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One-step Heck Reaction Generates Nonimmunosuppressive FK506 Analogs for Pharmacological BMP Activation

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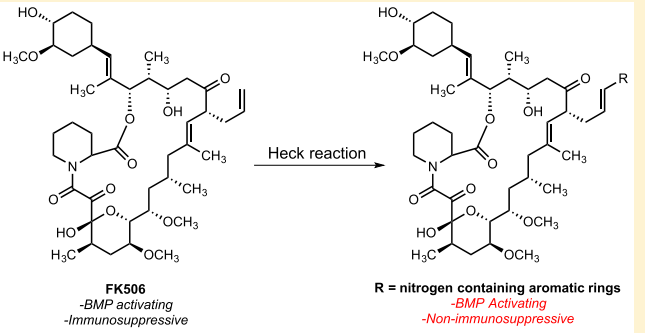
Supporting Information

ABSTRACT: FKBP12 ligands such as FK506 have been shown to activate the BMP signaling pathway and facilitate tissue regeneration. However, the immunosuppressive activity of FK506 limits its clinical application. Using Heck reaction, we generated nonimmunosuppressive analogs of FK506 by fusing heterocycles to the calcineurin (CN) binding domain of FK506. Structure−activity relationships provided novel mechanistic insights into the FK506−CN interaction that can be exploited for rational design of future analogs.



KEYWORDS: *FKBP ligands, regenerative medicine, nonimmunosuppressive, BMP signaling, SMAD1/5, FKVP, calcineurin*

FK506 (tacrolimus), a 23-membered macrocyclic natural product isolated from *Streptomyces tsukubaensis*, has been widely used as an immunosuppressant for prevention of allogeneic transplant rejection.[1,2] The immunosuppressive activity of FK506 results from its inhibition of T cell receptor-mediated signal transduction. At a molecular level, FK506 binds FK506-binding proteins (FKBPs) upon entering cells.[3] The resultant FKBP12−FK506 complex forms a ternary complex with the protein phosphatase calcineurin, allosterically blocking access to its active site,[4−6] preventing dephosphorylation of the nuclear factor of activated T cells (NFAT) by calcineurin and its ensuing nuclear translocation and transcriptional activation of IL-2 and some other cytokine genes.[4]

In addition to facilitating the interaction between FK506 and calcineurin, FKBP12 has been shown to interact with several other proteins including TGF-β/BMP type 1 receptors.[7−10] FK506 has been shown to exhibit unique pharmacological activities unrelated to immunosuppression and has potential for treating diseases such as pulmonary hypertension[11] and bladder cancer.[12]

Recently, we synthesized a novel nonimmunosuppressive FK506 analog, named FKVP (FK506-C40-py), that retained FKBP binding and lacked calcineurin inhibition activity (Figure 1). FKVP was found to activate Bone Morphogenic Protein (BMP) signaling in lymphocytes and endothelial cells through disruption of the FKBP12−BMPR1 interaction. Moreover, the combination of FKVP and AMD3100, an antagonist of the CXCR4 receptor that is capable of mobilizing hematopoietic stem cells from the bone marrow, was found to accelerate wound healing in diabetic rats in a BMP-dependent manner.[13]

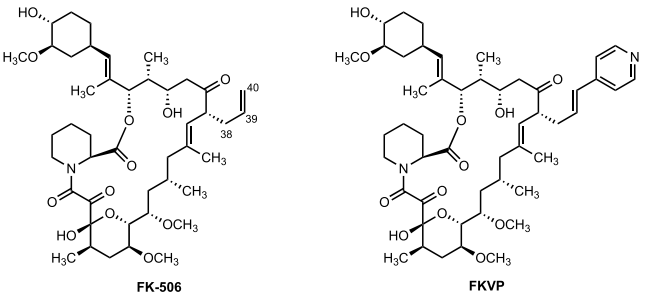


Figure 1. Structures of FK-506 and FKVP.

In the past, we and others have relied on ruthenium-catalyzed cross metathesis (CM) reaction to directly modify the terminal alkene of FK506 (C40) to generate non-immunosuppressive FK506 analogs.[14−17] Unfortunately, FKVP and other nitrogen-containing analogs could only be obtained in very low yields (<10%) under CM reaction conditions. This is most likely due to the nitrogen lone electron pair that competitively coordinates to the ruthenium metal center.[18] The use of soluble tosylated salts of amines could improve yields,[19] but it did not help in the synthesis of FKVP. Another reported functionalization method toward FK506 terminal olefin is thiol-ene "click" reaction,[20] which

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requires more than one step to synthesize the nitrogen-containing FK506 analogs.

Aside from the ruthenium-catalyzed CM reaction, the palladium-catalyzed Heck reaction also allows for modifications of terminal alkenes, making the Heck reaction a viable alternative for synthesizing FKVP. Importantly, palladium catalysts used in the Heck reaction are compatible with nitrogen-containing heterocycles such as pyridine.[21−24] Moreover, halogen-containing heterocycles are commercially available and inexpensive. After optimizing the reaction conditions for the production of FKVP (1b),[25] we found that reacting FK506 with 4-iodopyridine (2.0 equiv) in the presence of $Pd(OAc)_2$ (10 mol %) and P(*o*-tol)$_3$ (20 mol %) in DMF at 100 °C gave the best yield (66%, Table S1). These conditions can be applied toward production of several new FKVP analogs.

It was previously reported that when the terminal olefin (C40) of FK506 is linked to small aromatic groups such as phenyl and β-naphthyl group, the resulting FK506 analogs retained most of the inhibitory activity against calcinerurin.[15] In contrast, FKVP lost most of its activity against calcineurin with negligible effect at concentrations up to 10 μM,[13] even though pyridine is smaller than naphthalene. To further explore this apparent paradoxical observation, we conducted a structure−activity relationship study to gain new insight into the interactions between FK506 analogs and calcineurin. Using FKVP as a starting point, we selected three pyridinyl-halides, three quinolyl-halides, and four aniline-halides as substrates for the Heck reaction. To our delight, all halide substrates were successfully coupled to FK506 with moderate-to-good yields (Table 1).

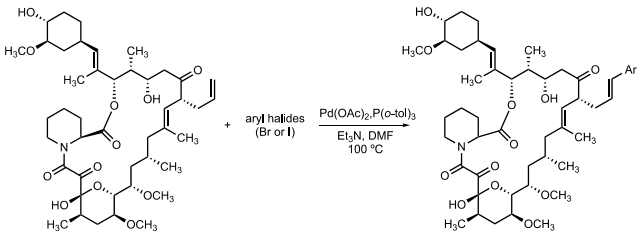
The halide substrates displayed distinct reactivity in the Heck reaction. First, bromides and iodides gave similar yields. Second, electron-withdrawing groups on the pyridines and quinolines appeared to increase the yields (entry 4,6,7). Third, unprotected anilines gave the lowest yields (entry 9−11). Importantly, the unreacted FK506 starting material in the reaction mixture was easily separated from the more polar nitrogen-containing products with flash chromatography, which posed a major problem for the purification of other related FK506 analogs.[15]

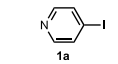
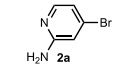
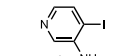
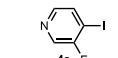
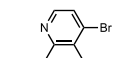
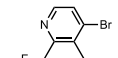
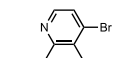
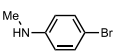
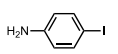
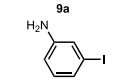
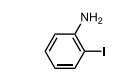
With FK506 analogs in hand, we assessed their effects on cell viability, BMP activation, and NFAT activation at two concentrations, 1 μM and 10 μM (Figure 2). In a cell viability assay using donor-pooled human umbilical vein endothelial cells (HUVEC), we found that quinoline analogs (**5b**−**7b**) inhibited cell proliferation at 10 μM (Figure 2a), while other compounds were comparable to FK506 in their cytotoxicity.

We next used a BMP-response-element (BRE) pathway reporter (luciferase under the control of the ID1 promoter) in Jurkat T cells to determine whether the new analogs were capable of activating the BMP signaling pathway.[11] Initial screening of the compounds showed that most analogs had similar activity as FK506 or FKVP. This is somewhat expected, as we have previously shown that FKBP12 binding is necessary and sufficient for activation of BMP signaling (Figure 2b).

To determine the effects of the analogs on calcineurin, we employed a PMA/ionomycin-activated Nuclear Factor of Activated T-cells (NFAT) reporter in Jurkat T cells[15] (luciferase under the control of the IL-2 promoter) (Figure 2c). Two analogs (**2b**, **3b**) did not cause significant inhibition of the NFAT-Luciferase reporter at concentrations up to 10 μM, similar to FKVP (**1b**). Surprisingly, most other analogs

Table 1. Heck Reaction of FK-506 with Aryl Halides[a]



Entry	Aryl Halide	Product (Ar =)	Time / h	Yield (%)[b]
1	 **1a**	 **1b**	24	66
2	 **2a**	 **2b**	24	42 (52)[c]
3	 **3a**	 **3b**	24	40 (54)[c]
4	 **4a**	 **4b**	24	78
5	 **5a**	 **5b**	18	72
6	 **6a**	 **6b**	18	70
7	 **7a**	 **7b**	18	68
8	 **8a**	 **8b**	20	62
9	 **9a**	 **9b**	12	36
10	 **10a**	 **10b**	12	42
11	 **11a**	 **11b**	12	45

[a]Reaction condition: FK-506 (0.050 mmol), aryl halide (0.10 mmol), $Pd(OAc)_2$ (0.0050 mmol), P(*o*-tol)$_3$ (0.010 mmol), and Et_3N (0.10 mL) in DMF (1.0 mL) at 100 °C under Ar. [b]Isolated yield. [c]Yield in parentheses is based on FK-506 recovery.

showed either partial or nearly complete inhibition of the NFAT reporter at 1 μM (Figure 2c). It is noteworthy that some of the immunosuppressive analogs, including **6b** and **7b**, have bulkier substituents than **2b** and **3b** due to the presence of a fused aromatic ring. How those bulkier groups are accommodated at the binding site of calcineurin remains to be determined.

We determined the EC_{50} values of the three non-immunosuppressive analogs (**1b**−**3b**) in the BMP luciferase assay. All three analogs were found to be slightly more potent than FK506 (Figure 3a, Table S2), likely attributable to increased solubility of the more polar pyridine substituents. In addition, we observed dose-dependent induction of SMAD1/5 phosphorylation by compounds **2b** and **3b** in Jurkat T cells,

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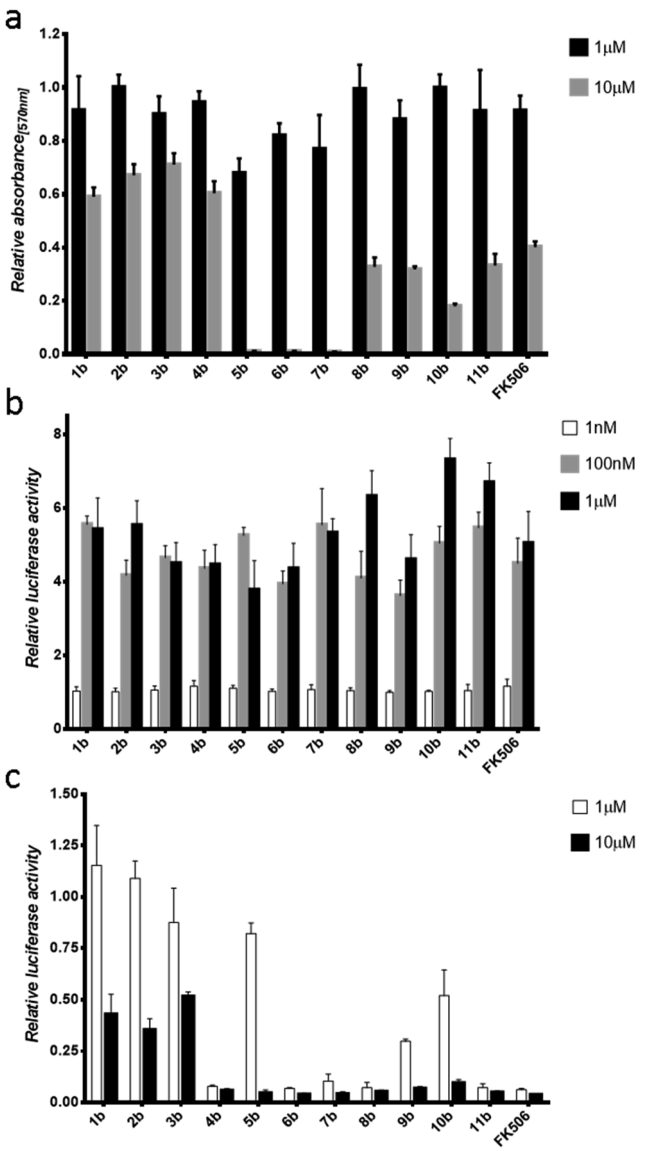

Figure 2. FK506 analogs display variable immunosuppressive qualities. (a) Cell viability after 72 h analog treatment in HUVEC cells. (b) All analogs activate a BMP pathway reporter in Jurkat cells with similar potency to FK506. (c) Derivatives show structure-dependent effects in NFAT reporter inhibition in Jurkat cells. Error bars represent standard deviation from the mean for all measurements (n = 3), and absorbance/luminescence values were normalized to DMSO-treated cells.

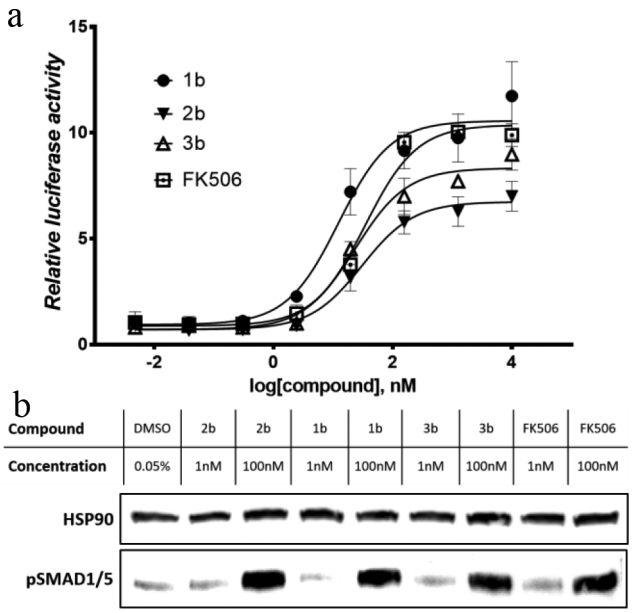

Figure 3. (a) Dose–response curves for BMP reporter activation by three nonimmunosuppressive analogs (**1b**, **2b**, and **3b**) and FK506. (b) Activation of SMAD1/5 phosphorylation in Jurkat T cells by selected analogs. Cells were treated with the compounds for 2 h. HSP90 was used as a loading control.

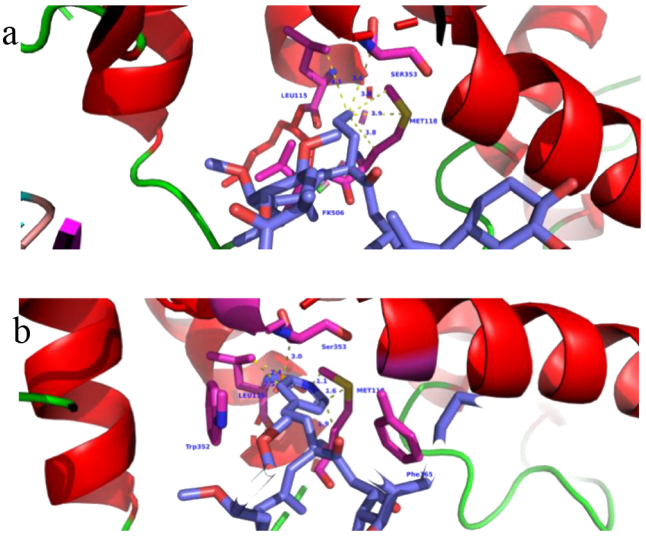

Figure 4. Binding detail toward calcineurin. (a) Close-up of FK-506 terminal olefin with calcineurin. (b) Steric effect of FKVP with calcineurin.

consistent with the previously observed effect of both FK506 and FKVP (Figure 3b).

The structure of the FKBP12–FK506–calcineurin complex was previously determined by X-ray crystallography.[5,6,15] In this complex, the terminal alkene of FK506 fits into a binding pocket in calcineurin formed by hydrophobic amino acids (Figure 4a). When modeled in place of FK506, the pyridine moiety in FKVP (**1b**) shows a steric clash with the side chain of M118 of calcineurin (Figure 4b), which may explain the elimination of calcineurin binding by analogs **1b**–**3b**. It was previously shown that both phenyl and β-naphthyl substitution at C40 of FK506 allowed for significant retention of calcineurin inhibition.[15] How the β-naphthyl analog, which contains bulkier substituents than FKVP at the same position, remains active against calcineurin cannot be explained by steric clash alone.

As steric hindrance cannot explain the difference in calcineurin inhibitory activity among the existing analogs, we turned our attention to electrostatic status of the basic nitrogen-containing analogs. We noticed that heterocycles with higher pK_a values (**1b**, **2b**, **3b**, and **5b**) showed less calcineurin inhibition. Conversely, those with a lower pK_a caused by electron-withdrawing groups were all immunosuppressive at 1 μM (**4b**, **6b**, and **7b**).[26] These observations suggest that the formation of positively charged pyridinium and quinolinium appendage at the terminal alkene of FK506 play a more important role in disrupting the interaction

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between the terminal alkene of FK506 and the hydrophobic pocket in calcineurin. These observations reveal an alternative and complementary mechanism for the loss of calcineurin inhibition in nonimmunosuppressive FK506 analogs, which in the past has been rationalized by a large molecular "bump" to sterically hinder calcineurin binding.[15] It is likely that the same binding pocket in calcineurin has significant conformational flexibility to accommodate noncharged bulky aromatic rings such as those present in **4b**, **6b**, and **7b**.

It is important to note that the predominant electrostatic effects of C40 substituents on inhibition of calcineurin only holds true within the same family of heterocycles containing the pyridine core. The pK_a value of aniline (**9b**−**11b**) is slightly higher than methylaniline (**8b**), but methylaniline with the highest pK_a (**8b**) showed the strongest calcineurin inhibition at 1 μM. It is possible that the additional methyl group may forge favorable interaction with the hydrophobic pocket of calcineurin, overcoming the unfavorable electrostatic effect.

In summary, we have developed a one-step synthesis of FK506 analogs to couple FK506 with small nitrogen-containing heterocycles using the Heck reaction. We have identified a new class of nonimmunosuppressive analogs with diverse structures and high potency in activating the BMP signaling pathway. Moreover, these basic nitrogen-containing FK506 analogs are easy to separate from FK506, allowing for bulk synthesis of the analogs for future preclinical studies. Elimination of calcineurin inhibition rendered those analogs not only nonimmunosuppressive but also free of nephrotoxicity and neurotoxicity, which are caused by inhibition of calcineurin.[27,28] In the SAR study, we found that electrostatic effect can play a dominant role in disrupting FK506−calcineurin interaction among the heterocycle series. Given the role of BMP signaling in wound healing and tissue regeneration,[13] our newly established method for synthesizing and identifying nonimmunosuppressive BMP agonists will facilitate the development of nonimmunosuppressive analogs of FK506 for regenerative medicine and other BMP-related diseases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.9b00144.

Experimental procedures and characterization of compounds **1b**−**11b** (PDF)

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Author Contributions

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Notes

The authors declare no competing financial interest.

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(25) See details in the Supporting Information.
(26) We calculated the pK_a of each analog by Marvin software using QSPR algorithm. This prediction is , however, informative and relatively accurate. Liao, C.; Nicklaus, M. C. Comparison of Nine Programs Predicting pK_a Values of Pharmaceutical Substances. *J. Chem. Inf. Model.* **2009**, *49*, 2801−2812. pK_a is a measurement of proton affinity, which helps us evaluate the extent of electrostatic repulsion but should not be treated as experimental values especially in the protein microenvironment. pK_a calculated for substituted FKBP-: pyridine, 5.4; 3-amino pyridine, 5.98; 2-amino pyridine,7.14; 3-fluoro pyridine, 3.47; quinoline, 4.87; *o*-aniline, 4.04.
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