FEBS Letters 525 (2002) 48–52 FEBS 26340

4-Isoavenaciolide covalently binds and inhibits VHR, a dual-specificity phosphatase

Kazunori Ueda[a,b], Takeo Usui[a], Hiroshi Nakayama[c], Masashi Ueki[a], Koji Takio[c], Makoto Ubukata[b], Hiroyuki Osada[a,*]

[a] *Antibiotics Laboratory, RIKEN, Hirosawa 2-1, Wako-shi, Saitama 351-0198, Japan*
[b] *Laboratory of Biofunctional Chemistry, Biotechnology Research Center, Toyama Prefectural University, Kosugi-machi, Toyama 939-0398, Japan*
[c] *Biomolecular Characterization Division, RIKEN, Hirosawa 2-1, Wako-shi, Saitama 351-0198, Japan*

Received 8 April 2002; revised 18 May 2002; accepted 18 June 2002

First published online 15 July 2002

Edited by Richard Marais

Abstract A potent inhibitor of a dual-specificity protein phosphatase, VHR (vaccinia H1 related), was isolated during a screening of microbial metabolites. This inhibitor was identified as 4-isoavenaciolide (4-iA), and was determined to irreversibly inhibit VHR phosphatase activity with a 50% inhibitory concentration of 1.2 μM. Detailed tandem mass spectrometry analyses of proteolysed fragments revealed that two molecules of 4-iA bound a molecule of VHR at the two different fragments: one containing the catalytic domain and the other containing the α6 helix positioned surface domain. As 4-iA possesses a reactive exo-methylene moiety, it is possible that 4-iA inhibits VHR through the direct binding to the cysteine residue in the catalytic site (Cys124). Furthermore, 4-iA inhibited dual-specificity protein phosphatases and tyrosine phosphatases, but did not inhibit serine/threonine phosphatases. These results suggest that 4-iA is a cysteine-targeting inhibitor of protein phosphatases with a common HCX_5RS/T motif in the catalytic site.

Key words: Dual-specificity phosphatase; 4-Isoavenaciolide; Michael addition; Liquid chromatography–tandem mass spectrometry

1. Introduction

Protein phosphorylation is a fundamental regulation mechanism of intracellular signal transduction and is involved in important cellular events such as growth and differentiation. Phosphorylation level of proteins is strictly controlled by various protein kinases and protein phosphatases [1,2]. Dual-specificity phosphatases (DSPases) are a subfamily of protein tyrosine phosphatases (PTPases) and hydrolyze not only phosphotyrosine but also phosphoserine/threonine. VHR (vaccinia H1 related) is a human DSPase [3] and is thought to be a negative regulator of the mitogen-activated protein kinases extracellular signal-regulated kinase (ERK) 1 and ERK2, which are involved in growth factor signaling [4]. The structure of VHR was determined by X-ray crystallography and shown to consist of six α-helices and five β-strands [5,6]. PTPases and DSPases share a common consensus amino acid sequence, HCX_5RS/T, in the catalytic domain that is different from the corresponding domain of protein serine/threonine phosphatases [1,7]. In the case of VHR, the cysteine residue (Cys124) in the active site is located between β5 strand and α4 helix and it is known that chemical modification of this cysteine residue abolishes the DSPase activity [8].

Specific phosphatase inhibitors are proving to be useful tools for revealing phosphatase-mediated signal transduction cascades. For example, the potent protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) inhibitors, okadaic acid, phoslactomycin and tautomycin, have been used to investigate the physiological role of serine/threonine phosphorylation during signal transduction [9–11]. However, to date only a few DSPase inhibitors, such as sodium arsenate, phenylarsine oxide, and RK-682, have been reported [12,13]. Recently, we described the development of a potent inhibitor of VHR based on the structure activity relationship between VHR and RK-682 [14]. However, more selective and potent DSPase inhibitors are still required to provide a full range of compounds for inhibitory studies.

During the screening for specific VHR inhibitors from microbial metabolites, 4-isoavenaciolide (4-iA) was identified as an inhibitor of VHR. Turner et al. first isolated avenaciolide as an antifungal antibiotic and later they isolated 4-iA as a stereoisomer of avenaciolide [15,16]. There were several papers on the biological activity of avenaciolide [17–19]. Since avenaciolide possesses a reactive exo-methylene group moiety, it was thought that an adduct with a target protein is formed by Michael addition and that this leads to inhibition of glutamate transport [20]. However, there is no report on the inhibition of phosphatases, moreover, no direct evidence of adducts formation between the compound and its target protein. In this paper, we investigated the inhibitory mechanism of 4-iA against VHR and found that the compound covalently binds to Cys124 at the active site of VHR. Therefore we have provided structural information to account for the inhibitory action of 4-iA.

*Corresponding author. Fax: (81)-48-462-4669.
E-mail address: antibiot@postman.riken.go.jp (H. Osada).

Abbreviations: IC_{50}, 50% inhibitory concentration; PTPase, protein tyrosine phosphatase; DSPase, dual-specificity phosphatase; VHR, vaccinia H1 related; *p*NPP, *p*-nitrophenyl phosphate; LC–MS/(MS), liquid chromatography–(tandem) mass spectrometry; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; ERK, extracellular signal-regulated kinase; 4-iA, 4-isoavenaciolide

0014-5793/02/$22.00
PII: S0014-5793(02)03065-X

2. Materials and methods

2.1. Screening microbial extracts for inhibition of VHR

Microbial extracts (5 μl) dissolved in 50% methanol were added to 50 μl of the reaction mixture (50 mM sodium succinate (pH 6.0), 1.0 mM EDTA, 150 mM NaCl, 0.04 μM VHR, 0.05% Tween 20). The phosphatase reaction was started by addition of 50 μl of 20 mM *p*-nitrophenyl phosphate (*p*NPP). After incubation for 30 min at 37°C, absorbance at 405 nm was measured by a multiplate reader (Wallac ARVO, Amersham Pharmacia Biotech).

2.2. Isolation and identification of the VHR inhibitor

In the course of screening for VHR inhibition, a fungus strain BAUA-3130 was found to produce an active compound in the culture broth. Based on morphological characterization, the strain BAUA-3130 was assigned to the genus *Neosartorya* [21]. The active compound was extracted with acetone from mycelia and was purified by ethyl acetate extraction, and a successive column chromatography on silica gel and preparative high-performance liquid chromatography (HPLC).

The molecular formula of the compound was determined by high-resolution mass spectrometry to be $C_{15}H_{22}O_4$ (molecular weight 266). This compound was subsequently identified as 4-iA [16,22] using extensive analyses with various nuclear magnetic resonance experiments.

2.3. Measurement of various phosphatase activities

Inhibitory activity of 4-iA against various kinds of protein phosphatases was measured. Assay conditions of phosphatase activities (VHR [13], Cdc25B [23], laforin [24], PTP1B [25], CD45 [26], PP1 and 2A [10]) were performed as previously described.

Time-dependent VHR inhibition by 4-iA was determined by incubating VHR (0.75 μM) with 4-iA at concentrations of 0, 1.0, 1.5 and 3.5 μM for 0–240 min followed by measurement of VHR activity. To examine the reversibility of 4-iA-dependent VHR inhibition, VHR (0.9 μM) was incubated with 1.9 μM 4-iA at 30°C for 180 min followed by dialysis against 1000-fold of reaction buffer. The residual activity of VHR after dialysis was compared with a control sample that had not been dialyzed and a sample that had not been incubated with 4-iA.

2.4. Binding site determination by liquid chromatography–mass spectrometry (LC–MS) and LC–tandem MS (MS/MS)

A complex of 4-iA and VHR protein was prepared by incubation of 3.75 μM VHR with 6.0 μM 4-iA in reaction buffer lacking NaCl for 180 min at 30°C. Under this condition, approximately 40% of the VHR activity was inhibited.

The molecular weight of the VHR protein incubated with and without 4-iA was determined by LC–MS. The protein was desalted and purified by reversed phase HPLC (Capcell Pak Phenyl column, 1.5×35 mm; Shiseido, Tokyo, Japan) with a diode-array detector at a flow rate of 30 μl/min. VHR was eluted by a linear gradient of two solvent systems, A (H_2O/acetonitrile/trifluoroacetic acid at 100:0:0.09) and B (H_2O/acetonitrile/trifluoroacetic acid at 20:80:0.075). The molecular weight of the eluted VHR was analyzed using a Finnigan LCQ ion trap mass spectrometer with an ESI probe.

The proteins were digested with *Achromobacter* protease I (E/S = 1/100 (w/w)) at 37°C for 4 h. The generated peptides were separated on a Mightysil C18 column (1×50 mm; Kanto Chemical, Tokyo, Japan) by a linear gradient of 2–60% solvent A to solvent B in 30 min and analyzed by LC–MS and LC–MS/MS. MS/MS sequencing was performed by selection and fragmentation of the precursor ion with mass differences corresponding to addition of 4-iA.

3. Results

3.1. Isolation and identification of 4-iA

Microbial metabolites were screened for their ability to inhibit VHR activity. The screening used an in vitro enzyme assay with highly purified VHR as a target enzyme and *p*NPP as a substrate. Culture broths of approximately 650 fungi were screened and an extract of a strain, *Neosartorya* sp. BAUA-3130, showed strong inhibitory activity against VHR. The inhibitor was isolated and identified as 4-iA (dihydro-3-methylene-4-octyl-furo[3,4-b]furan-2,6(3H,4H)-dione (3a*R*,4*S*,6a*R*), Fig. 1).

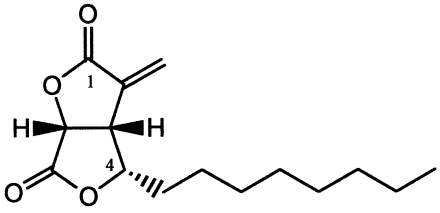

Fig. 1. Structure of 4-iA.

3.2. 4-iA is an irreversible inhibitor of VHR

In order to determine the inhibitory mechanism of 4-iA against VHR, we first characterized the inhibitory activity under several different conditions. The compound inhibited VHR activity in a time-dependent manner (Fig. 2A). Complete inhibition by 3.5 μM 4-iA was reached at 180 min of incubation. Subsequently, we tested whether such inhibition was reversible or not. After incubation of VHR with 4-iA, the reaction mixture was diluted and dialyzed against phosphate buffer. It would be predicted that if 4-iA is a reversible inhibitor, VHR activity should be recovered following dialysis. However, we found that the VHR activity of the dialysate was the same as that of an undialyzed sample, indicating that irreversible inhibition (Fig. 2B). Furthermore, this inhibition by 4-iA was completely abolished in the presence of the reducing reagent, such as β-mercaptoethanol (data not shown). These results strongly suggest that the inhibitory action of 4-iA is due to the covalent bond formation between the inhibitor and the target protein, VHR.

3.3. Binding site assignments

Our initial analyses suggested that 4-iA could be covalently bound to VHR and that this may provide the mechanism of inhibition. Therefore to verify these observations and to gain more precise information as to the nature of the covalent linkage, we measured the molecular weight shift of VHR be-

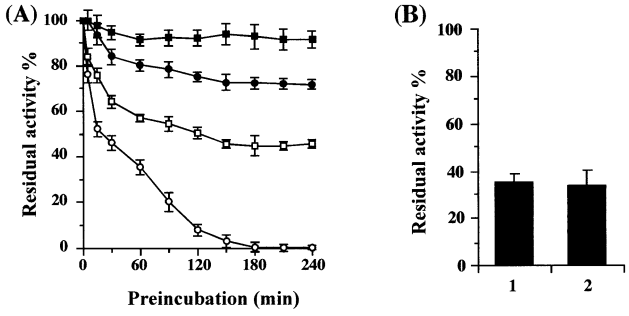

Fig. 2. Inhibition properties of 4-iA on VHR. A: Time-dependent inhibition by 4-iA. VHR (0.75 μM) was incubated with various concentrations of 4-iA (0, closed squares; 1.0 μM, closed circles; 1.5 μM, open squares; 3.5 μM, open circles) for 0–240 min and the residual activities measured at the time points indicated. B: Irreversible inhibition of VHR by 4-iA. VHR (0.9 μM) was incubated for 180 min with 1.9 μM 4-iA followed by dialysis against drug-free buffer. Then, residual activities before (1) and after (2) dialysis are measured (mean ± standard deviation obtained from three independent experiments). VHR activity without the drug treatment represents the 100% value.

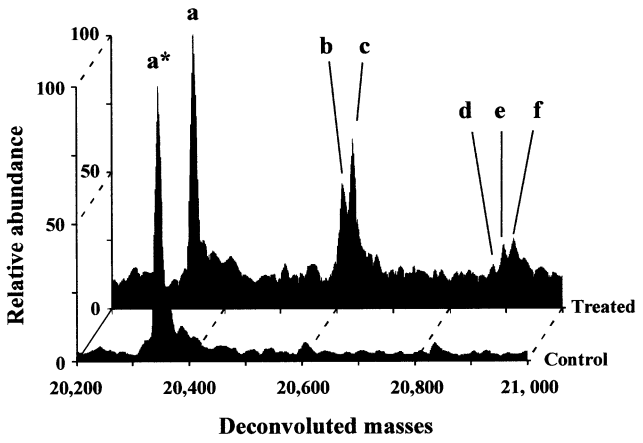

Fig. 3. 4-iA covalently binds to VHR. Molecular weight changes of VHR by treatment with 4-iA as determined by LC–MS. A: Mass spectrum of control and 4-iA-treated VHR. a*: control, and a–f: 4-iA-treated VHR ion peaks. Molecular weights were determined as 20 345 (a* and a), 20 610 (b), 20 628 (c), 20 877 (d), 20 895 (e), and 20 913 (f).

fore and after 4-iA treatment using LC–MS. The molecular weight of VHR was determined to be 20 345 (Fig. 3, peak a*) and this is in agreement with that predicted from its amino acid sequence. However, a range of molecular weights for 4-iA-treated VHR was obtained (Fig. 3). These were a: 20 345 (VHR), b: 20 610 (VHR+4-iA), c: 20 628 (VHR+water adduct-4iA), d: 20 877 (VHR+2×4-iA), e: 20 895 (VHR+4-iA+water adduct-4iA) and f: 20 913 (VHR, 2×water adduct-4iA). These results suggested that one or two molecules of 4-iA (molecular weight 266) or water adduct-4iA (molecular weight 284) are able to covalently bind to a VHR molecule under conditions in which VHR activity is inhibited.

Following the demonstration that two molecules of 4-iA are able to covalently bind to VHR, we decided to precisely determine the 4-iA binding sites. VHR was incubated in the presence or absence of 4-iA, and digested with *Achromobacter* protease I. Then, the resulting peptides were analyzed by LC–MS. The amino acid sequences of the digested peptides were deduced based on the molecular masses. We detected doublet extra masses with the 4-iA-treated protein difference between calculated and observed masses. These masses corresponded to peptides #9 and #10 with 4-iA (Δ 266.5, 265.6) and water-adducted 4-iA (Δ 284.9, 284.8), respectively. Using more detailed analyses of these peptides by MS/MS, fragment ions were detected and assigned. In peptide #9, b_{11}, b_{16} and y_{26}

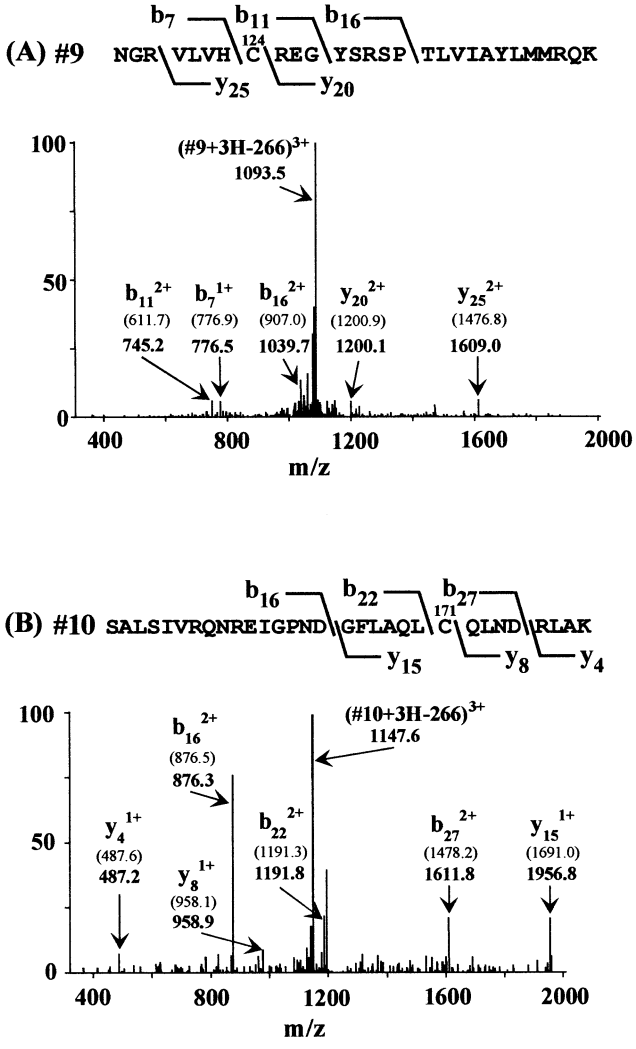

Fig. 4. 4-iA binding sites assignment. 4-iA binding sites were determined by LC–MS/MS. A: MS/MS spectrum of triply charged peptide #9 (*m/z*, 1094.0). Cleaved peptide fragments (singly charged b7 and doubly charged b11, b16, y20 and y25) were identified. Predicted peptide fragments are given parentheses. B: MS/MS spectrum of triply charged peptide #10 (*m/z*, 1148.0). Cleaved peptide fragments (singly charged y4, y8, and y15 and doubly charged b16, b22, and b27) were identified.

obtained fragments (*m/z* 745.2 (b_{11}^{2+}), 1039.7 (b_{16}^{2+}), and 1609.0 (y_{25}^{2+})) increased about 266 more than the predicted fragments (*m/z* 611.7 (b_{11}^{2+}), 907.0 (b_{16}^{2+}), and 1476.8 (y_{25}^{2+})) which account for the peptide containing 4-iA (molecular weight 266). Other

Table 1
Theoretical peptides and mass (Da) after digestion by *Achromobacter* protease I

No.	Predicted peptide sequence	Calculated (Da)	Observed (Da)	Δ (Da)
1:	P	115.1	–	–
2:	LK	259.4	–	–
3:	EGK	332.4	–	–
4:	LQK	387.5	–	–
5:	MDVK	491.6	491.0	0.6
6:	DSGITYLGIK	1066.2	1066.5	0.3
7:	LGITHVLNAAEGRSMHVNTNANFYK	2905.3	2906.2	0.9
8:	ANDTQEFNLSAYFERAADFIDQALAQK	3077.3	3076.8	0.5
9:	NGRVLVHCREGYSRSPTLVIAYLMMRQK	3278.9	3545.4 (3563.8)	266.5 (284.9)
10:	SALSIVRQNREIGPNDGFLAQLCQLNDRLAK	3440.9	3706.5 (3726.7)	265.6 (284.8)
11:	SGSFELSVQDLNDLLSDGSGCYSLPSQPCNEVTPRIYVGNASVAQDIPK	5172.7	5171.7	1.0

Δ (Da) is the difference between calculated and observed masses in Da. 4-iAv and water-adducted 4-iAv were observed in both peptides 9 and 10.

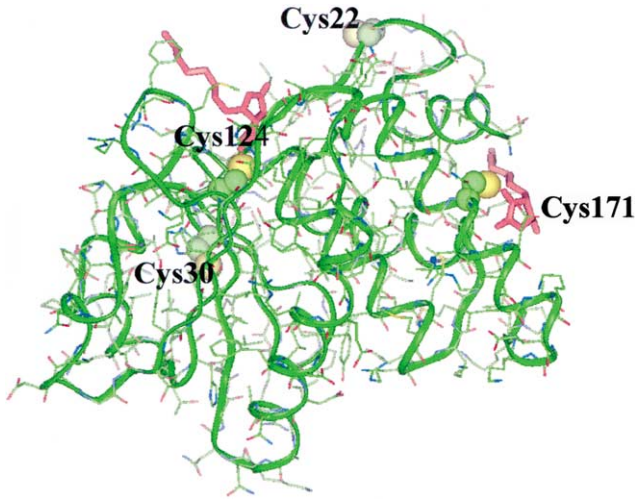

Fig. 5. Proposed binding image of 4-iA on VHR.

fragments (b_7^{1+} and y_{20}^{2+}) did not change after treatment with 4-iA (Fig. 4A). Likewise in peptide #10, b_{27} and y_{15} obtained fragments (*m/z* 1611.8 (b_{27}^{2+}) and 1956.8 (y_{15}^{1+})) increased the number more than the predicated fragments (*m/z* 1478.2 (b_{27}^{2+}), and 1691.0 (y_{15}^{1+})). Other fragments (b_{16}, b_{22}, y_8 and y_4) did not change after treatment with 4-iA (Fig. 4B). These results demonstrated that the binding sites of 4-iA to VHR were Cys124 on peptide #9 and Cys171 on peptide #10, respectively (Fig. 4A,B). Interestingly Cys124 is located in the catalytic site of VHR and therefore covalent binding of 4-iA at this site would indeed be predicted to inhibit phosphatase activity.

3.4. 4-iA inhibits DSPases and PTPases, but not serine/threonine phosphatases

The inhibitory activity of 4-iA is rather potent against VHR with a 50% inhibitory concentration (IC_{50}) value of 1.2 μM. To determine whether the inhibitory activity of 4-iA is specific for VHR, we tested its inhibitory activity against other protein phosphatases (Table 2). RK-682 and arsenate are known PTPase inhibitors and inhibited DSPases and PTPases, but had no inhibitory activity against PP1 or PP2A. Tautomycin, known to be a potent PP1/PP2A inhibitor, inhibited PPases, but not DSPases and PTPases. 4-iA potently inhibited DSPases (IC_{50} 1.2–9.4 μM) and PTP1B (IC_{50} 10.7 μM), and weakly inhibited CD45 (IC_{50} 245 μM). However, PPases (PP1 and PP2A) were not inhibited by 4-iA even at a concentration of 250 μM. These results suggested that 4-iA is a potent DSPase and PTPase inhibitor.

4. Discussion

Protein phosphatase inhibitors are useful tools to reveal signal transduction pathways of immune response as well as to cure immunological diseases [27,28]. In this report, we identified 4-iA as a potent VHR inhibitor and investigated the effect of 4-iA on VHR, a well-characterized member of DSPases. Avenaciolide was originally reported as an antifungal compound (minimal inhibitory concentration 10 μg/ml = 40 μM) [15] and its target was predicted to be a component of the mitochondria (IC_{50} 12.5 μg/ml = 47 μM) [17–20]. Subsequently 4-iA was isolated as a stereoisomer of avenaciolide and synthesized [16,22], however, its biological activity and mechanism of action were not reported. In this work, we have isolated 4-iA from culture broth of *Neosartorya* sp., which is teleomorph of some species of *Aspergillus*, then we have characterized the inhibitory mode of action of 4-iA on VHR. Inhibition of VHR activity by 4-iA seemed to be irreversible (Fig. 2B). Analyses of LC–MS profiles suggested that two molecules of 4-iA covalently bound to a VHR molecule (Fig. 3). To determine the binding sites of 4-iA on the VHR, peptides were prepared by protease digestion (Table 1). Analyses of LC–MS/MS suggested the two 4-iA binding sites were located on Cys124 in the catalytic domain (residues 123–131) and Cys171 in the α6 helix (residues 163–179) on the surface of VHR (Fig. 4). The data suggest that 4-iA covalently binds to the SH group of cysteine residues by 1,4-addition (Michael addition) in the same manner as avenaciolide [20]. As shown in Fig. 5, there are four cysteine residues in a VHR molecule. We anticipated that 4-iA might bind to any cysteine residue without selectivity, because the exo-methylene moiety should be highly reactive. However, 4-iA bound to Cys124 in the active site and Cys171 on the surface of VHR (α6), but not to Cys22 and Cys30. These observations suggest that a hydrophobic cleft near a cysteine residue is required for the adduct formation between 4-iA and the target protein. This assumption is consistent with the previous report, moreover, 4-iA derivatives with a shorter alkyl chain at the 4-position have weaker inhibitory activity (data not shown).

4-iA potently inhibited DSPases and PTP1B with a IC_{50} range of 1–10 μM. However, the inhibitory activity of 4-iA is rather weak to CD45 (IC_{50} 245 μM) and LAR [29] (IC_{20} 30 μM, data not shown). Apparently, 4-iA may potently inhibit non-receptor type phosphatases (VHR, Cdc25 etc.) more than receptor type phosphatases (CD45 and LAR), but there is more clear selectivity of 4-iA in respect of tyrosine phosphatases (DSPase, PTPase) vs. serine/threonine phosphatases. The catalytic center of DSPases and PTPases has a consensus sequence, HCX_5RS/T [1,7], which is a target site of 4-iA. On the

Table 2
IC_{50} values (μM) for various phosphatases

Compound	DSPases			PTPases		PPases	
	VHR	Cdc25B	Laforin	PTP1B	CD45	PP1	PP2A
4-iA	1.2 ± 0.1	9.4 ± 1.0	1.8 ± 0.4	10.7 ± 0.8	244.6 ± 3.3	> 250	> 250
RK-682	11.6 ± 0.5	10.4 ± 0.8	40.4 ± 5.8	39.2 ± 0.5	13.4 ± 0.2	> 250	> 250
Arsenate	65.0 ± 5.5	212.0 ± 18.5	35.1 ± 4.5	76.9 ± 2.5	90.8 ± 0.9	> 250	> 250
Tautomycin	> 200	> 200	> 200	> 200	> 200	0.03 ± 0.0	0.05 ± 0.1

IC_{50} values for various phosphatases by 4-iA are shown. The mean ± standard deviation from three independent experiments for VHR (0.75 μM) and Cdc25B (0.05 μM), and the mean ± standard deviation from two independent experiments for laforin (0.01 μM), PTP1B (0.07 μM), DC45 (0.02 μM), PP1 (0.05 U) and PP2A (0.005 U) are shown.

contrary, since the catalytic site of PPases is different from that of DSPases, 4-iA did not inhibit PPases even at 250 μM (Table 2). This is the first report revealing the covalent binding of 4-iA to a protein phosphatase, VHR and the amino acid sequences involved in the adduct formation.

Acknowledgements: This work was supported by a Grant-in-Aid for Scientific Research to T.U. and H.O. from the Ministry of Education, Science, Sports, Culture and Technology, Japan, and Basic Scientific Research Project, Multibioprobe in RIKEN. The authors thank S. Kanazawa (Inst. Biotech. Appl. Soil Eumycetes) for a gift of the producing strain of 4-iA and S. Uchida and S. Chonan (Taisho Pharm. Co.) for the bioassay of tyrosine phosphatases. We are grateful to H. Ohrui (Tohoku University) and to N. Kanoh (RIKEN) for their valuable suggestions concerning the structure of 4-iA.

References

[1] Fauman, E.B. and Saper, M.A. (1996) Trends Biochem. Sci. 21, 413–417.
[2] Stone, R.L. and Dixon, J.E. (1994) J. Biol. Chem. 269, 31323–31326.
[3] Ishibashi, T., Bottaro, D.P., Chan, A., Miki, T. and Aaronson, S.A. (1992) Proc. Natl. Acad. Sci. USA 89, 12170–12174.
[4] Todd, J.L., Tanner, K.G. and Denu, J.M. (1999) J. Biol. Chem. 274, 13271–13280.
[5] Yuvaniyama, J., Denu, J.M., Dixon, J.E. and Saper, M.A. (1996) Science 272, 1328–1331.
[6] Assignments of region for α-helices and β-sheets were modified: PDB Id 1VHR.
[7] Denu, J.M., Stuckey, J.A., Saper, M.A. and Dixon, J.E. (1996) Cell 87, 361–364.
[8] Zhou, G., Denu, J.M., Wi, L. and Dixon, J.E. (1994) J. Biol. Chem. 1994, 28084–28090.
[9] Ishihara, H., Martin, B.L., Brautigan, D.L., Karaki, H., Ozaki, H., Kato, Y., Fusetani, N., Watabe, S., Hashimoto, K., Uemura, D. and Hartshorne, D.J. (1989) Biochem. Biophys. Res. Commun. 159, 871–877.
[10] Usui, T., Marriott, G., Inagaki, M., Schwarp, G. and Osada, H. (1999) J. Biochem. 125, 960–965.
[11] MacKintosh, C. and Klumpp, S. (1990) FEBS Lett. 277, 137–140.
[12] Garcia-Morales, P., Minami, Y., Luong, E., Klausner, R.D. and Samelson, L.E. (1990) Proc. Natl. Acad. Sci. USA 87, 9255–9259.
[13] Hamaguchi, T., Sudo, T. and Osada, H. (1995) FEBS Lett. 372, 54–58.
[14] Usui, T., Kojima, S., Kidokoro, S., Ueda, K., Osada, H. and Sodeoka, M. (2001) Chem. Biol. 8, 1209–1220.
[15] Brookes, D., Tidd, B.K. and Turner, W.B. (1963) J. Chem. Soc. 1963, 5385–5391.
[16] Aldridge, D.C. and Turner, W.B. (1971) J. Chem. Soc. 1971, 2431–2432.
[17] Wimhurst, J.M. and Harris, E.J. (1976) Biochim. Biophys. Acta 437, 51–61.
[18] Hoek, J.B. and Njogu, R.M. (1980) J. Biol. Chem. 255, 8711–8718.
[19] Njogu, R.M. and Hoek, J.B. (1983) FEBS Lett. 152, 222–226.
[20] Meyer, J. and Vignais, P.M. (1973) Biochim. Biophys. Acta 325, 375–384.
[21] Malloch, D. and Cain, R.F. (1972) Can. J. Bot. 50, 2621–2621.
[22] Ohrui, H. and Emoto, S. (1975) Tetrahedron Lett. 42, 3657–3660.
[23] Gottlin, E.B., Xu, X., Epstein, D.M., Burke, S.P., Eckstein, J.W., Ballou, D.P. and Dixon, J.E. (1996) J. Biol. Chem. 271, 27445–27449.
[24] Ganesh, S., Agarwala, K.L., Ueda, K., Akagi, T., Shoda, K., Usui, T., Hashikawa, T., Osada, H., Delgado-Escueta, A.V. and Yamakawa, K. (2000) Hum. Mol. Genet. 9, 2251–2261.
[25] Liu, F., Hill, D.E. and Chernoff, J. (1996) J. Biol. Chem. 271, 31290–31295.
[26] Pacitti, A., Stevis, P., Evans, M., Trowbridge, I. and Higgins, T.J. (1994) Biochim. Biophys. Acta 1222, 277–286.
[27] Hamaguchi, T., Takahashi, A., Kagamizono, T., Manaka, A., Sato, M. and Osada, H. (2000) Bioorg. Med. Chem. Lett. 10, 2657–2660.
[28] Hamaguchi, T., Takahashi, A., Manaka, A., Sato, M. and Osada, H. (2001) Int. Arch. Allergy Immunol. 126, 318–324.
[29] Tsai, A.Y., Itoh, M., Streuli, M., Thai, T. and Saito, H. (1991) J. Biol. Chem. 266, 10534–10543.