Peptide-based activity-based probes (ABPs) for target-specific profiling of protein tyrosine phosphatases (PTPs)†

Karunakaran A. Kalesh,[a] Lay Pheng Tan,[b] Kai Lu,[b] Liqian Gao,[c] Jigang Wang[b] and Shao Q. Yao*[abc]

Received (in Cambridge, UK) 22nd September 2009, Accepted 2nd November 2009
First published as an Advance Article on the web 17th November 2009
DOI: 10.1039/b919744c

Synthesis of a novel unnatural amino acid (2-FMPT) for the solid-phase synthesis of peptide-based probes suitable for target-specific activity-based profiling of protein tyrosine phosphatases from crude proteomes is reported.

Reversible protein dephosphorylation at the tyrosine residues plays a pivotal role in signal transduction.[1] *In vivo*, it is regulated by the opposite action of protein tyrosine phosphatases (PTPs) and protein tyrosine kinases (PTKs).[2] In humans, there are well over 100 known PTPs, which, together with several hundred protein kinases, control the phosphorylation of as many as 1–2% of all human proteins. In order to maintain an intricate balance of this highly complex phosphoproteome network, PTPs carry out dephosphorylation with a high degree of specificity inside the cell, although *in vitro* they have shown only moderate specificity towards synthetic peptide substrates.[3] Consequently, chemical and biological methods that report not only the global PTP activities, but more importantly the precise enzymatic activity of individual PTPs, may offer unprecedented views on how these enzymes carry out their biological functions under native physiological settings. Activity-based protein profiling (ABPP) is a chemical proteomics strategy that makes use of mechanism-based small molecule probes to detect active enzymes present in a crude proteome, and has been successfully applied to different classes of enzymes.[4] In order to develop activity-based probes (ABPs) that detect global PTP activities, Lo *et al.* first explored small molecules containing a 4-fluoromethyl phenyl phosphate (**FMPP**) moiety which serves as a phosphotyrosine mimic, and upon dephosphorylation by a PTP, generates a highly reactive quinone methide intermediate that subsequently alkylates nucleophiles present in the PTP active site.[5a] We made similar PTP probes using 2-difluoromethyl phenyl phosphate (**DFPP**) which presumably works *via* a similar mechanism as **FMPP**.[5b] Unfortunately, due to the diffusible nature of the quinone methide generated, both classes of probes possess poor PTP specificity and cross-react with other nearby proteins in the crude proteome. Zhang *et al.* recently developed a PTP probe that uses α-bromobenzylphosphonate as a non-hydrolyzable phosphotyrosine (*p*Tyr) mimic.[5c,d] The probe was shown to be specific towards PTPs in a proteomic experiment, but the highly reactive/unstable nature of the probe renders it impractical for widespread use. The same group developed a newer version of PTP probes by using phenyl vinyl sulfone/sulfonate as the enzyme-targeting warhead.[5e] Vinyl sulfones/sulfonates are, however, well-established irreversible inhibitors of other cysteine-utilizing enzymes (*i.e.* cysteine proteases), and are likely to cross-label them as well. Notwithstanding, all four existing classes of PTP probes have so far failed to address one of the most important issues in PTP biology—how can one monitor/profile the active state of individual PTPs in a crude proteome, where these enzymes' native cellular environment is closely emulated?

Herein, we report the design and chemical synthesis of a novel unnatural amino acid, **2-FMPT**, and its successful incorporation into peptide-based probes for potential target-specific, activity-based profiling of individual PTPs (Fig. 1(a)). Apart from an additional 2-fluoromethyl group located in the aromatic ring, **2-FMPT** is structurally identical to *p*Tyr and should cause minimal disruption in PTP recognition. The most significant advantage of **2-FMPT** over other existing PTP probes is that, with its N- and C-terminus (as in the case of naturally occurring amino acids), essential peptide recognition elements which occupy the proximal positions of *p*Tyr in a naturally occurring PTP substrate could be introduced. With this, the corresponding peptide-based probes could achieve target-specific binding to/profiling of different PTPs. As shown in Fig. 1(b), the peptide-based probe works by first binding to the active site of the target PTP in a sequence-specific manner,

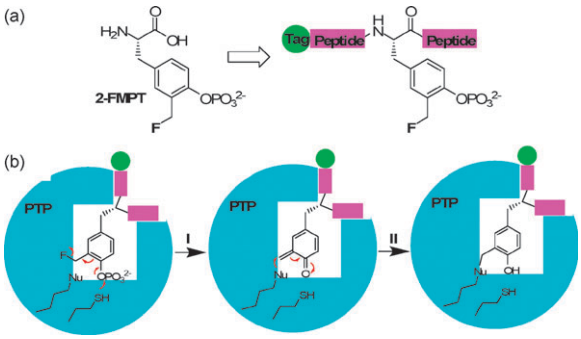

Fig. 1 (a) Structures of the unnatural amino acid, **2-FMPT**, and its corresponding peptide-based ABPs. (b) Proposed mechanism of the sequence-specific, activity-based labeling of PTPs using the peptide-based probes.

[a] *Department of Chemistry, National University of Singapore, 3 Science Drive 3, Singapore 117543. E-mail: chmyaosq@nus.edu.sg; Fax: (65) 67791691; Tel: (65) 65162925*
[b] *Department of Biological Sciences, National University of Singapore, 3 Science Drive 3, Singapore 117543*
[c] *NUS MedChem Program of the Life Sciences Institute, National University of Singapore, 3 Science Drive 3, Singapore 117543*
† Electronic supplementary information (ESI) available: Detailed experimental procedures and results. See DOI: 10.1039/b919744c

and is subsequently dephosphorylated (I; as in the case of a synthetic PTP substrate). The ensuing charge delocalization followed by spontaneous elimination of –F generates the highly reactive quinone methide intermediate which, upon attack by a nearby nucleophilic residue in the enzyme, would give rise to the covalent enzyme-probe complex (II). We anticipated that, in our newly developed probes, introduction of the additional peptide fragments specific towards individual PTPs should improve probe binding affinity as well as specificity, thus overcoming the diffusion problem observed in the previous ABPs (*i.e.***FMPP**/**DFPP**).

The synthesis of the Fmoc-protected **2-FMPT** (**10** in Scheme 1) was carried out from Boc-L-tyrosine. Briefly, formylation of **2** at the *ortho* position of the phenolic group by Reimer–Tiemann reaction gave **3** in moderate yield (39%).[6] Subsequently, **3** was protected to give the corresponding benzyl ester **4** (80%), followed by phosphorylation with chlorodiethyl-phosphate to give **5** (70%). Reduction of the CHO group in **5** with $NaBH_4$ gave **6** (69%) followed by fluorination with DAST, giving **7** (74%). Finally, deprotection of the benzyl ester in **7**, followed by Boc-to-Fmoc conversion gave **10**. To show that **10** could be conveniently incorporated into peptides using standard SPPS protocols, eleven peptide probes, designed against five different PTPs (PTP1B, TCPTP, SHP1, SHP2 and LMWPTP), were synthesized using Rink amide resin (Scheme 1, Table 1). For comparison, ten corresponding phosphopeptides were also synthesized, in which the naturally occurring *p*Tyr was used in place of **2-FMPT** (see ESI†).

Under standard labeling conditions, the probes were found to label only PTPs in acivity-dependant manner (see ESI†). Next, activity-based labeling of the ten rhodamine-containing probes, **P1–P10**, with five different PTPs was tested to generate the so-called activity-based fingerprints.[7] As shown in Fig. 2, clearly distinctive labeling profiles of individual PTPs were observed, and to a large extent corroborated well with their known substrate preferences (Table 1 in Scheme 1). For example, the top-5 probes (**P2**, **P3**, **P7**, **P9** and **P10**) which generated the strongest labeling against PTP1B, were derived expectedly from known PTP1B substrates. TCPTP, known to have similar substrate preferences as PTP1B, gave a similar activity-based fingerprint. For SHP1, two of the most active probes are **P1** and **P6**. Of the two SHP2-specific probes (**P8** and **P10**), one was derived from its known substrate. Similarly, LMWPTP also gave a distinctive labeling profile, which is in good agreement with its substrate preference.[8] Further characterizations of the labeling profiles were carried out by kinetic measurements of PTP1B inactivation in the presence of the ten probes, as well as measurements of PTP1B-mediated dephosphorylation with ten corresponding phospho-peptides (see ESI†). Analysis of pseudo-first-order inactivation rate constants, k_{obs}, as a function of probe concentration showed time- and concentration-dependant inactivation with saturation kinetics (typical of irreversible inhibition) and followed the Kitts–Wilson relationship. The maximal inactivation rate k_i and the dissociation constant K_i for the ten probes were determined. The dissociation constants of the probes reflect their substrate preferences, with highest $1/K_i$ values correlated well with the most active probes (in their fluorescence labeling), as well as the most preferred phosphopeptide substrates (in the amount of PO_3^{2-} release). All these lines of evidence suggest our peptide-based activity probes can be used to report not only PTP enzymatic activities, but more importantly the substrate specificities of individual PTPs.

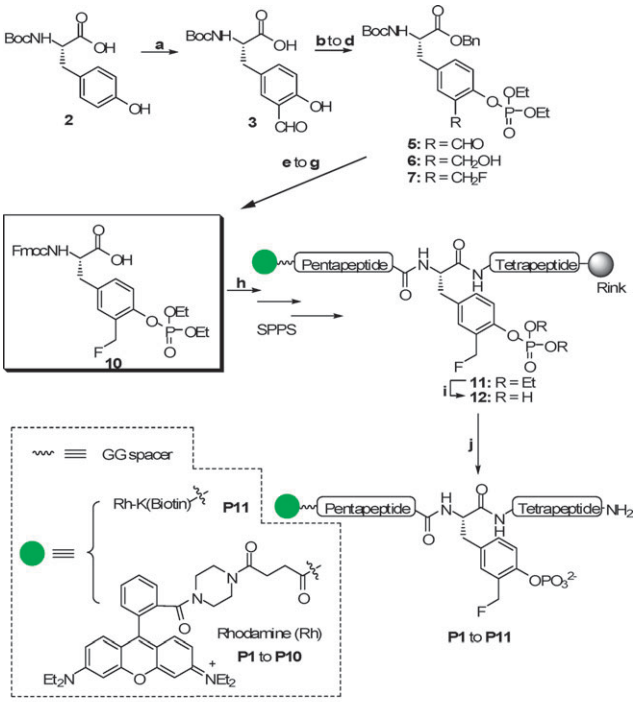

Table 1. The eleven probes used in this study.

Probe	Putative Target	Original Protein Source[1]	Sequence[3]
P1	SHP1	Siglec	Rh-GGDEGIH**X**SELI-NH_2
P2	PTP1B/TCPTP	Stat3	RhGGGSAAP**X**LKTK-NH_2
P3	PTP1B	Stat5b	Rh-GGKAVDG**X**VKPQ-NH_2
P4	LMWPTP	Zap70	Rh-GGLNSDG**X**TPEP-NH_2
P5	SHP2	Ptk2b	Rh-GGLPPEG**X**VVVV-NH_2
P6	SHP1/SHP2	CD31	Rh-GGNSDVQ**X**TEVQ-NH_2
P7	PTP1B	Tyk2	Rh-GGPEGHE**X***p*YRVR-NH_2
P8	SHP2/PTP1B	Janus	Rh-GGPQDKE**X**YKVK-NH_2
P9	SHP2/PTP1B	EGFR	Rh-GGVDADE**X**LIPQ-NH_2
P10	PTP1B	Zhang's[2]	Rh-GGELEF**X**MDE-NH_2
P11	PTP1B	Stat5b	Rh-K(Biotin)-KAVDG**X**VKPQI-NH_2

[1] Obtained from www.hprd.org; [2] Obtained from reference 3b; [3] **X** = **2-FMPT.**

Scheme 1 Synthesis of **10** and solid-phase peptide synthesis (SPPS) of the eleven probes. *Reagents and conditions*: (a) $CHCl_3$, NaOH, H_2O, reflux, 39%; (b) BnOH, EDC, DMAP, DCM, 0 °C to RT, 80% to give **4**, then chlorodiethylphosphate, DBU, DCM, 0 °C to RT, 70% to give **5**; (c) $NaBH_4$, EtOH–THF (4 : 1), 0 °C, 69%; (d) DAST, DCM, 0 °C to RT, 74%; (e) H_2, Pd–C, MeOH, 96% to give **8**; (f) TFA–DCM (1 : 1), 90% to give **9**; (g) FmocOSu, $NaHCO_3$, THF–water (1 : 1), 76%; (h) standard Fmoc-based SPPS conditions; (i) TMSI, DCM, RT; (j) TFA–H_2O–TIS (95 : 2.5 : 2.5), RT.

Finally, the target-specific, activity-based PTP profiling of these peptide-based probes was evaluated in crude bacterial and mammalian proteomes using the **P3** probe. First, the high specificity of this probe towards PTP1B was unambiguously confirmed by a spike experiment with a bacterial proteome

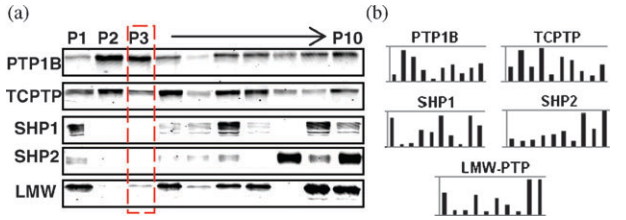

Fig. 2 (a) Fluorescence labeling profiles (in gray scale) of five different PTPs with the ten peptide probes (left to right). The labeling profiles of **P3** were highlighted (boxed in red). (b) The fluorescence intensity of each band is quantified using ImageQuant software and the relative fluorescence intensity (*y*-axis) of labeling of each PTP were plotted against the panel of 10 probes (*x*-axis, **P1** to **P10** from left to right in each graph).

(free of endogenous PTP activities); as shown in Fig. 3(a), as low as 5 ng of PTP1B (0.05% total proteome) could be readily detected with the probe. Next, total cell lysates from two different mammalian cells (HEK293T and NIH3T3) were prepared and treated with **P3** (Fig. 3(b)). The resulting labeled lysates were visualized by in-gel fluorescence scanning (left panel) and Western blotting using *anti*-PTP1B antibody (right panel); Western blotting indicated the presence of endogenously expressed PTP1B in HEK293T (~52 kDa) but not in NIH3T3 cells. A corresponding fluorescent band was observed in the fluorescence gel from the HEK293T lysate, but not from the NIH3T3 lysate (lanes 1 and 2, respectively, highlighted in red box). This is in agreement with previous reports that extremely low or undetectable expression levels of endogenous PTP1B in NIH3T3 cell lines were observed.[9] To further validate the labeling of endogenous PTP1B, we performed pull-down experiments using **P11**—the

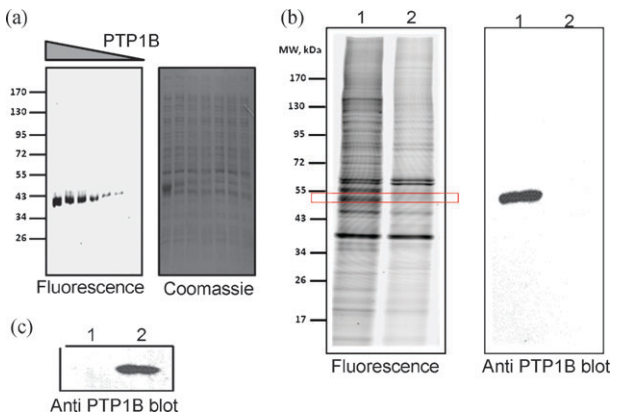

Fig. 3 (a) Labeling of spiked PTP1B in the presence of bacterial proteome. Different amounts (left to right: 1000 ng, 400 ng, 200 ng, 100 ng, 20 ng, 5 ng and 0.5 ng) of recombinant PTP1B was spiked to 10 μg BL21 bacterial proteome and the labeling was performed with 10 μM probe **P3** for 1 h. (b) Labeling of PTPs in mammalian cell lysates. Left panel, in-gel fluorescence analysis of global PTP activity profiles obtained from total cell lysates of HEK293T and NIH3T3 cells (20 μg total proteins/lane) with probe **P3**. Right panel, Anti PTP1B blot of the two labeled lysates detecting endogenous PTP1B at ~52 kDa from HEK293T cells (lane 1) but not from NIH3T3 cells (lane 2). (c) Pull-down results using the biotinylated probe **P11** with HEK293T lysates followed by enrichment with NeutrAvidin beads. Anti-PTP1B WB of the probe treated HEK293T cell lysate after pull-down experiments detected the endogenous PTP1B at ~52 kDa (lane 2) while no PTP1B was detected from the untreated pull-down fraction (lane 1).

biotinylated version of **P3**. As shown in Fig. 3(c), upon labeling with **P11**, the HEK293T cell lysate was subjected to affinity enrichment and subsequently analyzed by Western blotting; endogenous PTP1B was successfully detected (lane 2). This experiment thus demonstrates the feasibility of our peptide-based activity-based probes for target-specific profiling of endogenous PTP activities in cellular lysates. We noticed that in Fig. 2(b), in addition to the endogenous PTP1B, the probe also labeled a number of other proteins in the HEK293T lysate. We speculate they might have originated from other endogenous PTPs which were present in the cell lysate and accepted **P3** as a substrate. We are currently carrying out large-scale proteomic/mass spectrometric experiments to identify these labeled proteins and will report our findings in due course.

In conclusion, we have successfully synthesized a novel unnatural amino acid (**2-FMPT**) which is a close mimic of phosphotyrosine. With the peptide-based probe design, we were able to demonstrate target-specific profiling of PTPs present in mammalian cell lysates. Further improvement of our probes may be achieved by incorporation of longer peptide recognition sequences or by genetically or semi-synthetically incorporating the unnatural amino acid into suitable protein-based substrates of PTPs.[10] Ultimately, it might be possible to extend our strategy to *in vivo* profiling of PTP/substrate interactions in living cells.

Funding support was provided by the tier-2 grant (R-143-000-394-112) from the Ministry of Education of Singapore.

Notes and references

1 (*a*) L. N. Johnson and R. J. Lewis, *Chem. Rev.*, 2001, **101**, 2209–2242; (*b*) T. Hunter, *Cell*, 2000, **100**, 113–127.
2 A. Alonso, J. Sasin, N. Bottini, I. Friedberg, I. Friedberg, A. Osterman, A. Godzik, T. Hunter, J. Dixon and T. Mustelin, *Cell*, 2004, **117**, 699–711.
3 (*a*) Z.-Y. Zhang, A. M. Thieme-Sefler, D. Maclean, D. J. McNamara, E. M. Dobrusin, T. K. Sawyer and J. E. Dixon, *Proc. Natl. Acad. Sci. U. S. A.*, 1993, **90**, 4446–4450; (*b*) S. W. Vetter, Y.-F. Keng, D. S. Lawrence and Z.-Y. Zhang, *J. Biol. Chem.*, 2000, **275**, 2265–2268; (*c*) C. Blanchetot, M. Chagnon, N. Dubé, M. Hallé and M. L. Tremblay, *Methods*, 2005, **35**, 44–53; (*d*) M. Köhn, M. Gutierres-Rodriguez, P. Jonkheijm, S. Wetzel, R. Wacker, H. Schroeder, H. Prinz, C. M. Niemeyer, R. Breinbauer, S. E. Szedlacsek and H. Waldmann, *Angew. Chem., Int. Ed.*, 2007, **46**, 7700–7703.
4 For recent reviews, see: (*a*) M. J. Evans and B. F. Cravatt, *Chem. Rev.*, 2006, **106**, 3279–3301; (*b*) M. Uttamchandani, J. Li, H. Sun and S. Q. Yao, *ChemBioChem*, 2008, **9**, 667–675.
5 (*a*) L. C. Lo, T. L. Pang, C. H. Kuo, Y. L. Chiang, H. Y. Wang and J. J. Lin, *J. Proteome Res.*, 2002, **1**, 35–40; (*b*) Q. Zhu, X. Huang, G. Y. J. Chen and S. Q. Yao, *Tetrahedron Lett.*, 2003, **44**, 2669–2672; (*c*) S. Kumar, B. Zhou, F. Liang, W.-Q. Wang, Z. Huang and Z.-Y. Zhang, *Proc. Natl. Acad. Sci. U. S. A.*, 2004, **101**, 7943–7948; (*d*) S. Kumar, B. Zhou, F. Liang, H. Yang, W.-Q. Wang and Z.-H. Zhang, *J. Proteome Res.*, 2006, **5**, 1898–1905; (*e*) S. Liu, B. Zhou, H. Yang, Y. He, Z.-X. Jiang, S. Kumar, L. Wu and Z.-Y. Zhang, *J. Am. Chem. Soc.*, 2008, **130**, 8251–8260.
6 M. E. Jung and T. I. Lazarova, *J. Org. Chem.*, 1997, **62**, 1553–1555.
7 (*a*) H. Sun, S. Chattopadhaya, J. Wang and S. Q. Yao, *Anal. Bioanal. Chem.*, 2006, **386**, 416–426; (*b*) R. Srinivasan, X. Huang, S. L. Ng and S. Q. Yao, *ChemBioChem*, 2006, **7**, 32–36.
8 (*a*) L. Bialy and H. Waldmann, *Angew. Chem., Int. Ed.*, 2005, **44**, 3814–3839; (*b*) S. Zhang and Z.-Y. Zhang, *Drug Discovery Today*, 2007, **12**, 373–381.
9 L. V. Ravichandran, H. Chen, Y. Li and M. J. Quon, *Mol. Endocrinol.*, 2001, **15**, 1768–1780.
10 (*a*) J. M. Xie and P. G. Schultz, *Nat. Rev. Mol. Cell Biol.*, 2006, **7**, 775–782; (*b*) T. W. Muir, *Annu. Rev. Biochem.*, 2003, **72**, 249–289.