does not alter its tight binding to FKBP12.¹⁹ Accordingly, we used microwave-assisted, Grubbs' cross metathesis chemistry²⁰ to install 4-pentenoic acid at the terminal alkene in one step with modest yield (3hr, 80°C, 60%). Likewise, we modified SLF at the aniline with excess succinic anhydride in anhydrous DCM to produce a modified SLF intermediate containing a terminal carboxylic acid in quantitative yield (Fig. 1a). These two compounds provided FKBP12-binding motifs for further coupling. On the opposite end of the molecule, we first assembled the core of the FDA-approved, HIV protease inhibitor, amprenavir. We chose this starting point because the core of amprenavir has modest efficacy against HIV protease ($K_i \approx 177$ nM) but its affinity is highly tunable by substitution at the nearby amine.^{21–23} For example, installation of a tetrahydrofuranyl urethane moiety (4-OMe-amprenavir) improves the K_i to 0.5 nM (Fig. S1a and S1b). Thus, by making substitutions in this region, we envisioned “tuning” the affinity for HIV protease. Accordingly, the core of amprenavir was synthesized by a known route,²⁴ after which amino acid linkers were incorporated using HATU amide coupling and subsequent deprotection (50 mg scale, 60 to 80% yield). Amino acids were chosen for the linker because the side chains could be used to readily introduce functional groups with different hydrophobicities, flexibilities, geometries and chain lengths (Fig. 1a). Based on well-established structure-activity relationships (SAR) for HIV protease inhibitors,^{21–23} we hypothesized that small alkyl and benzyl substitutions would be tolerated, while the proline and lysine analogues might not. Finally, the modified amprenavir derivatives were coupled to the free acid of the modified FK506 or SLF motifs and the final products purified by HPLC to yield twelve bi-functional molecules (**1a-f** and **2a-f**) in 20 to 40% overall yield (Fig. 1b).

Biochemical characterization of bi-functional molecules

With this library in-hand, we first measured the affinity of the compounds for purified FKBP12 *in vitro*. In these studies, we determined K_i values by competition with a fluorescent polarization (FP) tracer **6** that has tight affinity for FKBP12 ($K_D = 9.6 \pm 1.6$ nM; Fig. S1c).^{25, 26} We found that the K_i values were principally determined by the identity of the FKBP12 ligand (either FK506 or SLF) (Fig. 2a). Specifically, compounds **1a-f** had K_i values ranging between 19 and 29 nM, while **2a,b,d-f** had values ranging from 40 to 93 nM. These affinities were similar to those of unmodified FK506 or SLF (Fig. S1d), suggesting that neither the linker nor the pendant HIV protease inhibitor dramatically impacted apparent affinity. This result might be expected because the binding site on FKBP12 is highly exposed^{27, 28} and many studies on bi-functional FKBP12 ligands have shown similar modularity.^{12–15} Because of non-specific tracer binding, we had to measure the affinity of **2c** for FKBP12 using a surface-plasmon resonance (SPR) platform instead of FP. Using this approach we found that **2c** had a similar affinity for FKBP12 as the other molecules in the series (~50 nM). In this setting, we also decided to test the affinity of **2c** for immobilized HIV protease, revealing an affinity of 42 ± 28 nM (Fig. S2a). This platform also allowed us to ask whether bi-functional molecules could bind HIV protease and FKBP12 simultaneously, because the increase in the molecular mass from binding to the proteins is expected to be significantly larger than that provided by the small molecule. Adding a combination of FKBP12 and **1b** to immobilized HIV protease showed that FKBP12 doesn't

bind to HIV protease in the absence of **1b**, but that a stable ternary complex is formed in the presence of the molecule (Fig. S2b).

To analyze binding of the full compound collection to HIV protease, we employed a standard enzymatic cleavage assay.²⁹ Compounds **1a-f** and **2a-f** were incubated with HIV protease and a FRET-labelled substrate and inhibition curves generated from endpoint measurements (Fig. 2b). Consistent with previous SAR,^{21–23} we found that the identity of the linker had a substantial impact on activity. Specifically, the K_i values varied by more than two orders-of-magnitude; compound **2a** had a K_i of 1.3 ± 0.3 nM, while compound **1d** had a K_i of 170 ± 39 nM. Replacing FK506 with SLF did not seem to significantly impact anti-protease activity, likely because that portion of the molecule is too far from the active site, even when FKBP12 is introduced into the assay (Fig. S3a). The inhibitory activity of protease inhibitors is known to correlate with binding affinity,³⁰ thus, compounds **1a-f** and **2a-f** appear to provide a range of affinity values for each protein target.

Before progressing into cellular models, we first explored the membrane permeability of the compounds using a standard parallel artificial membrane permeability assay (PAMPA). Consistent with their large size, the bi-functional molecules exhibited modest passive diffusion rates ($1\text{--}2 \times 10^{-6}$ cm/s, Fig. S4a). However, these permeability values are only 2- to 5-fold less than the parent compounds: amprenavir and FK506 (5.7×10^{-6} and 2.2×10^{-6} cm/s, respectively), suggesting that the modifications had relatively modest effects on permeability. With these baselines established, we added recombinant human FKBP12 to one side of the PAMPA membrane and measured the partitioning of a subset of compounds. We found that FKBP12 enhanced partitioning by 2- to 5-fold compared to control wells (Fig. S4b), providing proof-of-concept that protein binding could retain the molecule on one side of a membrane. The partitioning of amprenavir was unaffected by FKBP12 (Fig. S4b), as expected. We desired to see if HIV protease could enhance partitioning in a similar fashion, but found that the buffers tolerated in the PAMPA system were not compatible with HIV protease solubility. Thus, we turned to a cellular assay.

Tuning cyto-selectivity of bi-specific molecules

To measure HIV protease binding in cells, we adapted an assay that uses a GFP-HIV protease reporter.^{31, 32} This method relies on the observation that expression of HIV protease culminates in rapid cytotoxicity and cell death in mammalian cells.^{33, 34} However, when a protease inhibitor is added, the cells become fluorescent and are able to proliferate. In this way, the extent of partitioning and HIV protease binding can be estimated from the measured GFP levels. In early studies, we found that transient transfection with the standard GFP-HIV protease reporter was not sufficiently reproducible, so we generated stable HEK293 T-REx cell lines under the control of a Tet repressor. As in the standard assay, we found that HIV protease was auto-proteolyzed following doxycycline induction in the absence of HIV protease inhibitor, leading to cell death (Fig. 3a and S5a). However, treatment with 4-OMe-amprenavir or **1a** (5 μ M), but not FK506 or DMSO, could rescue cell viability and increase fluorescence (Fig. 3b and S5b), as observed by fluorescence and light microscopy. Next, we measured the effects of compounds **1a-f** and **2a-f** on GFP expression by flow cytometry to provide a more quantitative and high throughput assay. These studies

showed that the best compounds, such as compounds **1a-c** or **2a-c**, also had potent anti-protease activity in the cells. Satisfyingly, the most potent molecules from this experiment were also those with the best anti-protease activity in the biochemical assays. For example, compound **1a** was significantly better than **1f** (Fig 3c). We also noticed that there was an apparent “activity cliff” in the results. In other words, compounds **1a-c** were active in the GFP-HIV protease assay, while **1d-f** were largely inactive rather than simply weaker. This type of threshold response has been observed previously for HIV protease inhibitors and is likely due to an inability to stabilize the active site.³⁵ The second observation from our studies was that compounds **1a-c**, bearing the FK506 group, were significantly more potent than the equivalent compounds with the SLF moiety, **2a-c** (all $IC_{50} > 5000$ nM) (Fig. 3c and S5c). These results suggest that the affinity for FKBP is critical to cellular partitioning. FKBP12 is more abundantly expressed (concentration estimated to be high micromolar)³⁶ than HIV protease, consistent with FKBP12 being a major determinant of compound retention in the cytosol. To more closely explore the role of partitioning in this assay, we created control compounds, Ac-Ala-amprenavir and propionyl-amprenavir (see Fig S1a–b), that matched the permeability and *in vitro* HIV protease inhibitory activity of **1a**. These compounds allowed us to directly compare the effects of FKBP-driven partitioning on apparent activity in the GFP-HIVp assay without adjusting for other features, such as potency. Consistent with the model, we found that **1a**, but not **2a**, was significantly more active than the control molecules (Fig. S5f).

These results suggested that the availability of FKBP12 may tune partitioning. As a test of this idea, we saturated available FKBP12 sites using free FK506 or SLF as direct competitors. Indeed, these molecules dramatically reduced fluorescence in the GFP-HIV reporter cells, with FK506 being more potent than the weaker binding SLF (Fig. 4a). To test this idea in the opposite direction, we increased the available pool of FKBP12 through transient over-expression of a mCherry-FKBP12 construct (Fig. S5b and d). After the transfected cells were allowed to recover, we treated with **1a** and used microscopy to reveal that GFP fluorescence was substantially increased in cells that also had high levels of mCherry-FKBP12 (Fig. 4b and S5b). 4-OMe-amprenavir did not show this dependence on mCherry-FKBP12 expression, showing that bi-valency was required. Using flow cytometry, we quantified the effect of FKBP12 on partitioning. Specifically, we isolated the subpopulation of cells (approximately 50%) that had high levels of mCherry expression. In these cells, the EC_{50} for **1a-c** was substantially improved ($EC_{50} = 610$ nM in the FKBP12^{low} cells vs. $EC_{50} = 100$ nM in the FKBP12^{high} cells), while co-treatment with free FK506 reversed the enhanced partitioning effect (Fig. 4c, 4d and S5e), demonstrating that it is indeed due to the availability of FKBP12. Finally, we performed live virus HIV-infectivity assays. Consistent with our cell-based reporter assay, we observed that **1a** was significantly more potent than **2a** (Fig. 5a and S6), further supporting the idea that the affinity for FKBP determines relative partitioning. Together, these results show that bi-specific molecules take advantage of dual expression of two proteins to gain enhanced cyto-selectivity.

The selectivity of a drug for its target cell in an animal helps determine its maximum safe dose by limiting damage to bystander cells. In this work, we synthesized bi-functional inhibitors in which the affinities for two target proteins, HIV protease and FKBP12, were

systematically varied. We gained control over the FKBP12 binding by using either FK506 or the weaker ligand, SLF, while affinity for HIV protease was tuned by installing linkers at a region that was known to impact binding. Using *in vitro* assays, we confirmed that the affinities sampled a wide range and then found that the bi-specific molecules were sequestered on one side of a membrane by FKBP12. This partitioning partially overcame the poor permeability of the molecules, emphasizing that target engagement impacts equilibrium across membranes.³⁷ Then, using a modified version of a cell-based GFP reporter assay, we confirmed that interactions with FKBP12 and HIV protease also control partitioning in cells. Binding to FKBP12 dominated the cyto-selectivity effect, likely because it represents a greater pool of available binding sites. To further explore the role of FKBP12, we used pharmacological competitors and over-expression to systematically control the number of free sites. Consistent with a key role for this binding partner, over-expression of a mCherry-FKBP12 construct could further enhance partitioning (and anti-HIV protease activity), while competitors neutralized this effect. These results suggest that the key design principles in engineering cyto-selective small molecules are the abundance of the protein partners and the avidity of the interactions, reminiscent of what has been found for bi-specific antibodies and cell surface antigens.²

In part, we chose the HIV protease and FKBP12 model system because HIV infection commonly occurs in lymphocytes. As mentioned previously, lymphocytes are a major tissue target of FK506, likely because of the unusually high concentration of FKBP12 in these cells. Thus, we envisioned that the selectivity of HIV protease inhibitors might be improved by directing them to the relevant cell population – cells expressing both HIV protease and FKBP12. Such a strategy might improve activity by concentrating the active molecule into the relevant cell subpopulation, which in this case would be HIV-infected lymphocytes. This concept is supported by the results of the live viral assays, in which compound **1a** was found to be much more potent than **2a**. However, we anticipate that this strategy may be beneficial in other situations in which therapeutics need to be guided to a specific cell type with precision. For example, many transformed cells express high levels of at least two possible targets, such as oncoproteins, cell cycle proteins and molecular chaperones. Using bi-specific molecules might enhance selectivity for these cancer cells and reduce accumulation in healthy cells. Also, recent work has established ways of degrading target proteins, including FKBP12, by recruiting the cereblon E3 ligase.^{38–40} The results presented here might inform ways of thinking about how these bi-specific molecules might take advantage of dual binding to generate cyto-selectivity. The clear disadvantages of this approach are that the resulting molecules are large and complex, with predicted physical properties that do not correlate well with oral bioavailability.⁴¹ Like the original inspiration, FK506, effective bi-specific molecules may require cyclization^{42, 43} or further engineering to incorporate the favorable valency features into a more amenable scaffold

Methods

Fluorescence Polarization Assay

Binding to FKBP12 was measured by fluorescence polarization. The assay was performed at 60 μ L final volume in PBS (Gibco) with 0.01% Triton X-100 using Corning 384-well flat-

bottom black plates. For initial binding analysis of the fluorescent compound, recombinant FKBP12 was serially diluted 2-fold from 80 μ M initial concentration and then 15 μ L added to each well for a top concentration of 20 μ M. Fluorescent ligand 6 was initially dissolved in DMSO at 100 μ M then dissolved 1000-fold into assay buffer and 15 μ L of this solution was added to each well to give a final concentration of 25 nM. Finally, 30 μ L of assay buffer with 4% DMSO was added to each well to give a final volume of 60 μ L with 2% DMSO. The plate was covered and equilibrated at room temperature for 30 minutes then read on a SpectraMax M5 at wavelength 488/515 ex/em.

Competition experiments were performed under similar conditions. Separate solutions of FKBP12 and fluorescent ligand 6 at 100 μ M were prepared and then 15 μ L of both was added to each well. Under these conditions, the majority of the fluorescent ligand would be bound to FKBP12. Compounds were initially dissolved in DMSO to 250 μ M, serially diluted 3-fold, then diluted to 10 μ M with assay buffer and 30 μ L was added to each well to a final top concentration of 5 μ M and 2% DMSO. The assay was equilibrated at room temperature for 30 minutes and then read as before.

HIV protease Enzymatic Cleavage Assay

The HIV protease cleavage assay was performed as previously described.²⁹ Protease Substrate 1, RE(edans)SQNYPIVQK(dabcyl)R, was purchased from Sigma and Corning Low Volume Round-Bottom Black plates were used. In brief, recombinant HIV protease was diluted into Buffer P (100 mM sodium acetate, 1 M NaCl, 1 mM EDTA, 1 mM DTT, 20% Glycerol, 0.1% w/v CHAPS, pH 4.7) to an initial concentration of 60 nM and 5 μ L was added to each well. Compounds were 4-fold serially diluted from 1 mM in DMSO and then diluted 100-fold with assay buffer to 10 μ M top concentration before 2 μ L was added to each well. After compound addition, either 1 μ L ddH₂O or PEG-400 (final concentration 0.2% v/v to improve solubility) was added to each well and plates were incubated for 30 minutes at room temperature. After incubation, a fluorescent substrate solution (5 μ M) was prepared in assay buffer and 12 μ L was rapidly added to each well. The assay plate was immediately shaken for 5 seconds in a SpectraMax M5 plate reader and monitored at 340/490 nm with a 475 cutoff filter for 30 minutes. Inhibition curves were plotted from the final timepoint after background subtraction of the fluorescent signal at assay initiation.

Generation of GFP-HIV protease Cell Lines

HEK293 T-REx cells were grown in DMEM supplemented with 10% FBS at 37 °C in 5% CO₂. pcDNA5/FRT/TO containing the GFP-HIV protease fusion was transiently transfected into cells with different ratios of pOG44 (Invitrogen) with Lipofectamine 3000. Clones incorporating GFP-HIVp at the FRT site were selected with Hygromycin B (Roche) then checked for Zeocin sensitivity and expression levels after doxycyclin induction via fluorescent microscopy.

Flow Cytometry Analysis of Fluorescent Construct Expression

HEK293 cells incorporating the GFP-HIVp fusion construct were plated at 20,000 cells/well in clear 96-well TC-treated plates and grown for 24 hours. Compounds were titrated down in DMSO, diluted to 5X final concentration with PBS and 10 μ L was added to each well to a

final volume of 200 μ L and 1% DMSO. Doxycycline was then added to each well at 1 μ g mL^{-1} to induce GFP-HIVp expression. Cells were incubated for 36 hours at 37 °C in 5% CO_2 , media aspirated, washed once with PBS and trypsinized. Cells were then re-suspended in ice-cold PBS and analyzed on a BD LSRii cytometer for GFP fluorescence. Median fluorescence for each well was determined. To analyze FKBP12 levels, cells positive for mCherry fluorescence were gated and median GFP fluorescence was measured.

Live HIV Spreading Assay

Jurkat (E6-1) cells (NIH AIDS Reagent Program) were cultured in RPMI media supplemented with 25 mM HEPES pH 7.4, 10% heat-inactivated fetal calf serum (Hyclone), and 1% penicillin/streptomycin. 2.5×10^5 Jurkat cells were inoculated with 250pg p24 in 250 μ L media in a 96-well flat bottom polystyrene cell culture microplate. Input virus was removed after 18 hours via washing cells with phosphate-buffered saline (PBS). Cells were resuspended in 250 μ L of media containing compounds at the described concentrations. Cells were incubated at 37 °C, 5% CO_2 and supernatant samples were removed every 48 hours and replaced with fresh media containing the compounds. Viral replication was quantified using ELISA to quantify p24 viral capsid protein in the culture supernatant. Mouse monoclonal and rabbit polyclonal α -p24 antibodies used in the ELISA were obtained from the NIH AIDS Reagent Program. Toxicity was monitored using the MTT cell viability assay.⁴⁴

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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(a) Bifunctional molecules composed of three major components

(b) Schematic representation

Figure 1.

Design of bi-specific small molecules. (a) Modular methods were used to assemble molecules containing either FK506 (series 1) or SLF (series 2) attached to an amprenavir core. (b) Schematic of the modular, bi-specific compounds.

(a) Affinity for FKBP12 is dominated by the FK506 or SLF portion of the molecule

(b) Apparent affinity for HIV protease varies by at least 100-fold

(c) Quantification of the binding studies

Ki Values (nM)				
FKBP12 Binding		HIVp Inhibition		
Series 1	Series 2	Series 1	Series 2	
a	29 ± 9	93 ± 24	5.6 ± 1.0	1.3 ± 0.3
b	22 ± 4	40 ± 9	4.6 ± 0.7	2.9 ± 0.3
c	24 ± 5	n.d.	2.7 ± 0.3	1.7 ± 0.2
d	19 ± 5	41 ± 9	170 ± 40	84 ± 15
e	21 ± 5	75 ± 17	23 ± 5	13 ± 2
f	23 ± 11	75 ± 28	40 ± 9	26 ± 9

n.d. = not determined (see text)

Figure 2.

Binding of molecules to FKBP12 and HIV protease in vitro. (a) Competition for binding of an SLF tracer to human FKBP12. (b) Inhibition of HIV protease (HIVp) activity measured using a fluorescent reporter. (c) Quantification of the half-maximal values from (a) and (b). Results are the average of at least three independent experiments performed in triplicate. Error bars represent standard error of the mean (SEM). Some bars are smaller than the symbols.

Figure 3.

Bi-specific molecules bind HIV protease and FKBP12 in cells. (a) Schematic of the GFP-HIV protease reporter assay. After doxycycline induction, cell viability and fluorescence is dependent on the cellular concentration of HIV protease inhibitor. (b) Confirmation of the HIV protease reporter assay. As expected, both **1a** and the control, 4-OMe-amprenavir (5 µM), were active based on viability and GFP fluorescence. (c) Relative potency measured using flow cytometry. Results are the average of at least three experiments in which fluorescence from 10,000 cells per concentration were quantified. Error bars were calculated from the three independent experiments.

Figure 4.

Altering FKBP12 levels tunes the partitioning of bi-specific molecules. (a) Competition with free FK506 (5 μ M) reduces partitioning of **1a** and **2a**, with the weaker inhibitor, SLF, being less potent. Results are the average of at least three experiments in which 10,000 cells were quantified and the error bars are SEM. (b) After transient transfection with mCherry-FKBP12, cells express variable amounts of the protein. Cells with high mCherry signal tended to have more GFP (arrows). (c) Shift in dose-response curves and (d) quantification of the ability of FKBP12 to tune partitioning, as judged by flow cytometry. See text for details. Results are the average of at least three experiments in which 10,000 cells were quantified. Error bars are SEM and statistical analysis was conducted using a One-Way ANOVA ($p < 0.001$) and post-hoc Dunnett's multiple comparisons test with **1a** as a reference (* = $p < 0.05$, *** = $p < 0.001$).

(a) **1a** has greater efficacy than **2a** in a live virus infectivity model

Figure 5.

Affinity for each bi-valent interaction mediates cellular activity of bi-functional molecules. (a) Viral replication is inhibited by **1a** and **2a** six and eight days post-infection. **1a** exhibits greater efficacy due to the tighter affinity for FKBP12 despite identical protease inhibitor motifs. Infectivity curves are representative of experiments performed twice in triplicate. Error bars are SD and IC₅₀ values are shown with calculated standard error.