Photoaffinity Labeling

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Affinity-Based Labeling of Cytohesins with a Bifunctional SecinH3 Photoaffinity Probe**

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Dedicated to Professor Manfred T. Reetz on the occasion of his 65th birthday

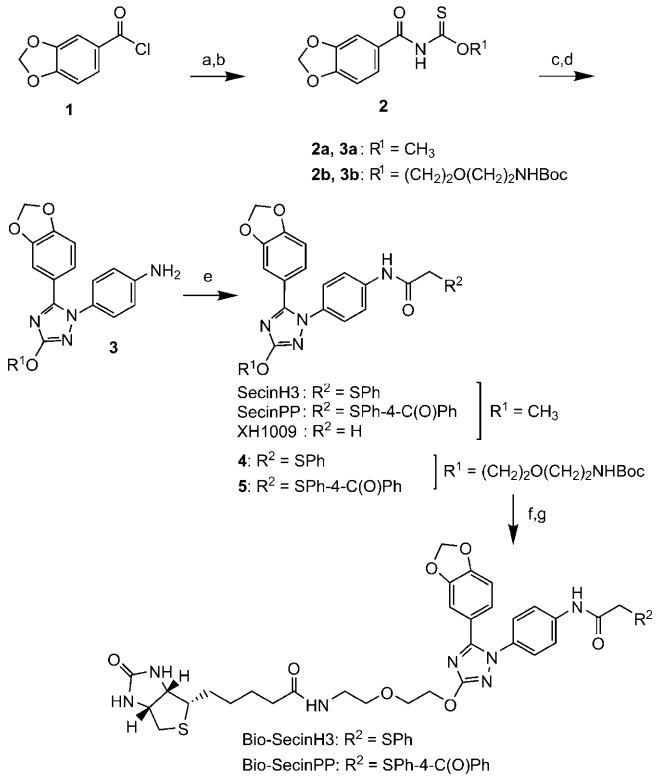
We recently identified the 1,2,4-triazole derivative SecinH3 (see Scheme 1) as the first small-molecule inhibitor of a class of cytoplasmic regulatory proteins called cytohesins by using an aptamer-displacement assay.[1,2] Cytohesins are small guanine nucleotide exchange factors (GEFs) that stimulate ADP (adenosine diphosphate) ribosylation factors (Arfs), ubiquitously expressed Ras-like GTPases which control various cellular regulatory networks ranging from vesicle trafficking to integrin activation.[3] The four highly homologous cytohesins known in mammals share the same domains: a Sec7 domain, which harbors the GEF activity, a pleckstrin-homology (PH) domain, and a coiled-coil (CC) domain. SecinH3 targets the Sec7 domain of cytohesins 1, 2 (also known as "Arf nucleotide-binding-site opener" (ARNO)), and 3, and inhibits their guanine-nucleotide-exchange activity. Its application in human liver cells, flies, and mice led to impaired insulin signaling in each case. Thus, cytohesins associated with the insulin-receptor complex appear to be essential components of this central cellular signaling pathway.[1a,4]

Two classes of Arf-GEFs are known and can be distinguished by their size. Cytohesins belong to the small 47 kDa Arf-GEFs; the large Arf-GEFs, which also contain a Sec7 domain, have a mass of about 200 kDa.[5] Unlike the large Arf-GEFs, the small SecinH3-responsive cytohesins are insensitive to the fungal metabolite brefeldin A (BFA). The specificity of SecinH3 for cytohesin GEFs has so far been tested only for a limited set of proteins by means of isothermal titration calorimetry (ITC).[1a]

A powerful method for assessing the specificity of small-molecule modulators for their targets is photoaffinity labeling,[6] as it enables the analysis of a large number of individual proteins or protein mixtures in parallel.[6a,b] The combination of photoaffinity labeling and mass spectrometry has been established as an efficient approach for the identification of the binding site of modulator compounds to their protein ligands.[6c,d] Reporter-tagged derivatives of small molecules can also serve as probes for activity-based protein profiling (ABPP).[7]

Herein we report the design, synthesis, and application of SecinH3 photoaffinity probes. By applying them to a wide range of GEFs and their small GTPase substrates, we demonstrate that Secin derivatives, which enable the direct detection of the covalent linkage between the photoaffinity probe and the cytohesin Sec7 domain, exhibit high specificity for cytohesins.

Preliminary structure–activity-relationship (SAR) studies of SecinH3 showed that the terminal thiophenyl group is critical for inhibitory activity.[8] The deletion of this residue in XH1009 (Scheme 1) resulted in a dramatic loss of inhibition



Scheme 1. Synthesis of the Secin derivatives used: a) KSCN, acetone, 60 °C; b) R^1OH, acetone, 50 °C, 83%; c) 4-nitrophenylhydrazine, EtOH, 80 °C, 70%; d) Fe/HCl, NH_4Cl, $MeOH/H_2O$, 65 °C, 64%; e) R^2CH_2COOH, HBTU/NEt_3, DMF, 22 °C, 57%; f) TFA/CH_2Cl_2, 94%; g) D-(+)-biotin, HBTU/NEt_3, DMF, 69%. Boc = *tert*-butoxycarbonyl, DMF = *N*,*N*-dimethylformamide, HBTU = *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, TFA = trifluoroacetic acid.

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of Sec7-catalyzed guanine-nucleotide exchange on [Δ17]Arf1 (Figure 1). In contrast, modification of the phenyl ring had little effect on the inhibitory activity, or was even beneficial.

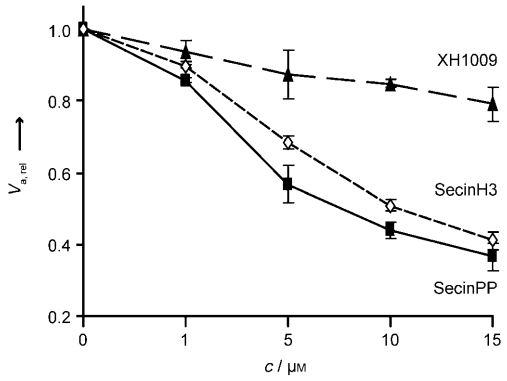

Figure 1. Inhibition by SecinH3, SecinPP, and XH1009 of guanine-nucleotide exchange on [Δ17]Arf1 catalyzed by the Sec7 domain of cytohesin 2 (ARNO; tryptophan fluorescence assay).[10] c = inhibitor concentration, $v_{a,rel}$ = relative initial rate.

To convert SecinH3 into a photoaffinity probe, we took advantage of this finding by substituting a photolabile benzophenone (Bp) group for the terminal phenyl ring, as in SecinPP (Scheme 1). The advantages of Bp as the photophore are its chemical stability, specificity of insertion sites during photoactivation, and ability to be excited repeatedly by UV light without loss of reactivity.[9] The attachment of the biotin group with the aminoethoxyethanol spacer in Bio-SecinPP (Scheme 1) was based on preliminary SAR studies, according to which the methoxy group at the 3-position of the triazole ring can be extended without significant loss of inhibitory activity.[8] As a control, we synthesized biotin-labeled Bio-SecinH3.

The synthesis of the Secin derivatives (Scheme 1) begins with the transformation of the benzodioxole carboxylic chloride **1** into the thiocarbamoyl acid *O*-alkyl ester **2** with KSCN and the respective alcohol. Treatment with 4-nitrophenylhydrazine and subsequent reduction afforded the 1,2,4-triazole compounds **3**. The attachment of phenylsulfanyl- or benzophenonesulfanylacetic acid derivatives onto **3a** gave SecinH3 and SecinPP, respectively, whereas compounds **4** and **5**, respectively, were obtained by the attachment of the same derivatives onto **3b**. The acetylation of **3a** afforded XH1009. The deprotection of **4** and **5**, followed by acylation with D-(+)-biotin, gave Bio-SecinH3 and Bio-SecinPP in good yields.

SecinPP showed a similar inhibition curve to that of SecinH3 in the ARNO Sec7-catalyzed GDP/GTP-exchange assay on [Δ17]Arf1, which suggests that the two compounds bind the ARNO Sec7 domain similarly. Compound XH1009 was used as a negative control (Figure 1). Next, we examined the concentration- and time-dependent efficiency of the labeling of ARNO-Sec7 by Bio-SecinPP. For this purpose, the Sec7 domain of ARNO was incubated at a concentration of 2.5 μM with Bio-SecinPP (5 μM) at ambient temperature for 2–3 min. Afterwards, the samples were subjected to UV irradiation at 366 nm for varying amounts of time (0–5 min) at an energy output of 200 mW. After separation of the proteins by 12.5% SDS-PAGE, the modified protein was detected with a horseradish peroxidase–streptavidin conjugate. As all proteins used in this study contained a hexahistidine (His6) tag, a His6-specific antibody was used to normalize the amount of loaded protein. The amount of labeled protein increased steadily as a function of time; after irradiation for 3 min, the amount of labeled protein was 30 times greater than that observed for the nonirradiated background (Figure 2B). The photoaffinity labeling at a fixed concentration of the protein (2.5 μM) and constant UV irradiation for 3 min was found to be concentration-dependent when Bio-SecinPP was used at concentrations between 0 and 5 μM (Figure 2C). When Bio-SecinH3 (2.5 μM) was subjected to the same conditions, the signal intensity was similar to that of the background signal (Figure 2C, lane 7), which shows that the labeling depends on the presence of the photophore. We also performed a competition experiment (Figure 2D) to verify that the binding mode of Bio-SecinPP was similar to that of SecinH3. For this purpose, SecinH3 (2.5 μM) was first incubated with ARNO-Sec7 for 3 min at ambient temperature, and then the same amount of Bio-SecinPP was added; the resulting mixture was irradiated for 1 min and then analyzed by western blotting. In the presence of an equimolar amount of SecinH3, the amount of labeled protein decreased significantly (Figure 2D, lane 4). In contrast, XH1009 at this concentration did not affect the efficiency of photolabeling

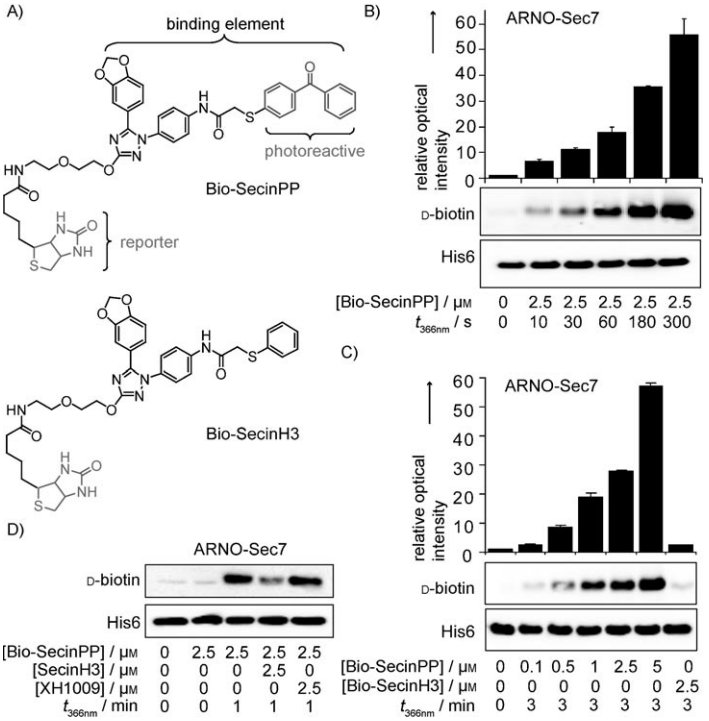

Figure 2. A) Structures of Bio-SecinPP and Bio-SecinH3. B),C) Photoaffinity labeling of ARNO-Sec7 with Bio-SecinPP with increasing irradiation time (B) and increasing concentration (C). D) Competition experiment with an equal amount of SecinH3 or XH1009.

by Bio-SecinPP (Figure 2D, lane 5). These results indicate that Bio-SecinPP and SecinH3 bind to ARNO at the same site.

Having verified that Bio-SecinPP could be used as a bifunctional probe, we examined the labeling specificity by using a range of Arf-GEF Sec7 domains, GEFs without Sec7 domains, and small GTPases (Figure 3). The Sec7 domains of

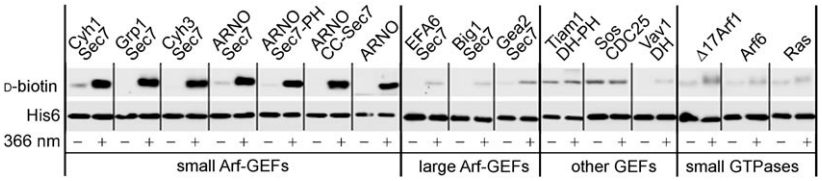

Figure 3. Photoaffinity labeling of GEF functional domains and small GTPases (2.5 μM) with Bio-SecinPP (2.5 μM). Irradiation: 100 mW, 1 min.

cytohesins 1 and 3 and of the *Drosophila melanogaster* cytohesin Steppke (Grp1 Sec7) were all labeled in the same way as ARNO-Sec7 with high efficiency after treatment with Bio-SecinPP and UV irradiation for 1 min. Full-length ARNO and several truncated ARNO constructs, including ARNO-Sec7-PH and ARNO-CC-Sec7, were labeled as efficiently as the Sec7 domain alone. Thus, the other cytohesin domains did not affect the labeling reaction. The efficient labeling of full-length ARNO indicates that Bio-SecinPP is a promising ABPP probe for cytohesins.

The Sec7 domains of the medium and large Arf-GEFs, such as "exchange factor for Arf6" (EFA6), "brefeldin A inhibited guanine nucleotide-exchange protein 1" (Big1), and "guanine nucleotide exchange factor for Arfs 2" (Gea2) from yeast showed no or only background modification. Likewise, the Sec7-unrelated "Dbl homology" (DH) domain of the Rho GEF Vav1 did not react with Bio-SecinPP. The DH–PH domain of the Rac GEF "T-lymphoma invasion and metastasis-inducing protein 1" (Tiam1) and the CDC25-like GEF domain of the Ras GEF "son of sevenless" (Sos) exhibited a slight nonspecific interaction with the peroxidase–streptavidin conjugate. This interaction was not dependent on irradiation (Figure 3) or Bio-SecinPP (data not shown). The small GTPases Arf1, Arf6, and Ras were not labeled with Bio-SecinPP. Taken together, these results demonstrate that the labeling by Bio-SecinPP is cytohesin-specific.

In conclusion, we have designed, synthesized, and applied a photoactive probe for cytohesins by integrating a benzophenone group into SecinH3 in such a way that its inhibitory activity was maintained. Further functionalization with a detectable biotin tag led to Bio-SecinPP, an activity-based protein-profiling (ABPP) reagent for cytohesins. The application of this probe to a variety of GEFs and GTPases provided cogent evidence for the specific binding of SecinH3 to the Sec7 domain of the members of the cytohesin family. We can now define reaction conditions that might enable the proteome-wide, affinity-based profiling of cytohesin complexes in whole cells or even organisms.

Experimental Section

The photoaffinity labeling of ARNO-Sec7 with Bio-SecinPP in a time-course experiment is described exemplarily: Bio-SecinPP (2.5 μM) was incubated with ARNO-Sec7 (2.5 μM) in phosphate-buffered saline (PBS; pH 7.4, 50 μL) with 1% dimethyl sulfoxide at 23 °C for 2–3 min. The mixture was then transferred into a glass tube (50 × 10 mm, thickness: 0.65 mm) and irradiated with UV light (home-built) for 0–5 min at 366 nm and 100 mW. After UV irradiation, the proteins were denatured by adding 6X loading buffer and heating for 5 min at 96 °C. The proteins were separated by 12.5% SDS-PAGE and subsequently blotted onto a nitrocellulose membrane (Whatman). For biotin detection, the membrane was treated for 1 h with TBST (Tris-buffered saline 0.1% Tween (Sigma); pH 7.6, 10 mL; Tris = tris(hydroxymethyl)aminomethane) and 5% bovine serum albumin (BSA) at room temperature, washed twice with TBST (10 mL), and then incubated with horseradish peroxidase (HRP) conjugated streptavidin (1:20000; Rockland) in 5% BSA/TBST (10 mL) at 4 °C for 16 h. For detection of the His6 tag, the membrane was blocked in 5% nonfat powdered milk in TBST at 23 °C for 1 h, and then incubated at 4 °C for 16 h with a His6-specific antibody (1:2000; Santa Cruz Biotechnology). After extensive washing, the membrane was incubated at 23 °C for 1 h with a goat antimouse IgG conjugated to HRP (Santa Cruz Biotechnology; IgG = immunoglobulin G). Proteins were visualized by enhanced chemiluminescence (Millipore). Experiments were performed in duplicate.

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