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Synthesis of orthogonally reactive FK506 derivatives via olefin cross metathesis

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ABSTRACT

Chemical inducers of dimerization (CIDs) are employed in a wide range of biological applications to control protein localization, modulate protein–protein interactions and improve drug lifetimes. These bifunctional chemical probes are assembled from two synthetic modules, which each provide affinity for a distinct protein target. FK506 and its derivatives are often employed as modules in the syntheses of these bifunctional constructs, owing to the abundance and favorable distribution of their target, FK506-binding protein (FKBP). However, the structural complexity of FK506 necessitates multi-step syntheses and/or multiple protection–deprotection schemes prior to installation into CIDs. In this work, we describe an efficient, one-step synthesis of FK506 derivatives through a selective, microwave-accelerated, cross metathesis diversification step of the C39 terminal alkene. Using this approach, FK506 is modified with an array of functional groups, including primary amines and carboxylic acids, which make the resulting derivatives suitable for the modular assembly of CIDs. To illustrate this idea, we report the synthesis of a heterobifunctional HIV protease inhibitor.

1. Introduction

The natural product FK506 is a powerful immunosuppressant that is used to prevent allograft rejection in transplant patients.[1] This compound blocks immune cell function through an intriguing mechanism in which it first interacts with the FK506-binding protein (FKBP) and then this complex binds tightly to the phosphatase calcineurin.[2,3] This unusual binding property has been mirrored in synthetic tools termed chemical inducers of dimerization (CIDs).[4–6] Like FK506, these compounds are composed of two, non-overlapping chemical domains, which enables them to bridge two protein targets. CIDs have been employed in a broad range of applications, many focused on understanding signaling pathways, subcellular localization and protein–protein interactions.[7–14] In addition, this platform has been used in drug discovery applications to modulate specificity, lifetime and affinity.[15–19]

Despite this versatility, methods for the modular construction of CIDs have not been extensively explored. One notable exception is a study by the Verdine group that explored the systematic synthesis of compounds coupled to the synthetic ligand for FKBP (SLF).[20] SLF is an attractive chemical component because it is a non-immunosuppressive analog of FK506 that can be attached to other molecules via pendant anilines[18] or carboxylates.[11,21–23] Although SLF has been used to synthesize many useful CIDs, there are limitations to this approach, including modest aqueous solubility and decreased affinity for FKBP (~10-fold compared to FK506).[24] In addition, SLF is challenging to use on a large scale because its synthesis requires ~12 steps, with modest overall yield.[21,23,24] One alternative is to employ FK506 as a commercially available FKBP-binding ligand. In fact, this strategy was used to create the first CID, a homodimer of FK506 termed FK1012.[7] However, because of its structural complexity, manipulations of FK506 are challenging; for example, reported methods for preparing reactive intermediates require five steps with ~50% yield.[7]

We reasoned that an improved entry into reactive FK506 derivatives might involve selectively modifying the C39 alkene via cross metathesis (CM) using Grubbs' second generation ruthenium catalyst (GII).[25] The GII catalyst was chosen, in part, because it is tolerant of the many functional groups found in FK506, such as alcohols and ketones. In support of this idea, both the Wender and Schreiber groups have diversified complex natural products via CM at a terminal alkene.[26,27] Another attractive feature of this approach is that the extracyclic C39 position of FK506 is crucial for binding calcineurin. Thus, modifications at this site would be expected to block immunosuppression without impacting the affinity for FKBP.[28] Consistent with this model, Clemons et al. used CM to selectively append bulky groups to the C39 position on FK506 to interrupt binding to calcineurin[27] and Diver and Schreiber used CM to produce FK1012-like homodimers, which are non-immunosuppressive but able to dimerize chimeric FKBP fusions.[29] Based on these studies, we sought to explore CM as a means to develop modular building blocks for the rapid creation of FKBP-binding CIDs.

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2. Results and discussion

Our initial efforts focused on optimizing the microwave-accelerated coupling between FK506 and a representative olefin, 3,4-epoxy-1-butene (Fig. 1). Recently, Chapman et al. identified a narrow window of conditions for efficient GII-mediated, microwave-accelerated, ring-closing metathesis on peptide substrates.[30] Guided by this effort, we systematically varied the reaction conditions and found that microwave irradiation for 5 min yielded good conversion rates (40–64%), while shorter (1–2 min) or longer (10–20 min) irradiation times reduced the yields (Fig. 2A). Additionally, by varying temperature, we identified a clear peak of efficiency at 150 °C. Higher temperatures resulted in lower yields and greater side-product formation, while reactions at lower temperatures consisted mostly of unreacted starting materials (Fig. 2B). Importantly, when a 20-fold molar excess of the olefin was used, these reactions proceeded readily on unprotected FK506, while lower ratios favored formation of FK1012-like homodimers.

As the success of microwave-accelerated reactions is dependent on absorption of microwave energy by the reaction medium, we examined a variety of solvents and monitored production of the desired CM product (**1**) by liquid chromatography–mass spectrometry (LC–MS). Using this screening approach, we found that 1,2-dichlorobenzene (DCB) and 1,2-dichloroethane (DCE) produced the highest yields (61–64%; Table 1). Other solvents with strong dielectric constants, such as ethanol, were poor choices because of reagent insolubility.

With these results in hand, our next goal was to evaluate a series of target olefins. Structural and chemical diversity were key in our selection criteria, as we hoped to produce a range of FK506 intermediates containing orthogonally reactive electrophiles and nucleophiles for the synthesis of CIDs. Accordingly, we prepared a series of reactions using 21 different target olefins and 10% GII catalyst irradiated in a sealed tube at 150 °C for 5 min in DCB (Table 2). Based on the work of Chatterjee et al., we hypothesized that target olefins bearing relatively bulky and electron-withdrawing groups might provide the highest yielding CM reactions.[31] In agreement with this concept, some of the compounds with carbonyls or carboxylates close to the reacting olefinic center had the highest conversion efficiency. For example, acrylate gave an excellent yield (99%), as did olefins with acrylic or benzoic acids (79–99%). Some of the halogenated olefins, such as allyl bromide, had low yields, which MS experiments confirmed was due to the propensity of the unhindered allyl halide to undergo rapid, non-productive homodimerization. Poor yields were also observed for free amines, such as allylamine and 3-butenylamine (less than 5%), while protecting with Boc anhydride significantly improved reaction yield for the allylamine reaction (50%). Finally, for a subset of olefins that were only mildly soluble in DCB, we explored isopropanol as an alternative solvent. This strategy improved the solubility of all the chosen substrates and the yield of the CM reaction involving acryloyl chloride improved from 6% to 88%; however, this solvent did not significantly improve the reaction efficiency for the other olefins.

From these studies, we selected FK506 derivatives with the most favorable combination of properties (e.g., yield, solubility and reactive functionality) for re-synthesis and expanded studies. No effort was made to separate *E* and *Z* isomers, because these

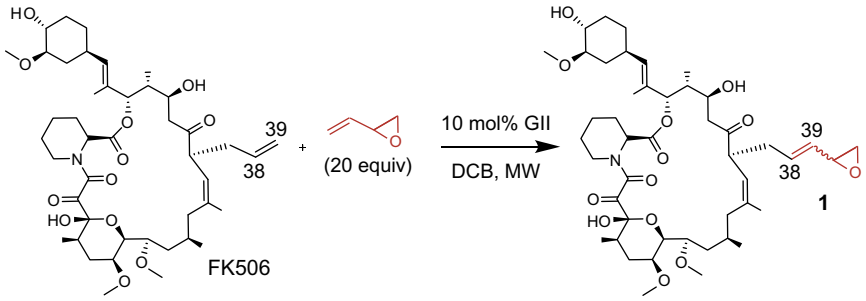


Figure 1. Cross metathesis of 3,4-epoxy-1-butene to FK506 creates a reactive derivative (1). Grubb's ruthenium catalyst (GII), FK506 and the epoxide-bearing olefin were irradiated in 1,2-dichlorobenzene (DCB).

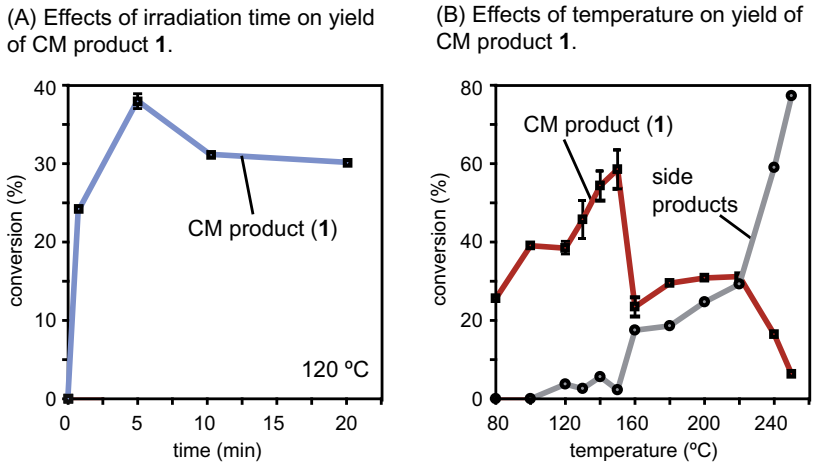


Figure 2. Varying the reaction time and temperature reveals optimal CM conditions. Results are the average of three independent reactions with the standard error shown. Yields were estimated from quantitative LC–MS profiles, as described in Section 4. DCB was used as the solvent in all the reactions.

Table 1
Summary of cross metathesis reactions between FK506 and 3,4-epoxy-1-butene in different solvents (5 min, 150 °C)

Solvent	Yield (%)
1,2-DCB	64
1,2-DCE	61
DCM	Trace
DMF	20
ACN	3
Isopropanol	42
1-Butanol	18
EtOH	5
MeOH	4
DMSO	2

Table 2
Summary of cross metathesis reactions between FK506 and functionalized olefins

Olefin	Solvent	Yield (%)
None	DCB	4 (FK1012)
Acrylamide	DCB	99
Acrylic acid	DCB	99
Acrylic acid, succinimidyl ester	DCB	25
Acryloyl chloride	DCB	6
	Isopropanol	88
Allylamine	DCB	Trace
Allylbromide	DCB	11
Allylchloroformate	DCB	12
2-Allylchlorohexanone	DCB	44
Allylisothiocyanate	DCB	27
Allylisocyanate	DCB	22
N-Boc allylamine	DCB	50
3-Butene-1-ol	DCB	39
3-Butenylamine	DCB	5
	Isopropanol	2
3,4-Epoxy-1-butene	DCB	56
5-Hexenitrile	DCB	36
Methyl acrylate	DCB	97
5-Norbonene-2-carboxylic acid	DCB	8
	Isopropanol	7
4-Pentenoyl chloride	DCB	25
4-Pentynoic acid	DCB	Trace
	Isopropanol	Trace
4-Vinylaniline	DCB	5
4-Vinylbenzoic acid	DCB	79
	Isopropanol	9

have been found to be equally active in biological assays.[29] Following HPLC purification to remove trace amounts of unmodified FK506, a small collection of derivatives (**1**–**6**) suitable for the generation of CIDs was produced (Fig. 3).

Because these molecules are intended for use in the exploration of biological systems, we were also interested in finding efficient ways to remove ruthenium byproducts. Even trace amounts of these organometallic complexes can lead to olefin isomerization, product decomposition and cell toxicity. Several relevant purification methods have already been explored, including the use of tris-(hydroxymethyl)phosphine, triphenylphosphine oxide, and dimethyl sulfoxide.[32–34] However, these procedures require multiple reagents, subsequent column chromatography, and relatively long exposure times for maximal efficacy. Here, we explored a quick and efficient strategy using silica-bonded equivalents of the metal scavenger, 1-propanethiol. We found that treatment of the crude reaction mixture with 5 equiv of resin for 30 min reduced the levels of the ruthenium byproducts by greater than 50-fold (Fig. 4). This simple method compared favorably to other approaches, especially in the minimal handling steps and short times required.

One of the potential advantages of using FK506 as a module in the synthesis of bifunctional molecules is its superior affinity for FKBP, especially when compared to SLF.[24] We recently reported that appending SLF to an HIV protease inhibitor partitions the resulting molecule into blood cells and prolongs its lifetime 20-fold in vivo.[35] Evidence suggests that this improved persistence results from the modified compound being sequestered into the cytoplasm of the blood cells, which is an environment that contains high levels of FKBP but is nearly devoid of the P450 enzymes that metabolize xenobiotics. Based on this model, we reasoned that cellular partitioning and drug lifetime might be sensitive to the affinity for FKBP.[19] Therefore, as a proof-of-concept for the present work, we sought to generate a fusion between FK506 and an HIV protease inhibitor, which would allow us to directly compare SLF- and FK506-conjugated molecules. Towards that goal, we coupled the reactive FK506 derivative **2** in a single step to an amprenavir-like core to yield the bifunctional molecule **7** (Fig. 5A). Coupling of the core inhibitor to the FK506 derivative occurred through a pendant amine, which is known to be a well tolerated attachment point (Fig. 5B).[35,36]

To test the influence of FKBP binding on cellular partitioning, we first examined the distribution of 4-methoxy amprenavir in whole blood and, consistent with previous studies, we found that it distributed evenly between the plasma and cellular compartments (Fig. 6). Next, we confirmed that the corresponding SLF-modified derivative **8** showed a strong (~9-fold) preference for the cellular fraction (Fig. 6).[35] Remarkably, the FK506-modified derivative **7** was concentrated by more than ~22-fold in the blood cell compartment. Thus, replacing SLF with FK506 exaggerated cellular partitioning by more than twofold, consistent with an important role for FKBP-binding affinity. These results clearly illustrate one advantage of using orthogonally reactive FK506 derivatives to synthesize new CIDs.

3. Conclusions

The goal of this effort was to generate reactive building blocks for CID applications. Accordingly, we systematically investigated several reaction variables and identified conditions suitable for microwave-accelerated CM and diversification at the C39 position of FK506. These efforts afforded reactive analogs (**1**–**6**), which present either electrophiles or nucleophiles. These derivatives are designed for facile installation into parent compounds bearing the corresponding functionality. One key feature of this strategy is that the reactive intermediates are accessed in a single step using unprotected FK506. During the course of these studies, we also explored a scavenger resin-based method for removing ruthenium byproducts, which may be particularly advantageous for parallel syntheses of CIDs. Finally, as demonstrated by the synthesis of the bifunctional protease inhibitor **7**, these methods are expected to provide a convenient and modular platform for creating FKBP-binding CIDs with interesting new properties.

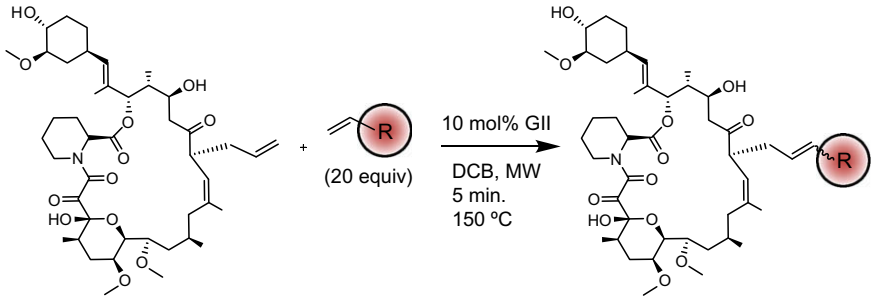
4. Experimental methods

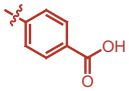
4.1. Synthetic methods

Solvents and olefins were purchased from Sigma or ThermoFisher and used without further purification. FK506 was purchased from LC Labs. All mass spectrometry results collected on a Shimadzu LC–MS using an APCI probe in negative mode. All NMR spectra collected on a Varian 600 MHz system, with the samples dissolved in DMSO-*d*.

4.2. General procedure for cross metathesis

To a solution of FK506 (2.0 mg; 0.00249 mmol) in dichlorobenzene (2.0 mL) was added the target olefin (20 equiv; 0.0498 mmol)



cmpd	R =	yield	cmpd	R =	yield
1		55-60%	4		75-80%
2		>95%	5		35-40%
3		>95%	6*		45-50%

*following Boc deprotection

Figure 3. Synthesis of reactive FK506 derivatives. From the screen of 21 olefins, these FK506 derivatives were chosen for re-synthesis, scale up and characterization. Yields are representative of at least two independent syntheses.

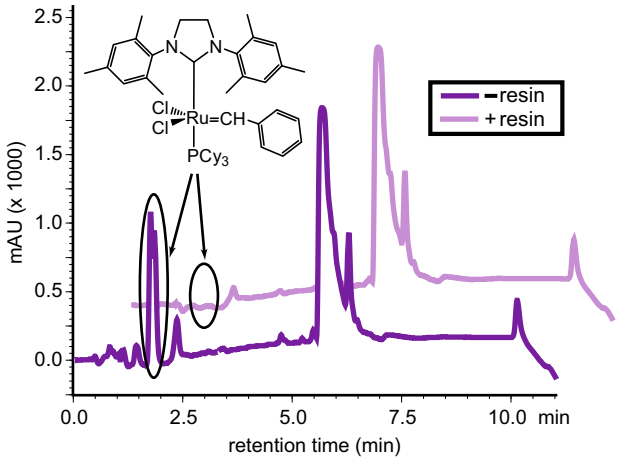


Figure 4. Removal of ruthenium byproducts by scavenger resin. LC–MS traces of a CM reaction between FK506 and 3,4-epoxy-1-butene is shown before and after treatment with 5 equiv of resin (see Section 4). The peak area of the ruthenium byproducts was reduced by greater than 50-fold, without any appreciable reductions in the product peak. Results are representative of the traces from other reactions.

followed by catalyst (0.1 equiv; 0.211 mg; 0.249 μmol) in a microwave vial. The vial was then sparged with nitrogen, sealed, and placed in a Biotage Initiator EXP microwave reactor. The mixture was stirred for 30 s, followed by irradiation at the desired temperature and time. To achieve the desired temperature, the microwave power was typically ~20 W. To quantify reaction progress, crude mixtures were placed in 1.5 ml vials and analyzed with a Shimazdu (Columbia, MD) series 2010EV Liquid Chromatograph Mass Spectrometer equipped with an APCI probe to minimize ion suppression. Samples (5.0 μL) were injected onto 1.8 μm ZORBAX Eclipse XDB-C18 column (Agilent), and elution was performed with a stepwise 10–100% gradient of acetonitrile over 11 min. Products eluted between 5.5 and 6 min, while the peak for unreacted FK506 was found at 6.7 min. Quantification was performed using LCMSolution Version 2.05 using external standards. For the CM reactions that were selected for further analysis, the scale was increased 10-fold and the resulting crude reactions were treated with silica-immobilized, 1-propanethiol (5 equiv; Biotage; Isolute Si-Thiol) for 30 min, filtered and then the product was purified by preparative chromatography on a Waters Spherisorb S10 ODS2 column (10 × 250 mm). For compound **6**, the crude product was additionally treated with 5 equiv of 1:1 TFA:DCM, followed by dilution into DCM, neutralization with 5% sodium bicarbonate and concentration by roto-evaporation prior to HPLC. See the Supplementary data for characterization (^{1}H NMR and mass spectrometry).

4.3. Synthesis of bifunctional HIV protease inhibitor (7)

As a test of the modular assembly of bifunctional molecules, the reactive FK506 derivative **2** (1.0 equiv; 15.8 mg; 0.0186 mmol) was added to the advanced HIV protease inhibitor (1.2 equiv; 9.1 mg; 0.0224 mmol) in 3.0 mL of DMF with DIC (10.0 equiv; 29.2 μL; 0.1863 mmol), HOBt (1.0 equiv; 2.5 mg; 0.1863 mmol) and DMAP (1.0 equiv; 2.3 mg; 0.1863 mmol). This reaction was stirred at room temperature overnight and the resulting product **7** purified in 75% yield by HPLC as described above. The blood partitioning experiments were performed as described.[35] The synthesis of the amprenavir core structure and compound **8** have been reported.[35] See the Supplementary data for characterization (^{1}H NMR and mass spectrometry). FK506 coupled to HIV protease inhibitor (**7**); molecular formula $C_{66}H_{97}N_3O_{17}S$; expected mass (*m/z*) = 1236.55; actual mass (*m/z*) = 1235.95. δ 7.74(d), δ 7.63(s), δ 7.57(d), δ 7.51(s), δ 7.44(d), δ 7.36(s), δ 7.28(t), δ 7.19(s), δ 7.11(t), δ 6.9(d), δ 6.86(d), δ 5.42(s), δ 5.36(s), δ 5.27(s), δ 5.125(dd), δ 5.04(d), δ 4.8(m), δ 4.7(d), δ 4.6(s), δ 4.48(s), δ 4.24(d), δ 3.9(s), δ 3.87(d), δ 3.81(s), δ 3.78(s), δ 3.71(s), δ 3.62(d), δ 3.54(d), δ 3.41(dd), δ 3.33(d), δ 3.28(d), δ 3.22(s), δ 2.9–3.08(m), δ 2.91(d), δ 2.78(m), δ 2.72(m), δ 2.27(s), δ 2.1(s), δ 2.0(m), δ 1.92(s), δ 1.86(m), δ 1.8(d), δ 1.72(d), δ 1.65(d), δ 1.62(s), δ 1.55(s), δ 1.48(m), δ 1.39(s), δ1.26(t), δ 1.21(t), δ 1.14(t), δ 1.03(m), δ 0.86(d), δ 0.81(d), δ 0.79(d), δ 0.73(d).

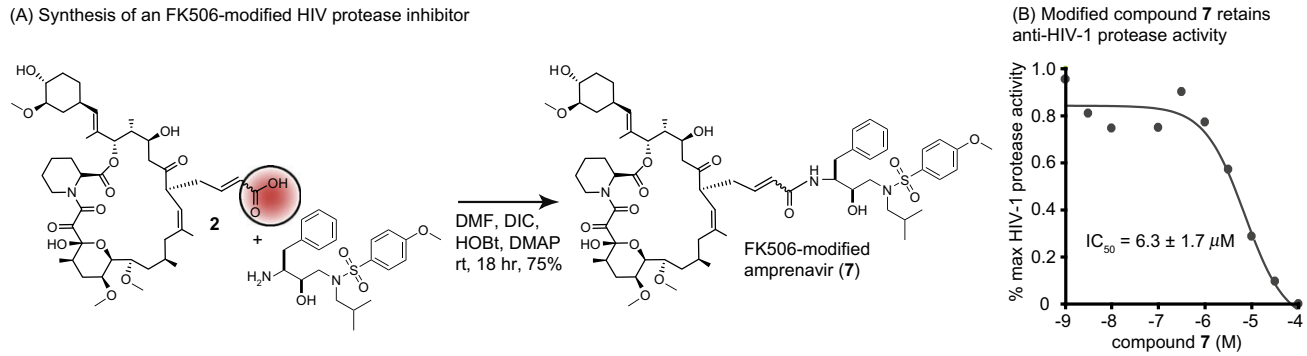


Figure 5. Modular synthesis of a modified HIV protease inhibitor by installation of FK506. (A) Reactive intermediate **2** was coupled to the known core of an HIV protease inhibitor to produce the bifunctional molecule **7** in good purified yield. (B) The FK506-modified inhibitor **7** retains anti-protease activity in vitro. Results are the average of triplicates.

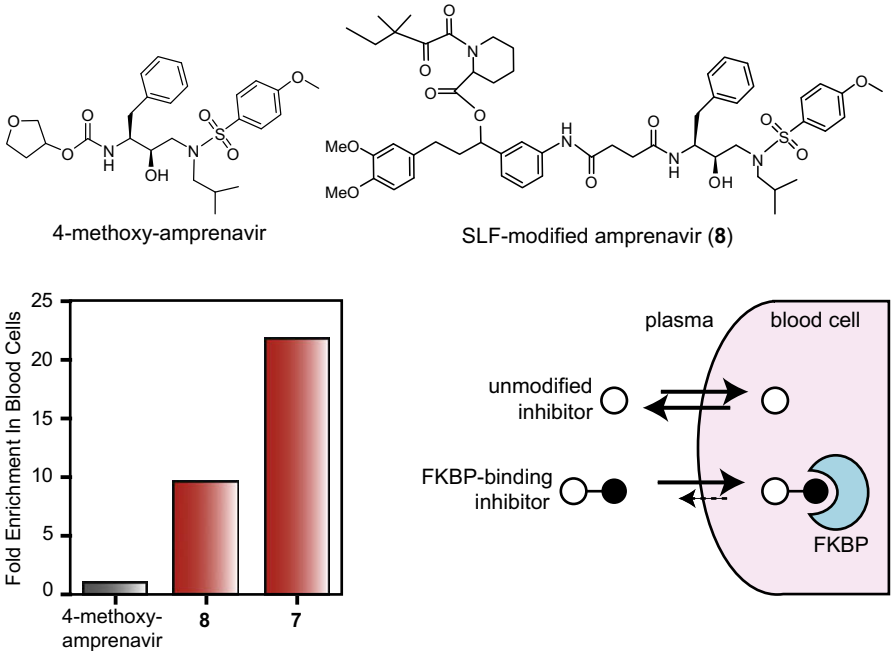


Figure 6. An FK506-coupled HIV protease inhibitor is partitioned into blood cells. Fresh whole blood from male C57BL/6 mice was treated with either 4-methoxyamprenavir, SLF-conjugated amprenavir (**8**) or FK506-modified amprenavir (**7**) at 100 μM. After 6 h at 37 °C, whole blood was separated into plasma and cellular components by centrifugation and the compound in each fraction quantified by LC–MS. The fold partitioning was determined by dividing the cellular concentration by the concentration in the plasma. Error of triplicate experiments was approximately 10%. The schematic shows the model for how high affinity binding to cytoplasmic FKBP might deplete modified compounds from plasma.

4.4. HIV protease assay

To determine the inhibitory potency of compound **7**, we used a commercially available, FRET-based assay (Bachem, Torrance, CA), as previously reported.[35] Briefly, HIV-1 protease was preincubated with inhibitor for 60 min at 37 °C, followed by addition of 30 nM HIV-1 protease substrate 1 (Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(dabcyl)-Arg; Molecular Probes, Eugene OR). Substrate cleavage was measured for 60 min. at 37 °C by monitoring fluorescence at 490 nm on a SpectraMax M5 plate reader (Molecular Devices, Mountain View, CA).

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Supplementary data

Synthesis and ^{1}H NMR spectra of modified FK506 derivatives and a complete record of all the reactions. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.07.030.

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