Scientific paper

Tacrolimus as Antifungal Agent

Lyudmyla Antypenko,* Zhanar Sadykova, Fatuma Meyer, Leif-Alexander Garbe and Karl Steffens

Department of Agriculture and Food Science, Neubrandenburg University Brodaer Str. 2, 17033, Neubrandenburg, Germany

* Corresponding author: E-mail: antypenkol@gmail.com

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Abstract

Tacrolimus (FK506) is an immunosuppressant drug widely used to avoid organ rejection in transplant patients. It has a profound influence on the cellular stress response by interfering with the calmodulin-calcineurin signaling pathway. In this context FK506 also became a valuable antifungal drug in medical care. Here it is shown *in vitro* that tacrolimus has a potent growth inhibition activity against 11 fungi and 3 oomycetes of agricultural importance. The significance of this finding is discussed with respect to crop protection. The *in silico* molecular docking to 6 major antifungal enzymes determined UDP-*N*-acetylmuramoyl-*L*-alanine: *D*-glutamate ligase (MurD) as the main target by the best affinity score.

Keywords: Tacrolimus, Antiphytofungal activity

1. Introduction

Presently azoles, echinocandines, pyrimidine analogs and polyenes are among the most common antifungals in human medical care and crop protection. The search for new potent antifungal agents will remain an urgent task at the background of an ongoing emergence of drug resistance.1 Especially resistant pathogenic fungi rise concerns human medical care. For instance, immunocompromised patients after HIV infection or organ transplantation, who have overcome cryptococcal meningitis infection, may need a life-time fluconazole prophylaxis.² Here, management of infections become extremely difficult if azole resistant pathogens get involved. Also the arise of fluconazole-resistant mutants among Cryptococcus neoformans clinical isolates was observed.³ In search for new targets for antifungals the calmodulin-calcineurin signaling cascade came into focus. A stress response of a fungal cell starts by the uptake of Ca²⁺, which then binds to the four binding sites of calmodulin. After a conformational change Ca²⁺ occupied calmodulin forms a ternary complex together with calcineurin subunits CnA and CnB, thereby gaining a phosphatase activity; in turn the complex dephosphorylates transcription factor Crz1. Genes activated by dephosphorylized Crz1 are involved in drug resistance, growth and cell wall integrity. Generally, calcineurin is an important part of the stress response of fungal cells.4

Searching for novel immunosuppressive agents a 822-kD 23-membered macrolide lactone named tacrolim-

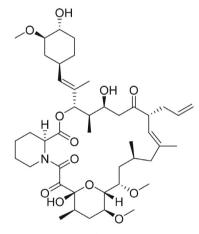


Figure 1. Chemical structure of tacrolimus (FK506).

us (FK506, Fujimycin, etc.) was isolated and characterized from *Streptomyces tsukubaensis* (Fig. 1).⁵

Tacrolimus in capsules for oral intake and as solution for injection is used for prophylaxis against organ rejection in patients receiving liver, kidney or heart transplants. As a topical ointment it is applied as a second-line therapy for the short-term and non-continuous chronic treatment of moderate to severe atopic dermatitis in non-immunocompromised adults and children. FK506 has a favorable toxicological profile: no evidence of mutagenicity was observed *in vitro* in the CHO/HGPRT assay (the Chinese hamster ovary cell assay). Also it did not cause unsched-

uled DNA synthesis in rodent hepatocytes. LD_{50} in rat oral test was 134 mg/kg for tacrolimus hydrate.⁷

Experimental evidence suggests that tacrolimus binds to the intracellular protein FKBP12 and thereby interferes with the calcineurin signal pathway, which is a key element in regulation of intracellular Ca²⁺ concentration.⁴ FKBP12-tacrolimus inhibits the phosphatase activity of the calmodulin-CnA-CnB complex, which in turn silences transcription factor Crz1 and corresponding stress related genes. As a consequence, inhibitors of calcineurin like tacrolimus may function as potent antifungals. In combination with standard antifungal drugs FK506 may reverse resistance against them or increase their efficacy.^{8,9} Also phytopathogenic fungi rely on the calcineurin pathway securing cell wall and membrane

integrity, virulence 10 or formation of infectious appressoria. 11

Practically all of tacrolimus studies are related to the development of supportive antifungal therapy to improve regimes of infected patients. Here we describe novel antifungal efficacy of FK506 towards eleven pathogenic fungi and three *Phytophthora* oomycetes of agricultural importance.

2. Results and Discussion

2. 1. Known Antifungal Data

All reported antifungal literature data were summarized below (Table 1) to show its found minimum inhibitory concentrations (MIC).

Table 1. Summary of reported antifungal activity of tacrolimus (FK506)

Fungus	Reported strain name	MIC_{100} , $\mu g/mL$
	AF293, DUMC 119.00,	1.56 ¹²
	DUMC 153.90, DUMC 101.01	1.56
A an ancillus funcicatus	DUMC 165.86	6.25
Aspergillus fumigatus	DUMC 182.99	0.39
	DUMC 168.95	3.125
	DUMC 131.00	0.78
	6 strains	$0.025 \text{-} 0.4^{13}$
Aspergillus fumigantus	AF1 - AF10	>256 ¹⁴
	TIMM0063	$\leq 0.008^{15}$
A	2 strains	$0.006 - 0.012^{13}$
Aspergillus niger	ATCC6275	$\leq 0.008^{15}$
A	4 strains	$0.1 - 0.2^{13}$
Aspergillus flavus	AFL1 - AFL8	>256 ¹⁴
4	4 strains	0.025-0.113
Aspergillus terreus	AT1, AT2	>25614
Aspergillus ustus	3 strains	>0.414
Aspergillus versicolor	1 strain	>0.414
	SC5314, echinocandin-res.:	
	Fks1 S645Y, Fks1 F641S,	
Candida albicans	Fks1 S645F, Fks1 S645P;	>16 ¹⁶
	azole-res.: 2–76,	
	2–86, 12–99.	
	SC5314, cnb1/cnb1 (DAY364),	
	cnb1/cnb1 CNB1 (MCC85),	>3.121
	CNB1-1/CNB1 (YAG237),	
	rbp1/rbp1 (YAG171)	7
	azole-susc. CA5, CA8, CA12,	
	CA14, CA129; azole-res. CA10,	512^{18}
	CA15, CA16, CA135, CA137.	
	TIMM1623	
Candida tropicalis	TIMM0313	>128 ¹⁵
Candida kefyr	ATCC28838	

Malassezia globose36 strains $0.016-0.25^{22}$ Malassezia restrica28 strains $0.016-0.25^{22}$ Malassezia sympodialis19 strains 0.125^{22} Malassezia obtuse7 strains 0.03^{22} Malassezia slooffiae10 strains 0.016^{22} Malassezia japonica2 strains 0.016^{22} Malassezia nana4 strains 0.016^{22} Malassezia pachydermatis6 strainsMalassezia yamatoensis2 strainsMucor spp. b4 strains 0.012 to $>0.4^{13}$ Paecilomyces licasinusb5 strains $>0.4^{13}$ Paecilomyces varioti b4 strains 0.025 to $>0.4^{13}$ Rhizomucor spp. b2 strains $0.012 - 0.025^{13}$ Rhizopus spp. b5 strains 0.1 to $>0.4^{13}$ Scedosporium apiospermumb4 strains 0.05 to $>0.4^{13}$ Scedosporium prolificansb4 strains >0.05 to $>0.4^{13}$ Scopulariopsis spp. b4 strains >0.05 to $>0.4^{13}$ YFK005 wild type YFK012 (fkr1), YFK256 (PDR) YFK257 (nkt 4), YFK258 (nkt 4), YFK257 (nkt 4), YFK258 (nkt 4), YFK257 (nkt 4), YFK257 (nkt 4), YFK257 (nkt 4), YFK257 (nkt 4), YFK258 (nkt 4), YFK257 (Fungus	Reported strain name	MIC ₁₀₀ , μg/mL	
Part	Candida alahrata ^a		256^{19}	
Cryptococcus neoformans $2015 \\ -0.09^{11}, 12.5^{20} \\ -0.09$		CG4, CG8, CG2, CG3	512	
Cryptococcus neoformans H99 C20, C21 C20 C21 C20F1 C20F2 C20F2 C21F2 C21F3 C20F3 C20F		EP1551	•	
$ \begin{array}{c} C20, C21 \\ C20F1 \\ C20F2 \\ C20F2 \\ C20F2 \\ C21F3 \\ C21$				
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$ \begin{array}{c ccccccccccccccccccccccccccccccccccc$	Cryptococcus neoformans			
C21F3 Acnal mutant, Afril mutant 2521 $>252^{30}$ Cunninghamella spp.b 2 strains 0.05-0.213 Fusarium spp.b 6 strains >0.4413 Lichtheimia spp.b 2 strains 0.2-0.413 Malassezia furfur 8 strains 0.53215 0.0322 Malassezia furfur 26 strains 0.016-0.2524 Malassezia globose 36 strains 0.016-0.2524 Malassezia globose 36 strains 0.016-0.2524 Malassezia sympodialis 19 strains 0.12522 Malassezia obtuse 7 strains 0.0322 Malassezia slooffiae 10 strains 0.01622 Malassezia padruse 2 strains 0.01622 Malassezia japonica 2 strains 0.01622 Malassezia pachydermatis 6 strains 0.01622 Malassezia pachydermatis 6 strains 0.012 to >0.413 Malassezia yamatoensis 2 strains 0.012 to >0.413 Paecilomyces licasinusb 5 strains 0.012 to >0.413 Paecilomyces varioti b 4 strains 0.025 to >0.413 Rhizopus sp				
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Paecilomyces varioti b 4 strains $0.025 \text{ to } > 0.4^{13}$ Rhizomucor spp. b 2 strains $0.012 - 0.025^{13}$ Rhizopus spp. b 5 strains $0.1 \text{ to } > 0.4^{13}$ Scedosporium apiospermum b 4 strains $0.05 \text{ to } > 0.4^{13}$ Scedosporium prolificans b 4 strains $> 0.4^{13}$ Scopulariopsis spp. b 4 strains $0.05 \text{ to } > 0.4^{13}$ YFK005 wild type 15^{23} YFK007 wild type 22 YFK012 (fkr1), YFK256 (PDR) > 80 YFK041 (fkr2), YFK258 (PDR) > 80 YFK093 (fkr3) > 50 YFK223-5C (pdrl-3) > 6 YFK222-2A (pdrl-7) > 35 Sporonthrix brasilliensis CBS 133021 1^{24} Sporonthrix schenckii CBS 132984 $> 0.025 \text{ to } > 0.04^{13}$	Mucor spp.b	4 strains	0.012 to >0.4 ¹³	
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Rhizopus $spp.^{b}$ 5 strains $0.1 \text{ to } > 0.4^{13}$ Scedosporium apiospermumb 4 strains $0.05 \text{ to } > 0.4^{13}$ Scedosporium prolificansb 4 strains $> 0.4^{13}$ Scopulariopsis $spp.^{b}$ 4 strains $0.05 \text{ to } > 0.4^{13}$ YFK005 wild type 15^{23} YFK007 wild type 22 YFK012 (fkr1), YFK256 (PDR) > 80 YFK257 (pdrl-3), YFK259 (pdrl-7) > 80 YFK014 (fkr2), YFK258 (PDR) 45 YFK093 (fkr3) 50 YFK223-5C (pdrl-3) 6 YFK222-2A (pdrl-7) 35 Sporonthrix brasilliensis CBS 133021 1^{24} Sporonthrix schenckii CBS 132984 2^{24}	Paecilomyces varioti ^b	4 strains	0.025 to >0.4 ¹³	
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Scopulariopsis spp. b 4 strains $0.05 \text{ to } > 0.4^{13}$ YFK005 wild type 15^{23} YFK007 wild type 22 YFK012 (fkr1), YFK256 (PDR) > 80 YFK257 (pdrl-3), YFK259 (pdrl-7) > 80 YFK014 (fkr2), YFK258 (PDR) 45 YFK093 (fkr3) 50 YFK223-5C (pdrl-3) 6 YFK222-2A (pdrl-7) 35 Sporonthrix brasilliensis CBS 133021 1^{24} Sporonthrix schenckii CBS 132984 2^{24}	Scedosporium apiospermum ^b	4 strains	0.05 to >0.4 ¹³	
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YFK007 wild type 22 YFK012 (fkr1), YFK256 (PDR) >80 YFK257 (pdrl-3), YFK259 (pdrl-7) >80 YFK014 (fkr2), YFK258 (PDR) 45 YFK093 (fkr3) 50 YFK223-5C (pdrl-3) 6 YFK222-2A (pdrl-7) 35 Sporonthrix brasilliensis CBS 133021 124 Sporonthrix schenckii CBS 132984 224	Scopulariopsis spp. ^b	4 strains	0.05 to >0.4 ¹³	
Saccharomyces cerevisiaeb YFK012 (fkr1), YFK256 (PDR) >80 YFK257 (pdrl-3), YFK259 (pdrl-7) >80 YFK014 (fkr2), YFK258 (PDR) 45 YFK093 (fkr3) 50 YFK223-5C (pdrl-3) 6 YFK222-2A (pdrl-7) 35 Sporonthrix brasilliensis CBS 133021 124 Sporonthrix schenckii CBS 132984 224				
Saccharomyces cerevisiaeb YFK257 (pdrl-3), YFK259 (pdrl-7) >80 YFK014 (fkr2), YFK258 (PDR) 45 YFK093 (fkr3) 50 YFK223-5C (pdrl-3) 6 YFK222-2A (pdrl-7) 35 Sporonthrix brasilliensis CBS 133021 124 Sporonthrix schenckii CBS 132984 224				
Saccharomyces cerevisiae YFK014 (fkr2), YFK258 (PDR) 45 YFK093 (fkr3) 50 YFK223-5C (pdrl-3) 6 YFK222-2A (pdrl-7) 35 Sporonthrix brasilliensis CBS 133021 124 Sporonthrix schenckii CBS 132984 224				
YFK093 (fkr3) 50 YFK223-5C (pdrl-3) 6 YFK222-2A (pdrl-7) 35 Sporonthrix brasilliensis CBS 133021 1 ²⁴ Sporonthrix schenckii CBS 132984 2 ²⁴	Saccharomyces cerevisiae ^b			
YFK223-5C (pdrl-3) 6 YFK222-2A (pdrl-7) 35 Sporonthrix brasilliensis CBS 133021 1 ²⁴ Sporonthrix schenckii CBS 132984 2 ²⁴				
YFK222-2A (pdrl-7) 35 Sporonthrix brasilliensis CBS 133021 1 ²⁴ Sporonthrix schenckii CBS 132984 2 ²⁴				
Sporonthrix schenckii CBS 132984 2 ²⁴				
	Sporonthrix brasilliensis	CBS 133021	1 ²⁴	
Trichosporon asahii CBS 2479 >64 ²⁵	Sporonthrix schenckii	CBS 132984	2^{24}	
	Trichosporon asahii	CBS 2479	>64 ²⁵	

Reported strain name	MIC ₁₀₀ , μg/mL	
VUT-77011	>100 ²⁶	
VUT-97010	0.781^{26}	
VUT-00001	6.25^{26}	
VUT-00002	25^{26}	
VUT-00003	12.5^{26}	
TIMMl189	>12815	
EP594	15	
EP596	>128 ¹⁵	
	VUT-77011 VUT-97010 VUT-00001 VUT-00002 VUT-00003 TIMM1189	

aMIC₈₀, bMIC₅₀

Odom at al. revealed, that in vitro FK506 was toxic to Cryptococcus neoformans at 37 °C, but not at 24 °C, because at the higher temperature growth and virulence were dependent on the protein phosphatase calcineurin.²¹ It was found, that tacrolimus inhibited CDR1 and CaM-DR1 genes in *C. albicans*, which are thought to play a role in the antifungal resistance to azole derivatives.⁸ Along with the high level of stress that was induced by an influx Ca2+ and Na+ the membrane distortion caused by the azole interference of the ergosterol metabolism gave rise to an enhanced intracellular FK506 concentration.^{9,27} In vitro studies with fluconazole-resistant C. glabrata isolates revealed a distinct synergistic effect when FK506 was combined with ketoconazole (77%), itraconazole (73%), voriconazole (63%) and fluconazole (60%).²⁸ It has also been shown that tacrolimus dramatically induced Ca2+ uptake in C. glabrata cells in the presence of fluconazole²⁹ or terbinafine and fenpropimorph.¹⁷ The addition of FK506 or the disruption of calcineurin gene CMP2 specifically reversed the β-1,3-glucan synthase Fks2-mediated resistance of C. glabrata to echinocandin³⁰ as well as to posaconazole in C. albicans. 16 Li et al. 19 mentioned the decrease in ERG11 and SNQ2 gene expression levels and the inhibition of fluconazole efflux by FK506 in Candida glabrata. Poeta et al.20 demonstrated that it exhibits marked synergistic activity with the H+ATPase inhibitor bafilomycin A₁ and pneumocandin MK-0991/caspofungin acetate in C. neoformans cells. Steinbach et al. 12 confirmed that FK506 and cyclosporin exhibit an inherent activity against Aspergillus fumigatus, generally delaying filamentation and producing smaller hyphae. Borba-Santos²⁴ observed an increase of itraconazole and fluconazole efficiency when applied in combination with FK506 in therapy regime for sporotrichosis, which is caused by pathogenic fungi Sporonthrix brasiliensis or S. schenckii. Ozawa et al.²⁶ also revealed synergistic activity of tacrolimus with itraconazole against Trichophyton mentagrophytes. In contrast FK506 showed no high activity against Trichosporon asahii strains (MIC > 64.0 μg/mL).²⁵ However, a larger synergistic interaction was observed by the combinations FK506 + amphotericin B (96.67%) and FK506 + caspofungin (73.33%) against fluconazole-susceptible isolates of Trichosporon asahii. The activity of

FK506 in combination with azole antifungals against *Malassezia* species was investigated too. 15,22

Also FK506 was thoroughly tested against fungal biofilms of A. fumigatus¹⁴ and 60 selected clinical fungal isolates:¹³ A. flavus, A. terreus, A. ustus, A. niger, A. versicolor, Purpureocillium lilacinus, Paecilomyces variotii, Scopulariopsis spp., Rhizopus spp., Mucor spp., Rhizomucor spp., Lichtheimia spp., Cunninghamella spp., Fusarium spp., Scedosporium apiospermum and S. prolificans. Besides FK506 was reported to effect growth of Paecilomyces variotii,¹³ Cryptococcus neoformans,²¹ Neurospora crassa³¹ and Coccidioides immitis.³²

2. 2. Antifungal Studies

Considering the above-mentioned data, we decided to investigate antifungal activity of tacrolimus in concentrations of 1, 0.05 and 0.001 µg/mL against not studied before ten phytopathogenic fungi and three *Phytophthora* oomycetes: *A. alternata*, *F. graminearum*, *B. cinerea*, *C. higginsianum*, *F. equiseti*, *F. fujikuroi*, *F. oxysporum*, *P. digitatum*, *V. lecanii*, *M. indicus*, *P. infestans* p-4, GC-1 and p-3. (Table 2). And also *A. niger*, that to our knowledge was the only one fungus treated earlier with FK506 (Table 1).¹³

So, it was found that at 1 µg/mL all fungi were susceptible to FK506, except M. indicus. A near complete inhibition of 99.0 % was detected with B. cinerea. Whereas lowest effect (21.1%) was observed with V. lecanii. Moreover, FK506 was always more active against any strain than the strong antifungal reference cyproconazole (2-(4-chlorphenyl)-3-cyclopropyl-1-(1H-1,2,4-triazol-1-yl)butan-2-ol)³³ in the same concentration. And the results against the different strains of the same specie had the similar inhibition zones when treated with 1 µg/mL of tacrolimus.

Lowering the concentration to 0.05 µg/mL still conferred an inhibition of all strains. *A. alternata*, *F. graminearum*, *B. cinerea*, *C. higginsianum* and *A. niger* were the most sensitive ones and were effected more than 70%.

Even at a 1000 times lower concentration (0.001 μ g/mL) moderate effects were still observed against *F. graminearum* (41.0 %), *A. alternata* (36.1%), *C. higginsianum* (27.1%), *P. infestans* p-4 (23.7%) and *A. niger* (15.9 %), showing high efficiency of FK506 antifungal activity.

Numbers represent means of experiments carried out in triplicate. Cyproconazole was used as positive control. Experimental procedures were as described in materials and methods. The strains are Table 2. Growth inhibition rates (%) of tacrolimus (FK506)

#	Dosage					Growt	h inhibition	Growth inhibition rates of fungi or oomycetes, $\% \pm SD$; or oomyce	tes, % ± SD				
	(µg/mL)	BC	AA	FF	FG	FE	СН	AN	FO	PI 4	PI 3	PI 1	PD	ΛΓ
	1	99.0 ± 0	99.0 ± 0 98.5 ± 2.1	94.4 ± 1.1	89.4 ± 1.0	89.4 ± 1.0 89.4 ± 2.1	87.8 ± 0	87.8 ± 0 86.5 ± 1.1 69.2 ± 1.1 64.8 ± 2.3 66.4 ± 1.0 60.5 ± 1.2 44.2 ± 6.5 21.1 ± 0	69.2 ± 1.1	64.8 ± 2.3	66.4 ± 1.0	60.5 ± 1.2	44.2 ± 6.5	21.1 ± 0
*	0.05	97.6 ± 1.0	94.6 ± 2.8	49.8 ± 1.2	77.7 ± 1.0	7.7 ± 1.0 68.9 ± 4.1	79.8 ± 1.2 85.1 ± 1.1	85.1 ± 1.1	63.4 ± 2.2	63.4 ± 2.2 59.9 ± 0.9 50.2 ± 2.5 69.2 ± 2.2 21.1 ± 6.5	50.2 ± 2.5	69.2 ± 2.2	21.1 ± 6.5	3.5 ± 5.0
	0.001	0.0	36.4 ± 0.9	1.6 ± 3.2	41.0 ± 2.1	41.0 ± 2.1 1.5 ± 2.9	27.1 ± 2.5 15.9 ± 2.9	15.9 ± 2.9	-3.4 ± 4.4	-3.4 ± 4.4 23.7 ± 0.7 -3.3 ± 1.2 2.2 ± 0 1.3 ± 0.9	-3.3 ± 1.2	2.2 ± 0	1.3 ± 0.9	3.5 ± 5.0
C	1	67.3 ± 1.9	67.3 ± 1.9 64.0 ± 3.1	80.7 ± 0.5	38.8 ± 1.0 84.8 ± 0.2	84.8 ± 0.2	78.4 ± 1.4 83.5 ± 2.4		42.8 ± 7.7	$42.8 \pm 7.7 62.0 \pm 1.4 32.4 \pm 1.0 56.8 \pm 5.6 0.0$	32.4 ± 1.0	56.8 ± 5.6	0.0	14.0 ± 5.0

4) and p-3 (PI 3), P. digitatum (PD) and V. lecanii (VL). M. indicus was insensitive to all tested substances (0%

2. 3. Molecular Docking Studies

In order to propose unstudied antifungal activity mechanism *in silico* molecular docking was done³⁴. The found affinity scores of tacrolimus and reference cyproconazole³³ to common antifungal targets³⁵ are presented in Table 3.

As it was expected the 14α -demethylase (CYP51) has shown the best affinity with triazole derivative cyproconazole according to its best score (-7.9 Kcal/Mol) (Table 3).

An interesting result was found for tacrolimus, because it fitted into the active sites of secreted aspartic proteinase (SAP2) and UDP-*N*-acetylmuramoyl-*L*-alanine: *D*-glutamate ligase (MurD) better then cyproconazole, and its affinity scores were much higher (–8.5 and –9.8 Kcal/Mol correspondingly).

The visualization of interaction (Figure 2) with the best target indicates that tacrolimus binds to MurD by four conventional hydrogen and three carbon hydrogen bonds. Also five hydrophobic alkyl and π -alkyl bonds are formed with LEU15, LEU416, ARG186 and PHE422 residues of enzyme (Table 4).

3. Experimental

The mycelial growth rate assay was used for antifungal studies.³⁶ Strains of filamentous fungi were obtained from the following sources: Aspergillus niger DSM 246, Altenaria alternata DSM 1102, Fusarium equiseti DSM 21725, F. graminearum DSM 1095, F. fujikuroi DSM 893, Verticillium lecanii, Penicillium digitatum DSM 2731 from DSMZ (Braunschweig, Germany); Fusarium oxysporum 39/1201 St. 9336 and Botrytis cinerea from the Technische Universität Berlin (Germany); Colletotrichum higginsianum MAFF 305635, originally isolated in Japan, via the Department of Biology, Friedrich-Alexander-Universität (Erlangen, Germany); Phytophthora infestans GL-1 01/14 wild strain, p-3 (4/91; R+) and p-4 (4/91; R-) strains were kindly donated by Julius Kühn-Institut (Quedlinburg, Germany). Potato Dextrose Agar (PDA) were purchased from C. Roth (Karlsruhe, Germany). Cyproconazole (99%) was obtained from (Sigma Aldrich, Germany). Tacrolimus (99 %) was purchased from Huaian Ruanke Trade, Ltd. (Huaian, China). Strains were cultivated on PDA for 6 d at 25 °C. Spores from each strain were gently harvested with a sterile glass rod from plate surfaces with deionized water. Spore concentration numbers in suspension were determined microscopically and adjusted to 7.5*106 spores/mL. The clear stock solutions of 1 mg/mL were made of 0.01 g of cyproconazole and tacrolimus in 10 mL of deionized sterile water as solvent. 10 mL of these stock solutions were mixed in situ into 99 mL of PDA prior to solidification to obtain a final concentration of 1 μg/mL. Additionally, from the same stock solution PDA solutions with final concentration of 0.05 and 0.001 µg/mL were made appropriately. 9 mL of each mixture were poured

Table 3. Affinity to binding sites

The calculated affinity of substances to binding sites of sterol 14α-demethylase (CYP51) 5TZ1, *N*-myristoyltransferase (NMT) 1IYL, secreted aspartic proteinase (SAP2) 1EAG, UDP-*N*-acetylmuramoyl-*L*-alanine: *D*-glutamate ligase (MurD) 1UAG, topoisomerase II (Topo II) 1Q1D, and *L*-glutamine: *D*-fructose-6-phosphate amidotransferase (GlcN-6-P) 1XFF.

Substance				Kcal/Mol			
	Candida albicans			Escherichia coli	Sacchromyces cerevisiae	Escherichia coli	
	5TZ1	1IYL	1EAG		1UAG	1Q1D	1XFF
Cyproconazole	-7.9	-7.8	-6.3		-6.9	-6.1	-5.5
Tacrolimus	-6.0	-6.7	-8.5		-9.8	-6.1	-5.4

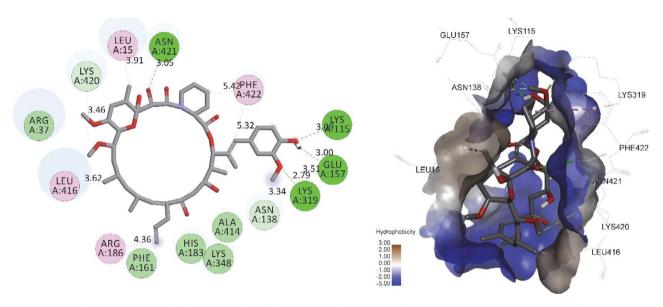


Figure 2. Visual representation (2D (left) and 3D (right)) of the tacrolimus showing bonds formation and position in the active site of UDP-N-acetylmuramoyl-L-alanine: D-glutamate ligase (MurD) 1UAG. Pale green – van der Waals interaction, green – classical conventional hydrogen bond, light green – non classical carbon hydrogen bond, pink – hydrophobic alkyl and π -alkyl bonds.

Table 4. The list of in silico calculated bonds formed between tacrolimus and UDP-N-acetylmuramoyl-L-alanine: D-glutamate ligase (MurD) active site

Name	Distance, Å	Category	Type
A:LYS115:NZ - :UNL1:O	3.09159	Hydrogen	Conventional
A:LYS319:NZ - :UNL1:O	2.78738	Hydrogen	Conventional
A:ASN421:ND2 - :UNL1:O	3.0506	Hydrogen	Conventional
:UNL1:H - A:GLU157:OE2	2.99646	Hydrogen	Conventional
:UNL1:C - A:LYS420:O	3.45662	Hydrogen	Carbon
:UNL1:C - A:ASN138:O	3.33652	Hydrogen	Carbon
:UNL1:C - A:GLU157:OE2	3.51104	Hydrogen	Carbon
:UNL1:C - A:LEU15	3.90689	Hydrophobic	Alkyl
:UNL1:C - A:LEU416	3.6241	Hydrophobic	Alkyl
:UNL1:C - A:ARG186	4.36225	Hydrophobic	Alkyl
A:PHE422 - :UNL1	5.42448	Hydrophobic	π-Alkyl
A:PHE422 - :UNL1:C	5.32047	Hydrophobic	π-Alkyl

into 6 cm diameter petri dishes. A central hole (diameter: 2.5 mm) was cut out and inoculated with 6.5 μ L spore suspension. Plates were incubated at 25°C (+/-1°C) for 6 d. Control plates containing only PDA and DMSO were prepared in the same way. Inhibitory effects (1%) were deter-

mined by analyzing growth zone diameters and calculated as described³⁶: I % = [(C-T)/(C-2.5 mm)])*100, where C (mm) represents the growth zone of control PDA, and T (mm) the average growth zone in presence of tacrolimus. All growth experiments were carried out in triplicate.

Means and standard deviations were calculated with software "Excel 2016" (Microsoft, USA). The strong antifungal agent was used as positive control.

Molecular docking. Macromolecular data of crystal structures were downloaded from the Protein Data Bank (PDB):³⁷ sterol 14α-demethylase (CYP51) 5TZ1, topoisomerase II (Topo II) 1Q1D, L-glutamine: D-fructose-6-phosphate amidotransferase (GlcN-6-P) 1XFF, secreted aspartic proteinase (SAP2) 1EAG, *N*-myristoyltransferase (NMT) 1IYL, and UDP-*N*-acetylmuramoyl-L-alanine: D-glutamate ligase (MurD) 1UAG; taken from *Candida albicans* (CYP51, NMT, SAP2), *Escherichia coli* (MurD, GlcN-6-P) and *Sacchromyces cerevisiae* (Topo II).³⁵ As reference cyproconazole (2-(4-chlorphenyl)-3-cyclopropyl-1-(1*H*-1,2,4-triazol-1-yl)butan-2-ol) was chosen.³³

Ligand preparation. Substances were drawn using MarvinSketch 6.3.0 and were saved in mol format. They were optimized by program Chem3D using molecular dynamics MM2 algorithm and saved as pdb-files. Molecular mechanics was used to produce more realistic geometry values for the majority of organic molecules owing to the fact of being highly parameterized. By using AutoDock-Tools-1.5.6 pdb-files were converted to PDBQT, and number of active torsions was set as default.

Protein preparation. Discovery Studio 4.0 was used to delete water molecules and ligand from crystal structures. The proteins were saved as pdb-files. In AutoDock-Tools-1.5.6 polar hydrogens were added and saved as PD-BQT. The Grid boxes were set as following: 5TZ1 center_x = 70.728, center y = 65.553, center z = 3.865, size x = 35, $size_y = 35$, $size_z = 35$; 1Q1D center_x = 28.5, center_y = 34.4, center_z = 32.7, size_x = 30, size_y = 30, size_z = 30; $1XFF center_x = 5$, $center_y = 28$, $center_z = 26$, $size_x = 26$ 30, size $_y = 30$, size $_z = 30$; 1UAG center $_x = 47$, center $_y = 47$ = -1.5, center_z = 15, size_x = 30, size_y = 30, size_z = 30, $1IYL center_x = 31$, $center_y = 74.5$, $center_z = 57$, $size_x$ = 30, size_y = 30, size_z = 30, 1EAG center_x = 42, cen $ter_y = 26$, $center_z = 11$, $size_x = 30$, $size_y = 30$, $size_z = 20$ 30. Vina was used to carry out docking. For visualization Discovery Studio Visualizer v17.2.0.16349 was used.

4. Conclusion

Considering a substantial role of FK506 in crop protection due to its distinct activity against phytopathogenic fungi, the availability in bulk quantities at affordable costs have to be taken into account. For the development of a formulation for agricultural usage purity standards may be much less demanding than for immunosuppressant manufacturing. Furthermore, quantities necessary of tacrolimus may be reduced if advantages of synergistic effects in blends with conventional antifungal agrochemicals (e.g. triazoles) can be generated as it is already described for medical care of patients infected with *C. albicans*. Investi-

gations to further explore the potential benefits of FK506 in agriculture must be accompanied by studies covering emergence of resistance, toxicity and environmental friendliness of this compound.

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Povzetek

Takrolimus (FK506) je imunosupresivno zdravilo, ki se pogosto uporablja za preprečevanje zavrnitve organov pri bolnikih s presaditvijo. Ima močan vpliv na celični stresni odziv z vmešavanjem v signalno pot kalmodulin-kalcinevrin. V tem kontekstu je FK506 postal tudi dragoceno protiglivično zdravilo v zdravstveni negi. Tu je *in vitro* prikazano, da ima takrolimus močan učinek zaviranja rasti proti 11 glivam in 3 oomicetom kmetijskega pomena. Pomen te ugotovitve je obravnavan v zvezi z zaščito pridelkov.



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