Inhibitors

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Cyplecksins Are Covalent Inhibitors of the Pleckstrin Homology Domain of Cytohesin**

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Dedicated to the Bayer company on the occasion of its 150th anniversary

Cytohesins are cytoplasmic multidomain proteins that act as guanine nucleotide exchange factors (GEFs) for small Ras-like GTPases called ADP-ribosylation factors (Arfs).[1] Their Sec7 domain catalyzes the exchange of guanosine-5′-diphosphate (GDP) for guanosine-5′-triphosphate (GTP), which activates Arf proteins like Arf1 and Arf6. Mammalian cells contain four highly homologous cytohesins (cytohesins 1–4) that are implicated in cellular processes such as β2-integrin-mediated cell adhesion and actin dynamics, including Arf-mediated functions, namely membrane trafficking, vesicle transport, endocytosis, and more.[2] Moreover, cytohesin-2 (ARNO) is a cytoplasmic activator of receptor tyrosine kinase signaling by the insulin receptor (IR)[3] and epidermal growth factor receptor (EGFR),[4] respectively. ARNO and Arf6 also contribute to the disruptive effects of interleukin-1β (IL-1β) on endothelial stability by binding to the adaptor protein MYD88.[5] These discoveries were greatly aided by the availability of SecinH3, an inhibitor of the cytohesin Sec7 domain.[3b,6]

C-terminal to their Sec7 domain, cytohesins contain another functional domain called the pleckstrin homology (PH) domain (Figure 1 a). Through their PH domain cytohesins are recruited to the inner leaflet of the plasma membrane and intracellular membranes by binding either to the phosphatidylinositol phosphates PIP_2 or PIP_3, or to activated Arf6-GTP.[2] Cytohesin Sec7-dependent integrin activation in cell adhesion requires the presence of the PH domain, and the PH domain exerts a certain level of inhibition on the Sec7 domain that is relieved upon membrane recruitment and interaction with PIPs.[7] These findings indicate that the functions of the cytohesin Sec7 and PH domains may be tightly interconnected. Inspired by the usefulness of SecinH3 as a chemical biology tool for elucidating previously unknown functions of cytohesin Sec7 domains, we sought to identify a small-molecule inhibitor for cytohesin PH domains. Here we report the discovery of a class of cytohesin PH domain inhibitors called Cyplecksins (*Cy*tohesin *plecks*trin homology domain *in*hibitor*s*) that act by a covalent mechanism.

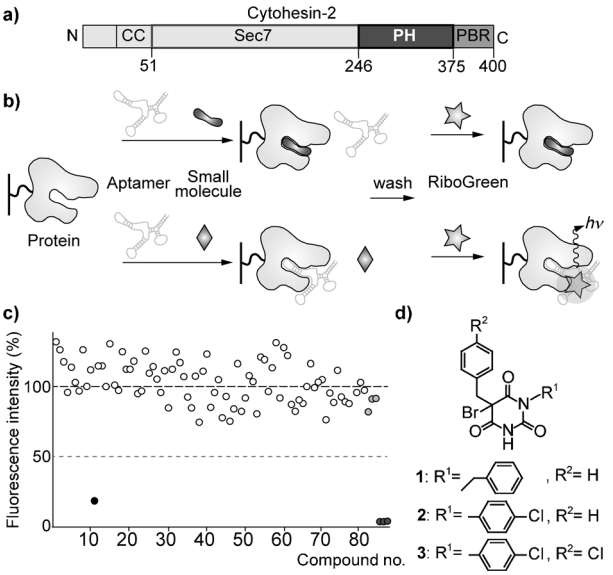

Figure 1. Aptamer displacement assay based on RiboGreen fluorescence capture. a) Domain structure of cytohesin-2. CC: Coiled coil; PBR: polybasic region. b) The immobilized cytohesin is incubated with the aptamer and small molecules. Nonbinding molecules are removed by a buffer wash. Remaining bound aptamer is detected by RiboGreen fluorescence (bottom right); reduced fluorescence is obtained upon aptamer displacement by the small molecule (top right). c) Representative primary screening plate with a hit compound (black dot; no. 11). Positive controls (dark gray dots; nos. 87–90) lacked cytohesin-1 coating; negative controls (light gray dots; nos. 84–86) did not contain any compound. d) Chemical structures of the most active hit compounds, derivatives of 5-bromopyrimidine-2,4,6-trione.

To develop an aptamer displacement assay for HTS, we first selected an RNA aptamer that bound the PH domain of cytohesin-1. After seven selection rounds, the enriched RNA library was cloned and sequenced, and clone 6.10 was identified as a cytohesin PH domain binder (Figure S1a in the Supporting Information (SI)). Clone 6.10 bound PH domains of cytohesins 1, 2, and 3 with K_d values between 0.3 and 0.7 μM, whereas no binding to the Sec7 domain and to related PH domains could be detected (Figure S1 b,c (SI)).

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The aptamer also inhibited the binding of cytohesins to PIP_3-doped liposomes in a concentration-dependent manner (Figure S1 d (SI)). Binding and inhibition required the presence of the triphosphate group at the 5′-end of the aptamer.

The aptamer displacement screen was established as illustrated in Figure 1 b. 96-well screening plates were coated with cytohesin-1 PH, and incubated with aptamer 6.10 in the presence of compound. Small molecules that compete with 6.10 for binding lead to a reduced level of aptamer complexation to the immobilized protein, whereas noncompeting compounds do not (Figure 1 b; middle). The plates are washed with buffer to remove nonbound aptamer, and then incubated with RiboGreen, a dye that exhibits an increase in fluorescence that is directly proportional to the amount of nucleic acid. Low fluorescence indicates a compound that has displaced the bound aptamer from its target protein (for possible false-positive scenarios see Figure S2 (SI)). A chemical library of roughly 12 000 diversity-based druglike small molecules was screened to identify potential cytohesin PH domain inhibitors. The assay quality parameter Z' was 0.69, which is compatible with HTS conditions (Figure 1 c and Figure S3 (SI)). The screening revealed the series of substituted 5-bromopyrimidine-2,4,6-triones **1**–**3** that competed with 6.10 for cytohesin PH domain binding (Figure 1 d).

We next performed microscale thermophoresis measurements to quantify the binding of Cyplecksins **1**–**3** and the related derivatives **4** and **5** (Figure 2 a), which lack the 5-bromo substituent, to the Alexa647-labeled cytohesin-1 PH domain (Figure 2 b). Increasing concentrations of **1**–**3** led to sigmoidal binding curves corresponding to K_d values around 2 μM. In contrast, neither **4** nor **5** showed any binding to the cytohesin-1 PH domain. To test the specificity of **1**–**3** for other PH domains we carried out the same binding test using the Alexa647-labeled DH-PH domain of the "T-lymphoma invasive and metastasis inducing protein 1" (Tiam1). No concentration-dependent change in thermophoresis was detected, indicating that the PH domain of the Tiam1 fragment is not recognized by **1**–**3** (Figure 2 c). We then investigated whether Cyplecksins **1**–**3** compete with PIP_2 binding to the cytohesin-1 PH domain in vitro (Figure 2 d). We used PIP_2 labeled with tetramethylrhodamine (TMR) and measured fluorescence polarization (FP) in the presence of increasing concentrations of **1**–**5**. The complex between TMR-PIP_2 and the cytohesin-1 PH domain leads to higher levels of FP than the free TMR-PIP_2. Indeed, increasing Cyplecksin concentrations led to inhibition of TMR-PIP_2 binding to the cytohesin-1 PH domain with IC_{50} values below 10 μM. Derivatives **4** and **5** again were inactive in this assay. The same experiment was done using the PH domains of GEP100, another Arf6-GEF (Figure 2 e), and of DAGK, ARHGAP25, IRS1, Pleckstrin, and full-length Akt2 (Figure S4 (SI)). Cyplecksins **1** and **2** either did not interfere at all with the binding of TMR-PIP_2 or TMR-PIP_3 to these proteins, or exhibited only weak inhibition with IC_{50} values at least above 100 μM. In the latter cases the precise IC_{50} values could not be determined because compound concentrations beyond the solubility limit would have been required. Only Cyplecksin **3** showed a somewhat less specific inhibitory profile.

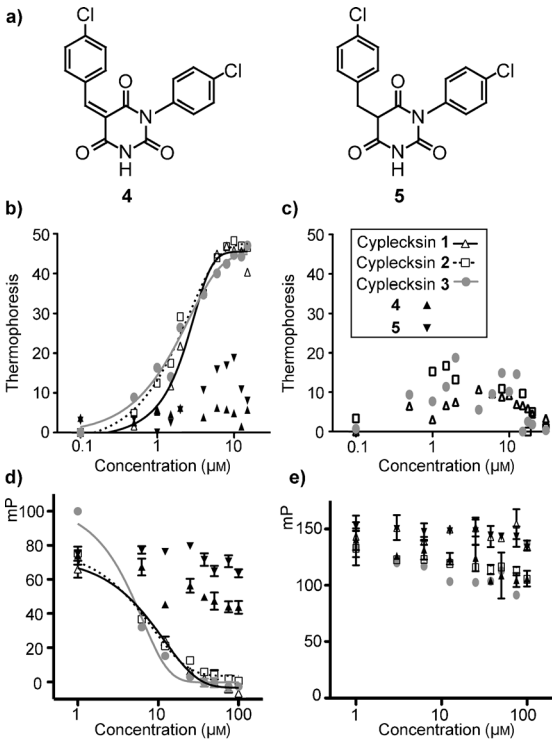

Figure 2. Binding behavior of Cyplecksins **1**–**3**, and their analogues **4** and **5**. a) Chemical structures of nonbinding Cyplecksin analogues **4** and **5**. b), c) Cyplecksins **1**–**3** bind specifically to cytohesin-1 PH but not to Tiam1-DH-PH. 100 nM Alexa647-labeled Cyth1-PH (b) and Tiam1-DH-PH domain (c) were incubated with compounds (0.1–20 μM), followed by microscale thermophoresis (MST) measurements. For color and symbols legend see box insert in (c). d), e) Cyplecksins specifically inhibit the binding of cytohesin to PIP_2. 250 nM Cyth1-PH (d) and 500 nM GEP100-PH (e) were incubated with compounds (1–100 μM) and 30 nM tetramethylrhodamine (TMR)-conjugated PIP_2. PIP_2 binding was quantified by fluorescence polarization. mP = millipolarization.

Cyplecksins **1**–**3**, but not **4** and **5**, also inhibited the binding of PIP_3 to cytohesin-1 PH, and to nearly full-length cytohesins-1 and -2 that lacked only the short polybasic region (Figure S5 (SI)). Taken together, these data indicate that Cyplecksins **1** and **2** exhibit a high degree of specificity for the PH domain of cytohesins.

The lack of inhibitory activity of the Cyplecksin analogues **4** and **5** suggests that the bromine substituent in **1**–**3** is crucial for binding and inhibition. In aqueous solution, Cyplecksins slowly hydrolyze within several hours into mixtures of derivatives including those that result from ring opening (data not shown). In the presence of amines, however, a substitution of the bromine for the amine occurs in related 5-methyl- or 5-phenyl-substituted pyrimidine-2,4,6-triones.[8] This suggests that upon binding of Cyplecksins to their binding site in the cytohesin PH domain, a substitution reaction may occur, in which either the C5 in **1**–**3** is attacked by a nucleophile (lysine or cysteine) to displace the Br atom, or one of the carbonyl C atoms in the heterocycle is attacked in a ring-opening reaction.[9] In any case, both mechanisms should lead to a covalent attachment of Cyplecksins to the cytohesin PH domains.

To test this hypothesis, we synthesized the probe **6**, a biotinylated analogue of Cyplecksin-1 (Figure 3a). The non-brominated variant **7** was used as a negative control. We then incubated the cytohesin-1 PH domain, full-length cytohesin-1, and the Sec7 domain of cytohesin-2, to which no binding of **6** should occur, with increasing concentrations of **6** and **7**. After denaturing polyacrylamide gel electrophoresis (PAGE) and western blotting, covalently attached biotin groups in the proteins were detected with neutravidin. Indicative of the formation of the covalent adduct, cytohesin-1 PH and full-length protein both showed concentration-dependent biotinylation with **6** but not with **7**, whereas cytohesin-2 Sec7 remained unmodified (Figure 3b).

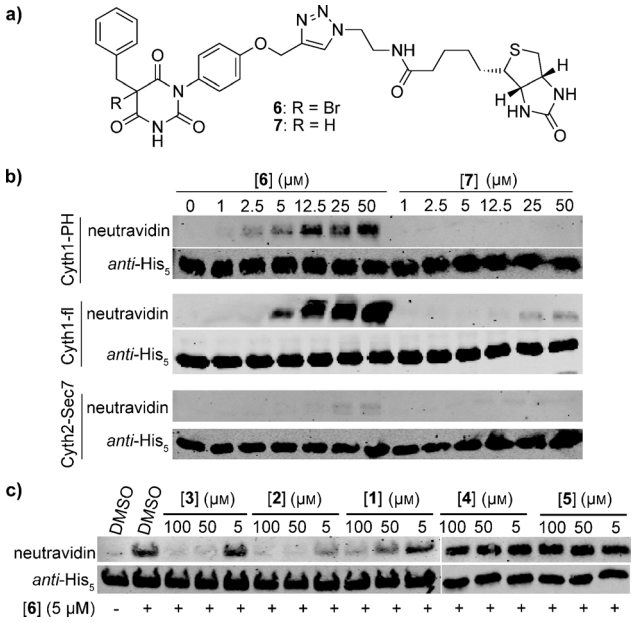

Figure 3. Cyplecksins bind covalently to cytohesins a) Chemical structure of biotinylated Cyplecksin-2 analogues **6** and **7.** b) Increasing concentrations of **6** and its inactive analogue **7** incubated with cytohesin-1 PH domain (top panel), full-length cytohesin 1 (Cyth1-fl; middle panel), and cytohesin-2 Sec7 domain (bottom panel), analyzed by denaturing PAGE. Covalently bound biotin is detected by neutravidin and total protein by an *anti*-His_5 antibody. Cytohesin-1 PH domain and full-length cytohesin-1, but not the cytohesin-2 Sec7 domain, were found to be biotinylated. In all experiments, compound **7** was inactive. c) Competition of biotinylated adduct formation by **6** using Cyplecksins **1**–**5**. Cyplecksins **1**–**3**, but not **4** and **5**, competed with **6** for binding to the cytohesin-1 PH domain; analysis was the same as in (b).

To further substantiate this result, we tested whether the biotinylation of the cytohesin-1 PH domain with **6** was competed by non-biotinylated Cyplecksins **1**–**3**. At concentrations of 50 μM or higher, Cyplecksins **1**–**3** resulted in a marked reduction of the biotinylation by **6**, and at 5 μM a reduction was already detectable. Neither **4** nor **5** were able to compete with **6** for cytohesin-1 PH domain binding, not even at 100 μM concentrations (Figure 3c). Altogether, these results can only be explained by a specific covalent modification of the cytohesin-1 PH domain with the biotinylated Cyplecksin-1 variant **6**.

As a first step to identify the Cyplecksin-reactive nucleophile in the protein, the cysteine residues of cytohesin-1 PH were alkylated by iodoacetamide. Neither the binding of TMR-PIP_2 nor its inhibition by Cyplecksins **1**–**3** were affected by the treatment, arguing against cysteine residues being the sites of the covalent attachment of Cyplecksins (Figure S6 (SI)). To unambigously identify the binding mode and binding site of Cyplecksins a combined mass spectroscopic/crystallographic approach would be required which, however, is beyond the scope of this work.

To analyze whether Cyplecksins inhibit membrane targeting of cytohesin-2 PH, nanodisks[10] were used as a membrane surrogate. Cytohesin-2 PH bound only to PIP_2-doped nanodisks, indicating that nanodisks are suitable to reliably detect the PIP_2-dependent membrane recruitment of cytohesins (Figure 4a). Cyplecksins **1**–**3** but not the control compounds **4** and **5** inhibited the binding.

Having shown that Cyplecksins inhibit the binding of cytohesin PH domains to PIP_2/PIP_3 phospholipids by a covalent mechanism in vitro we next sought to test the activity of these compounds in living cells. When HeLa cells are stimulated with insulin, insulin receptor (IR)-dependent signaling cascades lead to production of PIP_3, which stimulates the translocation of cytohesins to the inner leaflet of the plasma membrane by means of their PH domains. To analyze the effect of Cyplecksins on this process, we transfected HeLa cells with a cytohesin-2 construct that was fused to green fluorescent protein (GFP) (Figure 4b, top row, Cyth2-GFP). The cells were then analyzed for membrane recruitment of cytohesin-2 GFP by confocal fluorescence microscopy. Without insulin stimulation almost no Cyth2-GFP can be detected at the membrane (Figure 4b, column 1) but after insulin stimulation, Cyt2-GFP translocates to the membrane (white arrows, column 2) and colocalizes with membrane proteins stained by wheat germ agglutinin (white arrows, merge, column 2). In the presence of 50 μM Cyplecksins **1**, **2**, or **3**, however, no insulin-dependent Cyth2-GFP translocation to the plasma membrane can be detected (columns 3–5). In contrast, the inactive Cyplecksin analogues **4** and **5** at similar concentrations have no influence on the membrane recruitment of Cyth2-GFP (columns 6 and 7). The analysis of Cyth2-GFP membrane recruitment in a large number of cells revealed a statistically highly significant inhibition by Cyplecksins **1**–**3** (Figure 4c). These results clearly demonstrate that Cyplecksins **1**–**3** effectively inhibit the binding of PIP_3 to cytohesin PH domains also in the context of living cells.

In conclusion, our study demonstrates that the methodology of aptamer displacement screening is feasible for identifying small-molecule inhibitors of the cythohesin PH domain. Aptamer-directed screening assays have previously led to useful druglike inhibitors.[3b,6,11] Our screening assay, based on RiboGreen detection, revealed a series of substituted 5-bromopyrimidine-2,4,6-triones **1**–**3**, termed Cyplecksins, which inhibit phospholipid binding to PH domains of cytohesins. Within the subset of PH domains of other proteins tested here, Cyplecksins exhibited high selectivity for cyto-

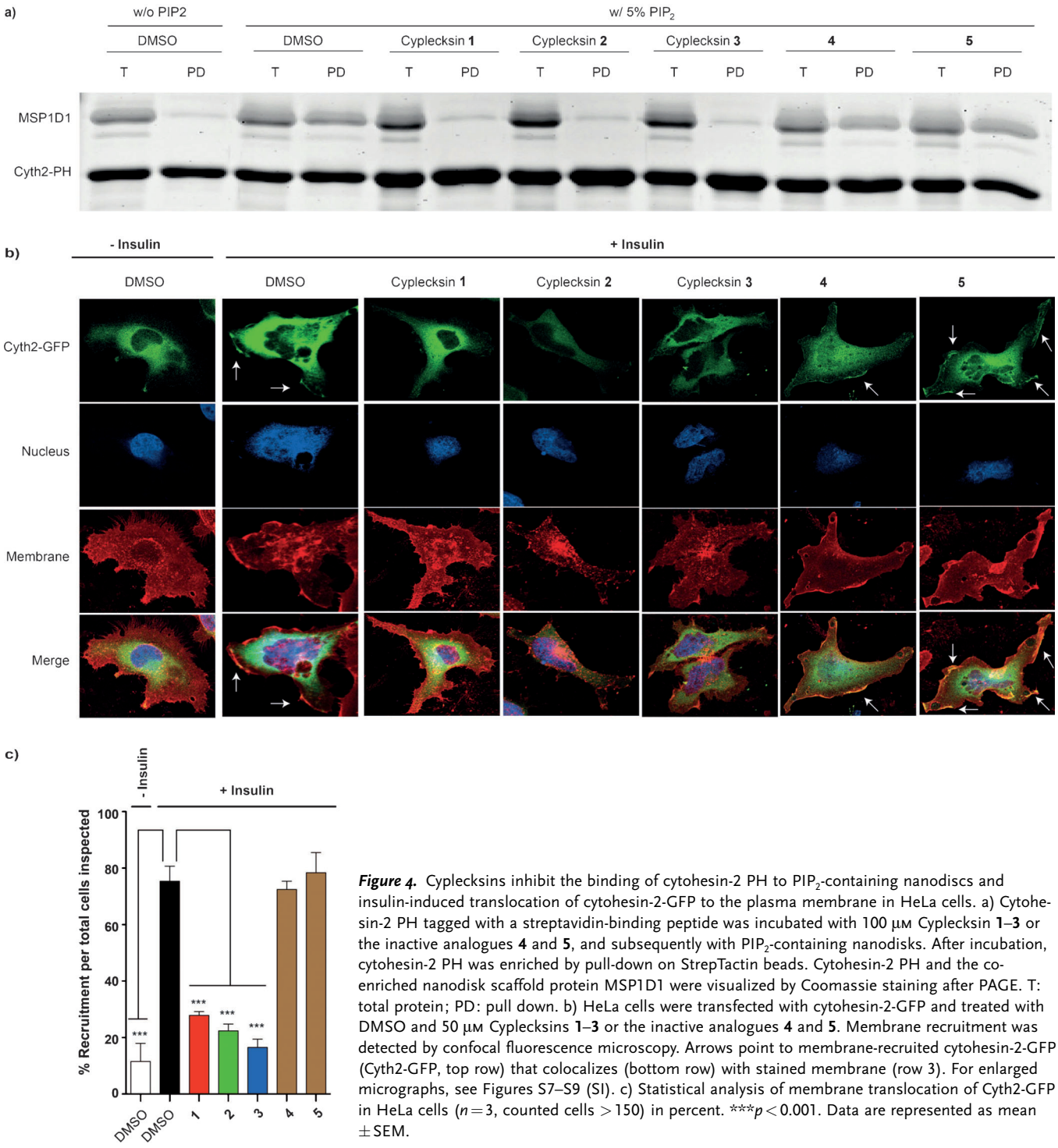

Figure 4. Cypleckxins inhibit the binding of cytohesin-2 PH to PIP_2-containing nanodiscs and insulin-induced translocation of cytohesin-2-GFP to the plasma membrane in HeLa cells. a) Cytohesin-2 PH tagged with a streptavidin-binding peptide was incubated with 100 μM Cypleckxin **1**–**3** or the inactive analogues **4** and **5**, and subsequently with PIP_2-containing nanodisks. After incubation, cytohesin-2 PH was enriched by pull-down on StrepTactin beads. Cytohesin-2 PH and the co-enriched nanodisk scaffold protein MSP1D1 were visualized by Coomassie staining after PAGE. T: total protein; PD: pull down. b) HeLa cells were transfected with cytohesin-2-GFP and treated with DMSO and 50 μM Cypleckxins **1**–**3** or the inactive analogues **4** and **5**. Membrane recruitment was detected by confocal fluorescence microscopy. Arrows point to membrane-recruited cytohesin-2-GFP (Cyth2-GFP, top row) that colocalizes (bottom row) with stained membrane (row 3). For enlarged micrographs, see Figures S7–S9 (SI). c) Statistical analysis of membrane translocation of Cyth2-GFP in HeLa cells ($n = 3$, counted cells > 150) in percent. $^{***}p < 0.001$. Data are represented as mean ± SEM.

hesin PH domains, but did not discriminate within the cytohesin family. In this respect, Cypleckxins behave similar to SecinH3, a Sec7 domain-specific cytohesin inhibitor that targets cytohesins 1–3 but not other Sec7 domains. Whether Cypleckxins are cytohesin-specific over the entire human proteome, which contains more than 250 proteins with PH domains,[12] remains to be investigated.

PH domains are widely distributed within the proteomes of higher organisms.[13] They share a common and highly conserved three-dimensional architecture without similarities in their primary sequence. Besides the common structural elements, PH domains contain variable loop regions that connect antiparallel β-sheets within a β-sandwich motif, and often contribute to ligand specificity to some extent.[14] Recently it was shown that certain PH domains possess the specificity required for discriminating between various inositol pentakisphosphate isomers, while others could not.[15] These structural differences may allow for recognition of

specific PH domains by a given inhibitor despite the overall similarity of this domain. Another study reported the identification of PITenins, a class of (thio)urea derivatives that inhibited PIP_3-binding of the Akt-PH domain, from the screening of roughly 50 000 compounds.[16] PITenins exhibited K_d values of 20–40 μM towards a distinct subset of PIP_3-specific PH domains, and affected cytohesin PH domain binding to PIP_3 with even lower affinities.

A remarkable feature of the Cyplecksins described here is the fact that they are covalent inhibitors. To our knowledge, Cyplecksins represent the first example of covalent inhibitors for PH domains, and even for any kind of GEF protein. This feature not only has potential advantages with respect to the prospect of future drug development of this class of compounds, particularly regarding prolonged pharmacodynamics, selectivity, and potency.[17] It is also an important property that will facilitate the use of Cyplecksins as research tools to further elucidate the biological function of cytohesins, especially the interplay between their Sec7 and PH domains, including structure-functional analysis, binding-site determination, and rational design.[18] Moreover, it will now become possible to chemically couple Cyplecksins with the cytohesin Sec7-domain-specific small-molecule inhibitors of the Secin class.[6,19] This might allow the simultaneous targeting of two distinct domains of the cytohesin family, thus opening up exciting new avenues in developing greatly improved cytohesin inhibitors for the validation of this interesting class of proteins as drug targets.

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