Organic & Biomolecular Chemistry

ROYAL SOCIETY OF CHEMISTRY

PAPER

View Article Online
View Journal

Cite this: DOI: 10.1039/d6ob00193a

Affinity enhancement of polo-like kinase 1 polo-box domain-binding peptides by *N*-methylation and lipidation†

Kohei Tsuji, *‡ Kenan Bai,‡ Xueyuan Huang‡ and Hirokazu Tamamura

Cell membrane permeability is one of the biggest issues for bioactive peptides targeting intracellular proteins because of their high polarity and flexibility. To overcome this issue, cell-penetrating peptides (CPPs) are often attached to peptides. Additional approaches include backbone modifications, such as *N*-methylation and lipidation, which increase structural rigidity and hydrophobicity. Herein, we synthesized and evaluated *N*-methylated or lipidated Plk1 PBD-binding peptides. *N*-Methylated and lipidated analogs showed up to 4-fold and 260-fold enhanced PBD affinities, respectively, as compared with the parent PBD-binding peptide. However, none of these analogs showed significant cytotoxicity against HeLa cells. These results suggest that further optimization is required to develop practical Plk1 PBD-binding inhibitors that exhibit cellular activities.

Received 3rd February 2026,
Accepted 9th March 2026

DOI: 10.1039/d6ob00193a

rsc.li/obc

Introduction

Peptides are considered to be attractive platforms for drug development. Numerous peptides and peptide-derived molecules have been approved for clinical use.[1–4] A key feature of peptidic drugs is their ability to target protein–protein interactions (PPIs) due to their extended interaction surface, which is generally difficult for targeting by small molecules.[2–5] Peptides are often of medium size, which limits their cell membrane permeability.[1,6] A noted exception is the orally administrable immunosuppressant ciclosporin A, which is an 11-mer cyclic peptide that has seven *N*-methylated amides in its backbone.[7,8] Cyclization of peptides can restrict conformational flexibility and thereby facilitate cell membrane penetration.[7] An alternative approach to fix the peptide conformation and increase hydrophobicity is by means of *N*-alkylation.[9,10] A further, often used, method to deliver peptides into the cells is to employ cell-penetrating peptides (CPPs).[11–13]

A serine/threonine kinase, polo-like kinase 1 (Plk1), is a key cell-cycle regulator during mitosis. The activity and localization of Plk1 are highly restricted at critical points in the cell cycle.[14,15] Overexpression of Plk1 can be associated with aggressiveness and a poor prognosis of cancers observed in various cancer types, such as non-small cell lung cancer, prostate cancer, colon cancer, and breast cancer.[16–22] Thus, Plk1 is considered to be an attractive target for the development of anti-cancer agents.[23–25]

Plk1 contains an N-terminal kinase domain (KD), a flexible internal domain linker (IDL), and a C-terminal polo-box domain (PBD).[14,26] The latter recognizes phosphoserine and phosphothreonine (pThr, pT)-containing sequences, which serve to regulate Plk1 function and localization spatiotemporally. Plk1 actions are also modulated *via* intramolecular PPIs of the KD and PBD (resulting in "autoinhibition"), although the mechanisms by which this is achieved are not clearly understood.[14,27] Taken together, the KD and PBD are widely regarded as being "druggable" targets. A variety of KD-targeting inhibitors have been developed, and some of these have entered clinical trials.[24] However, no Plk1 inhibitor is used clinically, so continued efforts to develop Plk1 inhibitors are warranted.

Various Plk1 PBD-binding inhibitors have been developed in recent decades. An example is the pentapeptide PLHSpT (**1**) (Fig. 1), which is derived from polo-box domain interacting protein 1 (PBIP1).[28] Modification of peptide **1** by attaching alkylphenyl groups to the His sidechain imidazole π-nitrogen leads to PLH*SpT (**2**) (H* represents modification of a $-(CH_2)_8Ph$ group on the His *N*3(π)-nitrogen, Fig. 1), which has demonstrated greater than 1000-fold enhanced affinity as compared with the parent peptide **1**.[29]

Guided by the crystal structure of PLH*SpT (**2**) bound to isolated Plk1 PBD (PDB: 3RQ7), several peptides with high Plk1 affinity and selectivity among three members of the Plk family (Plk1–3) were developed by tethering aryl and heteroaryl moieties from the His *N*3(π) nitrogen,[30–32] and by replacement of

Department of Medicinal Chemistry, Laboratory for Biomaterials and Bioengineering, Institute of Integrated Research, Institute of Science Tokyo, 2-3-10 Kandasurugadai, Chiyoda-ku, Tokyo 101-0062, Japan.
E-mail: ktsuji.mr@tmd.ac.jp

†Dedicated to Professor Akira Otaka on the occasion of his retirement from Tokushima University.

‡These authors contributed equally to this work.

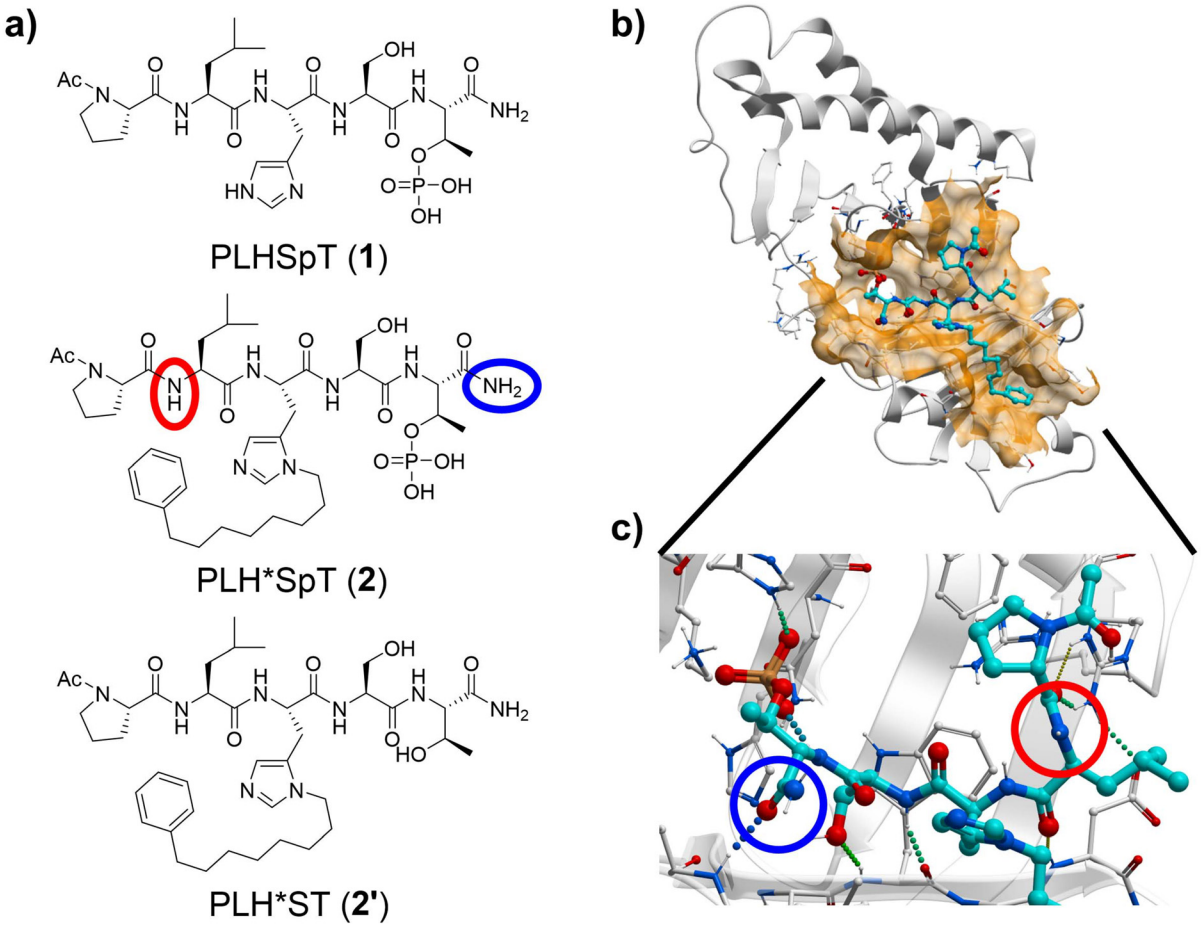


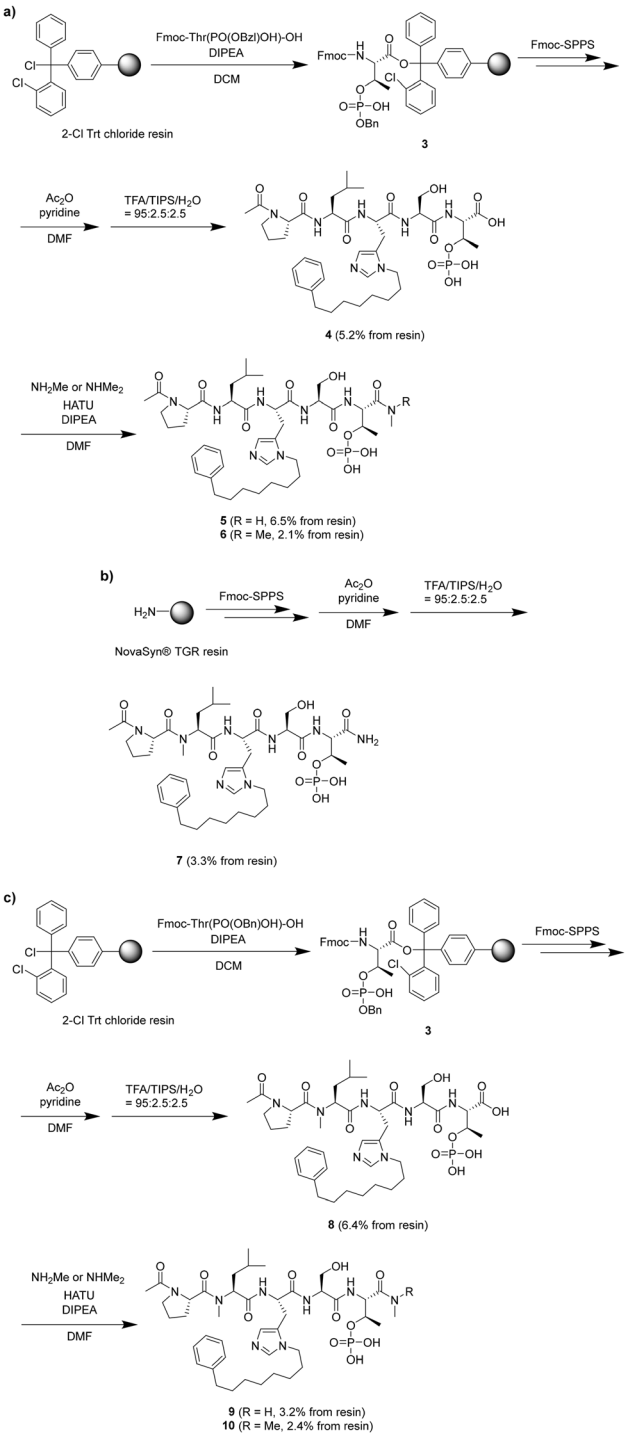
Fig. 1 Structures of pentapeptides **1**, **2**, and **2'**. (a) Chemical structures of **1**, **2**, and **2'**. (b) Co-crystal structure of Plk1 PBD and **2** (PDB: 3RQ7). (c) Enlarged structure of PBD-bound **2**. Protein shown with white ribbon and sticks: Plk1 PBD; orange surface: PBD-binding pocket of **2**; cyan stick: **2**; red, blue, and orange atoms: oxygen, nitrogen, and phosphorus atoms, respectively. Red and blue circles: modifiable amide protons.

the pT-2 histidine residue with other amino acids having long-chain alkylphenyl modifications on their side chains.[33] We have also examined the effects of cyclizing peptide **2**, utilizing a bis-alkylated His residue[34] and utilizing an orthogonally protected Glu residue.[35] Although peptide **2** and its derivatives involving cyclic peptides demonstrated extremely high affinities for Plk1 PBD in *in vitro* experiments, none of these peptides showed significant cytotoxicity, which may have been due to the low permeability of the cell membrane.[36] To overcome this issue, we developed a novel CPP-attached polo-like kinase 1 (Plk1) polo-box domain (PBD)-binding peptide, which showed significant cytotoxicity and mitotic arrest against the human histiocytic lymphoma cell line U937.[37] More recently, Gunasekaran and co-workers reported Plk1-directed proteolysis targeting chimeras (Plk1-PROTACs), DD-2 and NC1.[38–40] DD-2 and NC1 are composed of a pT-containing cyclic peptide and PLH*SpT (**2**), respectively, as Plk1 PBD ligands conjugated with 12-mer poly-arginine (R_{12}) as a dual function motif (CPP and N-degron) tethered by aminohexanoic acid. These Plk1-PROTACs demonstrated Plk1 degradation, potent cytotoxicity against several cancer cell lines, and *in vivo* anti-tumor activity in xenograft mouse models.[39,40] This work supports the potential value of overcoming issues with the cell membrane permeability of Plk1 PBD inhibitors as a viable approach to developing cancer therapeutics. Herein, we report the design, synthesis, and *in vitro* biological evaluation of *N*-methylated and lipidated peptide analogs of peptide **2** designed to achieve cell-active Plk1 PBD-targeting inhibitors.

Results and discussion

Design and synthesis of *N*-methylated analogs of peptide 2

The structure of Plk1 PBD-bound peptide **2**, displayed in Fig. 1b and c, shows that the amide protons of Ser and the N-terminus of pThr residue contribute to protein binding, while the amide protons of Leu, His, and the C-terminus of pThr are exposed to solvent. Accordingly, we examined the *N*-methylation of the Leu α-amino group and pThr C-terminal amide group (Scheme 1). The C-terminal carboxylic acid analog **4** was synthesized on 2-Cl trityl (Trt) chloride resin using standard Fmoc-based solid-phase peptide synthesis (Fmoc-SPPS) methodologies. When sidechains



Scheme 1 Synthesis of *N*-methylated peptide **2** analogs **4–10**. (a) Synthesis of peptides **4–6**. (b) Synthesis of peptide **7**. (c) Synthesis of peptides **8–10**. DIPEA: *N*,*N*-diisopropylethylamine; DCM: dichloromethane; DMF: *N*,*N*-dimethylformamide; TFA: trifluoroacetic acid; TIPS: triisopropylsilane; HATU: 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate.

of the Ser and pThr residues-protected C-terminal carboxylic acid peptide (Ac-PLH*S(^{t}Bu)T{PO(OBn)OH}-OH) was cleaved from the resin and subjected to coupling with *N*-methyl amine or *N*,*N*-dimethyl amine, a dehydroalanine-containing C-terminal *N*-alkyl peptide was formed, as evidenced by mass spectroscopy. These products possibly resulted from β-elimination of the phosphate group.[41–45] To suppress this side reaction, peptide **4**, having a free phosphate on the Thr sidechain, was used for the coupling with *N*-methyl amine or *N*,*N*-dimethyl amine. These couplings proceeded well to provide the desired *N*-alkyl peptides **5** and **6** (Scheme 1). Peptide **7**, having an *N*-Me-Leu residue, was synthesized by Fmoc-SPPS using Fmoc-*N*-Me-Leu-OH rather than Fmoc-Leu-OH. The *N*-Me-Leu-containing C-terminal carboxylic acid peptide **8**, its *N*-methyl amide analog **9**, and its *N*,*N*-dimethyl amide analog **10** were synthesized in fashions similar to the syntheses of peptides **4**, **5**, and **6**.

Design and synthesis of lipidated peptide 2 analogs

Lipidation is a common modification used in peptide drug development. Lipidation enhances cell membrane affinity, which improves cell membrane permeability.[46] It also enhances biological potency, as exemplified in the development of SARS-CoV-2 fusion inhibitor peptides.[47] Lipidation also improves the PK profiles of peptides by increasing binding to serum proteins.[48–50] The glucagon-like peptide-1 analog semaglutide is an orally administrable peptide drug for type-2 diabetes mellitus. It is used in combination with the oral absorption enhancer salcaprozate sodium (SNAC).[51,52] Semaglutide possesses a C_{18} fatty diacid on the Lys20 sidechain tethered by two 8-amino-3,6-dioxaoctanoic acid units (mini-PEG) and L-Glu (Fig. 2).[51] We envisioned that lipidation of Plk1 PBD inhibitor peptide **2** could enhance cellular activity. Previously, we found that modification of peptide **2** at the N- and C-termini was acceptable.[36,53–55] Therefore, we designed and synthesized the peptide **2** analogs **15–20** possessing semaglutide-like modifications. We also prepared their non-phosphorylated threonine derivatives **21–26** as negative controls to determine the non-specific cytotoxicity of the lipid moieties (Schemes 2 and 3). The C-terminally modified peptides **15** and **21** were synthesized on NovaSyn® TGR resin by standard Fmoc-SPPS protocols using 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)isovaleryl (ivDde)-protected Lys for C-terminal modification following chain elongation (Scheme 2a). Selective deprotection of the ivDde group on resins **11** and **12**, followed by coupling with two mini-PEG units and an L-Glu residue, gave the protected resins **13** and **14**. Subsequent global deprotection afforded peptides **15** and **21**. When the lipid moieties were attached to peptide resins **13** or **14**, the mainchain of the L-Glu residue was used as a connector, rather than the L-Glu side chain in semaglutide (Scheme 2b and c). Stearic acid or 18-(^{t}Bu)-18-oxooctadecanoic acid was coupled to resins **13** or **14**, respectively, and the resulting resins were subjected to deprotection and cleavage to afford the C-terminally lipidated peptides **16**, **17**, **22**, and **23**. The N-terminally modified peptides **18** and **24** were synthesized on NovaSyn® TGR resin by standard Fmoc-SPPS methodologies using ivDde-protected Lys for N-terminal modification following chain elongation (Scheme 3a). Lipidation of the resin **29** and **30** was achieved using stearic acid or 18-(^{t}Bu)-18-oxooctadecanoic acid, respect-

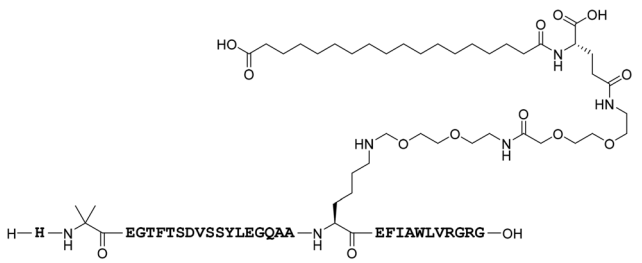
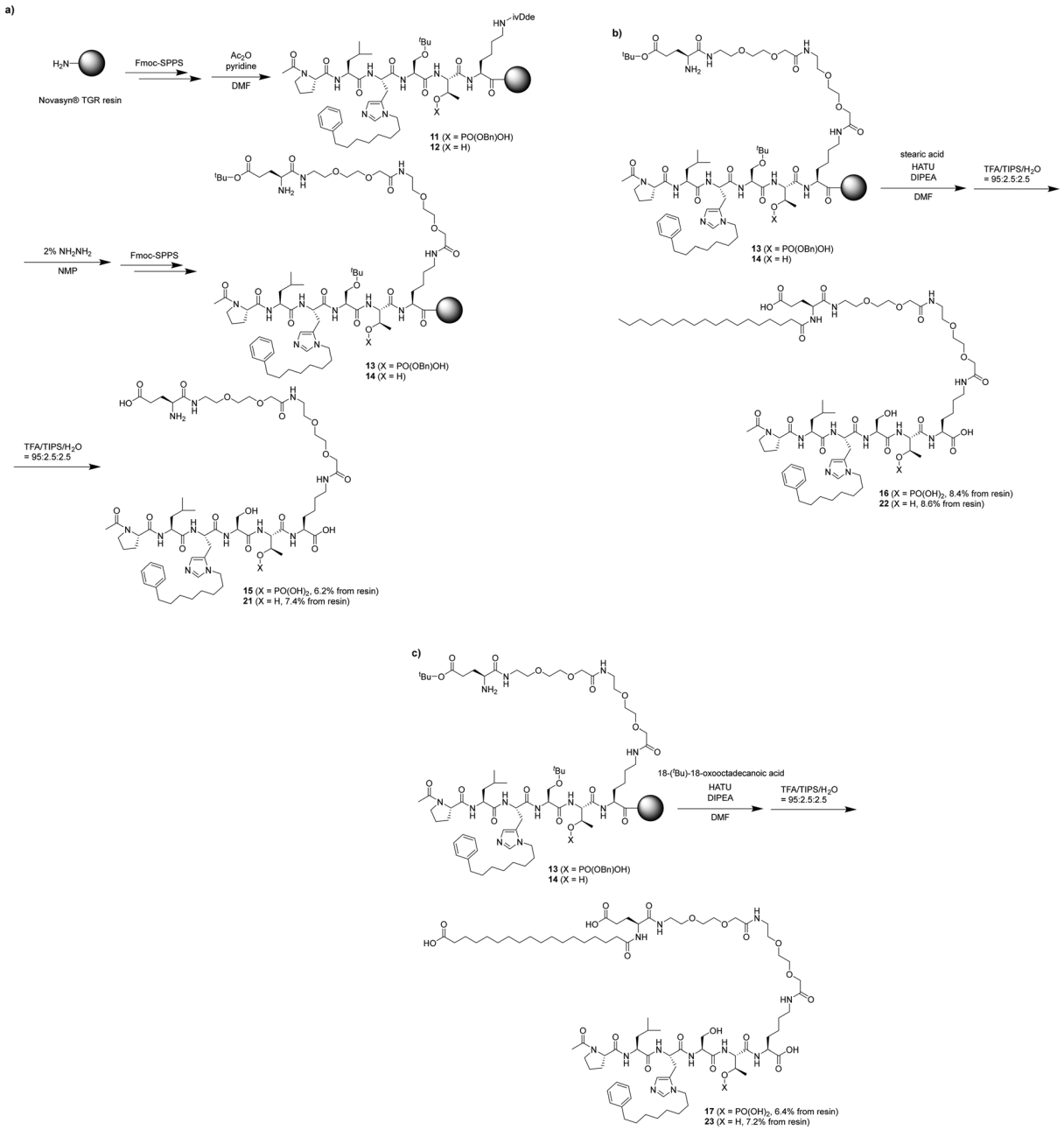


Fig. 2 Structure of semaglutide.

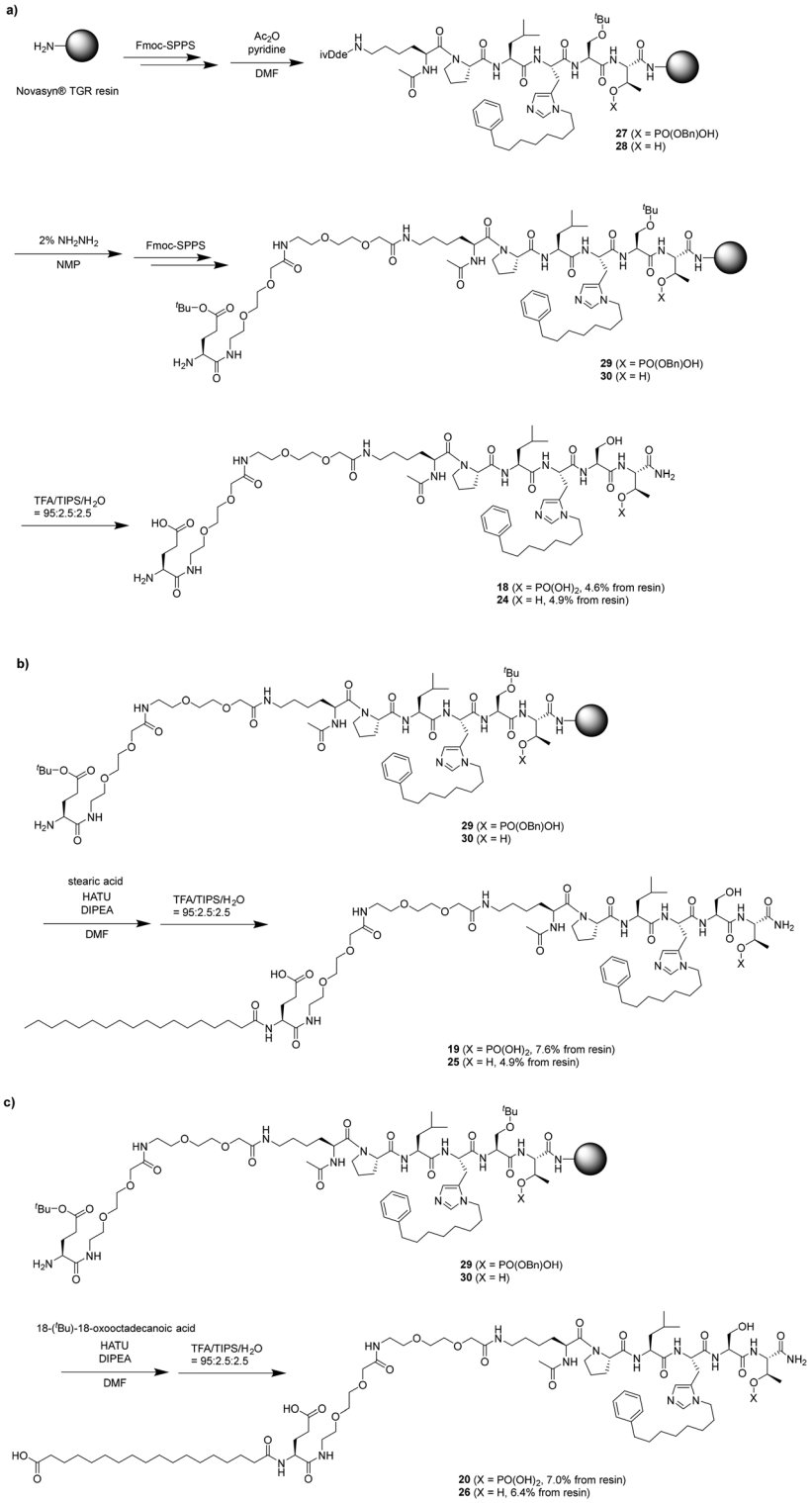
ively, to afford the N-terminally lipidated peptides **19**, **20**, **25**, and **26** (Scheme 3b and c).

Evaluation of Plk1 PBD-binding affinities and cytotoxicity of the *N*-methylated peptide 2 analogs 4–10

Plk1 PBD-binding affinities of synthesized peptide **2** analogs **4–10** were evaluated by in-house fluorescence polarization (FP) assays using a peptide **2**-based fluorescent probe (FITC-mini-PEG-PLH*SpT) and full-length Plk1.[36] The *N*-methylated analogs **4–10** showed improved affinities as compared with peptide **2** (Table 1 and Fig. S1). The



Scheme 2 Synthesis of C-terminally modified peptide **2** analogs **15–17** and **21–23**. (a) Synthesis of peptides **15** and **21**. (b) Synthesis of peptides **16** and **22**. (c) Synthesis of peptides **17–23**. NMP: *N*-methylpyrrolidone.



Scheme 3 Synthesis of N-terminally modified peptide **2** analogs **18–20** and **24–26**. (a) Synthesis of peptides **18** and **24**. (b) Synthesis of peptides **19** and **25**. (c) Synthesis of peptides **20** and **26**.

C-terminally di-methylated amide **6** showed approximately 4-fold affinity enhancement as compared with the parent peptide **2** in experiments 1 and 2. However, MTT assays using HeLa cells showed that none of the *N*-methylated analogs exhibited significant cytotoxicity below 100 μM (Fig. S2). We interpreted these data to suggest that *N*-methylation of peptide **2** did not contribute to improving cell membrane permeability.

Table 1 Results of FP assays examining the Plk1 PBD-binding affinities of the N-methylated peptide **2** analogs **4–10**

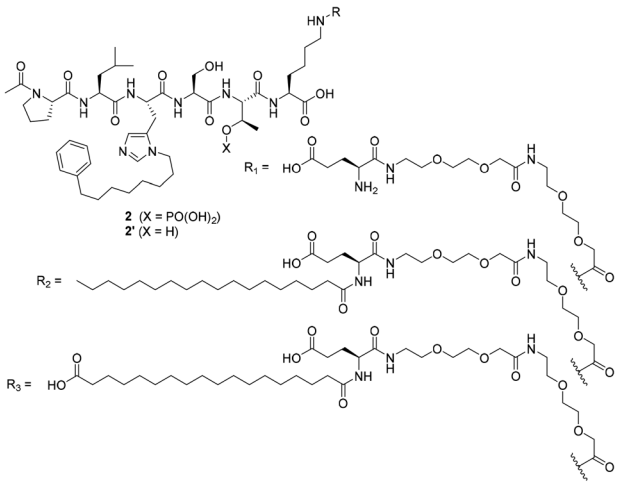
Compound	Modification residue	Plk1 PBD affinity[a] (nM)
PLH*SpT (**2**)	—	440 ± 37,[b] 290 ± 23[c]
PLH*ST (**2′**)	—	n.d.[d]
4	C-term, –COOH	190 ± 63,[b] 170 ± 4.0[c]
5	C-term, –CONH(Me)	150 ± 29,[b] 99 ± 18[c]
6	C-term, –CON(Me)$_2$	120 ± 2.5,[b] 62 ± 9.0[c]
7	*N*-Me-Leu	140 ± 30,[b] 85 ± 21[c]
8	*N*-Me-Leu C-term, –COOH	120 ± 19,[b] 97 ± 15[c]
9	*N*-Me-Leu C-term, –CONH(Me)	280 ± 18,[b] 110 ± 9.2[c]
10	*N*-Me-Leu C-term, –CON(Me)$_2$	190 ± 4.9,[b] 120 ± 11[c]

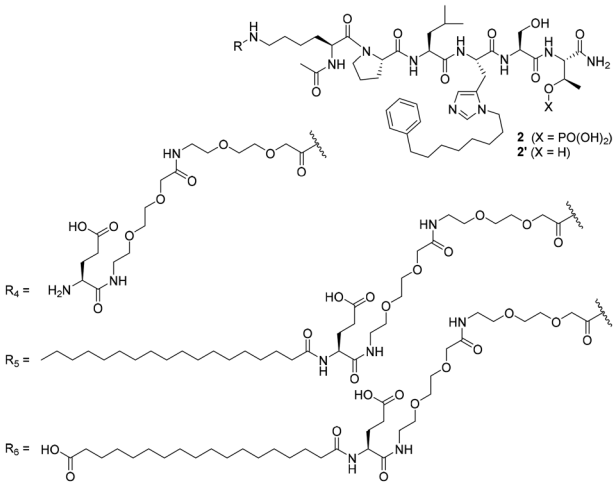
[a] FP assays using purified full-length Plk1 with FITC-mini-PEG-**2** as the probe. IC_{50} values were determined from three independent experiments and shown as the average IC_{50} ± SEM (nM). [b,c] Experiment 1 or 2, respectively. [d] Not determined.

Evaluation of Plk1 PBD-binding affinities and cytotoxicity of the lipidated peptide 2 analogs 15–26

Plk1 PBD-binding affinities of the synthesized peptide **2** analogs **15–26** were evaluated by in-house FP assays using the fluorescein-labeled probe (FITC-mini-PEG-**2** [36] or FITC-mini-PEG-FDPPLHSpTA[55]) and full-length Plk1 (Table 2 and Fig. S3). FP assays using different probes showed that peptide **15** with a mini-PEG linker and peptides **17** and **20** having semaglutide-like lipidation at C- and N-termini, respectively, showed higher PBD-binding affinities than parent peptide **2** and peptides **16**, **18**, and **19** having other modifications. Notably, peptide **17** demonstrated greater than 260-fold affinity enhancement. Although the reasons why the lipidated analogs showed improved affinities remain unclear, this tendency is partially consistent with our previous finding that the attachment of a mini-PEG unit on the C- or N-terminus of the PBD-binding peptide improved the affinity.[55] The non-phosphorylated threonine analogs **21–26** did not show any binding, suggesting specific binding of **15–26** to the Plk1 PBD. MTT

Table 2 Results of FP assays examining the Plk1 PBD-binding affinities of the lipidated peptide **2** analogs **15–26**





Compound	Peptide	Modification site	Modification	Plk1 PBD affinity[a] (nM) Probe: FITC-mini-PEG-**2**	Plk1 PBD affinity[a] (nM) FITC-mini-PEG-FDPPLHSpTA
PLH*SpT (**2**)	—	—	—	560 ± 130,[b] 300 ± 17[c]	60 ± 2.3
PLH*ST (**2′**)	—	—	—	n.d.[b,c,d]	n.t.[e]
15	**2**	C-term	R_1	4.3 ± 0.54[b]	8.1 ± 0.040
16	**2**	C-term	R_2	450 ± 61[b]	7.4 ± 0.060
17	**2**	C-term	R_3	2.1 ± 0.062[b]	1.5 ± 0.054
18	**2**	N-term	R_4	1300 ± 420[b]	19 ± 0.48
19	**2**	N-term	R_5	5400 ± 1300[b]	59 ± 0.35
20	**2**	N-term	R_6	26 ± 3.4[b]	2.9 ± 0.15
21	**2′**	C-term	R_1	n.d.[c]	n.t.
22	**2′**	C-term	R_2	n.d.[c]	n.t.
23	**2′**	C-term	R_3	n.d.[c]	n.t.
24	**2′**	N-term	R_4	n.d.[c]	n.t.
25	**2′**	N-term	R_5	n.d.[c]	n.t.
26	**2′**	N-term	R_6	n.d.[c]	n.t.

[a] FP assays using purified full-length Plk1 with FITC-mini-PEG-**2** [36] or FITC-mini-PEG-FDPPLHSpTA[55] as the probe. IC_{50} values were determined from three independent experiments and shown as the average IC_{50} ± SEM (nM). [b,c] Experiments 1 and 2, respectively. [d] Not determined. [e] Not tested.

assays using HeLa cells showed that none of the lipidated analogs with or without phosphorylation on their Thr residues had significant cytotoxicity below a concentration of 100 μM (Fig. S4). Taken together with the results of the FP assays, it was apparent that semaglutide-like lipidation of peptide **2** did not facilitate cellular activity.

The Plk family is composed of five members (Plk1–5). Plk1–3 have high homology in their PBDs.[31] In contrast to Plk1, both Plk2 and Plk3 function as tumor suppressors.[56,57] Therefore, Plk1 selectivity against Plk2 and Plk3 is an important consideration for the development of Plk1 inhibitors. There is no structural information about the PBD of Plk3, so we predicted the Plk3 PBD (312D–646A) structure using ColabFold,[58,59] and compared its structure with the PBDs of Plk1 and Plk2 (Fig. S5a–c). According to the superimposed structures of PBDs of Plk1–3, these structures were highly conserved, which might suggest the potencies of Plk1 PBD-binding peptides for binding to PBDs of Plk2 and Plk3 (Fig. S5d and e).

Conclusions

Targeting intracellular proteins is among the greatest challenges confronting the development of peptide-based agonists/antagonists. Plk1 is an established anti-cancer target, but there have been few reports of Plk1 PBD-directed peptides having significant cellular potencies.[36,37,39,40] Peptide **2** is one of the highest affinity Plk1 PBD-binding peptides, but it has poor cell membrane permeability and cellular potency.[29,36] We have undertaken sustained efforts to improve the cellular active Plk1 inhibitor peptides based on peptide **2** that include incorporating additional auxiliaries on the His side chain,[30–32] macro-cyclization,[34,35] and bivalent approaches.[54] However, the resulting peptides failed to show good cytotoxicity.

Herein, we examined *N*-methylation and lipidation using peptide **2** as a starting point. The *N*-methylated peptide **2** analogs **4–10** showed improved PBD-binding affinities, with peptide **6** exhibiting up to 4-fold affinity enhancement (Table 1). However, these peptides failed to show improved cytotoxicity against HeLa cells (Fig. S2). The lipidated peptide **2** analogs **15**, **17**, and **20** also showed from 20- to 260-fold enhanced PBD-binding affinities, but these peptides failed to show cytotoxicity (Table 2 and Fig. S4). These results suggest that there is room for further modification to improve cell membrane permeability. Additionally, the selectivity of the synthesized peptides against PBDs of Plk1–3 was not investigated in the present study. Therefore, investigations to develop cell-active Plk1 PBD inhibitors, including selectivity evaluation, are underway by our research team.

Experimental

Peptide synthesis

The synthetic methods for representative compounds **4–10** and **15–26** are described in Schemes 1–3 and the SI. The purities of all the final compounds were determined by analytical RP-HPLC as being >95%. Experimental procedures, including characterization data and analytical RP-HPLC charts of the purified compounds, are provided in the SI.

FP assays using purified full-length Plk1

FP assays to evaluate the PBD-binding affinities of test compounds using full-length Plk1 were run as previously reported.[36,54,55] Briefly, purified protein was prepared following methods described in previous reports[36,60] and diluted to a 2× working dilution in assay buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline with 0.05% Tween-20, 1 mM dithiothreitol (DTT), and 1 mM ethylenediaminetetraacetic acid (EDTA)) with the final protein concentration representing the approximate K_D values as determined for the probe FITC-mini-PEG-PLH*SpT and FITC-mini-PEG-FDPPLHSpTA, respectively. Inhibitors were serially diluted to generate 4× working dilutions in assay buffer. To each well of a 384-well plate was added 20 μL of 2× Plk1 solution (0% binding controls received 20 μL of assay buffer). A total of 10 μL of the 4× inhibitor solution (or assay buffer as blank) was added to the corresponding wells and allowed to pre-incubate at room temperature for 30 min with shaking. A fluorescent probe was diluted to 40 nM (4×) in assay buffer, and then 10 μL was added to each well. The plate was allowed to equilibrate at room temperature for 30 min with shaking. The FP was read using a TECAN Spark plate reader with 485/15 excitation and 528/15 emission. FP values were obtained in triplicate and normalized to 100% (no inhibitor) and 0% binding (no protein) controls. Normalized values were plotted *versus* concentration and analyzed using non-linear regression in GraphPad Prism 10 [log(inhibitor) *vs.* response – variable slope (four parameter) model]. IC_{50} values represent average ± standard error of the mean (SEM).

MTT assays using HeLa cells

MTT assays were performed as previously reported.[36] In brief, HeLa cells (JCRB9004, Japanese Collection of Research Bioresources (JCRB) Cell Bank, Osaka, Japan) were seeded in a 96-well plate (5×10^3 cells per well) with 100 μL of Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-Gln, 100 μg mL^{-1} of penicillin, and 100 μg mL^{-1} of streptomycin for each well. After 1-day incubation at 37 °C, the media were replaced with the serially diluted compounds containing media, and cells were incubated at 37 °C for an additional 2 days. Cells were washed with phosphate-buffered saline (PBS, 200 μL × 1) and the generated formazan was dissolved in 200 μL of 4 M HCl aq./2-propanol (0.1 : 10). The absorbance of each well at 565 nm was read using the iMark Microplate Reader (BIO-RAD). The absorbance was obtained in triplicate and normalized to 100% (no inhibitor) and 0% viability (no cell) controls. Normalized values were plotted *versus* concentration and analyzed using non-linear regression in GraphPad Prism 10 [log(inhibitor) *vs.* response – variable slope (four parameter) model]. IC_{50} values represent average ± SEM.

Author contributions

KT: conceptualization, funding acquisition, investigation, project administration, writing (original draft). KB: investigation; XH: investigation; HT: funding acquisition, supervision, writing (review and editing).

Conflicts of interest

There are no conflicts of interest to declare.

Data availability

Supplementary information (SI): synthetic experimental procedures, compound characterization data (analytical HPLC and LRMS), binding curves of the reported assay results, MTT assay results, and structural representations of Plk1–3 PBDs. See DOI: https://doi.org/10.1039/d6ob00193a.

Acknowledgements

This research was supported in part by JSPS KAKENHI (22K15243 and 25K08852 (K. T.) and 24K02144 (H. T.)), Japan Agency for Medical Research and Development (JP25ama121043 and JP24ama121043) (Research Support Project for Life Science and Drug Discovery, BINDS) (K. T. and H. T.), JST SPRING (JPMJSP2180 (X. H.)).

This work was also supported in part by MEXT Promotion of Development of a Joint Usage/Research System Project: Coalition of Universities for Research Excellence Program (CURE) (JPMXP1324134721). This research was conducted under the auspices of the Cooperative Research Project of the Research Center for Biomedical Engineering. The authors thank Dr Terrence R. Burke, Junior (NCI/NIH) for editing this manuscript.

References

1 A. Henninot, J. C. Collins and J. M. Nuss, *J. Med. Chem.*, 2018, **61**, 1382–1414, DOI: 10.1021/acs.jmedchem.7b00318.
2 L. Wang, N. Wang, W. Zhang, X. Cheng, Z. Yan, G. Shao, X. Wang, R. Wang and C. Fu, *Signal Transduction Targeted Ther.*, 2022, **7**, 48, DOI: 10.1038/s41392-022-00904-4.
3 K. Sharma, K. K. Sharma, A. Sharma and R. Jain, *Drug Discovery Today*, 2023, **28**, 103464, DOI: 10.1016/j.drudis.2022.103464.
4 W. Xiao, W. Jiang, Z. Chen, Y. Huang, J. Mao, W. Zheng, Y. Hu and J. Shi, *Signal Transduction Targeted Ther.*, 2025, **10**, 74, DOI: 10.1038/s41392-024-02107-5.
5 T. A. Cardote and A. Ciulli, *ChemMedChem*, 2016, **11**, 787–794, DOI: 10.1002/cmdc.201500450.
6 P. G. Dougherty, A. Sahni and D. Pei, *Chem. Rev.*, 2019, **119**, 10241–10287, DOI: 10.1021/acs.chemrev.9b00008.
7 A. Rüegger, M. Kuhn, H. Lichti, H. R. Loosli, R. Huguenin, C. Quiquerez and A. von Wartburg, *Helv. Chim. Acta*, 1976, **59**, 1075–1092, DOI: 10.1002/hlca.19760590412.
8 S. Noble and A. Markham, *Drugs*, 1995, **50**, 924–941, DOI: 10.2165/00003495-199550050-00009.
9 X. Li, N. Wang, Y. Liu, W. Li, X. Bai, P. Liu and C. Y. He, *Bioorg. Chem.*, 2023, **141**, 106892, DOI: 10.1016/j.bioorg.2023.106892.
10 Y. Hosono, S. Uchida, M. Shinkai, C. E. Townsend, C. N. Kelly, M. R. Naylor, H. W. Lee, K. Kanamitsu, M. Ishii, R. Ueki, T. Ueda, K. Takeuchi, M. Sugita, Y. Akiyama, S. R. Lokey, J. Morimoto and S. Sando, *Nat. Commun.*, 2023, **14**, 1416, DOI: 10.1038/s41467-023-36978-z.
11 L.-L. Zou, J.-L. Ma, T. Wang, T.-B. Yang and C.-B. Liu, *Curr. Neuropharmacol.*, 2013, **11**, 197–208, DOI: 10.2174/1570159X11311020006.
12 Z. Sun, J. Huang, Z. Fishelson, C. Wang and S. Zhang, *Biomedicines*, 2023, **11**, 1971, DOI: 10.3390/biomedicines11071971.
13 J. Xu, F. Wang, L. Ye, R. Wang, L. Zhao, X. Yang, J. Ji, A. Liu and G. Zhai, *J. Drug Delivery Sci. Technol.*, 2023, **84**, 104475, DOI: 10.1016/j.jddst.2023.104475.
14 V. Archambault, G. Lepine and D. Kachaner, *Oncogene*, 2015, **34**, 4799–4807, DOI: 10.1038/onc.2014.451.
15 R. F. Lera, G. K. Potts, A. Suzuki, J. M. Johnson, E. D. Salmon, J. J. Coon and M. E. Burkard, *Nat. Chem. Biol.*, 2016, **12**, 411–418, DOI: 10.1038/nchembio.2060.
16 B. D. Cholewa, X. Liu and N. Ahmad, *Cancer Res.*, 2013, **73**, 6848–6855, DOI: 10.1158/0008-5472.can-13-2197.
17 K. Strebhardt and A. Ullrich, *Nat. Rev. Cancer*, 2006, **6**, 321–330, DOI: 10.1038/nrc1841.
18 J.-M. Schvartzman, R. Sotillo and R. Benezra, *Nat. Rev. Cancer*, 2010, **10**, 102–115, DOI: 10.1038/nrc2781.
19 Z. Liu, Q. Sun and X. Wang, *Transl. Oncol.*, 2017, **10**, 22–32, DOI: 10.1016/j.tranon.2016.10.003.
20 W. Weichert, M. Schmidt, J. Jacob, V. Gekeler, J. Langrehr, P. Neuhaus, M. Bahra, C. Denkert, M. Dietel and G. Kristiansen, *Pancreatology*, 2005, **5**, 259–265, DOI: 10.1159/000085280.
21 Q. N. Hassan II, L. Alinari and J. C. Byrd, *J. Clin. Invest.*, 2018, **128**, 5206–5208, DOI: 10.1172/jci124919.
22 S. B. Jeong, J. H. Im, J. H. Yoon, Q. T. Bui, S. C. Lim, J. M. Song, Y. Shim, J. Yun, J. Hong and K. W. Kang, *Mol. Cancer Ther.*, 2018, **17**, 825–837, DOI: 10.1158/1535-7163.mct-17-0545.
23 K. S. Lee, T. R. Burke Jr., J.-E. Park, J. K. Bang and E. Lee, *Trends Pharmacol. Sci.*, 2015, **36**, 858–877, DOI: 10.1016/j.tips.2015.08.013.
24 R. E. A. Gutteridge, M. A. Ndiaye, X. Liu and N. Ahmad, *Mol. Cancer Ther.*, 2016, **15**, 1427–1435, DOI: 10.1158/1535-7163.mct-15-0897.
25 J.-E. Park, D. Hymel, T. R. Burke Jr. and K. S. Lee, *F1000Research*, 2017, **6**, 1024, DOI: 10.12688/f1000research.11398.1.
26 J. Xu, C. Shen, T. Wang and J. Quan, *Nat. Struct. Mol. Biol.*, 2013, **20**, 1047–1053, DOI: 10.1038/nsmb.2623.

27 A. E. H. Elia, P. Rellos, L. F. Haire, J. W. Chao, F. J. Ivins, K. Hoepker, D. Mohammad, L. C. Cantley, S. J. Smerdon and M. B. Yaffe, *Cell*, 2003, **115**, 83–95, DOI: 10.1016/s0092-8674(03)00725-6.
28 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 and K. S. Lee, *Nat. Struct. Mol. Biol.*, 2009, **16**, 876–882, DOI: 10.1038/nsmb.1628.
29 F. Liu, J.-E. Park, W.-J. Qian, D. Lim, M. Gräber, T. Berg, M. B. Yaffe, K. S. Lee and T. R. Burke Jr., *Nat. Chem. Biol.*, 2011, **7**, 595–601, DOI: 10.1038/nchembio.614.
30 X. Z. Zhao, D. Hymel and T. R. Burke Jr., *Bioorg. Med. Chem. Lett.*, 2016, **26**, 5009–5012, DOI: 10.1016/j.bmcl.2016.08.098.
31 X. Z. Zhao, D. Hymel and T. R. Burke Jr., *Bioorg. Med. Chem.*, 2017, **25**, 5041–5049, DOI: 10.1016/j.bmc.2017.02.063.
32 X. Z. Zhao, K. Tsuji, D. Hymel and T. R. Burke Jr., *Molecules*, 2019, **24**, 1488, DOI: 10.3390/molecules24081488.
33 W.-J. Qian, J.-E. Park, K. S. Lee and T. R. Burke Jr., *Bioorg. Med. Chem. Lett.*, 2012, **22**, 7306–7308, DOI: 10.1016/j.bmcl.2012.10.093.
34 D. Hymel, R. A. Grant, K. Tsuji, M. B. Yaffe and T. R. Burke Jr., *Bioorg. Med. Chem. Lett.*, 2018, **28**, 3202–3205, DOI: 10.1016/j.bmcl.2018.08.018.
35 D. Hymel, K. Tsuji, R. A. Grant, R. M. Chingle, D. L. Kunciw, M. B. Yaffe and T. R. Burke Jr., *Org. Biomol. Chem.*, 2021, **19**, 7843–7854, DOI: 10.1039/d1ob01120k.
36 K. Tsuji, D. Hymel, B. Ma, H. Tamamura, R. Nussinov and T. R. Burke Jr., *RSC Chem. Biol.*, 2022, **3**, 1111–1120, DOI: 10.1039/d2cb00153e.
37 S. E. Miller, K. Tsuji, R. P. M. Abrams, T. R. Burke Jr. and J. P. Schneider, *J. Am. Chem. Soc.*, 2020, **142**, 19950–19955, DOI: 10.1021/jacs.0c07921.
38 K. M. Sakamoto, K. B. Kim, A. Kumagai, F. Mercurio, C. M. Crews and R. J. Deshaies, *Proc. Natl. Acad. Sci. U. S. A.*, 2001, **98**, 8554–8559, DOI: 10.1073/pnas.141230798.
39 P. Gunasekaran, Y. S. Hwang, G.-H. Lee, J. Park, J. G. Kim, Y. K. La, N. Y. Park, R. Kothandaraman, M. S. Yim, J. Choi, H. N. Kim, I. Y. Park, S. J. Lee, M.-H. Kim, H. Cha-Molstad, S. Y. Shin, E. K. Ryu and J. K. Bang, *J. Med. Chem.*, 2024, **67**, 3307–3320, DOI: 10.1021/acs.jmedchem.3c01493.
40 P. Gunasekaran, S. C. Shin, Y. S. Hwang, J. Lee, Y. K. La, M. S. Yim, H. N. Kim, T. W. Kim, E. Yang, S. J. Lee, J. M. Yoon, E. E. Kim, S. Jeon, E. K. Ryu and J. K. Bang, *Pharmaceutics*, 2025, **17**, 1027, DOI: 10.3390/pharmaceutics17081027.
41 G. Riley, J. H. Turnbull and W. Wilson, *J. Chem. Soc.*, 1957, 1373–1379, DOI: 10.1039/JR9570001373.
42 T. Wakamiya, K. Saruta, J. Yasuoka and S. Kusumoto, *Chem. Lett.*, 1994, **23**, 1099–1102, DOI: 10.1246/cl.1994.1099.
43 T. Vorherr and W. Bannwarth, *Bioorg. Med. Chem. Lett.*, 1995, **5**, 2661–2664, DOI: 10.1016/0960-894X(95)00475-9.
44 G. Shapiro, D. Büchler, C. Dalvit, M. C. Fernandez, B. Gomez-Lor, E. Pombo-Villar, U. Stauss and R. Swoboda, *Bioorg. Med. Chem. Lett.*, 1996, **6**, 409–414, DOI: 10.1016/0960-894X(96)00044-3.
45 H. Waldmann, A. Heuser and S. Schulze, *Tetrahedron Lett.*, 1996, **37**, 8725–8728, DOI: 10.1016/S0040-4039(96)02041-2.
46 S. Trier, L. Linderoth, S. Bjerregaard, H. M. Strauss, U. L. Rahbek and T. L. Andresen, *Eur. J. Pharm. Biopharm.*, 2015, **96**, 329–337, DOI: 10.1016/j.ejpb.2015.09.001.
47 S. Xia, M. Liu, C. Wang, W. Xu, Q. Lan, S. Feng, F. Qi, L. Bao, L. Du, S. Liu, C. Qin, F. Sun, Z. Shi, Y. Zhu, S. Jiang and L. Lu, *Cell Res.*, 2020, **30**, 343–355, DOI: 10.1038/s41422-020-0305-x.
48 L. Yuan, J. Wang and W.-C. Shen, *Pharm. Res.*, 2005, **22**, 220–227, DOI: 10.1007/s11095-004-1189-z.
49 A. Zorzi, S. J. Middendorp, J. Wilbs, K. Deyle and C. Heinis, *Nat. Commun.*, 2017, **8**, 16092, DOI: 10.1038/ncomms16092.
50 E. M. Bech, S. L. Pedersen and K. J. Jensen, *ACS Med. Chem. Lett.*, 2018, **9**, 577–580, DOI: 10.1021/acsmedchemlett.8b00226.
51 J. Lau, P. Bloch, L. Schäffer, I. Pettersson, J. Spetzler, J. Kofoed, K. Madsen, L. B. Knudsen, J. McGuire, D. B. Steensgaard, H. M. Strauss, D. X. Gram, S. M. Knudsen, F. S. Nielsen, P. Thygesen, S. Reedtz-Runge and T. Kruse, *J. Med. Chem.*, 2015, **58**, 7370–7380, DOI: 10.1021/acs.jmedchem.5b00726.
52 V. R. Aroda, L. Blonde and R. E. Pratley, *Rev. Endocr. Metab. Disord.*, 2022, **23**, 979–994, DOI: 10.1007/s11154-022-09735-8.
53 W.-J. Qian, J.-E. Park, R. Grant, C. C. Lai, J. A. Kelley, M. B. Yaffe, K. S. Lee and T. R. Burke Jr., *Biopolymers*, 2015, **104**, 663–673, DOI: 10.1002/bip.22698.
54 K. Tsuji, H. Tamamura and T. R. Burke Jr., *RSC Chem. Biol.*, 2024, **5**, 721–728, DOI: 10.1039/d4cb00031e.
55 K. Tsuji, H. Tamamura and T. R. Burke Jr., *Bioorg. Med. Chem.*, 2025, **119**, 118055, DOI: 10.1016/j.bmc.2024.118055.
56 S.-Y. Lee, C. Jang and K.-A. Lee, *Dev. Reprod.*, 2014, **18**, 65–71, DOI: 10.12717/DR.2014.18.1.065.
57 M. V. R. Reddy, B. Akula, S. Jatiani, R. V.-D. Carpio, V. K. Billa, M. R. Mallireddigari, S. C. Cosenza, D. R. C. V. Subbaiah, E. V. Bharathi, V. R. Pallela, P. Ramkumar, R. Jain, A. K. Aggarwal and E. P. Reddy, *Bioorg. Med. Chem.*, 2016, **24**, 521–544, DOI: 10.1016/j.bmc.2015.11.045.
58 N. Jiang, X. Wang, M. Jhanwar-Uniyal, Z. Darzynkiewicz and W. Dai, *J. Biol. Chem.*, 2006, **281**, 10577–10582, DOI: 10.1074/jbc.M513156200.
59 M. Mirdita, K. Schütze, Y. Moriwaki, L. Heo, S. Ovchinnikov and M. Steinegger, *Nat. Methods*, 2022, **19**, 679–682, DOI: 10.1038/s41592-022-01488-1.
60 K. Tsuji, H. Tamamura and T. R. Burke Jr., *Biol. Pharm. Bull.*, 2024, **47**, 1282–1287, DOI: 10.1248/bpb.b24-00189.