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A calcineurin antifungal strategy with analogs of FK506

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ABSTRACT

A novel antifungal strategy targeting the inhibition of calcineurin is described. To develop a calcineurin based inhibitor of pathogenic fungi, analogs of FK506 were synthesized that were able to permeate mammalian but not fungal cells. Antagonists in combination with FK506 were not immunosuppressive and retained antifungal activity in *A. fumigatus*. To reduce the dosage burden of the antagonist, murine oral PK was improved an order of magnitude relative to previous FK506 antagonists.

Treatment options for systemic fungal infections are limited, with mortality rates for systemic aspergillosis reported to be over 50%.[1] To improve treatment and circumvent resistance, new antifungal therapies with novel mechanisms of action are needed. Calcineurin (CN), a calcium signaling protein is a Ca^{+} sensitive serine/threonine protein phosphatase. It is a heterodimeric protein consisting of a catalytic subunit, calcineurin A, and a Ca(2+)-binding regulatory subunit, calcineurin B. Calcineurin is essential for growth of *Cryptococcus neoformans* and important for virulence and pathogenicity in *Aspergillus fumigatus*, two important pathogenic fungi.[2] FK506 is a natural product that inhibits calcineurin signaling. It inhibits the growth of *C. neoformans* and *A. fumigatus* with MIC values of 0.01 and 0.025 μg/mL respectively.[2,3] FK506 inhibits calcineurin activity by a unique, small molecule-mediated, protein-protein interaction.[4] FK506 binds to FKBP12, and this binary complex binds to calcineurin and inhibits its phosphatase activity and downstream signaling.

FK506 is not effective as an antifungal therapy for systemic fungal infections because FK506, clinically known as Tacrolimus, is a potent immunosuppressant. An intact immune system is believed to be an essential component for clearing systemic fungal infections.[5] Tacrolimus is an essential clinical drug used to dampen the immune response for organ transplant patients.[6] A consequence of the inhibition of calcineurin is the abrogation of both the innate and adaptive human immune response via the inhibition of NFATsignaling.[7,8]

This study explores the utility of dosing a second molecule with FK506 that reverses or antagonizes the immunosuppressive effects of FK506 in human immune cells, but does not substantially lower the antifungal properties of FK506. In untreated cells, FKBP12 does not bind to calcineurin, and calcineurin is able to dephosphorylate the transcription factors, pNFAT (mammalian)[8a] or pCrz1 (fungal),[2g] leading to a normal immune response or to normal fungal growth respectively (Fig. 1A). In cells treated with FK506, a binary complex of FK506 and FKBP12, binds to calcineurin and inhibits its phosphatase activity (Fig. 1B). Inhibition of calcineurin's phosphatase activity results in immunosuppression (mammalian cells) and antifungal activity. In the presence of both FK506 and excess FK506 antagonist, the antagonist binds to human FKBP12 excluding the binding of FK506 and thus preventing its subsequent binding to calcineurin and inhibition of calcineurin activity (Fig. 1C).

Both human and fungal cells are permeable to FK506. The antagonists will be designed to be cell-permeable to mammalian cells but not to fungal cells, Fig. 2. This dichotomy will result in a functional immune system and antifungal activity when FK506

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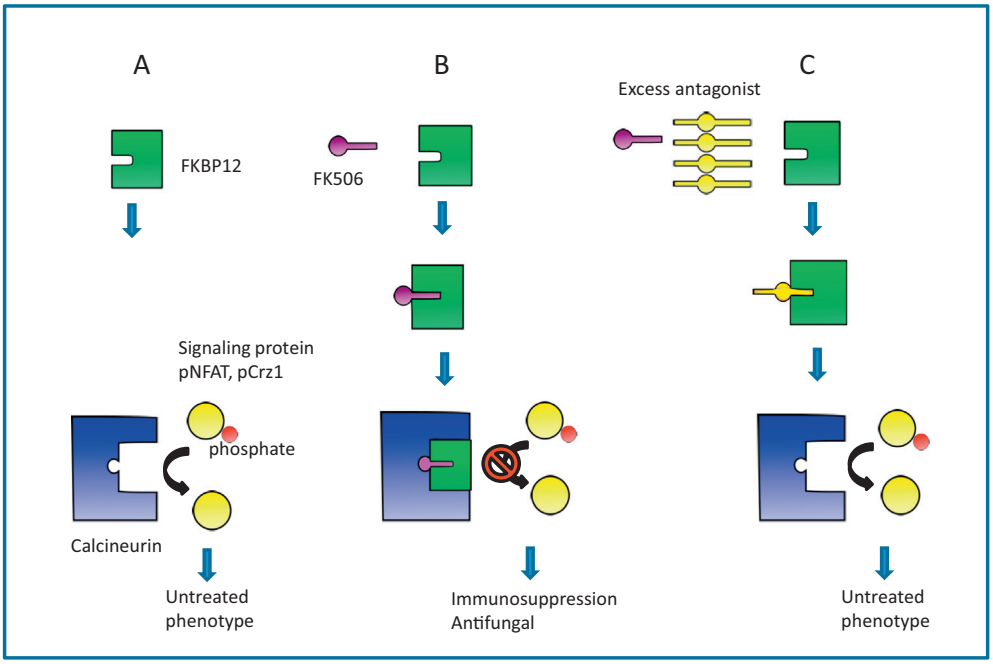


Fig. 1. A) FKBP12 does not interact with calcineurin. Calcineurin dephosphorylates pNFAT or pCrz1. B) FK506 binds to FKBP12, this binary complex then binds to calcineurin and blocks dephosphorylation of pNFAT (mammalian) or pCrz1 (fungal) leading to immunosuppression and antifungal phenotype in respective cell types. C) Addition of antagonists block FK506 from binding to FKBP12 in mammalian cells resulting in removal of immunosuppression.

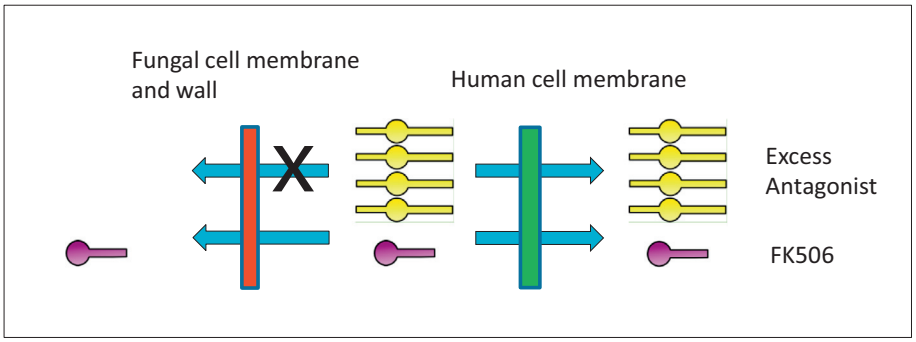


Fig. 2. Human and fungal cells are permeable to FK506. Human cells are permeable to antagonist. Fungal cells are not permeable to antagonist.

and antagonists are dosed in combination. Previous studies in our lab suggested that select FK506 analogs were less able to penetrate fungal cells compared to mammalian cells.

This study was inspired by the early work at Merck characterizing FK506 antagonists such as their C18 hydroxy FK506 antagonist compound, L-685,818, which binds to FKBP12 but does not inhibit calcineurin and is non-immunosuppressive, Fig. 3.[9,10] *In vitro* dosing of a 1000-fold excess of L-685, 818 with respect to FK506 reverses the immunosuppression caused by FK506 alone in mouse splenocytes.[10] An *in vivo* study showed that only a 10-fold excess of L-685,818 to FK506 completely reversed the depletion of mouse CD4 + 8− bearing thymocytes as observed with FK506 alone.[11]

Our studies confirmed the immunosuppressive antagonism of L-685,818 (**2**); however, it antagonized the antifungal effects of FK506 (Table 1) and possessed poor pharmacokinetic (PK) properties (Table 2). This work details the discovery of an antifungal FK506-antagonist that potently antagonizes FK506, has better cell-permeability in human cells vs. fungal cells, and shows improved PK properties compared to L-685,818 and FK506. We note that high FKBP12 binding potency and good PK are necessary to reduce the required dosing ratio of antagonist/FK506 to achieve the effects of low immunosuppression while maintaining antifungal activity.

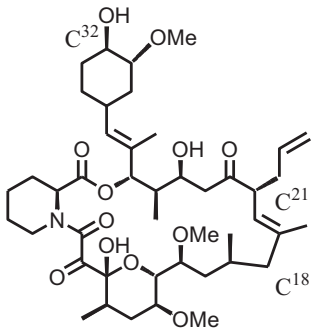
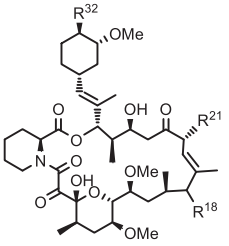
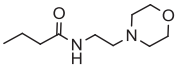
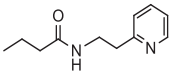
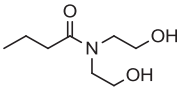
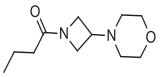
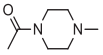
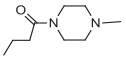
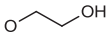
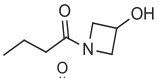
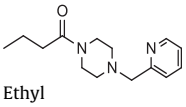
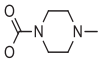
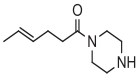
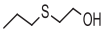
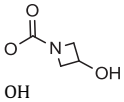
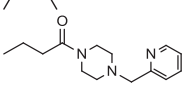
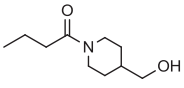
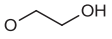


Fig. 3. FK506, L-685,818 (**2**) (OH at C18).

FK506 was chosen as the scaffold for the antagonist because it is an extremely potent binder of FKBP12. From X-ray structural analysis of the *Bos taurus* FK506/FKBP12/Calcineurin complex (1TCO),

Table 1
[a](standard deviation), [b] binding to human FKBP12, [c] binding to *A. fumigatus* FKBP12, [d]calcineurin activity, antagonists were co-incubated with FK506 (250 nM), FKBP12, calcineurin, calmodulin and phosphatase substrate, [e]IL-2 levels in stimulated Jurkat cells, [f]100-fold [antagonist] co-incubated with 10 nM FK506 with PBMCs, [g]100-fold [antagonist] co-incubated with 125 ng/ml FK506 with *A. fumigatus*, [h]only single measurement.



ID	R^{21}	R^{32}	R^{18}	FKBP12 human[b] IC_{50} nM	FKBP12 Asp[c] IC_{50} nM	CN competition human[d] IC_{50} nM	IL2[e] IC_{50} μM	Competition% proliferation PBMC[f]	Competition% proliferation *A. fumigatus*[g]
1	Allyl	OH	H	14.05 (1.13)[a]	18.45 (1.14)	NA	0.36 (0.06)	NA	NA
2	Ethyl	OH	OH	6.80 (1.22)	18.48 (1.15)	1314 (1.16)	>10	78.7 (3.1)	86.8 (4.4)
3		OH	H	11.48 (1.18)		824 (1.27)	>10	45.9 (6.3)	39.7 (2.2)
4		OH	H	14.30 (1.14)		849.8 (1.45)	>10	36.6 (2.0)	31.0 (2.0)
5		OH	H	13.39 (1.12)	22.52 (1.14)	988.6 (1.18)	>10	36.2 (6.9)	35.7[h]
6		OH	H	9.52 (1.18)	12.76 (1.14)	1077 (1.22)	>10	49.3 (1.2)	49.6 (12.3)
7		OH	H	12.61 (1.15)	16.81 (1.13)	1098 (1.17)	>10	102.8 (8.1)	43.1 (5.4)
8			H	19.1 (1.12)			>10	67.8 (5.7)	42.0[h]
9		OH	H	12.48 (1.16)		1111 (1.23)	>10	49.9 (5.2)	24.0 (1.3)
10		OH	H	11.19 (1.15)		1291 (1.09)	>10	88.3 (5.2)	47.4 (13.1)
11	Ethyl		H	10.99 (1.16)		1735 (1.09)	>10	79.8 (5.1)	68.0[h]
12		OH	H	12.59 (1.14)		1808 (1.38)	>10	27.5 (6.3)	36.5[h]
13		OH	H	27.00 (1.17)			>10	96.5 (4.7)	37.5[h]
14		OH	H	22.10 (1.14)			>10		90.5[h]
15			H	22.60 (1.14)			>10	63.0 (7.1)	22.0[h]
16		OH	H	19.20 (1.14)			>10	105.6 (12.5)	54.3 (20.0)
17		OH	H	11.19 (1.15)			>10	88.3 (5.2)	47.4[h]
18			H	23.60 (1.14)			>10	81.7 (3.3)	24.0[h]

and SAR work, vectors off C21 or C32 were expected to have little effect on FKBP12 binding. In addition, polar and larger substituents off C21 were expected to prevent binding of calcineurin. Substitutions off C32 were designed to block the known metabolic demethylation pathway[12] of the C31 methoxy group and to provide an additional handle for modifying physical and PK properties. Over one hundred analogs were synthesized with variations at these two sites.

Throughout our studies it was observed that small impurities of active calcineurin binding ligands such as FK506 or specific FK506

Table 2
Mouse PK data.

ID	IV-3mpk-CL-[L/h/kg]	IV-3mpk-V_ss-[L/kg]	IV-3mpk-Terminal_t1/2-[h]	IV-3mpk-AUC_last-[h*ng/mL]	Oral-10mpk-Tmax-[h]	Oral-10mpk-Cmax-[ng/mL]	Oral-10mpk-AUC_last-[h*ng/mL]
1	1.77	1.8	0.952	1617			
2	3.65	2.58	1.11	787	0.25	184	208
13	1.37	1.17	0.793	2114	0.5	1643	2709
14	1.37	1.01	0.714	2141			
15	0.964	1.35	1.36	2781	0.5	814	1858

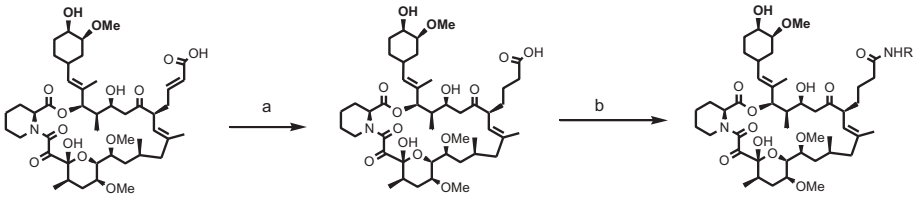


Fig. 4. Butyramides: reagents and conditions: (a) 10% Pd/C, H_2, 1 atm., 89%; (b) RNH_2, HATU, DIPEA, 30–56%.

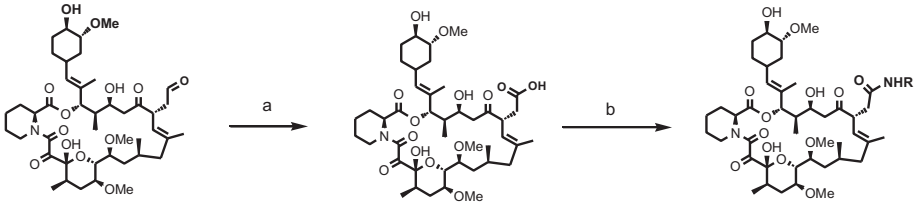


Fig. 5. Acetamides: reagents and conditions: (a) $NaClO_2$, KH_2PO_4, 2-methyl-2-butene, *t*-BuOH, 85%; (b) RNH_2, HATU, CH_2Cl_2, 30–55%.

analogs off C32 or C21 would adversely affect the interpretation of the antagonists' properties. For this reason only compounds that contained less than 0.1% calcineurin active impurities were tested. Any impurities present at this level would not affect the interpretation of the IL-2 assay's top concentration of 10 μM. Purity was assessed by LCMS set to the specific masses of the compounds of interest. Many compounds were not assayed because of the inability to meet this criterion of purity. For example, the polar C21 acrylic acid derivative of FK506 was unable to be suitably purified away from the much less polar FK506 after exhaustive chromatography and solid phase trapping efforts.

FK506 was modified at one or more accessible sites. The syntheses of the analogs are described in Figs. 4–7. Selective hydrogenation of the FK506 C21 butenoic acid[13] analogs with Pd/C followed by HATU mediated amide bond formation[14] resulted in substituted butyramides (Fig. 4). The FK506 C21-acetamides were synthesized from the Pinnick oxidation of the corresponding aldehydes[9] followed by HATU mediated amide bond formation[14] (Fig. 5). Radical initiated photoreaction of the C21 allyl group with thiols (thiol-ene reaction) is a mild and selective method to generate substituted mercaptans in good yield[15] (Fig. 6). Reductive amination[16] of the bis-TBS-protected aldehydes[9] led to tertiary amines (Fig. 7). The C32 carbamate (**11**, **15**) and the hydroxyethyl ether (**8**, **18**) combination analogs were made via the nitrophenylcarbamate intermediates[17] and from the TBS-oxyethyl triflate[18] respectively followed by substitution at C21. Selenium dioxide mediated hydroxylation[19] at C18 followed by carbamate formation led to the C18-hydroxy-C21-carbamate combinations.

A fluorescence polarization assay was implemented to determine binding of the antagonists to FKBP12 as previously described.[20] FK506 was determined to bind to human FKBP12 with an IC_{50} of 14 nM. The C21 vector of FK506 does not contact FKBP12,

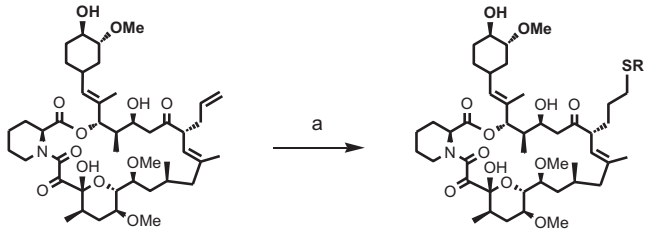


Fig. 6. Mercaptans: reagents and conditions: (a) RSH, 2-dimethoxy-2-phenylacetophenone, light, 50–80%.

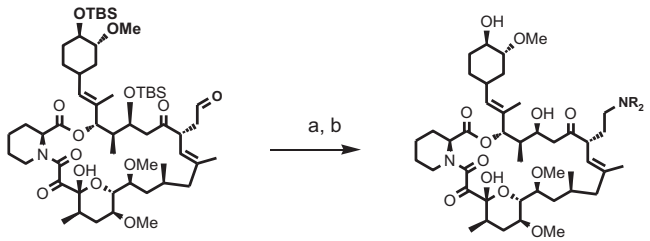


Fig. 7. Tertiary amines: reagents and conditions: (a) R_2NH, $NaBH(OAc)_3$; (b) 48% HF/ACN, 23%.

and substitution off that vector is anticipated to have a negligible effect on binding to FKBP12. As predicted, all the FK506 analogs with polar substituents off the C21 position bound tightly to FKBP12 with similar binding constants to FK506, ranging from 9 to 14 nM. A subset of compounds was tested for binding to *A. fumigatus* FKBP12. FK506 bound to *A. fumigatus* FKBP12 with an IC_{50} of

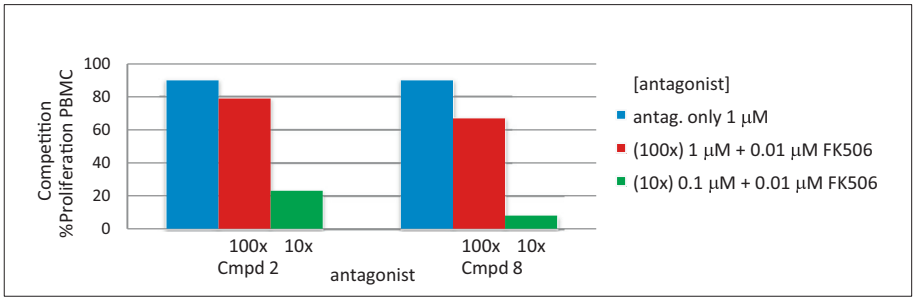


Fig. 8. Antagonism of the anti-proliferative effects of FK506 with compounds **2** and **8** on PBMCs: FK506 (0.01 μM) dosed with increasing levels of antagonist, **2** and **8**, (10× and 100×) with respect to proliferation of PBMCs.

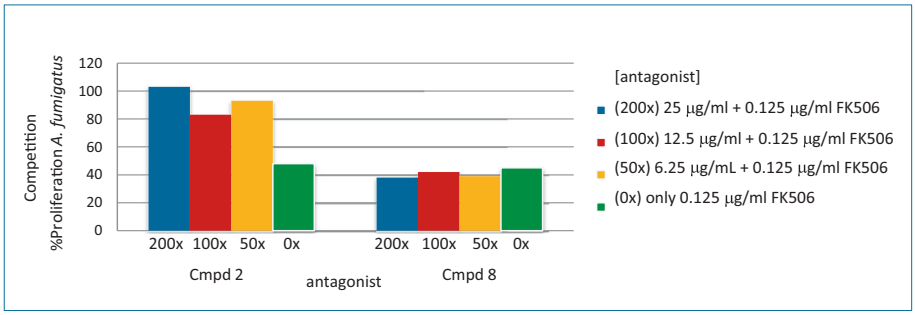


Fig. 9. Antagonism of the antifungal effect of FK506 with compound **2** and **8** on *A. fumigatus:* FK506 (0.125 μg/mL) dosed with increasing levels of antagonist with respect to proliferation of *A. fumigatus.*

19 nM. The antagonists all showed similar *A. fumigatus* binding constants relative to FK506 reinforcing that the C21 vector does not make contact with human or fungal FKBP12.

In addition, a biochemical competition assay was designed to assess the ability of an antagonist to compete with FK506 with respect to calcineurin phosphatase activity. The calcineurin (CN) inhibition assay was adapted from the R&D systems website protocol.[21] FK506 inhibits the phosphatase activity of calcineurin by binding to human FKBP12 and subsequently to the calcineurin/-calmodulin complex with an IC_{50} of 125 nM. The compounds antagonized FK506 binding with a range of IC_{50} values from 800 to 1800 nM (CN competition human IC_{50} nM, Table 1). The antagonists tested alone did not measurably inhibit phosphatase activity.

It is interesting that the antagonists' competition IC_{50} values were higher than the IC_{50} value of FK506 (125 nM) despite FKBP12 binding constants that were similar to FK506 (FKBP12 human IC_{50} nM, Table 1). The discrepancy may be attributed to the conformational differences between the FK506/FKBP12/CN/calmodulin and FK506/FKBP12 complexes with respect to FK506 and antagonist binding.

IL-2 secretion is dependent on calcineurin phosphatase activity and is a biomarker for an active immune system. To validate that the antagonists alone were non-immunosuppressive, the antagonists were evaluated in an IL-2 secretion assay in stimulated Jurkat cells according to established methods.[22] FK506 inhibited IL-2 secretion at 0.36 μM. The IL-2 assay IC_{50} values for all the antagonists were greater than 10 μM (Table 1), and were therefore non-immunosuppressive.

Initial efforts employing an LCMS method to directly assay for cell permeability were not successful due to poor mass recovery. Instead a cellular assay was developed in which Concanavalin A stimulated Peripheral Blood Mononuclear Cells,[23] PBMCs, were dosed with 10 nM of FK506 and an ascending dose of antagonist. FK506 alone inhibited PMBC proliferation, and antagonists that were able to permeate PMBCs reversed the FK506 inhibition of proliferation. Under cell free conditions, the compounds were shown to reverse the FK506 inhibition of calcineurin phosphatase activity in the CN competition assay, Table 1. The antagonists tested alone at 1–2 μg/mL showed no inhibition of proliferation.

Compounds when dosed at 100-fold excess of FK506 antagonized the anti-proliferative effects of FK506, and the data is shown in Figs. 8 and 9. Both the Merck compound L-685,818 (**2**), and compound **8** showed antagonism of FK506 at the 100x ratio, but little antagonism at 10x ratio (Fig. 8). Compounds **16** and **7** penetrated mammalian cells, and at 100-fold excess completely reversed the anti-proliferative effects of FK506. Fifteen additional compounds showed greater than 80% antagonism. A subset of these is represented by compounds **10**, **13** and **17,** which were all FK506 antagonists at the 80–90% level at 100-fold excess. It was unanticipated that mammalian cells were permeable to only a small subset of the antagonists. FK506 is a large macrocyclic natural product and is, by definition, an exception to Lipinski's rules.[24] Since natural products have been evolutionarily selected to permeate their target species, any changes to the structure or physical properties may result in poor penetration of the cell membrane.

To determine the ability of the antagonists to enter fungal cells, a similar competition assay was developed using *A. fumigatus* growth as an endpoint (Table 1**,** Fig. 9, competition% proliferation *A. fumigatus*). Briefly, *A. fumigatus* spores were prepared and plated as per CLSI methods[25] with some modifications. Spores were treated with FK506 together with increasing concentrations of antagonist. FK506 alone inhibited fungal growth of *A. fumigatus* with a MEC value of 0.1 μg/mL. For the competition study FK506 was dosed at 0.125 μg/mL in combination with 100-fold excess of antagonist.

The Merck antagonist, **2**, when dosed 50–200-fold over and in combination with FK506 (0.125 μg/mL) did not inhibit *A. fumigatus* proliferation, Fig. 9. The fungi grew up to 83% of the untreated controls at 100×, and thereby L-685,818 was likely permeable to the fungal cells and antagonistic to the antifungal effects of FK506. In contrast, under similar combination conditions compound **8**

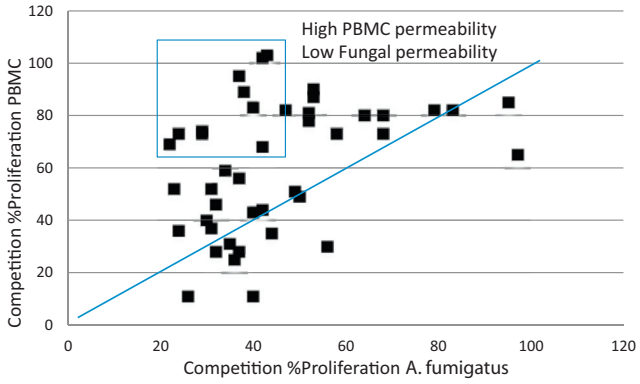


Fig. 10. Competition% Proliferation of *Aspergillus* vs. Human PBMC[26] Diagonal line represents Competition% Proliferation PBMC values equal to Competition% Proliferation *A. fumigatus*.

showed inhibition of *A. fumigatus* at all concentrations. The best compounds, **15** and **18**, in combination with FK506 inhibited *A. fumigatus* proliferation to 22 and 24% of the untreated controls, suggesting these compounds were relatively impermeable to fungal cells and allowed FK506 to freely inhibit calcineurin. Antagonists alone up to 25 μg/mL showed no appreciable antifungal activity.

A plot of the competition% proliferation *Aspergillus* vs. PBMC data (Fig. 10) represents a comparison of the antagonists properties with respect to cell-permeability in fungal and human immune cells. The plot shows that the antagonists are generally more cell-permeable to PBMCs vs. fungal cells as more compounds lie above the diagonal. The better compounds occupy the top leftmost quadrant of Fig. 10. Compounds **15** and **18** show the lowest levels of fungal permeability but are near 75% with respect to PBMC permeability. Alternatively, **7** and **13** have slightly higher fungal permeability values but show maximal PBMC permeability of 100%. It is yet undetermined which characteristics would lead to a more efficacious response with respect to an *in vivo* fungal infection model.

FK506 binds to FKBP12 and inhibits its peptidyl proline isomerase activity (PPIase). Previous studies have suggested that inhibiting this activity has few adverse effects because other related PPIases can compensate.[27] In this study, the dosing level of antagonist will be high enough to effectively inhibit the PPIase activity of FKBP12 and its isoforms. To build confidence in the validity of this strategy the antagonists were studied for cytotoxic effects on Vero76 and HepG2 cells.[28] All compounds tested showed an IC_{50} greater than 100 μM, and therefore were determined to be relatively non-toxic at the dosing levels of the antagonists used in these experiments.

Optimizing for PK proved challenging since attempts to filter potential compounds for animal PK studies by *in vitro* microsomal stability studies were unsuccessful. Poor correlation was observed between the *in vitro* and *in vivo* clearance data. Moreover, *in vitro* Caco-2 studies to model gut absorption were unsuccessful due to poor mass recovery. Despite these shortcomings, select compounds did show improved PK properties with respect to the model compound **2** (L-685,818) (Table 2). Compound **13** exhibited an oral AUC value thirteen times larger than of compound **2**. This greater AUC will allow lower dosing levels of the antagonists vs. FK506 in animal models of invasive aspergillosis, thereby improving the tractability of the experiment.

Inhibition of calcineurin has been established as a potential antifungal target, but efforts to exploit its antifungal properties have always failed due to its associated human immunosuppressive effects. In this paper we demonstrated a strategy to circumvent the immunosuppressive effects. Compounds were identified which bound FKBP12 as tightly as FK506 or better. Specifically all FK506 analogs with polar groups off C21 exhibited these properties. These same compounds lacked significant binding to calcineurin and did not show any *in vitro* immunosuppression. A subset of these compounds reversed the *in vitro* immunosuppressive effects of FK506 when dosed in 100-fold excess. A subset of these compounds was additionally identified that did not reverse the antifungal effects of FK506 when dosed in 100-fold excess. In mice, two compounds, (**13** and **15**), exhibited a thirteen and nine-fold better oral AUC value than the prototype compound L-685,818 respectively. All of the compounds tested exhibited IV AUC values from 2.7 to 3.5 times that of L-685,818. In conclusion, the combination of identified antagonists used in conjunction with FK506 has shown promise as a novel non-immunosuppressive antifungal therapy. Future proof of principle studies in a mouse aspergillosis infection model are needed.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.04.004.

References

1. Perfect JR, Cox GM, Lee JY, et al. The impact of culture isolation of Aspergillus species: a hospital-based survey of aspergillosis, Mycoses Study Group. *Clin Infect Dis*. 2001;33:1824–1833.
2. (a) Odom A, Del Poeta M, Perfect JR, Heitman J. The immunosuppressant FK506 and its nonimmunosuppressive analog L-685, 818 are toxic to *Cryptococcus neoformans* by inhibition of a common Target protein. *Antimicrob Agents Chemother*. 1997;41:156–161;
Cruz MC, Del Poeta M, Wang P, et al. Immunosuppressive and nonimmunosuppressive cyclosporine analogs are toxic to the opportunistic fungal pathogen cryptococcus neoformans via cyclophilin-dependent inhibition of calcineurin. *Antimicrob Agents Chemother*. 2000;143–149;
(c) Steinbach WJ, Reedy JL, Cramer Jr RA, Perfect JR, Heitman J. Harnessing calcineurin as a novel anti-infective agent against invasive fungal infections. *Nat Rev*. 2007;5:418–430;
(d) Odom A et al. Calcineurin is required for virulence of *Cryptococcus neoformans*. *EMBO J*. 1997;16:2576–2589;
f) Steinbach WJ et al. Calcineurin controls growth, morphology, and pathogenicity in *Aspergillus fumigatus*. *Eukaryot Cell*. 2006;5:1091–1103;
(g) Lev S, Desmarini D, Chayakulkeeree M, Sorrell TC, Djordjevic JT. The Crz1/Sp1 transcription factor of cryptococcus neoformans is activated by calcineurin and regulates cell wall integrity. *PLoS ONE*. 2012;7(12):e51403.
3. High K. The antimicrobial activities of cyclosporine, FK506, and rapamycin. *Transplantation*. 1994;57(12):1689–1700.
4. Liu J et al. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell*. 1991;66:807–815.
5. Greenblatt MB, Aliprantis A, Hu B, Glimcher LH. Calcineurin regulates innate antifungal immunity in neutrophils. *J Exp Med*. 2010;207(5):923–931.
6. Siekierka JJ, Signal NH. Immunosuppressive mechanism of action and beyond. *Curr Opin Immunol*. 1992;5:548–552.
7. Jain J, Rao A. The T-cell transcription factor NF-ATp is a substrate for calcineurin and interacts with Fos and Jun. *Nature*. 1993;365:352–355.
8. (a) Flanagan WM, Corthesy B, Bram RJ, Crabtree GR. Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. *Nature*. 1991;352:803–807;
(b) Northrop JR, Ullman KS, Crabtree GR. Characterization of the nuclear and cytoplasmic components of the lymphoid-specific nuclear factor of activated T-cells (NF-AT) complex. *Biol Chem*. 1993;268:2917–2923.
9. Organ HM, Holmes MA, Pisano JM, et al. *Bioorg Med Chem Lett*. 1993;13:651–662.
10. Dumont FJ, Staruch MJ, Koprak SL, et al. The immunosuppressive and toxic effects of FK-506 are mechanistically related: pharmacology of a novel antagonist of FK-506 and rapamycin. *J Exp Med*. 1992;176:751–760.
11. Koprak S, Sirotina A, Ok H, Dumont F. *Transplantation*. 1996;61(6):926–932.

12. Hosohata K, Uesugi M, Hashi S, et al. Association between CYP3A5 genotypes in graft liver and increase in tacromilus biotransformation from steroid treatment in living-donor liver transplant patients. *Drug Metab Pharamacokinet*. 2014;29 (1):83–89.
13. Marinec PS, Evans CG, Gibbons GS, Tarnowski MA, Overbeek DL, Gestwicki JE. Synthesis of orthogonally reactive FK506 derivatives via olefin cross metathesis. *Bioorg Med Chem*. 2009;17:5763–5776.
14. Carpino LA. 1-Hydroxy-7-azabenzotriazole, an efficient peptide coupling additive. *JACS*. 1993;115:4397–4398.
15. Guo Z-F, Zhang R, Liang F-S. Facile functionalization of FK506 for biological studies by the thiol-ene 'click' reaction. *RSC Adv*. 2014;4(22):11400–11403.
16. Abdel-Magid AF, Carson KG, Harris BD, Maryanoff CA, Shah RD. Reductive amination of aldehydes and ketones with sodium triacetoxyborohydride, studies on direct and indirect reductive amination procedures. *JOC*. 1996;61:3849–3862.
17. Hersperger R, Buchheit KH, Cammisuli S. *J Med Chem*. 2004;47(20):4950–4957.
18. Schlachter ST, Gaudino JJ, Burgess LE, Hunt KW, Macrocyclic Analogs for the Treatment of Immunoregulatory Disorders and Respiratory Diseases, US0160838, 2006.
19. Sinclair PJ, Wong F, Staruch MJ, et al. *Bioorg Med Chem Lett*. 1996;6 (18):2193–2196.
20. Kozany C, Marz A, Kress C, Hausch F. Fluorescent probes to characterize FK506-binding proteins. *ChemBioChem*. 2009;10:1402–1410.
21. R & D systems, Inhibition of Calcineurin Phosphatase Activity, https://www.rndsystems.com/resources/protocols/inhibition-calcineurin-phosphatase-activity, (accessed 04/01/2016), FK506 was added for competition.
22. Imboden JB, Weiss A, Stobo JD. *J Immunol*. 1985;134:663.
23. (a) Ambrosia GA, Keinonen S, Sanchez AM, Ferrari G, Denny TN, Moody AM. Leukopak PBMC sample processing for preparing quality control material to support proficiency testing programs. *J Immunol Methods*. 2014 Jul;409:99–106;
(b) Kruisbeek A.M., Shevach E, Thornton AM, Proliferative Assays for T Cell Function, Current Protocols in Immunology, Supplement 60, Current Protocols in Immunolog, John Wiley & Sons Inc, 2004, 3.12.1-3.12.20.;
(c) Jeurink PV, Vissers YM, Rappard B, Savelkoul Huub FJ. T cell responses in fresh and cryopreserved peripheral blood mononuclear cells: Kinetics of cell viability, cellular subsets, proliferation, and cytokine production. *Cryobiology*. 2008;57:91–103.
24. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev*. 2001;46(1–3):3–26.
25. Rex JH, Alexander BD, Andes D, et al. Espinel-Ingrofff, Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; Approved Standard-Second edition. 2008, CLSI document, vol 28, no. 16, M38-A2.
26. In the PBMC Competition,%Proliferation = 100× [OD (antagonist+FK506+ConA stimulated cells)]/[OD (vehicle+ ConA stimulated cells)], where baseline is adjusted to reflect FK506 treated cells alone by subtracting the FK506 treated cells OD from all other data values. In the *A. fumigatus* Competition% Proliferation = 100× OD (antagonist+FK506+cells)/OD (vehicle+cells).
27. Poulter MO, Payne KB, Steiner JP. Neuroimmunophilins: a novel drug treatment for the reversal of neurodegenerative disease. *Neuroscience*. 2004;128(1):1–6.
28. Konowalchuk J, Speirs JI, Stravic S. Vero response to a cytotoxin of *Escherichia coli*. *Infect Immun*. 1977;18:775–779.