

A Semisynthetic Fusicoccane Stabilizes a Protein-Protein Interaction and Enhances the Expression of K⁺ Channels at the Cell Surface

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SUMMARY

Small-molecule stabilization of protein-protein interactions is an emerging field in chemical biology. We show how fusicoccanes, originally identified as fungal toxins acting on plants, promote the interaction of 14-3-3 proteins with the human potassium channel TASK-3 and present a semisynthetic fusicoccane derivative (FC-THF) that targets the 14-3-3 recognition motif (mode 3) in TASK-3. In the presence of FC-THF, the binding of 14-3-3 proteins to TASK-3 was increased 19-fold and protein crystallography provided the atomic details of the effects of FC-THF on this interaction. We also tested the functional effects of FC-THF on TASK channels heterologously expressed in *Xenopus* oocytes. Incubation with 10 μ M FC-THF was found to promote the transport of TASK channels to the cell membrane, leading to a significantly higher density of channels at the surface membrane and increased potassium current.

INTRODUCTION

Development of pharmaceutical agents currently focuses on inhibitors of enzymes, G-protein-coupled receptors and ion channels. Recently, the field has been expanded to target protein-protein interactions (PPIs) with nearly all of the studies performed so far aiming at disruption of PPIs (Wells and McClendon, 2007; Thiel et al., 2012). Here, we show that the alternative approach of stabilizing PPIs may also be feasible and may present a useful strategy for the design of novel bioactive compounds. Previously, we reported a proof of concept of PPI stabilization by deciphering in structural detail how the natural compound fusicoccin A (FC-A), a diterpene glycoside, stabilizes the activated protein complex between the plant plasma membrane H⁺-ATPase (PMA2) and 14-3-3 adaptor proteins (Würtele et al., 2003; Ottmann et al., 2007a). The FC-A molecule binds to

the interface of the complex by simultaneously contacting both proteins and enhances the apparent affinity of the protein-protein interaction about 90-fold.

14-3-3 proteins are ubiquitous eukaryotic adaptor proteins involved in the regulation of cell-cycle control, signal transduction, protein trafficking, and apoptosis (Hermeking and Benzinger, 2006). They assemble as dimers with each of the subunits possessing an amphipathic groove that accommodates the phosphorylated interaction motifs of their clients' proteins (Yaffe et al., 1997). The mode 1 and mode 2 14-3-3 binding motifs, RXX-pS/T-XP and RXXX-pS/T-XP (Yaffe et al., 1997; Johnson et al., 2010), are often found in the middle of proteins like C-Raf (Freed et al., 1994; Molzan et al., 2010). In contrast, the mode 3 motif (X-pS/T-X-COOH) (Smith et al., 2011) is functional only when the polypeptide chain terminates at position +1 after the phosphorylated serine/threonine. Among the ~200 interaction partners of 14-3-3 proteins described so far (Johnson et al., 2010) are many disease-relevant proteins such as the protein kinase C-Raf (Freed et al., 1994; Molzan et al., 2010), the transcriptional modulator YAP (Vassilev et al., 2001; Schumacher et al., 2010a), and the tumor suppressor p53 (Rajagopalan et al., 2010; Schumacher et al., 2010b). 14-3-3 proteins have been implicated in a variety of human diseases: they play a role in the genesis of diverse forms of cancer (Hermeking 2003), they have been associated with the development of neurodegenerative diseases (Berg et al., 2003), and they modulate the virulence of human pathogenic organisms (Fu et al., 1993; Ottmann et al., 2007b). These versatile and widespread biological functions have stimulated a growing interest in 14-3-3 proteins as a target class for pharmacological intervention.

In light of the important roles of 14-3-3 proteins we looked for human 14-3-3 client proteins that interact with 14-3-3 via a mode 3 motif similar to that of plant PMA2 and that might be modulated by fusicoccanes or related compounds. In contrast to the more than 200 14-3-3-interacting proteins that display an internal mode 1- or mode 2-like 14-3-3 binding motif, only six 14-3-3 interaction clients are known to mediate their binding to 14-3-3 via a mode 3 motif (see Table S1 available online) (Johnson et al., 2010). Among these are the two-pore-domain (K_{2p}) potassium channels, TWIK-related acid sensitive K⁺-channels 1 and 3

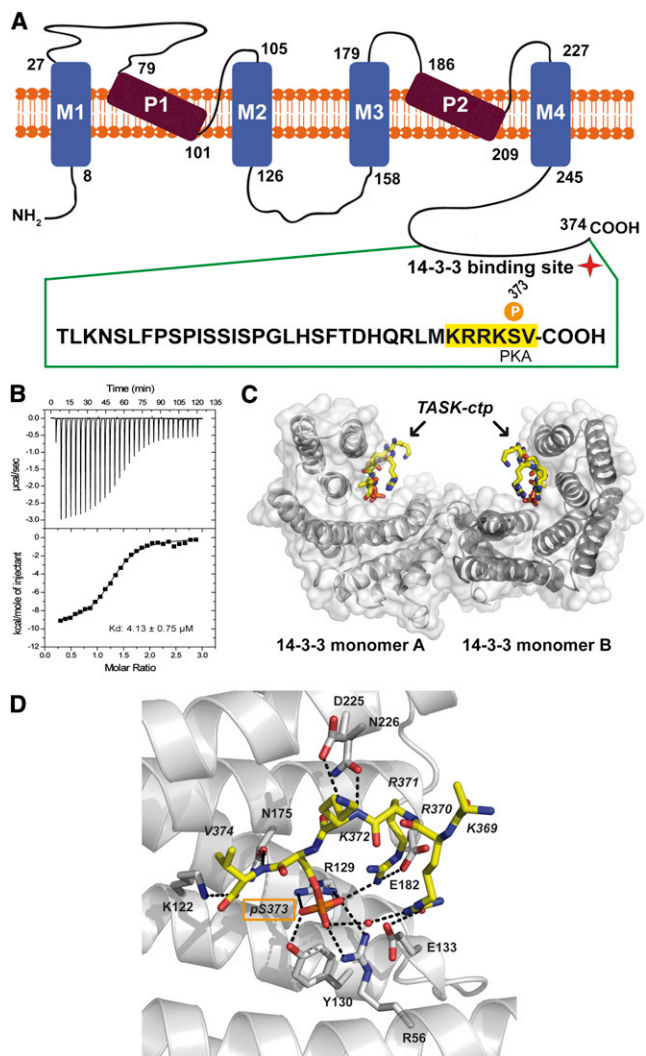


Figure 1. Crystal Structure of the 14-3-3 σ /TASK-ctp Complex

(A) Topology of the K⁺-channel TASK-3. M1–M4, membrane-spanning domains; P1 and P2, pore-forming domains. The end of the intracellular C terminus is shown as an amino acid sequence; the part visible in the crystal structure with 14-3-3 σ (TASK-ctp) is highlighted (yellow). See also Table S1. (B) Isothermal titration calorimetry of binding of TASK-ctp to 14-3-3 σ . (C) Cartoon plot with semitransparent surface of the 14-3-3 σ dimer (light gray) complexed with the TASK-ctp phosphopeptide (yellow sticks). (D) Polar contacts (dotted lines) between residues of 14-3-3 σ (light gray sticks and cartoon) and TASK-ctp (yellow sticks). A water molecule is shown as a red sphere. The italic labels are for the TASK-ctp peptide. See also Figures S1 and S2. See also Table S2.

(TASK-1 [Duprat et al., 1997] and TASK-3 [Rajan et al., 2000]). The subunits of these channels possess four transmembrane domains and two so-called pore-forming domains (Figure 1A); two of these subunits assemble as a functional channel (Lesage and Barhanin, 2011). Because K_{2P} channels play key roles in mammalian cells (Mathie, 2007), we decided to analyze a potential stabilization of the 14-3-3/TASK-3 interaction and the possible functional consequences of this modulation.

The K_{2P} channels TASK-1 and TASK-3 are functionally expressed in neurons and other cell types (Meuth et al., 2003)

and the number of TASK-1/3 channels inserted in the surface membrane of cells can be regulated at the transcriptional (Zanzouri et al., 2006; Cui et al., 2007) and at the posttranslational level (O’Kelly et al., 2002; Rajan et al., 2002). In this context, 14-3-3 proteins promote the functional expression of human TASK-1/3 channels by masking the basic ER retention signal, ³⁶⁹KRR³⁷¹, thereby allowing transport to the plasma membrane (O’Kelly et al., 2002; Rajan et al., 2002; Shikano et al., 2005; O’Kelly and Goldstein, 2008; Zuzarte et al., 2009), which leads to an increase in the number and density of these channels in the plasma membrane. It was shown in several studies that the TASK-3 protein is overexpressed in breast, lung, colon, and prostate cancer cells (Mu et al., 2003), and it may also be involved in K⁺-dependent apoptosis in neuronal cells (Lauritzen et al., 2003). In neuropathology, TASK channel activity has been linked to ischemia, inflammation, and epilepsy (Bittner et al., 2010). Pharmacological modulation of TASK-1 and TASK-3 channels could therefore be a valuable chemical biology tool to decipher its biological and pathophysiological role in greater detail and to explore the possibility of therapeutic interventions targeting these channels.

Here we report a semisynthetic fusicoccan derivative, FC-THF, that modulates the activity of the K⁺ channels TASK-1 and TASK-3 by stabilizing the interaction with the adaptor protein 14-3-3. We describe in detail the molecular mechanisms underlying the strict mode 3 specificity of this compound, demonstrating for the first time in structural detail the small-molecule stabilization of a human 14-3-3 PPI. Furthermore, we tested the effects of FC-THF in intact cells expressing TASK channels. In *Xenopus laevis* oocytes injected with cRNA encoding TASK channels, application of FC-THF produced an increase in the number of channels expressed at the surface membrane.

RESULTS

Binary Complex of 14-3-3 σ with the TASK C-Terminal Phosphopeptide

The 14-3-3 interaction motif in TASK-3 is located at the extreme C-terminal end of the K⁺-channel (Figure 1A), and interaction with 14-3-3 proteins is dependent on phosphorylation of the penultimate serine (Ser373). To study the mechanisms underlying the interaction of these two proteins we used a synthetic hexapeptide of TASK-3 containing the putative 14-3-3 interaction site (³⁶⁹KRRK-pS-V³⁷⁴-COOH); this peptide is termed TASK-C-terminal phosphopeptide (TASK-ctp) here. Using isothermal titration calorimetry (ITC), we found that TASK-ctp binds to 14-3-3 σ with a K_d of 4.13 ± 0.75 μM (Figure 1B), which is in the range of other known 14-3-3 interactions (Yaffe et al., 1997; Würtele et al., 2003; Molzan et al., 2010; Schumacher et al., 2010b). In order to answer the question whether the 14-3-3/TASK-ctp complex displays a binding pocket for fusicoccanes (as found at the interface of 14-3-3 with the plant PMA2 peptide ⁹⁵⁷QQSY-pT-V⁹⁵⁶-COOH) (Würtele et al., 2003), we solved its crystal structure to 1.4 Å resolution (Protein Data Bank [PDB] ID: 3p1n). We found interpretable density for all 231 residues of the 14-3-3 σ construct as well as for all six residues of the TASK-3 phosphopeptide (data and refinement statistics are summarized in Table S2). The 14-3-3 σ dimer displays the typical W-like shape, with each monomer forming a central binding groove that accommodates

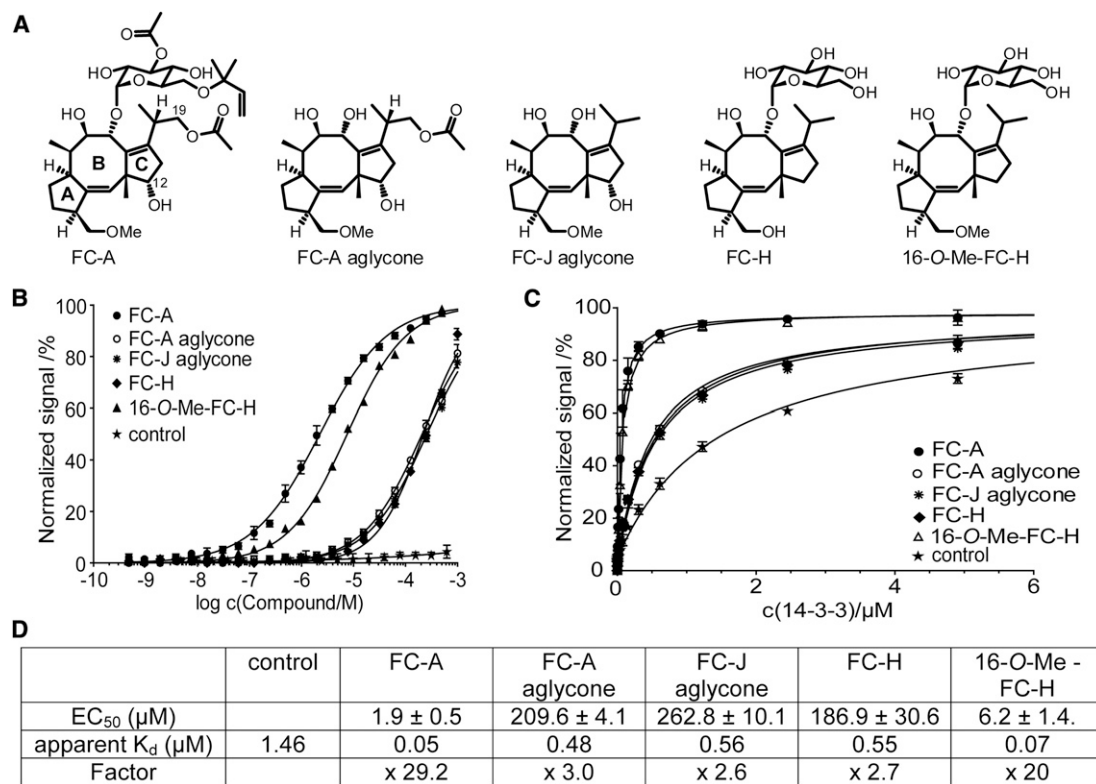


Figure 2. Stabilization of the 14-3-3/TASK-ctp Complex by Fusicoccin A and Other Naturally Occurring Fusicoccanes

(A) Structures of the naturally occurring fusicoccanes tested in this study.

(B) FP measurements of fluorescein-labeled FAM-TASK-ctp and 14-3-3σ titrated with different fusicoccanes to obtain EC₅₀ values for their stabilizing activity toward the 14-3-3σ/TASK-ctp complex. Error bars indicate the mean ± SD of at least three experiments.

(C) FP measurements of fluorescein-labeled FAM-TASK-ctp in the absence (control) or presence of 100 μM of the different fusicoccanes titrated with 14-3-3σ to obtain the apparent K_d of the 14-3-3σ/TASK-ctp interaction. Error bars indicate the mean ± SD of at least three experiments.

(D) Values of EC₅₀, apparent K_d, and the resulting stabilization factor of the 14-3-3σ/TASK-ctp interaction for the different fusicoccanes.

the phosphorylated interaction motif of TASK-3 (Figure 1C). The peptide occupies about half of the amphipathic groove of each monomer (Figures 1D and S1) and is bound in an extended conformation establishing a number of polar interactions (Figure 1D). The phosphate moiety of TASK-ctp forms electrostatic interactions with a positively charged pocket in 14-3-3σ consisting of Arg56, Arg129, and Tyr130. Further side-chain contacts are established between Arg371 of TASK-3 and Glu182 of 14-3-3σ, as well as between TASK-3 Lys372 and 14-3-3σ Asp225. Arg370 of TASK-3 forms a water-mediated contact to Glu133 of 14-3-3. Main-chain contacts of TASK-ctp are formed with Lys122, Asn175, and Asn226 from 14-3-3σ. One water-mediated and one direct intramolecular contact between the phosphate moiety of TASK-ctp and Arg370 and Arg371, respectively, coordinates the TASK-3 phosphopeptide in its binding to 14-3-3σ, highlighting the functional importance of these two consecutive arginines.

The 14-3-3 binding motif of TASK-3 is the only human 14-3-3 binding motif of the mode 3 type whose structure has been elucidated so far. Like the 14-3-3 binding motif of tobacco PMA2 (Würtele et al., 2003; Ottmann et al., 2007a), the polypeptide chain of TASK-3 ends at the position +1 distal to the phosphorylated amino acid. The last amino acid of TASK-ctp, Val374, is

coordinated by Lys122 and Asn175 of 14-3-3σ. To deduce structural prerequisites for the binding, stabilization, and activity modulation of the regulatory 14-3-3σ/TASK-3 complex by fusicoccanes, we compared it to the complex of tobacco 14-3-3-like protein C with a C-terminal phosphopeptide of tobacco PMA2 (PDB ID: 1o9f) (Würtele et al., 2003). We optimally superposed both 14-3-3 proteins with an emphasis on secondary structure matching (see Figure S2) and calculated the root-mean-square deviations (rmsds) of the phosphorylated residues (0.29 Å) and the C-terminal valines (0.42 Å). The very low rmsd of the phosphorylated residues is consistent with the highly conserved phosphobinding geometry of 14-3-3 proteins and the likewise low rmsd of the C-terminal valines indicates that fusicoccanes should be compatible with the 14-3-3σ/TASK-3 complex.

Fusicoccanes Stabilize the 14-3-3σ/TASK-ctp Interaction

The hypothesis that binding of FC-A (Figure 2A) may stabilize the 14-3-3σ/TASK-ctp complex was corroborated by fluorescence polarization (FP) experiments. FC-A stabilized the 14-3-3σ/TASK-ctp complex with an EC₅₀ of 1.90 ± 0.50 μM (Figure 2B). The apparent affinity of TASK-ctp to 14-3-3 was enhanced

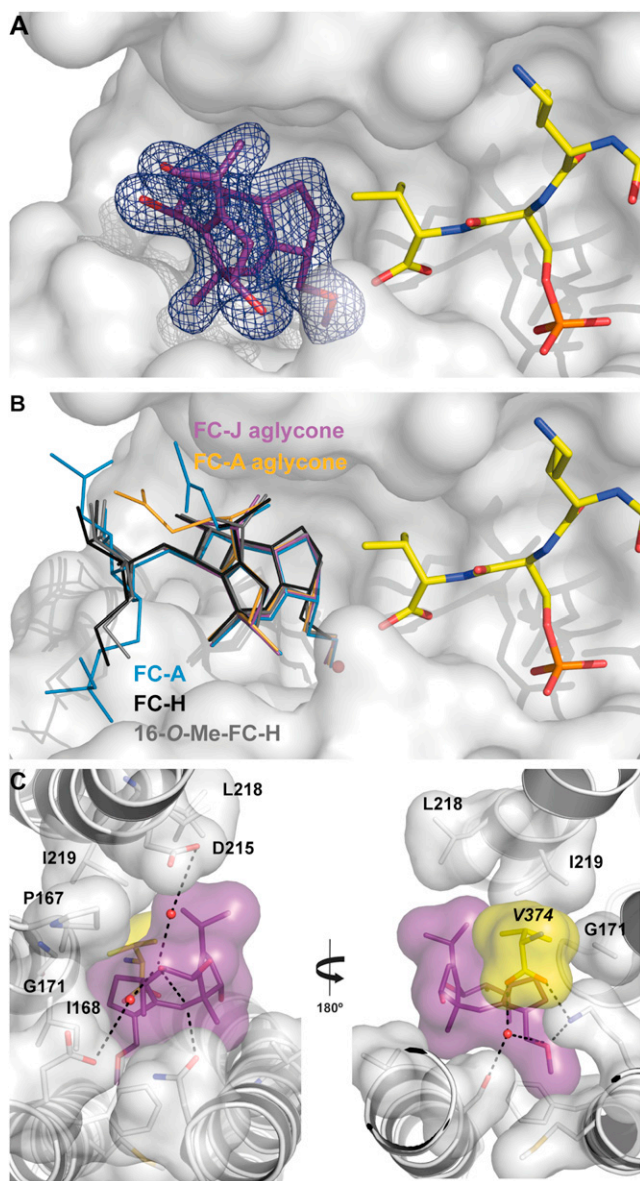


Figure 3. Crystal Structures of 14-3-3/TASK-ctp Complexes Stabilized by Fusicoccanes

(A) Binding of FC-J aglycone (purple sticks) and TASK-ctp (yellow sticks) to the 14-3-3 binding groove (light gray surface). Final $2F_o - F_c$ electron density map of FC-J aglycone (blue mesh, contoured at 1σ) is shown.

(B) Superposition of FC-A (blue sticks), FC-A aglycone (orange sticks), FC-H (black sticks), 16-O-Me-FC-H (gray sticks), and FC-J aglycone (purple sticks). The ternary structures of the 14-3-3 protein (light gray surface), the TASK-ctp peptide (yellow sticks), and the respective compound were overlaid. The coordination of the single FC-A, FC-A aglycone, and FC-H is shown in the Supplemental Information (see Figures S3–S6).

(C) Detailed view of the fusicoccan binding pocket composed of the 14-3-3 protein (light gray surface, sticks, and cartoon) and the TASK-3 peptide (yellow surface, sticks) with FC-J aglycone (purple surface, sticks). Polar contacts are indicated by black dotted lines and water molecules are displayed as red solid spheres.

See also Tables S3–S5.

approximately 30-fold, from a K_d of $1.46 \pm 0.06 \mu\text{M}$ to $0.05 \pm 0.002 \mu\text{M}$, when titrated in the presence of $100 \mu\text{M}$ FC-A (Figure 2C). Next we tested other fusicoccanes (de Boer and de Vries-van Leeuwen, 2012) for stabilization of the binary 14-3-3/TASK-ctp complex, among them fusicoccin H (FC-H), 16-O-Me-FC-H, and the aglycones of FC-A and fusicoccin J (FC-J) (Figure 2A). These natural products were also able to bind to and stabilize the 14-3-3/TASK-ctp complex to varying degrees with the aglycones showing considerably lower stabilization activity than the glycosides (Figure 2D). This observation might be explained by two effects. (1) The hydrophobic diterpene ring system of the glycosides is shielded from the water environment by the sugar moiety when bound to the 14-3-3/TASK-ctp complex. The aglycones, however, are in direct contact with the solvent, which can be assumed to result in energetically unfavorable interactions. (2) The glycosides display a considerably larger molecular surface and their binding to 14-3-3 is more strongly stabilized by van der Waals contacts with the protein partners.

Fusicoccanes Bind to the Interface of the 14-3-3/TASK-ctp Complex

In order to study the binding modes of the different fusicoccanes, we solved the structures of the ternary complexes by soaking the binary 14-3-3/TASK-ctp crystals with the individual compounds. Soaked crystals retained their high quality and diffracted to a maximum resolution of between 1.65 and 2.0 Å. All fusicoccanes bound to the same site adjacent to the C-terminal end of TASK-ctp, as exemplified by the aglycone of FC-J (Figure 3A; PDB ID: 3smm and 3smo; data and refinement statistics summarized in Table S4). The terpene moiety of the compounds inserts into a deep hydrophobic pocket that is mainly constituted by the 14-3-3 protein but has to be completed by the presence of Val374 of TASK-3. In the absence of TASK-ctp, the respective binding site is not fully functional, with a complete wall of the pocket missing (Figure S2). The 14-3-3/TASK-ctp structure with the aglycone of FC-J provides an explanation for the observation that the methoxymethyl substituent of ring A is important. The terminal O-methyl group of this substituent anchors the molecule in its binding pocket by contacting the hydrophobic side chains of Phe119 and Met123 of 14-3-3 (Figures 3B and 4C). These hydrophobic interactions help to form the stable complex together with hydrogen bonds of the ether oxygen to Lys122 and, via a water molecule, to Ser45. The only other polar ligand-protein interaction of this complex is the water-mediated hydrogen bond of one B ring hydroxyl group and the 14-3-3 residues Asn38, Glu115, and Asp215. In the case of FC-H (PDB ID: 3ux0, data and refinement statistics are summarized in Tables S3 and S4), the terminal O-methyl group is missing (Figure 2A). A highly defined water molecule occupies this position and mediates the necessary contacts (Figure 3B). Nevertheless, the missing methylation in FC-H results in an increase of the EC_{50} from $6.2 \pm 1.4 \mu\text{M}$ for the semisynthetic 16-O-Me-FC-H to $186.90 \pm 30.60 \mu\text{M}$ for FC-H. The major contributions of fusicoccan binding to the 14-3-3/TASK-3 complex are of a hydrophobic nature. In addition to the C-terminal Val374 of TASK-3, and Phe119 and Met123 of 14-3-3, the FC-J aglycone shares a hydrophobic surface contact with Val46, Pro167, Ile168, Gly171, Leu218, and Ile219 of 14-3-3 (Figure 3C). Besides FC-J, we

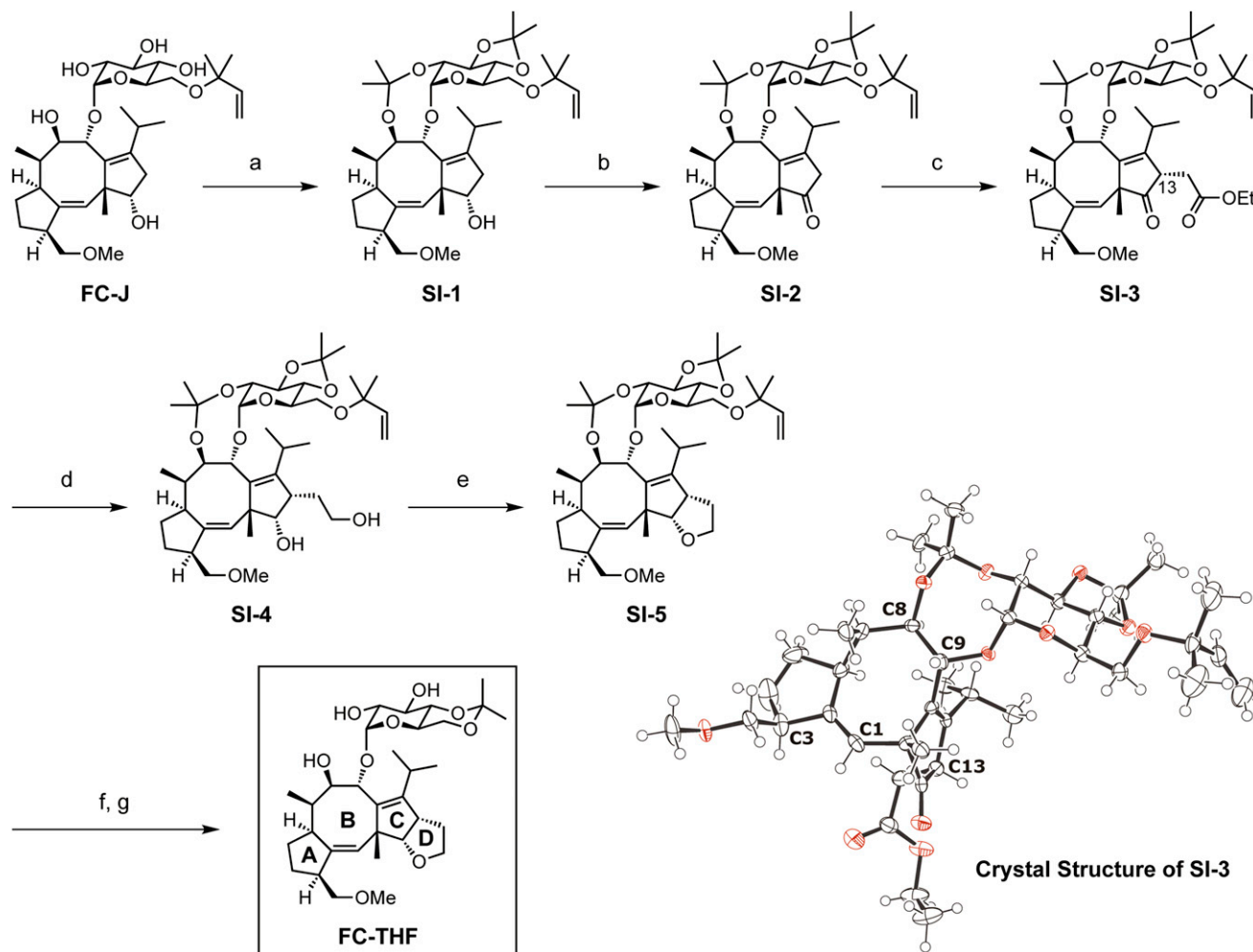


Figure 4. Semisynthetic Procedures for FC-THF from FC-J and the Crystal Structure of Synthetic Intermediate 3

Reagents and conditions: (a) 2-methoxypropene, camphorsulfonic acid/*N,N*-dimethylformamide, 40°C, 2 hr, 80%; (b) Dess-Martin periodinane, pyridine/ CH_2Cl_2 , room temperature (RT), 2 hr, 91%; (c) lithium diisopropylamide, hexamethylphosphoric triamide/THF, -70°C, 30 min, then ethyl 2-iodoacetate, -70°C ~RT, 13 hr, 89%; (d) LiAlH_4 /THF, 0°C, 1 hr, quantitatively; (e) PPh_3 , diethyl azodicarboxylate/THF, RT, 2 hr, quantitatively; (f) 1N HCl in EtOH, RT, 26 h; (g) 2,2-dimethoxypropane, pyridinium *p*-toluenesulfonate/ CH_2Cl_2 , RT, 16 hr, 84% in 2 steps. Ortep drawing of the crystal structure of SI-3: Nonhydrogen atoms are shown with 50% probability ellipsoids. For further information, see [Supplemental Information](#).

solved the crystal structures of 14-3-3 σ /TASK-ctp in complex with four other fusicoccane derivatives: FC-A (PDB ID: 3p1o, data and refinement statistics summarized in [Table S3](#)), FC-A aglycone (PDB ID: 3sml, [Table S4](#)), FC-H (PDB ID: 3ux0, [Table S4](#)), and 16-O-Me-FC-H (PDB ID: 4fr3, [Table S5](#)). The orientation of the 5-8-5 ring systems and the side chain of ring A superimposed perfectly with those of the FC-J aglycone in the binding groove ([Figure 3B](#)).

Semisynthesis and Structure of a 14-3-3/Mode-3-Specific Fusicoccane

It has been shown previously that FC-A acts as a stabilizer of the binary complex of 14-3-3 and a mode 3 peptide and forms a ternary complex with them ([Würtele et al., 2003](#); [Ottmann et al., 2007a](#)). Furthermore, FC-A cannot stabilize the complex composed of 14-3-3 and a canonical mode 1 and mode 2 binding site because of a steric clash between the invariant Pro+2

and a hydroxyl group at ring C of fusicoccanes ([Ottmann et al., 2009](#)). However, half of the known 14-3-3 clients display noncanonical 14-3-3 interaction motifs that do not include a proline at position +2 ([Johnson et al., 2010](#)). For example, Chibby carries a Ser at this position ($^{75}\text{PRKSApSLSNLH}^{25}$) ([Li et al., 2008](#)). To ensure the steric conflict with all (mode 1, mode 2, and noncanonical) 14-3-3 clients that extend beyond position +1, we devised the molecule FC-THF ([Figure 4](#)) as a specific stabilizer for the binary complex of 14-3-3 and mode 3 (X-pS/T-X₁-COOH) 14-3-3 recognition motifs. To achieve this, a five-membered tetrahydrofuran ring was constructed utilizing the 12-hydroxyl group of FC-J. This α -oriented tetrahydrofuran (THF) ring (ring D) of FC-THF is expected to produce steric repulsion with any residue at position +2.

As mentioned, we chose FC-J as the starting point of the semisynthesis ([Figure 4](#)). Alkylation on an enolate of synthetic intermediate 2 (SI-2) yielded an α -mono-alkylated product SI-3

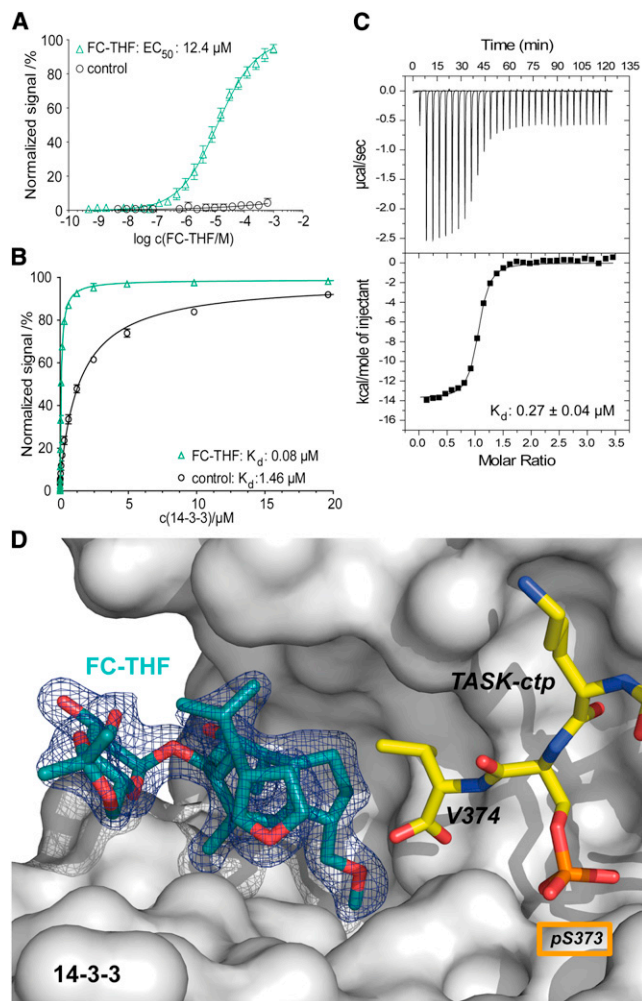


Figure 5. The Semisynthetic Fusicoccanes FC-THF Is a 14-3-3/TASK-3 Stabilizer

(A) FP measurements of fluorescein-labeled FAM-TASK-ctp and 14-3-3 σ titrated with FC-THF to obtain EC₅₀ values for its stabilizing activity toward the 14-3-3 σ /TASK-ctp complex. Error bars indicate the mean \pm SD of at least three experiments.

(B) FP measurements of fluorescein-labeled FAM-TASK-ctp in the absence and presence of 100 μ M FC-THF titrated with 14-3-3 σ to obtain the apparent K_d of the 14-3-3 σ /TASK-ctp interaction. Error bars indicate the mean \pm SD of at least three experiments.

(C) Isothermal titration calorimetry of binding of TASK-ctp to 14-3-3 σ in the presence of 100 μ M FC-THF.

(D) Crystal structure of FC-THF (teal sticks) bound to the complex of 14-3-3 σ (light gray solid surface) and TASK-ctp (yellow sticks). The final 2F_o - F_c electron density (blue mesh) for FC-THF (contoured to 1 σ) is shown. See also Table S6.

due to stereoelectronic preference in the addition reaction. The stereochemistry at the 13-position of **SI-3** was determined by X-ray crystallographic analysis (Figure 4) and the THF ring was constructed in a straightforward way by a Mitsunobu type of dehydration reaction on **SI-4** to furnish FC-THF.

To characterize the biochemical properties of FC-THF we tested the ability of FC-THF to bind to and stabilize the binary complex of 14-3-3 σ /TASK-ctp. Using FP experiments, we

measured the EC₅₀ of 12.4 \pm 2.3 μ M (Figure 5A). Furthermore FC-THF enhanced the apparent affinity of TASK-ctp to 14-3-3 σ 19-fold (from a K_d of 1.46 \pm 0.06 to 0.08 \pm 0.001 μ M), as measured via FP (Figure 5B). This observation was supported by isothermal titration calorimetry (ITC) measurements: In the absence of FC-THF, TASK-ctp was found to bind with a K_d of 4.13 \pm 0.75 μ M (compare Figure 1B); in the presence of FC-THF, the K_d value decreased to 0.27 \pm 0.04 μ M, revealing a 21-fold stabilization (Figure 5C). Soaking experiments were carried out to test the ability of FC-THF to bind to the preformed complex of 14-3-3 σ /TASK-ctp in the protein crystal and to solve the ternary structure to a resolution of 2.0 Å (Figure 5D; Table S6). The clear electron density for FC-THF allowed the determination of the spatial orientation of the molecule and thus its exact binding mode.

Because FC-THF was designed to selectively stabilize the interaction of 14-3-3 with mode 3 binding sites, we also tested its effect on the formation of the binary complex of 14-3-3 with the mode 2 motifs C-RafpS259 (²⁵⁴SQRQRST-pS-TPNVH²⁶⁴-COOH) and Cdc25BpS309 (³⁰²QLFRSP-pS-MPCSVIR³¹⁶), and with the nonproline internal motif of Chibby (¹⁵PRKSA-pS-LSNLH²⁵). FP measurements showed that FC-THF was not able to stabilize the binding of these motifs to 14-3-3, whereas TASK-ctp binding to 14-3-3 was clearly enhanced (Figure 6A). The superposition of the structures of 14-3-3 σ /TASK-ctp with a mode 1 (PDB ID: 1qjb) and a mode 2 (PDB ID: 1qja) motif illustrates the previously described potential for steric conflicts of ring D and the classical 14-3-3 binding motifs (mode 2 and mode 1; Figures 6B and 6D).

Effect of FC-THF on the Surface Expression of TASK Channels in *Xenopus* Oocytes

14-3-3 proteins are required for efficient intracellular transport of TASK-3 channels to the surface membrane (O'Kelly et al., 2002; Rajan et al., 2002; Zuzarte et al., 2009). The binding site for 14-3-3 (mode 3) is at the extreme C terminus of the channel protein (³⁷⁰RRK-pS-V³⁷⁴-COOH). It overlaps with a strong endoplasmic-reticulum (ER) retention signal (³⁶⁹KRR³⁷¹). The binding of 14-3-3 masks the ER retention signal; in this way the affinity of 14-3-3 binding to the channel protein controls the intracellular transport of the channel to the surface membrane and thus the number of functional channels at the cell surface (Zuzarte et al., 2009). Because FC-THF stabilizes the interaction between 14-3-3 proteins and their binding motif in TASK-3, we tried to elucidate the possible functional consequences of this stabilization by measuring the current flowing through heterogeneously expressed TASK-3 channels in the presence and absence of FC-THF. The cRNA encoding TASK-3 channels was injected into *Xenopus laevis* oocytes; 48 hr after cRNA injection, voltage-clamp experiments were performed to measure TASK-3 potassium currents at different potentials. The current-voltage relation showed the typical outward rectification expected under these experimental conditions (Duprat et al., 1997) (Figure 7A, dark blue curve). 50% of the oocytes in each batch were incubated with 10 μ M FC-THF for 48 hr after cRNA injection. The currents measured in these oocytes were significantly larger ($p < 0.01$; Figure 7A, light blue curve, and Figure 7B, blue bars) than in oocytes not incubated with FC-THF. Oocytes not injected with cRNA encoding the channels were used as

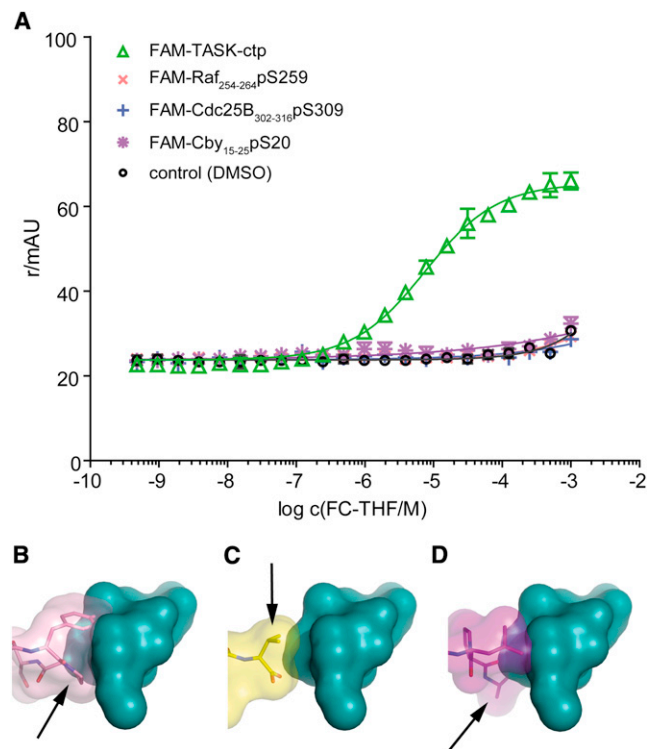


Figure 6. Selectivity of FC-THF for Mode 3 Binding Motifs

(A) FP titration with increasing concentrations of FC-THF to 14-3-3 α /FAM-TASK3-ctp (green open triangles) or different internal 14-3-3 recognition motifs. FP titration with increasing concentrations of DMSO to 14-3-3 α /FAM-TASK-ctp is shown as control (black circles). Error bars indicate the mean \pm SD of at least three experiments.

(B) Superposition of a mode 1 phosphopeptide (light purple sticks, PDB ID: 1qjb) with FC-THF (teal) See also Table S6.

(C) Relative positions of TASK-ctp (yellow sticks) and FC-THF from the experimental structure of this study.

(D) Superposition of a mode 2 phosphopeptide (purple sticks, PDB ID: 1qja) with FC-THF.

negative controls. Their current-voltage relation was virtually flat (Figure 7A, black curve).

To ascertain that the effects of FC-THF on current amplitude were mediated by the stabilization of the interaction between the channel and 14-3-3 proteins we carried out control experiments with a TASK-3 mutant in which the last five amino acids at the C terminus of the channel were removed (TASK-3 Δ C5). This mutant cannot interact with 14-3-3 proteins (Rajan et al., 2002). The amplitude of the currents measured with the Δ C5 mutant were smaller (Figure 7A, dark red curve), because the stimulatory effect of 14-3-3 proteins on intracellular transport to the cell surface was removed by the truncation (Zuzarte et al., 2009). We found that in the TASK-3 Δ C5 mutant, incubation with FC-THF caused a significant reduction of the relative current amplitude (Figures 7A and 7B). This reduction may be a nonspecific effect on the oocytes that is not related to the binding of 14-3-3. To test for this possibility we expressed another K_{2P}-channel, TREK-1, in *Xenopus* oocytes and applied 10 μ M FC-THF under exactly the same conditions as in the case of TASK-3. We found that the current carried by TREK-1 channels

was reduced in the presence of FC-THF by approximately the same percentage as the current carried by TASK-3 channels (Figure S7). As it has been shown previously that interaction with 14-3-3 proteins is required for efficient surface expression of TASK channels (O'Kelly et al., 2002; Rajan et al., 2002), our findings are consistent with the idea that FC-THF stabilized the interaction between 14-3-3 and the channels and, in addition, had some nonspecific effect that decreased the potassium current.

To confirm this hypothesis, we carried out a second series of experiments in which we measured the copy number of TASK-3 channels at the cell surface: a hemagglutinin-tag (HA-tag) was attached to the channels and surface expression was measured using an antibody-based luminometric assay (Zuzarte et al., 2009). We found that incubation of the oocytes with 10 μ M FC-THF increased the surface expression of TASK-3 channels by 45% (Figure 7C). In the corresponding Δ C5 mutant (which cannot interact with 14-3-3 proteins), incubation with 10 μ M FC-THF did not have a statistically significant effect, although the mean value was slightly reduced. Application of FC-THF to oocytes expressing TREK-1 also caused a slight reduction in surface expression that was not statistically significant (Figure S7). Thus, the results obtained with the luminometric surface expression experiments were in line with data obtained with the current measurements.

We then performed surface expression measurements with HA-tagged TASK-1 channels (Figure 7D), which share 59% amino acid identity with TASK-3 channels and interact with 14-3-3 proteins in exactly the same way as TASK-3 (O'Kelly et al., 2002; Zuzarte et al., 2009). Incubation with 10 μ M FC-THF increased the surface expression of TASK-1 by 48% and reduced the surface expression of the Δ C5 mutant by 40% (Figure 7D). We interpret these findings to indicate that (1) FC-THF increased surface expression of TASK-1 and TASK-3 by facilitating the interaction of the channels with 14-3-3 proteins; and (2) FC-THF had an additional nonspecific effect on the oocytes (unrelated to 14-3-3) that reduced the surface expression of the channel proteins and the amplitude of the outward currents in both wild-type and mutant channels.

DISCUSSION

In recent years, the use of small molecules modulating PPIs has been established as an alternative to active-site inhibitors of enzymes, GPCRs and ion channels (Wells and McClendon, 2007). However, currently known cases are almost exclusively based on the inhibition of PPIs. Prominent examples are the disruption of the interaction of p53 with its negative regulator MDM2 by the nutlins (Vassilev et al., 2004) and inhibition of the interaction of antiapoptotic Bcl-X_L with proapoptotic BAD or BAK by ABT-737 (Oltersdorf et al., 2005). Compared to inhibitors of PPIs, reports describing the search for small molecules that stabilize PPIs are scarce. Nonetheless, the principal feasibility of this concept for chemical biology as well as for therapeutic purposes is illustrated by the immunosuppressive natural compounds rapamycin and FK506, which, long after their identification as immunosuppressive agents, have been found to exert their physiological effects by stabilizing specific protein-protein interactions, i.e., the complexation of the small immunophilin

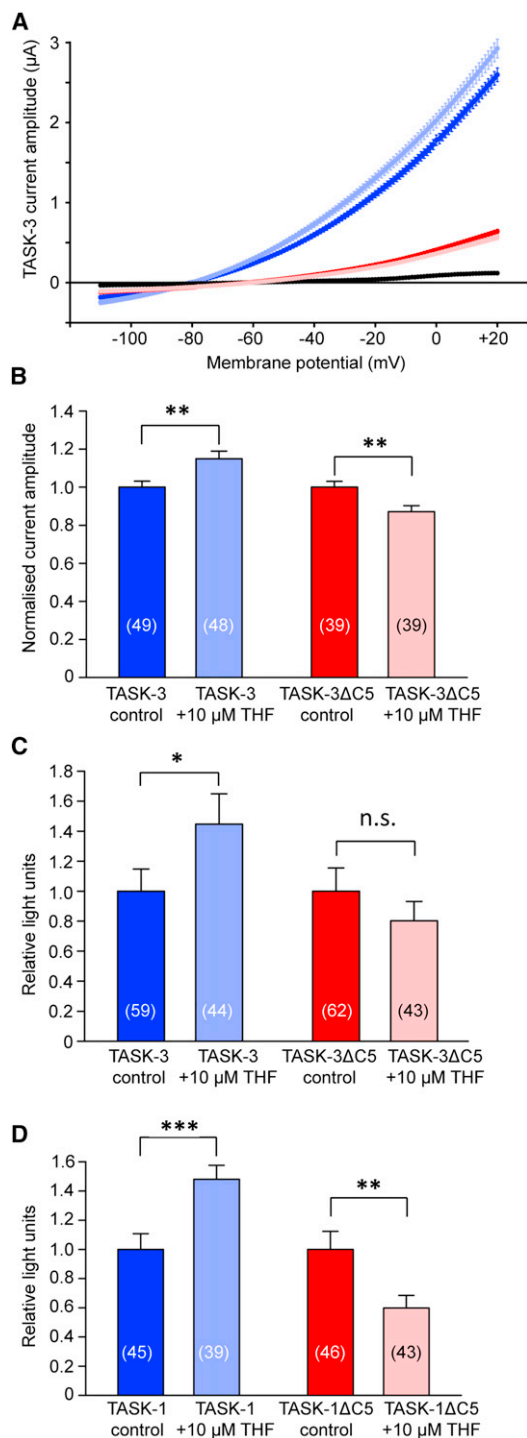


Figure 7. Effect of FC-THF on TASK Channels in *Xenopus laevis* Oocytes

(A) Current-voltage relations were measured in oocytes expressing TASK-3 channels (blue curves) or the TASK-3 ΔC5 mutant (red curves). The mean values \pm SEM of all data obtained in the absence (dark blue and dark red curves) and presence (light blue and pink curves) of 10 μM FC-THF are shown. The black curve shows the mean currents measured in noninjected oocytes. (B) Current amplitudes measured at 0 mV with wild-type TASK-3 (blue bars) and with the TASK-3 ΔC5 mutant (red bars) in the absence (blue and red bars) and presence (light blue and pink bars) of 10 μM FC-THF.

FKBP12 with mTOR or calcineurin, respectively (Choi et al., 1996; Griffith et al., 1995).

In our present study, we describe the pharmacological stabilization of a human 14-3-3 protein-protein interaction in atomic detail. We analyzed the interaction of 14-3-3 proteins with the human K⁺-channel TASK-3, which carries a C-terminal (mode 3) binding motif (X-pS/T-X-COOH) for 14-3-3 proteins. The K_d of the interaction between TASK-ctp and 14-3-3 was found to be in the low μM -range (4.16 μM). The crystal structure of the binary 14-3-3/TASK-ctp complex was solved to 1.4 Å, explaining the structural basis of this interaction.

We examined the ability of the fusicocanes FC-A, FC-H, 16-O-Me-FC-H, FC-A aglycone, and FC-J aglycone to stabilize the 14-3-3 σ /TASK-ctp interaction. As reported earlier (Ottmann et al., 2009), FC-A shows a steric conflict with the +2 proline in the mode 1 and mode 2 binding motifs when bound to 14-3-3. In the present study, we tried to devise a semisynthetic fusicocane with high mode 3 specificity by enhancing the steric hindrance with 14-3-3 binding motifs that extend beyond the +1 position. Using FC-J as the starting molecule, we obtained a mode-3-specific molecule (FC-THF) by adding a tetrahydrofuran ring to ring C (Figure 4). FC-THF bound to the 14-3-3/TASK-ctp complex in a mode similar to that observed for the natural fusicocanes (Figure 5D), and our FP and ITC measurements showed an approximately 19-fold increase in the apparent affinity of TASK-ctp toward 14-3-3 σ in the presence of FC-THF (Figure 5).

Finally, we studied the functional consequences of the PPI-stabilizing effect of FC-THF in a cellular system. Previous studies have clearly shown that the interaction of 14-3-3 with TASK-1 or TASK-3 is essential for efficient trafficking of the channels to the cell surface (O'Kelly et al., 2002; Rajan et al., 2002; Zuzarte et al., 2009). We found that the surface expression of our HA-tagged TASK-3 and TASK-1 channels in *Xenopus* oocytes was increased by \sim 45% and \sim 48%, respectively, when the oocytes were incubated with 10 μM FC-THF. In addition, we found that the outward current carried by TASK-3 channels in *Xenopus* oocytes was increased by \sim 15% when the oocytes were incubated with FC-THF. Because the binding of 14-3-3 proteins has no effect on the biophysical properties of TASK channels (Rajan et al., 2002), the increase in TASK 3 current was most likely also due to an increase in the number of channels at the cell surface. Thus, we have obtained evidence for a change in surface expression using two independent approaches with two different potassium channels that both interact with 14-3-3 via a mode-3 binding motif. Each approach has its own limitations: current measurements might, in principle, include possible direct effects of FC-THF on the open probability of the channels; the luminometric measurements are not strictly linearly related to the copy number of channels at the surface membrane and show a much larger scatter. Taking these limitations into account, the surface expression measurements are in

(C) Surface expression of HA-tagged TASK-3 channels measured using a luminometric assay.

(D) Surface expression of HA-tagged TASK-1 channels measured using a luminometric assay. The number of oocytes from which the data were derived is indicated in parentheses.

See also Figure S7.

reasonable agreement with the current measurements of TASK-3 channels.

Interestingly, the signal obtained after incubation with FC-THF was reduced in the $\Delta C5$ mutants of TASK-1 and TASK-3 (which do not interact with 14-3-3, Rajan et al., 2002). Because a very similar effect on current amplitude and channel surface expression was also found in TREK-1 (another K_{2P} -channel, which does not interact with 14-3-3 proteins), we think that this reduction is caused by a nonspecific side effect that is unrelated to the action of 14-3-3 proteins. It is likely that the side effect was also present in the full-length (wild-type) TASK-1 and TASK-3 channels. In other words, the “true” increase in surface expression caused by FC-THF was partially concealed by an additional unspecific effect of the drug. Thus, our measurements probably underestimate the effects of FC-THF on surface expression (and on the PPI between the channels and 14-3-3). Given that the concentration of FC-THF used (10 μ M) was near the biochemical EC_{50} , our results suggest that a substantial increase in the surface expression of the channels, by a factor of 2 or more, can be achieved by chemical stabilization of the interaction between the channel and 14-3-3 proteins in intact cells.

Our results provide a mechanistic rationale for the development of small molecules that specifically stabilize the few, but important, 14-3-3 interactions using mode 3 binding motifs, as exemplified by the case of the K^+ -channels TASK-1 and TASK-3. The critical specificity determinant of the fusicoccanes is the substitution pattern of ring C. Compatibility with binding to internal 14-3-3 binding motifs (e.g., mode 1 and mode 2) is given only when ring C is undecorated (Ottmann et al., 2009). Decoration of ring C causes steric conflicts with the main-chain constellation of residues +2 and +3 (Pro-X) of the classical mode 1 and mode 2, as well as other internal 14-3-3 binding motifs, and prevents binding to and stabilization of the corresponding 14-3-3 complexes (Figure 6). The bulky tetrahydrofuran substitution at ring C in FC-THF makes the semisynthetic compound specific for mode 3 14-3-3 binding motifs (X-pS/T-X).

SIGNIFICANCE

We present a semisynthetic stabilizer (FC-THF) of the interaction between the K^+ -channel TASK-3 (phosphorylated at Ser373) and 14-3-3 proteins. High-resolution protein crystallography revealed the atomic details of binding of FC-THF and related naturally occurring fusicoccanes to the 14-3-3/TASK-ctp complex. FC-THF displays half-maximal activity at 12 μ M and increases the apparent affinity of TASK-ctp binding to 14-3-3 about 19-fold at 100 μ M. In *Xenopus* expressing TASK-1 or TASK-3 channels, incubation with 10 μ M FC-THF caused a substantial increase in the number of channels at the cell membrane. Thus, we report a modulation of cell function by stabilization of a protein complex involved in the intracellular traffic of a membrane protein.

TASK-1 channels may play an important role in the electrical activity of the heart, in oxygen sensing by the carotid body, and in aldosterone secretion by the adrenal gland. Studies of human diseases and knockout mice have suggested that TASK-3 channels also play important roles in cognitive functions, depression, circadian rhythms, hypertension, and aldosterone secretion (Barel et al., 2008; Gua-

gliardo et al., 2012; Gotter et al., 2011). Thus, in view of numerous possible side effects, it is rather unlikely that global modulation of TASK-1 and TASK-3 channels by FC-THF or a similar substance will turn out to be a useful therapeutic approach. On the other hand, TASK-3 channels show genomic imprinting with silencing of the paternal gene (Ruf et al., 2007; Barel et al., 2008) and have been convincingly shown to play a causal role in the development of cancer (Pei et al., 2003; Mu et al., 2003) and in cellular apoptosis (Patel and Lazdunski, 2004). Thus, FC-THF, by modulating TASK-3 currents without activating any intercellular second messengers, may be a useful chemical biology tool for in vitro investigation of the mechanisms underlying the clonal expansion of cancer cells.

EXPERIMENTAL PROCEDURES

Protein Purification

Purification of His₆-tagged 14-3-3 σ protein is reported elsewhere (Schumacher et al., 2010a). The protein was dialyzed against 25 mM HEPES (pH 7.5), 100 mM NaCl, 10 mM MgCl₂, and 1 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and stored at -80° C. The phosphopeptides were synthesized by Biosyntan (Berlin) or Genscript (Piscataway, NJ, USA) and resuspended in distilled water.

Crystallization

Three 14-3-3 variants, the wild-type 14-3-3 σ , 14-3-3 σ C38V/N166H, and 14-3-3 σ C38N/N166H, were used for crystallization. For crystallization of the 14-3-3 σ /TASK-3 peptide complexes, protein and peptides were mixed at a 1:1.5 molar ratio in 25 mM HEPES (pH 7.5), 100 mM NaCl, 10 mM MgCl₂, and 1 mM TCEP and set up for crystallization in 0.1 M HEPES (pH 7.5), 0.2 M CaCl₂, 28% PEG 400, and 5% glycerol at 4 $^{\circ}$ C. Crystals grew within a week and could be directly flash-cooled in liquid nitrogen. See Supplemental Experimental Procedures for data collection, structure determination, and refinement.

Fusicoccanes Diterpenoids

Natural fusicoccanes were obtained as metabolites of *Phomopsis amygdali*. FC-THF was semisynthetically derived. Details are described in the Supplemental Information.

Fluorescence Polarization Binding Assay

Fluorescence-anisotropy-based affinity measurements were performed using a filter-based microplate reader (Infinite F500, Tecan, Männedorf, Switzerland) with a fluorescein filter set (λ_{ex} : 485 nm/20 nm, λ_{em} : 535 nm/25 nm) and an integration time of 50 μ s in black, flat-bottom 384 microwell plates (784076-25, Greiner, Kremsmuenster, Austria). Measurements were performed using 100 nM fluorescein-labeled ligand FAM-³⁷⁰RRK-pS-V³⁷⁴-COOH (TASK-3) or 20 nM FAM-²⁵²SQRQRST-pS-TPNVH²⁶⁴-COOH (C-Raf) in buffer containing 10 mM HEPES, 150 mM NaCl, 0.1% (v/v) Tween 20, and 0.1% (w/v) BSA.

First, the peptides were titrated with His-14-3-3 σ in order to obtain a K_d -value and select an appropriate concentration for the subsequent stabilization experiments. For the determination of EC_{50} -values, a solution comprising 100 nM TASK-3-peptide and 600 nM His-14-3-3 σ was titrated with the respective compound. The measured anisotropy values were normalized using FC-A as a positive control and plotted against logarithmic compound concentration. To obtain EC_{50} values, the resulting curve was fitted to a four-parameter logistic model (4PL) using GraphPad Prism 5.03 for Windows (GraphPad Software, La Jolla, CA).

Isothermal Titration Calorimetry

Experiments were carried out with a VP-ITC isothermal titration calorimeter (Microcal, Northampton, MA, USA) in buffer containing 25 mM HEPES (pH 6.5), 100 mM NaCl, 10 mM MgCl₂, and 0.5 mM TCEP at 30 $^{\circ}$ C. The 14-3-3 σ protein was dialyzed against the described buffer and stored at -80° C. In the sample cell, a solution of 60 μ M 14-3-3 protein was placed and titrated

stepwise with 8 μ l aliquots of a 1 mM solution of TASK-ctp (³⁶⁹KRRK-pS-V³⁷⁴-COOH). To measure the stabilizing potency of FC-THF, the sample cell was loaded with 25 μ M 14-3-3 protein and 100 μ M FC-THF. The concentration of the peptide was 500 μ M. Origin 7.0 software was used for data acquisition and analysis by a nonlinear least-squares routine using a single-site binding model with varying stoichiometry (N), association constant ($K_a = 1/K_d$), and molar binding enthalpy (ΔH°). The listed K_d value is the average of three independent measurements.

Compound Soaking

The ternary complexes with the different compounds were obtained via a soaking procedure. One microliter of 10 mM compound solution in ethanol was allowed to dry on a cover slide. After addition of 2 μ l reservoir solution to the dried compound, crystals of the binary 14-3-3 α /TASK-3 complex were transferred to the drop and incubated for 24 hr before flash-cooling in liquid nitrogen.

Voltage-Clamp Measurements in *Xenopus* Oocytes

Adult female African clawed frogs (*Xenopus laevis*) were anaesthetized by putting them in an induction tank with water containing 1 g/l tricaine, and stage-V oocytes were obtained from ovarian lobes. Anaesthesia and operation were carried out in accordance with the principles of German legislation with approval of the animal welfare officer of the Medical Faculty of Marburg University. The oocytes were incubated at 19°C in ND96 solution containing: 96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 5 mM HEPES (pH 7.5), supplemented with 100 μ g/ml gentamycin, 2.5 mM sodium pyruvate, and 0.5 mM theophylline. Either 24 hr or 48 hr after the operation, cRNA encoding TASK-3 (0.05 ng), TASK-1 (1.5 ng), or TREK-1c (1.0 ng), or the corresponding mutants, was injected into *Xenopus* oocytes using a nanoject II injector (Drummond Scientific, Broomall, PA, USA). Subsequently the oocytes were incubated again at 19°C for 48 hr in the ND96 solution described above. For one half of the oocytes, 10 μ M FC-THF was added to the incubation solution.

For two-microelectrode voltage-clamp experiments the cells were superfused with ND96 solution at 22°C. The voltage-clamp recordings were carried out with a Tec-10C amplifier (npi, Tamm, Germany) and an A/D converter (PC-MIO 16-XR-10, National Instruments, Austin, TX, USA), as described previously (Zuzarte et al., 2009). Current-voltage relationships were obtained with slow voltage ramps (40 mV s⁻¹) between -120 and +20 mV. To minimize the possible contribution of leakage currents, the value of steady-state current amplitude was determined at 0 mV.

Surface Expression Analysis in *Xenopus* Oocytes

The surface expression of TASK-1, TASK-3, or TREK-1 channel constructs in *Xenopus* oocytes was studied using an antibody-based chemiluminescence assay described previously (Zuzarte et al., 2009). In brief, a hemagglutinin (HA)-epitope (YPYDVPDYA), preceded and followed by a proline-glycine-glycine linker, was inserted in the extracellular P2-M4 loop of the channels. To avoid complications related to the binding of p11 (S100A10), we excised 20 amino acids in the proximal C terminus of TASK-1 (Renigunta et al., 2006). Two days after injection of cRNA, oocytes were incubated for 30 min in ND96 solution containing 1% BSA at 4°C to block nonspecific binding of antibodies. Subsequently, oocytes were incubated for 60 min at 4°C with 1 μ g/ μ l rat monoclonal anti-HA antibody (1:100 dilution; clone 3F10, Roche, Indianapolis, IN, USA) in 1% BSA/ND96, washed six times at 4°C with 1% BSA/ND96, and incubated with 2 μ g/ μ l peroxidase-conjugated affinity-purified F(ab')₂ goat anti-rat IgG antibody fragment (1:500 dilution; Dianova, Hamburg, Germany) in 1% BSA/ND96 for 60 min. Oocytes were washed thoroughly, initially in 1% BSA/ND96 (at 4°C for 30 min) and then in ND96 without BSA (at 4°C for 30 min). Individual oocytes were placed in 20 μ l SuperSignal Elisa Femto solution (Thermo Scientific, Rockford, IL, USA) and chemiluminescence was measured using a luminometer (GloMax 20/20, Promega, Madison, WI, USA).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, six tables, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.chembiol.2013.03.015>.

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