ACS chemical biology

Reviews

pubs.acs.org/acschemicalbiology

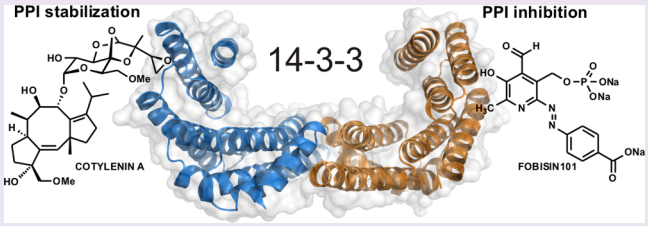
Stabilization and Inhibition of Protein−Protein Interactions: The 14-3-3 Case Study

Lech-Gustav Milroy,[†] Luc Brunsveld,[†] and Christian Ottmann*[,†,‡]

[†]Laboratory of Chemical Biology, Department of Biomedical Engineering, Technische Universiteit Eindhoven, Den Dolech, 5612 AZ Eindhoven, The Netherlands

[‡]Chemical Genomics Centre of the Max Planck Society, Otto-Hahn Straße 15, 44227 Dortmund, Germany

ABSTRACT: Small-molecule modulation of protein−protein interactions (PPIs) is one of the most exciting but also difficult fields in chemical biology and drug development. As one of the most important "hub" proteins with at least 200−300 interaction partners, the 14-3-3 proteins are an especially fruitful case for PPI intervention. Here, we summarize recent success stories in small-molecule modulation, both inhibition and stabilization, of 14-3-3 PPIs. The chemical breath of modulators includes natural products such as fusicoccin A and derivatives but also compounds identified *via* high-throughput and *in silico* screening, which has yielded a toolbox of useful inhibitors and stabilizers for this interesting class of adapter proteins. Protein−protein interactions (PPIs) are involved in almost all biological processes, with any given protein typically engaged in complexes with other proteins for the majority of its lifetime. Hence, proteins function not simply as single, isolated entities but display their roles by interacting with other cellular components. These different interaction patterns are presumably as important as the intrinsic biochemical activity status of the protein itself. The biological role of a protein is therefore decisively dependent on the underlying PPI network that furthermore can show great spatial and temporal variations. A thorough appreciation and understanding of this concept and its regulation mechanisms could help to develop new therapeutic agents and concepts.



The total number of PPIs in an organism, the "interactome", has been predicted to lie between 130,000[1] and 650,000[2] in humans, with the most recent estimation centered around 300,000.[3] Consequently, the successful addressing of PPIs with small, drug-like molecules will vastly expand our opportunities for pharmacological intervention. With up to 500 identified protein interaction partners in eukaryotic cells, the family of the so-called 14-3-3 proteins are an especially interesting case for small-molecule PPI modulation.

1. 14-3-3 PROTEINS

14-3-3 proteins are ubiquitous eukaryotic adapter proteins involved in the regulation of cell-cycle control, signal transduction, protein trafficking, and apoptosis.[4] They mediate their physiological effects by binding to other proteins, modulating their (clients') subcellular localization, enzymatic activity, or their ability to interact with further proteins.[5] 14-3-3 proteins are functional dimers with each of the monomers displaying a so-called amphipathic groove that accommodates the mostly phosphorylated interaction motifs of their partner proteins (see Figure 1).[6,7]

Among the several hundred 14-3-3 interaction partners described thus far are medicinally relevant proteins such as CRaf,[8,9] Serotonin-*N*-acetyl-transferase AANAT,[10] the transcriptional modulator YAP,[11,12] and the tumor suppressor p53.[13,14] 14-3-3 proteins have been implicated in a variety of human diseases. In addition to their participation in diverse cancers,[15,16] they have been associated with oocyte development,[17] the occurrence of neurodegenerative diseases,[18] or virulence of human pathogenic organisms.[19,20] The versatile and widespread biological functions have facilitated a growing interest in 14-3-3 proteins as a novel target class for pharmacological intervention.[15,16,21,22] This review manuscript, rather than reviewing all 14-3-3 PPIs, provides a focused analysis of the small molecule approaches taken for the modulation of 14-3-3′s. These compounds provide the entry point required both for the elucidation of the biology at hand and for pharmacological targeting of this important class of proteins.

2. STABILIZERS OF 14-3-3 PROTEIN−PROTEIN INTERACTIONS

In contrast to protein−protein inhibitors (*vide infra*), the use of protein−protein stabilizers is a vastly underexploited strategy for the modulation of protein interactions. This is arguably due to the added complexity of finding molecules capable of stabilizing a ternary complex, either *via* an allosteric mechanism through binding to only one of the partner proteins or *via*

Special Issue: Translational Chemical Biology

Received: November 4, 2012
Accepted: December 4, 2012
Published: December 4, 2012

 © 2012 American Chemical Society

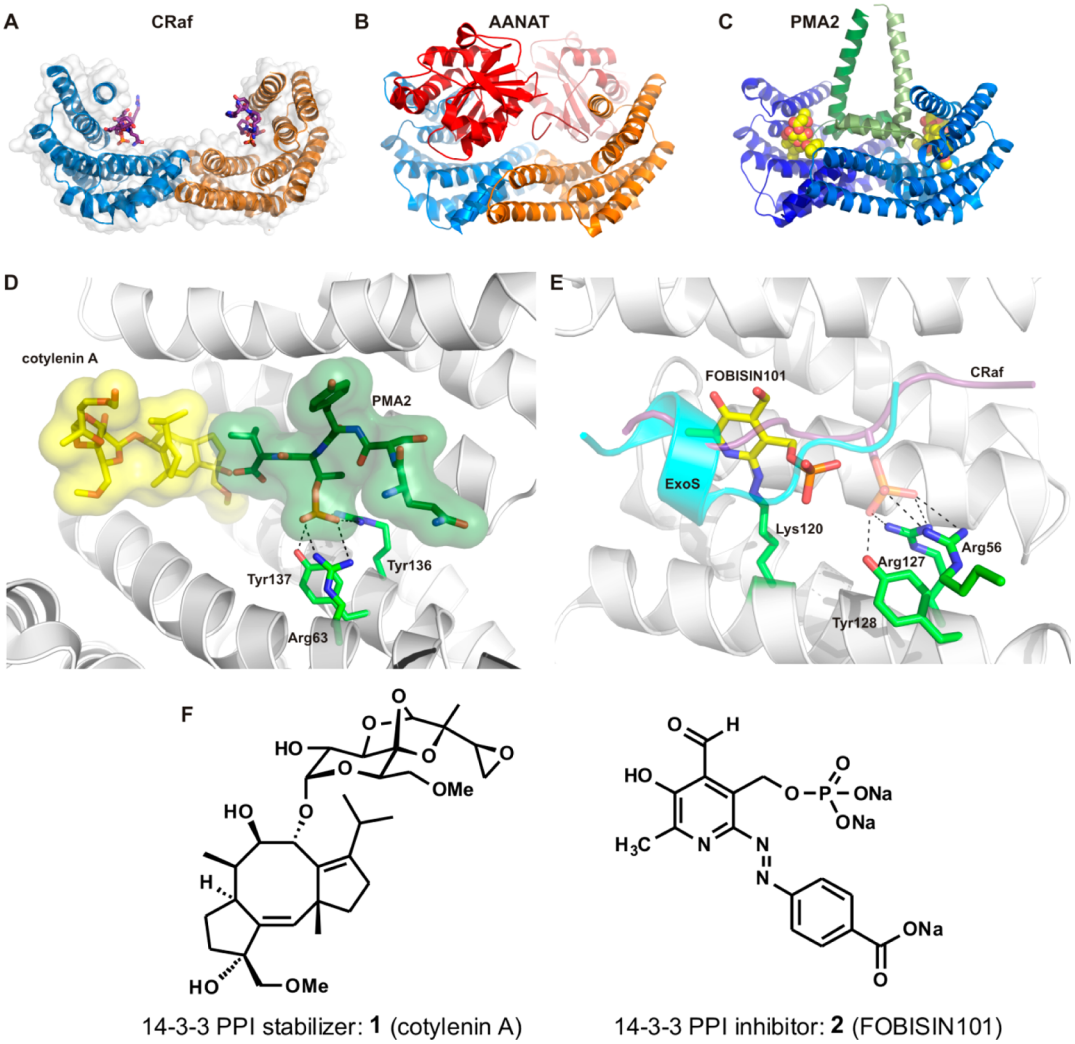


Figure 1. Small molecules acting on 14-3-3 proteins. (A) Co-crystal structure of the 14-3-3ζ dimer (blue and orange ribbon, semitransparent white surface) and the two inhibitory 14-3-3 binding sites of CRaf (purple sticks, PDB ID: 4FJ3). (B) Crystal structure of the 14-3-3ζ dimer (blue and orange ribbon) in complex with Serotonin-*N*-acetyltransferase (AANAT, light and dark red ribbon, PDB ID: 1IB1). (C) Co-crystal structure of the tobacco 14-3-3 isoforms T14-3c (light and dark blue ribbon) in complex with the regulatory C-terminus of the plant plasma membrane H^+-ATPase PMA2 (light and dark green ribbon) and fusicoccin A (yellow and red spheres, PDB ID: 2O98). (D) The 14-3-3 PPI stabilizer cotylenin A (**1**) bound to 14-3-3 (white ribbon) and the phosphorylated C-terminus of PMA2 (green sticks, PDB ID: 3E6Y). (E) The 14-3-3 PPI inhibitor FOBISIN101 (**2**) bound to 14-3-3 (white ribbon, PDB ID: 3RDH). As semitransparent ribbon the structure of Exoenzyme S (cyan, PDB ID: 2O02) and CRafpS259 (PDB ID: 4FJ3) is superimposed. (F) Chemical structures of **1** and **2**.

multiple simultaneous contact points with two (or more) partner proteins. For example, the contact amino acid residues of the interacting partners of protein interactions are themselves frequently lead structures for the design of novel protein−protein inhibitors. The same cannot be said for stabilizers, however, which is evidenced by the current lack of *de novo* protein−protein stabilizers in comparison to inhibitors. Nevertheless, nature has been a rich source of therapeutically relevant and naturally derived protein−protein stabilizers, such as the anticancer drug paclitaxel,[23] which stabilizes microtubules, and the immunosuppressants rapamycin[24] and FK506,[25] which bind to immunophilin FKBP12. In the latter case, the binary FKBP12:small molecule complex formation increases the affinity of binding to effector proteins calcineurin and mTOR. As for protein−protein inhibitors and stabilizers of other protein classes, the therapeutic payoff for discovering new 14-3-3 stabilizers is potentially very high. For example, in a number of cases 14-3-3 binding has been shown to increase the biostability and availability of numerous physiologically relevant proteins, including TASK3[26] and CFTR.[27] In the former case, the misregulation of TASK3, TWIK related-acid-sensitive K^+ channels, has been linked to cancer, inflammation, and epilepsy.[28] Small molecules that act to enhance the stability of the TASK3:14-3-3 interaction, behaving thus as the "molecular glue" that holds these two protein partners in place, are expected to increase the functional expression of TASK3 and therefore show promise as a basis for future anticancer, anti-inflammation, and anti-epilepsy treatments. The challenge of finding 14-3-3 stabilizers however remains great in that it requires the design of small molecules capable of making multiple simultaneous contact points with 14-3-3 and its partner protein. Fortunately, nature has found a way to do this job very effectively (*vide infra*) and in doing so has set very high standards for future studies in this direction.

2.1. Natural Products Fusicoccin A and Cotylenin A. *2.1.1. Isolation and Molecular Physiology.* Fusicoccin A (FC-

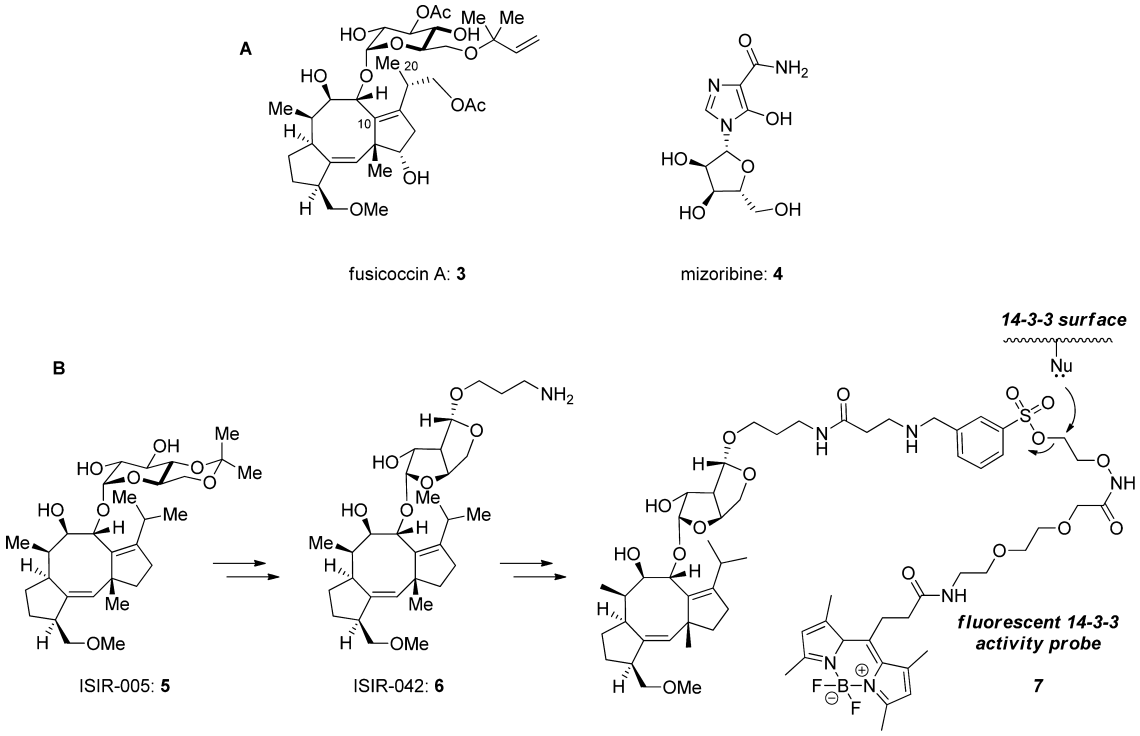


Figure 2. (A) Chemical structures of natural product stabilizers: fusicoccin A (**3**) and the imidazole nucleoside mizoribine (**4**), a naturally derived treatment for renal transplantation, lupus nephritis, and rheumatoid arthritis. (B) Synthetic analogues of fusicoccin H: ISIR-005 (**5**), ISIR-042 (**6**), and the fluorescently labeled activity probe, **7**. 'Nu' = · nucleophilic amino acid side chain residue, e.g. His or Ser.

A, Figure 2, **3**), a diterpene glycoside isolated from the phytopathogenic fungus *Phomopsis amygdali* (formerly *Fusicoccum amygdali*), was initially described in the mid-1960s to be a wilt-inducing toxin.[29] Studies on the molecular target resulted in the identification of a complex between the regulatory domain of the plasma membrane H^+-ATPase (PMA) and 14-3-3 adapter proteins as the primary receptor.[30] We have reported in two protein crystallography studies, that **3** binds to the rim of the interface of the 14-3-3/PMA complex, establishes contacts to both protein partners and increases their affinity about 90-fold.[31,32] In this regard, **3** is a "molecular glue" like the classical examples of FK506 and rapamycin.[24,25] However, in contrast to the aforementioned immunosuppressive natural products, **3** binds not to the single components of the protein complex with physiologically relevant affinity but exclusively targets the preformed binary complex.[31,32] Cotylenin A (Figure 1, **1**) was originally identified as a cytokinin-like bioactive substance against plants produced by the fungus *Cladosporium* sp. 501-7W.[33] This natural product was reported to induce differentiation in human acute myeloid leukemia in both cell culture and mouse models.[34,35]Additionally, the combination of **1** with other agents such as interferon α resulted in desirable anticancer properties.[36] The crystal structure of **1** bound to a complex of 14-3-3 with the phosphopeptide derived from the binding motif of the plant plasma membrane H^+-ATPase PMA2 (QQSYpTV-COOH) has been elucidated.[37] By comparing this structure with other 14-3-3/phosphopeptide complexes, it has been possible to explain why **1** is capable of stabilizing human, cancer-relevant 14-3-3 PPIs (in contrast to **3**).

Thus, the core fusicoccin structure provides a powerful entry point for the selective modulation of 14-3-3 signaling. Given that fusicoccin's stabilizing properties require the formation of multiple contact points with both protein partners of the ternary complex, facile access to designer fusicoccin analogues will be of high importance for the programming of selectivity in 14-3-3 ternary complex formation. There are three complementary approaches to accessing unnatural fusicoccin analogues, each with their own strengths: biosynthesis, total synthesis, and semisynthesis.

2.1.2. Fusicoccin Biosynthesis versus Total Synthesis. The isolation of **1**, **3**, and other related natural products fungii is currently the only entry to access these important compounds. In the years to come, however, an improved knowledge about the biosynthetic gene cluster, the chain of proteins responsible for the biosynthesis of these natural compounds in their respective host organisms, will ensure a sustainable supply of structurally unique analogues that would otherwise be difficult to obtain *via* any other means. Some important steps have already been made in this direction, toward the elucidation of the biosynthetic gene cluster of **1** and **3**. In 2007, Sassa and coworkers characterized the highly unusual fusicoccadiene synthase (PaFS) enzyme in the fusicoccin host fungus, *P. amygdali*, which displays combined prenyltransferase and terpene cyclase activities. PaFS is responsible for assembling the key 5-8-5 ring aglycon of the fusicoccin subclass, (+)-fusicocca-2,10-(14)-diene, from C5 isoprene units.[38] The same group has subsequently identified dioxygenases important for the installation of key oxidation motifs at the fusicoccin A and cotylenin A-type aglycon.[39] More recently, the gene encoding for a prenyltransferase, *papt*, was characterized, which is responsible for selective prenylation of the glucose unit of **3** at the 6′-position.[40] The total synthesis of fusicoccin, unrestrained in terms of the possibilities for structural diversity, will as well enable the preparation of unique fusicoccin analogues, which are otherwise beyond the reach of biosyn-

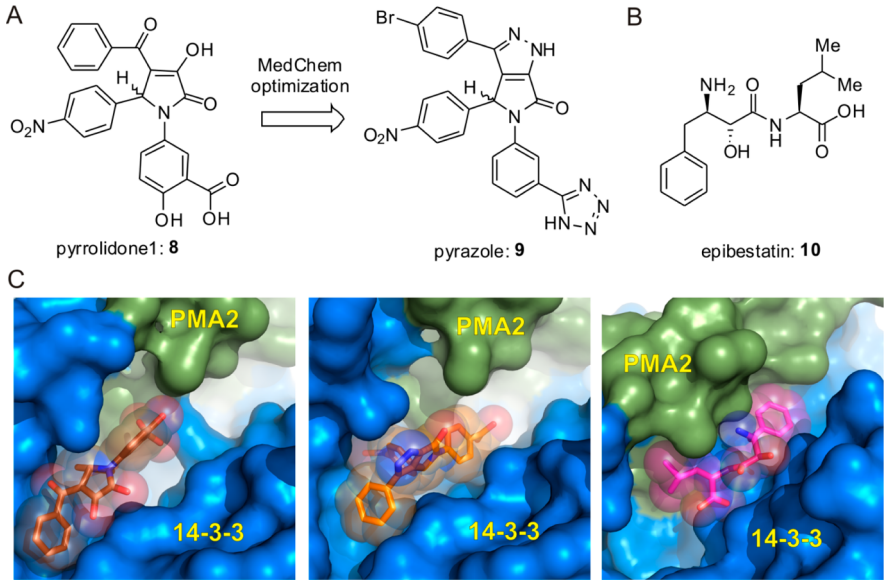


Figure 3. (A) Pyrrolidone1 (**8**), identified by high-throughput screening, and the pyrazole derivative **9** with improved 14-3-3 stabilizing properties. (B) Peptidase inhibitor epibestatin (**10**) forms a stable ternary complex with 14-3-3/PMA2. (C) Co-crystal structure of **8**, **9**, and **10** showing simultaneous contacts to both 14-3-3 and PMA2 in the binding cleft.

thesis. In this regard, biosynthesis and total synthesis can be considered complementary approaches to the synthesis of unnatural fusicoccin analogues. In anticipation of the first total syntheses of **1** or **3**, the highly instructive total syntheses of related secondary metabolites, such as albolic acid and ceroplastol II[41] and cotylenol,[42] are likely to be of benefit for the design and synthesis of related analogues.[43,44] Synthetic studies into fusicoccin's characteristic 5-8-5 hydrocarbon ring system[45−47] are sure to be helpful for the development of simplified modulators of the 14-3-3 ternary complex.

2.2. Strength of the Semisynthetic Approach: Natural Product Cotylenin A Analogues. With the biosynthesis and total synthesis approaches still yet to reach full maturity, the semisynthesis of unnatural fusicoccin analogues is currently the most reliable source of novel fusicoccine-derived chemotypes. The principle advantage of this approach is the sustainable access to gram quantities of advanced intermediates. A weakness, however, is the narrowed window for structural diversity given that much of the core structure has already been committed to a certain degree of shape and functional group complexity. Nevertheless, the semisynthesis approach has already been applied with great affect to the preparation of useful unnatural fusicoccin analogues as exemplified by the story of cotylenin A (Figure 1D and F, **1**). The limited supply of **1** from its natural sources and the presence of a metabolically labile epoxide ring have so far restricted its usefulness as a novel anticancer agent for clinical studies. Honma and co-workers[48] have cleverly circumvented these problems by using semisynthesis to prepare more stable cotylenin A derivatives starting from the more readily available fusicoccin H (Figure 2B). One of the highlights of this synthesis is the conversion of intermediate ISIR-005 (Figure 2, **5**) to ISIR-042 (**6**) where the pyranose sugar is made to undergo a base-induced rearrangement into a structurally interesting 5,5-fused furanose ring system with the simultaneous introduction of a useful amine linker group. Profiting from the efficient route to **6**, Ohkanda and co-workers subsequently reported on the development of a reactive fluorescent probe derived from ISIR-042 (Figure 2B),[49] for the phosphopeptide-dependent labeling of 14-3-3ζ proteins. Their approach incorporates a previously engineered bioorthogonal phenylsulfonylmethylene (tosylate) group, attached to **6** *via* amide coupling, to forge the reactive probe **7**. Formation of the FC-14-3-3-peptide ternary complex thus triggers covalent labeling through the entropically favored nucleophilic attack of proximal surface residues.

2.3. Mizoribine. Another naturally derived compound proposed to act as a stabilizer of a 14-3-3 protein−protein interaction is Mizoribine (Figure 2A, **4**).[50] This imidazole nucleoside, isolated from the culture media of *Eupenicillum brefaldianum* in Japan in 1974,[51] shows immunosuppressive activity[52] and has been approved in Japan for therapy after renal transplantation and for lupus nephritis and rheumatoid arthritis. In the absence of a structural basis for the interaction of **4** with 14-3-3, knowledge about its synthesis would clearly be useful for future structure−function studies.[53−56]

2.4. Non-natural 14-3-3 PPI Stabilizers. Stabilizers for 14-3-3 PPIs can be discovered *via* mechanism-based analysis of the action of natural products, as sketched above. Typically these natural compounds provide strong binding affinity and proven physiological activity. Their synthetic access is sometimes challenging, as sketched above. Therefore recently the first small-molecule PPI stabilizers were identified from high-throughput screening of a compound library.[57] Two compounds, the pyrrolidone, pyrrolidone **1** (Figure 3A, **8**), and the dipeptide epibestatin (Figure 3A, **10**), were found to stabilize the interaction of 14-3-3 proteins with the plasma membrane H^+-ATPase PMA2. The crystal structures of the ternary complexes of epibestatin and pyrrolidone1 with 14-3-3 and a 30-amino-acid fragment of PMA2 revealed that these compounds occupied different binding pockets in the rim of the protein−protein interface. The extent to which epibestatin and pyrrolidone1 contact the 14-3-3 protein and PMA2 simultaneously is different and reflects the disparities in potency of both compounds.

Very recently, a successful optimization of the 14-3-3-stabilizing activity of **8** was reported through conversion of the pyrrolidone scaffold into a more rigid pyrazole-derived ring system (**9**).[58] Based on crystallography studies, this mod-

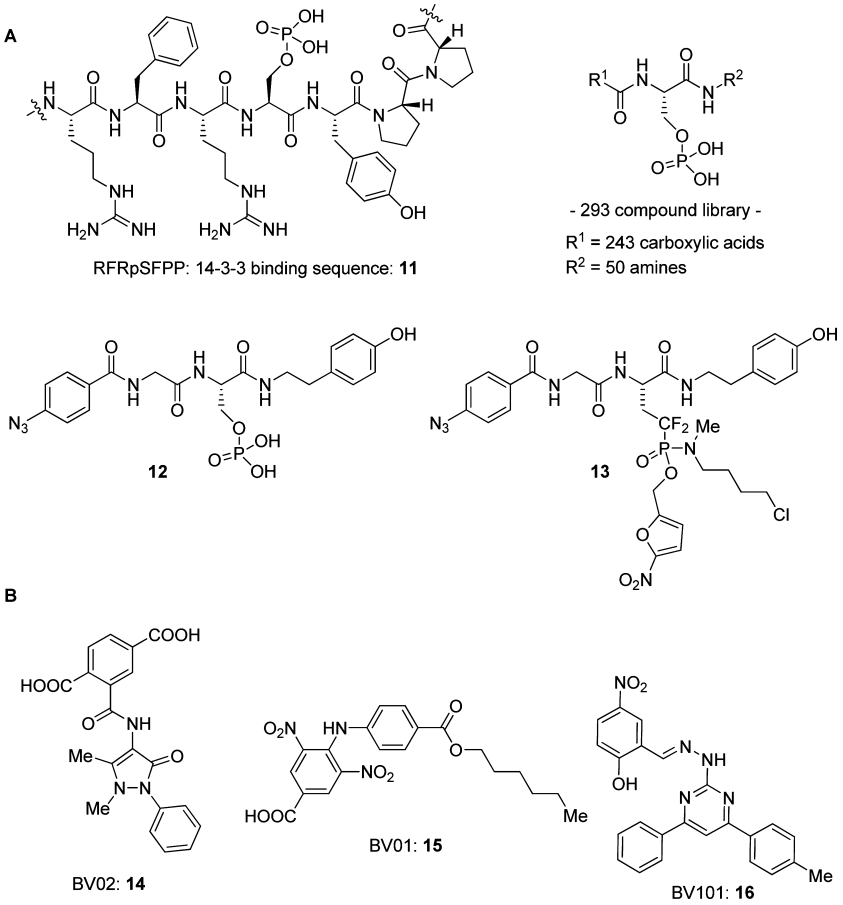


Figure 4. (A) Screening of small molecule mimetics of the 14-3-3 binding peptide sequence **11** identified phosphoserine derivative **12**; the difluoromethylenephosphoserine analogue **13** functions as a potent and cell-permeable 14-3-3 inhibitor. (B) Chemical structures of BV02 (**14**), BV01 (**15**), and BV101 (**16**), small molecule inhibitors of the c-Abl/14-3-3 interaction.

ification facilitated a deeper binding of the molecule at the rim of the interface between 14-3-3 and PMA2. The synthesis of **8** and **9** was achieved using a highly efficient one-pot Doebner condensation reaction, which also enabled the preparation of structurally diverse pyrrolidones and pyrazoles. Epibestatin (**10**) was originally developed nearly 30 years ago as a peptidase inhibitor and is derived from a bacterial metabolite, bestatin.[59] An efficient modular synthesis of epibestatin was recently reported using Sharpless asymmetric dixhydroxylation followed by a regioselective Mitsunobu stereoinversion to set the correct stereochemistry of the unusual α-hydroxy-β-amino acid segment.[60] The work presented on these two classes of molecules highlights the potential of identifying novel 14-3-3 inhibitors with unique modes of binding *via* high-throughput screening. Furthermore, due to the improved synthetic tractability of these compounds compared with the more complex fusicoccane structures, the activity of the compounds can be more readily optimized using a rational design approach guided by the co-crystal structure data and synthetic chemistry. This overall approach lays important foundations for the discovery of novel 14-3-3 stabilizers as 14-3-3 tools and future drug molecules.

3. INHIBITORS OF 14-3-3 PROTEIN–PROTEIN INTERACTIONS

The discovery of inhibitors of 14-3-3-mediated protein–protein interactions is in principle more straighforward than for 14-3-3 stabilizers, as the phosphorylated peptide sequence of the protein binding partner provides a useful anchor point for *de novo* design. As for 14-3-3 stabilizers, the therapeutic benefits of inhibiting the 14-3-3-mediated ternary complex are potentially significant, given the role of 14-3-3 as an adapter molecule for a range of disease-relevant proteins like pro-apoptotic BAD[61] or the FOXO transcription factors[62] both important in, *e.g.*, cancer biology. Thus the development of small molecule 14-3-3 inhibitors is essential to enable the elucidation of the mechanistic and biological significance of such interactions for cancer and other diseases.

3.1. Phage Peptide Inhibitor R18 and Dimeric Difopein. The first inhibiting agent of 14-3-3 protein–protein interactions was the 20-amino-acid peptide R18 (PHCVPRDLSWLDLEANMCLP) identified from a phage display by the Fu laboratory.[63] This peptide comprised the central sequence WLDLE that was shown in the 14-3-3 complex crystal structure to be coordinated by the highly conserved phospho-accepting pocket within the amphipathic groove of 14-3-3.[64] Later, it has been shown that expression of a 64-amino-acid peptide comprising a dimeric form of this 14-3-3 inhibitor peptide, so-called Difopein, resulted in apoptosis, sensitized cancer cells for the anti-neoplastic drug cisplatin, and suppressed tumor growth in mice.[65,66] Although certainly not a small molecule, R18 proved the principal feasibility of inhibiting 14-3-3 PPIs.

3.2. Cell-Permeable Phosphoserine Mimetics. As the next important step, the Yao group reported in 2010 a small-molecule microarray (SMM) technique for the identification of novel 14-3-3 inhibitors (Figure 4A).[67] Based on the amino-acid

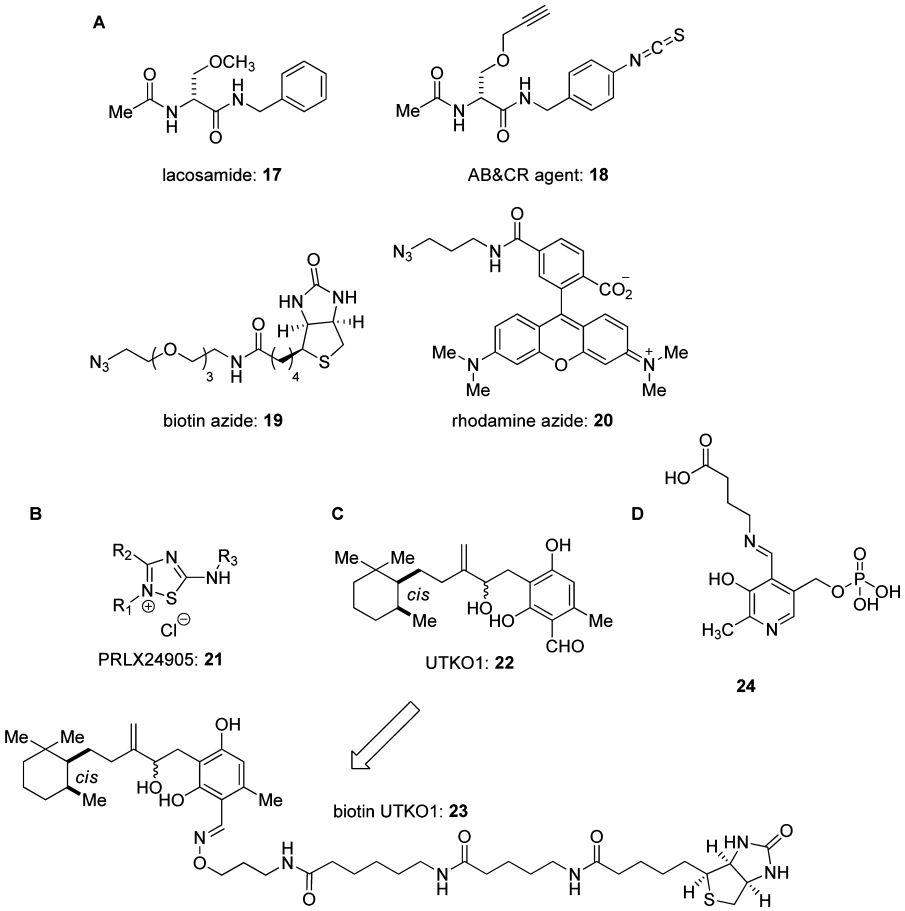


Figure 5. (A) Anti-epileptic drug lacosamide (**17**) is known to function as a 14-3-3 inhibitor on the basis of affinity probe target identification studies using the Affinity Bait and Chemical Reporter (AB&CR) agent (**18**), which enables functionalization with biotin (**19**) and a rhodamine dye (**20**). (B) General structure of potential small molecule inhibitor of the HSP20/14-3-3 interaction, **21**. (C) UTKO1 (**22**), selective inhibitor of the 14-3-3/RacGEF Tiam interaction, and biotinylated UTKO1 pull-down probe, **23**. (D) Pyridoxal-phosphate derivatives as 14-3-3 inhibitors: **24** (see also Figure 1D and F, **1**).

sequence RFRpSYPP (**11**), which is optimal for 14-3-3 binding, a library of small-molecule/peptide hybrids was synthesized in which either 50 diverse amines were coupled to the N-terminal peptide (RFRpS) or 243 diverse acid building blocks were introduced to the C-terminal peptide (pSYSPP). The resulting 293 single hybrids were spotted on a glass slide, and binding of fluorescently labeled GST-14-3-3 (isoform sigma) was measured. Five compounds, including **12** (Figure 4A), showed robust 14-3-3 binding, and the subsequent titration revealed a K_d of 0.25 μM for the control peptide (Biotin-GG-RLSH-pSLPG) and dissociation constants for the five "hit" compounds between 0.6 and 1.03 μM. In an orthogonal, fluorescence polarization (FP) competition assay, the entire compound library was again tested for 14-3-3 binding and the five "hit" compounds from the SMM were verified, yielding IC_{50} values comparable to the microarray-measured K_d values. Of these five compounds, three were structurally diverse in the N-terminal peptide region (Figure 4A, R^1) and two at the C-terminal peptide region (Figure 4A, R^2). The six possible combinations of these fragments were then tested in the FP competition assay giving rise to molecules with IC_{50} values between 2.6 and 3.6 μM. Very recently, a phosphoserine mimetic prodrug (Figure 4A, **13**), inspired by the structure of **12**, was reported to show potent 14-3-3 inhibitory activity in cells.[68]

3.3. BV01, BV02, and BV101. Botta's group reported the identification of a small-molecule inhibitor of the c-Abl/14-3-3 interaction by employing structure-based pharmacophore modeling, virtual screening, and molecular docking simulations (Figure 4B). They started with roughly 200,000 compounds, of which finally 14 compounds were tested in cellular and biochemical assays, resulting in the identification of one lead structure, BV02 (**14**).[69] However, in part motivated by the chemical instability of **14** under ambient conditions, the same group performed further computational studies that uncovered two additional promising "hit"compounds: dinitrobenzoic acid derivative BV01 (**15**) and aminopyrimidine BV101 (**16**). The 14-3-3-inhibiting properties of these two compounds were confirmed by NMR and biochemical methods.[70]

3.4. Lacosamide. Recently, the groups of Kohn and Liu reported 14-3-3ζ as a target protein for lacosamide (Figure 5A, **17**), an anti-epilectic drug that has been introduced in the USA and Europe for the adjunctive treatment of partial-onset seizures in adults.[71] The authors showed that effective interaction of lacosamide with 14-3-3 depended on the presence of xanthine. Employing mass spectrometry, K120 of 14-3-3ζ was identified to be covalently bound by the isothiocyanate group of (**18**), which was used as an affinity bait unit for target identification through attachment of biotin label **19** and rhodamine dye molecule **20**. The lysine at position 120 is proximal to the phospho-motif binding channel of 14-3-

3, making it plausible that lacosamide binding could directly modulate the interaction with a 14-3-3 partner protein.

3.5. HSP20/14-3-3. By screening a 58,019-compound library, researchers at Prolexys Pharmaceuticals and the Johns Hopkins Bloomberg School of Public Health identified 268 compounds that decreased the interaction of a phosphorylated heat shock protein 20 (HSP20) peptide with 14-3-3.[72] A small subset of these hits belonging to the scaffold PRLX24905 (Figure 5B, **21**) caused relaxation of isolated human airways smooth muscle (ASM) cells *in vitro* and attenuated active force development of intact tissue *ex vivo*. Although no direct binding to 14-3-3 has been shown for these compounds, the assay setup based on fluorescence polarization (FP) of a FAM-labeled 8-mer phosphopeptide derived from HSP20 to full-length 14-3-3γ (247 amino acids) strongly indicates that the 14-3-3 protein rather than the peptide is the primary binding partner for the PPI inhibitor.

3.6. Moverastin Analogue, UTKO1. In 2006, the Imoto group reported a natural product, moverastin, as an inhibitor of cancer cell migration with farnesyl transferases (FTases) identified as molecular targets.[73] Later, a moverastin derivative, UTKO1 (Figure 5C, **22**), with enhanced potency in migration inhibition was described.[74] Interestingly, this molecule showed no FTase inhibition. Instead, it was demonstrated by coprecipitation studies employing biotin-labeled UTKO1 (*e.g.*, **23** *via* oxime ligation of the corresponding biotinylated hydroxylamine linker group) that 14-3-3 proteins are the direct targets of this molecule. This result was confirmed by pull down assays with recombinant glutathione-*S*-transferase (GST)-14-3-3 fusions, and its physiological relevance was supported by siRNA experiments downregulating 14-3-3. Concerning the issue of which of the many 14-3-3 PPIs is relevant in the context of cell migration, the authors provided experimental evidence that UTKO1 probably acts by disrupting the interaction of 14-3-3 with the RacGEF Tiam.

3.7. Phosphonate-Type Inhibitors. In 2011, the first covalent 14-3-3 inhibitor was reported.[75] Fu's group identified from the LOPAC library a pyridoxal-phosphate derivative (Figure 1E and F, **2**) that they named FOBISIN101 (FOurteen-three-three BInding Small molecule INhibitor). This molecule inhibits binding of phosphorylated as well as unphosphorylated 14-3-3 partners by covalently attaching to Lys120 in the proximity of the highly conserved phosphoserine/threonine-accepting pocket in the amphipathic groove of 14-3-3. This unexpected mode of action was revealed by protein crystallography and the inhibitory potency was demonstrated by GST-14-3-3 affinity chromatography employing COS-7 cell lysates. In the presence of FOBISIN101, interaction of 14-3-3 with C-Raf or PRAS40 was dose-dependently inhibited. Furthermore, in a functional assay, the molecule also inhibited the phosphorylation-independent activation of ExoS by 14-3-3 proteins leading to an efficient impairment of ADP-ribosylation of soybean trypsin inhibitor (SBTI). Very recently, pyridoxal derivative **24** (Figure 5D) was identified as a 14-3-3 inhibitor, by means of virtual screening.[76] The crystal structure of **24** complexed to 14-3-3σ was solved to a resolution of 1.8 Å and was found to covalently bind 14-3-3 in a similar way to FOBISIN101 (Figure 1D and F) *via* a lysine residue located nearby to the phospho-accepting pocket.

4. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In recent years the 14-3-3s have been recognized as an important target class of proteins in chemical biology. Triggered by the elucidation of the mode of action of the natural products fusicoccin A and cotylenin A, specific small-molecule stabilization of distinct 14-3-3 PPIs was approached successfully with either semisynthetic derivatives or with new molecules identified from high-throughput screening. Likewise, the first small-molecule inhibitors have been identified and were shown to be useful molecules displaying physiological activity. Especially in the case of the 14-3-3 PPI stabilizers it can be envisioned that this kind of molecule could lead to the development of new and specific therapeutic agents for a variety of indications. Here, specificity is mediated by the compound's simultaneous binding to the highly variable rim of the PPI interface, opening up the unique opportunity for targeted manipulation of the extensive 14-3-3 interactome. In the case of inhibitors of 14-3-3 PPIs introduction of specifity will be more challenging; however, different approaches such as addressing the 14-3-3 surface outside the conserved phospho-accepting pocket might, for example, be a promising strategy. In conclusion and considering the recent advances, it can be expected that in the very near future additional molecules will soon be published both in the field of stabilization and inhibition of 14-3-3 PPIs, furthering this exciting field of chemical biology.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: c.ottmann@tue.nl.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Venkatesan, K., Rual, J. F., Vazquez, A., Stelzl, U., Lemmens, I., Hirozane-Kishikawa, T., Hao, T., Zenkner, M., Xin, X., Goh, K. I., Yildirim, M. A., Simonis, N., Heinzmann, K., Gebreab, F., Sahalie, J. M., Cevik, S., Simon, C., de Smet, A. S., Dann, E., Smolyar, A., Vinayagam, A., Yu, H., Szeto, D., Borick, H., Dricot, A., Klitgord, N., Murray, R. R., Lin, C., Lalowski, M., Timm, J., Rau, K., Boone, C., Braun, P., Cusick, M. E., Roth, F. P., Hill, D. E., Tavernier, J., Wanker, E. E., Barabási, A. L., and Vidal, M. (2009) An empirical framework for binary interactome mapping. *Nat. Methods 6*, 83−90.

(2) Stumpf, M. P., Thorne, T., de Silva, E., Stewart, R., An, H. J., Lappe, M., and Wiuf, C. (2008) Estimating the size of the human interactome. *Proc. Natl. Acad. Sci. U.S.A. 105*, 6959−6964.

(3) Zhang, Q. C., Petrey, D., Deng, L., Qiang, L., Shi, Y., Thu, C. A., Bisikirska, B., Lefebvre, C., Accili, D., Hunter, T., Maniatis, T., Califano, A., and Honig, B. (2012) Structure-based prediction of protein-protein interactions on a genome-wide scale. *Nature 490*, 556−560.

(4) Hermeking, H., and Benzinger, A. (2006) 14-3-3 proteins in cell cycle regulation. *Semin. Cancer Biol. 16*, 183−192.

(5) Aitken, A. (2006) 14-3-3 proteins: a historic overview. *Semin. Cancer Biol. 16*, 162−172.

(6) Yaffe, M. B., Rittinger, K., Volinia, S., Caron, P. R., Aitken, A., Leffers, H., Gamblin, S. J., Smerdon, S. J., and Cantley, L. C. (1997) The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell 91*, 961−971.

(7) Conklin, D. S., Galaktinov, K., and Beach, D. (1995) 14-3-3 proteins associate with cdc25 phosphatases. *Proc. Natl. Acad. Sci. U.S.A. 92*, 7892−7896.

(8) Freed, E., Symons, M., Macdonald, S., McCormick, F., and Ruggieri, R. (1994) Binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation. *Science 265*, 1713−1716.
(9) Molzan, M., Schumacher, B., Ottmann, C., Baljuls, A., Polzien, L., Weyand, M., Thiel, P., Rose, R., Rose, M., Kuhenne, P., Kaiser, M., Rapp, U. R., Kuhlmann, J., and Ottmann, C. (2010) Impaired binding of 14-3-3 to C-RAF in Noonan syndrome suggests new approaches in diseases with increased Ras signaling. *Mol. Cell. Biol. 30*, 4698−4711.
(10) Obsil, T., Ghirlando, R., Klein, D. C., Ganguly, S., and Dyda, F. (2001) Crystal structure of the 14-3-3zeta:serotonin N-acetyltransferase complex. a role for scaffolding in enzyme regulation. *Cell 105*, 257−67.
(11) Vassilev, A., Kaneko, K. J., Shu, H., Zhao, Y., and DePamphilis, M. L. (2001) TEAD/TEF transcription factors utilize the activation domain of YAP65, a Src/Yes-associated protein localized in the cytoplasm. *Genes Dev. 15*, 1229−1241.
(12) Schumacher, B., Skwarczynska, M., Rose, R., and Ottmann, C. (2010) Structure of a 14-3-3σ-YAP phosphopeptide complex at 1.15 A resolution. *Acta Crystallogr., Sect. F 66*, 978−984.
(13) Rajagopalan, S., Sade, R. S., Townsley, F. M., and Fersht, A. R. (2010) Mechanistic differences in the transcriptional activation of p53 by 14-3-3 isoforms. *Nucleic Acids Res. 238*, 893−906.
(14) Schumacher, B., Mondry, J., Thiel, P., Weyand, M., and Ottmann, C. (2010) Structure of the p53 C-terminus bound to 14-3-3: implications for stabilization of the p53 tetramer. *FEBS Lett. 584*, 1443−1448.
(15) Hermeking, H. (2003) The 14-3-3 cancer connection. *Nat. Rev. Cancer 3*, 931−943.
(16) Molzan, M., Weyand, M., Rose, R., and Ottmann, C. (2012) Structural insights of the MLF1/14-3-3 interaction. *FEBS J. 279*, 563−571.
(17) Rose, R., Rose, M., and Ottmann, C. (2012) Identification and structural characterization of two 14-3-3 binding sites in the human peptidylarginine deiminase type VI. *J. Struct. Biol. 180*, 65−72.
(18) Berg, D., Holzmann, C., and Riess, O. (2003) 14-3-3 proteins in the nervous system. *Nat. Rev. Neurosci. 9*, 752−762.
(19) Fu, H., Coburn, J., and Collier, R. J. (1993) The eukaryotic host factor that activates exoenzyme S of Pseudomonas aeruginosa is a member of the 14-3-3 protein family. *Proc. Natl. Acad. Sci. U.S.A. 90*, 2320−2324.
(20) Ottmann, C., Yasmin, L., Weyand, M., Veesenmeyer, J. L., Diaz, M. H., Palmer, R. H., Francis, M. S., Hauser, A. R., Wittinghofer, A., and Hallberg, B. (2008) Phosphorylation-independent interaction between 14-3-3 and exoenzyme S: from structure to pathogenesis. *EMBO J. 26*, 902−913.
(21) Morrison, D. K. (2009) The 14-3-3 proteins: integrators of diverse signaling cues that impact cell fate and cancer development. *Trends Cell Biol. 19*, 16−23.
(22) Andrews, R. K., Du, X., and Berndt, M. C. (2007) The 14-3-3zeta-GPIb-IX-V complex as an antiplatelet target. *Drug News Perspect. 20*, 285−292.
(23) Wani, M. C., Taylor, H. L., Wall, M. E., Coggon, P., and A. T. McPhail, A. T. (1971) Plant antitumor agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. *J. Am. Chem. Soc. 93*, 2325−7232.
(24) Brown, E. J., Albers, M. W., Shin, T. B., Ichikawa, K., Keith, C. T., Lane, W. S., and Schreiber, S. L. (1994) A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature 369*, 756−758.
(25) Liu, J., Farmer, J. D., Lane, W. S., Friedman, J., Weissman, I., and Schreiber, S. L. (1991) Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell 66*, 807−815.
(26) Rajan, S., Preisig-Müller, R., Wischmeyer, E., Nehring, R., Hanley, P. J., Renigunta, V., Musset, B., Schlichthörl, G., Derst, C., Karschin, A., and Daut, J. (2002) Interaction with 14-3-3 proteins promotes functional expression of the potassium channels TASK-1 and TASK-3. *J. Physiol. 545* (Pt 1), 13−26.
(27) Liang, X., Da Paula, A. C., Bozóky, Z., Zhang, H., Bertrand, C. A., Peters, K. W., Forman-Kay, J. D., and Frizzell, R. A. (2012) Phosphorylation-dependent 14-3-3 protein interactions regulate CFTR biogenesis. *Mol. Biol. Cell 23*, 996−1009.
(28) Bittner, S., Budde, T., Wiendl, H., and Meuth, S. G. (2010) From the background to the spotlight: TASK channels in pathological conditions. *Brain Pathol. 20*, 999−1009.
(29) Ballio, A., Chain, E. B., De Leo, P., Erlanger, B. F., Mauri, M., and Tonolo, M. A. (1964) Fusicoccin: a new wilting toxin produced by Fusicoccum amygdali del. *Nature 203*, 297.
(30) Oecking, C., Eckerskorn, C., and Weiler, E. W. (1994) The fusicoccin receptor of plants is a member of the 14-3-3 superfamily of eukaryotic regulatory proteins. *FEBS Lett. 352*, 163−166.
(31) Würtele, M., Jelich-Ottmann, C., Wittinghofer, A., and Oecking, C. (2003) Structural view of a fungal toxin acting on a 14-3-3 regulatory complex. *EMBO J. 22*, 987−994.
(32) Ottmann, C., Marco, S., Jaspert, N., Marcon, C., Schauer, N., Weyand, M., Vandermeeren, C., Duby, G., Boutry, M., Wittinghofer, A., Rigaud, J. L., and Oecking, C. (2007) Structure of a 14-3-3 coordinated hexamer of the plant plasma membrane H+ -ATPase by combining X-ray crystallography and electron cryomicroscopy. *Mol. Cell 25*, 427−440.
(33) Sassa, T., Tojyo, T., and Munakata, K. (1970) Isolation of a new plant growth substance with cytokinin-like activity. *Nature 227*, 379−381.
(34) Yamada, K., Honma, Y., Asahi, K. I., Sassa, T., Hino, K. I., and Tomoyasu, S. (2001) Differentiation of human acute myeloid leukaemia cells in primary culture in response to cotylenin A, a plant growth regulator. *Br. J. Hamaetol. 114*, 814−821.
(35) Honma, Y., Ishii, Y., Sassa, T., and Asahi, K. (2003) Treatment of human promyelocytic leukemia in the SCID mouse model with cotylenin A, an inducer of myelomonocytic differentiation of leukemia cells. *Leuk. Res. 27*, 1019−1025.
(36) Honma, Y., Ishii, Y., Yamamoto-Yamaguchi, Y., Sassa, T., and Asahi, K. (2003) Cotylenin A, a differentiation-inducing agent, and IFN-alpha cooperatively induce apoptosis and have an antitumor effect on human non-small cell lung carcinoma cells in nude mice. *Cancer Res. 63*, 3659−3666.
(37) Ottmann, C., Weyand, M., Sassa, T., Inoue, T., Kato, N., Wittinghofer, A., and Oecking, C. (2009) A structural rationale for selective stabilization of anti-tumor interactions of 14-3-3 proteins by cotylenin A. *J. Mol. Biol. 386*, 913−919.
(38) Toyomasu, T., Tsukahara, M., Kaneko, A., Niida, R., Mitsuhashi, W., Dairi, T., Kato, N., and Sassa, T. (2007) Fusicoccins are biosynthesized by an unusual chimera diterpene synthase in fungi. *Proc. Natl. Acad. Sci. U.S.A. 104*, 3084−3088.
(39) Ono, Y., Minami, A., Noike, M., Higuchi, Y., Toyomasu, T., Sassa, T., Kato, N., and Dairi, T. (2011) Dioxygenases, key enzymes to determine the aglycon structures of fusicoccin and brassicicene, diterpene compounds produced by fungi. *J. Am. Chem. Soc. 133*, 2548−2555.
(40) Noike, M., Liu, C., Ono, Y., Hamano, Y., Toyomasu, T., Sassa, T., Kato, N., and Dairi, T. (2012) An enzyme catalyzing O-prenylation of the glucose moiety of fusicoccin A, a diterpene glucoside produced by the fungus Phomopsis amygdale. *ChemBioChem 13*, 566−573.
(41) Kato, N., Kataoka, H., Ohnuchi, S., Tanaka, S., and Takeshita, H. (1988) Total synthesis of albolic acid and ceroplastol II, 5−8−5-membered tricyclic insect sesterterpenoids, via a lactol-regulated silyloxy−Cope rearrangement. *J. Chem. Soc., Chem. Commun. 5*, 354−356.
(42) Kato, N., Okamoto, H., and Takeshita, H. (1996) Total synthesis of optically active cotylenol, a fungal metabolite having a leaf growth activity. Intramolecular ene reaction for an eight-membered ring formation. *Tetrahedron 52*, 3921.
(43) Li, F., Kato, N., Mori, A., Takeshita, H., and Sassa, T. (1997) Synthesis of 9-deoxycotylenol derivatives and their seed germination-stimulating activity. *Chem. Lett. 12*, 1207−1208.
(44) Song, D., McDonald, R., and West, F. (2006) Diastereoselective [4 + 4]-photocycloaddition reactions of pyran-2-ones: rapid access to functionalized 5−8-5 skeletons. *Org. Lett. 8*, 4075−4078.

(45) Molander, G., and Harris, C. (1995) Sequenced reactions with samarium(ii) iodide. Tandem intramolecular nucleophilic acyl substitution/intramolecular Barbier cyclizations. *J. Am. Chem. Soc. 117*, 3705−3716.
(46) Williams, D. R., Robinson, L. A., Nevill, C. R., and Reddy, J. P. (2007) Strategies for the synthesis of fusicoccanes by Nazarov reactions of dolabelladienones: total synthesis of (+)-fusicoauritone. *Angew. Chem., Int. Ed. 46*, 915−918.
(47) Paquette, L., Sun, L., Friedrich, D., and Savage, P. (1997) Total synthesis of (+)-epoxydictymene. Application of alkoxy-directed cyclization to diterpenoid construction. *J. Am. Chem. Soc. 119*, 8438−8450.
(48) Kawakami, K., Hattori, M., Inoue, T., Maruyama, Y., Ohkanda, J., Kato, N., Tongu, M., Yamada, T., Akimoto, M., Takenaga, K., Sassa, T., Suzumiya, J., and Honma, Y. (2012) A novel fusicoccin derivative preferentially targets hypoxic tumor cells and inhibits tumor growth in xenografts. *Anti-Cancer Agents Med. Chem. 12*, 791−800.
(49) Takahashi, M., Kawamura, A., Kato, N., Nishi, T., Hamachi, I., and Ohkanda, J. (2012) Phosphopeptide-dependent labeling of 14-3-3 ζ proteins by fusicoccin-based fluorescent probes. *Angew. Chem., Int. Ed. 51*, 509−512.
(50) Takahashi, S., Wakui, H., Gustafsson, J. A., Zilliacus, J., and Itoh, H. (2000) Functional interaction of the immunosuppressant mizoribine with the 14-3-3 protein. *Biochem. Biophys. Res. Commun. 274*, 87−92.
(51) Mizuno, K., Tsujino, M., Takada, M., Hayashi, M., and Atsumi, K. (1974) Studies on bredinin. I. Isolation, characterization and biological properties. *J. Antibiot. 27*, 775−782.
(52) Yoshioka, H., Nakatsu, K., Hayashi, M., and Mizuno, K. (1975) Studies on bredinin. II The molecular structure of bredinin. *Tetrahedron Lett. 46*, 4031−4033.
(53) Hayashi, M., Hirano, T., Yaso, M., Mizuno, K., and Ueda, T. (1975) Studies on bredinin III. Chemical synthesis of bredinin. *Chem. Pharm. Bull. 23*, 245−246.
(54) Shuto, S., Haramuishi, K., Fukuoka, M., and Matsuda, A. (2000) Synthesis of sugar-modified analogs of bredinin (mizoribine), a clinically useful immunosuppressant, by a novel photochemical imidazole ring-cleavage reaction as the key step. *J. Chem. Soc., Perkin Trans. 1*, 3603−3609.
(55) Franchetti, P., Pasqualini, M., Cappellacci, L., Petrelli, R., Vita, P., Grifantini, M., and Jayaram, H. N. (2005) Ribose-modified mizoribine analogues: synthesis and biological evaluation. *Nucleosides Nucleotides Nucleic Acids 24*, 2023−2027.
(56) Humble, R. W., Middleton, D. F., Banoub, J., Ewing, D. F., Boa, A. N., and Mackenzie, G. (2011) A synthesis of bredinin (Mizoribine®) from an acyclic precursor. *Tetrahedron Lett. 52*, 6223−6227.
(57) Rose, R., Erdmann, S., Bovens, S., Wolf, A., Rose, M., Hennig, S., Waldmann, H., and Ottmann, C. (2010) Identification and structure of small-molecule stabilizers of 14-3-3 protein-protein interactions. *Angew. Chem., Int. Ed. 49*, 4129−4132.
(58) Richter, A., Rose, R., Hedberg, C., Waldmann, H., and Ottmann, C. (2012) An optimized small-molecule stabiliser of the 14-3-3-PMA2 protein-protein interaction. *Chemistry 18*, 6520−6527.
(59) Umezawa, H., Aoyagi, T., Suda, H., Hamada, M., and Takeuchi, T. (1976) Bestatin, an inhibitor of aminopeptidase B, produced by actinomycetes. *J. Antibiot. 29*, 97−99.
(60) Richter, A., and Hedberg, C. (2010) A practical and convenient synthesis of the protease inhibitor epibestatin. *Synthesis 12*, 2039−2042.
(61) Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell 87*, 619−628.
(62) Van Der Heide, L. P., Hoekman, M. F., and Smidt, M. P. (2004) The ins and outs of FoxO shuttling: mechanisms of FoxO translocation and transcriptional regulation. *Biochem. J. 380* (Pt 2), 297−309.
(63) Wang, B., Yang, H., Liu, Y. C., Jelinek, T., Zhang, L., Ruoslahti, E., and Fu, H. (1999) Isolation of high-affinity peptide antagonists of 14-3-3 proteins by phage display. *Biochemistry 38*, 12499−12504.
(64) Petosa, C., Masters, S. C., Bankston, L. A., Pohl, J., Wang, B., Fu, H., and Liddington, R. (1998) 14-3-3zeta binds a phosphorylated Raf peptide and an unphosphorylated peptide via its conserved amphipathic groove. *J. Biol. Chem. 273*, 16305−16310.
(65) Masters, S. C., and Fu, H. (2001) 14-3-3 proteins mediate an essential anti-apoptotic signal. *J. Biol. Chem. 276*, 45193−45200.
(66) Cao, W., Yang, X., Zhou, J., Teng, Z., Cao, L., Zhang, X., and Fei, Z. (2010) Targeting 14-3-3 protein, difopein induces apoptosis of human glioma cells and suppresses tumor growth in mice. *Apoptosis 15*, 230−241.
(67) Wu, H., Ge, J., and Yao, S. Q. (2010) Microarray-assisted high-throughput identification of a cell-permeable small-molecule binder of 14-3-3 proteins. *Angew. Chem., Int. Ed. 49*, 6528−6532.
(68) Arrendale, A., Kim, K., Choi, J. Y., Li, W., Geahlen, R. L., and Borch, R. F. (2012) Synthesis of a phosphoserine mimetic prodrug with potent 14-3-3 protein inhibitory activity. *Chem. Biol. 19*, 764−771.
(69) Corradi, V., Mancini, M., Manetti, F., Petta, S., Santucci, M. A., and Botta, M. (2010) Identification of the first non-peptidic small molecule inhibitor of the c-Abl/14-3-3 protein-protein interactions able to drive sensitive and Imatinib-resistant leukemia cells to apoptosis. *Bioorg. Med. Chem. Lett. 20*, 6133−6137.
(70) Corradi, V., Mancini, M., Santucci, M. A., Carlomagno, T., Sanfelice, D., Mori, M., Vignaroli, G., Falchi, F., Manetti, F., Radi, M., and Botta, M. (2011) Computational techniques are valuable tools for the discovery of protein-protein interaction inhibitors: the 14-3-3σ case. *Bioorg. Med. Chem. Lett. 21*, 6867−6871.
(71) Park, K. D., Kim, D., Reamtong, O., Eyers, C., Gaskell, S. J., Liu, R., and Kohn, H. (2011) Identification of a lacosamide binding protein using an affinity bait and chemical reporter strategy: 14-3-3ζ. *J. Am. Chem. Soc. 133*, 11320−11330.
(72) An, S. S., Askovich, P. S., Zarembinski, T. I., Ahn, K., Peltier, J. M., von Rechenberg, M., Sahasrabudhe, S., and Fredberg, J. (2011) A novel small molecule target in human airway smooth muscle for potential treatment of obstructive lung diseases: a staged high-throughput biophysical screening. *J. Respir. Res. 13*, 12−18.
(73) Takemoto, Y., Watanabe, H., Uchida, K., Matsumura, K., Nakae, K., Tashiro, E., Shindo, K., Kitahara, T., and Imoto, M. (2005) Chemistry and biology of moverastins, inhibitors of cancer cell migration, produced by Aspergillus. *Chem. Biol. 12*, 1337−1347.
(74) Kobayashi, H., Ogura, Y., Sawada, M., Nakayama, R., Takano, K., Minato, Y., Takemoto, Y., Tashiro, E., Watanabe, H., and Imoto, M. (2011) Involvement of 14-3-3 proteins in the second epidermal growth factor-induced wave of Rac1 activation in the process of cell migration. *J. Biol. Chem. 286*, 39259−39268.
(75) Zhao, J., Du, Y., Horton, J. R., Upadhyay, A. K., Lou, B., Bai, Y., Zhang, X., Du, L., Li, M., Wang, B., Zhang, L., Barbieri, J. T., Khuri, F. R., Cheng, X., and Fu, H. (2011) Discovery and structural characterization of a small molecule 14-3-3 protein-protein interaction inhibitor. *Proc. Natl. Acad. Sci. U.S.A. 108*, 16212−16226.
(76) Röglin, L., Thiel, P., Kohlbacher, O., and Ottmann, C. (2012) Covalent attachment of pyridoxal-phosphate derivatives to 14-3-3 proteins. *Proc. Natl. Acad. Sci. U.S.A. 109*, E1051−1053.