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Review

Calcineurin inhibitors: *status quo* and perspectives

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Abstract

Despite the fact that cyclosporin A (CsA) and tacrolimus (FK506) are very potent drugs in the treatment of serious autoimmune diseases and in the prevention of graft vs. host reactions or tissue rejections after allo- or xenotransplantations, modern transplantation medicine attempts to develop alternative medication regimes without these calcineurin inhibitors. The primary motivation for this endeavor is the high incidence of dramatic side effects upon immunosuppressive therapy. CsA and FK506 target not only the calcineurin/NFAT pathway, but they also bind and inhibit members of distinct peptidyl-prolyl *cis/trans* isomerase families, which are involved in numerous important signal transduction pathways. Therefore, the development of a potent calcineurin inhibitor that discriminates between calcineurin and other protein phosphatases and peptidyl-prolyl *cis/trans* isomerases, respectively, should improve the drug safety in clinical use and represent a valuable tool in basic research to investigate calcineurin modulated pathways. This review gives a current overview about novel calcineurin inhibitors, which were identified by screening of compound libraries and in natural materials or were derived from known inhibitors in the past decades. Thereby, we focus on their structure, properties and biological effects.

Keywords: calcineurin; drug discovery; immunosuppression; inhibitor; NFAT.

Introduction: calcineurin – a key protein in immune response

The protein phosphatase calcineurin (PPP3, formerly known as PP2B) was first isolated from bovine brain by Klee and Krinks in 1978 (1). In the following years it was shown that calcineurin is an ubiquitous protein across lower and higher eukaryotes with a heterodimeric composition, consisting of an approximately 59 kDa catalytic subunit and a 19 kDa calcium binding regulatory subunit (2). In human, three different isoforms of the catalytic subunit (α, β, γ) and two different regulatory subunits (α and β) were identified (3–6). Their tissue-specific distribution and differential substrate specificity indicate individual physiological functions (7). Calcineurin possesses an unique position among the families of Ser/Thr protein phosphatases because its enzyme activity is triggered by binding of calcium ions and calmodulin. The phosphatase only becomes activated if both effectors are present simultaneously (8, 9). Binding of calcium ions and calmodulin induces conformational changes in protein and unmasks the active site that was previously blocked by the autoinhibitory domain (10, 11). Thus, phosphatase activity of calcineurin is closely linked with the cytosolic calcium level and the phosphorylation state of substrate proteins is changed as response to an altered intracellular calcium concentration. These unique biochemical properties allocate calcineurin a central role in many Ca^{2+}-dependent physiological and pathological processes, including regulation of immune response, apoptosis, skeletal and heart muscle differentiation and development, bone formation and neuronal signaling (12–17).

The role of calcineurin in immune response was the subject of intense research and is therefore best understood (18). After activation of the T cell receptor by specific antigens and the initiation of a signal cascade, phospholipase Cγ becomes activated and hydrolyzes phosphatidylinositol-4,5-bisphosphate to the second messengers diacylglycerol and inositol-1,4,5-trisphosphate (IP_3). Binding of IP_3 to its endoplasmic reticulum localized receptor causes calcium release into the cytoplasm. Subsequently, the formation of a ternary calcium/calmodulin/calcineurin complex leads to calcineurin activation. Phosphorylated substrates as nuclear factor of activated T cells (NFAT) become dephosphorylated by calcineurin and mediate their specific functions (19). Members of NFAT transcription factor family represent in their hyperphosphorylated form the major substrates of calcineurin in immune cells, cardiomyocytes and skeletal muscle cells. Dephosphorylation of NFATc1–c4 leads to conformational changes resulting in demasking of a nuclear translocation signal and additionally to an increased affinity for specific DNA sequences. Binding of NFAT onto regulatory elements of genes stimulates the expression of a set of cytokines (e.g., IL-2, IL-3, IL-4, IL-5, TNFα and IFNγ) and chemokines (e.g., IL-8 or MIP-1α), which play an important role in the initiation and progress of immune response (20) (Figure 1). Therefore, the application of calcineurin inhibitors represents an effective strategy to prevent transplantation rejections and other serious immune reactions.

Determination of calcineurin phosphatase activity

The precise determination of calcineurin phosphatase activity is crucial for the identification of calcineurin inhibitors. In

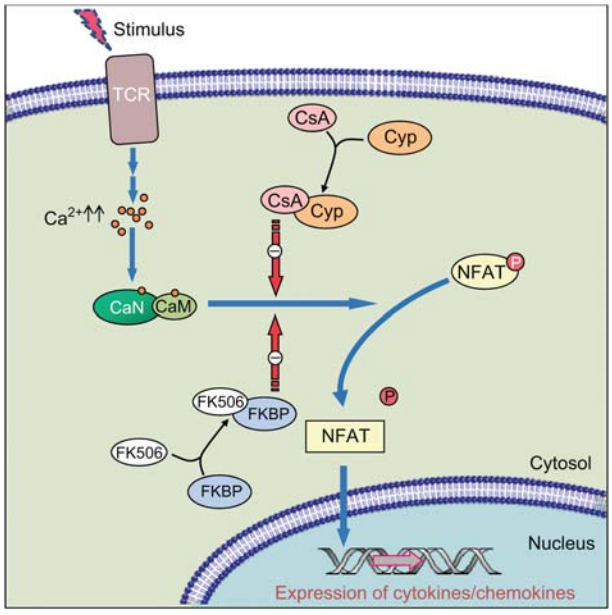


Figure 1 The gain-of-function mechanism of CsA/FK506 inhibition.
The cyclic undecapeptide CsA and the macrolide FK506 bind to and inhibit the phosphatase activity of calcineurin only after interaction with their respective peptidyl-prolyl *cis/trans* isomerases, cyclophilins and FK506 binding proteins through a gain-of-function mechanism. Owing to calcineurin inhibition, dephosphorylation of NFAT is blocked and therefore IL-2 production decreased.

addition to recombinant calcineurin, purified authentic enzyme from bovine brain is also commercially available. The small 4-nitrophenyl phosphate (pNPP) is used as artificial chromogenic substrate in phosphatase assays to generally determine phosphatase activity in samples. By contrast, the RII phosphopeptide, a phosphorylated 19-residue peptide of the regulatory subunit of type II cAMP-dependent protein kinase, represents a specific substrate for calcineurin. In the case of [^{32}P] or [^{33}P] radioactively labeled RII phosphopeptide, the hydrolysis of phosphoester bond can be monitored by scintillation measurements (21, 22). Otherwise the released phosphate is detectable spectrophotometrically as molybdenum blue. The dye is developed after reduction of the formed phosphomolybdate complex. The low sensitivity of the assay can be slightly improved by forming a phosphomolybdate/dye complex with malachite green (23). Most commercial available *in vitro* calcineurin assays exploit this principle. Both assays, the scintillation and chromogenic assays, are feasibly in 96- or 384-well plates and thereby allow automatization and high-throughput screening (HTS) of large compound libraries. Alternatively, radioactive labeled proteins, e.g., casein, DARPP-32, Elk-1 or NFAT, were also used as calcineurin substrates (7, 24–26). However, the determination of calcineurin activity in cells is rather difficult, because less than 5% of whole protein phosphatase activity in cell extracts is mediated by calcineurin. Therefore, the dephosphorylation of the transcription factor NFAT, a calcineurin activity triggered cellular event, is investigated in most cases. The transcriptional activity of NFAT, which can be quantified easily in a NFAT driven luciferase reporter gene assay, correlates very well with the calcineurin phosphatase activity in cells (27, 28). Other cellular assays, such as the mixed lymphocyte reaction, do not reflect in every case the activity of calcineurin because additional pathways contribute here to the effect.

Cyclosporin A (CsA) and FK506 as typical calcineurin inhibitors

In 1976, the natural compound cyclosporin A (CsA) was first isolated from extract of fungus *Tolypocladium inflatum* (Gams) and its immunosuppressive properties were demonstrated in several mice and rat models for arthritis, graft-versus-host disease and allergic encephalomyelitis (29). In contrast to classic immunosuppressive and cytostatic drugs (e.g., methotrexate or azathioprine) the cyclic undecapeptide CsA shows strong immunosuppression and only weak myelotoxicity. Following FDA approval the new drug has found very fast entry into clinical use (International Nonproprietary Name, INN: Ciclosporin; Sandimmune®). In the following years CsA has revolutionized transplantation medicine (30, 31). In 1987, a new compound from fermentation broth of *Streptomyces tsukubaensis* No. 9993 was extracted and characterized (32). The new compound has a macrolide structure and was designated as FK506 (INN: Tacrolimus; Prograf®, Protopic®). FK506 acts synergistic to CsA and exhibits even a stronger immunosuppressive effect *in vitro* and *in vivo* (33, 34). However, the molecular basis of immunosuppression was unclear until the protein phosphatase calcineurin was identified as target of both immunosuppressive drugs (35). CsA and FK506 bind only in complex with their respective major intracellular acceptor proteins cyclophilin A (Cyp18) and FK506 binding protein 12 (FKBP12) onto calcineurin and inhibit its protein phosphatase activity (36). CsA and FK506 do not represent active calcineurin inhibitors by themselves. For activation by a gain-of-function mechanism, both drugs need prior binding to immunophilins, individual endogenous cyclophilin or FKBP proteins (Figure 2A and B) (37). Immunophilins are members of the enzyme class of peptidyl-prolyl *cis/trans* isomerases (PPIases), which are involved in protein folding processes and contribute to many other cellular functions (38). The formation of binary complexes of CsA and cyclophilins or FK506 and FKBP are prerequisite for inhibition of calcineurin. Concomitantly, the PPIase activity of both immunophilin families is also inhibited (39, 40). This could represent one reason for many serious side effects, which were observed in clinical use of both drugs such as nephrotoxicity, hypertension, hyperlipidemia, neuropathies and an increased risk for diabetes mellitus type II (41–45). Therefore, in the past years intensive research was undertaken to identify new calcineurin inhibitors in libraries of natural or synthetic compounds or to modify known protein phosphatase inhibitors to increase their specificity for calcineurin.

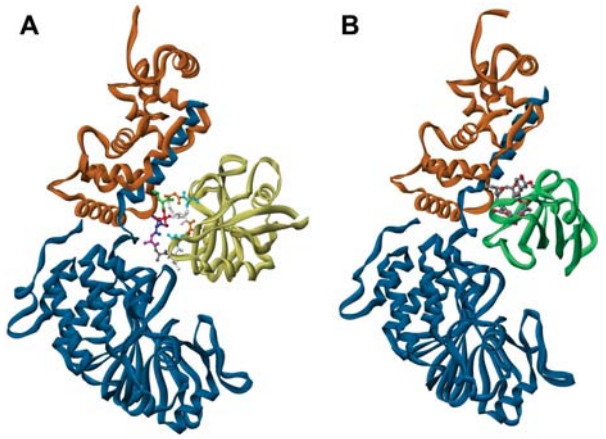


Figure 2 Structure of calcineurin in complex with CsA/Cyp18 and FK506/FKBP12.
The structure of the human calcineurin α isoform is shown in complex with (A) CsA/Cyp18 (PDB entry 1MF8) and (B) FK506/FKBP12 (PDB entry 1TCO). The C-terminal truncated catalytic subunit is indicated in blue and the regulatory subunit in brown. CsA (each amino acid individually colored as ball and stick) was complexed with human cyclophilin 18 (yellow) and FK506 (also as ball and stick) was shown in complex with human FKBP12 (green).

Cyclosporin A (CsA)

In 2002, the three-dimensional structure of the CsA/Cyp18/calcineurin complex was determined by the groups of Huai et al., and Jin and Harrison, respectively (46, 47). Figure 2A shows the X-ray crystal structure of the ternary complex and reveals that CsA/Cyp18 complex binds not at the active site of calcineurin. Residues 3–9 of CsA and Trp121 of Cyp18 form a composite surface for interaction with the catalytic and regulatory subunits of calcineurin and limit the accessibility of active site for peptide and protein substrates. Therefore, the CsA/Cyp18 complex masks the docking site for the NFATc LxVP motif and acts as non-competitive inhibitor (48). This fact explains why CsA/Cyp18 does not affect other protein phosphatases (PP1, PP2A or PP2C) although their active sites are very similar (49). Nevertheless, the small artificial phosphatase substrate pNPP still becomes dephosphorylated in the presence of Cyp18 complexed CsA. As shown in Figure 3A, CsA is a cyclic undecapeptide containing a cyclophilin binding site (residues 9, 10, 11, 1 and 2) and an interaction surface for calcineurin (residues 4–7). In the past years, several CsA derivatives were synthesized, which were modified in almost all positions, e.g., 3 ([(*S*)α-methylthiosarcosine^3]-CsA), 6 ([*N*-methyl-Ala^6]-CsA), and 8 ([D-diaminopropyl^8]-CsA and [D-diaminobutyryl^8]-CsA) (50, 51). All compounds do not inhibit calcineurin, although most of these derivatives are still inhibitors of cyclophilin 18. The only exception of this set are the *R* enantiomer of [α-methylsarcosine^3]-CsA and [dimethylaminoethylthiosarcosine^3]-CsA ([DAT-Sar^3]-CsA) (52). Both position 3 modified derivatives inhibit phosphatase activity of calcineurin on its own, without prior formation of a drug/cyclophilin 18 complex. Unfortunately, in cells both compounds inhibit the PPIase activity of cyclophilins at the nanomolar concentration range and are therefore unsuitable as a monospecific calcineurin inhibitor. Moreover, modifications of residues in the cyclophilin binding region (e.g., MeBmt at position 1) by substitution or elongation of the side chain alter only the cyclophilin inhibition, but impair the immunosuppressive potency only marginally, as observed for [MeBm$_2$t^1]-CsA,

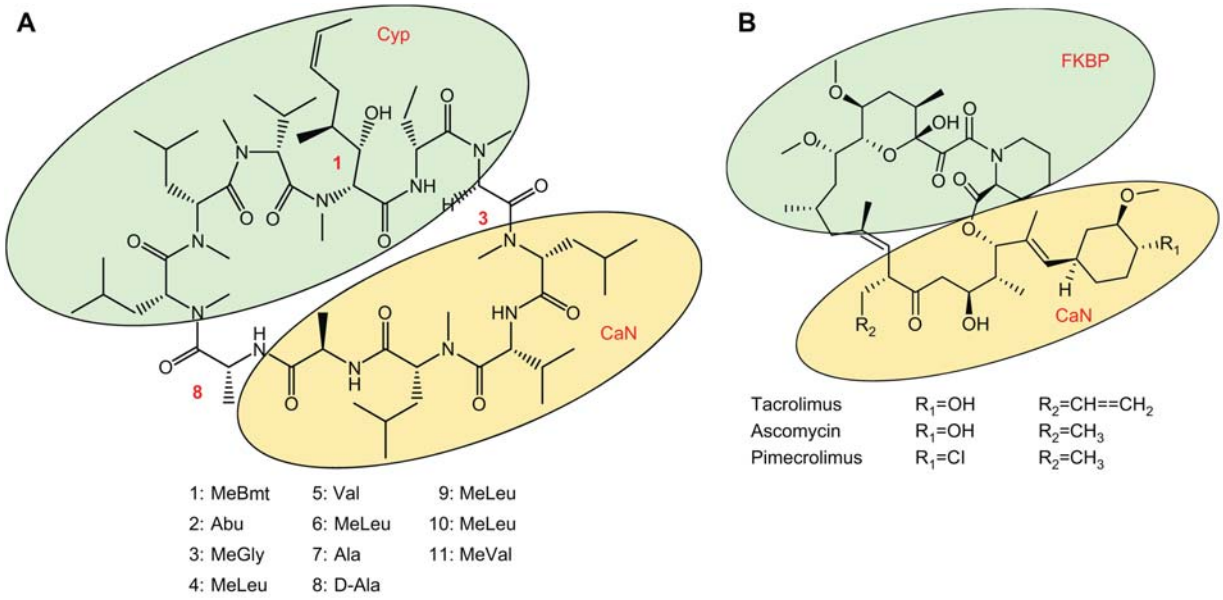


Figure 3 Structures of CsA (A) and FK506 derivatives (B).
The immunosuppressive drug CsA contains nine non-proteinogenic amino acids and four *N*-methylated peptide bonds. Residues 9 to 2 are important for cyclophilin binding (green) and 7 to 4 for interaction with calcineurin (yellow). The calcineurin binding region of FK506 is also indicated in yellow and the FKBP binding region in green.

[MeBth^1]-CsA, [MeByt^1]-CsA and voclosporin (ISA247) (53, 54). A completely new regulation mechanism of CsA inhibitory potency was realized with a CsA derivative containing a biotin moiety connected by an azobenzene tether (55). The irradiation induced *trans*-azo to *cis*-azo photoisomerization causes only a minor intensification on cyclophilin and calcineurin binding/inhibition, respectively. However, the influence of irradiation was dramatically increased, when the biotinyl moiety interacts with streptavidin. The augmentation of inhibition by photoswitching using protein surface borrowing strategy was realized *in vitro* and also in Jurkat T cells.

Tacrolimus (FK506)

The three-dimensional structure of the FK506/FKBP12/calcineurin complex was also investigated by X-ray crystal structure analysis (Figure 2B) (56). Before structural information was available, derivatives of FK506 and ascomycin (FK520) were synthesized from different research groups and companies (36). Most derivatives are modified at the hydroxyl moiety in position 32 of FK520 (e.g., CP-123.369, A-119435, L-732.531, L-733.725, SDZ MDL987, SDZ ASM981, ABT-281) or at the cyclohexane ring substituted derivative (SDZ-281-240) and exhibit a different immunosuppressive potential. Only one of these compounds – SDZ ASM981 (INN: Pimecrolimus; Elidel®, Douglan®) – is established in clinical routine for topic treatment of atopic eczema (57). However, the ascomycin derivative L-585.818, which contains an additional OH in position 18, and the truncated FK506 derivative V-10.367 are selective FKBP inhibitors, which do not affect calcineurin (58, 59). Noteworthy, the macrolidic FKBP ligand rapamycin (INN: Sirolimus; Rapamune®), isolated from *Streptomyces hygroscopicus* is a very effective immunosuppressive and antiproliferating drug, which acts far away from the calcineurin/NFAT pathway by influencing mTOR/Akt in the IL-2 receptor signal transduction pathway (60, 61).

Low-molecular weight calcineurin inhibitors

As mentioned before, the active site of calcineurin exhibits a high degree of structural and conformational similarities to other members of Ser/Thr protein phosphatases (e.g., PP1, PP2A and PP5). Therefore, active site inhibitors of these phosphatases also inhibit calcineurin. Examples for this behavior include the highly reactive compound 4-(fluoromethyl)phenyl phosphate (FMPP), few tyrphostins as tyrosine kinase inhibitors, and norcantharidin (62–64). Moreover, the natural compound okadaic acid or the pesticide endothall possess the ability to inhibit calcineurin at micromolar concentrations. However, PP1 and PP2A are inhibited at the nanomolar range (65, 66). Endothall is structurally related to cantharidin, the natural defensive toxin of blister beetles. Cantharidin is a potent inhibitor of PP1 and PP2A, but only a weak inhibitor of calcineurin. Contrary to an earlier report, class II pyrethroid insecticides (cypermethrin, deltamethrin and fenvalerate) are unable to inhibit calcineurin (67, 68). A summary of low-molecular weight calcineurin inhibitors is shown in Table 1.

1,5-Dibenzoyloxymethyl-norcantharidin is a semi-synthetic compound derived from monoterpene cantharidin. Starting from known PP1 and PP2A inhibitor cantharidin and by using a structure based approach, (nor)cantharidin derivatives were synthesized and investigated regarding their calcineurin inhibition (69, 70). The compound binds to calcineurin, but not to PP1 or PP2A. Although *in vitro* inhibition data with pNPP and RII phosphopeptide are available, no data from cell experiments are published.

The *O*- and *S*-containing barbiturates pentobarbital, secobarbital, thiopental and thiamylal are very weak calcineurin inhibitors (71). In millimolar concentrations they inhibit the calcineurin catalyzed dephosphorylation of RII phosphopeptide and calcineurin-triggered cell events as NFAT translocation and cytokine production in human T cells. This fact might explain the observation that application of frequently used barbiturates for the treatment of intracranial hypertension after brain injuries are associated with a profound increase in the infection rate. Thus, Correa-Sales et al. demonstrated that the antigen-specific lymphocyte proliferation and IL-2 production by peripheral blood lymphocytes from patients under thiopental anesthesia are significantly reduced (72). Regardless, the $GABA_A$ receptor, a ligand triggered Cl^- channel, represents the major pharmacological target of barbiturates. There exists no connection of the receptor signaling with the observed immunosuppressive effects.

The 2,6-diaryl-substituted pyrimidine derivative CN585 was obtained by screening of a substance library and by rational design of a lead compound (22). CN585 specifically inhibits calcineurin in a noncompetitive and reversible manner without affecting other Ser/Thr protein phosphatases or peptidyl-prolyl *cis/trans* isomerases. CN585 is cell-permeable and shows only low cytotoxicity against Jurkat T cells. Moreover, CN585 shows potent immunosuppressive effects, as inhibition of NFAT nuclear translocation and transactivation, cytokine production and T cell proliferation.

In 1996, the phenolic compound dibefurin was isolated in Abbott Laboratories from fungal culture AB 1650I-759 and structurally and biochemically characterized (73). Dibefurin suppresses calcineurin phosphatase activity in a fluorescence coupled assay with 4-methylumbelliferyl phosphate as substrate as well as mixed lymphocyte reaction in a Balb/c and C57BL/6 mouse model.

In a fluorescence polarization-based assay, the thrombocyte aggregation blocking drug dipyridamole was identified as disruptors of the RCAN1/calcineurin A complex (74). The RCAN1 (formerly known as DSCR1) region, spanning amino acids 198–218 is responsible for inhibition of the calcineurin-NFAT signaling pathway *in vivo*. Despite the fact that dipyridamole does not affect calcineurin phosphatase activity against RII phosphopeptide *in vitro*, it suppresses ionomycin induced NFAT nuclear translocation in a U-2 osteosarcoma cell line, and minimizes NFAT-triggered reporter gene and cytokine expression in Jurkat T cells. Moreover, dipyridamole is an efficient inhibitor of phosphodiesterase V (PDE5), which leads to an increase in cGMP level and subsequently

Table 1 Low-molecular weight inhibitors of calcineurin.

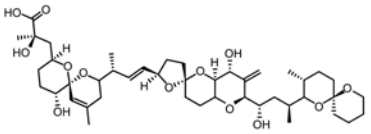
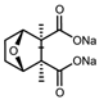
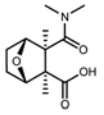
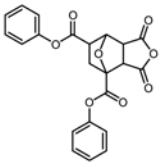
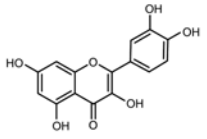
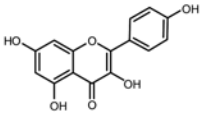
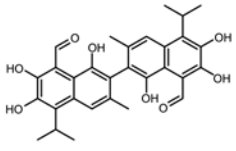
Trivial name	Structure with IUPAC name	Inhibition	Ref.
Okadaic acid	 (2*R*)-2-hydroxy-3-((2*S*,5*R*,6*R*)-5-hydroxy-8-((2*R*,*E*)-4-((2*R*,5*R*,6′*S*,8′*R*,8a′*S*)-8′-hydroxy-6′-((1*S*,3*S*)-1-hydroxy-3-((3*R*,6*S*)-3methyl-1,7-dioxaspiro[5.5]undecan-2-yl)butyl)-7′-methyleneoctahydro-3*H*,3′*H*-spiro[furan-2,2′-pyrano[3,2-*b*]pyran]-5-yl)but-3-en-2-yl)-10-methyl-1,7-dioxaspiro[5.5]undec-10-en-2-yl)-2-methylpropanoic acid	IC_{50}=4.6 μM	(66)
Endothall (Norcantharidin)	 (1*R*,4*S*)-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic anhydride	K_i=3.33 μM K_i=11.5 μM	(64) (65)
Cantharidin	 (1*R*,2*S*,3*R*,4*S*)-2,3-dimethyl-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic anhydride	K_i=10.8 μM	(123)
Cantharidin compound **3b**	 sodium (1*R*,2*S*,3*R*,4*S*)-2,3-dimethyl-7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylate	K_i=3.02 μM	(123)
Cantharidin compound **6**	 (1*S*,2*R*,3*S*,4*R*)-3-(dimethylcarbamoyl)-2,3-dimethyl-7-oxabicyclo[2.2.1]heptane-2-carboxylic acid	K_i=2.78 μM	(123)
1,5-Dibenzoyloxymethyl-norcantharidin	 1,5-Dibenzoyloxymethyl-7-oxabicyclo[2,2,1]heptan-2,3-dicarboxylic anhydride	IC_{50}=7 μM	(70)
Quercetin	 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one	IC_{50}=11.2 μM	(98)
Kaempferol	 3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one	IC_{50}=51 μM	(87)
Gossypol	 1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl-2,2′-binaphthyl-8,8′-dicarbaldehyde	IC_{50}=17 μM	(21)

Table 1 (*Continued*)

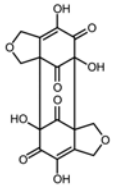
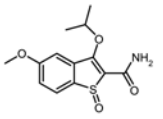
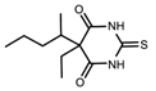
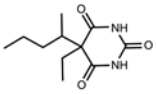
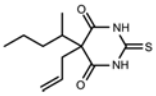
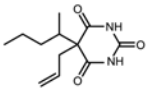
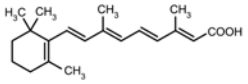
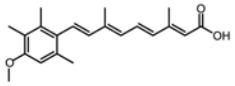
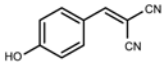
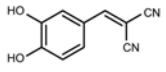
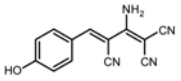
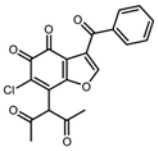
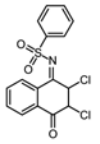
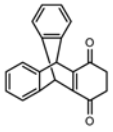
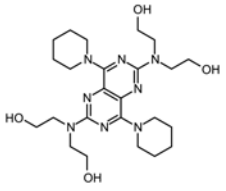
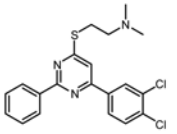
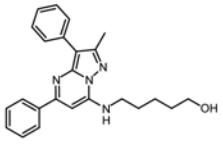
Trivial name	Structure with IUPAC name	Inhibition	Ref.
Dibefurin	 (3a*R*,4*R*,9a*R*,10*S*)-4,6,10,12-tetrahydroxy-9,10-dihydro-3a, 10:4,9a-dimethanocyclodeca[1,2-*c*;6,7-*c*']difuran-5,11,13,14(1*H*,3*H*,4*H*,7*H*)-tetraone	IC_{50}=46 μM	(73)
PD144795	 3-isopropoxy-5-methoxybenzo[*b*]thiophene-2-carboxamide-1-oxide	IC_{50}=4 μM	(97)
Thiopental	 5-ethyl-5-(pentan-2-yl)-2-thioxodihydropyrimidine-4,6(1*H*,5*H*)-dione	IC_{50}≈2.5 mM[a]	(71)
Pentobarbital	 5-ethyl-5-(pentan-2-yl)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione	IC_{50}≈7 mM[a]	(71)
Thiamylal	 5-allyl-5-(pentan-2-yl)-2-thioxodihydropyrimidine-4,6(1*H*,5*H*)-dione	IC_{50}≈3 mM[a]	(71)
Secobarbital	 5-allyl-5-(pentan-2-yl)pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione	IC_{50}≈6 mM[a]	(71)
Retinoic acid	 (2*E*,4*E*,6*E*,8*E*)-3,7-dimethyl-9-(2,6,6-trimethylcyclohex-1-enyl)nona-2,4,6,8-tetraenoic acid	IC_{50}≈40 μM	(99)
Acitretin	 (2*E*,4*E*,6*E*,8*E*)-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethylnona-2,4,6,8-tetraenoic acid	IC_{50}≈350 μM[a]	(99)
Tyrphostin A8	 2-(4-hydroxybenzylidene)malononitrile	IC_{50}=21 μM	(63)
Tyrphostin A23	 2-(3,4-dihydroxybenzylidene)malononitrile	IC_{50}=62 μM	(63)
Tyrphostin A48	 (*Z*)-2-amino-4-(4-hydroxyphenyl)buta-1,3-diene-1,1,3-tricarbonitrile	IC_{50}=30 μM	(65)

Table 1 (*Continued*)

Trivial name	Structure with IUPAC name	Inhibition	Ref.
INCA1	 3-benzoyl-6-chloro-7-(2,4-dioxopentan-3-yl) benzofuran-4,5-dione	K_D=0.5 μM	(84)
INCA2	 (*E*)-*N*-(2,3-dichloro-4-oxo-3,4-dihydronaphthalen-1(2*H*)-ylidene)benzenesulfonamide	K_D=0.12 μM	(84)
INCA6	 (9*s*,10*s*)-14,15-dihydro-9,10-[1,2]benzeno anthracene-13,16(9*H*,10*H*)-dione	K_D=0.8 μM	(84)
Dipyridamole	 2,2′,2″,2‴-(4,8-di(piperidin-1-yl)pyrimido[5,4-*d*]pyrimidine-2,6-diyl)bis(azanetriyl)tetraethanol	IC_{50} ≈ 10 μM[a]	(74)
CN585	 2-(6-(3,4-dichlorophenyl)-2-phenylpyrimidin-4-ylthio)-*N*,*N*-dimethylethanamine	K_i=3 μM	(22)
NCI3	 5-(2-methyl-3,5-diphenylpyrazolo[1,5-*a*]pyrimidin-7-ylamino)pentan-1-ol	IC_{50}=5 μM	(89)

[a]Interpolated or extrapolated data from graph.

abolished Ca^{2+} oscillations in cells (75). The altered intracellular calcium concentration can also modulate the Ca^{2+}/calmodulin regulated protein phosphatase calcineurin.

The natural compound gossypol, a polyphenolic aldehyde isolated from cotton seed (*Gossypium arboretum* and *Gossypium herbaceum*), is a non-competitive inhibitor of calcineurin, but not of PP1, PP2A and PP2C (21). Moreover, the observed inhibition is reversible and not dependent on the presence of immunophilins or other proteins. Similar to other calcineurin inhibitors, T cell receptor signaling stimulated by PMA/ionomycin is suppressed by gossypol in a dose-dependent manner, as demonstrated by blocking NFAT translocation from the cytosol into the nucleus as well as NFAT-triggered luciferase reporter gene activity. In 2007, a calmodulin-competing effect was postulated for gossypol as a possible mode of inhibition (76). However, this mechanism can only marginally contribute to the inhibition, because increased concentrations of calmodulin have no effect on calcineurin inhibition by gossypol, demonstrated in enzyme kinetic assays. In mice, gossypol significantly inhibits the proliferation of PMA/ionomycin stimulated lymphocytes in a dose-dependent manner (77). The lymphoblastic transfor-

mation of both T and B lymphocyte subsets was significantly suppressed by gossypol. Additionally, the immunosuppressive effects in mice were improved by induction of apoptosis. The antiproliferating effect of gossypol was tested in male antifertilization and contraceptive therapy (78, 79). Nevertheless, the known inactivation of other cellular enzymes as dehydrogenases, protein kinases, proteases, phospholipases and topoisomerases might also contribute to the inhibition of tumor cell proliferation (80–83).

A screening of a small organic compound library has led to identification of non-peptide inhibitors of NFAT-calcineurin association (inhibitor of NFAT/calcineurin association, INCA) (84, 85). The scaffolds of INCA1, INCA2 and INCA6 are chemically unrelated. All three compounds interact in a covalent manner with Cys266 at the active site of calcineurin and induce conformational changes, which mask the binding site for NFAT and VIVIT peptide. Interestingly, the calcineurin/INCA binding is chemically labile, because treatment with excess DTT largely reversed the inhibitory effect, as demonstrated for INCA2. Despite the fact that only INCA2 inhibits calcineurin activity against RII phosphopeptide, both INCA2 and INCA6 block the dephosphorylation and nuclear import of NFAT in a stimulated murine T cell line. All INCA compounds exhibit a marked cytotoxicity, excluding their use in human.

Kaempferol is an ubiquitous polyphenolic compound, which can be isolated from a multitude of plants (86). The flavonol inhibited enzyme activity of calcineurin when pNPP or RII phosphopeptide were used as substrate (87). Studies on kaempferol/calcineurin interaction by spectroscopic methods and docking experiments indicate that kaempferol binds in a 1:1 stoichiometry onto the catalytic domain of calcineurin (88). Interestingly, neither PP1 nor the alkaline phosphatase was affected. Moreover, an inhibitory activity against IL-2 gene expression in activated Jurkat T cells was detected by using RT-PCR.

The pyrazolopyrimidine NCI3 was also identified by screening a compound library for new calcineurin inhibitors (89). Although the phosphatase activity of calcineurin against pNPP or RII phosphopeptide substrate is not affected by NCI3, the compound inhibits NFATc2 dephosphorylation, following nuclear translocation, cytokine production and cell proliferation in Jurkat or primary human T cells. Because the VIVIT peptide, a 5-meric oligopeptide derived from NFATs PxIxIT-calcineurin recognition motif, is partially displaced by NCI3, an interaction of the pyrazolopyrimidine with the calcineurin substrate binding interface *in vivo* was hypothesized as mode of action.

In addition to the calcineurin phosphatase activity against phosphoseryl and phosphothreonyl residues, there is some evidence for its phosphatase activity against phosphotyrosyl residues in various proteins (90, 91). The observation that the active site of calcineurin tolerates and interacts with the relatively large phosphotyrosyl residue has inspired Martin to test tyrphostin derivatives (63). Tyrphostin are members of a big family of tyrosine kinase inhibitors (92, 93). Tyrphostins were first characterized as possible inhibitors of calcineurin using pNPP as the substrate, but tyrphostins also inhibit the dephosphorylation of small phosphopeptides in the lower micromolar range.

It is known for a few years that several benzothiophene derivatives exhibit anti-inflammatory and anti-HIV effects (94–96). To investigate the molecular basis for these properties PD 144795, a benzothiophene-2-carboxamide, was tested (97). PD 144795 inhibits calcineurin activity in Jurkat T cells and also in phosphatase assay, measured with [^{32}P]-labeled phosphopeptide, in a concentration-dependent manner. Furthermore, the compound blocks transcriptional activity of p53 and NF-κB, whereby the latter and the calcineurin inhibition could both contribute to the anti-inflammatory effect.

Quercetin, another ubiquitous flavonol, was identified by Wang et al. as a non-competitive inhibitor of calcineurin (98). The inhibition was determined *in vitro* with RII phosphopeptide as substrate as well as in a Jurkat T cell model. Quercetin reduces the mRNA level of IL-2 in a concentration-dependent manner, as observed semiquantitatively in an agarose gel and quantitatively in RT-PCR.

Based on the findings that tyrphostins inhibit calcineurin activity and that inhibition was improved by the presence of unsaturated and conjugated side chains as substituent of the aromatic ring of the tyrphostin, it was concluded that the conjugated chains were important for inhibition of calcineurin. One group of biologically active and pharmacological interesting molecules containing conjugated chains is the retinoids. Retinoids are members of the vitamin A family, which count as effectors in various biological signaling events, such as cell differentiation and proliferation. In addition to their important role in nutrition, retinoids are often used locally or systemically for the treatment of dermatological diseases, including acne and psoriasis. Out of all therapeutically used retinoids, only Tretinoin (all-*trans* retinoic acid), as a member of the first generation retinoids (non-aromatic retinoids), and acitretin, as a member of the second retinoid generation, were investigated by Spannaus-Martin and Martin (99). Both compounds inhibit the calcineurin catalyzed dephosphorylation of pNPP in micromolar range. Unfortunately, no data about the influence of retinoids on dephosphorylation of physiological substrates or calcineurin driven *in vivo* effects are available.

Inhibitory peptides and proteins

In addition to the effect of intracellular calcium level, calcineurin phosphatase activity is also influenced by the interaction with other proteins or small peptides representing an additional regulation mechanism. New calcineurin inhibitory peptides were derived from calcineurin interaction partners targeting the active site or binding motifs for substrates or effectors. For the identification of peptidic inhibitors large peptide libraries with peptide length and amino acid variations were synthesized. With this approach it is possible to encircle binding motifs, improve the affinity of peptides, find new binding partners and elucidate signaling pathways. Unfortunately, inhibitory peptides and proteins are protease

sensitive and can usually not cross the cell membrane – facts which impede their therapeutic use.

PxIxIT as general calcineurin binding motif and VIVIT

The Rao group identified a conserved calcineurin binding motif, the PxIxIT sequence. This sequence exists alongside NFATc2, and also in RCAN1, CABIN1, AKAP79, the yeast transcription factor Crz1 and the viral modulator protein A238L (100–102). PxIxIT containing peptides compete with NFATc for binding to calcineurin and diminish the dephosphorylation of NFATc1, NFATc2 and NFATc4 in a cell-free system (103). However, the dephosphorylation of other substrates, which do not use the calcineurin docking site for PxIxIT, is not affected. Based on a SPRIEIT containing 16-meric peptide of NFATc2, new peptides with an optimized amino acid sequence were synthesized and tested. The VIVIT peptide exhibits (with a K_d value of 0.5 μM) a 24-fold higher efficiency for the inhibition of NFATc dephosphorylation when compared with the SPRIEIT peptide. In contrast to other calcineurin inhibitors, the VIVIT peptide acts in a very selective manner. In Jurkat T cells, it inhibits only the calcineurin-NFAT interaction and dephosphorylation without affecting NF-κB or other pathways.

Autoinhibitory domain of calcineurin

Similar to some other proteins, the multidomain protein calcineurin has its own inhibitor inside. The last 97 amino acids of the C-terminus (CaN $A\alpha^{424-521}$) form the autoinhibitory domain that interacts with the active site of the enzyme in a competitive manner (104). Sagoo et al. have determined in a pNPP-coupled phosphatase assay a K_i value of 5 μM and against RII phosphopeptide an IC_{50} of 2.5 μM (105). Three years later, Perrino found that removal of residues 457–482, the core motif of the autoinhibitory domain, does not result in complete Ca^{2+}/calmodulin-independent activity (11). Moreover, peptides spanning residues 420–511 and 328–511 of CaN Aα were three- to four-fold more potent inhibitors of calcineurin phosphatase activity in comparison with the CaN $A\alpha^{457-482}$. These results indicate that additional autoinhibitory elements are present outside the described core motif.

AKAP79

The A-kinase anchoring protein (AKAP79) is a multivalent anchoring protein that holds in addition to calcineurin, the protein kinase A, and protein kinase C at the postsynaptic membrane of excitatory synapses, where it is recruited into complexes with NMDA- or AMPA-subtype glutamate receptors (106, 107). Dell'Acqua et al. have demonstrated that $AKAP79^{315-360}$ is necessary and sufficient for AKAP79-calcineurin anchoring in COS7 cells (108). Several residues in this peptide mediate the binding onto CaN A subunit and inhibit phosphatase activity against RII phosphopeptide. Thereby, $AKAP79^{315-360}$ antagonizes calcineurin anchoring *in vitro* and targeting in transfected COS7 cells. By using a peptide mapping approach, aa 330–357 were identified as the core calcineurin interaction motif (109). $AKAP79^{330-357}$ also contains the known PxIxIT calcineurin binding motif and inhibits calcineurin phosphatase activity with an IC_{50} value of 1.5 μM. Moreover, AKAP79 disrupts the calcineurin-dependent modulation of GluR1 receptor currents in HEK293 cells.

Cain/Cabin1

A novel endogenous calcineurin binding protein, Cain (calcineurin inhibitor), was identified in 1998 by Lai et al. in a yeast two-hybrid screening of a rat hippocampal cDNA library (110). In a phosphatase assay using RII phosphopeptide a K_i value of 440 nM was determined. Simultaneously, the same protein, here designated as Cabin1 (calcineurin binding protein 1), was described by the Liu group as a negative regulator for calcineurin signaling in T lymphocytes (111). They demonstrated that a truncated Cabin inhibits dephosphorylation of NFAT by calcineurin *in vivo* and that the expression of full length Cabin 1 or its C-terminal fragment blocks PMA/ionomycin stimulated IL-2 promoter activity.

Carabin

Carabin is a 49.7 kDa protein, which was described as a dual inhibitor of calcineurin and Ras (112). It plays a role in feedback regulation of adaptive immunity and is unrelated to Cain/Cabin1. The C-terminal domain of Carabin ($Carabin^{406-446}$) mediates the interaction with calcineurin. In a phosphatase assay with RII phosphopeptide as substrate, an IC_{50} value of 151 nM was measured. Cabin inhibits the dephosphorylation of NFATc2 and blocks the nuclear translocation of a green fluorescent protein (GFP)-tagged NFATc4 in response to stimulation with ionomycin. Moreover, in human T cells it leads to the inhibition of the IL-2 luciferase reporter gene activation in a dose-dependent manner. The downregulation of Carabin expression in primary T cells by using lentivirus harboring shRNA technology caused a more than fivefold increase in IL-2 production compared with control T cells supporting the negative TCR signaling regulator theory.

RCAN1

In 2007, the members of the DSCR1/Adapt78 gene family were summarized to RCAN (regulators of calcineurin) (113). RCAN1, formerly known as DSCR1, MCIP1 or calcipressin1, exist as several splice variants (114). All isoforms posses an identical sequence at the C-terminus, but have different N-termini. $RCAN1^{236-241}$, encoded in exon 7 and containing the PxIIxT motif, resembles the PxIxIT motif of NFAT proteins that binds to calcineurin without blocking the active site (115). However, kinetic analysis with the pNPP substrate demonstrated a competitive mode of inhibition, which suggests a direct interaction with the active site. For the full length RCAN1, a K_i value of 68.6 nM and for $RCAN1^{236-241}$ a K_i value of 51.6 nM was measured. Binding

studies with a truncated calcineurin A subunit (CaN $A\alpha^{\Delta 347}$) indicate the physical interaction between RCAN1 and the catalytical core (116). In BHK cells, it was shown that $RCAN1^{236-241}$ is necessary and sufficient to inhibit calcineurin-triggered nuclear translocation of GFP-NFATc3 (117). In a later study, two independent calcineurin interaction sites were encircled by *in vitro* binding assays and $RCAN1^{198-210}$ (KYELHAXaaTX$_{aa}$X$_{aa}$TPS motif) and $RCAN1^{236-241}$ (PxIxxT motif) were identified.

African swine fever virus protein A238L

In 1998, Miskin and colleagues have identified in a yeast two-hybrid screening A238L, a protein from African swine fever virus (family of *Asfarviridae*) as binding partner of the porcine calcineurin A subunit (118). An N-terminal truncated A238L ($A238L^{157-238}$) containing the known PxIxIT sequence (here as PKIIITG) binds onto calcineurin. The calcineurin interaction motif in A238L was identified by mutational analysis and a 14-amino acid region ($A238L^{200-213}$) was described (119). Although peptides corresponding to this domain of A238L bind to calcineurin with a K_d value of 600 nM, they do not inhibit its phosphatase activity. It was shown that residues 32–350 of calcineurin A subunit are involved in A238L binding and residues 1–347 for NFAT binding (120). Therefore, A238L inhibits calcineurin phosphatase activity, whereas NFAT proteins are the substrates. The 28 kDa protein A238L, also known as p28, contains a 4-ankyrin-repeat domain as a structural feature, which prevents binding of the transcription factor NF-κB subunits RelA and NF-κB3 to specific DNA sequences. Moreover, the viral protein inhibits acetylation and transcriptional activation of NFATc2, NF-κB and c-Jun through a mechanism involving PKCθ-mediated upregulation of N-terminal transactivation domain of p300 (121, 122). The ability of the A238L to inhibit both calcineurin phosphatase activity and activation of NF-κB, two key signaling pathways involved in immune response, could represent potent mechanisms to evade the host immune system.

Expert opinion

Calcineurin inhibitors have been in use since 1983 as immunosuppressive drugs. Because of their outstanding effectiveness, they represent the most widely used drugs in preventing organ rejection. Apart from transplantation medicine, calcineurin inhibitors are also used in psoriasis, severe atopic dermatitis, pyoderma gangrenosum, several autoimmune diseases, and, in severe cases, in rheumatoid arthritis. However, calcineurin inhibitors show a range of toxic adverse effects resulting in drugs with a narrow therapeutic window. Therefore, the primary objective of the development of novel immunosuppressive calcineurin inhibitors should consist of the improvement of side effect profiles, the optimization of the ease-of-use, and the reduction of nephrotoxicity in addition to the more traditional goal of improved short-term outcomes. One approach to accomplish these aims consisted of the development of monofunctional calcineurin inhibitors. Thereby, several new synthetic and natural compounds were identified in the past decades and characterized concerning their phosphatase inhibition and immunosuppressive effect. Although some of these compounds proved to be effective in immunosuppression, not a single one of the substances has been introduced in clinical trials. The identification and development of proteinogenic and peptidic calcineurin inhibitors provided new insights in the field of calcineurin research and represented an important tool to investigate calcineurin-mediated signaling; however, they were not suitable for use as immunosuppressive drugs, because of their protease sensitivity and poor cell permeability. The development of new calcineurin inhibitors as immunosuppressive drugs often fails on the nonexistent specificity for calcineurin among the PPP family of protein phosphatases. The missing specificity can be explained by the high degree of homology of the active sites of this phosphatase family. One approach to circumvent this fact might represent the development of noncompetitive calcineurin inhibitors, which bind to regions remote to the active site. CsA and FK506 represent suchlike compounds. However, they need prior binding to their respective immunophilins, which is thought to be a reason for their toxic side effects and thus their narrow therapeutic window.

Outlook

Based on the proven effectiveness of calcineurin inhibitors as immunosuppressive drugs, the primary focus of further research should consist of the development of highly specific compounds. One promising strategy to accomplish this goal might represent the synthesis of new FK506 and CsA analogs. These analogs should potently inhibit the phosphatase activity of calcineurin but not the peptidyl prolyl isomerase activity of their respective immunophilins. Moreover, new compounds should exhibit increased cell permeability as well as an improved bioavailability and ease-of-use. There are some FK506 or CsA based drugs currently under investigation in clinical trials with diminished nephrotoxicity but highly efficient calcineurin inhibition. One compound is voclosporin, a cyclosporin A derivative with reduced nephrotoxicity. Voclosporin has been studied as a possible alternative to cyclosporine A for psoriasis, organ transplant, uveitis, an eye disorder. Therefore, the market authorization by the European Medicines Agency (EMA) for voclosporin for the treatment of chronic non-infectious uveitis is pending. In addition to FK506 (tacrolimus), which is EU-wide authorized to treat moderate to severe atopic dermatitis in patients who cannot use or do not respond to standard treatments, such as topical corticosteroids; SDZ ASM981 (pimecrolimus) was authorized in the European Union for the treatment of mild or moderate atopic dermatitis. HTS of synthetic and natural compound libraries, combined with structure-assisted design of small molecules, should also provide new candidates for evaluation of the use as immunosuppressive drugs in the future.

Highlights

Calcineurin plays a prominent role in many physiological and pathophysiological cellular events, such as immune response, schizophrenia, diabetes mellitus, Down syndrome, cardiac hypertrophy and Alzheimer's disease. Therefore, inhibitors of calcineurin represent not only a valuable tool for the investigation of several signaling pathways but also attract large commercial interest of pharmaceutical companies. Thus, cyclosporin A has successfully been applied as an immunosuppressive drug for almost three decades, and several new compounds were approved in the past decade or are currently in Phases I, II, or III of clinical trials.

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