Bioorganic & Medicinal Chemistry 17 (2009) 6188–6195

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Preparation of conophylline affinity nano-beads and identification of a target protein

Eriko Suzuki [a], Hideki Ogura [a], Kuniki Kato [a], Izumi Takei [b], Yasuaki Kabe [c], Hiroshi Handa [c], Kazuo Umezawa [a,*]

[a] Department of Applied Chemistry, Faculty of Science and Technology, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama 223-0061, Japan
[b] Tokyo Dental College Ichikawa General Hospital, 5-11-13 Kanno, Ichikawa 272-8513, Japan
[c] Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuda, Midori-ku, Yokohama 226-8503, Japan

ARTICLE INFO

Article history:
Received 19 June 2009
Revised 23 July 2009
Accepted 24 July 2009
Available online 29 July 2009

Keywords:
Conophylline
Amination
Nano-beads
Affinity-purification
ARL6IP

ABSTRACT

Conophylline, a vinca alkaloid extracted from the tropical plant *Ervatamia microphylla*, has been shown to induce the differentiation of insulin-producing β-cells in cultured cells and in animals. However, its mechanism of action and the molecular target have remained unclear. Therefore, we prepared a fishing probe with conophylline to identify the target protein by using latex nano-beads, which are newly innovated tools for affinity-purification. With these conophylline-linked nano-beads, we found that conophylline directly interacted with ARL6IP. ARL6IP may thus be involved in the mechanism of cellular differentiation of β-cells, and this probe should be useful to find other target proteins.

1. Introduction

Conophylline (**1**) isolated from the leaves of *Tavertaemontana divaricata* of Malaysia is a low-molecular-weight compound having a vinca alkaloid structure.[1,2] Also, we isolated it as a *Ras* function inhibitor from the leaves of *Ervatamia microphylla* collected in Thailand.[3] Moreover, recently we also found that conophylline showed antidiabetic activity in vivo, as it decreased the blood glucose level in diabetic model mice. Its mechanism of action is unique, since conophylline induces the differentiation of insulin-producing cells from precursor cells in culture[4] and in animals.[5] So it may be useful for the regeneration therapy of type-2 diabetes mellitus. Recently, conophylline was also shown to induce the differentiation of insulin-producing cells from mouse bone marrow cells when added together with betacellulin-d4,[6] thus suggesting its usefulness for transplantation therapy of type-1 diabetes. However, its molecular mechanism of action and the target molecule have not yet been well documented. Especially, identification of the target molecule(s) of conophylline should give us new insights into the function of the identified protein, and also into the molecular mechanism of its β-cell differentiation-promoting activity.

Latex nano-beads, termed SG beads, are a new innovative tool for affinity purification-based identification of the target molecules of bioactive compounds.[7] SG beads are composed of a glycidylmethacrylate (GMA) and styrene copolymer core with a GMA polymer surface, and have several advantages over conventional affinity-purification supports; for example, their extremely large surface area results in a relatively high binding capacity, and their lack of pores facilitates the efficient removal of residual proteins. SG beads have been used successfully to purify various proteins, including transcription factors and several target proteins for various chemical compounds. DNA affinity latex beads were used to purify sequence-specific DNA-binding proteins directly from crude cell extracts.[8] The target proteins of several bioactive compounds have been identified using this device, as in the example of the anti-NF-κB agent E3330.[7] The beads were also used to show that deoxycytidine kinase, an enzyme that acts in the salvage pathway of nucleotide biosynthesis, is another target of methotrexate.[9] Moreover, by use of affinity latex beads, the mitochondrial transporter of 2-oxoglutarate, was shown to bind to PdTCPP, a phosphorescent porphyrin derivative; and PdTCPP was found to inhibit 2-oxoglutarate uptake into mitochondria.[10]

Thus, we prepared conophylline-ligated latex nano-beads and used them to screen for and identify the target protein of conophylline.

* Corresponding author.
E-mail address: umezawa@applc.keio.ac.jp (K. Umezawa).

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doi:10.1016/j.bmc.2009.07.062

2. Results

2.1. Synthesis of conophylline amino-derivative

In screening for target proteins by affinity-purification using nano-beads, the bait for the target protein should be immobilized. Either a carboxyl or amino-group of the bioactive compound under study can be employed for the immobilization on the surface of SG beads through amide-bond formation. Therefore, we synthesized an amino derivative of conophylline according to the reactions depicted in Figure 1. We introduced an amino function at the site of the oxirane ring by performing the ring opening reaction.

At first, when conophylline (**1**) was treated with trimethylsilyl azide and $ZnCl_2$ in 1,2-dichloroethane, an azide group was introduced to give the silylated azido-alcohol (**2**) by a radioselective opening of the oxirane ring, as shown in Figure 1. Then, after cleavage of the trimethylsilyl ether groups with trifluoroacetic acid to give 14′-azidoconophylline (**3**), this compound was hydrogenated under an H_2 atmosphere in the presence of Lindlar's catalyst to provide 14′-aminoconophylline (**4**, conophylline–NH_2). The structure of 14′-azidoconophylline (**3**) was determined from its MS and NMR spectra (chemical shift assignments are shown in Table 1). The stereochemistry at 14′ and 15′ was elucidated by using 2D NMR, ROESY, and differential NOE. The ^{13}C NMR data except for the 14′ and 15′ positions for conophylline azide were very similar to the reference data on conophylline. The coupling constant (*J*) of 14′-H and 15′-H showed 10 Hz. The NOEs were observed between 15′-H and 21′-H and between 14′-H and 17′-H. Therefore, the stereochemistry of 14′-N_3 and 15′-OH was determined to be *trans*. The structure of 14′-aminoconophylline (**4**, conophylline–NH_2) was determined from its MS and NMR spectra. The chemical shift assignments are shown in Table 1.

2.2. Biological activity of conophylline amino-derivative

We next examined whether conophylline–NH_2 (**4**) would possess the same biological activity as conophylline. We employed rat pancreatic carcinoma AR42J cells, since conophylline showed a cytostatic effect on and induced endocrine differentiation in this cell line.[4] Cytotoxicities of conophylline and conophylline–NH_2 toward AR42J cells were determined by using the trypan blue dye exclusion assay. Although the toxic concentration of conophylline–NH_2 for AR42J cells after 48 h of treatment was about 10 times higher than that of conophylline (Fig. 2), conophylline–NH_2 was confirmed to possess biological activity comparable to that of conophylline.

2.3. Immobilization of conophylline (8)

SG beads with carboxyl groups at their terminus were prepared as previously described.[7] As shown in Figure 3, after activation of the carboxyl group with succinic anhydride, the amino-group of conophylline–NH_2 (**4**) was reacted to give immobilized conophylline on the beads via amide-bond formation. After treatment with 2-aminoethanol, non-reacted carboxyl groups were eliminated. The control beads were prepared by using the same protocol, except that the reaction with conophylline–NH_2 was omitted. The quantity of immobilized conophylline on the beads was estimated by quantification of the succinic anhydride released during the reaction by using high-performance liquid chromatography. The content of ligands was approximately 100 nmol/mg beads. Hence, we succeeded in immobilizing conophylline on the SG beads with high efficiency.

2.4. Affinity-purification using the conophylline-immobilized beads

Then, using the prepared beads, affinity-purification was performed by the batch method. Besides affecting AR42J cells, conophylline also induces cell death at low concentrations in HeLa (unpublished results) and K-ras-NRK cells.[3] Therefore, these 3 cell lines were employed for the preparation of cell extracts for affinity-purification. About 500 mg of cytosol extracts or nuclear extracts of AR42J-B13 cells prepared by the Dignam method[11] were subjected to affinity-purification with 0.5 mg of control or conophyl-

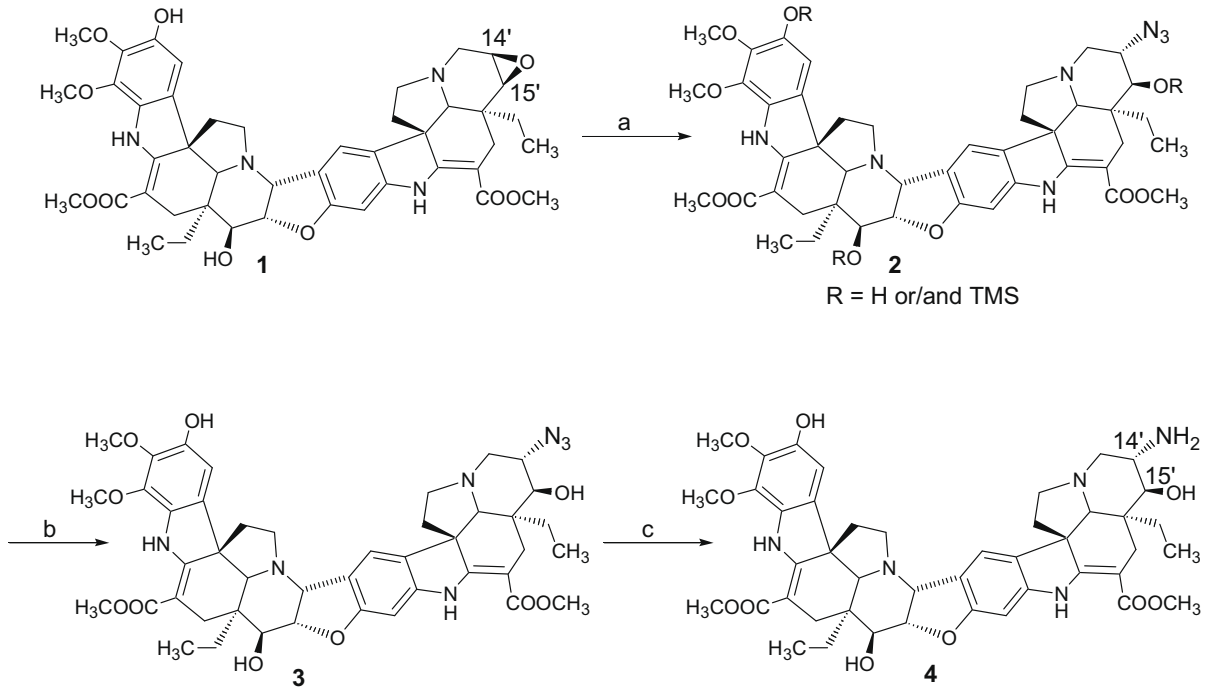

Figure 1. Synthesis of conophylline amino-derivative. Conophylline–NH_2 was prepared by employing oxirane ring opening via the azide derivative. Conophylline was firstly converted to 14′-azidoconophylline with trimethylsilyl azide (64.7% yield). Then, 14′-azidoconophylline was reduced into 14′-aminoconophylline with H_2 and Lindler's catalyst (64.9% yield). Reagents and conditions: (a) $TMSN_3$, $ZnCl_2$, $ClCH_2CH_2Cl$, reflux, 12 h; (b) 50% CF_3COOH, rt, 1 h; (c) 5% $Pd/BaSO_4$, H_2, EtOH.

Table 1
NMR data for conophylline derivatives

	Conophylline–N_3		Conophylline–NH_2	
	^{13}C	^{1}H	^{13}C	^{1}H
1-NH		8.78		8.78
2	164.7		164.7	
3	59.5	4.78	59.6	4.78
4(N)				
5	46	2.82, 2.97	46	2.84, 2.97
6	41.9	1.69, 1.99	41.9	1.69, 2.00
7	54.8			
8	133.5			
9	104	5.53	104.2	5.57
10	143.5		143.6	
11	138.7		138.8	
12	136.8		136.8	
13	128.8		128.7	
14	85.2	5.03	85.2	5.03
15	69.6	4.15	69.6	4.15
16	90.7		90.6	
17	22.1	2.39, 2.73	22.2	2.40, 2.73
18	7.4	0.7	7.4	0.7
19	26.4	0.84, 1.15	26.4	0.85, 1.15
20	44.8		44.8	
21	65.3	2.63	65.2	2.65
10-OH		5.14		
11-OMe	61	3.82	61	3.81
12-OMe	60.5	3.86	60.5	3.86
15-OH		2.54		
16-CO	168.8		168.8	
16-CO_2Me	51.1[a]	3.77	51[b]	3.77
1′-NH		8.95		8.95
2′	167.3		167.6	
3′	51.6	2.49, 3.37	56.6	2.30, 3.24
4′ (N)				
5′	50.9	2.70, 2.95	51.1	2.65, 2.91
6′	46.6	1.72, 2.10	46.6	1.71, 2.10
7′	55.2		55.3	
8′	129.9		130.4	
9′	118.8	7.13	118.8	7.14
10′	114		113.8	
11′	161.1		161	
12′	93.6	6.35	93.5	6.35
13′	145.2		145.2	
14′	62.1	3.75	51.6	3.02
15′	79.6	3.6	81.7	3.8
16′	92.2		92.5	
17′	22.7	2.51, 2.77	22.9	2.52, 2.73
18′	9	0.81	9	0.83
19′	27.6	1.3	27.9	1.3
20′	44		43.1	
21′	70	2.75	70.5	2.76
15′-OH		2.13		
16′-CO	169.1		169.2	
6′-CO_2Me	51.0[a]	3.77	50.9[b]	3.77

[a, b] Assignments are exchangeable.

line-immobilized beads. After the beads had been washed, the bound proteins were eluted with an elution buffer containing 1 M NaCl. Residual proteins on the beads were also collected by boiling the beads with Laemmli dye. These samples obtained were subjected to SDS–PAGE, and the proteins were detected by silver staining. A protein band having a molecular weight of approximately 23 kDa was detected only with the conophylline-immobilized beads from both the cytosol and nuclear extracts (Fig. 4A). Using SDS–polyacrylamide gel electrophoresis, we confirmed that the electrophoretic mobility of the bands obtained with both cytosol and nuclear extracts were the same (data not shown), indicating that these bands were of the same protein origin. Subsequently, the band was proved to be conophylline-specific by drug-competition analysis (Fig. 4B). Moreover, the 23-kDa band was also observed when the cytosol extracts of both HeLa cells and K-ras-NRK cells were examined (Fig. 4C). Thus, we identified this 23-kDa protein as a positive candidate for the target protein of conophylline.

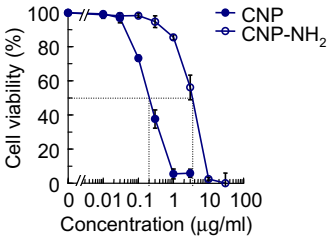

Figure 2. Biological activity of conophylline amino-derivative. Effect of conophylline–NH_2 on cell viability. AR42J cells were treated with the indicated concentrations of conophylline or conophylline–NH_2 for 48 h. The cell viability was assessed by trypan blue dye exclusion. Each value is the mean ± S.D. of quadruplicate determinations.

2.5. Identification of ARL6IP and preparation of the recombinant protein

Next we subjected the 23-kDa protein to MS/MS analysis for identification. To obtain a sufficient amount of the protein, we used nuclear extracts of AR42J-B13 cells (20 mg protein in total) for the affinity-purification. Since the protein could not be fully eluted without boiling, as was shown in Figure 4A, all the treated beads were immediately boiled with the Laemmli dye. The protein band of 23 kDa was detected again on the acrylamide gel by SYPRO-Ruby staining (Fig. 5A). Then, the band was taken out, trypsin-digested, and then used for the MS/MS analysis. As a result, the peptide sequences of 2 fragments were determined. These sequences matched those in the amino acid sequence of ADP-ribosylation factor-like 6 interacting protein (ARL6IP) (accession; AAP83442 in GenPept). The amino acid sequence of the 23-kDa protein is shown in Figure 5B. Subsequently, this protein in the nuclear extract was confirmed to be ARL6IP by immuno-blotting using commercially available anti-ARL6IP antibody (Fig. 5C). The affinity-purified proteins from the cytosol extracts of AR42J-B13, HeLa, and K-ras-NRK cells were also confirmed to be ARL6IP by immuno-blotting. Additionally, ARL6IP was detected more strongly in nuclear extracts than in cytosol extracts by immuno-blotting analysis (Fig. 5D).

To examine whether ARL6IP directly interacts with conophylline, a binding assay was carried out by using recombinant ARL6IP protein. ARL6IP cDNA (accession; AY316590 in GenBank) was cloned from a rat brain cDNA library, and the protein was prepared as described in Section 4. Since it is a membrane protein, we adopted the mammalian cell expression system rather than using *Escherichia coli* for the preparation of the recombinant protein. Briefly, FLAG-tagged ARL6IP was transiently over-expressed in HeLa cells. Then the cells were collected, and after lysis the cell lysate was immuno-precipitated by using the anti-FLAG antibody-conjugated agarose beads. As expected, the recombinant ARL6IP protein strongly interacted with the conophylline-immobilized beads (Fig. 5E), whereas no interaction with ARL6IP was observed with the control beads. Thus, we confirmed that ARL6IP could physically interact with conophylline in vitro.

2.6. Inhibition of ARL6IP /ARL6 interaction by conophylline in vitro

As ARL6IP is known to bind to ARL6, we examined whether the ARL6IP/ARL6 interaction would be inhibited by conophylline. First, we prepared ARL6-ligated Sepharose 4B beads. Subsequently, in the presence or absence of conophylline the beads were mixed with Flag-tagged ARL6IP that had been purified from the lysate of over-expressing cells. The ARL6-ligated Sepharose 4B beads were then centrifuged, and the pellet was incubated with anti-Flag antibody. As a result, the interaction was observed in the absence

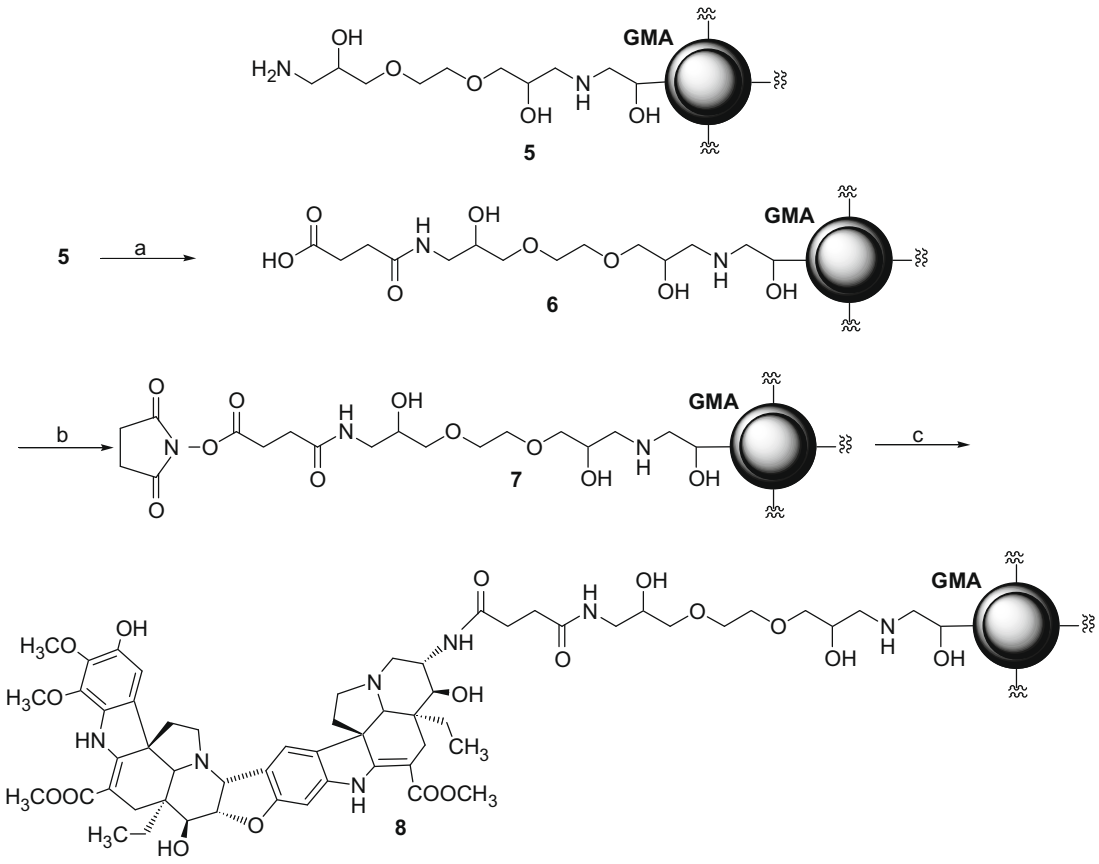

Figure 3. Immobilization of conophylline. The latex nano-beads, termed SG beads, are composed of glycidylmethacrylate (GMA) on the surface and a styrene copolymer core. The SG beads previously prepared[11] were firstly converted to the carboxylic-type SG beads. Then, the carboxyl groups on SG beads were activated with succinic anhydride. Next, the amino-group of conophylline–NH_2 was reacted with these activated carboxyl groups to give immobilized conophylline on the beads leaving the succinic anhydride moiety. An excess amount of 14′-amimoconophylline was added. We estimated the portion of reacted aminoconophylline by the production of liberated succinic anhydride. About 10% of the conophylline used was incorporated into the beads; and the approximate content of conophylline moiety in the beads was 100 nmol/mg beads. Reagents and conditions: (a) (i) succinic anhydride, Et_3N, DMF, (ii) 1 N aq NaOH; (b) *N*-hydroxysuccinimide (HOSu), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), dioxane, rt, 2 h; (c) (i) 4, DMF, rt, 24 h, (ii) $HOCH_2CH_2NH_2$, DMF, rt, 2 h.

of conophylline, whereas in its presence the interaction of ARL6IP with ARL6 was clearly inhibited (Fig. 6).

3. Discussion

Conophylline shows antidiabetic and anticancer activities in vivo; however, its molecular target has not been elucidated. In the present research, we identified ARL6IP as the target protein of conophylline by affinity-purification from both cytosolic and nuclear extracts derived from conophylline-sensitive cells. Subsequent immuno-blotting analysis revealed that ARL6IP tended to be present more in the nuclear extract than in the cytosolic one, which suggests that the protein was localized in organelles such as the endoplasmic reticulum linked to the nuclear membrane. We also prepared recombinant ARL6IP, and confirmed the physical interaction of ARL6IP with conophylline (Fig. 5).

ADP ribosylation factors (ARFs) is involved in vesicle formation and intracellular transport. ADP-ribosylation-like factor 6 (ARL6) is a member of the ARL subfamily of small GTPases, and is also likely to be involved in intracellular trafficking.[12] By use of the yeast two-hybrid system, ARL6IP was identified as one of the interacting proteins of ARL6.[13] Pettersson et al.[14] showed that ARL6IP is an endoplasmic reticulum (ER) integral membrane protein with 4 predicted transmembrane domains. Although its function has not been well described, ARL6IP was also identified by the mRNA differential display method to be a gene dramatically down-regulated during cytokine-induced hematopoietic cell differentiation.[14] ARL6IP is highly expressed in brain and primary hematopoietic tissues, such as bone marrow and thymus, all of which contain many immature and undifferentiated cells. Because ARL6IP is predominantly localized in the intracytoplasmic membranes, it has been suggested that ARL6IP would be involved in protein transport, membrane trafficking or cell signaling during differentiation.[14]

Besides the predicted role of ARL6IP in cell differentiation, Lui et al.[15] suggested a possible role for it in cell survival. They showed that overexpression of ARL6IP protected HT1080 fibrosarcoma cells from apoptosis by multiple apoptotic inducers including serum starvation, UV irradiation, tumor necrosis factor-α, and ER stressors such as brefeldin A; therefore, ARL6IP was also designated as an apoptotic regulator in the membrane of the endoplasmic reticulum (ARMER). However, the molecular mechanism of its anti-apoptotic function is not yet understood. Proteins in the Bcl-2 family including Bcl-X_L and Bcl-2 are central regulators of apoptosis. ARL6IP was also shown by screening to be a protein that interacts with Bcl-X_L.[16]

If conophylline inhibits the function of ARL6IP, inhibition of the anti-apoptotic function may partly explain the mechanism of the anticancer activity of conophylline. Since ARL6IP was identified by its physical interaction with ARL6,[13] conophylline may have an effect on this interaction and on the ARL6 function. Actually conophylline inhibited the interaction between ARL6IP and ARL6 (Fig. 6). ADP-ribosylation factors (ARFs) have been reported to play an important role in intracellular membrane trafficking.[12] ARF-like

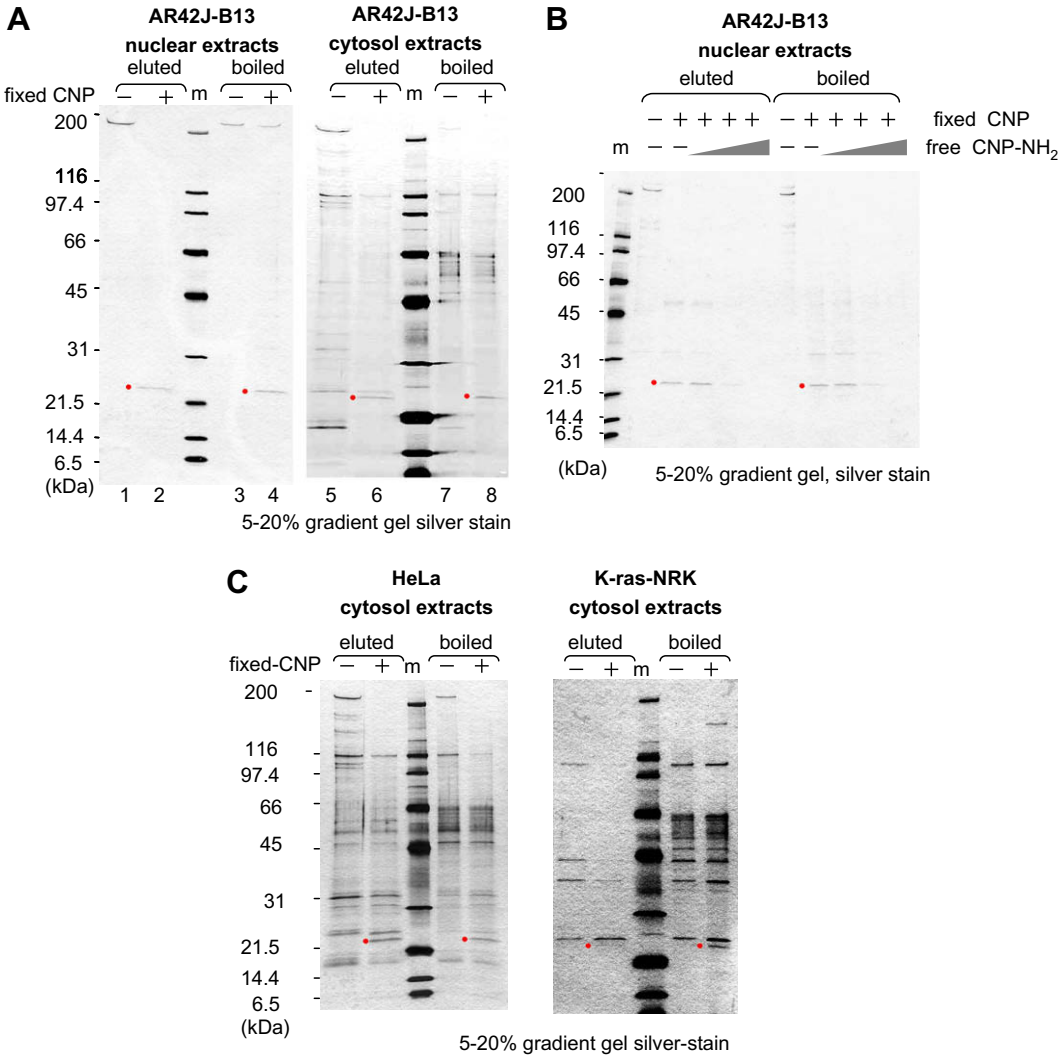

Figure 4. Affinity-purification using the conophylline-immobilized beads. (A) Identification of a 23-kDa band protein specifically purified from AR42J-B13 cells extracts with the affinity beads. Cytosol extracts (lanes 1–4) or nuclear extracts (lanes 5–8) were subjected to affinity-purification with control or conophylline-ligated latex beads. The beads were precipitated and washed, and bound proteins were eluted with elution buffer (lanes 1–2 and 5–6). Proteins remaining on the beads were liberated by boiling the beads (lanes 3–4 and 7–8). Samples were subjected to SDS–PAGE, and proteins detected by silver staining. Red dots indicate the 23-kDa bands. (B) Competition analysis with conophylline–NH_2. Nuclear extracts of AR42J-B13 cells were pretreated with increasing concentrations of conophylline–NH_2, and subjected to affinity-purification using conophylline-fixed latex beads. After incubation for 1 h at 4 °C, the bead were washed and analyzed by SDS–PAGE followed by silver staining. (C) Identification of the specific 23-kDa band obtained with cytosol extracts of HeLa and K-ras NRK cells. Cytosol extracts of HeLa and K-ras-NRK cells were subjected to affinity-purification using conophylline-fixed latex beads. Eluted or boiled samples were analyzed by SDS–PAGE followed by silver staining.

proteins (ARLs) are very similar to ARFs in terms of amino acid sequence. Although ARL1-13 proteins have been cloned,[17] their biological function remains unclear. Conophylline may be a useful tool to study the role of ARL6IP and ARL6.

In conclusion, we prepared conophylline-ligated affinity nanobeads for screening, and using this method we discovered ARL6IP as the target protein of conophylline. Conophylline may thus be a useful ligand to study the roles of ARL6IP and ARL.

4. Experimental procedures

4.1. Materials

Conophylline was isolated in our laboratory from the leaves of *E. microphylla*,[2] and stored at –20 °C in benzene at 1 mg/ml. For use, the solution was dried; and the conophylline was dissolved in MeOH at 10 μg/ml.

Recombinant human HGF was purchased from Sigma–Aldrich, Inc. (St. Louis, MO). Recombinant human activin A was obtained from R&D Systems (Minneapolis, MN). Western lightning™ chemiluminescence reagents came from PerkinElmer, Norwalk, CT. Horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse IgGs from donkey were obtained from Amersham Life Science, UK.

4.2. Spectroscopy

General procedures: Optical rotations were taken on a Perkin–Elmer 241 polarimeter. MS spectra were measured on a JEOL JMS-T100LC mass spectrometer; and NMR spectra, on a JEOL JNM-A500 spectrometer in $CDCl_3$ using TMS ($\delta = 0$) as the internal standard. ^{1}H NMR spectra were recorded at 500 MHz; and ^{13}C NMR spectra, at 125 MHz.

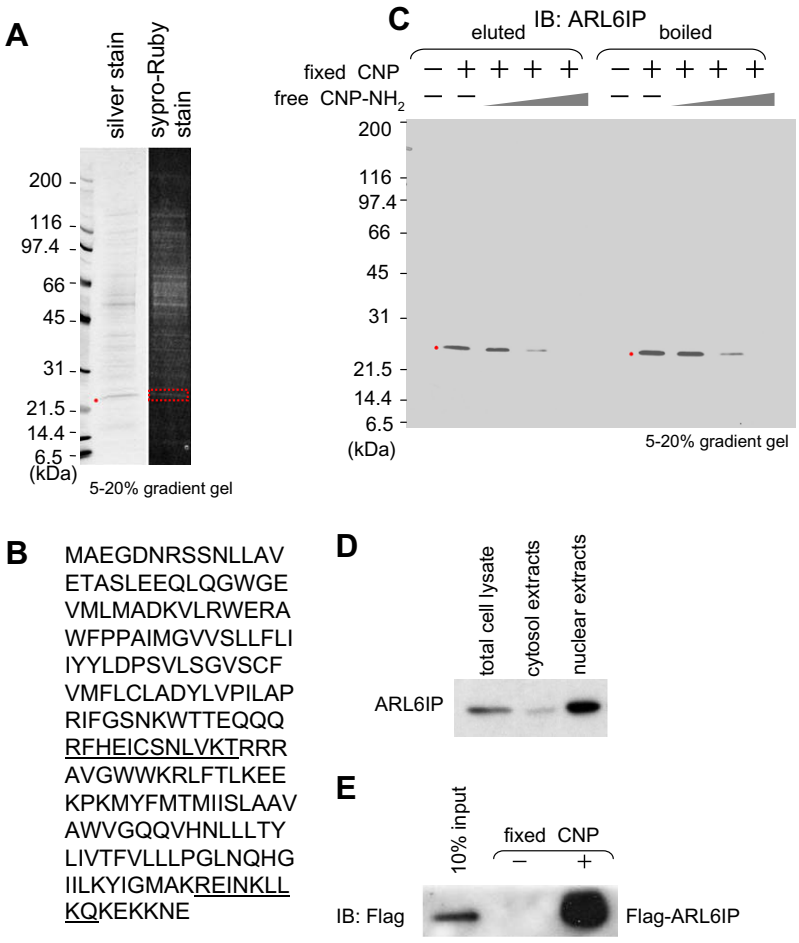

Figure 5. Identification of the conophylline-binding protein as ARL6IP. (A) Isolation of the 23-kDa protein. Affinity-purified proteins from nuclear extracts of AR42J-B13 cells were analyzed by SDS–PAGE and stained with SyproRubyGold. Protein bands were detected under UV light, and the band at 23 kDa was removed and subjected to MS/MS analysis. (B) Amino acid sequence of ARL6IP. Using an amino acid sequence database we identified the 23-kDa protein as ARL6IP. Two peptides derived from fragments of the 23-kDa protein are underlined. (C) Immunostaining of affinity-purified ARL6IP. Affinity-purified proteins from nuclear extracts of AR42J-B13 cells were analyzed by SDS–PAGE followed by immuno-blotting using anti-ARL6IP antibody. (D) Intracellular localization of ARL6IP. Nuclear and cytosol extracts from AR42J-B13 cells were prepared and analyzed by immuno-blotting using anti-FLAG antibody. (E) Binding of FLAG-labeled ARL6IP to the affinity beads. Recombinant ARL6IP proteins were subjected to the binding assay using conophylline-fixed latex beads. The protein was detected by using anti-FLAG antibody.

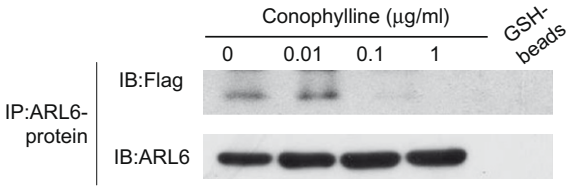

Figure 6. Inhibition of ARL6IP/ARL6 interaction by conophylline. The Flag-ARL6IP-overexpressing HeLa cells were collected and lysed. The lysates were subjected to immuno-precipitation using anti-Flag antibody. The precipitated proteins were eluted with Flag peptides to release FLAG-ARL6IP. Then, the ARL6 binding Sepharose4B beads with or without conophylline were added to the eluted proteins. After the incubation for overnight, the beads were washed and boiled. Then, the samples were subjected to SDS–PAGE and immuno-blotted with anti-Flag and anti-ARL6 antibodies.

4.2.1. Preparation of 14′-azidoconophylline (3)

To a solution of conophylline (229.3 mg, 0.289 mmol) in 1,2-dichloroethane (15 ml) was added trimethylsilyl azide (0.383 ml, 2.89 mmol) and $ZnCl_2$ (315 mg, 23.1 mmol) at room temperature, and the mixture was heated at refluxing temperature for 12 h. The reaction mixture was then concentrated in vacuo. The residue was dissolved in MeOH (100 ml) and 50% CF_3COOH (50 ml), and the mixture was stirred at room temperature for 1 h and thereafter concentrated in vacuo. The residue was dissolved in EtOAc (100 ml), washed sequentially with 5% $NaHCO_3$ and saturated NaCl, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was purified by preparative TLC with hexane/acetone (1:1) to give 14′-azidoconophylline (156.2 mg, 64.7%), with ESIMS (pos) m/z 838 $(M+H)^+$ and $[\alpha]_D$ − 169 (c 0.7, $CHCl_3$). The NMR data are shown in Table 1.

4.2.2. Preparation of 14′-aminoconophylline (4)

A suspended solution of compound **3** (150 mg, 0.179 mmol) and Lindlar's catalyst (150 mg) in EtOH (10 ml) was hydrogenated under an H_2 atmosphere for 20 h. The reaction mixture was filtered through a Celite pad, and the filtrate was concentrated in vacuo. The residue was purified by preparative TLC with $CHCl_3$/MeOH/28% NH_4OH (40:10:1) to give conophylline–NH_2 (97.0 mg, 64.9%), with ESIMS (pos) m/z 812 $(M+H)^+$ and $[\alpha]_D$ − 179 (c 0.35, $CHCl_3$). NMR data are shown in Table 1.

4.2.3. Preparation of conophylline-immobilized latex nano-beads (8)

The SG beads (10 mg) prepared as described[11] were centrifuged in 2-ml tubes at 15,000 rpm for 5 min at 20 °C, and the supernatant removed. Next, *N,N*-dimethylformamide (DMF, 500 ml) was added

to the beads, which were then centrifuged again. After having been washed three times, they were suspended in DMF (300 ml). Triethylamine (100 ml) and 500 mM succinic anhydride (600 ml) were then added to this suspension, and the mixture was incubated for 24 h at room temperature. Then, the beads were washed with DMF three times and suspended and kept in DMF (430 ml) with triethylamine (50 ml) and 500 mM succinic anhydride (20 ml) for 2 h at room temperature. After centrifugation, the supernatant was removed, and DMF (100 ml) and water (500 ml) were added. After another centrifugation, the beads were washed with water, suspended in water (450 ml) with 1 N NaOH (50 ml), and agitated for 30 min at room temperature. After the reaction the beads were washed three to five times to afford the carboxylic-type SG beads.

After the beads had been washed with MeOH, they were suspended in MeOH (100 ml) and dioxane (500 ml). The supernatant was removed by centrifugation. After having been washed twice with dioxane, the beads were resuspended in dioxane (200 ml), to which suspension HOSu (23 mg in dioxane) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (38.4 mg) were added. After 2 h at room temperature, the supernatant was obtained by centrifugation and discarded. After the beads had been washed with DMF five times, they were reacted with 1 mM conophylline–NH_2 in DMF for 24 h. After centrifugation, the beads were washed with DMF (500 ml), and then reacted with 1 M 2-ethanolamine in DMF (500 ml) for 2 h at room temperature. After the centrifugation, the beads were washed with DMF three times followed by 3 washings with 50% methanol. Then the beads were suspended in 50% methanol (500 ml) and kept at 4 °C. Control beads were prepared by the same protocol without the reaction with conophylline–NH_2.

4.3. Affinity-purification

The conophylline-immobilized SG beads (0.5 mg) or control beads in 2-ml tubes were washed with a binding buffer, 15 mM Tris–HCl (pH 7.0), containing 75 mM NaCl, 15 mM EDTA, 1.5 mM DTT, 7.5% glycerol, 0.3% NP-40, and 0.25 mg/ml BSA. The cellular extract was diluted by adding sufficient binding buffer to yield 1 mg/ml protein. After centrifugation at 15,000 rpm for 15 min at 4 °C, the supernatant was taken into the tube containing the beads; and the mixture was incubated for 4 h at 4 °C. Then, the mixture was centrifuged at 15,000 rpm for 5 min at 4 °C. After removal of the supernatant, the pellet was washed with the binding buffer five times, suspended in 30 ml of elution buffer containing 20 mM HEPES (pH 7.9), 10% glycerol, 0.1 M KCl, 0.2 M EDTA, and 1 mM DTT, on ice for 15 min. After centrifugation at 15,000 rpm for 5 min at 4 °C, the supernatant was taken into a 1.5-ml tube. Binding buffer (30 ml) was added to the pellet, which was resuspended and moved to a new 1.5-ml tube, which was heated at 98 °C with the Laemmli dye (10 ml). The eluates were subjected to electrophoresis in a 5–20% SDS–polyacrylamide gradient gel, and visualized with silver staining.

4.4. Cell culture

Rat pancreatic acinar carcinoma AR42J-B13 cells, a subline of AR42J cells, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 mM HEPES–NaOH (pH 7.4), 100 mg/l kanamycin, 0.6 g/l glutamine, 100 k units/l penicillin G, 5 mM $NaHCO_3$, and 10% FBS at 37 °C in a humidified atmosphere with 5% CO_2.

4.5. Trypan blue dye exclusion

AR42J or HT1080 cells were seeded into 24-well plates at 1×10^5 cells/well and treated as described in the legend of Figure 2. Then the cells were stained with trypan blue, and the numbers of total and stained cells were counted.

4.6. Preparation of rat ARL6IP recombinant protein

AR42J cells were cultured in 100-mm dishes until 70–80% confluence had been achieved. On the next day, the cells were transfected with 10 mg pCMV-Tag2-flagged-ARL6IP plasmid by using Lipofectamine2000 (Invitrogen, San Diego, CA). After the medium had been removed, the cells were further cultured for 48 h. Then the cells were collected and lysed with FP buffer, which was 10 mM Tris–HCl (pH 7.4) containing 100 mM NaCl, 1 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 10% glycerol, 0.5% Triton X-100, and 1% protease inhibitor cocktail. After centrifugation at 14,000 rpm for 20 min at 4 °C, the supernatant was transferred into a new tube. The cell lysate (1 mg/ml) was immuno-precipitated with anti-flag antibody-conjugated agarose beads, and then the precipitated ARL6IP was eluted with 3 × flag peptides. After centrifugation at 5000 rpm for 30 s at 4 °C, the supernatant was transferred into a new tube as prepared recombinant protein. Samples were subjected to SDS–PAGE, and ARL6IP was detected by immuno-blotting using anti-flag antibody.

4.7. Construction of plasmids

Plasmids were prepared by PCR using rat ARL6IP cDNA (accession no.; AY316590 in GenBank) as template, forward primer 5′-TTTT GTC GAC CTA TTC GTT TTT CTT TTC TTT TTG C-3′, and reverse primer 5′-TTTT GGA TCC ATG GCC GAG GGG GAT AAC C -3′. These primers were introduced at *Bam*HI and *Sal*I sites at the 5′ and 3′ ends, respectively, in the extracellular ARL6IP domain. PCR products were cloned into the corresponding sites of the pCMV-Tag2B plasmid (Invitrogen, San Diego, CA), resulting in pCMV-Tag2-flagged-ARL6IP. The *Bam*H-*Xho*I fragments including ARL6IP domain in pCMV-Tag2- flagged-ARL6IP were cloned into the corresponding sites of pcDNA/TO (Invitrogen, San Diego, CA), resulting in pcDNA4/TO-ARL6IP.

4.8. ARL6IP and ARL6 binding assay

Firstly, we prepared ARL6-ligated Sepharose4B beads. ARL6 cDNA was inserted into the pGEX-1 vector, and *E. coli* BL21 were transfected with this vector. The bacteria containing pGEX-ARL6 were cultured for 4 h with ampicillin at 30 °C and then induced with 0.1 mM IPTG. After incubation for another 4 h at 25 °C, the cells were collected by centrifugation at 5000 rpm for 30 min. The cell pellet was resuspended in Ca^{2+}, Mg^{2+}-free PBS (PBS^-) and lysed with the sonication buffer (PBS^- with 0.2% Triton X-100, 1% protease inhibitor cocktail, 1 mM DTT) on ice. The lysate was centrifuged at 14,000 rpm for 10 min to remove cell debris. Glutathione–Sepharose 4B beads were added to the supernatant, and the supernatant was incubated at 4 °C for 1 h. After incubation the beads were washed with PBS^-, and the purity of ARL6-GST was checked by CBB staining and immuno-blotting. Flag-tagged ARL6IP was prepared from the ARL6IP-overexpressing HeLa cells. The HeLa cell pellet was lysed by using Flag buffer (10 mM Tris containing 0.5% Triton X-100, 100 mM NaCl, 10% glycerol, 1 mM $MgCl_2$, 0.2 mM EDTA, 1 mM PMSF, 1% protease inhibitor cocktail, and 1 mM DTT) on ice. The lysate was centrifuged at 14,000 rpm for 10 min to remove cell debris. Anti-Flag beads were added to the supernatant, which was then incubated at 4 °C for 4 h. After incubation, the beads were washed with Flag buffer. The beads were next resuspended in Flag buffer with 3 × Flag peptide and incubated at 4 °C overnight. After incubation, the lysate was centrifuged at 14,000 rpm for 10 min to remove the beads; and the supernatant was used as Flag-tagged

ARL6IP. Finally, the ARL6-ligated Sepharose4B beads were mixed with Flag-tagged ARL6IP with or without conophylline and incubated overnight at 4 °C. After incubation, the mixture was centrifuged at 14,000 rpm for 10 min to remove the supernatant; and the beads were washed with Flag buffer. The beads were resuspended in Flag buffer with loading buffer. Then, after electrophoresis the immuno-blotting was carried out.

Acknowledgments

This work was financially supported in part by grants from the programs Grants-in-Aid for Scientific Research on Priority Areas and Grants-in-Aid for the Global Centers of Excellence (GCOE) Program (2007–2012 to Keio University) of the Ministry of Education, Culture, Sports, Science, and Technology (MEXT). This work was also supported by the High-Tech Research Center Project for Private Universities: matching fund subsidy from MEXT, 2006–2010.

References and notes

1. Kam, T. S.; Loh, K. Y.; Wei, C. *J. Nat. Prod.* **1993**, *56*, 1865.
2. Kam, T. S.; Pang, H. S.; Lim, T. M. *Org. Biomol. Chem.* **2003**, *1*, 1292.
3. Umezawa, K.; Ohse, T.; Yamamoto, T.; Koyano, T.; Takahashi, Y. *Anticancer Res.* **1994**, *14*, 2413.
4. Umezawa, K.; Hiroki, A.; Kawakami, M.; Naka, H.; Takei, I.; Ogata, T.; Kojima, I.; Koyano, T.; Kowithayakorn, T.; Pang, H. S.; Kam, T. S. *Biomed. Pharmacother.* **2003**, *57*, 341.
5. Ogata, T.; Li, L.; Yamada, S.; Yamamoto, Y.; Tanaka, Y.; Takei, I.; Umezawa, K.; Kojima, I. *Diabetes* **2004**, *53*, 2596.
6. Hisanaga, E.; Park, K. Y.; Yamada, S.; Hashimoto, H.; Takeuchi, T.; Mori, M.; Seno, M.; Umezawa, K.; Takei, I.; Kojima, I. *Endocr. J.* **2008**, *55*, 535.
7. Shimizu, N.; Sugimoto, K.; Tang, J.; Nishi, T.; Sato, I.; Hiramoto, M.; Aizawa, S.; Hatakeyama, M.; Ohba, R.; Hatori, H.; Yoshikawa, T.; Suzuki, F.; Oomori, A.; Tanaka, H.; Kawaguchi, H.; Watanabe, H.; Handa, H. *Nat. Biotechnol.* **2000**, *18*, 877.
8. Wada, T.; Takagi, T.; Yamaguchi, Y.; Kawase, H.; Hiramoto, M.; Ferdous, A.; Takayama, M.; Lee, K. A.; Hurst, H. C.; Handa, H. *Nucleic Acids Res.* **1996**, *24*, 876.
9. Uga, H.; Kuramori, C.; Ohta, A.; Tsuboi, Y.; Tanaka, H.; Hatakeyama, M.; Yamaguchi, Y.; Takahashi, T.; Kizaki, M.; Handa, H. *Mol. Pharmacol.* **2006**, *70*, 1832.
10. Kabe, Y.; Ohmori, M.; Shinouchi, K.; Tsuboi, Y.; Hirao, S.; Azuma, M.; Watanabe, H.; Okura, I.; Handa, H. *J. Biol. Chem.* **2006**, *281*, 31729.
11. Dignam, J. D.; Lebovitz, R. M.; Roeder, R. G. *Nucleic Acids Res.* **1983**, *11*, 1475.
12. Chavrier, P.; Goud, B. *Cell Biol.* **1999**, *11*, 466.
13. Ingley, E.; Williams, J. H.; Walker, C. E.; Tsai, S.; Colley, S.; Sayer, M. S.; Tilbrook, P. A.; Sarna, M.; Beaumont, J. G.; Klinken, S. P. *FEBS Lett.* **1999**, *459*, 69.
14. Pettersson, M.; Bessonova, M.; Gu, F. H.; Groop, C. L.; Jonsson, J. *Genomics* **2000**, *68*, 351.
15. Lui, M. H.; Jun, C.; Wang, L.; Naumovski, L. *Cancer Res.* **2003**, *1*, 508.
16. Wong, C.; Naumovski, L. *Anal. Biochem.* **1997**, *252*, 33.
17. Kahn, R. A.; Volpicelli-Daley, L.; Bowzard, B.; Shrivastava-Ranjan, P.; Li, Y.; Zhou, C.; Cunningham, L. *Biochem. Soc. Trans.* **2005**, *33*, 1269.