FR225659-binding Proteins: Identification as Serine/Threonine Protein Phosphatase PP1 and PP2A Using High-performance Affinity Beads

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FR225659 was originally isolated as a novel gluconeogenesis inhibitor produced by fungal strain *Helicomyces* sp. No. 19353. To identify the target protein of FR225659, we synthesized high-performance affinity latex beads that immobilized FR225659 derivative FR253761 or FR259383. Using these beads, we identified FR225659 binding proteins as serine/threonine protein phosphatase type1 (PP1) and type2A (PP2A) from rat hepatocyte crude extract. FR225659 and its synthetic derivatives were strongly inhibited the enzyme activities of purified catalytic subunits of PP1 and PP2A *in vitro*.

To date, a number of naturally occurring pharmacologically active compounds have been screened and discovered without elucidation of their target molecules or precise pharmacological mechanisms. Despite their unique biological activities, many of these compounds were not used for clinical purposes, because of their undesirable side effects. If the target molecules of these natural products are identified, it may be possible that the more potent or less toxic compound is discovered from the structure based rational screening or drug design.

Although affinity purification is a well-established technique as a powerful method of ligand binding proteins, widespread use of the technique has been limited by the poor characteristics of commercially available matrices. We have previously developed the latex beads which consist of polystyrene and polyglycidyl methacrylate copolymer that were originally used for the affinity purification of DNA-binding proteins^{1~3}). The hydrophilic polyglycidyl surface shows relatively few non-specific adsorptions of proteins

and can be reacted with amino group to load ligands. Furthermore, the large total surface area is especially effective in purifying the receptor from a small amount of cell lysate. We have recently reported that receptor purification of a small molecules such as E-3330 and FK506 using this latex particles⁴).

FR225659 (**1**) was originally isolated as potent gluconeogenesis inhibitor produced fungal strain *Helicomyces* sp. No. 19353⁵). This compound possesses a very unique structure that consists of a novel acyl-group and three unusual amino acids⁶). **1** inhibits glucagon-stimulated gluconeogenesis in primary rat hepatocytes *in vitro*, and shows hypoglycemic activity in two different *in vivo* models⁷). This compound does not inhibit two major rate limiting enzymes of gluconeogenesis pathway, *i.e.* glucose-6-phosphatase and fructose-1,6-bisphosphatase. **1** also does not interfere the binding of glucagon and its cell surface receptor. So the target molecule of **1** is still unclear.

For the aim of target molecule identification of **1**, we

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Fig. 1. Chemical structure of FR225659 (1) and its synthetic derivatives.

synthesized bioactive derivatives of **1**, FR253761 (**2**), FR259383 (**3**) and FR259384 (**4**) (Fig. 1), and prepared high-performance affinity beads that immobilized **2** or **3** previously⁸⁾. Using these beads we screened binding proteins of **1** from rat hepatocyte crude extract. We describe here, identification of **1** binding proteins as serine/threonine protein phosphatase type1 (PP1) and type2A (PP2A), and direct inhibition of PP1 and PP2A enzyme activity by **1** and its synthetic derivatives.

Materials and Methods

Preparation of Rat Hepatocyte Extracts

Rat hepatocytes were isolated by the collagenase perfusion method⁹⁾ for 24 hour fasted male Wister rats (200~220 g). Nuclear extract and cytoplasmic fraction of hepatocytes were prepared as described¹⁰⁾.

In Vitro Gluconeogenesis Assay

Measurement of *in vitro* gluconeogenesis activities of isolated rat hepatocyte was described previously⁷⁾.

Preparation of Drug Immobilized Matrices

Latex beads for affinity purification (SG beads) was prepared as described previously¹⁾. Chemical synthesis of compound **2**, **3** and **4**, plus preparation of latex beads which immobilized **2** or **3** were previously described⁸⁾. The amount of immobilization of **2** or **3** on latex beads were 38.9 nmol/mg or 15.0 nmol/mg, respectively.

Affinity Purification of Drug Binding Proteins

0.1 mg of latex beads which immobilized **2** or **3** were equilibrated with buffer E (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM MgCl₂, 0.2 mM EDTA, 10% Glycerol, 0.1% Nonidet P-40, 1 mM CaCl₂, 0.5 mM DTT), mixed with 200 μ l of cytoplasmic fraction (0.45 mg protein) or nuclear extract (0.2 mg protein) of rat hepatocytes and incubated at 4°C for one hour with occasional agitation. After washing buffer E, binding proteins were eluted with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and subjected to 12.5% SDS-PAGE followed by silver staining. For competition analysis, cytoplasmic fraction or nuclear extract was pre-incubated with indicated concentration of **1** or **2** for one hour at 4°C with occasional agitation followed by affinity purified.

To estimate the binding proteins, protein bands that competes free ligands were digested with trypsin, followed by peptide-mass fingerprinting was performed using metrics-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

Immunoblotting

Immunoblotting was performed as described¹¹⁾ using anti-PP1C α , anti-PP1C β , anti-PP1C γ , anti-PP2A catalytic subunit and anti-PP2A regulatory subunit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Protein Phosphatase Assay

Purified catalytic subunits of PP1 and PP2A were purchased from Upstate Biotechnology, Inc (Lake Placid, NY). Okadaic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Protein phosphatase activities were

measured using 10 μM of ^{32}P -labelled glycogen phosphorylase a as substrate and assayed according to previously described procedure¹²⁾.

Results

Inhibition of Hepatic Gluconeogenesis

The abilities of **1**, **2**, **3** and **4** to inhibit glucagon-stimulated gluconeogenesis in primary rat hepatocytes *in vitro* are summarized in Table 1. The 50% inhibitory concentration (IC_{50}) of **1** was 0.19 μM . Compound **2**, **3** or **4** also possess inhibitory activities with IC_{50} values 0.83 μM , 1.3 μM or 6.0 μM , respectively. This result indicates that synthetic derivatives could also bind to the target molecule of **1** in similar concentration.

Affinity Purification of Drug Binding Proteins

Fig. 2A shows the existence three peptides which specifically binds to **2** immobilized latex beads and these binding are dose dependently competed by the addition of free **1** in rat hepatocyte cytoplasmic fraction (indicated solid arrows). Presumed molecular weight of these peptides

are 36 kDa, 37 kDa, and 60 kDa. As the result of peptide mass finger printing, we estimated these proteins were PP1 catalytic subunits (PP1Cs), PP2A catalytic subunit and PP2A regulatory subunit (Fig 2B).

Immunoblot Analysis

Immunoblot analysis indicated that these peptides which bind to drug immobilized affinity beads were PP1 α , PP1 β , PP1 γ , PP2A regulatory subunit and PP2A catalytic subunit in rat hepatocyte cytoplasmic fraction (Fig. 3 lane 2). In nuclear fraction, we identified PP1 α ,

Table 1. Inhibitory effects of **1**, **2**, **3** and **4** on glucagon induced gluconeogenesis.

Compound	Inhibition of hepatocyte gluconeogenesis (μM)
1	0.52
2	0.83
3	1.3
4	6.0

Fig. 2. Affinity purification and estimation of **1** binding protein.

(A) Affinity purification and competition analysis of **1** binding proteins. A 200 μl samples of rat hepatocyte cytoplasmic fraction was incubated in the presence (lanes 3~6) or absence (lanes 1, 2) of **1**. Then 0.1 mg of **2** immobilized beads (lanes 2~6) or beads alone (lane 1) were added. After incubation for 1 hour at 4°C, the beads were washed and analyzed 12.5% SDS-PAGE followed by silver staining.

(B) Estimation of **1** binding proteins as serine/threonine phosphatase type1 catalytic subunits (PP1Cs), type2A (PP2A) catalytic subunit and PP2A regulatory subunit by peptide-mass fingerprinting.

Fig. 3. Immunoblot analysis shows that the **1** binding proteins are PP1 and PP2A.

Compound **2** immobilized latex beads (lanes 2~4, 6~8), and latex beads alone (lanes 1, 5) were used for affinity purification as described in Figure 2. For competition analysis 10 μg/ml of **1** (lanes 3, 7) or **2** (lanes 4, 8) were added to the protein samples. Eluted fractions were resolved by 12.5% SDS-PAGE and immunoblotted with anti-PP1Cs, anti-PP2A catalytic subunit and anti-PP2A regulatory subunit antibodies.

PP1Cβ, PP1Cγ as binding proteins (Fig. 3 lane 6). These proteins bind to the latex by drug immobilization specific manner, this binding were inhibited by existence of 10 μg/ml of free **1** (Fig. 3 lanes 3, 7) or **2** (Fig. 3 lanes 4, 8).

Inhibition of Protein Phosphatase Activity

IC₅₀ values and PP2A/PP1 ratio of IC₅₀ of compounds **1** to **4** and Okadaic acid are summarized in Table 2. All compounds potently inhibited phosphatase activities of PP1 and PP2A.

Discussion

In this paper, we describe the affinity purification and identification of naturally occurring gluconeogenesis inhibitor **1** binding proteins as PP1 and PP2A, and direct inhibition of PP1 and PP2A enzyme activity by **1** and its synthetic derivatives.

For the purpose of searching for **1** binding protein using affinity beads technology, we previously designed and synthesized biologically active derivatives which have

suitable amine moiety and stable structure for immobilization to the matrices⁸⁾. As shown in Fig. 1, compound **2**, **3** and **4** have a primary amine group, which is used for immobilizing to metrics, at the end of linker attached to phenolic hydroxyl group in carboxy-terminal acyl-group. 3-chloro-4-hydroxy arginine residue originally exists in **1** is substituted for arginine or lysine residue because of improvement of their chemical stability. Because of **3** which has two primary amine group (ε-amino group of lysine residue and amine spacer attached to carboxy-terminal phenolic hydroxyl group), we prepared **4** immobilized latex followed by deprotection of Boc group to prepare **3** immobilized latex⁸⁾. All synthetic compounds possess inhibitory activities against glucagon induced gluconeogenesis *in vitro* (Table. 1). Although *in vitro* activities of **2**, **3** or **4** are less effective than **1**, we think it would be enough to identify their binding proteins.

Using the latex beads immobilized **2**, we screened binding proteins from crude rat liver extract. From cytoplasmic fraction, we detected three peptides which bind to specifically **2** immobilized latex. Their binding to the latex is competed with free compound **1** by concentration dependent manner (Fig. 2A indicated solid arrows). The

same result was obtained that compound **2** was used for competition. As results of peptide-mass finger printing, we estimated these proteins were PP1Cs, PP2A catalytic subunit and PP2A regulatory subunit (Fig. 2B). On the other hand, from rat liver nuclear extract, only putative PP1C peptides seems to bind to affinity beads (data not shown). When used the affinity latex immobilized **3**, we detected essentially the same peptides in cytoplasmic fraction and nuclear extract which compete free **1** or **3** by concentrate dependent manner (data not shown).

Immunoblot analysis used specific antibodies for PP1C subtypes and PP2A subunits indicated that the peptides which binding to drug immobilized affinity beads were PP1C α , PP1C β , PP1C γ , PP2A regulatory subunit and PP2A catalytic subunit in cytoplasmic fraction (Fig. 3 lane 2). Whilst in nuclear fraction, we identified PP1C α , PP1C β , PP1C γ as binding proteins (Fig. 3 lane 6). These proteins were bound to the latex beads by drug immobilization dependent manner and their binding was inhibited by the addition of free compound **1** or **2** (10 μ g/ml). When we used 10 μ g/ml of compound **2** as a competitor, binding of PP2A catalytic and regulatory subunits to drug immobilized latex was not completely inhibited while same concentration of **1** completely inhibited (Fig. 3 lane 3, 4). This result indicates binding affinity to PP2A subunits of **2** is much weaker than **1**, and is parallel to the IC₅₀ values of PP2A inhibition of these compounds (Table 2). Similar results were obtained when using **3** immobilized latex beads (data not shown).

To clarify that compound **1**, and its synthetic analogues affect to enzymatic activities of PP1 or PP2A catalytic subunits, we assayed inhibitory effects of phosphatase activity purified PP1 or PP2A catalytic subunits. As indicated in Table 2, all compounds strongly inhibited protein phosphatase activities of PP1 and PP2A when ³²P-labelled glycogen phosphorylase α as substrate. According to the ratio of IC₅₀ value of PP1 and PP2A, compound **1** to **4** are more specific inhibitor to PP1 than PP2A. Especially, synthetic derivatives **2**, **3** and **4** are extremely specific to PP1 with the ratio of IC₅₀ ranged 600 to 1500. In comparison with gluconeogenesis inhibitory activity summarized in Table 1, specific inhibition of PP1 may be more critical for inhibition of hepatic gluconeogenesis. Indeed PP2A specific inhibitor Okadaic acid did not inhibit hepatic gluconeogenesis at non-cytotoxic concentration, and synthetic derivatives of **1** which did not have inhibitory activity of gluconeogenesis are also non-potential against PP1 activity (data not shown).

Protein phosphatases regulate many cellular functions and signal transduction pathways in cooperation with

Table 2. Inhibitory activities of **1** and its synthetic derivatives on PP1 and PP2A.

Compound	IC ₅₀ (nM)		Ratio of PP2A/PP1
	PP1	PP2A	
1	0.10	2.2	21.7
2	0.36	260	720
3	0.30	200	667
4	0.037	58	1573
Okadaic acid	25	0.88	0.035

protein kinases^{13,14}). PP1 and PP2A are two of four major protein serine/threonine phosphatases that regulate diverse cellular events such as cell division, transcription, translation, muscle contraction, glycogen metabolism, and neuronal signaling¹⁵). Because of PP1 catalytic subunits are encoded by three different genes and associated with a number of targeting subunits, for the investigation of detailed cellular functions of PP1, it is useful that usage of selective inhibitors of these enzymes.

To date, a number of naturally occurring structurally diverse families of inhibitors which inhibit PP1 as well as PP2A, have been identified and purified. Most of these compounds inhibit PP1 and PP2A activity at same potency, with the exception of compounds such as Okadaic acid, Thyriferyl 23-acetate (TF-23A), fostriecin, and tautomycin. Okadaic acid, TF-23A and fostriecin selectively inhibit PP2A activity^{16–18}), and their selectivity has made it possible to analyze PP2A function in living cells. Tautomycin is recently reported PP1 selective inhibitor. The PP1/PP2A ratio of IC₅₀ of tautomycin was reported to be within range of 23 to 40¹⁹). **1** and its derivatives are structurally unrelated to all reported PP1/PP2A inhibitors and possess extreme specificity to PP1 with the ratio of IC₅₀ ranged from 20 to 1500. Therefore **1** and its derivatives maybe useful to clarify the roles of protein phosphatases especially PP1 in cellular functions including glucose metabolism in liver, and to develop a novel gluconeogenesis inhibitor which improved potency and safety than **1**.

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References

- 1) WADA, T.; H. WATANABE, H. KAWAGUCHI & H. HANDA: DNA affinity chromatography. *Methods Enzymol.* 254: 595~604, 1995
- 2) INOMATA, Y.; H. KAWAGUCHI, M. HIRAMOTO, T. WADA & H. HANDA: Direct purification of multiple ATF/E4TF3 polypeptides from HeLa cell crude nuclear extracts using DNA affinity latex particles. *Anal Biochem.* 206: 109~114, 1992
- 3) INOMATA, Y.; T. WADA, H. HANDA, K. FUJIMOTO & H. KAWAGUCHI: Preparation of DNA-carrying affinity latex and purification of transcription factors with the latex. *J. Biomater. Sci. Polym. Ed.* 5: 293~302, 1994
- 4) SHIMIZU, N.; K. SUGIMOTO, J. TANG, T. NISHI, I. SATO, M. HIRAMOTO, S. AIZAWA, M. HATAKEYAMA, R. OHBA, H. HATORI, T. YOSHIKAWA, F. SUZUKI, A. OOMORI, H. TANAKA, H. KAWAGUCHI, H. WATANABE & H. HANDA: High-performance affinity beads for identifying drug receptors. *Nature Biotech.* 18: 877~881, 2000
- 5) OHTSU, Y.; H. SASAMURA, Y. TSURUMI, S. YOSHIMURA, S. TAKASE, M. HASHIMOTO, T. SHIBATA, M. HINO & T. FUJII: The novel gluconeogenesis inhibitors FR225659 and related compounds that originate from *Helicomyces* sp. No. 19353. I. Taxonomy, fermentation and physico-chemical properties. *J. Antibiotics* 56: 682~688, 2003
- 6) ZENKOH, T.; Y. OHTSU, S. YOSHIMURA, N. SHIGETAMATSU, H. TAKASE & M. HINO: The novel gluconeogenesis inhibitors FR225659 and FR225656 from *Helicomyces* sp. No. 19353. III. Structure determination. *J. Antibiotics* 56: 694~699, 2003
- 7) OHTSU, Y.; H. SASAMURA, T. SHIBATA, H. NAKAJIMA, M. HONO & T. FUJII: The novel gluconeogenesis inhibitors FR225659 and related compounds that originate from *Helicomyces* sp. No. 19353. II. Biological profiles. *J. Antibiotics* 56: 689~693, 2003
- 8) ZENKOH, T.; H. HATORI, H. TANAKA, M. HASEGAWA, M. HATAKEYAMA, Y. KABE, H. SETOI, H. KAWAGUCHI, H. HANDA & T. TAKAHASHI: Design and synthesis of a solid-supported FR225659 derivative for its receptor screening. *Organic Letters*, in press.
- 9) SEGLEN, P. O.: Preparation of isolated rat liver cells. *Methods Cell Biol.* 13: 29~83, 1976
- 10) DIGNAM, J. D.; R. M. LEVOBITZ & R. G. ROEDER: Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11: 1475~1489, 1983
- 11) HIRAMOTO, M.; N. SHIMIZU, K. SUGIMOTO, J. TANG, Y. KAWAKAMI, M. ITO, S. AIZAWA, H. TANAKA, I. MAKINO & H. HANDA: Nuclear targeted suppression of NF- κ B activity by the novel quinine derivative E3330. *J. Immunol.* 160: 810~819, 1998
- 12) COHEN, P.; S. ALEMANY, B. HEMMINGS, T. J. RESIK, P. STRALFORS & H. Y. TUNG: Protein phosphatase-1 and proteinphosphatase-2A from rat skeletal muscle. *Methods Enzymol.* 159: 390~408, 1988
- 13) HUNTER, T.: Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell* 80: 225~236, 1995
- 14) COHEN, P. T.: Novel serine/threonine phosphatases: variety is the spice of life. *Trends Biochem. Sci.* 22: 245~251, 1997
- 15) WERA, S. & B. A. HEMMINGS: Serine/threonine protein phosphatases. *Biochem. J.* 311: 17~29, 1995
- 16) GUPTA, V.; A. K. OGAWA, X. DU, K. N. HOUK & R. W. ARMSTRONG: A model for binding of structurally diverse natural product inhibitors of protein phosphatases PP1 and PP2A. *J. Med. Chem.* 40: 3199~3206, 1997
- 17) MATSUZAWA, S.; T. KAWAMURA, S. MITSUHASHI, T. SUZUKI, Y. MAYSUO, M. SUZUKI, Y. MIZUNO & K. KIKUCHI: Thyrsiferyl 23-acetate and its derivatives induce apoptosis in various T- and B-leukemia cells. *Bioorg. Med. Chem.* 7: 381~387, 1999
- 18) CONNOR, J. H.; T. KLEEMAN, S. BARIK, R. E. HONKANEN & S. SHENOLIKAR: Importance of the β 12- β 13 loop in protein phosphatase-1 catalytic subunit for inhibition by toxins and mammalian protein inhibitors. *J. Biol. Chem.* 274: 22366~22372, 1999
- 19) MITSUHASHI, S.; N. MATSUURA, M. UBUKATA, H. OIKAWA, H. SHIMA & K. KIKUCHI: Tautomycetin is a novel and specific inhibitor of serine/threonine protein phosphatase type1, PP1. *Biochem. Biophys. Res. Commun.* 287: 328~331, 2001