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Original Article

A high-throughput screening system for G-protein-coupled receptors using β-lactamase enzyme complementation technology

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Aim: To establish a system for monitoring the activation of G-protein-coupled receptors (GPCRs) using β-lactamase enzyme fragment complementation (EFC) technology.
Methods: Two inactive β-lactamase deletion fragments, bla(a) and bla(b), were fused to β-arrestin and GPCR, respectively. A stable cell line named HEK/293-β2a2, which expressed two fusion proteins, GPCR/bla(b) and β-arrestin2/bla(a), was generated under antibiotic selection. A natural compound library of high performance liquid chromatography (HPLC)-fractionated samples from the ethanol extracts of Chinese medicinal herbs was used for high-throughput screening (HTS) of β2-adrenoceptor (β2AR) agonists against the cell line HEK/293-β2a2. The interested hits were validated by the measurement of second-messenger cyclic adenosine monophosphate (cAMP) production.
Results: The stable cell line HEK/293-β2a2 responded to β2AR agonist/antagonist in a dose-dependent manner. The EC_{50} value obtained for isoproterenol was 15.5 nmol/L, and the IC_{50} value obtained for propranolol was 51.9 nmol/L. Furthermore, HTS was performed to identify β2AR agonists from the natural compound library we established. The Z′ factor value was determined to be 0.68. Three hits were identified from primary screening and found to be as potent as isoproterenol in a cAMP assay.
Conclusion: A cell-based high-throughput functional assay was established to directly monitor the activation of GPCRs based on the interaction between agonist-activated GPCR and β-arrestin using β-lactamase EFC technology, which can be used to search for leads in the natural compound library.

Keywords: G-protein-coupled receptor; β-lactamase; enzyme fragment complementation; high-throughput screening

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Introduction

G-protein-coupled receptors (GPCRs) are the largest known family of transmembrane receptors. They bind to and transduce signals for a huge variety of endogenous ligands, and they are involved in most physiological functions, such as metabolism, neuronal transmission, embryonic development, hormonal homeostasis and cancer invasion[1, 2]. Of all currently marketed drugs, over 30% target GPCRs[3], and the development of new technologies for GPCR-based drug discovery promises to keep this field active for years to come.

Recently, several cell-based drug discovery strategies were developed using β-arrestin as a surrogate marker for GPCR function. β-arrestins are ubiquitously expressed in all cells and tissues. They bind with high affinity to the agonist-activated GPCR carboxyl-terminal tail. Assays developed based on GPCR-β-arrestin interaction, such as transfluor assay[4, 5], bioluminescence resonance energy transfer assay[6], PathHunter™ β-arrestin Assays[7] and Tango assay,[8] were widely used in the investigation of many GPCRs[9–15]. These achievements have demonstrated that monitoring GPCR-β-arrestin interaction can offer a direct indication of GPCR activation.

Here we describe a cell-based protein-protein interaction assay based on β-lactamase complementation technology that uses GPCR and β-arrestin as protein partners to monitor GPCR activation. The β-lactamase-based enzyme fragment complementation (EFC) assay was pioneered in 2002[16, 17]. The properties of β-lactamase provide significant advantages in mammalian cells. β-lactamase is monomeric and relatively

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small[18], and it can be expressed in many different cell types and diverse cellular compartments[19]. In addition, there is no endogenous β-lactamase activity. A highly sensitive cell-permeable fluorescent substrate, CCF4/AM, also offers great potential for the quantification of results in intact cells[20]. In our research, we successfully applied this system to monitor GPCRs activation. Two inactive β-lactamase deletion fragments, bla(a) and bla(b), were fused to β-arrestin and GPCR, respectively. The two β-lactamase fragments complement each other and recover activity by agonist-promoted interaction between GPCR and β-arrestin. The recovery of β-lactamase activity was then determined with the cell-permeable fluorescent substrate, CCF4/AM, to demonstrate GPCR activation.

In this study, we chose β2-adrenoceptor (β2AR) as a model. β2AR is a typical GPCR that has been thoroughly characterized. The β2-adrenoceptor agonists are the most widely used agents in the treatment of asthma owing to their bronchodilator actions[21]. Chinese medicinal herbs have been used to treat many diseases, and they have demonstrated effectiveness for thousands of years. Our lab established a natural compound library containing high performance liquid chromatography (HPLC)-fractionated samples from the ethanol extracts of Chinese medicinal herbs. This library was successfully used to find inhibitors for Severe Acute Respiratory Syndrome-associated coronavirus (SARS-CoV) and agonists for dopamine receptors[22, 23]. In the following, we describe in detail the application of β-lactamase-based EFC assay to monitor GPCR activation and to screen for agonists of the β2-adrenoceptor from the natural compound library we constructed.

Materials and methods

Materials

Isoproterenol and propranolol were purchased from Sigma (St Louis, USA). CCF4/AM was obtained from Invitrogen (Carlsbad, CA, USA).

Plasmid construction

β-lactamase gene fragments bla(a) and bla(b) were amplified and ligased to pEGFP-N1 vector (Clontech, Mountain View, CA, USA). The primers used were the forward primers [5′-CGG GGTACCGGAGGAGGAGGAAGTGGAGGAGGAG-GAAGTGGAGGAGGAGGAAGTCACCCAGAAACGCT-GGTG-3′ for bla(a) and 5′-CCCAAGCTTGGTGGTGGTG-GTAGTGGTGGTGGTGGTAGTGGTGGTGGTGGTAGTC-TACTTACTCTAGCTTCC-3′ for bla(b)] and the reverse primers [5′-ATTTGCGGCCGC TCAGCCAGTTAATAGTTTG-3′ for bla(a) and 5′-ATTTGCGGCCGCTTACCAATGCTTAAT-CAGT-3′ for bla(b)]. A flexible $(G_4S)_3$ linker was inserted in front of each β-lactamase fragment. Human β-arrestin2 and β2AR cDNA without a stop codon were cloned in front of the β-lactamase fragments bla(a) and bla(b), respectively, to generate pβarr2-bla(a) and pβ2AR-bla(b), which are two expression vectors. The primers used were the forward primers (5′-CTAGCTAGCACCATGGGGGAGAAACCCGGGAC-3′ for β-arrestin2 and 5′-CTAGCTAGCACCATGGGGCAACCCGG-GAAC-3′ for β2AR) and the reverse primers (5′-CGGGGTAC-CCTAGCAGAGTTGATCATC-3′ for β-arrestin2 and 5′-CCCA-AGCTTCAGCAGTGAGTCATTTGTAC-3′ for β2AR).

Cell culture and stable cell line selection

HEK-293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 2 mmol/L *L*-glutamine. The stable cell line HEK/293-β2a2 was generated by co-transfection of two expression constructs, pβarr2-bla(a) and pβ2AR-bla(b), into HEK-293 cells. Cells were selected by the addition of 0.8 g/L G418 into the culture medium for 15 d. The isolation of HEK/293-β2a2 cell clones was performed by the stimulation of cellular clones with 1 μmol/L isoproterenol. The clones, which turned from green to blue under fluorescence microscopy detection, were individually isolated.

CCF4/AM-based β-lactamase assay

To perform the CCF4/AM assay, cells were loaded with 1 μmol/L CCF4/AM according to the dye provider's protocol and then incubated at room temperature for 30 min. A Flexstation II (Molecular Devices, Sunnyvale, CA, USA) was used to determine the blue/green ratio by setting the excitation at 409 nm and the emission at 460 nm (for blue fluorescence) or 530 nm (for green fluorescence). The blue/green ratio was calculated following the manufacturer's instructions.

Induction/inhibition of β2AR receptors

HEK/293-β2a2 cells (4×10^4 cells/well) were seeded in a 96-well black matrigel-coated microplate with a clear-bottom and cultured for 24 h. For agonist dose response, cells were then incubated with isoproterenol in serum-free DMEM for 5 min at 37 °C. For antagonist dose response, cells were first pre-incubated for 10 min in serum-free DMEM containing different concentrations of propranolol at room temperature. Then 1 μmol/L of isoproterenol was added and incubated for another 5 min at 37 °C. CCF4/AM was then loaded to perform the β-lactamase assay. The blue/green ratio was determined by a Flexstation II. Images were captured by fluorescence microscopy (Olympus, Tokyo, Japan).

Preparation of the natural compound library

Herbal plants with therapeutic indications were collected from an herb market in Anguo, Hebei, China. Briefly, the dry Chinese herbs were dissolved in 90% ethanol for 12 h and sonicated for 0.5 h. The remains were then soaked with 50% ethanol for 12 h and extracted for 0.5 h. The ethanol extract was filtered and lyophilized. Products after lyophilization were re-dissolved in 90% ethanol for high performance liquid chromatography (HPLC) isolation. A total of 16 gradients were obtained by HPLC isolation for each Chinese herb extract. All gradients were lyophilized, dissolved in dimethyl sulfoxide (DMSO) and kept in a -80 °C freezer.

High-throughput screening

Stably transfected HEK/293-β2a2 cells were seeded at 4×10^4

cells per well in a 96-well black matrigel-coated microplate with a clear-bottom. The cells were cultured for 24 h before treatment. Culture media were replaced with serum-free DMEM containing 0.01 g/L diluted testing compounds. Positive and negative controls were designed in each plate. After incubation at 37 °C for 5 min, β-lactamase activity was quantitated with CCF4/AM.

cAMP assay

HEK/293-β2a2 cells were plated (4×10^4 cells/well) in a 96-well plate for 24 h. Compounds were diluted with phosphate-buffered saline and incubated with the cells for 15 min prior to measuring the cAMP level with cAMP-Glo™ Assay (Promega, Madison, WI, USA), according to product instructions. Luminescence was determined using an Envision 2100 (PerkinElmer, Waltham, MA, USA).

Data analysis and calculations

Data were presented as mean±SD. Statistical significance was evaluated with Student's *t*-test, and the difference was considered statistically significant at $P<0.05$. The *Z′* factor was calculated as described previously by Zhang *et al*[24]. For concentration-response determinations, a curve was fitted to calculate EC_{50} and IC_{50} using GraphPad Prism 5 software.

Results

Recombinant cell line generation

To study GPCR activation with a β-lactamase EFC assay, stable cell line HEK/293-β2a2 was established by the co-transfection of plasmids encoding human β-arrestin2/bla(a) and β2AR/bla(b), two fusion constructs (Figure 1A), into HEK-293.

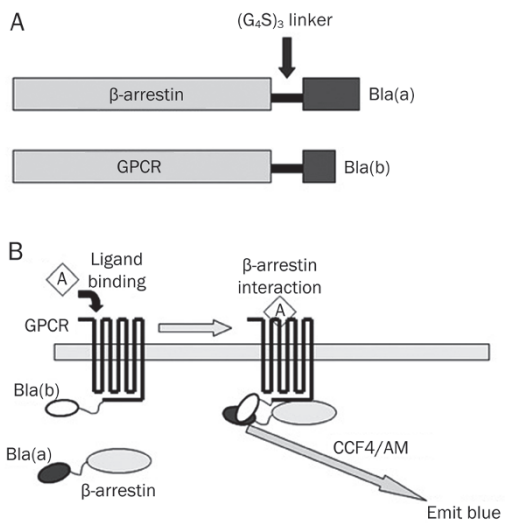

Figure 1. β-lactamase EFC based receptor/β-arrestin interaction assay. (A) Vector construction. β-lactamase fragment was fused to target protein with a flexible $(G_4S)_3$ linker. (B) Illustration of β-lactamase EFC based receptor/β-arrestin interaction assay. Agonist (A) activated GPCR recruits β-arrestin to the cell membrane, which brings two β-lactamase fragments proximity to reconstitute and recover its activity. The recovery of β-lactamase activity was then determined with CCF4/AM assay.

They were selected as described in materials and methods. The cell clone with the best signal to background ratio was chosen and named HEK/293-β2a2. In this clone, β2AR/bla(b) and β-arrestin2/bla(a), two fusion constructs, were highly expressed. The two β-lactamase deletion mutants, bla(a) and bla(b), are individually inactive and have a low affinity for each other. Ligand stimulation caused the conformation change of β2AR and recruited β-arrestins to the cell membrane. The interaction between β2AR and β-arrestin brought the two inactive β-lactamase fragments together to reconstitute a functional enzyme. The recovery of β-lactamase activity, as determined with the CCF4/AM assay, was a direct indicator of agonist-induced GPCR activation via the β-arrestin recruitment (Figure 1B).

Characteristics of the stable cell line HEK/293-β2a2

Agonist stimulation in cells co-expressing β2AR/bla(b) and β-arrestin2/bla(a) caused the complementation of two β-lactamase fragments. The reconstituted β-lactamase hydrolyzed the substrate CCF4/AM and emitted blue fluorescence when excited at 409 nm. The generation of blue fluorescent cells could be attributed to the activation of G-protein-coupled receptors (Figure 2A, top). The agonist effect could be inhibited by the β2AR antagonist, propranolol. When pre-incubated with propranolol, the percentage of cells that emitted blue fluorescence after agonist stimulation was similar to untreated cells (Figure 2A, middle and bottom). Dose response curves for isoproterenol and propranolol were also calculated using GraphPad Prism 5 software. As shown in Figure 2B, HEK/293-β2a2 cells responded to the known β2AR agonist, isoproterenol, with a dose-dependent increase in β-lactamase activity. The EC_{50} value of isoproterenol was found to be 15.5 nmol/L, which is not only similar to previously reported data for HEK-293 cells (11 nmol/L) using an isoproterenol-stimulated β2AR sequestration assay[25] but also data obtained in C2C12 cells (27 nmol/L) using a β-galactosidase based EFC assay[7]. Dose-dependent inhibition was demonstrated under the treatment of the β2AR antagonist, propranolol, in the presence of 1 μmol/L isoproterenol (Figure 2C). The IC_{50} of 51.9 nmol/L obtained for propanolol using β-lactamase EFC assay was also consistent with reported data[26].

Screening of β2AR agonists from natural compound library

To see if this cell-based assay was suitable for high-throughput screening, the *Z′* factor value was calculated as described previously by Zhang *et al*[24]. Half of the plate was treated with 1 μmol/L isoproterenol, and half of the plate was untreated. Four wells (A1, B1, G12, and H12) were designed for reagent control to calculate the blue/green ratio for the demonstration of β-lactamase activity. The *Z′* factor was 0.68 in our experiment (Figure 3A). To further assess the value of this cell model for searching agonists targeting β2-adrenoceptor, we screened 352 HPLC-fractionated samples from the ethanol extracts of Chinese medicinal herbs in a 96-well format. Isoproterenol was used as the positive control, and 1% DMSO was used as the negative control. Cells were incubated with

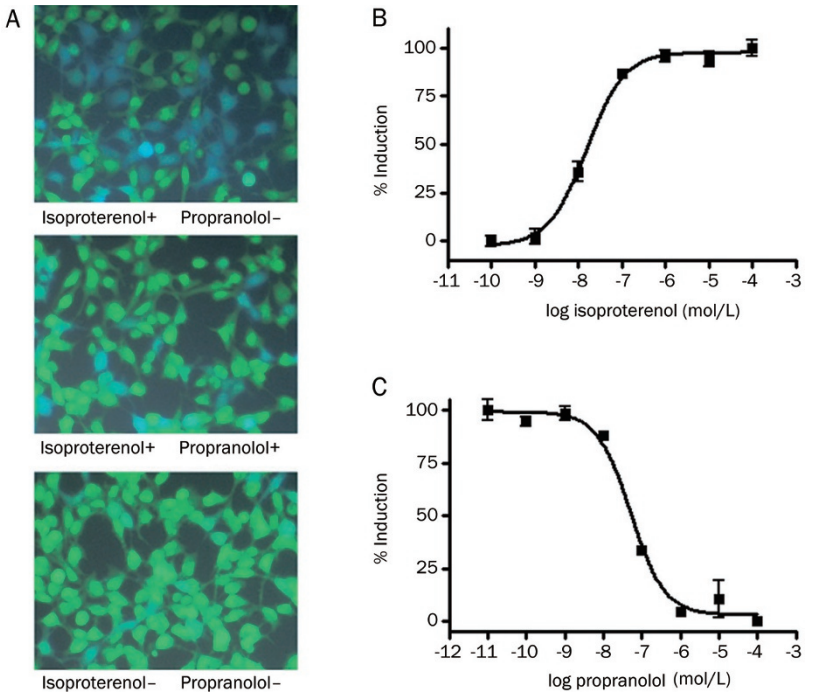

Figure 2. Characteristics of stable cell line HEK/293-β2a2. (A) Immunofluorescence assay of β-lactamase activity. Stable cell line HEK/293-β2a2 treated with 1 μmol/L isoproterenol (top); pre-incubated with 10 μmol/L propranolol and then treated with 1 μmol/L isoproterenol (middle); untreated (bottom) shown clear difference in blue cells percentage imaged by fluorescence microscope (magnification×100). (B) and (C) Dose response curves for isoproterenol and propranolol. Data represent means±SD for triplicate samples.

fractionated samples for 5 min in a serum-free medium. All wells were treated identically, and all contained 1% DMSO, a concentration which has been determined to have no adverse effect on the cells or the assay (data not shown). The fraction activity was expressed as the percentage of maximal β-lactamase induction by 1 μmol/L isoproterenol. As shown in Figure 3B, three fractions each exhibited a significant effect on the induction of β-lactamase activity (>40%). The effect of the other fractions on the induction of β-lactamase complementation is also shown in Figure 3. These fractions, which had activities below three times of standard deviation, were considered insignificant.

Hits validation

To confirm the activity of the primary screen hits, we repeated the receptor/β-arrestin interaction assay in triplicate (Figure 4A). Fractions 10-B11 and 10-C11 were identified to have >50% of maximum induction while fraction 10-D11 showed >40% of maximum induction. Three positive hits were further validated with the melanocortin receptor 4 (MC4R) cell line, which was also generated based on the β-lactamase complementation assay. As expected, they all had no agonist effect on the MC4R cell line (Figure S1). β2-adrenoceptor is a classic G-protein-coupled receptor used for investigation. Once the ligand binds to a β2-adrenoceptor, the receptor will change the conformation and couple to a heterotrimeric G-protein, resulting in an elevation of cAMP levels. Thus, hits obtained from the primary screening were also validated with another functional assay to measure the induction of cAMP expression. As shown in Figure 4B, three fractions had nearly the same intensity of cAMP induction as the positive control, isoproterenol. These two experiments confirm the effect of the three fractions on β2-adrenoceptor activation. Three positive fractions

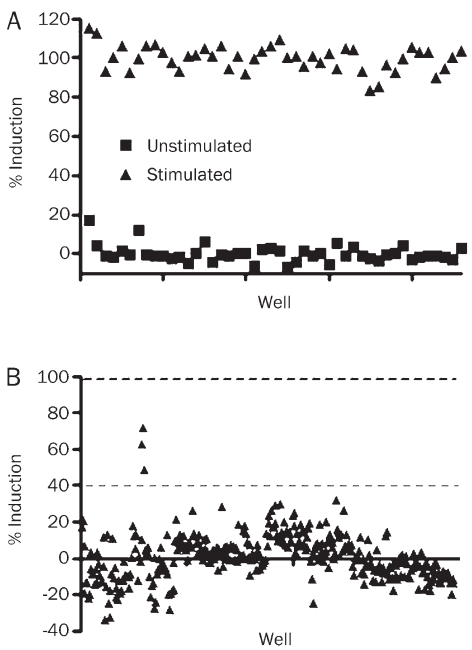

Figure 3. Z′ factor determination and high-throughput screening. (A) Z′ factor determination. This experiment was representative of three experiments. (B) High-throughput screening from natural compound library. Each triangle represents the reading from one well. The induction of β-lactamase activity was expressed as a percentage of isoproterenol induction (performed in the same plate).

were consecutively obtained from *Fructus Aurantii Immaturus*. HPLC chromatograms of fractions 10-B11, 10-C11, and 10-D11 are shown in Figure 5. In addition, the methyl thiazolyl tetrazolium (MTT) test showed that fraction treatment at test-

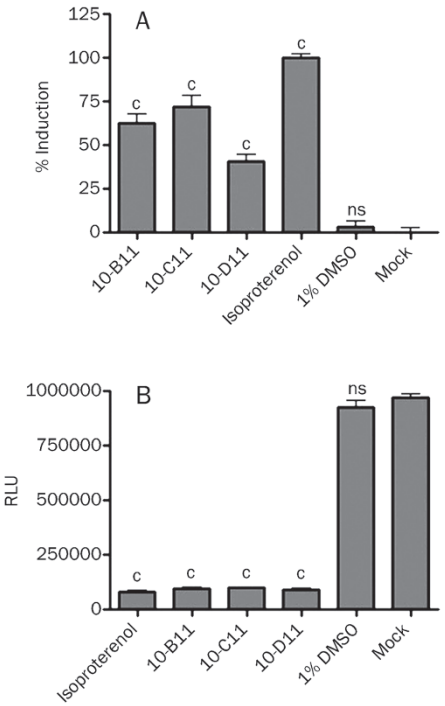

Figure 4. Positive hits validation. (A) Repeat of the receptor/β-arrestin interaction assay. Data represent means±SD for triplicate samples. (B) Positive hits validation by cAMP quantitation. Three hits have similar potential compared with positive control isoproterenol in elevating cAMP expression. Data represent means±SD for triplicate samples. $^{c}P<0.01$ *vs* Mock.

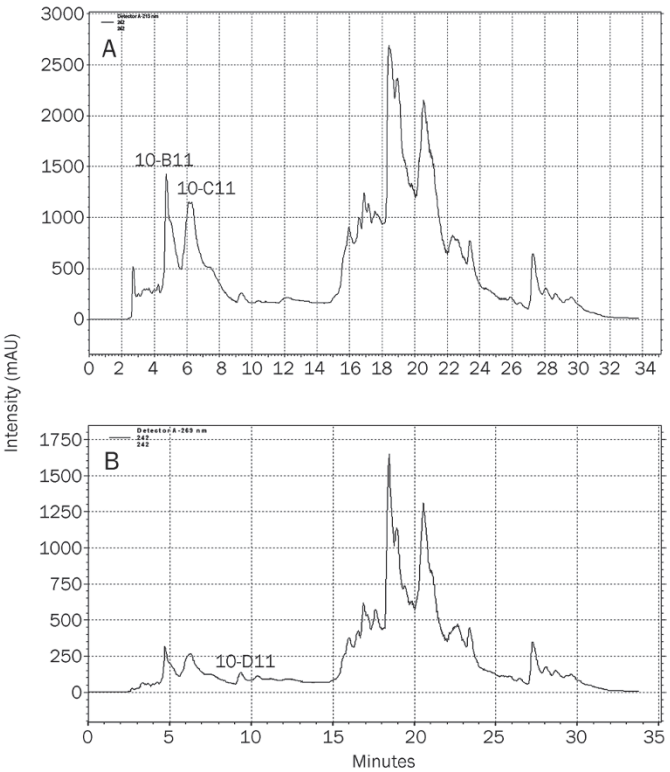

Figure 5. HPLC chromatograms of three positive fractions. (A) Fraction 10-B11 and 10-C11 (Detector A-215 nm). (B) Fraction 10-D11 (Detector A-269 nm). The arrows indicated the positions of active fractions.

ing condition did not decrease cell viability (data not shown). All these results suggest that a cell-based functional assay system based on β-lactamase complementation technology has high selectivity for agonist identification. It is a useful tool for high-throughput screening and monitoring GPCR activation.

Discussion

We established a cell-based high-throughput screening assay system for monitoring G-protein-coupled receptor activation using β-lactamase complementation technology. Detection of β-lactamase activity recovery with CCF4/AM could be observed and imaged in intact cells by a fluorescence microscope. This system is also adapted for sorting or analyzing by flow cytometry. The two fragments of β-lactamase are only about 10 kDa and 19 kDa. They are relatively smaller than many other tag proteins, and their size decreases the possibility of altering the function of chimeric fusion proteins. The small size, monomeric characteristic, and availability of a cell-permeable fluorescent substrate suggest that a system based on the β-lactamase enzyme complementation has wide potential in biological studies and drug discovery.

GPCR activation leads to signal transduction through two mediators: G-proteins and β-arrestins. Agonists bind to GPCR and activate heterotrimeric G-proteins. This activation promotes second-messenger signaling and subsequently regulates different physiological functions. Another pathway for GPCR signal transduction is mediated by β-arrestins. The activated receptors can be phosphorylated by G-protein-coupled receptor kinases and recruit β-arrestins, which terminate signal transduction through G-proteins. Receptors move into clathrin-coated pits with β-arrestins from cell membranes, which cause receptor internalization and desensitization. Meanwhile, β-arrestins also serve as a scaffold for numerous signaling molecules that are stimulated by receptor agonists, which initiate a second parallel set of signals[27]. Traditionally, it was thought that ligand binding stimulated or inhibited all receptor functions to an equal extent, but in recent years, researchers have found "biased ligands" in many GPCRs[28–30]. Perfect biased ligands stimulate one receptor activity without stimulating other known receptor activity. The development of β-arrestin-biased ligands might provide opportunities for novel therapies[31]. Our cell-based model provided a tool for the measurement of agonist-promoted interactions between GPCRs and β-arrestins. Along with assays that measure second-messenger production (for example, Ca^{2+} or cAMP), our model remains promising in the search for a biased ligand specific to the GPCR signal pathway that can activate only G-protein- or β-arrestins-mediated signal transduction.

Traditional Chinese medicinal herbs are effective in the treatment of many diseases. In this study, we screened a traditional Chinese medicinal herb library for hits that could activate β2-adrenoceptor, recruit β-arrestin and cause the

complementation of a β-lactamase fragment. Three hits were found from 352 fraction samples. Validated with the measurement of cAMP production, the three herbal extracts are each possible agonists of β2-adrenoceptor.

In summary, our achievement demonstrated that a cell-based assay using β-lactamase complementation technology was suitable for ligand screening. This assay was also validated on two other GPCRs: melanocortin receptor 4 and dopamine receptor 5 (data not shown). Our approach may provide an alternative strategy for scientists interested in this area.

Acknowledgements

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Author contribution

Chuan-ke ZHAO designed and performed the research, analyzed the data and wrote the paper; Qi YIN discussed and revised the paper; and Shi-you LI revised the paper.

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Erratum

A major error was found in the title of our recent publication in Acta Pharmacol Sin 2010 Sep; 31 (9): 1026–30. Epub 2010 Aug 2.

The false title is “Non-peptidic **glucose-like peptide-1** receptor agonists: aftermath of a serendipitous discovery”.

The correct title is “Non-peptidic **glucagon-like peptide-1** receptor agonists: aftermath of a serendipitous discovery”.

The authors felt very sorry for this oversight and an unforgivable mistake.

The editorial office and the authors appreciated Prof George HOLZ very much for his great help.