ChemPubSoc Europe

DOI: 10.1002/cbic.201500535

CHEMBIOCHEM Full Papers

SPECIAL ISSUE

Development of Bifunctional Inhibitors of Polo-Like Kinase 1 with Low-Nanomolar Activities Against the Polo-Box Domain

Andrej Scharow,[a] Daniel Knappe,[b] Wolfgang Reindl,[c, d] Ralf Hoffmann,[b] and Thorsten Berg*[a]

Polo-like kinase 1 (Plk1), a validated cancer target, harbors a protein–protein interaction domain referred to as the polo-box domain (PBD), in addition to its enzymatic domain. Although functional inhibition either of the enzymatic domain or of the PBD has been shown to inhibit Plk1, so far there have been no reports of bifunctional agents with the potential to target both protein domains. Here we report the development of Plk1 inhibitors that incorporate both an ATP-competitive ligand of the enzymatic domain, derived from BI 2536, and a functional inhibitor of the PBD, based either on the small molecule poloxin-2 or on a PBD-binding peptide. Although these bifunctional agents do not seem to bind both protein domains simultaneously, the most potent compound displays low-nanomolar activity against the Plk1 PBD, with excellent selectivity over the PBDs of Plk2 and Plk3. Our data provide insights into challenges and opportunities relating to the optimization of Plk1 PBD ligands as potent Plk1 inhibitors.

Introduction

The serine/threonine kinase polo-like kinase (Plk1) is a key regulator of mitosis.[1] It is upregulated in numerous tumors and has been identified as a negative prognostic marker for tumor patients.[2] Because tumor cells are thought to be more dependent on Plk1 than non-transformed cells, much effort has been directed to the development of small-molecule inhibitors of Plk1 enzyme activity.[3] One of the most advanced inhibitors of the enzyme's ATP binding sites is the compound BI 2536 (Figure 1, below),[4] which has been investigated in clinical trials.[5] BI 2536 displays low-nanomolar potency against the kinase domain of Plk1 (IC_{50} = 0.83 nM), but is also active against the kinase domains of Plk2 (IC_{50} = 3.5 nM) and Plk3 (IC_{50} = 9.0 nM).[4b] Activity profiling of BI 2536 by a chemical proteomics approach identified additional kinases inhibited by BI 2536, including death-associated protein kinases (DAPKs) and calcium/calmodulin-dependent protein kinase kinase 2 (CAMKK2).[6] This illustrates the key problem of ATP-competitive protein kinase inhibitors: because of the large number of protein kinases (ca. 500) and the presence of other ATP hydrolases, all of which contain conserved ATP binding pockets, in the proteome, even highly optimized compounds suffer from limited specificity against other kinases.[7]

In the light of these considerations, targeting the polo-box domain (PBD) has emerged as a promising alternative approach to the inhibition of Plk1.[8] The Plk1 PBD mediates protein–protein interactions of Plk1, enabling correct localization of Plk1 and docking of the enzyme to a subset of its substrates. To date, only four proteins are known to possess a PBD (Plk1, Plk2, Plk3, and Plk5).[9] This places Plk1 PBD inhibitors in a privileged position in terms of specificity. Functional inhibition of the Plk1 PBD by small-molecule agents has been shown to induce mitotic arrest and apoptosis in tumor cells,[10] and so has been validated as a useful approach to Plk1 inhibition. We presented the natural product derivative poloxin[10a] as the first small-molecule inhibitor of the Plk1 PBD, and recently reported on its development to the optimized analogue poloxin-2, displaying superior activity and selectivity for the Plk1 PBD.[11] Additional demonstration of the suitability of the Plk1 PBD as a target for Plk1 inhibition has been provided by peptide-based agents,[12] many of which display low-nanomolar activities against the Plk1 PBD and high selectivities over the PBDs of Plk2 and Plk3.[13]

Here we propose a new concept for the design of Plk1 inhibitors offering the potential for particularly high activity and selectivity for Plk1. We present synthetic methodology for the generation of bifunctional Plk1-targeting agents incorporating both an inhibitor of its PBD and a ligand of the Plk1 ATP bind-

[a] Dr. A. Scharow, Prof. Dr. T. Berg
Leipzig University, Institute of Organic Chemistry
Johannisallee 29, 04103 Leipzig (Germany)
E-mail: tberg@uni-leipzig.de

[b] Dr. D. Knappe, Prof. Dr. R. Hoffmann
Leipzig University, Institute of Bioanalytical Chemistry
Center for Biotechnology and Biomedicine (BBZ)
Deutscher Platz 5, 04103 Leipzig (Germany)

[c] Dr. W. Reindl
Max Planck Institute of Biochemistry, Department of Molecular Biology
Am Klopferspitz 18, 82152 Martinsried (Germany)

[d] Dr. W. Reindl
Present address: Evotec AG, Manfred Eigen Campus
Essener Bogen 7, 22419 Hamburg (Germany)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201500535.

This manuscript is part of a joint Special Issue between ChemBioChem and ChemMedChem on Protein–Protein Interactions.

ing site. We designed two classes of target molecules, based either on a peptidic ligand of the Plk1 PBD or on a non-peptidic functional inhibitor of the Plk1 PBD (Figure 1). The ATP-competitive Plk1 ligand BI 2536[4b] was incorporated into both classes of target molecules (Figure 1 A). The X-ray structure of the Plk1 kinase domain in complex with BI 2536 shows that the methylpiperidino moiety protrudes from the binding pocket (Figure 1 B),[14] and this suggests this part of the molecule as a suitable attachment point for the placement of a linker molecule. For the peptide-ligand-based molecules, we chose the peptide sequence PLHSpTA (pT = phosphothreonine, Thr(PO)$(OH)_2$), which has been reported as a selective Plk1 PBD ligand.[15] The X-ray structure of the Plk1 PBD in complex with the peptide Ac-PLHSpT demonstrates that the N terminus of the peptide extends beyond the PBD (Figure 1 C and D).[15] Therefore, we envisioned the synthesis of bivalent molecules in which the ATP-competitive ligand would be coupled as a carboxylic acid to the N terminus of the peptide sequence (Figure 1 E). For the non-peptide-based inhibitors, we chose the small molecule poloxin-2 (Figure 1 C, E).[11] Because it has proved challenging to obtain structural information about the binding site of poloxin[16] and its derivatives,[11] a large range of linker lengths had to be explored in order to increase the chances of achieving simultaneous binding of both functional parts of the molecule to Plk1.

Results and Discussion

The building blocks based on the ATP-competitive ligand BI 2536 were prepared by starting with the reported synthesis.[17] D-2-Aminobutyric acid (**1**) was converted into its methyl ester **2**, which was subjected to reductive amination conditions in the presence of cyclopentanone, providing the secondary amine **3** (Scheme 1). Nucleophilic aromatic substitution of 2,4-dichloro-5-nitropyrimidine with **3** yielded **4**, which was cyclized to the dihydropteridinone **5** by treatment with Fe in acetic acid. N-Methylation of **5** provided **6**, which was converted into **7** by treatment with 4-amino-3-methoxybenzoic acid.[17] Amide coupling between **7** and 4-amino-1-Boc-piperidine provided **8**. N-Deprotection of **8** afforded the amine **9** (overall yield 22% over eight steps), which served as the BI 2536-based building block for the generation of poloxin-2-based bifunctional Plk1 inhibitors. Further modification of **9** with 8-bromooctanoyl benzoate (**10**) afforded **11**, which was debenzylated by catalytic hydrogenolysis to afford **12** (overall yield 14% over eleven steps). Compound **12** was used as the BI 2536-based building block for the synthesis of the peptide-based bifunctional Plk1 ligands.

Synthesis of the building block **20**, based on the Plk1 PBD inhibitor poloxin-2, was carried out in a six-step procedure (Scheme 2). 4-Bromo-2-methylbenzoic acid (**13**) was converted into its methyl ester **14** and subsequently into the nitrile **15**. Reduction of the nitrile function by hydrogenolysis afforded the amine **16**, which was subsequently coupled to pent-4-ynoic acid. After ester hydrolysis of **17**, the acid **18** was coupled to the oxime **19**, providing the desired building block **20** in 18% total yield.

The three linker molecules **24**, **28**, and **30** were designed for connecting the poloxin-2-based alkyne **20** and the BI 2536-derived amine **9** (Scheme 3). The chemical nature of the linker molecules was guided by our aim to keep the polarity of the target molecules low to facilitate their purification on silica,

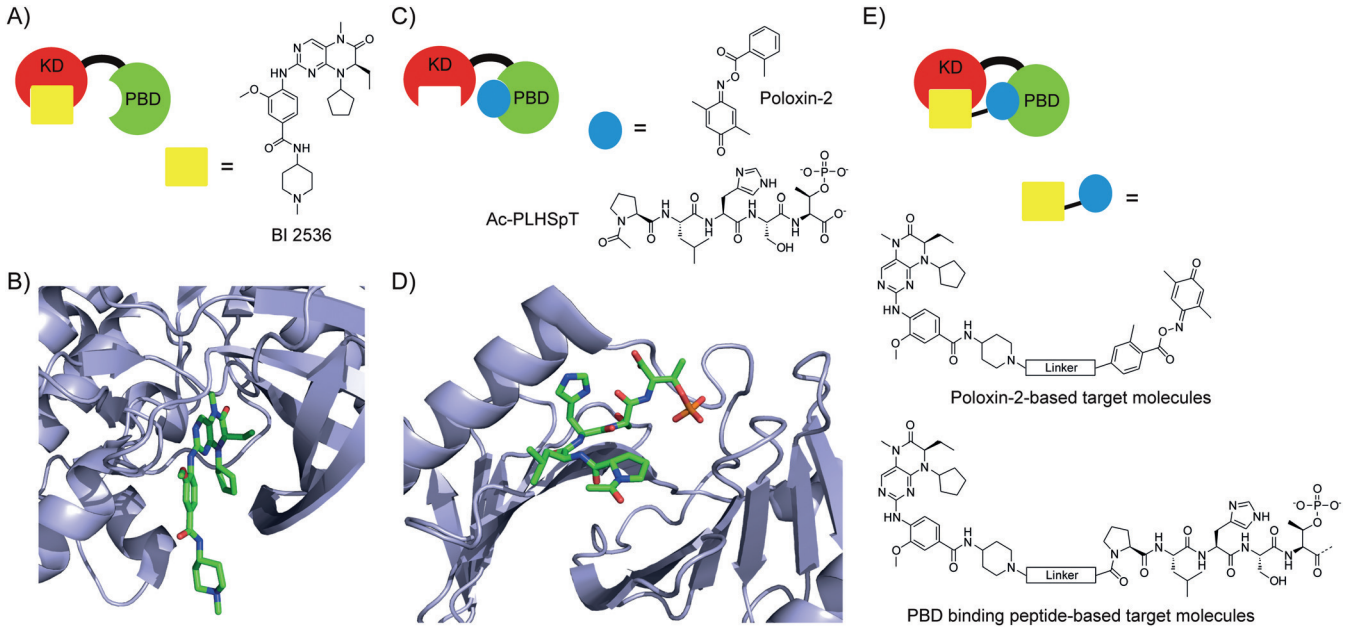
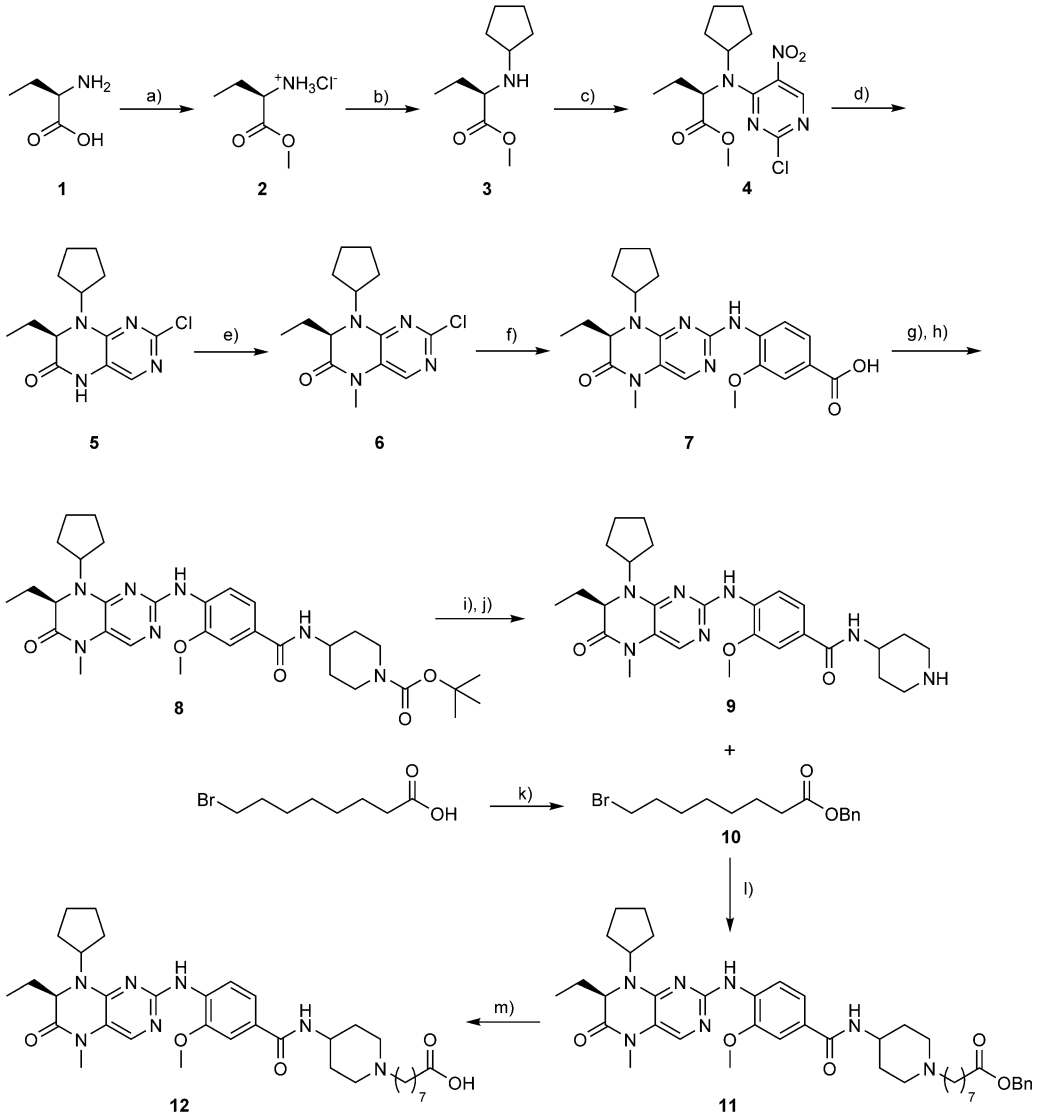


Figure 1. Design principle of bifunctional Plk1 inhibitors. A) ATP-competitive ligands such as BI 2536 bind to and inhibit the Plk1 kinase domain (KD). B) Binding of BI 2536 to the kinase domain of Plk1, based on PBD entry 2RKU.[14] C) Both poloxin-2 and the peptide PLHSpT are functional inhibitors of the polo-box domain. D) Binding mode of Ac-PLHSpT, based on PBD entry 3HIK.[15] E) Bifunctional ligands incorporating ligands of both the kinase domain and the PBD have the potential to inhibit the functions of both domains simultaneously. General structures of the target compounds based on poloxin-2 and the PBD-binding peptide are shown.

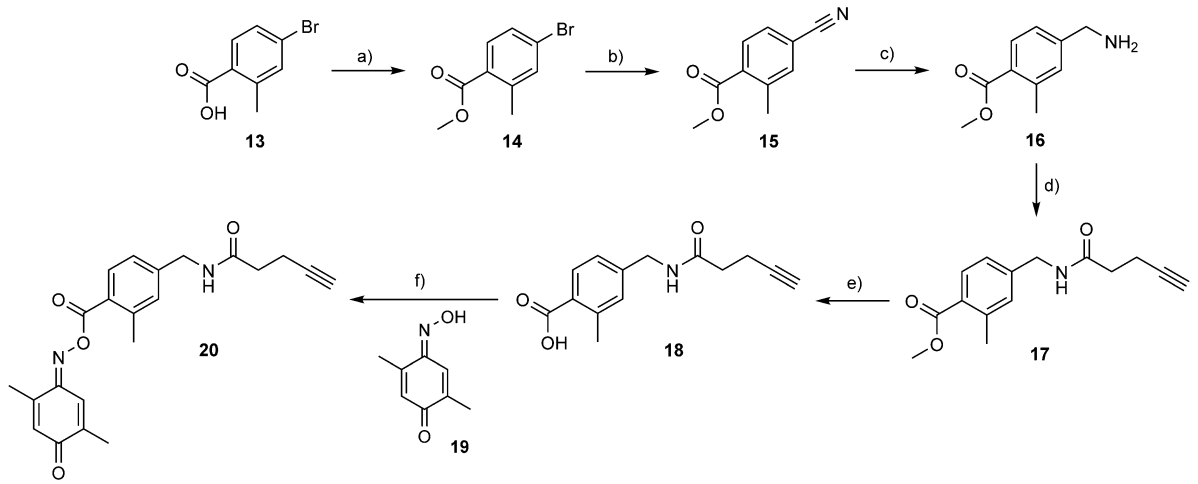


Scheme 1. Synthesis of the building blocks **9** and **12** based on BI 2536, a ligand of the ATP-binding-pocket ligand of Plk1. a) $SOCl_2$, MeOH, 0 °C to reflux, 90 min (quant.); b) cyclopentanone, NaOAc, $NaB(OAc)_3H$, CH_2Cl_2, RT, 16 h (quant.); c) 2,4-dichloro-5-nitropyrimidine, K_2CO_3, acetone, 0 °C to RT, 16 h (54%); d) Fe powder, HOAc, 70–110 °C, 3.75 h (52%); e) DMA, NaH, MeI, −10 °C to RT, 90 min (quant.); f) 4-amino-3-methoxybenzoic acid, EtOH, H_2O, HCl_{conc}, reflux, 48 h (85%); g) DMF, HBTU, DIA, RT, 30 min; h) 4-amino-1-Boc-piperidine, RT, 3 h (92%); i) TFA, CH_2Cl_2, 0 °C, 3 h; j) K_2CO_3, MeOH, 0 °C, 10 min (quant.); k) BnBr, DMF, K_2CO_3, RT, 16 h (90%); l) DMF, K_2CO_3, RT, 36 h (63%); m) EtOH, H_2, Pd/C, RT, 30 min (quant.).

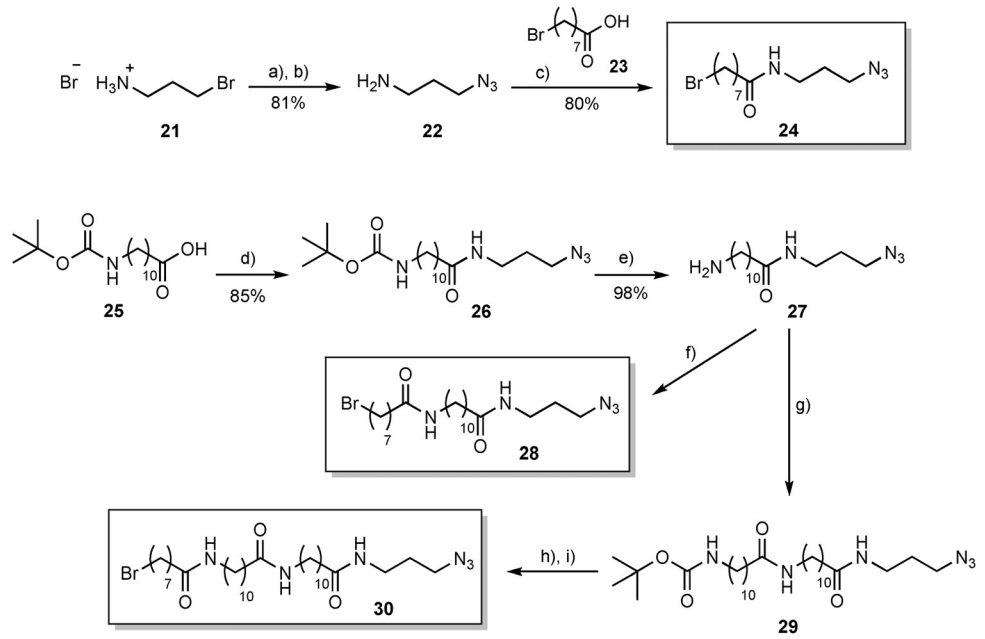
whilst exploiting the reliability of amide coupling chemistry. Synthesis of the linkers was carried out by use of standard coupling techniques in total yields of 65% (for **24**), 67% (for **28**), and 29% (for **30**).

Coupling of the two functional parts of the molecule through the linkers was carried out in a two-step synthesis. Firstly, the BI 2536-derived amine **9** was coupled with the bromoazides **24**, **28**, and **30**, to provide the azides **31**, **32**, and **33**, respectively (Scheme 4). Subsequently, the alkyne-bearing poloxin-2 derivative **20** was coupled to the azides, resulting in the final bifunctional ligands **34**–**36**. In these bifunctional molecules, the piperidine nitrogen atom (located at the surface of the ATP-binding pocket) and the poloxin-2 derivative's ester carbonyl group (thought to be attacked by a nucleophilic amino acid in the Plk1 PBD) are separated by 25 (compound **34**), 37 (compound **35**), or 49 (compound **36**) C–C or C–N bonds, corresponding to maximum distances of approximately 31 to 62 Å in an extended conformation. The synthesis of longer linkers was hampered by insufficient solubility. The optimal linker length is not known, because there is at present no available crystal structure of full-length Plk1 from which the distance between the ligands of the two domains might be assessed. In addition, the binding site of poloxin and its derivatives on the PBD has not been identified.

For the synthesis of the peptide-based building block, the known PBD ligand PLHSpTA[15] was synthesized by the Fmoc/*t*Bu-strategy on Tentagel R PHB resin (Scheme 5). A glycine residue was added to the N terminus to facilitate coupling of linkers consisting of one, five, 12, and 20 ethylene glycol units. Ethylene-glycol-based linkers were chosen for the peptide-



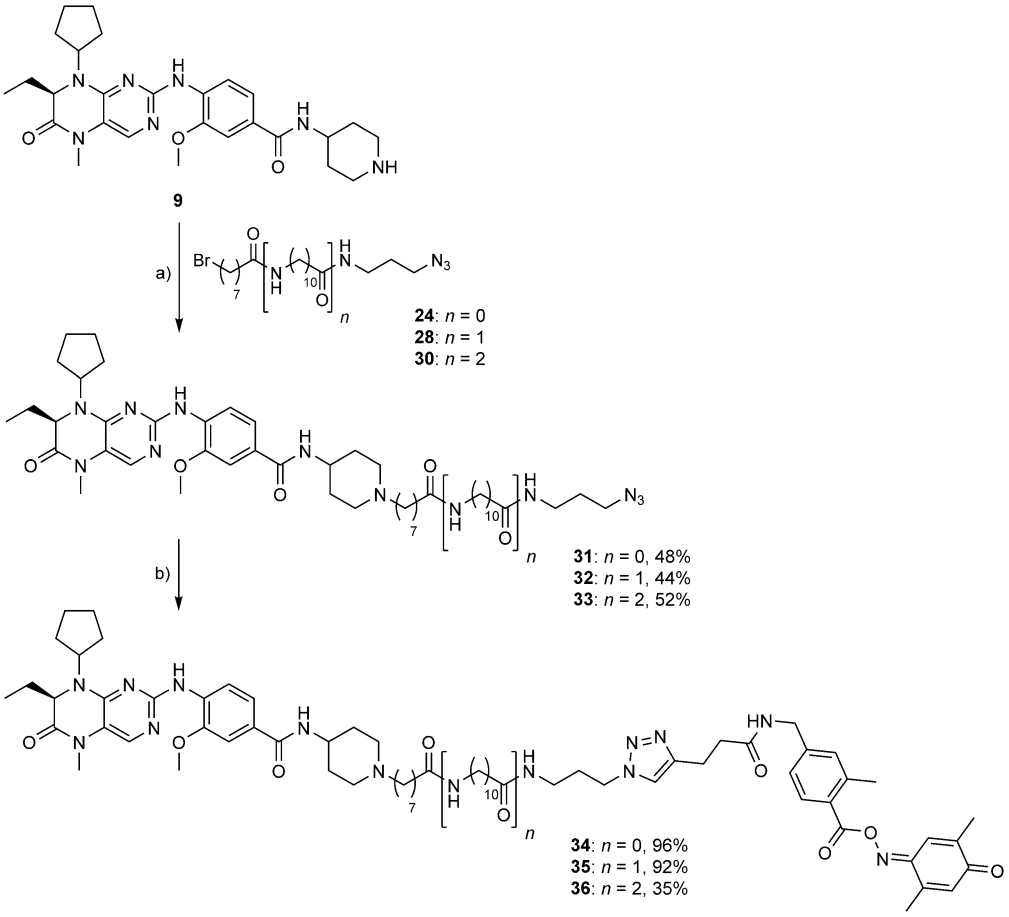
Scheme 2. Synthesis of the alkyne-functionalized poloxin-2 derivative **20**. a) $SOCl_2$, MeOH, 0 °C to 50 °C, 14 h, 96%; b) $Zn(CN)_2$, $Pd(PPh_3)_4$, DMF, 100 °C, 2 h, 88%; c) H_2, Pd/C, MeOH, RT, 12 h, 36%; d) pent-4-ynoic acid, DCC, DMAP, CH_2Cl_2, RT, 14 h, 72%; e) 1 M NaOH, THF, RT, 14 h, 93%; f) DCC, DMAP, CH_2Cl_2, RT, 14 h, 87%.



Scheme 3. Synthesis of the linker molecules **24**, **28**, and **30**. a) NaN_3, H_2O, reflux, 7 h; b) KOH, −15 °C, 10 min; c) EDC, HOBt, DIPEA, CH_2Cl_2, RT, 14 h; d) **22**, EDC, HOBt, DIPEA, RT; e) TFA, CH_2Cl_2, 0 °C, 2.5 h; f) **23**, EDC, HOBt, DIPEA, CH_2Cl_2, RT, 14 h, 81%; g) **25**, EDC, HOBt, DIPEA, CH_2Cl_2, RT, 14 h, 65%; h) 4 M HCl, MeOH, RT, 1 h, 99%; i) **23**, EDC, HOBt, DIPEA, CH_2Cl_2, RT, 14 h, 54%.

based bifunctional compounds to facilitate compound purification by reversed-phase HPLC and to promote high solubility of the final products in aqueous buffer. The BI 2536 derivative **12** was coupled to the amino group of the linkers. After cleavage from the resin, HPLC purification afforded the target compounds **37a–d** in high purities. Depending on the linker lengths, the distances between the nitrogen atom of the piperidine moiety and the carboxylic acid function of the glycine residue range from 18 C–C/C–N bonds (**37a**) to 75 bonds (**37d**) C–C/C–N, corresponding to maximum distances of approximately 23 to 95 Å in an extended conformation.

Analysis of the biological activities of the bifunctional compounds was carried out in a competitive binding assay for the Plk1 PBD based on fluorescence polarization, with full-length Plk1. Simultaneous binding of the bifunctional ligands both to the PBD and to the ATP-binding pocket should manifest itself in increased inhibitory activity against the Plk1 PBD, relative to the corresponding PBD ligand alone. However, the bifunctional ligands **34–36** were less active than the poloxin-2-based building block **20** (Figure 2A), thus suggesting that the BI 2536-derived part of the molecule does not interact with its target site when the PBD inhibitor binds to the PBD. The alkyne function-

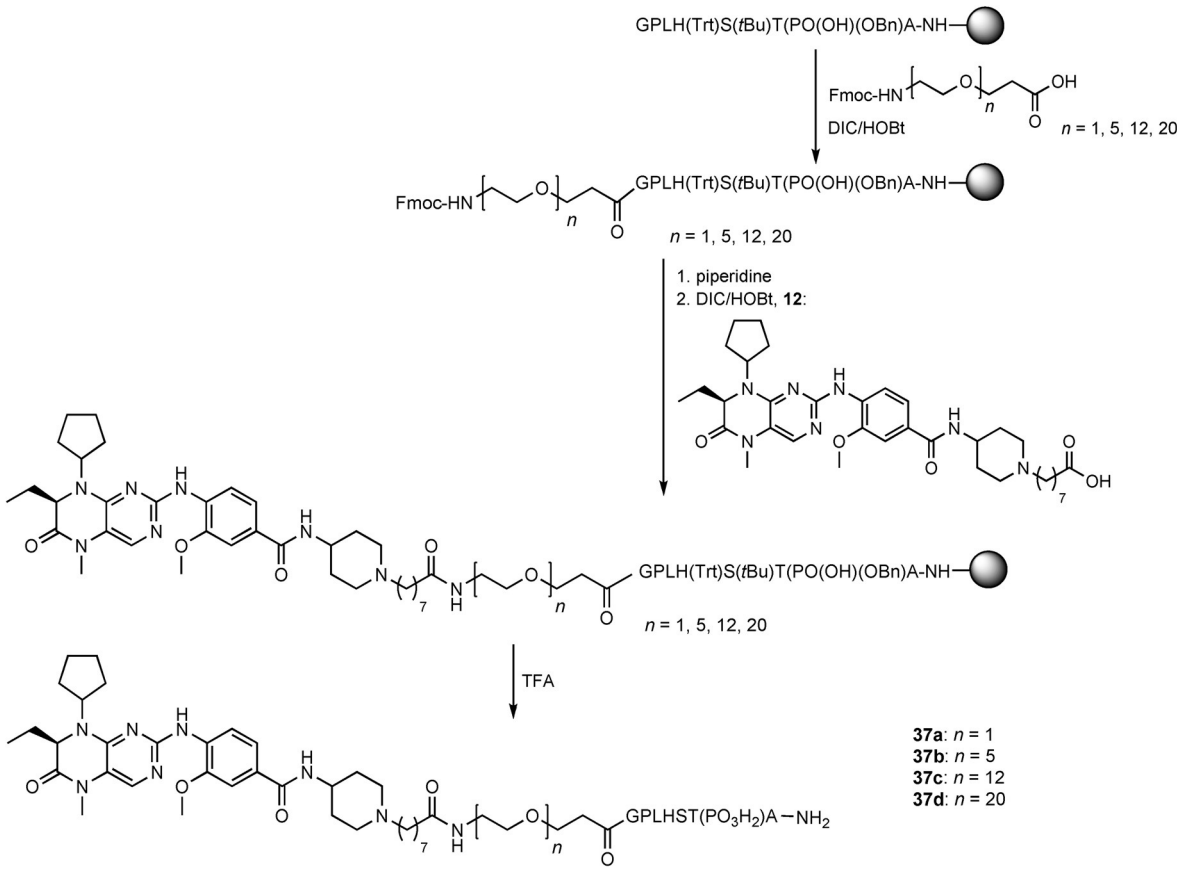


Scheme 4. Synthesis of the poloxin-2-based bifunctional ligands **34**–**36**. a) K_2CO_3, DMF, RT, 24 h; b) **20**, CuI, DIPEA, HOAc, CH_2Cl_2, RT, 2.5–14 h.

ality in **20** did not negatively affect the inhibitory activity against the Plk1 PBD, because **20** displayed inhibitory activity similar to that of the corresponding poloxin derivative **38** (Figure S1 in the Supporting Information).[11] One potential explanation for the observation that the activity of **34**–**36** against the Plk1 PBD is lower than the activity of the control compound **20** is an increased entropic penalty upon binding of **34**–**36** to Plk1 through their PBD targeting moiety, which, in the absence of simultaneous binding of the BI 2536-derived part of the molecules, is not compensated by an enthalpic gain.

In contrast to the poloxin-based molecules **34**–**36**, analysis of the peptide-based bifunctional molecules **37** against full-length Plk1 revealed that all bifunctional compounds **37a**–**d** displayed significantly higher activities than the control peptide Ac-GPLHSpTA-NH_2 (Figure 2B and Table 1). Notably, the activities of compounds **37a**–**d** increased with decreasing linker length, which argues against the possibility of nonspecific interactions between the linker and the protein as the driving force for activity. The shortest bifunctional molecule **37a** ($IC_{50} = 38 \pm 2$ nM) was an order of magnitude more active than the peptide Ac-GPLHSpTA-NH_2 ($IC_{50} = 369 \pm 15$ nM) in terms of IC_{50} values, representing a more than 25-fold increase in terms of the inhibition constants [K_i (**37a**) $= 6 \pm 1$ nM; K_i (Ac-GPLHSpTA-NH_2) $= 166 \pm 8$ nM]. Thus, fusion of the ATP-competitive moiety to the peptide is a valid means to improve activity of PBD ligands against the Plk1 PBD. Simultaneous titration of the peptide Ac-GPLHSpTA-NH_2 in the presence of 160 nM BI 2536 was less effective than titration with the peptide alone ($IC_{50} = 567 \pm 80$ nM). This observation can be explained by the concept of autoinhibition, by which the catalytic domain and the polo-box domain bind to and thereby inhibit one another.[8b] Addition of BI 2536 is expected to relieve inhibition of the Plk1 PBD by the enzyme's active site, rendering the PBD more susceptible to binding of ligands. Consistently, BI 2536 increased binding between full-length Plk1 and 5-carboxyfluorescein-GPMQSpTPLNG (Figure S2). It is currently not understood why the relief of PBD inhibition by the kinase domain increases the affinity of the PBD for the fluorophore-labeled peptide 5-carboxyfluorescein-GPMQSpTPLNG by a larger extent than for the peptide Ac-GPLHSpTA-NH_2. However, the data exclude the possibility that the increased activities of **37a**–**d** for the PBD are merely caused by relief of autoinhibition in the presence of an ATP binding site ligand.

To analyze whether the improved binding of the bifunctional ligands **37a**–**d** to the Plk1 PBD was caused by simultaneous binding to the ATP-binding site, we carried out competition assays with the bifunctional ligands **37a**–**d** at a fixed concentration of 160 nM, corresponding to 60–80% inhibition of the PBD, together with varying concentrations of BI 2536. If simultaneous bivalent binding of **37a**–**d** to both protein domains of



Scheme 5. Synthesis of bivalent compounds **37a–d**.

Plk1 occurs, titration of free BI 2536 should decrease the activities of **37a–d** to a significant extent. However, in the presence of up to 5 μM of BI 2536, only a minor reduction in activity against the Plk1 PBD was observed (Figure S3). Because Ac-GPLHSpTA-NH_2, the peptide part of **37a–d**, is also slightly less active in the presence of BI 2536 (Figure 2 and Table 1), these data suggest that the bifunctional agents **37a–d** were not simultaneously targeting the ATP-binding site and the Plk1 PBD.

To interrogate the binding mode of **37a–d** in more detail, we analyzed their effects on the enzymatic activity of full-length Plk1. Simultaneous inhibition both of the PBD and of the kinase domain should lead to an increased inhibitory activity of the bivalent molecules against the enzymatic activity of Plk1 and/or improved selectivity. To analyze this question, we tested the ability of Plk1, Plk2, and Plk3 to phosphorylate a suitable peptide substrate on a specific serine residue in the presence of the test compounds. The control compound BI 2536 inhibited the enzymatic activity of Plk1 with an IC_{50} of 0.78 ± 0.06 nM (Table 2 and Figure S4). Plk2 and Plk3 were inhibited to lesser extents [IC_{50} (Plk2): 5.9 ± 0.1 nM; IC_{50} (Plk3): 13.8 ± 0.1 nM], corresponding to selectivity factors of 7 and 17 for Plk1 against Plk2 and Plk3, respectively. The bifunctional molecules **37a–d** displayed slightly reduced activities against Plk1 relative to the reference compound BI 2536, which are likely to be caused by the attachment of the linker groups. The activities of the bifunctional molecules against the enzymatic function of Plk2 and Plk3 were reduced to a similar extent as their activities against Plk1, resulting in specificity profiles similar to that of the control compound BI 2536. These data argue against simultaneous binding of both functional parts of **37a–d** to the enzymes' ATP binding site and the PBD. However, the possibility remains that binding of **37a–d** might be bivalent after all, but the large number of rotatable bonds between the peptide sequence and the BI 2536-derived part might prevent an additive or synergistic effect of simultaneous binding to both protein domains.

Activity analysis of the most potent agent **37a** against the isolated Plk1 PBD in a binding assay based on fluorescence polarization[19] also revealed high potency (IC_{50} = 0.054 ± 0.004 μM, Figure 3 and Table S1), very similar to that obtained against full-length Plk1 (IC_{50} = 0.038 ± 0.002 μM, Figure 2C and Table 1). In addition, compound **37a** was found to display more than 400-fold selectivity for the Plk1 PBD (K_i = 0.012 ± 0.001 μM) over the Plk2 PBD (K_i = 5.5 ± 0.7 μM) and more than 300-fold selectivity over the Plk3 PBD (K_i = 3.7 ± 0.2 μM).[20] The control peptide Ac-GPLHSpTA-NH_2 was also found to be highly selective for the Plk1 PBD (Figure S5 and Table S1), confirming the high selectivity of the peptide motif PLHSpTA for the Plk1 PBD as reported in the literature.[15]

The improved activities of the bifunctional agents **37a–d** against the PBD of full-length Plk1 (Figure 2C, Table 1) and of **37a** against the isolated PBD (Figure 3, Table S1) might be ex-

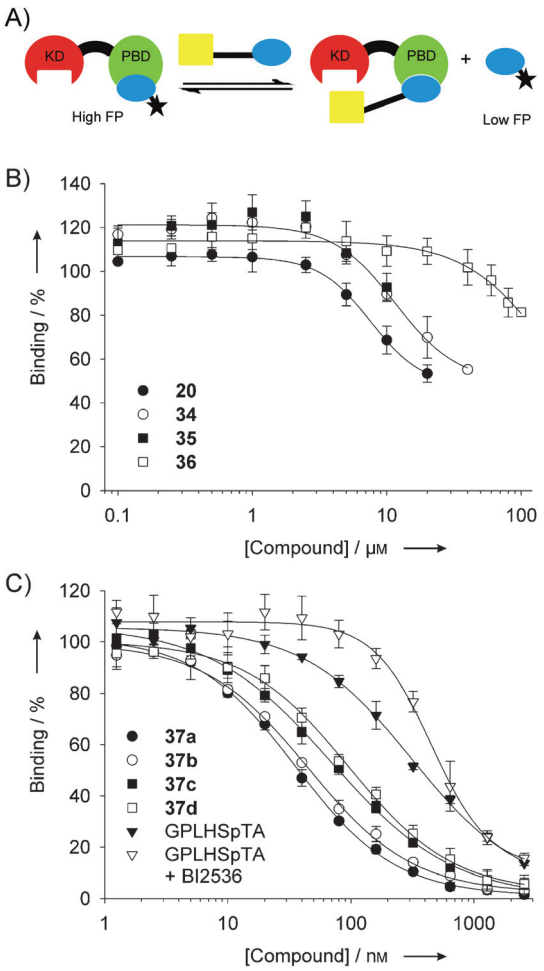


Figure 2. Activities of bivalent ligands against the PBD of full-length Plk1 as analyzed in fluorescence polarization (FP) assays. A) Principle of the assay. B) Activities of poloxin-2-based ligands **34**–**36** relative to the control compound **20**. C) Activities of peptide-based ligands **37a**–**d** relative to the PBD-binding peptide sequence GPLHSpTA.

Table 2. Activities of the bivalent agents **37a**–**d** and BI 2536 against the enzymatic function of full-length Plk1-3.

Cmpd	IC_{50} [nM] Plk1	Plk2	Plk3	Selectivity factor for Plk1 over Plk2/Plk3
BI 2536	0.78 ± 0.06	5.9 ± 0.1	13.8 ± 0.1	8/18
37a	1.95 ± 0.43	13.0 ± 1.0	26.0 ± 0.5	7/13
37b	1.84 ± 0.42	18.7 ± 1.5	41.9 ± 0.5	10/23
37c	2.62 ± 0.41	23.4 ± 1.1	61.2 ± 1.2	9/23
37d	3.53 ± 0.38	39.0 ± 2.7	80.8 ± 4.0	11/23

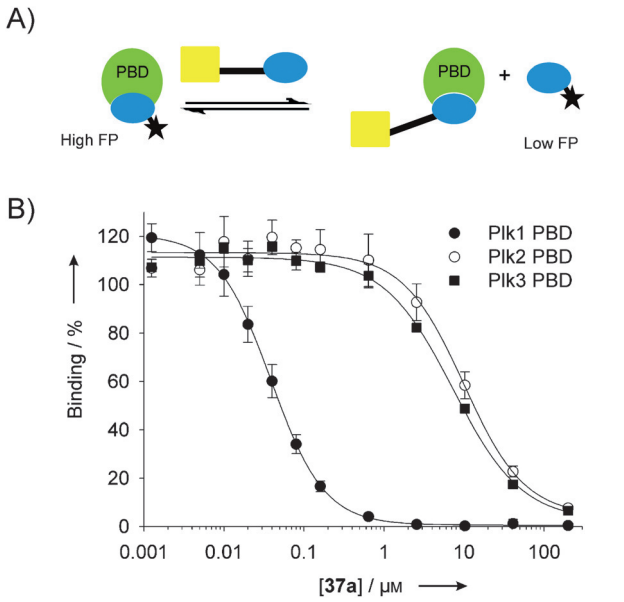


Figure 3. The bifunctional peptide **37a** selectively inhibits the function of the Plk1 PBD as analyzed by fluorescence polarization assay. A) Principle of the assay. The protein constructs used in this assay contain the PBD of the corresponding Plk only. B) Activity of **37a** against the PBDs of Plk1, Plk2, and Plk3.

Table 1. IC_{50} values against the function of the PBD in full-length Plk1 and inhibition constants (K_i) analyzed in a binding assay based on fluorescence polarization. Conversion of IC_{50} values into K_i values was carried out by use of the published equation.[18]

Compound	IC_{50} Plk1 [nM]	K_i Plk1 [nM]
Ac-GPLHSpTA-NH_2	369 ± 15	166 ± 8
37a	38 ± 2	6 ± 1
37b	57 ± 13	15 ± 6
37c	81 ± 8	27 ± 4
37d	102 ± 11	37 ± 5
Ac-GPLHSpTA-NH_2 + 160 nM BI 2536	567 ± 80	262 ± 39

plained by binding of the linker and/or the aliphatic chain of **37a**–**d** to a hydrophobic channel adjacent to the peptide-binding groove of the Plk1 PBD. Occupation of such channels has previously been reported for peptides bearing hydrophobic motifs attached to the side chains of the histidine,[13a] and also to the proline[13c] residue of the peptide sequence PLHSpT, which is also contained in **37a**–**d**. Structural analysis would be required to clarify the binding mode of the bivalent compounds **37a**–**d**.

Conclusion

In this study we present a synthetic methodology for the generation of two classes of bifunctional agents targeting both protein domains of Plk1. The bifunctional agents **34**–**37** each consist of a chemical moiety derived from the ATP-binding-pocket ligand BI 2536, and either a peptidic or a poloxin-2-based inhibitor of the Plk1 PBD (Figure 1). Although **34**–**37** do not appear to bind to both of the two protein domains simultaneously, fusion of BI 2536 to the PBD-binding peptide motif GPLHSpTA leads to agents with significantly improved activities against the Plk1 PBD. The most potent agent **37a** displays low-nanomolar activity both against the PBD of full-length Plk1 and against the isolated Plk1 PBD, together with more than 400-fold selectivity over the Plk2 PBD, and more than 300-fold selectivity over the Plk3 PBD. Once the crystal structure of full-length Plk1 becomes available, after which the length and

chemical nature of a suitable linker should be ascertainable, our methodology can be utilized for the rational design of bifunctional Plk1 inhibitors displaying simultaneous binding of both ligands to their associated protein domains. Because many protein kinases harbor functional domains in addition to their enzymatic domain, and simultaneous targeting of two domains is a means by which to overcome specificity problems of ATP-competitive kinase inhibitors, we expect our study to stimulate further research directed towards the development of bifunctional inhibitors of protein kinases.

Experimental Section

Protein expression and purification: Expression and purification of the PBDs of Plk1, Plk2, and Plk3 has been described.[10a,b,19–20] Full-length Plk1 for use in binding assays based on fluorescence polarization was expressed from Sf9 cells.

Binding assays based on fluorescence polarization: Binding assays for the PBDs of Plk1, Plk2, and Plk3 were performed essentially as described.[10a,b,19,20] In brief, proteins were incubated with the poloxin-2-based compounds at the indicated concentrations for 60 min. Subsequently, 5-carboxyfluorescein-labeled peptides (final concentration 10 nM) were added, and fluorescence polarization was analyzed after another 60 min by use of a Tecan Infinite F500 plate reader. The following carboxyfluorescein-labeled (CF-labeled) peptides were used: 5-CF-GPMQSpTPLNG-OH for Plk1, 5-CF-GPMQTSpTPKNG-OH for Plk2, and 5-CF-GPLATSpTPKNG-OH for Plk3. Compounds were tested at the following final concentrations of buffer components: NaCl (50 mM), Tris (pH 8.0, 10 mM), EDTA (1 mM), Nonidet P-40 substitute (0.1%, *v/v*), and DMSO (2%, *v/v*). Proteins were used at the following concentrations corresponding to their K_d values: 20 nM for Plk1 PBD and 50 nM for full-length Plk1 from Sf9 cells. Peptide-based compounds were tested as described above, but with additional dithiothreitol (1 mM) in the assay buffer, and were analyzed after addition of the carboxyfluorescein-labeled peptides. Proteins were used at the following concentrations: 15 nM for Plk1 PBD, 60 nM for Plk2 PBD, 200 nM for Plk3 PBD, and 40 nM for full-length Plk1 from Sf9 cells. All experiments were performed in triplicate. Inhibition curves were fitted with SigmaPlot (SPSS). Conversion of IC_{50} values into K_i values was carried out by use of the published equation.[18]

Enzyme activity assays: The IC_{50} values of BI 2536 and compounds **37a–d** against the enzymatic function of Plk1, Plk2, and Plk3 were determined by use of the Z′-LYTE Ser/Thr 16 peptide kinase assay kit (Invitrogen) according to the manufacturer's instructions. The kinase reaction buffer contained additional DTT (1 mM) and DMSO (2%). Full-length enzymes were used at the following concentrations: 1.25 ng μL^{-1} for Plk1, 2.5 ng μL^{-1} for Plk2, and 1.25 ng μL^{-1} for Plk3. The final concentration of the Z′-LYTE control phosphopeptide (ELMEFpSLKDQEA) and the Z′-LYTE peptide (ELMEFSLKDQEA) was 2 μM. ATP was used at the following concentrations: 20 μM for Plk1, 60 μM for Plk2, and 20 μM for Plk3. The phosphorylation reaction was allowed to proceed for 3 h at room temperature, followed by addition of development reagent and incubation for 1 h at room temperature. The reaction was then terminated with use of the stop reagent. Fluorescence intensities of coumarin and fluorescein moieties attached to the termini of the peptides were measured with a Tecan Infinite F500 plate reader (λ_{ex} = 405 nm, λ_{em} = 430 and 525 nm). Inhibition curves were fitted by use of SigmaPlot (SPSS). All experiments were performed in triplicate.

The synthesis of poloxin-2-based molecules **34**–**36** is described in the Supporting Information.

Peptide synthesis: Peptides were synthesized by the Fmoc/*t*Bu-strategy as C-terminal amides on Tentagel R PHB resin (0.18 mmol g^{-1}) with the aid of a multiple synthesizer (SYRO2000, MultiSynTech). Amino acid derivatives (10 equiv) were coupled by use of HBTU (10 equiv) and DIPEA (24 equiv). PEG1 and PEG5 (8 equiv), PEG12 and PEG20 (4 equiv), and compound **12** (6 equiv) were coupled manually by in situ activation with DIC/HOBt (8 equiv). Peptides were cleaved with TFA containing 12.5% (*v/v*) of a scavenger mixture (ethane-1,2-dithiol/*m*-cresol/thioanisole/water 1:2:2:2, *v/v/v/v*) for 2 h, precipitated with cold diethyl ether, and dried. Purification was by RP-HPLC with a Jupiter C18 column (10×250 mm) with use of a linear gradient of aqueous acetonitrile in the presence of TFA (0.1%) as ion pair reagent. RP-HPLC was used to evaluate peptide purities with use of a Poroshell SB-C18 column (2×100 mm) and typically a linear gradient from 3 to 57% acetonitrile in 30 min and a column temperature of 60 °C. Peptides were detected by absorbance at 214 nm. Monoisotopic masses were determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF-MS; 4700 proteomic analyzer; Applied Biosystems, GmbH, Darmstadt, Germany) with α-cyano-4-hydroxycinnamic acid [CHCA; 4 g L^{-1} in aqueous acetonitrile (60%) containing TFA (0.1%)] as matrix.

Acknowledgements

Work in the group of T.B. was generously supported by the Deutsche Forschungsgemeinschaft (BE 4572/1–1) and the Department of Molecular Biology at the Max Planck Institute of Biochemistry. We extend our thanks to Angela Berg for critical reading of the manuscript.

Keywords: bioorganic chemistry · inhibitors · peptides · protein kinases · protein–protein interactions

[1] M. Petronczki, P. Lénárt, J. M. Peters, *Dev. Cell* **2008**, *14*, 646–659.
[2] R. Knecht, C. Oberhauser, K. Strebhardt, *Int. J. Cancer* **2000**, *89*, 535–536.
[3] K. Strebhardt, *Nat. Rev. Drug Discovery* **2010**, *9*, 643–660.
[4] a) P. Lénárt, M. Petronczki, M. Steegmaier, B. Di Fiore, J. J. Lipp, M. Hoffmann, W. J. Rettig, N. Kraut, J. M. Peters, *Curr. Biol.* **2007**, *17*, 304–315; b) M. Steegmaier, M. Hoffmann, A. Baum, P. Lenart, M. Petronczki, M. Krssak, U. Gurtler, P. Garin-Chesa, S. Lieb, J. Quant, M. Grauert, G. R. Adolf, N. Kraut, J. M. Peters, W. J. Rettig, *Curr. Biol.* **2007**, *17*, 316–322.
[5] P. Schoffski, *Oncologist* **2009**, *14*, 559–570.
[6] M. Raab, F. Pachl, A. Krämer, E. Kurunci-Csacsko, C. Dötsch, R. Knecht, S. Becker, B. Küster, K. Strebhardt, *Cell. Res.* **2014**, *24*, 1141–1145.
[7] H. Daub, K. Specht, A. Ullrich, *Nat. Rev. Drug Discovery* **2004**, *3*, 1001–1010.
[8] a) A. E. Elia, L. C. Cantley, M. B. Yaffe, *Science* **2003**, *299*, 1228–1231; b) A. E. Elia, P. Rellos, L. F. Haire, J. W. Chao, F. J. Ivins, K. Hoepker, D. Mohammad, L. C. Cantley, S. J. Smerdon, M. B. Yaffe, *Cell* **2003**, *115*, 83–95.
[9] G. de Cárcer, G. Manning, M. Malumbres, *Cell Cycle* **2011**, *10*, 2255–2262.
[10] a) W. Reindl, J. Yuan, A. Krämer, K. Strebhardt, T. Berg, *Chem. Biol.* **2008**, *15*, 459–466; b) W. Reindl, J. Yuan, A. Krämer, K. Strebhardt, T. Berg, *ChemBioChem* **2009**, *10*, 1145–1148; c) N. Watanabe, T. Sekine, M. Takagi, J. Iwasaki, N. Imamoto, H. Kawasaki, H. Osada, *J. Biol. Chem.* **2009**, *284*, 2344–2353; d) H. M. Shan, Y. Shi, J. Quan, *ChemMedChem* **2015**, *10*, 158–163; e) G. Srinivasrao, J. E. Park, S. Kim, M. Ahn, C. Cheong, K. Y. Nam, P. Gunasekaran, E. Hwang, N. H. Kim, S. Y. Shin, K. S. Lee, E. Ryu, J. K. Bang, *PLoS One* **2014**, *9*, e107432.

[11] A. Scharow, M. Raab, K. Saxena, S. Sreeramulu, D. Kudlinzki, S. Gande, C. Dotsch, E. Kurunci-Csacsko, S. Klaeger, B. Kuster, H. Schwalbe, K. Strebhardt, T. Berg, *ACS Chem. Biol.* **2015**, *10*, 2570 – 2579.

[12] S. Richter, I. Neundorf, K. Loebner, M. Graber, T. Berg, R. Bergmann, J. Steinbach, J. Pietzsch, F. Wuest, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 4686 – 4689.

[13] a) F. Liu, J. E. Park, W. J. Qian, D. Lim, M. Graber, T. Berg, M. B. Yaffe, K. S. Lee, T. R. Burke, Jr., *Nat. Chem. Biol.* **2011**, *7*, 595 – 601; b) F. Liu, J. E. Park, W. J. Qian, D. Lim, A. Scharow, T. Berg, M. B. Yaffe, K. S. Lee, T. R. Burke, Jr., *ChemBioChem* **2012**, *13*, 1291 – 1296; c) F. Liu, J. E. Park, W. J. Qian, D. Lim, A. Scharow, T. Berg, M. B. Yaffe, K. S. Lee, T. R. Burke, Jr., *ACS Chem. Biol.* **2012**, *7*, 805 – 810; d) W. J. Qian, J. E. Park, D. Lim, C. C. Lai, J. A. Kelley, S. Y. Park, K. W. Lee, M. B. Yaffe, K. S. Lee, T. R. Burke, Jr., *Biopolymers* **2014**, *102*, 444 – 455; e) P. Sledz, C. J. Stubbs, S. Lang, Y. Q. Yang, G. J. McKenzie, A. R. Venkitaraman, M. Hyvonen, C. Abell, *Angew. Chem. Int. Ed.* **2011**, *50*, 4003 – 4006; *Angew. Chem.* **2011**, *123*, 4089 – 4092; f) P. Sledz, S. Lang, C. J. Stubbs, C. Abell, *Angew. Chem. Int. Ed.* **2012**, *51*, 7680 – 7683; *Angew. Chem.* **2012**, *124*, 7800 – 7803; g) M. Ahn, Y. H. Han, J. E. Park, S. Kim, W. C. Lee, S. J. Lee, P. Gunasekaran, C. Cheong, S. Y. Shin, Sr. , H. Y. Kim, E. K. Ryu, R. N. Murugan, N. H. Kim, J. K. Bang, *J. Med. Chem.* **2015**, *58*, 294 – 304.

[14] M. Kothe, D. Kohls, S. Low, R. Coli, G. R. Rennie, F. Feru, C. Kuhn, Y. H. Ding, *Chem. Biol. Drug Des.* **2007**, *70*, 540 – 546.

[15] S. M. Yun, T. Moulaei, D. Lim, J. K. Bang, J. E. Park, S. R. Shenoy, F. Liu, Y. H. Kang, C. Liao, N. K. Soung, S. Lee, D. Y. Yoon, Y. Lim, D. H. Lee, A. Otaka, E. Appella, J. B. McMahon, M. C. Nicklaus, T. R. Burke, Jr., M. B. Yaffe, A. Wlodawer, K. S. Lee, *Nat. Struct. Mol. Biol.* **2009**, *16*, 876 – 882.

[16] Z. Yin, Y. Song, P. H. Rehse, *ACS Chem. Biol.* **2013**, *8*, 303 – 308.

[17] a) G. Munzert, M. Steegmaier, *US-2006/0074088A1*, **2006**; b) G. Budin, K. S. Yang, T. Reiner, R. Weissleder, *Angew. Chem. Int. Ed.* **2011**, *50*, 9378 – 9381; *Angew. Chem.* **2011**, *123*, 9550 – 9553.

[18] Z. Nikolovska-Coleska, R. Wang, X. Fang, H. Pan, Y. Tomita, P. Li, P. P. Roller, K. Krajewski, N. G. Saito, J. A. Stuckey, S. Wang, *Anal. Biochem.* **2004**, *332*, 261 – 273.

[19] W. Reindl, K. Strebhardt, T. Berg, *Anal. Biochem.* **2008**, *383*, 205 – 209.

[20] W. Reindl, M. Gräber, K. Strebhardt, T. Berg, *Anal. Biochem.* **2009**, *395*, 189 – 194.

Manuscript received: October 14, 2015

Final article published: December 4, 2015