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Intracellular Generation of a Diterpene-Peptide Conjugate that Inhibits 14-3-3-Mediated Interactions

Prakash Parvatkar,[†] Nobuo Kato,[‡] Motonari Uesugi,[†] Shin-ichi Sato,[†] and Junko Ohkanda*[,†]

[†]Institute for Chemical Research and Institute for Integrated Cell-Material Sciences (WPI-iCeMS), Kyoto University, Gokasho, Uji, Kyoto 611-0011, Japan

[‡]Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan

Supporting Information

ABSTRACT: Synthetic agents that disrupt intracellular protein−protein interactions (PPIs) are highly desirable for elucidating signaling networks and developing new therapeutics. However, designing cell-penetrating large molecules equipped with the many functional groups necessary for binding to large interfaces remains challenging. Here, we describe a rational strategy for the intracellular oxime ligation-mediated generation of an amphipathic bivalent inhibitor composed of a peptide and diterpene natural product, fusicoccin, which binds 14-3-3 protein with submicromolar affinity. Our results demonstrate that co-treatment of cells with small module molecules, the aldehyde-containing fusicoccin **1** and the aminooxy-containing peptide **2**, generates the corresponding conjugate **3** in cells, resulting in significant cytotoxicity. In contrast, chemically synthesized **3** is not cytotoxic, likely due to its inability to penetrate cells. Compound **3**, but not **1** or **2**, disrupts endogenous 14-3-3/cRaf interactions, suggesting that cell death is caused by inhibition of 14-3-3 activity. These results suggest that intracellular generation of large-sized molecules may serve as a new approach for modulating PPIs.

Protein−protein interactions (PPIs) play crucial roles in signaling networks that regulate many physiologic processes. Controlling intracellular PPIs using synthetic agents remains challenging,[1] as dysregulated proteins found in diseases are often implicated in intracellular signaling circuits (e.g., kinase/phosphatase-signal transduction).[2] However, the large, featureless, dynamic, and even frequently disordered interfaces[3] of PPIs limit the development of typical small-molecule-based cell-penetrating agents, although there are some notable successes.[1,4] Larger agents are often necessary to target a correspondingly large surface, and thus, many researchers have focused on developing new delivery approaches.[5] We anticipated that we may provide a solution to this obstacle by delivering two small molecules that would assemble as a larger molecule through their interaction with a common intracellular protein target. Here, we describe the rational design of two small reactive modules based on the structure of a diterpene natural product and a phosphopeptide for oxime ligation, which produces a bivalent diterpene-peptide conjugate capable of disrupting 14-3-3 protein-mediated interactions in cells.

The 14-3-3 proteins are a family of dimeric proteins that are expressed in all eukaryotic cells and play key regulatory roles in a diverse array of physiologic processes mediated by serine/threonine kinases and phosphatases.[6] Each of the highly helical monomers possesses a conserved amphipathic groove (∼25 Å in length) that binds to a consensus phosphopeptide motif containing either phosphoserine (pS) or phosphothreonine (pT) residues.[6b,c] Hundreds of intracellular protein ligands have been identified, including key oncogenic regulators, such as Raf.[7] A number of studies have implicated dysregulation of 14-3-3 interactions in cancer[8] and neurologic diseases,[9] suggesting 14-3-3 as a new therapeutic target.[2,10]

A previous study conducted by screening a phage display library identified a 20-mer peptide as a potent 14-3-3 inhibitor.[11] This peptide, R18, binds 14-3-3 with a K_d of ∼80 nM. R18 occupies the amphipathic groove and thereby interferes with 14-3-3/native ligand interactions.[11] Moreover, expression of a dimer form of R18 in cells induces apoptosis,[12] thus validating the potential of 14-3-3 as a therapeutic target. To date, several hydrophobic small-molecule-based 14-3-3 inhibitors have been identified by library screenings,[13] however, these compounds show moderate activity in cells in the submillimolar range. The characteristic shallow amphiphilic binding groove of 14-3-3 increases the difficulty of developing potent inhibitors.

We therefore aimed at developing a strategy to generate large amphipathic agents based on the intracellular ligation of small hydrophobic and hydrophilic fragments containing complementary reactive groups (Figure 1A, red and blue, respectively). Such intracellularly generated conjugate (Figure 1A, green) would bind to the large 14-3-3 binding groove and thereby disrupt 14-3-3 PPIs, enabling the assembly of a nonpermeable large molecule using smaller cell penetrating fragments. Manetsch et al.[14] reported an *in vitro* approach in which Bcl-xL combines thioacids and sulfonylazides into acylsulfonamides. Screening of the resulting chemical library identified a potent Bcl-X_L inhibitor, confirming that this target-guided assembly approach[15] is a robust strategy for PPI-oriented drug discovery. We wanted to facilitate the in-cell synthesis of PPI inhibitors by leveraging structure-based design utilizing fragment modules capable of penetrating cells.

Toward this end, we focused on the diterpene natural product, fusicoccin A[16] (FC, Figure 1B) as the hydrophobic

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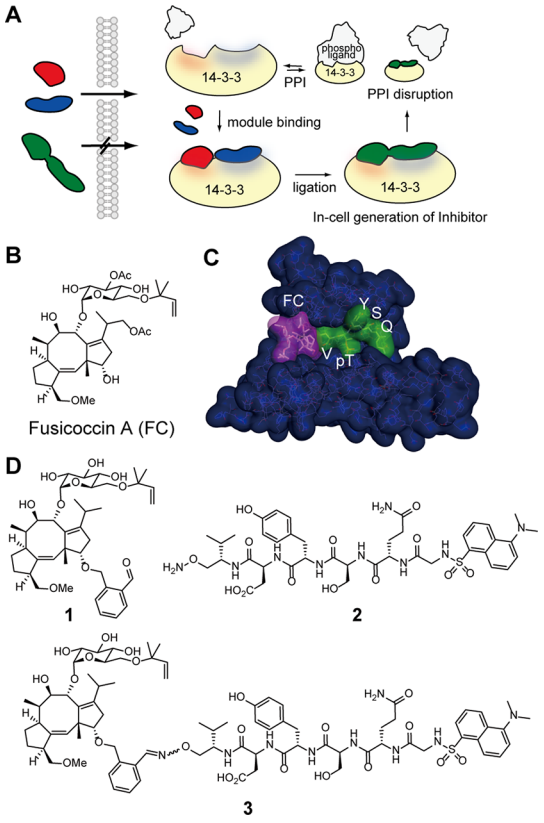

Figure 1. (A) Schematic of intracellular generation of the 14-3-3 inhibitor (green) by ligation of hydrophobic (red) and hydrophilic (blue) modules. (B) Chemical structure of fusicoccin A (FC). (C) Crystal structure of the ternary complex of 14-3-3, FC, and QSYpTV.[17] (D) Chemical structures of the modules (**1**, **2**) and conjugate product (**3**).

module. FC is a phytotoxin produced by the pathogenic fungus *Phomopsis amygdali*. FC binds to 14-3-3/ligand complexes and enhances the interaction,[10c] although FC itself is a weak ligand for 14-3-3 ($K_d \sim 66\ \mu M$).[17] The reported crystal structure of plant 14-3-3 bound to FC and the PMA2 phosphopeptide (QSYpTV; the 14-3-3-binding consensus motif found in the C-terminal of plant H^+-ATPase) clearly revealed that FC binds in the hydrophobic cavity adjacent to the peptide and forms a ternary complex (Figure 1C).[17] Notably, formation of this ternary complex is driven by van der Waals interactions between FC and the valine residue of the PMA2 peptide, resulting in an increase in the affinity of both ligands for 14-3-3 of nearly 2 orders of magnitude. This stabilization effect is thought to account for the toxicity of FC in plants.[17] Moreover, synthetic analogues of FC have been found to interact with human 14-3-3[18] and exhibit unique antitumor activity in animals.[19] These data suggested that FC would be a suitable motif for development of the proposed amphiphilic bivalent 14-3-3 inhibitors.

In our modular design strategy, we wanted to maximize the advantages derived from cooperative formation of the ternary complex of 14-3-3 bound to FC and the PMA2 peptide. In our earlier study toward 14-3-3-mediated ligation,[20] we attempted an epoxide-opening reaction using a modified FC derivative attached to the epoxide group by a long and flexible spacer, however, the moderate reaction efficiency hampered further evaluation in cells. Therefore, in order to exploit a proximity effect for the ligation upon the binding to 14-3-3, a simply modified FC derivative (**1**) containing an *o*-formyl benzyl group, and peptide **2**, in which the carboxy terminus of the PMA2 peptide was modified to an oxyamino group, was designed for oxime ligation (Figure 1D). To enhance membrane penetration and stability in cells, the pT residue was replaced with aspartic acid.

Oxime ligation has been widely used in both *in vitro*[21] and *in vivo*[22] studies, as this bioorthogonal condensation produces stable oxime derivatives under physiologic conditions. Moreover, previous studies by Rideout et al.[23] demonstrated that assembling of bioactive compounds utilizing hydrazone formation reaction resulted in a synergetic effect on cell-based activity. Thus, we hypothesized that the two modules could enter cells and bind 14-3-3, which in turn would template oxime ligation to yield the amphiphilic diterpene-peptide conjugate **3** (M_W = 1582 Da), which is capable of binding to 14-3-3 more tightly than either module alone due to the additive effect of binding energies.[24] Compound **3** was readily prepared by mixing **1** and **2** in methanol with a catalytic amount of trifluoroacetic acid (see Supporting Information [SI]).

To assess whether the modules were appropriately designed for 14-3-3-templated ligation, the docking of **1** and **2** to human 14-3-3ζ (see SI for details) was computationally modeled. The model showed that the benzyl group at the 12-position of FC favorably projects the aldehyde at the *ortho* position toward the ammonium group of **2**, which forms a hydrogen bond with the aldehyde oxygen (2.03 Å, Figure 2A), suggesting that the

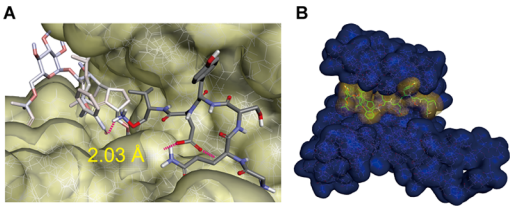

Figure 2. Docking models of (A) **1** (pale pink stick) and **2** (gray stick, dansyl-Gly was omitted for calculation), with predicted hydrogen bonds indicated by red dashed lines, and (B) **3** (green stick with yellow surface) to 14-3-3 (Glide in Schrödinger Suites 2015-2).

aldehyde and ammonium groups readily react to give the desired oxime derivative. The model also showed that the carboxylate of the aspartic acid residue in **2** binds to the phosphate-binding site of 14-3-3 interacting with lysine49 and arginine129, validating the mimicry of the phospho group (Figure 2A). Furthermore, the model indicated that conjugate **3** preserves the original conformation of **1** and **2** with only minimal changes, suggesting that the oxime ligation does not cause apparent steric repulsion between 14-3-3 and **3** (Figure 2B). These data suggest that **3** exhibits a complementary structure and size appropriate for binding to the 14-3-3 groove (Figure 2B).

In vitro evaluations of the reaction of **1** and **2** (300 μM each) and the template effect of recombinant 14-3-3ζ (300 μM) were carried out by HPLC in HEPES buffer (Figure 3, see SI for details). Ligation under 14-3-3-free conditions was found to be very slow, yielding only a small amount of conjugate **3** by 24 h (Figure 3, inset, left). In contrast, addition of an equimolar amount of 14-3-3ζ significantly accelerated the production of **3** (Figure 3, inset, right), with the yield at 1 h more than 50 times that observed under 14-3-3-free conditions (Figure 3, red and

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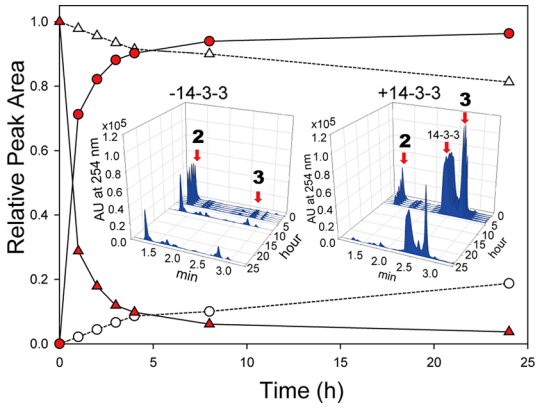

Figure 3. Changes in **2** (300 μM, triangle) and **3** (300 μM, circle) over time in the absence (white) or presence (red) of 14-3-3ζ (300 μM) in HEPES (100 mM, pH 7.3), 150 mM NaCl, 0.05% (v/v) Tween 20, 25 °C. Relative peak area = $a/(a + b)$ or $b/(a + b)$, where a and b are the peak areas of **2** and **3**. Insets: HPLC profiles of the reactions in the absence (left) and presence (right) of 14-3-3.

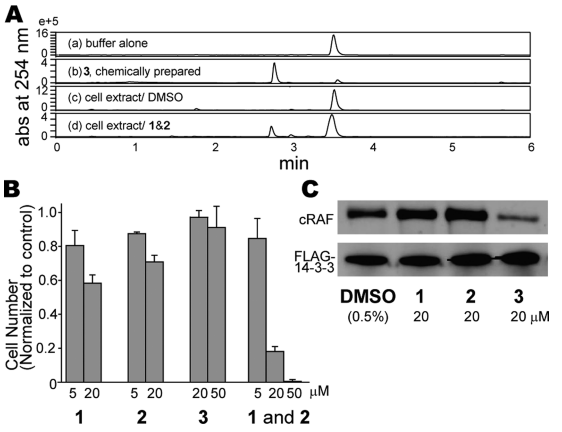

Figure 4. (A) HPLC analysis of oxime ligation in cells: (a) lysis buffer, (b) chemically synthesized **3**, (c) lysate of cells treated with DMSO (0.1% [v/v]), (d) lysate of cells treated with **1** and **2** (50 μM, respectively). (B) Trypan blue assay for HEK293 cells stably expressing FLAG-14-3-3ζ ($n \geq 3$). (C) Western blot of co-immunoprecipitated proteins using anti-cRaf and -FLAG antibodies.

white circles). The significant difference in reaction rates in the presence/absence of 14-3-3 is suggestive of a proximity effect associated with binding to 14-3-3. R18 peptide, a general 14-3-3 inhibitor, reduced the rate of **3** generation by ~80%, supporting the hypothesis that the 14-3-3 binding groove is involved in the ligation reaction (Figure S1).

In order to evaluate the additive effect of binding energies on ligation, isothermal titration calorimetry (ITC) of **2** and **3** with 14-3-3ζ was performed.[25] First, we tested the PMA2 peptide and confirmed its binding to 14-3-3ζ, with a K_d value of 1.24 ± 0.02 μM (Figure S2A), which was consistent with a previously reported value (K_d = 2.5 μM).[17] We then evaluated peptide fragment **2** and observed no apparent binding to the protein (Figure S2B), presumably due to the lack of a phosphorylated group, as a previous study showed that dephosphorylation of a 14-3-3 ligand peptide, SWpTY (K_d = 0.17 μM), significantly reduces its affinity, by more than 600-fold (SWTY, K_d > 100 μM).[26] In contrast, the conjugate **3** exhibited clear 1:1 binding to 14-3-3ζ, with a K_d value of 0.37 ± 0.14 μM ($\Delta G = -1.96$, $\Delta H = -1.33$, $-T\Delta S$ = 0.63 kcal/mol; Figure S2C), an affinity even greater than that of PMA2. This submicromolar affinity of the conjugate **3** was further confirmed by the competitive fluorescence anisotropy titration (K_d = 0.62 ± 0.35 μM; for more details, see SI). These results demonstrate that attachment of the diterpene moiety to **2** converts it to an amphiphilic compound, remarkably improving affinity due to the bivalency introduced by the covalent linking of **1** and **2**.

In order to test whether the ligation reaction could be carried out intracellularly, we prepared HEK293 cells stably expressing FLAG-14-3-3ζ. Cells (2.5×10^5) were treated with **1** and **2** (50 μM, 25 nmol, each), incubated for 12 h, and then washed with medium (3×) and buffer (4×). A cell lysate was obtained, lyophilized, extracted with methanol, and then analyzed by HPLC (for more details, see SI). The HPLC results clearly showed a peak corresponding to **3**, indicating that the conjugate was generated in the cells (Figure 4A). The amount of **3** generated was estimated at ~11 nmol (yield ~45%; for more details, see SI), indicating that the oxime ligation proceeded to produce **3** at a decent yield. The potential limitation of the cell permeation of **2** could be one of the reason for the moderate yield. Furthermore, treatment of cells with an equimolar mixture of **1** and **2** for a longer period (24 h) resulted in dose-dependent cytotoxicity, whereas treatment with either compound alone resulted in considerably less cytotoxicity (Figure 4B). Moreover, chemically prepared **3** was found to have no effect on cell proliferation, even at high concentrations (Figure 4B). Previous studies revealed that expression of a dimer form of R18 peptide in cells leads to cell death.[12] Thus, these results suggest that **3** generated intracellularly binds to and inhibits 14-3-3, resulting in significant cytotoxicity, whereas chemically synthesized **3** is inactive, most likely because it cannot penetrate cells due at least in part to its large molecular size.

Finally, we examined whether **3** can disrupt interactions between 14-3-3 and its protein ligands. cRaf is an oncogenic kinase that is regulated by 14-3-3[7] and is a well-studied potential target for cancer therapeutics.[27] Thus, we conducted a co-immunoprecipitation experiment with FLAG-14-3-3ζ to examine the effect of the compounds on the binding of cRaf to 14-3-3. Lysates of HEK293 cells expressing FLAG-14-3-3ζ were treated with anti-FLAG antibody affinity beads in the presence of test compounds (20 μM), and bound proteins were eluted using 3× FLAG peptide and analyzed by Western blotting using anti-cRaf and -FLAG antibodies. No significant differences in the cRaf bands were observed between the DMSO control and **1** or **2**, whereas **3** significantly reduced the intensity of the cRaf band (Figure 4C), demonstrating that the conjugate **3**, but not **1** or **2**, indeed disrupts the endogenous cRaf/14-3-3 interaction. As **3** was designed to occupy the entire binding groove of 14-3-3, this compound most likely acts as a general inhibitor, disrupting 14-3-3 interactions globally, which would account for its significant cytotoxicity.

In conclusion, this work presents a new approach for generating 14-3-3 inhibitors by oxime ligation between diterpene and peptide modules that are designed based on the co-crystal structure of 14-3-3. We demonstrated intracellular generation of **3**, which induces cell death, presumably by inhibiting 14-3-3 PPIs. Importantly, chemically synthesized **3** does not induce cytotoxicity, likely due to its inability to penetrate cells, validating the strategy of constructing large, nonpenetrating agents within cells as an alternative means of modulating the 14-3-3 signaling network. To the best of our knowledge, the diterpene conjugate **3** is one of the strongest

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nonpeptidic ligands for 14-3-3 reported to date. Therefore, this work also suggests that the series of fusicoccins hold a promise as scaffolds for further development of inhibitors of 14-3-3, which has received intense research attention as a potential new drug target for treating cancers and neurodegenerative diseases. In humans, there are seven isoforms of 14-3-3 proteins with a highly preserved amphipathic binding groove. A potential limitation of our 14-3-3 inhibitor is that it may likely inhibit all 14-3-3 isoforms as it is designed according to the structure of common binding groove. In order to advance our understanding of the biology of 14-3-3 proteins, isoform-selective inhibitors/modulators would therefore be desirable. Intracellular generation of the diterpene conjugates that can specifically target 14-3-3 protein isoforms may allow to achieve this goal. Work toward this end is currently in progress in our laboratory. Nonetheless, this study supports a new concept for modulating PPIs using intracellularly assembled synthetic agents, a concept that may expand to include other clinically relevant protein targets.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.5b09817.

Synthetic and biological experimental procedures and data (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*johkanda@scl.kyoto-u.ac.jp

Notes

The authors declare no competing financial interest.

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