Discovery of Novel Adenylyl Cyclase Inhibitor by Cell-Based Screening

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We screened 2400 compounds to find novel inhibitors of the adenylyl cyclase (AC)–protein kinase A (PKA)–cAMP response-element-binding protein (CREB) signaling pathway (AC/PKA/CREB pathway). Using a multistep cell-based screening system employing split luciferase technique, we narrowed down the candidates effectively from 2400 chemical compounds and identified a novel AC inhibitor (compound 1). Since dysregulation of the AC/PKA/CREB pathway is known to cause diseases not only in the nervous system but also in other organs, compound 1 is expected to be developed as a medicine for these diseases.

Key words adenylyl cyclase; high-throughput screening; inhibitor; protein kinase A; cAMP response element binding protein

The adenylyl cyclase (AC)–protein kinase A (PKA)–cAMP response-element-binding protein (CREB) signaling pathway (AC/PKA/CREB pathway) is considered important for the expression of the proteins that are required for memory formation.[1–3] Inhibition of this pathway blocks the consolidation of long-term memory.[4] On the other hand, activation of this pathway is involved in drug addiction.[5] Deficiency of type 5 AC induces Parkinson's disease-like symptoms in mice; conversely, excitotoxicity of neurons is alleviated in type 1 AC knockout mice.[6] Furthermore, CREB activation is considered to be important in oncogenesis,[7] and some tumor cells show overexpression or constitutive activation of CREB.[8–10] These reports indicate that dysregulation of the AC/PKA/CREB pathway may induce neurodegenerative diseases and cancers, and the compounds that modulate the AC/PKA/CREB pathway are expected to be developed as medicines for these diseases.

In our previous study, we employed the split luciferase technique to monitor the activation of the AC/PKA/CREB pathway in live cells.[11] In this technique, firefly luciferase was cleaved into N-terminal and C-terminal segments. Each segment was fused with the kinase-inducible domain (KID) of CREB and the interacting domain (KIX) of the CREB-binding protein (CBP). Following the phosphorylation of KID, KID and KIX interact with each other, and split segments (KID–KIX probes) complement each other to be a functional luciferase that can emit light. We found that these probes could be used for small-scale (120 samples) screening for herbal extracts that up-regulate the AC/PKA/CREB pathway.[11] In this study, we attempted to screen 2400 chemical compounds to find novel inhibitors of the AC/PKA/CREB pathway by multistep screening using the KID–KIX probe, cytomegalovirus promoter (CMV)–luciferase, and cAMP response element (CRE)–luciferase.

MATERIALS AND METHODS

Materials The 2400 chemical compounds were from the University of Tokyo (Core Library, Open Innovation Center for Drug Discovery). Forskolin was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). All reagents were diluted with culture medium or phosphate buffered saline (PBS) from a 10 mM stock solution prepared in dimethyl sulfoxide (DMSO).

Plasmids We constructed a plasmid that expressed the KID–KIX probe. The open reading frames of the probes, which were referred to as C6 and D5 probes in our previous report,[11] and the internal ribosome entry sites (IRES) sequence of pIRES2-EGFP (Clontech, Palo Alto, CA, U.S.A.) were amplified by polymerase chain reaction using primers containing restriction enzyme sites. The DNA fragments of D5, IRES, and C6 were inserted between the *Nhe*I and *Not*I sites of pEGFP-N1 (Clontech) in this order using a conventional molecular biological method and this insertion removed the enhanced green fluorescent protein (EGFP) coding region from pEGFP-N1. For the screening using wild-type luciferase driven by the CMV promoter, we inserted the open reading frame of luc2 (Promega, Madison, WI, U.S.A.) between the *Nhe*I and *Not*I sites of pEGFP-N1. For the CRE–luciferase assay we used pGL4.29 (Promega).

Cell Culture, Transfection, and Generation of Stable Expression Cell Lines Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum at 37°C in an incubator with 5% CO_2. The HEK293 cells were transfected with the plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.) in accordance with the manufacturer's instruction. For the establishment of stable cell lines, we selected G418 (0.4 mg/mL)-resistant colonies after 2 weeks of culture. Established HEK293 cells stably expressing the KID–KIX probe and HEK293 cells stably expressing CMV–luciferase were used for the first- and second-step screening, respectively. HEK293 cells transiently expressing the CRE–luciferase reporter gene were used for the third-step screening.

Cell-Based Screening For first-step screening, HEK293 cells stably expressing the KID–KIX probe were plated onto white 96-well plates (Nunc, Roskilde, Denmark) (1.5×10^4 cells/well) and incubated for 24 h at 37°C. Then the culture medium was replaced with L15 medium containing 0.5 mM D-

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luciferin (AAT Bioquest, Sunnyvale, CA, U.S.A.). The culture plate was incubated in a dark box for 1.5 h at room temperature. Compounds were added to the wells at 10 μM, which is same to the concentration used in previously reported screening using *Renilla* luciferase,[12] and incubated for 4 h at room temperature (first treatment). Subsequently, the cells were stimulated with 10 μM forskolin for 2 h at room temperature (second treatment). Control cells were treated with DMSO at the same concentration (0.1% in the first treatment and 0.2% in the second treatment) as in cells treated with compounds or forskolin. During this procedure, the intensity of light emission from each well was measured at three time points, namely, immediately prior to compound addition, 4 h after compound addition, and 2 h after forskolin addition. The second- and third-step screening were performed with the same procedure as in the first-step screening using HEK293 cells stably expressing luciferase driven by the CMV promoter and HEK293 cells transiently expressing luciferase under the control by the CRE sequence, respectively.

Measuring Light Intensity in Cultured Cells The Aequoria-2D/C8600 system (Hamamatsu Photonics, Hamamatsu, Japan) and Wasabi software (U9304-02) were used for data acquisition and analysis as described in our previous report.[13]

Quantification of cAMP HEK293 cells stably expressing KID–KIX probe were treated with compound **1** for 4 h (first treatment), followed by forskolin for another 15 min (second treatment). The cells were rinsed twice with PBS, and lysed with dilution buffer. Intracellular cAMP concentration was measured in accordance with the manual of a DetectX Direct Cyclic AMP Enzyme Immunoassay kit (ARBOR ASSAYS, Ann Arbor, MI, U.S.A.). After the reaction was stopped, optical density was read on a microtiter plate reader at 450 nm. The obtained optical density was fitted to the standard curve and cAMP concentration was calculated.

PKA Activity Assay The activity of PKA was detected by a method using a PepTag Non-Radioactive Protein Kinase Assays kit (Promega) with dye-labeled Kemptide (LRRASLG) as a substrate, in accordance with the manufacturer's instructions. Briefly, the cells treated with compound **1** were rinsed with PBS and collected. The cells were lysed with lysis buffer (20 mM 3-morpholinopropanesulfonic acid (MOPS), 5 mM ethylene glycol tetraacetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA), 1% NP-40, 1 mM dithiothreitol) containing a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) and a phosphatase inhibitor (Nacalai Tesque). The total protein concentration of each cell lysate was determined by bicinchoninic acid (BCA) assay and 10 μg of total protein was used for reaction with the substrates. The reaction was stopped by heating at 95°C for 10 min and the substrates were separated on a 0.8% agarose gel at 100 V for 15 min. The phosphorylated and nonphosphorylated substrates moved in opposite directions owing to the difference in charge. The phosphorylated and nonphosphorylated peptide substrate signals were detected using the LAS-4000 system (FUJIFILM, Tokyo, Japan), and quantitative determination was performed with Multi Gauge v3.1 software (FUJIFILM). The percentage of phosphorylated substrate was calculated from the signal intensity of phosphorylated substrate/signal intensity of total substrate.

Statistical Analyses Data are presented as mean±standard error of the mean (S.E.M.). Two-tailed Student's *t*-test was used to determine the statistical significance of differences.

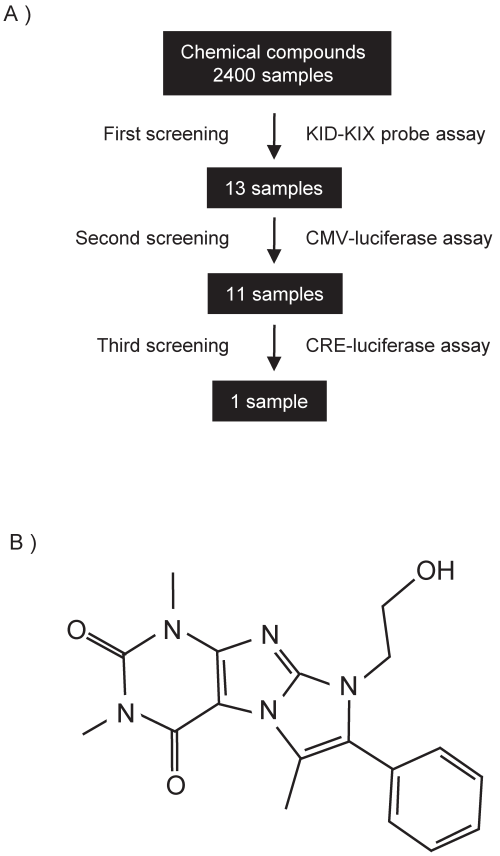

Fig. 1. Cell-Based Screening for Compounds That Affect the AC/PKA/CREB Pathway

A) Schematic diagram of screening process. The process consists of three steps. B) Chemical structure of compound **1** identified by cell-based screening.

RESULTS AND DISCUSSION

For the first screening, HEK293 cells stably expressing the KID–KIX probe were treated with compounds (10 μM) from a chemical library and then with 10 μM forskolin which is known to activate AC to up-regulate the AC/PKA/CREB pathway. Because we previously found that the light intensity in cells expressing the KID–KIX probe increased after forskolin treatment, we screened for the compounds that inhibited the forskolin-induced increase in light intensity. The screening process is shown in Fig. 1A. We found 13 compounds that cancelled the forskolin-induced increase in light intensity. In the next step, we added the 13 compounds (10 μM) to the medium of HEK293 cells expressing wild-type luciferase driven by the CMV promoter (CMV–luciferase). This step was performed to exclude the compounds that affect the activity of luciferase directly, and we found two that reduced the intensity of light from wild-type luciferase. We excluded these two compounds and the 11 remaining compounds were tested in the next step.

We examined whether the compounds cancelled the increase in light intensity induced by forskolin in HEK293 cells expressing CRE–luciferase in the third screening. The CRE is target sequence of CREB, and the transcription of the downstream gene of CRE is activated by CREB activation. There-

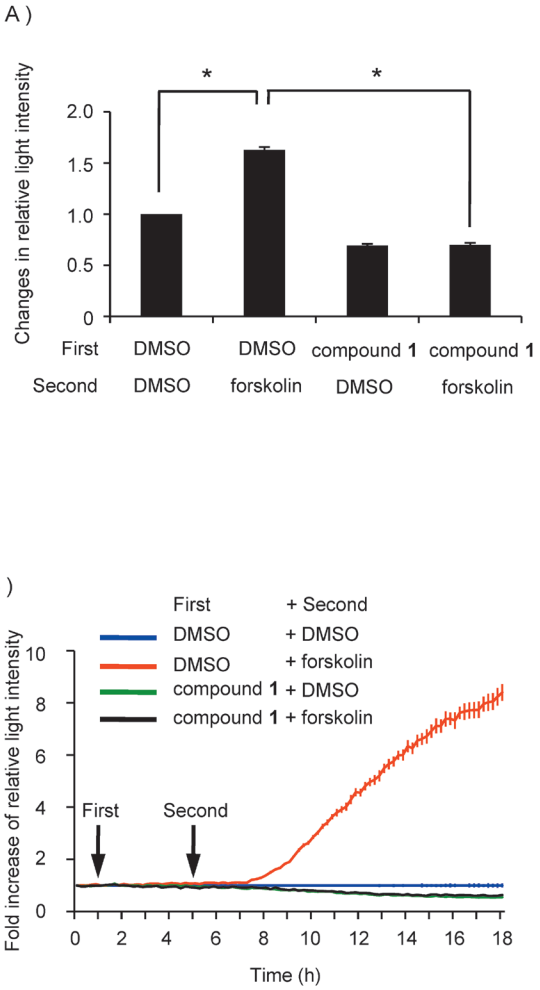

Fig. 2. Inhibition of Increase in Intensity of Light Emission from KID–KIX Probe and CRE–Luciferase by Compound **1**

A) Effect of compound **1** on intensity of light emission from KID–KIX probe. Light intensity in HEK293 cells stably expressing the KID–KIX probe was measured 4 h after treatment with dimethyl sulfoxide (DMSO) or 10 μM compound **1** (first treatment). Next, light intensity was measured 2 h after treatment with DMSO or 10 μM forskolin (second treatment). The vertical axis represents the changes in light intensity (second/first). Data are presented as relative intensity (mean±S.E.M., n=4). *p<0.05, with Student's t-test. B) Compound **1**-induced inhibition of increase in CRE-mediated gene expression level in response to forskolin treatment. HEK293 cells were transfected with the CRE–luciferase reporter plasmid. The cells were treated with DMSO or 10 μM compound **1** followed by DMSO or 10 μM forskolin treatment. Light intensity was measured every 10 min for 18 h. Arrows indicate the time points of first and second treatments. Data are presented as mean±S.E.M., n=4.

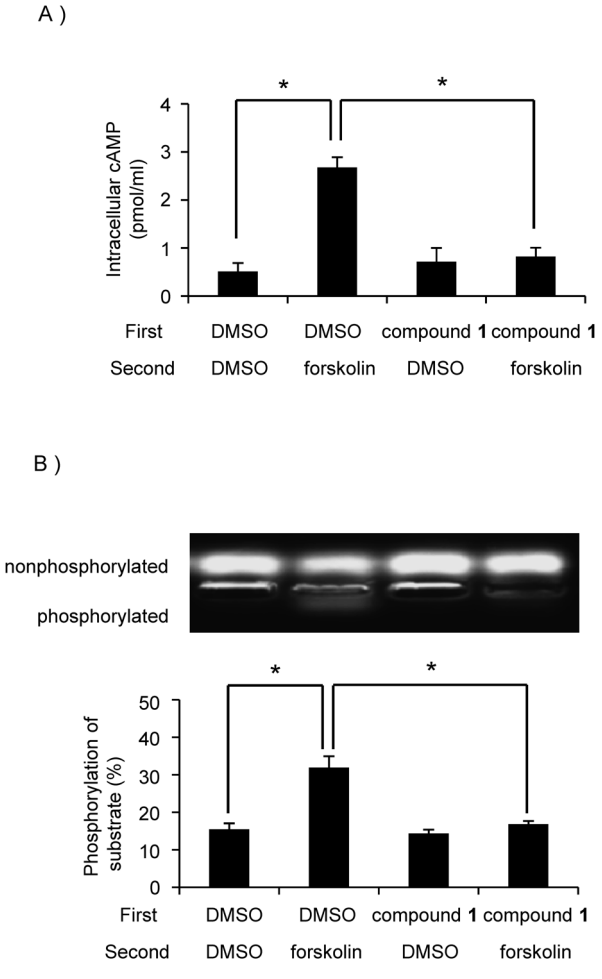

Fig. 3. Effects of Compound **1** on Changes in Intracellular cAMP Concentration and PKA Activity

HEK293 cells were treated with 10 μM compound **1** for 4 h (first treatment), and followed by 10 μM forskolin treatment for 15 min (second treatment), and then the cells were collected and lysed. Intracellular cAMP concentration (A) and relative PKA activity (B) were immediately measured. The upper panel in (B) shows a photograph of the representative gel electrophoresis of the PKA substrate, and the quantification of that assay is shown in the lower panel. Data are presented as percentage of phosphorylated substrate (mean±S.E.M., n=4). *p<0.05, with Student's t-test.

fore, the 11 compounds were expected to cancel the increase in light intensity induced by forskolin. As a result, we found a compound (compound **1**, Fig. 1B) whose IPUC name was 8-(2-hydroxyethyl)-1,3,6-trimethyl-7-phenyl-1*H*-imidazo[2,1-*f*]purine-2,4(3*H*,8*H*)-dione, suppressed forskolin-induced increase in light emission from CRE–luciferase, however other 10 compounds did not. Then compound **1** was subjected to subsequent analysis.

For the validation of the results of the screening, we evaluated the effect of compound **1** on the light emission from the KID–KIX probe and CRE–luciferase again. We confirmed that compound **1** inhibited the forskolin-induced increase in the intensity of light emission from the KID–KIX probe (Fig. 2A). This finding indicates that the interaction between CREB and CBP is inhibited in the presence of compound **1**. We observed no forskolin-induced increase in light intensity in the cells expressing CRE–luciferase in the presence of compound **1**, whereas a long lasting increase in light intensity was detected after forskolin treatment without compound **1** (Fig. 2B). Then we analyzed compound **1** by NMR to confirm the structure, and by mass spectrometry to confirm the purity. According to the obtained results, we concluded that compound **1** had the structure drawn in Fig. 1B and high purity (supplemental data), which indicated that inhibitory effects were due to compound **1** itself.

The KID–KIX probe and CRE–luciferase were designed to monitor the activity of CREB-CBP binding and CRE-mediated transcription, respectively. Thus, compound **1** may inhibit CREB directly, and at same time it is possible that compound **1** inhibits upstream enzymes such as PKA and AC. To determine the target protein of compound **1**, we then analyzed the effect of compound **1** on concentration of intracellular cAMP and PKA activity. We found that compound **1** treatment prior to forskolin treatment significantly inhibited forskolin-induced cAMP up-regulation (Fig. 3A). This finding indicates that compound **1** is an inhibitor of AC. Next, we examined the

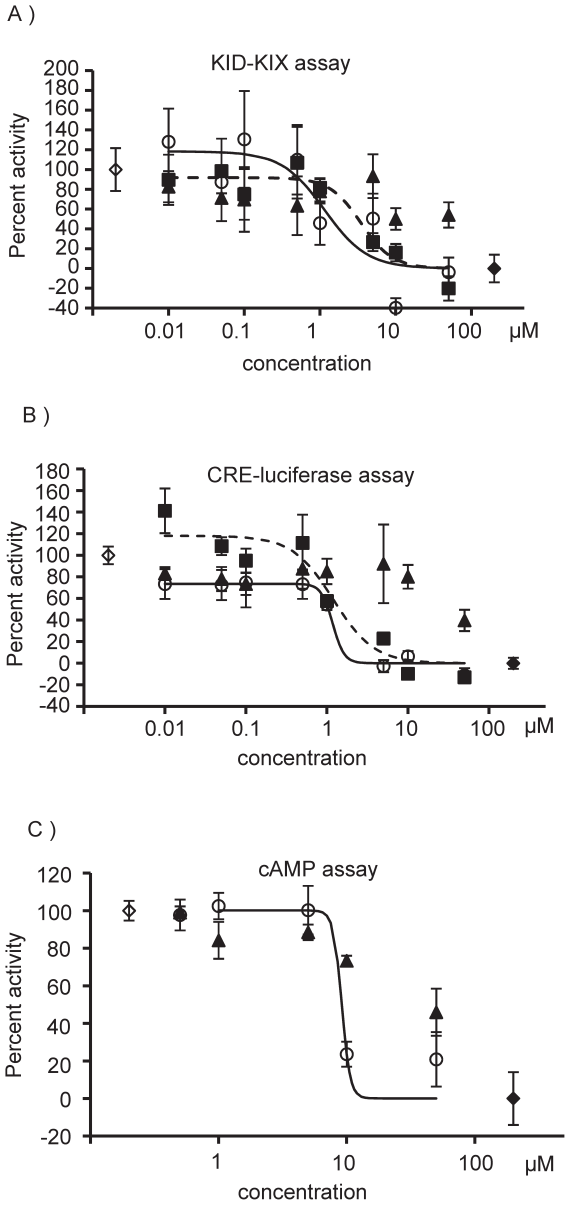

Fig. 4. Dose-Dependent Inhibitory Effect of Compound **1** on KID–KIX, CRE–Luciferase, and cAMP Assays

Inhibitory effect of compound **1** and other known inhibitors of AC/PKA/CREB pathway on KID–KIX assay (A), CRE–luciferase assay (B), and cAMP assay (C) were tested. In each graph, vertical axis represents percent change of forskolin induced increase in light emission (A, B) and amount of intracellular cAMP (C) comparing to the positive control that was treated with forskolin in the absence of inhibitors. Each mark represents compound **1** (○), PKA inhibitor H89 (■), AC inhibitor SQ22536 (▲), positive control (◇), and negative control (◆) which were not treated with forskolin or inhibitors. Solid and dotted lines represent fitted curves for compound **1** and H89, respectively. Data are presented as mean±S.E.M., n=4 (A, B), n=3 (C).

effect of compound **1** on PKA activity, which was induced by the increased concentration of cAMP. We observed that the increase in PKA activity induced by forskolin treatment was cancelled by pretreatment with compound **1** (Fig. 3B). This finding supports the idea that compound **1** is an inhibitor of AC.

We then examined whether compound **1** showed dose-responsive inhibition on KID–KIX assay, CRE–luciferase assay, and endogenous cAMP assay (Figs. 4A–C). Forskolin-induced increase in light emission from KID–KIX probe and CRE–luciferase plasmid expressing cells were cancelled depending on the concentration of compound **1**. Increased level of cellular cAMP induced by forskolin was also suppressed by compound **1** dose-responsively. IC_{50} values of compound **1** in these three assays were calculated by fitting of the Hill equation to the dose–response data. Determined IC_{50}s of compound **1** in KID–KIX assay, CRE–luciferase assay and cAMP assay were 1.15 μM, 1.18 μM, and 9.15 μM, respectively. A PKA inhibitor, H89 inhibited the increase in light intensity induced by forskolin with IC_{50}s of 3.48 μM and 1.20 μM in KID–KIX assay and CRE–luciferase assay, respectively. We did not determine the IC_{50}s of an AC inhibitor SQ22536 because it did not show maximum inhibition at the concentrations we tested.

Previously, some groups have attempted to screen the compounds that interacted with KID or KIX using NMR-based method[14] or split *Renilla* luciferase techniques *in vitro*.[12] In our report, we showed that multistep cell-based screening using bioluminescence probes was effective to narrow down the number of candidate compounds to identify the inhibitor of enzymes in AC/PKA/CREB pathway. Usage of live cells is a characteristic of our screening method comparing to the methods reported before.

We performed the second- and third-step screening using the hit compounds from the first-step screening. Although the first-step screening was effective in narrowing down the number of candidates from the library, there were some pseudopositive compounds. Such pseudopositive compounds were excluded in the second- and third-step screening. It is considered that the compounds excluded in the second-step screening affect the activity of luciferase. The pseudopositive compounds in the third-step screening may interact only with split fragments of luciferase but not with KID, KIX and wild-type luciferase. It can explain these compounds suppressed the increase in light emission only in the first screening. The existence of pseudopositive compounds indicates that solely cell-based split luciferase assay is not sufficient for identifying a compound to be a specific inhibitor, and it requires multistep screening and confirmation experiments.

A possible reason why single compound **1** treatment did not decrease the concentration of cAMP is that the efficacy of compound **1** to inhibit AC increases when AC is activated. This feature is also observed in the P-site inhibitor, which is a type of AC inhibitor that mimics the cAMP·PPi transition state.[15,16] It should be elucidated whether compound **1** is a P-site inhibitor, and which AC isoform (AC1-9) can be interacted with compound **1** in the future research.

Activation of the AC/PKA/CREB pathway in the nucleus accumbens is involved in addiction behavior such as cocaine self-administration,[17–19] and it is also important in oncogenesis.[7] Deficiency of type 1 AC up-regulates the resistance to glutamate-induced neurotoxicity in the nervous system,[20] and β-adrenergic receptor stimulation-induced myocyte apoptosis in the heart is attenuated in type 5 AC knockout mice.[6,21] Hence, compound **1** is expected to be a leading compound for developing medicines for above-mentioned conditions.

In conclusion, we have screened 2400 chemical compounds to find an inhibitor of the AC/PKA/CREB pathway. By employing multistep screening, we successfully screened for and found a compound that inhibited AC.

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