Reversible Inhibition of Calcineurin
by the Polyphenolic Aldehyde Gossypol

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SUMMARY
The reversible inhibition of calcineurin (CaN), which is the only Ca\(^{2+}\)/calmodulin dependent protein Ser/Thr phosphatase, is thought to be a key functional event for most cyclosporin A-(CsA) and FK506-mediated biological effects. Beside CaN inhibition, however, CsA and FK506 have multiple biochemical effects due to their action in a gain of function model that requires prior binding to immunophilic proteins. We screened a small molecule library for direct inhibitors of calcineurin using calcineurin mediated dephosphorylation of \([^{33}\text{P}]\)-labelled 19-residue phosphopeptide substrate (RII phosphopeptide)\(^1\) as an assay and found the polyphenolic aldehyde gossypol as a novel calcineurin inhibitor. Unlike CsA and FK506, gossypol does not require a matchmaker protein for reversible calcineurin inhibition with an IC\(_{50}\) value of 15 µM. Gossypolone, a gossypol analog, showed improved inhibition of both RII phosphopeptide and p-nitrophenylphosphate dephosphorylation with an IC\(_{50}\) of 9 µM and 6 µM, respectively. In contrast, apogossypol hexaacetate was inactive. Gossypol acts noncompetitively, interfering with the binding site for the Cyp18/CsA-complex in calcineurin. In contrast to FK506 and cyclosporin A, gossypol does not inactivate the peptidyl prolyl cis/trans isomerase (PPIase) activity of immunophilins. Similar to CsA and FK506, NFATC1 translocation from the cytosol into the nucleus in response to stimulation by PMA/ionomycin is inhibited by gossypol in a dose-dependent manner, and T cell signaling is suppressed dose-dependently in a NFAT-luciferase reporter gene assay.
Introduction

In vivo inhibition by membrane-penetrable low-molecular mass compounds plays an important role in evaluating the biological function of enzymes involved in protein phosphorylation/dephosphorylation. The functional discrimination with specific inhibitors of the four major protein Ser/Thr phosphatases, protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), protein phosphatase 2B (PP2B, calcineurin, CaN) and protein phosphatase 2C (PP2C) has proven to be successful (1). Okadaic acid and microcystin are strong inhibitors of PP1 and PP2A, but exhibit poor inhibition of the calcium/calmodulin regulated CaN and PP2C. It was difficult to identify CaN function in cell signaling until the membrane-penetrable cyclopeptide cyclosporin A (CsA) and the peptidomacrolide FK506 were found to inhibit CaN specifically under certain conditions (2). Currently, most reports about CaN involvement in cellular processes are based on CsA and FK506 susceptibility of the appropriate bioassays. CaN inhibition by these drugs is characterized by their prior binding to the 18-kDa cyclophilin (Cyp18) and 12-kDa FK506-binding protein (FKBP12), respectively, indicating a gain-of-function mechanism (3). These mammalian prototypes of two different families of the enzyme class of peptidyl prolyl cis/trans isomerases (EC 5.2.1.8; PPIases) function as molecular matchmakers because the drugs cannot bind to CaN on their own. The prototypic PPIases are themselves tightly inhibited in the course of formation of the CaN-inhibitory PPIase/drug complex (4).

It is essential to recognize the uncertainties in the interpretation of the biological effects found for the application of CsA and FK506 in cellular assays. They include the above-discussed multifunctional biochemical properties released through the PPIase/drug interaction and the often-unknown cellular content and isoenzyme composition of the matchmaker proteins. Recent analyses of the human genome revealed the existence of 18 cyclophilins and 16 FKBPs, at least, most of which having the potential to be enzymatically active and to form PPIase/drug complexes with mostly unknown affinity for CaN (5, 6, 7). In fact, the CaN-inhibitory complexes of different affinity for CaN are formed from the matchmaker proteins in competition for the limiting amounts of the drug rendering inhibition
less predictable. Other drug effects may result from the facilitated dissociation of receptor/PPIase complexes (8, 9) and the slow rate of formation of the PPIase/drug complexes (10, 11). In addition, the PPIase-mediated intracellular accumulation of the drugs prevents the reliable analysis of dose-response curves. Although the molarity of the administered dose is known the variable intracellular PPIase concentration does not permit calculations of the biologically effective dose.

It has now become apparent that the demonstration of an influence of CsA and FK506 on the signal of any biological assay does not by itself constitute final proof as to the involvement of CaN in cell signaling.

While the use of drug derivatives lacking the CaN effector domain has permitted the application of CaN inactive PPIase inhibitors (12, 13, 14, 15), corresponding experiments with specific small-molecule CaN inhibitors, which are inactive toward PPIases, are still missing. The results of biological studies using pyrethroid insecticides as CaN inhibitors (16) were at variance with recent investigation which could not find any CaN inhibition with this class of compounds (17, 18). The tyrphostin class of tyrosine kinase inhibitors possesses CaN inhibitory potency in the micromolar range but lacks CaN specificity. The CsA-like anti-human immunodeficiency virus-type 1 (HIV-1) replication inhibition of ring-substituted benzo thiophen-2-carboxamide was attributed to its CaN inactivating properties (19).

However, CaN inactivation has not been characterized with purified enzyme, and CsA does not act on HIV-1 replication via CaN inhibition (13).

Irreversible CaN inactivation by 4-(fluoromethyl) phenyl phosphate is active in the millimolar range but does not exhibit CaN specificity (20). A similar disadvantage must be considered for cantharidin and endothall as CaN inhibitors, because they cannot differentiate between CaN and PP1 or PP2A (21, 22). The inhibition of CaN-mediated dephosphorylation RII phosphopeptide by the dihydroisobenzofuran dibefurin exhibits an IC_{50} of 46 µM but the specificity of inhibition remained unknown. Interestingly, this compounds was active in the mixed lymphocyte reaction assay (23).
Alternatively, CaN-inhibitory polypeptides as a 25 residue oligopeptide excised from the autoinhibitory CaN domain (IC$_{50}$= 10 µM) (24), the 97 amino acid residue autoinhibitory oligopeptide of CaN (IC$_{50}$= 5 µM) (25), and the AKAP79 protein (IC$_{50}$= 4.2 µM) (26) may serve as possible functional probes but have high molecular masses and transport limitations.

To provide a more reliable tool for investigating the role of CaN in cellular processes, we screened a compound library for low-molecular mass inhibitors of CaN-mediated dephosphorylation of RII phosphopeptide. The experimental approach is based on scintillation proximity concept with streptavidin-coated scintillation wells. The polyphenolic aldehyde gossypol was identified to be a reversible inhibitor that is specific for CaN among the Ser/Thr phosphatases, and does not require prior binding to a molecular matchmaker. Furthermore, we have characterized the ability of gossypol to inhibit CaN-mediated cellular dephosphorylation of the cytoplasmic component of the transcription factor NFAT by using western blotting and the NFAT-luciferase reporter gene assay.
Experimental Procedures

Materials

Streptavidin coated scintillation wells were purchased from Wallac (Turku, Finland). The biotinylated and nonbiotinylated 19-residue peptides of a partial sequence of the subunit of the bovine cAMP-dependent protein kinase (DLDVIPGRFDRRVSVAE-OH) were synthesized. The purity of the peptides was assessed by analytical reversed-phase HPLC. Peptides were characterized by ESI-MS. Recombinant human Cyp18, human FKBP12 and human Pin1 have been prepared as described elsewhere (27, 28) The catalytic subunit of bovine heart cAMP dependent protein kinase (PKA) was obtained from Roche (Mannheim, Germany). Protein Phosphatase 1 (recombinant rabbit muscle α-isoform) was purchased from Calbiochem (Bad Soden, Germany) and Protein Phosphatase 2C (recombinant human α-isoform) from Upstate biotechnology (Biomol, Hamburg, Germany). Trimeric Protein Phosphatase 2A with a subunit composition of Cα/β Aα Bα was kindly provided by A. Werner (University Halle, Germany). Calmodulin, (+/-)-gossypol, (+/-)-gossypolone, (+/-)-apogossypol hexaacetate, buffers and salts were purchased from Sigma (Taufkirchen, Germany). Expression and purification of the recombinant human CaN α (rhCaN) from the Escherichia coli strain BL21-(pLysS)/pETCNα/pBB131 was performed as published previously (29).

The compound library „Natural Products Pool“ (Hans-Knöll-Institute for Natural Products Research, Jena, Germany) at the time comprising approximately 5,000 pure compounds has been used (30).

Enzyme activity assays

RII phosphopeptide

The biotinylated and nonbiotinylated 19-residue peptides of a partial sequence of the subunit of the bovine cAMP-dependent protein kinase (PKA) were phosphorylated according to a procedure previously described (31). In brief, the reaction mixture contained the following concentrations 700 µM peptide, 100 µCi [γ-33P]ATP with a specific activity of approximately
3000 Ci/mmol from a stock solution of 10 µCi/µl (ICN, Eschwege, Germany), and 125 µM ATP in a final volume of 100 µl buffer (20 mM MES, pH 6.5, 0.4 mM EDTA, 0.2 mM EGTA, 50 µM CaCl₂ and 5 mM MgCl₂). The phosphorylation of the peptides in the reaction mixture were performed with 10 mU PKA at 30°C for 1h. Then, the peptides and ATP were separated by a 1ml RP-C2 clean-up extraction column (Amchro, Sulzbach, Germany). The peptides was eluted with 70% acetonitril/water, then freeze-dried, and dissolved in water prior to use. The level of peptide phosphorylation was 24 %. The calculation takes into account the total amount of radioactivity in the peptide fraction, the specific activity of incorporated [³³P] and the ratio of radioactive and nonradioactive ATP in the reaction mixture.

RII-phosphopeptide based calcineurin activity assay

The scintillation proximity concept (32, 33) has been applied to measure CaN activity using scintillation wells coated with streptavidin. Preincubation of calmodulin (50 nM), CaN (1.32 nM), and inhibitor at the required concentrations in assay buffer (40 mM Tris/HCl, pH 7.5; 100 mM NaCl, 6 mM MgCl₂, 0.5 mM DTT, 1 mM CaCl₂, 0.1 mg/ml BSA) was carried out at 22°C for 30 min in a 96-well microtiter plate (Costar, Bodenheim, Germany). 10 pmol biotinylated [³³P] RII phosphopeptide were added to each well in a total assay volume of 100 µl. After dephosphorylation of the modified RII phosphopeptide by CaN at 30°C for 20 min, a 90-µl sample of the reaction mixture was transferred to a scintillation well coated with streptavidin. Biotinylated RII phosphopeptide was allowed to bind to streptavidin for 20 min at 22°C. The well was washed once with water, and the RII phosphopeptide associated [³³P] radioactivity was measured in a MicroBeta top-counter (Wallac, Turku, Finland).

Assay of protein phosphatases using [³²P]-labeled protein substrates

Protein phosphatase 1 and 2A were assayed by using [³²P]-labeled phosphorylase a as described in (34). For CaN and protein phosphatases 2C [³²P] casein was used as substrate
Procedures for $[^{32}\text{P}]$-labeling are detailed in ref. 36 and 37. Phosphorylase b, phosphorylase kinase and casein were purchased from Sigma (Taufkirchen, Germany).

**Inhibition of calcineurin**

Stock solutions of substances from the product library (10 mg/ml DMSO) tested for CaN inhibition were stored at -70°C. Each substance was assayed at a final concentration of 100 µg/ml in the reaction mixture.

The CaN inhibition was measured in a concentration range of 0.5 to 200 µM of gossypol and gossypol-derivatives at optimal Ca$^{2+}$ and calmodulin concentrations. The obtained data were fitted and computed with the SigmaPlot program (SPSS Inc., San Rafael, U.S.A.).

For competition experiments, the CaN/gossypol mixture was equilibrated in the assay buffer at 22°C for 30 min. Subsequent use of the incubation mixture in the RII phosphopeptide-based CaN assay yielded a final concentration of 1.3 nM CaN/12 µM gossypol. Residual CaN activity was determined in the simultaneous presence of gossypol and 10 µM CsA at varying concentrations of rhCyp18, and under similar conditions for CsA and gossypol alone.

CaN activity was referenced to the assay lacking additional compounds.

For kinetic analyses, biotinylated $[^{32}\text{P}]$ RII phosphopeptide and nonbiotinylated RII phosphopeptide were mixed at a concentration ratio of 1:200 in the assay buffer. A total RII phosphopeptide concentration of 5 to 10 µM was used for each concentration of gossypol (5-20 µM).

**pNPP-based calcineurin activity assay**

Phosphatase activity was measured at room temperature using p-nitro phenylphosphate (pNPP) as substrate in phosphatase assay buffer (see above). After preincubation of CaN and calmodulin with the inhibitor in the assay buffer at room temperature for 20 min, the reaction was initiated by the addition of pNPP to final concentrations up to 25 mM. The release of p-nitrophenol was continuously measured on a Dynatec MR7000 micotiterplate reader at 410 nm for 30 min.
**PPLase assay**

PPLases activity was determined with oligopeptide substrates using protease-coupled assays as described elsewhere (38, 39). Typically, experiments were performed with the PPLase concentrations in the low nM range and gossypol (120 µM) within the assay. The effect of gossypol on the PPLase activity was calculated from the remaining activity after preincubation of the enzyme and the inhibitor for 20 min.

**Reversibility of calcineurin inhibition**

The equilibrated gossypol/CaN mixture (120 µM/13.2 nM stock solution) in assay buffer containing calmodulin (50 nM) was used to examine the reversibility of inhibition by dialysis experiments. The mixture was placed on a Pierce system microdialyser equipped with a Mr-3000 cut-off dialysis membrane and dialyzed against the assay buffer at 4°C. Reference activity was determined with a CaN sample treated similarly but lacking gossypol. Aliquots of 10 µl dialyzed enzyme or gossypol/enzyme complex were assayed using RII phosphopeptide substrate. Ferrous ammonium sulfate at a final concentration of 50 to 500 µM was used to study the ability to recover the activity of gossypol-inhibited CaN according to the procedure previously described (40). CaN activity was assayed using the pNPP substrate.

**Elution of calcineurin from a Cyp18/CsA affinity column by gossypol**

For preparation of the column 500 µg rhCyp18 were immobilized on 250 µg Affi-Gel 10 (Biorad). Remaining reactive groups of the gel were blocked by addition of 1 M Tris/HCl pH 7.5. Subsequently the column was preincubated with 300 nmol CsA for 1 h. Before and after binding of 30 µg calcineurin on the column the beads were washed several times with PBS. Elution of calcineurin was performed by incubation for 10 minutes with 100 µM apogossypol hexaacetate, following 100 µM gossypol and 15 µM preformed Cyp18/CsA-complex. Eluates
were analysed by western blotting using in-house rabbit anti human polyclonal antibodies specific for CaN.

**Inhibition of other protein Ser/Thr phosphatases**

The activities of the three protein phosphatases PP1, PP2A, and PP2C were measured with RII phosphopeptide, as described for CaN. For assaying PP2C a final concentration of 30 mM MgCl₂ was also included. The protein phosphatase concentrations were adjusted to an activity level of approximately 80% dephosphorylation of 10 pmol RII phosphopeptide within 20 min. Protein phosphatase inhibition was evaluated for gossypol at a concentration range of 1 to 100 µM.

**T cell purification and cell culture**

Human PBMC were obtained from healthy volunteers using Ficoll-Hypaque gradient centrifugation. CD4 and CD8 T cells (>97% pure) were purified by positive selection using magnetic Multisort-MicroBeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

The selected cells were cultured at 3 x 10⁷ /ml in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10 % FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin overnight. Then, lymphocytes were split and preincubated with one of the following compounds at various concentrations as indicated: CsA (AWD, Dresden, Germany), apogossypol hexaacetate or gossypol at 37°C for 10 min. Stock solutions of the three compounds in DMSO (Sigma, Taufkirchen, Germany) were added to a final concentration of 0.5 % DMSO in each cell sample. T cells were then stimulated with PMA (40 nM) and ionomycin (2 µM) at 37°C for 20 min. Cells were lysed immediately in hypotonic lysis buffer (10 mM HEPES, pH 7.5, 0.1 mM EDTA, 10 mM KCl and 0.625 % NP-40) freshly supplemented with Complete Protease Inhibitor Mixture (Boehringer Mannheim, Germany) and 1 mM DTT on ice for 20 min and cleared by centrifugation.
Western Blotting

The proteins were separated in 10% SDS-PAGE gels and transferred onto polyvinylidene difluoride membranes (Schleicher & Schuell, Germany). Membranes were probed with monoclonal antibodies against NFATC1 and Actin (Santa Cruz Biotechnology, CA) or polyclonal antibodies against CaN followed by horseradish peroxidase-labeled secondary antibodies and visualized with the enhanced chemiluminescence reaction (Amersham Pharmacia Biotech).

Luciferase reporter gene assay

Sorted T cells transfected with the NFAT-luciferase reporter plasmid (Stratagene, Netherlands) by electroporation were cultured in RPMI 1640 with 10% FCS for 16 h at 37°C in 5% CO₂. The cells were incubated with CsA, gossypol, or apogossypol hexaacetate for 30 min and then stimulated with 40 nM PMA and 2 µM ionomycin for 5 h. The level of the extracted luciferase from these cells was determined by bioluminescence measurement using the luciferase assay system (Promega, Mannheim, Germany).

RESULTS

Calcineurin activity assay

Scintillation wells coated with streptavidin-bound biotinylated [³³P] RII phosphopeptide were used to determine human recombinant CaN activity. About 15 pmol/well biotinylated RII phosphopeptide saturated the binding capacity of the streptavidin wells. All experiments were performed at 10 pmol/well RII phosphopeptide. Figure 1A shows the amount of dephosphorylated RII phosphopeptide for a constant incubation time at different CaN concentrations. The arrow depicts the CaN concentration of 1.32 nM, which was used in all other experiments. {insert Fig. 1A and 1B} As could be inferred from Figure 1B the linear range of the dephosphorylation rate for RII phosphopeptide at 1.32 µM CaN covers the 15 min period of incubation. Although the time course of RII dephosphorylation deviates by 8% from linearity for the 20 min dephosphorylation time point the IC₅₀ values determined agreed
within experimental error with the IC$_{50}$ values at lower incubation times. However, the larger amount of product produced after 20 min ensures a high signal-to-noise ratio throughout the experiments. A calmodulin concentration of 50 nM ensures sufficient CaN activation (data not shown). A standard deviation less than 7% was obtained for the data points of Figure 1 when using the above assay protocol. The low CaN concentration of the scintillation proximity CaN assay is an essential prerequisite for the kinetic evaluation of effective inhibition as found for Cyp23/CsA (IC$_{50}$=50 nM) or FKBP12/FK506 (IC$_{50}$=50 nM) (5). The advantages of this described screening assay lies at its high sensitivity by using $^{33}$P-labeled RII phosphopeptide and thus small amounts of CaN are needed. In contrast to pNPP-assay colored natural compounds of the pool do not interfere in this test.

Characterization of CaN inhibition by gossypol

In the library screened for CaN inhibition the cottonseed oil product gossypol turned out to be the only compound active in the lower micromolar range. Gossypol inhibits CaN with an IC$_{50}$-value of 17±1 µM with the RII phosphopeptide as a substrate and 14±1 µM with pNPP as a substrate. Gossypolone displays a higher inhibitory potency (IC$_{50}$=9±1 µM and 6±1 µM respectively), whereas apogossypol hexaacetate is less active in inhibiting CaN (Fig. 2). CaN recovery via dialysis from inhibition in the presence of 120 µM gossypol was obtained with a final yield of 72 % of enzyme activity of a gossypol-free control treated similarly. This finding indicates that inhibition was reversible. After a prolonged preincubation (> 2h) of gossypolone and CaN, an addition irreversible term of inhibition of about 15 % was observed. Therefore, all kinetic experiments have been performed with a preincubation time of 30 min. On the other hand, 500 µM ferrous ammonium sulfate did not reverse the degree of CaN inactivation in the presence of 20 µM gossypol indicating that the inhibitor does not act via complexation of divalent metal ions.

The alternative CaN substrate pNPP revealed a similar inhibitory potency for gossypol and, when assayed at different pNPP concentrations, gives the first indication of noncompetitive inhibition (Fig. 3).
To examine the type of CaN inhibition for peptide dephosphorylation, a series of kinetic experiments with a mixture of $^{33}$P-labeled biotinylated and unlabeled RII phosphopeptides (1: 200 concentration ratio) were performed. This mixture exhibits a $K_M$ value of $20\pm3$ µM when determined by a Lineweaver-Burk plot (data not shown) that corresponds to the $K_M$ of 23 µM (41) and 25 µM (21) reported for nonbiotinylated RII phosphopeptide under similar reaction conditions. As in the pNPP-based assay, noncompetitive inhibition of CaN by gossypol was seen based on the Dixon plot (Fig. 4). Owing to the complexity of the reaction mixture deviations from the linear behaviours of the plot becomes visible which are more prominent in the absence of gossypol. However, the $K_i$ value of 17 µM estimated from the Dixon plot is similar to the aforementioned IC$_{50}$ values of 17 µM and 14 µM (Fig. 2).

To determine whether or not gossypol and Cyp18/CsA complex share common CaN binding sites, we assayed the residual RII phosphopeptide dephosphorylating CaN activity at 12 µM gossypol in the presence of different concentrations of the Cyp18/CsA complex (Fig. 5). The concentration of the Cyp18/CsA complex was assumed to be identical to the Cyp18 concentration in the assay buffer at 10 µM CsA because of the high affinity binding of CsA to Cyp18 with a $K_i$ value of 2.6 nM (38). The IC$_{50}$ value of 210 nM for the Cyp18/CsA complex calculated from the data of Figure 5 agrees with the reported $K_i$ value of 270 nM (42). The simultaneous presence of 12 µM gossypol and 10 µM CsA at varying Cyp18 concentrations allows the inhibitors to be bound at either identical or independent binding sites (Fig. 5). Obviously, inhibition increases in the presence of both compounds. However, the magnitude of residual CaN activity is different for both inhibition models where a lower degree of inhibition can be expected for competing binding sites. The feature of the curve shapes based on either the experimental or the calculated data points (Figure 5) indicates the inhibition model with common drug interaction sites. A close fit of the experimental and the calculated curves (Fig. 5) can be achieved by minor changes of our experimental IC$_{50}$ values used for calculating the theoretical curve with this model only.
To directly test this possibility, the Cyp18/CsA was formed by adding CsA to affigel-bound Cyp18 (2), followed by elution with various inhibitors. CaN was eluted from the column only by gossypol or Cyp18/CsA, but not by apogossypol hexaacetate (Fig. 6). The influence of calmodulin on CaN inhibition by gossypol is shown in Figure 7. The average IC$_{50}$-value was (18±2 µM) for four different calmodulin concentrations (25–150 nM) indicating that gossypol does not target calmodulin for CaN inhibition. 

**Specificity of gossypol**

Gossypol was tested as an inhibitor of other protein phosphatases utilizing the biotinylated RII phosphopeptide as a general substrate of protein phosphatases. Despite the lower efficiency relative to CaN, PP2A, and PP2C readily dephosphorylate the RII phosphopeptide (43). In the case of PP1 phosphorylase a was chosen as a substrate, because phosphorylated proteins are superior to phosphopeptide substrates regarding both specific activity and higher signal to noise ratio. Except for CaN, gossypol at up to 100 µM concentrations has no inhibitory effect on the other protein phosphatases (Table 1). These results were confirmed by using the protein substrates $[^{32}P]$-labeled phosphorylase a for PP2A, and $[^{32}P]$-labeled casein for CaN and PP2C (data not shown). To exclude the possibility that in cells gossypol inactivates PPIases, human prototypic members of the three PPIase families were tested at a concentration of 120 µM gossypol in PPIase assays. Residual activities of 86% (Cyp18), 92 % (FKBP12) and 87 % (Pin1) were found.

**Inhibition of NFAT translocation in activated T cells**

To investigate the effect of gossypol on CaN in cells, we examined the NFATC1 disappearance from the cytosol after PMA/ionomycin activation of T cells. The transcription factor NFAT is present in the cytoplasm of resting T cells and translocates into the nucleus only after dephosphorylation by CaN, which is activated immediately after T cell stimulation with a specific antigen, anti CD3/CD28 antibodies or PMA/ionomycin (44).
Human T cells were purified to 98% from human PBMC by magnetic cell sorting of CD4 or CD8 positive cells. The isolated cells were preincubated with cyclosporin A, apogossypol hexaacetate or gossypol and then stimulated with PMA/ionomycin in the presence of these compounds. The cytosolic portion of NFATC1 is detected in the cytosolic extracts by Western blot analysis. Both CsA and gossypol inhibited NFATC1 disappearance from the cytosol in a concentration-dependent manner (Fig. 8). In contrast, apogossypol hexaacetate neither inhibited CaN in vitro (Fig. 2) nor prevented NFATC1 disappearance from cytosol after T cell stimulation (Fig. 8).

The NFAT-luciferase reporter gene assay was used to show, that the cytoplasmic disappearance of NFAT after stimulation indeed corresponds to NFAT activation by NFAT dephosphorylation, and therefore its nuclear appearance with concomitant, DNA binding. It was confirmed by luciferase activity measurement, that gossypol and CsA inhibit this NFAT activation but apogossypol hexaacetate did not (Fig. 9).
DISCUSSION

In search for direct small molecule inhibitors of calcineurin, the naturally occurring polyphenolic aldehyde gossypol (C_{30}H_{30}O_{8}) was found to inhibit CaN reversibly with a potency in the micromolar range (Fig. 10). There was no previous evidence for the interaction of gossypol with phosphatases. Furthermore, an about two-fold increase in inhibitory potency was observed for the chinoid metabolic derivative gossypolone in comparison with gossypol, with an IC_{50} value of 7 \mu M. Both CsA and FK506 noncompetitively inhibit CaN with IC_{50}-values of 270 nM (42) and 57 nM (45), respectively, but each requires the prior binding of a matchmaker protein. Despite the significant homology between the catalytic subunits of the Ser/Thr protein phosphatases the CaN specificity of the PPlase/CsA(FK506) complexes among the four protein phosphatase families is high because the inhibitory complex does not involve the active site (46, 47). Similarly, gossypol is highly specific for CaN among the Ser/Thr protein phosphatase families including PP1, PP2A, and PP2C as judged by its specific inhibition of CaN at concentrations up to 100 \mu M (Table 1). Unlike CsA and FK506, however, gossypol directly binds to CaN independent of any matchmaker proteins. The ability of gossypol to inhibit CaN without affecting the enzyme activity of members of the three PPlase families makes it potentially more specific probes for calcineurin function than CsA and FK506 for which inhibition of PPlases is a prerequisite for inhibition of CaN.

CaN is a binuclear Fe-Zn metallophosphatase, which is activated by Ca^{2+}, calmodulin and bivalent metal ions such as Mn^{2+} and Ni^{2+}. The catalytically active form of Fe in the active center of CaN is still under debate. It is assumed to be either Fe^{2+} (40) or Fe^{3+} (48). CaN has been reported to be inactivated by oxidative processes and can be reactivated by ferrous ammonium sulfate (40), DTT or the dithiol oxidoreductase thioredoxin (49). Current hypotheses assume that either the binuclear metal center (40) or modification of cysteine residues (49) can be the target for the oxidative inactivation of CaN. From our experiments, we conclude that gossypol does not inactivate CaN by a redox mechanism, since (i) high concentrations of DTT (5 mM) did not prevent inactivation, (ii) ferrous ammonium sulfate
failed to reverse the inactivation, and (iii) dialysis recovered a major part of CaN activity. In contrast, gossypol in the micromolar concentration range inactivates sperm adenylate cyclase by chelating the essential metal ion of the enzyme. High manganese concentration protects this enzyme from inactivation.

Gossypol inhibits CaN independent of the nature of the three substrates used here. This finding is in contrast with the effect of the Cyp18/CsA (FKBP12/FK506) complexes, which inhibits the dephosphorylation of the RII phosphopeptide but activated the phosphatase activity of CaN towards a small substrate pNPP by 2-3 fold (2, 5).

Like Cyp18/CsA (31), gossypol inhibits CaN noncompetitively, raising the possibility that it may bind to a site overlapping that for Cyp18/CsA. In fact, the competition experiment (Figure 5) implies that both complexes might exhibit similarity in the way these inhibitors block CaN-catalyzed dephosphorylation. The specific elution by gossypol of CaN from a matrix-bound ternary complex Cyp18/CsA/CaN (Fig. 6) supports this inhibition model. The structure of the CaN/Cyp18/CsA complex has not been published so far but may relate to the ternary inhibitory complex of FK506 (47). Here, the CaN-interacting parts of the binary FKBP12/FK506 complex are remote to the active site of the phosphatase. Because the active sites of CaN, PP1, PP2A and PP2C display considerable similarities inhibitory specificity requires inhibitor binding at remote sites. Accordingly, the observed specificity of gossypol among the protein phosphatases is in accordance with the noncompetitive type of inhibition. Active-site directed CaN inhibitors like microcystin or the mixed type inhibitor okadaic acid do not show CaN specificity among the protein phosphatases (31).

Because the use of pNPP substrate in the CaN assay revealed gossypol to inhibit CaN with IC₅₀ = 14±1 µM, which is similar to the IC₅₀ value with the RII phosphopeptide as a substrate, a binding regime closer to the active site is implied, but specificity is still retained.

In addition to pNPP and the RII phosphopeptide, we also examined the effect of gossypol and analogs on the dephosphorylation of a protein substrate in vivo. Thus, the CaN dependent activation of the transcription factor NFAT was investigated in the presence of
gossypol and apogossypol hexaacetate in human PBMCs. Gossypol is advantageous over peptidic inhibitors (25, 26) for cellular experiments as it is cell permeable (50). In our assay, the phosphoprotein NFATC1 migrating with an apparent molecular mass of 90-115 kDa in SDS-polyacrylamide gels serves as the CaN substrate. It was previously shown that the Cyp18/CsA (or FKBP12/FK506) complex-mediated inhibition of dephosphorylation of NFAT is responsible for the inhibition of IL-2 transcription, and thus T-cell proliferation (46, 51). Gossypol, but not apogossypol hexaacetate, prevented the dephosphorylation and nuclear translocation of NFATC1 by CaN in a concentration-dependent manner. This effect is similar to that observed for the Cyp18/CsA complex observed previously (51) (Fig. 8) The NFAT-luciferase reporter gene assay was used to show, that the cytoplasmic disappearance of NFAT after stimulation indeed corresponds to NFAT activation by NFAT dephosphorylation, and therefore its nuclear appearance with concomitant DNA binding and gene transcription. It was confirmed by luciferase activity measurement that gossypol and CsA inhibit the NFAT activation but apogossypol hexaacetate did not (Fig. 9).

Inhibition of CaN by gossypol was found to be independent of CaN activation by calmodulin suggesting that neither cofactor requirement nor Calmodulin binding site competition was characteristic of gossypol-mediated inhibition even thought gossypol was also reported to bind to calmodulin at an independent site (52).

The discovery of gossypol as a specific CaN inhibitor is also expected to shed light on how this compound plays the well-known role in male antifertilization, inhibition of proliferation of tumor cells, and how it mediates side effects of the contraceptive therapy (53, 54, 55, 56). Gossypol has been already reported to inactivate intracellular dehydrogenases, protein kinases, steroidogenic adrenal enzymes, cathepsin L, and topoisomerase II. Noncovalent enzyme/gossypol complexes are formed by PKC (IC$_{50}$=100 µM) (57), cyclic AMP-dependent protein kinase (IC$_{50}$=10 µM) (58), and at the NADH cofactor binding site of LDH (IC$_{50}$=1 µM) (59). Rapid binding of gossypol followed by slow covalent protein modification by Schiff base formation at the N-terminal amino group results in the inactivation of phospholipase A$_2$ (58).
Typically, gossypol concentrations ranging from 10 to 20 µM were antiproliferative in cancer cell lines (60) irreversibly blocking cells in S phase. Spermicidal activity and alteration in morphology of carcinoma cells can also be found with gossypolone, although it is less efficient than gossypol (60, 61) and therefore suggest a CaN independent pathway. Yet there exist few studies that examine the impact of gossypol on protein phosphorylation. Treatment of human cancer cell lines with gossypol decreased the ratio of phosphorylated to unphosphorylated cell cycle regulatory retinoblastoma protein at inhibitor concentrations compatible with CaN inhibition but suggests a protein kinase as a putative gossypol target (62). On the other hand, it is interesting to note that the increase in the activity of ornithine decarboxylase, and subsequent proliferation following prolactin-stimulation of Nb 2 rat lymphoma cells can be blocked with both CsA and gossypol. (63).

In conclusion, the novel protein Ser/Thr phosphatase inhibitor gossypol, which is specific for CaN among different members of the phosphatase superfamily, allows CsA(FK506)-mediated CaN and PPIase effects to be differentiated. Previous attempts to distinguish among the enzymes were limited to monofunctional PPIase inhibitors that have no effect on CaN. Now the possibility has been extended to a monofunctional CaN inhibitor that does not require the mediation of any PPIases.

Our experiments with gossypol derivatives showed that gossypol may serve as a lead for the development of specific and potent CaN inhibitors active in the nanomolar range, and with improved specificity for CaN while discriminating against other gossypol-sensitive enzymes.
REFERENCES


**ACKNOWLEDGMENTS**

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The abbreviations used are: CsA, cyclosporin A; FK506, tacrolimus; rhCyp18, recombinant human cyclophilin 18; FKBP12, FK506-binding protein 12; PPIase, peptidyl prolyl cis/trans isomerase; PP1, Ser/Thr protein phosphatase type 1; PP2A, protein phosphatase type 2A; PP2B, protein phosphatase type 2B or calcineurin; PP2C, protein phosphatase 2C; pNPP, p-nitrophenyl phosphate; RII phosphopeptide, 19-residue phosphopeptide of the regulatory subunit of type II cAMP-dependent protein kinase; PKC, protein kinase C; LDH, lactate dehydrogenase; PMA, phorbol 12-myristate 13-acetate; PBMC, peripheral blood mononuclear cells; NFAT, nuclear factor of activated T cell
FIGURE LEGENDS

Fig. 1:

Assay characterization.

The Ca\(^{2+}\)/calmodulin stimulated recombinant human CaN activity was determined using 10 pmol/well biotinylated RII phosphopeptide substrate at pH 7.5, 30°C.

(A) Initial rate of dephosphorylation of the RII phosphopeptide at different CaN concentrations. The arrow depicts the CaN concentration of 1.32 nM, which was used in all experiments. Initial rates were determined by measuring the amount of dephosphorylated substrate after a 20 min incubation.

(B) Time course of CaN-catalyzed (1.32 nM) dephosphorylation of biotinylated RII phosphopeptide. Dephosphorylation was terminated by addition of EGTA to a final concentration of 10 mM.

The arrow depicts the incubation time of 20 min, which was constantly used in the experiments. The data presented are means ± SD of triplicates from two independent experiments.

Fig. 2:

Inhibition of CaN by gossypol and its derivatives.

Gossypol (square), gossypolone (triangle), and apogossypol hexaacetate (circle) were preincubated with CaN (1.32 nM) and calmodulin (50 nM) for 30 min at 22°C.

(A) The CaN phosphatase activity was measured using RII phosphopeptide substrate as described in Materials and Methods.

(B) 10 mM p-nitro phenylphosphate was used to determine protein phosphatase activity

Data are expressed as the activity, relative to reference values without inhibitor. IC\(_{50}\) values were calculated by four-parameter curve fitting using the program Sigma Plot. The data presented are mean ± SD of three independent experiments.
Fig. 3:

**Inhibition of CaN by gossypol using the pNPP substrate.**

CaN and calmodulin were preincubated with gossypol (see Fig. 2) and then CaN phosphatase activity was measured with pNPP substrate as described (see "Materials and Methods"). The IC$_{50}$ values at pNPP concentrations of 1 mM (circle), 5 mM (square), and 25 mM (triangle) were calculated. The data presented are mean ± SD of three independent experiments.

Fig. 4:

**Dixon plot of CaN inhibition by gossypol.**

Inhibition of CaN by gossypol was measured at a substrate concentration of 5 µM (circle), 6 µM (triangle), 8 µM (square), and 10 µM (diamond) RII phosphopeptide (mixture of biotinylated and nonbiotinylated RII phosphopeptide by 1 : 200) and a CaN concentration of 0.66 nM. Each graph represents the average of 4 independent experiments. The data presented are mean ± SD of three independent experiments.

Fig. 5:

**Simultaneous inhibition of CaN by Cyp18/ CsA and gossypol.**

Inhibition mediated by gossypol (12µM; squares), CsA (10µM; circles) and a mixture of CsA and gossypol (10µM and 12µM, respectively; triangles) in dependence of the Cyp18 concentration was plotted. Residual CaN activity (1.32 nM CaN, 50 nM calmodulin) was measured using the RII phosphopeptide substrate, and calculated relative to a reference experiment lacking the effectors. Preincubation of CaN with effectors was performed at 22°C for 30 min. The data presented are the mean ± SD of three independent experiments. Graphs were calculated in terms of residual activity assuming either identical (dash-dot-dot) or different (short dash) binding sites of gossypol and Cyp18/CsA.
The inhibitory Cyp18/CsA complex is formed in relation to the amount of Cyp18 applied according to the binding constant of Cyp18 and CsA.

Fig. 6:

Elution of CaN by gossypol from a Cyp18/CsA affinity column.

A Cyp18/CsA affinity column was incubated with 30 µg calcineurin for 1 hour. Next the column was washed with PBS (lane 1, last washing step). Subsequently calcineurin was eluted with the following reagents: 100 µM apogossypol hexaacetate (lane 2), 100 µM gossypol (lane 3) and 15 µM preformed Cyp18/CsA-complex (lane 4) after incubation for 10 minutes. Lane 5, western blot of a reference sample of CaN.

Fig. 7:

Effect of calmodulin on CaN inhibition by gossypol.

CaN was preincubated with gossypol and incubated with the RII phosphopeptide in presence of 25 nM (square), 37.5 nM (triangle up), 50 nM (circle), and 150 nM (triangle down) calmodulin. Residual CaN activity was plotted against gossypol concentration. The IC$_{50}$ value for each calmodulin concentration was calculated. The data presented are means ± SD of three independent experiments.

Fig. 8:

Translocation of NFATC1 from the cytosol in T cells.

Purified human T cells were preincubated with 1 µM CsA (lane 3), 30 µM apogossypol hexaacetate (lane 4) or gossypol at a concentration of 1 µM, 15 µM and 30 µM (lane 5, 6, 7) at 37°C for 10 min. Then cells were stimulated with 40 nM PMA /2 µM ionomycin (lane 2 to 7) in the presence of either the compounds or DMSO as control (lane 2) at 37°C for 20 min. Depletion of NFATC1 from the cytosol was detected by immunoblotting with anti-NFATC1, and actin monoclonal antibodies.
Luciferase reporter gene assay.

Sorted T cells transfected with a NFAT-luciferase reporter plasmid were stimulated with PMA plus ionomycin for 5 h after incubation with CsA, gossypol, or apogossypol hexaacetate for 30 min. Data are expressed as luminescence activity of the extracted luciferase. The data presented are mean ± SD of three independent experiments.

Structures of gossypol and gossypol analogues.
Table 1: Inhibitory effect of gossypol on enzyme activity of the major classes of protein Ser/Thr phosphatases measured against RII phosphopeptide and in the case of PP1 against phosphorylase a. The results are expressed as % of the respective phosphatase activity without inhibitor (SD ≤ 7%).

<table>
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<tr>
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<tr>
<td></td>
<td></td>
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<td>20µM</td>
<td>100µM</td>
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<tr>
<td>PP1*</td>
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<td>97</td>
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<td>PP2A</td>
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<td></td>
<td>104</td>
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<tr>
<td>PP2B (CaN)</td>
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<td></td>
<td>38</td>
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<td>PP2C</td>
<td></td>
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<td>85</td>
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*phosphorylase a as substrate
Figure 1

A

Calcineurin activity (pmol/min)

Calcineurin [nM]

0 2 4 6 8 10 12 14

B

Dephosphorylation (pmol/well)

Time (min)

0 20 40 60 80
Figure 2

A

B
Figure 3

Gossypol [µM]
0.1 1 10 100 1000

Calcineurin activity (% of control)
0 20 40 60 80 100

Gossypol [µM]

Calcineurin activity (% of control)
Figure 4

The figure shows a graph with the x-axis labeled as "Gossypol [µM]" and the y-axis labeled as "1/v (pmol·s⁻¹·min⁻¹)." The graph plots data points and trend lines indicating the relationship between Gossypol concentration and the reciprocal of the reaction rate. The data points are represented by different symbols and error bars, suggesting variability in the measurements.

The graph is used to illustrate the effect of Gossypol concentration on the reaction rate, typically seen in enzyme kinetics experiments. The trend lines suggest a non-linear relationship, which is common in Michaelis-Menten kinetics when the substrate concentration is not in the range of the Michaelis constant (Km).
Figure 5
Figure 6

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CaN subunit A

CaN subunit B
Figure 7

Gossypol [µM]

Calcineur activity [pmol/ min]
Figure 8

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Figure 9

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Figure 10

Gossypol

Gossypolone

Apogossypol hexaacetate
Reversible inhibition of calcineurin by the polyphenolic aldehyde gossypol
Ria Baumgrass, Matthias Weiwad, Frank Erdmann, Jun O. Liu, Dirk Wunderlich, Susanne Grabley and Gunter Fischer

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