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RK-682, a potent inhibitor of tyrosine phosphatase, arrested the mammalian cell cycle progression at G_1phase

Takuya Hamaguchi, Tatsuhiko Sudo, Hiroyuki Osada*

Antibiotics Laboratory, The Institute of Physical and Chemical Research (RIKEN), Hirosawa 2-1, Wako-shi, Saitama 351-01, Japan

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Abstract A specific inhibitor of protein tyrosine phosphatase (PTPase), RK-682 (3-hexadecanoyl-5-hydroxymethyl-tetronic acid) was isolated from microbial metabolites. In vitro, RK-682 inhibited dephosphorylation activity of CD45 and VHR with IC_{50} 54 and 2.0 μM, respectively. In situ, sodium orthovanadate and RK-682 enhanced the phosphotyrosine level of Ball-1 cells, a human B cell leukemia, but not the phosphoserine/threonine level. The PTPase inhibitors, however, had the different arrest point on the cell cycle progression. Sodium orthovanadate inhibited the cell cycle progression at G_2/M boundary phase, on the other hand, RK-682 inhibited the G_1/S transition.

Key words: Tyrosine phosphatase inhibitor; Cell cycle; cdc25B; CD45; VHR; Microbial product

1. Introduction

Reversible phosphorylation on tyrosine residues of proteins is one of the earliest and important events in the signal transduction pathways leading to the stimulation of cell proliferation. Because many oncogenes encode protein tyrosine kinases (PTKs), much attention had been paid to PTK compared with protein tyrosine phosphatases (PTPases). Recently, it was reported that PTPase played an important role in the signal transduction [1]. Mitosis is initiated following the activation of cdc2-cyclin B kinase complex, so-called M-phase promoting factor or maturation promoting factor (MPF) [2]. The key step in the activation of MPF is dephosphorylation on Thr-14 and Tyr-15 of cdc2 protein by cdc25, a kind of dual-specificity protein phosphatases [3]. VHR (VH1-related human protein), which was isolated from a human fibroblast has the dual-specificity phosphatase activity towards phosphotyrosine and phosphoserine/threonine like cdc25 [4].

The receptor-type PTPase, CD45 (a leukocyte common antigen) activates protein tyrosine kinase $p56^{lck}$ [5] and $p59^{fyn}$ [6,7], which are associated with the intracellular domain of the T cell surface glycoproteins, CD4 and CD8. CD45 is involved in T cell [8] and B cell [9] development and activation.

Specific PTPase inhibitors should be useful as a biological tool to reveal the signal transduction. Okadaic acid [10] and tautomycin [11], which are known to inhibit serine/threonine protein phosphatases (PPases), are valuable tools to test the physiological role of serine/threonine phosphorylation in the signal transduction. On the contrary, only a few PTPase inhibitors, for example, sodium orthovanadate (vanadate), phenylarsine oxide (PAO) [12] and dephostatin [13] have been known. A more potent and selective PTPase inhibitor is still required.

To discover specific PTPase inhibitors, we have started the screening of microbial metabolites. In this paper, we describe the isolation of RK-682, 3-hexadecanoyl-5-hydroxymethyl-tetronic acid, as a potent PTPase inhibitor from the fermentation of *Streptomyces* sp. 88-682 and elucidate the inhibitory activity of RK-682 to PTPases.

2. Materials and methods

2.1. Isolation of RK-682

Streptomyces sp. 88-682, isolated in Kuroishi City, Aomori, Japan, was cultured in 36 liters of a production medium (glucose 2.0%, soy bean meal 2.5%, soluble starch 1.0%, dry yeast 0.4%, meat extract 0.1%, NaCl 0.2% and K_2HPO_4 0.005%) for 96 h at 28°C. The mycelial cake was collected from the fermentation broth and extracted with acetone. After removal of acetone, the residual solution was extracted with ethyl acetate under acidic condition. The extract was concentrated to dryness and applied to a silica gel (Kiesel gel 60, Merck; 8 diam. × 60 cm), which was eluted with a mixture of methanol/chloroform (1:9 to 2:8). The active fractions were collected and applied to a column (6 diam. × 80 cm) of Sephadex LH-20 (Pharmacia) equilibrated with methanol. The eluted active fraction from Sephadex LH-20 was further purified by preparative HPLC using an ODS column (Capcell pak; 20 diam. × 250 mm; Shiseido, Tokyo, Japan) with 40% tetrahydrofuran/water to yield 700 mg of RK-682 (retention time; 14.1 min, flow rate; 6.0 ml/min).

2.2. Cell culture

A human B cell leukemia cell line, Ball-1 was obtained from Cell Bank, RIKEN and maintained in RPMI1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% FCS; fetal calf serum (Gibco). A mouse hybridoma 1G2 cell line was provided from ATCC (Rockville, MD, USA).

2.3. Phosphatase assay

GST-VHR fusion protein (VHR) was prepared as described [4]. The plasmid to express GST-cdc25B fusion protein (cdc25B) was constructed by insertion of the AatI fragment (nucleic acids 1394–1844) of the human cdc25B cDNA [14] into the *Sma*I site of the pGEX1 vector. Dephosphorylation activity of VHR and cdc25B to *p*-nitrophenylphosphate (*p*Npp) was measured in the assay buffer (25 mM MOPS, pH 6.5, 5 mM EDTA, 1 mM dithiothreitol) [15]. A CD45 fraction was prepared from Ball-1 cells by the method as described [16], and its PTPase activity to phosphotyrosine was measured by colorimetric assay [9].

2.4. Immunoblotting

Western blotting was performed as described below. Ball-1 cells (1×10^6) were treated with phosphatase inhibitors for 20 h at 37°C. The cell protein was subjected to 8% SDS-PAGE, electrophoretically transferred to a nitrocellulose membrane, and incubated for 1 h with a monoclonal antibody to phosphotyrosine (obtained from mouse hybridoma cells, 1G2) or phosphothreonine (Biomakor, Rehovot, Israel). The membrane was incubated with 0.5 μg/ml of horseradish peroxidase conjugated anti-mouse IgG (Kirkegaard & Perry Lab., Gaithersburg, MD, USA) and visualized with a chemiluminescence system (ECL, Amersham).

*Corresponding author. Fax: (81) (48) 462-4669.

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2.5. Cell cycle assay

Ball-1 cells were cultured in a 12-well plate at a concentration of 2×10^5 cells/well in 1 ml of 10% FCS-RPMI1640 medium with inhibitors at 37°C for 20 h. The cells were then washed with PBS (phosphate-buffered saline), stained with propidium iodide (Sigma) and analyzed by flow cytometry (Coulter, Epics Profile II, Hialeah, FL, USA).

3. Results

3.1. Identification and characterization of RK-682

We have screened about 350 strains of soil actinomycetes for PTPase inhibitors and found that *Streptomyces* sp.88-682 produced RK-682. The isolation of RK-682 was monitored by the inhibitory activity towards VHR (Table 1). The chemical structure of RK-682 was identified as 3-hexadecanoyl-5-hydroxymethyl tetronic acid (Fig. 1) by means of 2D-NMR and HR-FABMS.

We investigated the inhibitory activity of RK-682 to CD45 and VHR. RK-682 inhibited the dephosphorylation activity of CD45 to phosphotyrosine with IC_{50} 54 μM (Fig. 2A). RK-682 was more potent than vanadate (IC_{50} = 200 μM). On the other hand, okadaic acid and tautomycin had no detectable activity toward CD45. RK-682 and vanadate inhibited the dephosphorylation activity of VHR in vitro with IC_{50} 2.0 and 16 μM, respectively (Fig. 2B).

The inhibitory kinetics of RK-682 and vanadate against VHR substrate (*p*Npp) were measured and shown in the Lineweaver-Burk plot (Fig. 3A and B). Whereas RK-682 was a typical noncompetitive inhibitor with K_i of 2.5 μM, vanadate was a competitive inhibitor with K_i of 36 μM. These results suggest that vanadate mimics a substrate, but RK-682 is not a *p*Npp mimetic compound.

RK-682 was slightly soluble to MeOH, $CHCl_3$ and ethyl acetate, but hardly soluble to H_2O. When cells were incubated with 10 μM RK-682, incorporated RK-682 was not detectable within 5 h and reached only 10 pM (0.1% of total amount) after 20 h (data not shown). The inhibitory concentration of RK-682 in situ was much higher than that in vitro, because the membrane permeability of the inhibitor was poor.

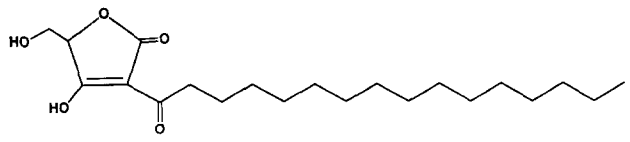

Fig. 1. Structure of RK-682.

3.2. Effect of RK-682 on the cellular phosphoprotein of Ball-1 cells

RK-682 inhibited dual-specificity protein phosphatase VHR activity. To determine the specificity of RK-682 in situ, we investigated the effect of the inhibitor on the phosphorylation state of cellular phosphoproteins in Ball-1 cells. Ball-1 cell lysates treated with phosphatase inhibitors were analyzed by Western blotting with anti-phosphotyrosine (Fig. 4B) or anti-phosphothreonine antibody (Fig. 4C). Okadaic acid (lanes 2 and 3) did not increase the tyrosine phosphorylation level of any cellular phosphoprotein but markedly increased the threonine phosphorylation level. On the contrary, vanadate (lanes 4 and 5) and RK-682 (lanes 6, 7 and 8) increased the only tyrosine phosphorylation level of proteins (approximately 110 and 90 kDa). These results showed that RK-682 specifically inhibited PTPase, but not PPase. Moreover, RK-682 and vanadate might inhibit the different cellular tyrosine phosphatases, which caused the differential phosphorylation level of 60-kDa protein indicated by an arrow.

3.3. Effect of RK-682 on the cell cycle progression

VHR is a dual-specificity phosphatase like cdc25 which is a cell cycle regulating phosphatase. Therefore, we next investigated the effect of RK-682 on the cell cycle of Ball-1 cells. RK-682 had an inhibitory effect on the cell cycle progression at G_1 phase (Fig. 5C). On the other hand, vanadate did not inhibit G_1 phase but G_2 phase (Fig. 5B). These results suggest that two PTPase inhibitors inhibit different phosphatases which regulate the cell cycle progression.

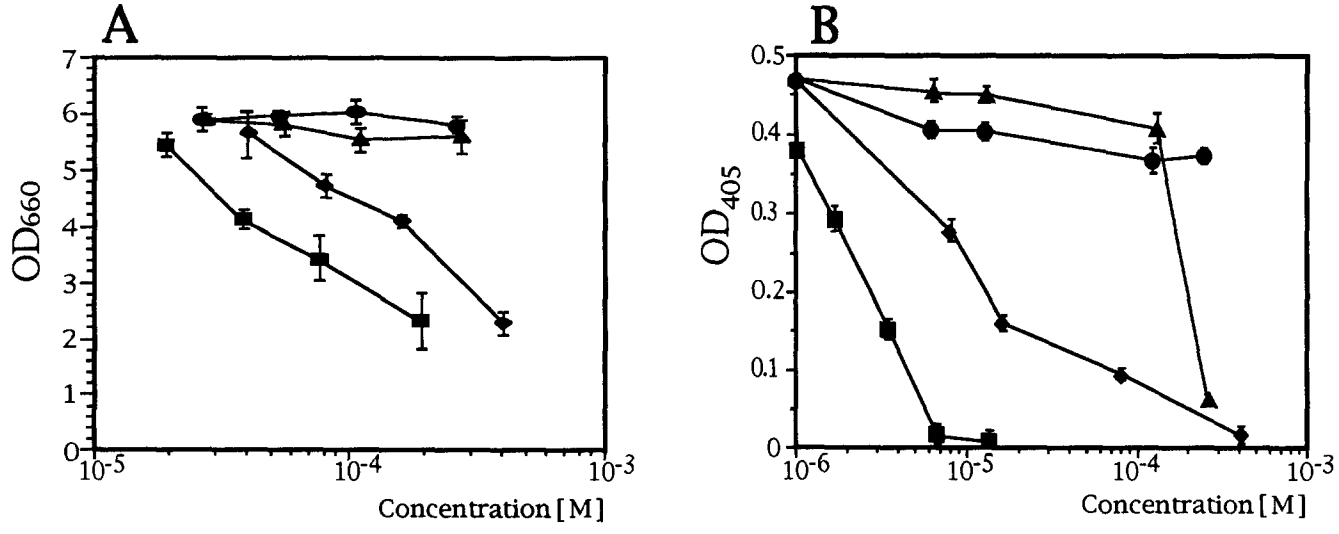

Fig. 2. The effect of phosphatase inhibitors to the dephosphorylation activity of CD45 and VHR. Inhibitory activity of okadaic acid (●), tautomycin (▲), vanadate (◆) and RK-682 (■) to CD45 (A) and GST-VHR (B). Data are the average of three independent experiments.

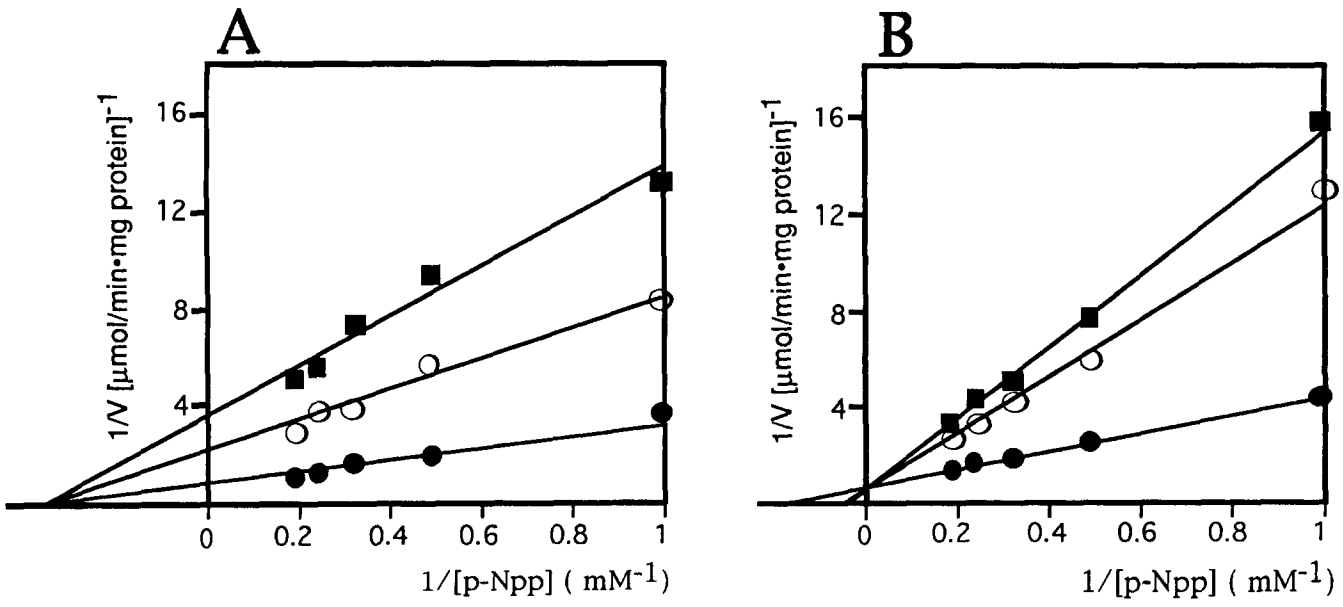

Fig. 3. The Lineweaver-Burk plot of RK-682 (A) and vanadate (B) to *p*Npp. The concentrations of RK-682 were 2.7 (●), 4.1 (○), 5.4 (■) μM. VHR (20 μg/ml) hydrolyzed *p*Npp (1, 2, 3, 4, 5 mM). Vanadate concentrations were 0 (●), 0.16 (○), 0.25 (■) mM.

3.4. Effect of PTPase inhibitors on cdc25B in vitro

We investigated the inhibitory activity of vanadate and RK-682 against the bacterially expressed cdc25B phosphatase (Fig. 6), which regulated G_2/M progression by dephosphorylation of MPF. Vanadate inhibited the phosphatase activity of cdc25B in vitro (IC_{50} = 30 μM) but RK-682 did not. The specificity of RK-682 to PTPases was different from that of vanadate, and they showed different arrest points of the cell cycle.

4. Discussion

Recently, it was reported that tyrosine phosphatases (PTPases) and dual-specificity phosphatases were key enzymes in the signal transduction pathway for a wide range of cellular processes [17]. Dual-specificity phosphatases, for example, cdc25, HVH1 and 3CH134 are known to be involved in cell cycle progression [3], MAP kinase inactivation [18] and growth factor stimulation [19], respectively. The specific inhibitor of the dual-specificity phosphatase will be a valuable tool to reveal the signal transduction. The bacterially expressed VHR had stronger dephosphorylation activity to *p*Npp compared with the bacterially expressed cdc25B in vitro. Therefore, we used VHR for the screening of inhibitors from microbial metabolites.

In this paper, we described the screening of PTPase inhibitors and the isolation of RK-682 from *Streptomyces* sp. 88-682. The chemical structure of RK-682 was already described as a HIV-1 protease inhibitor by other researchers [20]. However, RK-682 inhibited PTPase at a lower concentration compared with HIV protease reported in the paper. RK-682 consists of a tetronic acid and a saturated fatty acid moiety. It was known that fatty acids showed various non-specific inhibitory activities against purified enzymes. RK-682 derivatives which have a part of the structure showed only weak inhibitory activity to VHR, but not

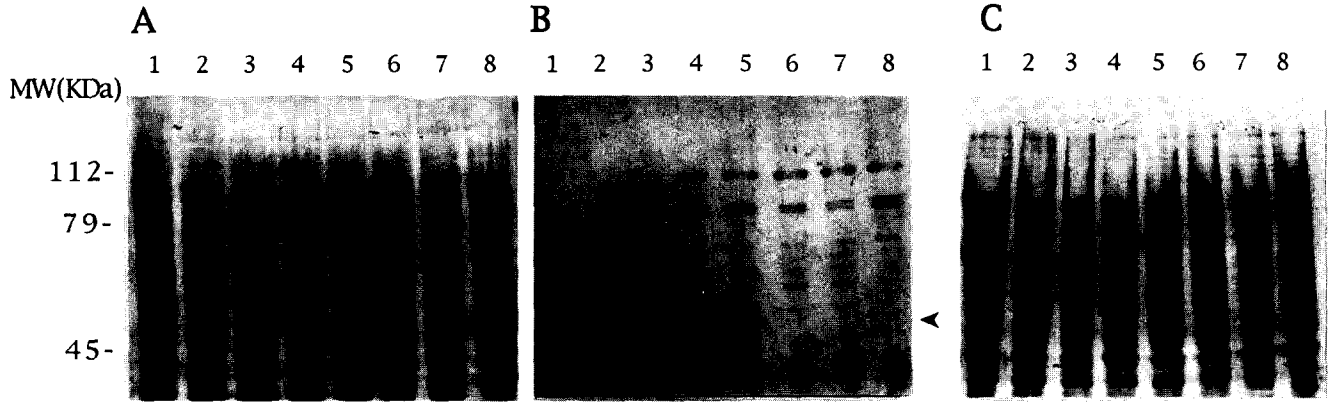

Fig. 4. The effect of phosphatase inhibitors on tyrosine phosphorylation in Ball-1 cells. Thirty micrograms of protein were loaded per lane, then stained by Coomassie blue (A). Ten micrograms of protein per lane were loaded for Western blotting with anti-phosphotyrosine (B) or anti-phosphothreonine (C). Phosphatase inhibitors okadaic acid (5, 10 μM; lanes 2 and 3), vanadate (250, 500 μM; lanes 4 and 5) and RK-682 (40, 80 and 160 μM; lanes 6, 7 and 8) were added to Ball-1 cells. Lane 1 is a control without inhibitor.

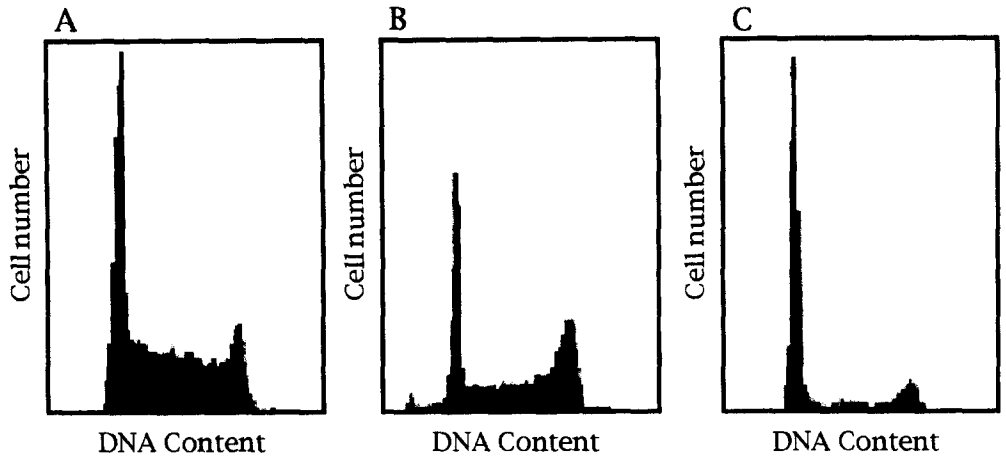

Fig. 5. The influence of PTPase inhibitors on cell cycle progression. After Ball-1 cells (1×10^5) were treated with vanadate 80 μM (B) and RK-682 30 μM (C), DNA content was analyzed by flow cytometry. The cells without treatment were shown in (A).

at all to CD45 (data not shown). Therefore, the whole structure of RK-682 is necessary to recognize and inhibit the protein phosphatases.

Vanadate is a well known and useful inhibitor to investigate the role of PTPase in the signal transduction. In this paper, we found that noticeable differences between vanadate and RK-682. First, RK-682 was more potent inhibitor (approximately 20 times stronger) compared with vanadate toward VHR and CD45 (Fig. 2). Second, the inhibitory profile of RK-682 and vanadate to VHR substrate *p*Npp are noncompetitive and competitive, respectively (Fig. 3). Third, vanadate enhanced the phosphotyrosine level of 60 kDa protein in Ball-1 cell, whereas RK-682 enhanced that of 110 kDa protein (Fig. 4). Finally, these PTPase inhibitors had the different arrest point on the cell cycle progression of Ball-1 cell (Fig. 5) and a mouse tsFT210 cell [21] (data not shown).

The mammalian cell cycle progression is regulated by cyclin dependent kinases (cdks) [2,3,22–24]. At the G_2/M transition, the activation of the cdc2–cyclin B complex depends on the activities of cdc25B [14] and cdc25C [25]. In fact, vanadate inhibited the cdc25B in vitro, but RK-682 did not (Fig. 6). The G_1/S transition requires cdk2 activity [22]. The cdk2–cyclin E complex was essential during S-phase for the phosphorylation of cdc25A [23]. Cdi1 (cyclin-dependent kinase interactor) is a dual-specificity phosphatase and negatively regulates cdk2 [24]. However, the principal which dephosphorylates and activates cdk2–cyclin E has not yet been cleared. RK-682 inhibited the cell cycle progression at G_1 phase (Fig. 5C) but not the phosphatase activity of cdc25B (Fig. 6), and the elucidation of this observation is a further investigation.

In this paper, RK-682 was described as a specific inhibitor of PTPases and used as a biochemical probe to investigate the mammalian cell cycle. The specific inhibitor of PTPase should be an useful tool to reveal the importance of protein phosphorylation and dephosphorylation in various mammalian cells [26].

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Table 1
Purification of RK-682

Fraction	Total amount [g]	Activity* Total [U]	Specific [U/g]	Yield [%]	Purification -fold
Ethyl acetate extract	47.3	9500	200	100	1.0
Silica gel	11.0	9100	830	95	4.2
Sephadex LH-20	1.1	9000	8200	94	41.5
HPLC (ODS)	0.17	8000	47000	83	237.0

The isolation of RK-682 was monitored by means of its inhibitory activity towards VHR and the procedure was revealed in section 2.1.
*One unit is the amount of RK-682 required to inhibit to dephosphorylation activity of GST-VHR (1 μg).

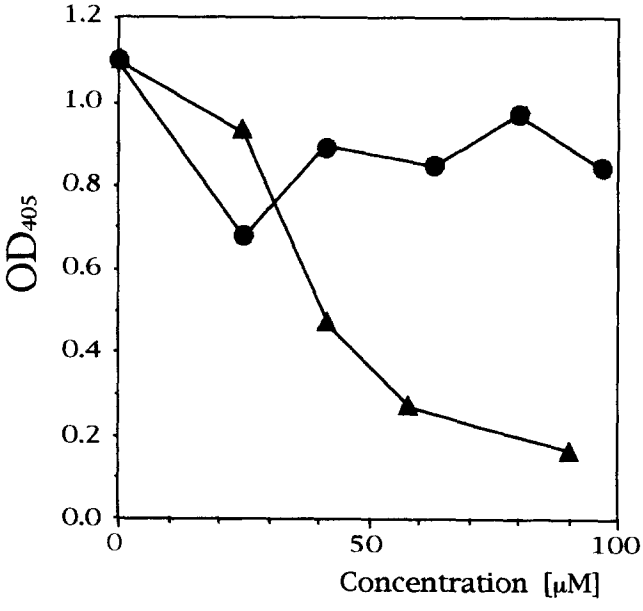

Fig. 6. Effect of PTPase inhibitors on cdc25B in vitro. Inhibitory activity of RK-682 (●) and vanadate (▲) against the phosphatase activity of cdc25B to *p*Npp.

References

[1] Sun, H. and Tonks, N.K. (1994) Trends Biochem. Sci. 19, 480–485.
[2] Murray, A.W. and Kirschner, M.W. (1989) Science 246, 614–621.
[3] Dunphy, W.G. and Kumagai, A. (1991) Cell 67, 189–196.
[4] Ishibashi, T., Bottaro, D.P., Chan, A., Miki, T. and Aaronson, S.A. (1992) Proc. Natl. Acad. Sci. USA 89, 12170–12174.
[5] Mustelin, T., Coggeshall, K.M. and Altman, A. (1989) Proc. Natl. Acad. Sci. USA 86, 6302–6306.
[6] Mustelin, T., Morikawa, T.P., Autero, M., Gassmann, M., Andersson, L.C., Gahmberg, C.G. and Burn, P. (1992) Eur. J. Immunol. 22, 1173–1178.
[7] Shiroo, M., Goff, L., Biffen, M., Shivnan, E. and Alexander, D. (1992) EMBO J. 11, 4887–4897.
[8] Cahir Mcfarland, E.D., Hurley, T.R., Pingel, J.T., Sefton, B.M., Shaw, A. and Thomas, M.L. (1993) Proc. Natl. Acad. Sci. USA 90, 1402–1406.
[9] Lin, J., Brown, V.K. and Justement, L.B. (1992) J. Immunol. 149, 3182–3190.
[10] 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.
[11] Magae, J., Osada, H., Fujiki, H., Saido, T.C., Suzuki, K., Nagai, K., Yamasaki, M. and Isono, K. (1990) Proc. Jap. Acad. 66, Ser B, 209–212.
[12] Morales, P.G., Minami, Y., Luong, E., Klausner, R.D. and Samelson, L.E. (1990) Proc. Natl. Acad. Sci. USA 87, 9255–9259.
[13] Imoto, M., Kakeya, H., Sawa, T., Hayashi, C., Hamada, M., Takeuchi, T. and Umezawa, K. (1993) J. Antibiot. 46, 1342–1346.
[14] Honda, R., Ohba, Y., Nagata, A., Okayama, H. and Yasuda, H. (1993) FEBS Lett. 318, 331–334.
[15] Tonks, N.K., Diltz, C.D. and Fischer, E.H. (1988) J. Biol. Chem. 14, 6731–6737.
[16] Mustelin, T., Coggeshall, K.M. and Altman, A. (1989) Proc. Natl. Acad. Sci. USA 86, 6302–6306.
[17] Stone, R.L. and Dixon, J.E. (1994) J. Biol. Chem. 269, 31323–31326.
[18] Zheng, C.F. and Guan, K.L. (1993) J. Biol. Chem. 268, 16116–16119.
[19] Charles, C.H., Sun, H., Lau, L.F. and Tonks, N.K. (1993) Proc. Natl. Acad. Sci. USA 90, 5292–5296.
[20] Roggo, B.E., Petersen, F., Delmendo, R., Jenny, H.-B., Peter, H.H. and Roesel, J. (1994) J. Antibiot. 47, 136–142.
[21] Mineo, C., Murakami, Y., Ishimi, Y., Hanaoka, F. and Yamada, M. (1986) Exp. Cell. Res. 167, 53–62.
[22] Tsai, L.-H., Lees, E., Faha, B., Harlow, E. and Riabowol, K. (1993) Oncogene 8, 1593–1602.
[23] Hoffmann, I., Draetta, G. and Karsenti, E. (1994) EMBO J. 4302–4310.
[24] Gyuris, J., Golemis, E., Chertkov, H. and Brent, R. (1993) Cell 75, 791–803.
[25] Hoffmann, I., Clarke, P.R., Marcote, M.J., Karsenti, E. and Draetta, G. (1993) EMBO J. 12, 53–63.
[26] Fujii, S., Kato, H., Furuse, H., Ito, K., Osada, H., Hamaguchi, T. and Kuroda, Y. (1995) Neurosci. Lett. 187, 130–132.