Synthesis and Cytotoxic Evaluation of Cycloheximide Derivatives as Potential Inhibitors of FKBP12 with Neuroregenerative Properties[▽]

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On the basis of the new finding that the protein synthesis inhibitor cycloheximide (**1**, 4-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-2,6-piperidinedione) is able to competitively inhibit hFKBP12 (K_i = 3.4 μM) and homologous enzymes, a series of derivatives has been synthesized. The effect of the compounds on the activity of hFKBP12 and their cytotoxicity against eukaryotic cell lines (mouse L-929 fibroblasts, K-562 leukemic cells) were determined. As a result, several less toxic or nontoxic cycloheximide derivatives were identified by N-substitution of the glutarimide moiety and exhibit IC_{50} values in the range of 22.0−4.4 μM for inhibition of hFKBP12. Among these compounds cycloheximide-*N*-(ethyl ethanoate) (**10**, K_i = 4.1 μM), which exerted FKBP12 inhibition to an extent comparable to that of cycloheximide (**1**), was found to cause an approximately 1000-fold weaker inhibitory effect on eukaryotic protein synthesis (IC_{50} = 115 μM). Cycloheximide-*N*-(ethyl ethanoate) (**10**) was able to significantly speed nerve regeneration in a rat sciatic nerve neurotomy model at dosages of 30 mg/kg.

Introduction

Peptidyl-prolyl *cis/trans* isomerases (PPIases; E.C. 5.2.1.8) are enzymes that catalyze the *cis/trans* isomerization of peptidyl-prolyl amide bonds in peptides and unfolded proteins.[1,2] Until today, three distinct PPIase families have been identified according to amino acid sequence homology and to the characteristics of inhibition by drugs of microbial origin: cyclophilins, FK506-binding proteins (FKBPs),[3] and parvulins.[4] Members of each of these families are evolutionarily conserved from prokaryotic to eukaryotic organisms and are localized in virtually all cell types.[5,6]

PPIases are reported to play an important role in a range of biological processes. Beside the cyclophilins, various FKBPs were shown to have regulatory functions as stable or dynamic part of heterooligomeric complexes containing physiologically relevant proteins.[7] As an example, FKBP52 seems to be functionally associated with untransformed steroid hormone receptors.[8] Recently it was found that FKBP12 binds to and modulates the properties of transforming growth factor β-type 1 (TGF-β1) receptors[9] and inhibits epidermal growth factor (EGF) receptor tyrosine autophosphorylation.[10] Several studies have demonstrated the coordination of gating of major intracellular Ca^{2+} release channels, e.g., IP_3 receptors and ryanodine receptors (RyR) of the sarcoplasmic reticulum of skeletal and cardiac muscles, by FKBP12, whereas FKBP12.6 is associated with cardiac RyR2.[11] Using a knockout model, Shou et al.[12] were able to show that FKBP12-deficient mice display severe dilated cardiomyopathy and ventricular septal defects mimicking human congenital heart disorders.

Initially published reports of inhibitors of FKBPs were focused on the macrolactone-type agents FK506[13] and rapamycin.[14] These compounds originally attracted scientific and clinical interest because of their immunosuppressive properties.[15] It is believed that complexes of FK506−FKBP12 bind to and thus inactivate the Ca^{2+}-activated protein serine/threonine phosphatase calcineurin, leading to a block of Ca^{2+}-dependent T- and B-lymphocyte responses,[16] whereas rapamycin−FKBP12 targets homologues of the yeast TOR molecules including the human RAFT/FRAP.[17] In addition to FK506 and rapamycin, various small molecule FKBP12 inhibitors devoid of immunosuppressive activity were described, containing the α-dicarbonyl amide key structural element of the FKBP binding domain, which is supposed to interact with the active site of the PPIase.[18] Among these compounds, a number of pipecolate and *N*-(glyoxyl)prolyl FKBP12 ligands were characterized as causing submicromolar FKBP12 inhibition ($K_{i,app}$ = 1−100 nM).[18b,d] Extremly potent inhibitors of this type carry bulky hydrophobic alkyl groups such as 1,1-dimethylpropyl and 3,4,5-trimethoxyphenyl as substituents for the pyranose ring region of FK506 as well as simple alkyl or alkylaryl esters instead of the lead cyclohexylethyl moiety. However, based on results obtained by free energy perturbation techniques in Monte Carlo statistical mechanics simulations, Lamb and Jorgensen[18e] recently brought into question reported binding data for some of these α-dicarbonyl amides, especially those containing a 3-(3-pyridyl)propyl moiety such as GPI-

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[▽] Abbreviations: EDL, extensor digitorum longus muscles; FKBP, FK506-binding protein; pNA, 4-nitroanilide; rhCyp18, recombinant human cytosolic cyclophilin with a molecular mass of 18 kDa; IP_3, inositol 1,4,5-trisphosphate; PPIase, peptidyl-prolyl *cis/trans* isomerase.

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1046. Replacement of the diketo portion by other functionalities led to less potent peptidic[19] as well as sulfonamide[20] and urea linked[21] FKBP12 inhibitors with generally micromolar affinity for the enzyme.

Most recently, FK506 and FKBP12 ligands such as V-10,367[22] were shown to increase axonal regeneration in neuronal cell cultures and different animal models via a yet unknown calcineurin-independent mechanism.[18d,23] Furthermore, FKBP inhibitors were proven to exert powerful antineurodegenerative effects on damaged central and peripheral neurons.[18d,24,25] General neuroprotective and neurotrophic properties of GPI-1046, published by Steiner and Hamilton,[25] are, however, controversially discussed as Harper and coworkers[26] failed to reproduce corresponding in vitro and in vivo activities.

Taking into account the clinical potential of selectively acting nonimmunosuppressive FKBP inhibitors, especially for the treatment of human nerve injuries and neurodegenerative disorders, such as Parkinson's and Alzheimer's Diseases, we performed a screening of effectors of the enzyme activity of hFKBP12. A collection of structurally diverse, pure secondary metabolites was used to identify new lead structures. Surprisingly, cycloheximide (**1**)[27] was found to specifically inactivate the PPIase activity of hFKBP12. The glutarimide antibiotic cycloheximide (**1**), which is produced by several strains of *Streptomyces*, is exclusively applied in biochemical research as a potent inhibitor of eukaryotic protein synthesis.[28] We therefore became interested in the synthesis of cycloheximide derivatives which would allow to discriminate between the contributions of the inhibition of eukaryotic translation and of FKBP by **1** to observed biological effects. Since neuroprotective properties of both inhibitors of FKBP[22−25] and subtoxic dosages of cycloheximide (**1**)[29] have been shown, we chose the rat sciatic nerve neurotomy model[30] as a representative system to evaluate the possible role of FKBP inhibition by active cycloheximide derivatives, which do not affect protein synthesis, for neuronal regeneration.

Chemistry

A number of synthetic approaches were pursued to create cycloheximide derivatives. To obtain the oxime **2**, cycloheximide (**1**) was treated with hydroxylamine in pyridine. The acetyl derivative **3** of cycloheximide (**1**) was prepared by selective acetylation of the hydroxyl group with acetic acid anhydride under basic conditions.[31]

In the literature there is precedence for the partial alkylation of cycloheximide analogues to *N*-alkylcycloheximide derivatives under basic conditions[32] starting from alkyl halides. This method proved to be a facile route to provide N-alkylated cycloheximide derivatives with various alkyl substitutions at the nitrogen atom of the glutarimide moiety. A number of useful alkyl halides containing several functionalities are available commercially. The synthesis of all the *N*-alkyl derivatives, except **5**, was achieved in a straightforward manner starting from bromoalkanes in the presence of K_2CO_3 and catalytic amounts of 18-crown-6. The derivative **5** containing a methyl group on the ring nitrogen was prepared from methyl iodide in the presence of

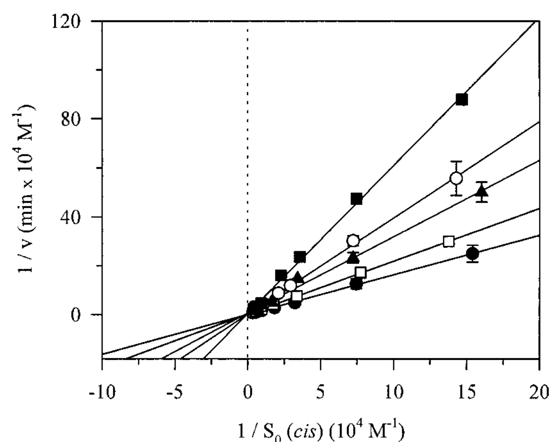


Figure 1. Lineweaver−Burk plot for the inhibition of hFKBP12 by cycloheximide (**1**). The initial velocity v of the PPIase-catalyzed reaction was determined by means of the protease-coupled PPIase assay as described in the Experimental Section using α-chymotrypsin (410 μg/mL) as a protease. hFKBP12 (14.2 nM) was incubated with concentrations of compound **1** of 0.0 (●), 0.9 (□), 1.8 (▲), 3.6 (○), and 7.2 (■) μM in 35 mM HEPES (pH 7.8) for 10 min. Remaining PPIase activity was monitored immediately after reactions were started by addition of 0.023−1.0 mM Suc-Ala-Leu-Pro-Phe-pNA.

K_2CO_3.[33] Under the used conditions N-1 and C-10 reactions cannot change the sterochemistry of the four stereocenters of cycloheximide (**1**). Similarly, the configuration on C-8 was not altered during synthesis of the acetyl derivative **3** as confirmed by ^{1}H NMR (dd, J = 8.2, 3.2 Hz, 1H, 8-H).

Results and Discussion

In the course of screening for new inhibitors of hFKBP12, using the protease-coupled PPIase assay developed by Fischer et al.,[1] a striking inhibitory activity of cycloheximide (**1**) was observed. As cycloheximide (**1**) differs completely from known FKBP12 ligands in terms of structural composition, it was chosen as a novel lead structure for detailed binding studies as well as for the investigation of structure−activity relationships in a series of synthesized cycloheximide derivatives in relation to cytotoxicity against eukaryotic cells.

On the basis of standard gel permeation chromatography methods,[34] the reversible character of the cycloheximide−hFKBP12 interaction was demonstrated. Evaluation of the inhibition kinetics by the Lineweaver−Burk plot revealed a competitive mode of inhibition of hFKBP12 by **1** (Figure 1). Using the formula of a competitive inhibitor model, a respecting K_i value of 3.4 μM was calculated (Table 1). To characterize whether enzyme inhibition by cycloheximide (**1**) expresses specificity with regard to different PPIases, we incubated members of three PPIase families with compound **1** (Table 1). Compared to the effect on hFKBP12, cycloheximide (**1**) caused a 2−60-fold weaker inhibition of the activity of further members of the FKBP family of PPIases such as *E. coli* FKBP26 (K_i = 10.3 μM), rabbit FKBP52 (K_i = 24.2 μM), FKBP22 from *Photobacterium sp.* (K_i = 76.2 μM), and *L. pneumophila* FKBP25 (Mip) (K_i = 124.0 μM). Except for a slight inhibitory effect on *E. coli* parvulin (K_i = 187.0 μM), no deterioration of the PPIase activity of tested FKBP homologous enzymes *E. coli* SlyD and *E. coli* trigger factor, the cyclophilin

Table 1. Specificity of Cycloheximide (**1**) and Its Effect on Different PPIases

PPIase family	PPIase[a]	K_i (μM)[b]
FKBPs	hFKBP12	3.4 ± 0.7
	E. coli FKBP26	10.3 ± 1.8
	rabbit FKBP52	24.2 ± 2.2
	Photobacterium sp. FKBP22	76.2 ± 6.2
	L. pneumophila FKBP25 (Mip)	124.0 ± 10.1
	E. coli trigger factor	>200
	E. coli SlyD	>200
cyclophilins	hCyp18	>200
parvulins	hPin1	>200
	E. coli parvulin	187.0 ± 12.4

[a] Concentrations of enzymes were 14 nM, 52 nM, 1 μM, 41 nM, 40 nM, 12 nM, 2 μM, 2 nM, 4 nM, and 6 nM for hFKBP12, rabbit FKBP52, *E. coli* FKBP26, *Photobacterium sp.* FKBP22, *L. pneumophila* FKBP25 (Mip), *E. coli* trigger factor, *E. coli* SlyD, hCyp18, hPin1, and *E. coli* parvulin, respectively. [b] Inhibition constants K_i for the inhibition of PPIase activity by compound **1** were determined according to the Experimental Section. Using these methods, a K_i value of 0.5 ± 0.3 nM was measured for FKBP12 inhibition by FK506 in agreement with previous reports.[15b,21,38] Data are reported as mean ± SD for three determinations.

hCyp18, and the parvulin hPin1 was observed (IC_{50} > 200 μM). Thus, cycloheximide (**1**) exhibits a certain inhibitory specificity for FKBP-like PPIases, which have been differentiated previously by their ability to bind FK506.

To identify the critical functional groups in the pharmacophore of cycloheximide (**1**) for hFKBP12 inhibition, it was necessary to prepare derivatives with modifications in positions R_1, R_2, and R_3 (Table 2). In addition, the effect of selected derivatives on the eukaryotic cell lines L-929 and K-562 was investigated by means of standard proliferation assays.[35] As expected, cycloheximide (**1**) was revealed to be extremely cytotoxic against both cell lines, thus indicating a relatively low selectivity for hFKBP12 inhibition (Table 2).

It was found that the oxime derivative **2**, obtained by modification of the C-10 carbonyl group, shows a ~260-fold lower cytotoxicity than **1** but a dramatically reduced inhibitory activity on hFKBP12. Inability to inhibit hFKBP12 was observed in the case of the *O*-acetyl derivative **3**, which displayed only a slightly decreased cytotoxic effect on L-929 and K-562 cells compared to **1**, and for the nontoxic O,N-substituted compound **4**. These results indicate a possible involvement of the C-10 carbonyl and the C-8 hydroxyl group in the binding of cycloheximide (**1**) to the active site of hFKBP12. In contrast, the calculated inhibitor constants for several of the N-substituted derivatives are in the same order of magnitude as the IC_{50} value of **1**. Therefore, the structural composition of position R_3 is presumably less important for inhibitory activity on hFKBP12.

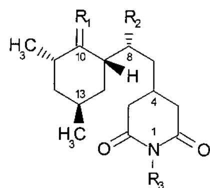
Exceptions are compounds with hydrophobic substituents in position R_3 such as methyl (**5**) and benzyl (**6**), reflected by low values of hFKBP12 IC_{50} of 124.1 and 116.7 μM, respectively. Changing the functional group at R_3 to 4-cyanobenzyl (**7**) yielded a compound with measurable inhibitory activity on hFKBP12 in the middle micromolar range that considerably affected the proliferation of K-562 and L-929 cells. Similar toxic properties but a ~3-fold lower hFKBP IC_{50} value of 21.6 μM were demonstrated by the acetic acid amide **8**. A derivative with a comparable inhibitor constant but a ~26-fold lower cytotoxicity against eukaryotic cells was obtained by substitution of the N-1 hydrogen atom of **1** by ethyl butanoate (**9**). A further reduction of the hFKBP IC_{50} to a value of 4.4 μM was observed for cycloheximide-*N*-(ethyl ethanoate) (**10**). Thus, ethyl ethanoate **10**, which caused at least an ~150-fold weaker cytotoxic effect against the eukaryotic cell lines L-929 and K-562 compared to the lead structure cycloheximide (**1**) but had a similar K_i value of 4.1 μM, was selected for further investigations.

Direct evidence for the profound deterioration of the ability of cycloheximide-*N*-(ethyl ethanoate) (**10**) to inhibit protein translation was obtained as a result of comparative studies of the influence of **1** and **10** on protein synthesis of the α-1 mating pheromone (α-factor) from *S. cerevisiae* by means of a rabbit reticulocyte type I translation assay. As illustrated in Figure 2, both compounds differ significantly in their inhibitory activity on protein translation by 3 orders of magnitude. The discrepancy in the calculated IC_{50} values for cycloheximide (**1**, 0.1 μM) and cycloheximide-*N*-(ethyl ethanoate) (**10**, 115.0 μM) confirms the above-described difference in cytotoxicity against eukaryotic cells.

On the basis of these results, cycloheximide-*N*-(ethyl ethanoate) (**10**), which satisfied required criteria for a differential cycloheximide-derived FKBP inhibitor, was selected for in vivo evaluation of neuroregenerative efficacy. The neurotrophic effect of compound **10** was studied employing the rat sciatic nerve neurotomy model[30] using FK506 as a positive control. Thereby the influence of both compounds on axonal regeneration was indirectly approached by assessment of walking behavior following neurotomy for 8 and 10 weeks. Additionally, the weight differences between the afterward prepared, sciatic nerve-innervated extensor digitorum longus muscles (EDL) of the left operated hind limbs and the corresponding right unlesioned legs were measured. The established animal model allowed us to reproduce the published enhancement in rate of axonal regeneration in rats treated with 1 mg/kg FK506 compared to placebo-treated controls (Table 3). Analysis of the walking behavior according to the criteria given in the Experimental Section showed a significant earlier onset of normal walking, reflected by a 100 ± 48% increased rate of functional recovery. On the basis of the evaluation of the differences of muscle weights, an 18 ± 12% positive regenerative tendency was seen for FK506-treated rats.

Before neuroregenerative testing of cycloheximide-*N*-(ethyl ethanoate) (**10**), the acute toxicity on 15-day-old embryonized hen's eggs was determined according to Nishigori et al.,[36] to get a crude measure for in vivo tolerated dosages. The obtained value of the maximal tolerated dosage (MTD) of compound **10** indicated an in vivo digestibility for dosages up to 50 mg/kg. Therefore, subtoxic cycloheximide-*N*-(ethyl ethanoate) (**10**) dosages of 30 mg/kg were administered to the site of the sciatic nerve lesion to get an analyzable effect. Assessment of walking behavior for a time interval of 8 weeks following neurotomy revealed an 46 ± 20% increase of functional recovery in cycloheximide-*N*-(ethyl ethanoate)-treated rats, whereas a less marked effect was obvious after 10 weeks (14 ± 7%). These results of behavioral studies agreed well with the 20 ± 1% and 13 ± 3% faster rebuilding of degenerated EDL muscles

Table 2. Evaluation of the Effect of Cycloheximide (**1**) and Cycloheximide Derivatives on the Activity of hFKBP12 and Cytotoxicity Against Eukaryotic Cell Lines L-929 and K-562



compd no.	R_1	R_2	R_3	hFKBP12 IC_{50} (μM)	cytotoxicity IC_{50} (μg/mL) L-929	K-562
1	O	OH	H	3.6	<0.39	<0.39
2	NOH	OH	H	177.0	86.9	116.5
3	O	OC(O)CH_3	H	>200	9.3	1.1
4	O	OC(O)NH-1-adamantane	$CH_2C(O)OC_2H_5$	>200	nd	nd
5	O	OH	CH_3	124.1	>200	132.6
6	O	OH	CH_2Ph	116.7	135.1	81.6
7	O	OH	*p*-CNPhCH_2	68.2	1.4	0.8
8	O	OH	$CH_2C(O)NH_2$	21.6	5.3	4.6
9	O	OH	$(CH_2)_3C(O)OC_2H_5$	22.3	144.9	109.2
10	O	OH	$CH_2C(O)OC_2H_5$	4.4	76.6	64.9

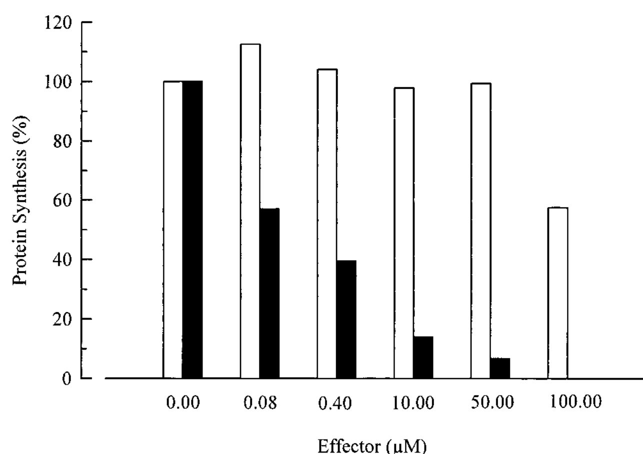


Figure 2. Effect of cycloheximide (**1**) and cycloheximide-*N*-(ethyl ethanoate) (**10**) on eukaryotic protein translation. Synthesis of *S. cerevisiae* α-factor in rabbit reticulocyte lysate was followed in the presence of 0.0−100 μM **1** (■) and **10** (□), respectively, by measuring incorporation of [^{35}S]Met for 60 min at 30 °C as stated in the Experimental Section.

Table 3. Effect of FKBP12 Inhibitors FK506 and **10** on Axonal Regeneration Following Sciatic Nerve Neurotomy

compd	dosage (mg/kg)	functional recovery (%)[a] walking behavior 8 weeks	walking behavior 10 weeks	Δm_{EDL}[b] 8 weeks	Δm_{EDL}[b] 10 weeks
FK506	1	nd	+100 ± 48	nd	+18 ± 12
10	30	+46 ± 20	+14 ± 7	+20 ± 1	+13 ± 3

[a] Neuroregenerative activities were assessed relative to placebo-caused effects as described in the Experimental Section. Data are reported as mean ± SD of *n* determinations as noted. [b] Weight differences between EDL muscles of axotomized left hind limbs and corresponding right controls.

of the lesioned hind limbs sampled after 8 and 10 weeks, respectively, compared to placebo-treated animals. Thus, cycloheximide-*N*-(ethyl ethanoate) (**10**) seems to effectively accelerate regeneration of the rat sciatic nerve at dosages of 30 mg/kg. Further experiments are in progress to determine the neuroregenerative efficacy in case of additional daily repeated subcutaneous injections of cycloheximide-*N*-(ethyl ethanoate) dosages for different time intervals. This will include a complete dose-dependence study.

Conclusion

In summary, the present results characterize the inhibition of FKBP12 by cycloheximide (**1**), which represents a new biological property of the compound. Cycloheximide (**1**) originally became the subject of pharmaceutical interest because of its fungicidal effectiveness, especially against yeasts and parasitic fungi, as well as its antiviral activity.[28a] Further studies were extended to the investigation of the potential pharmaceutical suitability of cycloheximide (**1**) and synthetic analogues for the prevention of proliferative skin diseases and epileptical disorders.[32] Today, exclusively the ability of cycloheximide (**1**) to inhibit eukaryotic protein synthesis is utilized in biochemical areas.[28] However, cycloheximide (**1**) concentrations in the range of 0.1−100 μM routinely used in biological experiments[37] may also cause a significant inhibition of FKBPs which has to be considered for the interpretation of results. As an example, the neuroregenerative capacity of cycloheximide-*N*-(ethyl ethanoate) (**10**) was demonstrated as a cycloheximide derivative ineffective in terms of translational inhibition. Thus, a possible contribution of the FKBP inhibition to neuroprotective effects of micromolar concentrations of cycloheximide (**1**) shown in different animal models[29] can be hypothesized.

Experimental Section

General Methods. 4-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-2,6-piperidinedione (cycloheximide) (**1**) and reagents were purchased from Aldrich Chemical Co. (Steinheim, Germany). Mass spectra were obtained on a Hewlett-Packard MALDI-TOF MS system G2025A. NMR spectra (Bruker Avance DPX 300, DRX 500; TMS as internal standard; ^{1}H, 300 or 500 MHz; ^{13}C, 75 MHz; $CDCl_3$ unless otherwise noted) were recorded for all compounds. Melting points were determined on a Büchi melting point B-545. TLC sheets of silica gel 60 F_{254} from Merck (Darmstadt, Germany) were used for qualitative information. Chromatographical purification was carried out on silica gel 60 M (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Elemental analysis was performed on a CHN-O-Rapid from Foss Heraeus.

4-[2-(3,5-Dimethylcyclohexyl 2-oxime)-2-hydroxyethyl]-2,6-piperidinedione (2). To a solution of 1.0 g (3.56 mmol) of cycloheximide (**1**) in 6.0 mL of pyridine was added 2.0 mL of a saturated solution of [NH_3OH]Cl in water. The reaction mixture was stirred at 35 °C for 2 h. After concentration in vacuo and addition of 5.0 mL of water, a white precipitate was observed, which was filtered, washed twice with water and CH_2Cl_2, and dried to yield 863 mg (82.3%) of the oxime **2**: mp = 210−213 °C dec; MALDI-TOF-MS $[M + H]^+$ = 297.0 *m*/*e*; ^1H NMR (500 MHz) (DMSO-d_6) δ 10.59 (s, 1H), 10.20 (s, 1H), 4.74 (d, 1H, *J* = 7.1 Hz), 3.66 (q, 1H, *J* = 8.8 Hz), 3.20 (dd, 1H, *J* = 9.6, 5.4 Hz), 2.59−2.43 (comp, 3H), 2.30−2.13 (comp, 4H), 2.08 (d, 1H, *J* = 13.0 Hz), 1.93 (mult, 1H), 1.53 (d, 1H, *J* = 12.5 Hz), 1.44 (mult, 1H), 1.24−1.13 (comp, 2H), 1.01 (d, 3H, *J* = 7.6 Hz), 0.92 (d, 3H, *J* = 7.5 Hz); ^{13}C NMR (75 MHz) δ 173.4, 173.2, 160.5, 67.0, 41.2, 38.5, 36.3, 34.7, 34.6, 26.7, 26.5, 22.1, 21.4, 20.1, 17.3. Anal. ($C_{15}H_{24}N_2O_4$) C, H, N.

4-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-acetoxyethyl]-2,6-piperidinedione (3). To a solution of 1.0 g (3.56 mmol) of cycloheximide (**1**) in 7.0 mL of pyridine was added 2.5 mL (26.5 mmol) of acetic acid anhydride. The reaction mixture was stirred at 25 °C for 1.5 h. After concentration in vacuo the crude product was purified by recrystallization from ethanol to yield 947 mg (82.3%) of compound **3** as white crystals: mp = 144−145 °C; MALDI-TOF-MS $[M + H]^+$ = 324.5 *m*/*e*; ^1H NMR (500 MHz) δ 8.63 (s, 1H), 5.28 (dt, 1H, *J* = 8.2, 3.2 Hz), 2.86 (dd, 1H, *J* = 17.3, 3.0 Hz), 2.66−2.49 (comp, 3H), 2.35−2.18 (comp, 2H), 2.12 (mult, 2H), 2.00 (s, 3H), 1.83 (mult, 2H), 1.69−1.52 (comp, 4H), 1.19 (d, 3H, *J* = 7.2 Hz), 0.93 (d, 3H, *J* = 6.4 Hz); ^{13}C NMR (75 MHz) δ 212.0, 172.2, 172.0, 170.4, 69.4, 49.0, 42.6, 40.6, 38.6, 38.2, 36.8, 36.1, 27.2, 26.6, 20.8, 18.0, 14.0. Anal. ($C_{17}H_{25}NO_5$) C, H, N.

4-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-(oxycarbonyl-1-adamantylamino)ethyl]-2,6-piperidinedione-1-(ethyl ethanoate) (4). To a solution of 300 mg (0.82 mmol) of 4-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-2,6-piperidinedione-1-(ethyl ethanoate) (**8**) in 5.0 mL of dry CH_2Cl_2 was added 145 mg (0.82 mmol) of 1-adamantyl isocyanate. The reaction mixture was stirred at 25 °C for 3 h. After concentration in vacuo the crude product was purified by column chromatography on silica gel (CH_2Cl_2/EtOAc (5:1)) to give 394 mg (88.2%) of compound **4** as a white solid: mp = 73−74 °C; MALDI-TOF-MS $[M + H]^+$ = 545.9 *m*/*e*; ^1H NMR (300 MHz) δ 5.07 (mult, 1H), 4.49 (s, 2H), 4.16 (q, 2H, *J* = 7.1 Hz), 3.00 (dd, 1H, *J* = 17.2, 3.0 Hz), 2.82−2.37 (comp, 6H), 2.27 (mult, 1H), 2.16 (mult, 1H), 2.07 (br, 3H), 1.92 (br, 8H), 1.73−1.55 (comp, 10H), 1.31−1.20 (comp, 6H), 0.98 (d, 3H, *J* = 6.4 Hz); ^{13}C NMR (75 MHz) δ 212.5, 171.7, 171.5, 167.9, 61.4, 50.8, 49.8, 42.9, 41.8, 40.8, 40.6, 38.9, 38.6, 37.9, 36.5, 36.3, 29.4, 26.8, 26.6, 18.2, 14.2, 14.1. Anal. ($C_{30}H_{44}N_2O_7$) C, H, N.

4-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-1-methyl-2,6-piperidinedione (5). To a solution of 300 mg (1.07 mmol) of cycloheximide (**1**) and 170 mg (1.2 mmol) of CH_3I in 3.0 mL of acetone were added 10 mg (0.04 mmol) of 18-crown-6 and 200 mg (1.45 mmol) of K_2CO_3. The reaction mixture was stirred at 25 °C for 48 h. After filtration and concentration in vacuo the crude product was purified by column chromatography on silica gel (CH_2Cl_2/EtOAc (3:1)) to give 147 mg (46.5%) of compound **5** as a colorless, viscous oil: MALDI-TOF-MS $[M + H]^+$ = 296.5 *m*/*e*; ^1H NMR (300 MHz) δ 4.12 (d, 1H, 10.7 Hz), 3.03 (s, 3H), 2.87−2.68 (comp, 3H), 2.55 (mult, 1H), 2.42 (mult, 1H), 2.37−2.21 (comp, 3H), 2.12 (mult, 1H), 1.91−1.72 (comp, 3H), 1.61−1.44 (comp, 2H), 1.19−1.07 (comp, 4H), 0.90 (d, 3H, *J* = 6.4 Hz); ^{13}C NMR (75 MHz) δ 216.0, 172.3, 172.1, 66.2, 50.0, 42.4, 40.3, 39.2, 38.0, 33.0, 26.5, 26.4, 26.0, 18.1, 14.0. Anal. ($C_{16}H_{25}NO_4$) C, H, N.

4-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-1-benzyl-2,6-piperidinedione (6). In a typical experiment 300 mg (1.07 mmol) of cycloheximide (**1**), 205 mg (1.2 mmol) of 4-benzyl bromide, 10 mg (0.04 mmol) of 18-crown-6, and 200 mg (1.45 mmol) of K_2CO_3 in 3.0 mL of acetone were stirred at 25 °C for 2 days. After filtration and concentration in vacuo the crude product was purified by column chromatography on silica gel (CH_2Cl_2/EtOAc (3:1)) to yield 186 mg (46.8%) of compound **6** as a colorless, viscous oil: MALDI-TOF-MS $[M + H]^+$ = 372.9 *m*/*e*; ^1H NMR (300 MHz) δ 7.37−7.19 (comp, 5H), 4.92 (s, 2H), 4.16 (dt, 1H, *J* = 10.8, 2.5 Hz), 2.86 (d, 2H, *J* = 12.7 Hz), 2.61 (mult, 1H), 2.49−2.30 (comp, 5H), 2.18 (mult, 1H), 1.96−1.72 (comp, 3H), 1.66−1.51 (comp, 2H), 1.22 (d, 3H, *J* = 7.1 Hz), 1.05−1.15 (mult, 1H), 0.97 (d, 3H, *J* = 6.4 Hz); ^{13}C NMR (75 MHz) δ 216.3, 172.0, 171.8, 140.8, 137.2, 128.7, 128.3, 127.3, 66.4, 50.0, 42.6, 42.5, 40.4, 39.6, 38.3, 37.8, 32.9, 26.6, 26.4, 18.3, 14.1. Anal. ($C_{22}H_{29}NO_4$) C, H, N.

4-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-1-(4-cyanobenzyl)-2,6-piperidinedione (7). In a typical experiment 300 mg (1.07 mmol) of cycloheximide (**1**), 235 mg (1.2 mmol) of 4-cyanobenzyl bromide, 10 mg (0.04 mmol) of 18-crown-6, and 200 mg (1.45 mmol) of K_2CO_3 in 3.0 mL of acetone were stirred at 25 °C for 2 days. After filtration and concentration in vacuo the crude product was purified by column chromatography on silica gel (CH_2Cl_2/EtOAc (3:1)) to yield 163 mg (38.4%) of compound **7** as a colorless, viscous oil: MALDI-TOF-MS $[M + H]^+$ = 397.8 *m*/*e*; ^1H NMR (300 MHz) δ 7.67−7.35 (comp, 4H), 4.94 (s, 2H), 4.19 (d, 1H, *J* = 10.9 Hz), 3.49 (s, 2H), 2.89 (d, 2H, *J* = 12.8 Hz), 2.63 (m, 1H), 2.56−2.32 (comp, 3H), 2.21 (mult, 1H), 1.99−1.73 (comp, 3H), 1.69−1.52 (comp, 2H), 1.23 (d, 3H, *J* = 7.1 Hz), 1.18−1.07 (mult, 1H), 0.98 (d, 3H, *J* = 6.4 Hz); ^{13}C NMR (75 MHz) δ 216.8, 172.1, 171.9, 138.6, 133.5, 132.4, 131.2, 129.2, 118.6, 112.6, 66.6, 50.1, 44.8, 42.0, 39.6, 38.4, 38.1, 31.5, 26.6, 26.0, 18.3, 14.1. Anal. ($C_{23}H_{28}N_2O_4$) C, H, N.

4-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-2,6-piperidinedione-1-(acetic acid amide) (8). To a solution of 500 mg (1.78 mmol) of cycloheximide (**1**) and 275 mg (2.0 mmol) of bromoacetic acid amide in 5.0 mL of acetone were added 10 mg (0.04 mmol) of 18-crown-6 and 300 mg (2.18 mmol) of K_2CO_3. The reaction mixture was stirred at 25 °C for 3 days. After filtration and concentration in vacuo the crude product was purified by column chromatography on silica gel (CH_2Cl_2/EtOAc (3:1)) to yield 214 mg (35.5%) of compound **8** as a colorless, viscous oil: MALDI-TOF-MS $[M + H]^+$ = 339.9 *m*/*e*; ^1H NMR (500 MHz) δ 6.03 (mult, 2H), 4.43 (s, 2H), 4.16 (d, 1H, *J* = 10.8 Hz), 2.95−2.80 (comp, 2H), 2.62 (comp, 2H), 2.54−2.41 (comp, 4H), 2.19 (mult, 1H), 1.93−1.83 (comp, 3H), 1.60 (td, 2H, *J* = 13.3, 4.2 Hz), 1.33−1.26 (m, 1H), 1.22 (d, 3H, *J* = 7.1 Hz), 0.97 (d, 3H, *J* = 6.4 Hz); ^{13}C NMR (75 MHz) δ 216.3, 172.3, 172.1, 169.5, 66.5, 50.3, 42.6, 41.6, 40.8, 38.9, 38.0, 37.8, 33.4, 26.7, 26.4, 18.3, 14.2. Anal. ($C_{17}H_{26}N_2O_5$) C, H, N.

4-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-2,6-piperidinedione-1-(4-ethyl butanoate) (9). To a solution of 500 mg (1.78 mmol) of cycloheximide (**1**) and 390 mg (2.0 mmol) of 4-bromobutanoic acid ethyl ester in 10 mL of dimethylformamide was added 414 mg (3.0 mmol) of K_2CO_3. This reaction mixture was stirred at 25 °C for 3 days. Subsequently, the solvent and other volatile organics were removed under low pressure. The remaining residue was dissolved in a mixture of 7.0 mL of $CHCl_3$ and 3.0 mL of EtOAc. After filtration and concentration in vacuo the crude product was purified by column chromatography on silica gel ($CHCl_3$/EtOAc (2:1)) to yield 197 mg (27.8%) of compound **9** as a colorless, viscous oil: MALDI-TOF-MS $[M + H]^+$ = 397.8 *m*/*e*; ^1H NMR (300 MHz) δ 4.20 (d, 1H, *J* = 10.8 Hz), 3.91 (t, 2H, *J* = 6.7 Hz), 3.68 (s, 1H), 3.45−3.23 (comp, 3H), 3.10−1.50 (comp, 16H), 1.33−0.95 (comp, 10H). Anal. ($C_{21}H_{33}NO_6$) C, H, N.

4-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]-2,6-piperidinedione-1-(ethyl ethanoate) (10). To a solution of 5.0 g (17.8 mmol) of cycloheximide (**1**) and 3.0 mL (27.0 mmol) of bromoacetic acid ethyl ester in 35 mL of acetone were added 100 mg (0.38 mmol) of 18-crown-6 and 3.0 g (21.6 mmol) of K_2CO_3. The reaction mixture was stirred at 25 °C for 3 days. After the potassium salts were filtered off, the filtrate was concentrated in vacuo. The oil was purified by column chromatography on silica gel ($CHCl_3$/EtOAc (2:1)). Recrystallization from EtOAc afforded 4.3 g (65.7%) of compound **10** as white crystals: mp = 100−101 °C; $[\alpha]_D$= +8.7° (pyridine); MALDI-TOF-MS $[M + H]^+$ = 368.8 *m*/*e*; ^1H NMR (300 MHz) δ 4.50 (s,

2H), 4.27–4.13 (comp, 3H), 2.97–2.81 (comp, 3H), 2.63 (mult, 1H), 2.55–2.43 (comp, 4H), 2.21 (mult, 1H), 2.02–1.78 (comp, 3H), 1.69–1.55 (comp, 2H), 1.33–1.22 (comp, 7H), 0.99 (d, 3H, $J = 6.4$ Hz); ^{13}C NMR (75 MHz) δ 216.5, 171.7, 171.6, 167.9, 66.6, 61.4, 50.1, 42.6, 40.5, 39.1, 37.7, 37.6, 33.0, 26.7, 26.4, 18.3, 14.1, 14.0. Anal. ($C_{19}H_{29}NO_6$) C, H, N.

In Vitro Assays. PPIases were kindly provided by Dr. J.-U. Rahfeld (rhFKBP12, *E. coli* FKBP26, FKBP22 from *Photobacterium sp.*, rhPin1, *E. coli* parvulin), Dr. B. Schmidt (*L. pneumophila* FKBP25 (Mip)), S. Hottenrott (*E. coli* SlyD), Dr. T. Zarnt (rabbit FKBP52), and Dr. G. Stoller (*E. coli* trigger factor; Halle, Germany). Human Cyp18 was produced recombinantly in *E. coli*.[4c] All substrates were synthesized by Dr. M. Schutkowski (Halle, Germany). PPIase activities were measured according to Fischer et al.[1] using α-chymotrypsin (Merck, Darmstadt Germany; 410 μg/mL) as isomer-specific protease and the peptide Suc-Ala-Phe-Pro-Phe-pNA (24.0 μM) as a substrate. In the case of the *E. coli* SlyD and hPin1, the protease trypsin (Boehringer/Mannheim, Germany; 210 μg/mL) and the peptides Suc-Ala-Phe-Pro-Arg-pNA (34.0 μM) and Ac-Ala-Ala-Ser(P)-Pro-Arg-pNA (26.0 μM), respectively, were applied instead. In general, the test was performed by observing the released 4-nitroaniline at 390 nm with a Hewlett-Packard 8452A diode array UV/vis spectrophotometer at 10 °C. For inhibition experiments, the enzymes were incubated with the effectors (0.01–200 μM) in 1200 μL of 35 mM HEPES (pH 7.8) for at least 5 min. Effectors were freshly diluted from 10–20 mM stock solutions in DMSO. The amount of organic solvent was kept constant within each experiment, usually below 0.1% (v/v). The reactions were started by addition of 2–3 μL of a peptide stock solution (10 mg/mL in DMSO). Under the condition $[S_0] \ll K_m$, recorded progression curves can be described by first-order kinetics. From the observed rate constant k_{obs} a first-order rate constant k_{enz} for enzymatic catalysis of *cis* to *trans* isomerization can be calculated as $k_{obs} = k_0 + k_{enz}$, where k_0 represents the first-order rate constant for the uncatalyzed *cis/trans* isomerization. Inhibitor constants (IC_{50}, K_i) were determined by analyzing the concentration dependence of each inhibitor on PPIase activity. Apparent K_i values were obtained by fitting the data by nonlinear regression to a mass action ratio-based equation for competitive binding inhibition. The Michaelis–Menten kinetics for the inhibition of hFKBP12 by cycloheximide (**1**) was determined according to the method of Kofron et al.[38] For the assay a 100 μg/mL solution of the substrate Suc-Ala-Leu-Pro-Phe-pNA (final concentration 0.02–1.0 mM) in 0.5 M LiCl/trifluoroethanol was used, thereby ensuring a proportion of peptide molecules in *cis* conformation of ~40%. Data were analyzed by means of the software programs SigmaPlot Scientific Graphing System version 4.0 (Jandel Corp., Chicago, IL) and TREND (Martin-Luther-University, Halle-Wittenberg, Germany).

The eukaryotic translation assay was performed using a rabbit reticulocyte type I translation kit (Boehringer/Mannheim, Germany) according to the manufacturer's instructions with the following changes: α-factor mRNA, generously provided by Dr. S. Panzner (Halle, Germany), was chosen as a substrate. To inhibit degradation of sample mRNA, Rnasin ribonuclease inhibitor (40 U/μL; Promega, Heidelberg, Germany) was added to the reaction mixtures containing reticulocyte lysate, 1 mM amino acid mixture without Met, 10 mCi/mL [^{35}S]Met, 100 μg/mL RNA, and 100–0.08 μM cycloheximide (**1**) or cycloheximide-*N*-(ethyl ethanoate) (**10**) diluted in nuclease-free H_2O, respectively.

In Vivo Studies. For the sciatic nerve neurotomy experiment,[30] 4-month-old female rats (HAN:Wist) were anesthetized by intramuscular application of 16 mg/kg RompunTS (Bayer, Leverkusen, Germany) and 125 mg/kg Ketavet (Pharmacia & Upjohn GmbH, Erlangen, Germany). The sciatic nerve of the left hind leg was exposed; the three fascicles were separated and then dissected. Immediately after dissection the nerve was microsurgically reconstructed by three interfascicular anastomoses with Ethilon 10/0 (Ethicon, Hamburg, Germany). FK506 ($n = 4$), kindly provided by Dr. A. Lawen (Clayton, Australia), and cycloheximide-*N*-(ethyl ethanoate) (**10**; $n = 7$) were applied directly to the anastomoses in dosages of 1 and 30 mg/kg in 5% DMSO, respectively. The reconstructed nerve was covered with a tube of BActerial SYnthesized Cellulose (BASIC)[39] serving as a drug depot. Corresponding dosages of the dipeptide Ala-Ala-OH ($n = 6$) were used as a placebo. All animals showed a complete paralysis of the concerned leg after neurotomy. Functional recovery was studied by weekly assessment of the walking behavior during 8 and 10 weeks, respectively. Following criteria were applied: usage and movement coordination of the operated leg, foot and toe posture, toe movement. The situation was quantified blindly using a point system: +1, positive; 0, inconspicuous; −1, negative. Obtained data were evaluated by calculation of the percentage of clinical signs of functional recovery relative to the placebo; 8 and 10 weeks after neurotomy the animals were again anesthetized and the EDL from both hind legs were prepared and removed. The muscle weights were determined in relation to protein concentration, and the percentages of weight differences of EDL muscles of the left neurotomized hind limbs in comparison to the corresponding right unlesioned legs were calculated relative to the placebo.

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