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A system for screening agonists targeting β_2-adrenoceptor from Chinese medicinal herbs*

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Abstract: In order to develop a model for screening the agonists of human β_2-adrenoceptor from Chinese medicinal herbs extracts, we used a cell-based functional assay based on a common G protein-coupled receptor (GPCR) regulation mechanism and destabilized enhanced green fluorescent protein (d_2EGFP) reporter gene technique. The positive cell clone was confirmed by real-time polymerase chain reaction (PCR) and imaging analysis. To assess the value of this model, we screened over 2000 high performance liquid chromatography (HPLC)-fractionated samples from the ethanol extracts of Chinese medicinal herbs. Six fractions (isolated from *Panax japonicus*, *Veratrum nigrum*, *Phellodendron amurense*, *Fructus Aurantii Immaturus*, *Chaenomeles speciosa*, and *Dictamnus dasycarpus*) showed significant effects on active reporter gene expression, three of which (isolated from *Phellodendron amurense*, *Fructus Aurantii Immaturus*, and *Chaenomeles speciosa*) were selected for further concentration response analysis and the half maximal effective concentration ($EC_{1/2\ max}$) values were 4.2, 2.7, and 4.8 μg/ml, respectively. Therefore, this reporter gene assay was suitable for screening β_2-adrenoceptor agonists. The results suggest that the six herbal extracts are the possible agonists of β_2-adrenoceptor.

Key words: β_2-adrenoceptor, Enhanced green fluorescent protein (EGFP), Chinese medicinal herbs, Screening
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INTRODUCTION

β_2-adrenoceptor is the first G protein-coupled receptor (GPCR) to be cloned (Kobilka *et al*., 1987), and belongs to the superfamily of GPCRs, which contain a conserved structure of seven transmembrane helices linked by three alternating intracellular and extracellular loops (Barki-Harrington *et al*., 2004). Once the ligand binds to the receptor, the receptor will change the conformation and couple to a heterotrimeric G protein that consists of an α subunit binding a guanosine triphosphate (GTP), a β subunit and a γ subunit. The G protein undergoes an activation-inactivation cycle to convey a signal from an activated receptor to an effector. The stimulatory G (Gs) protein- or inhibitory G (Gi) protein-coupled receptors activate adenylyl cyclase (AC) that in turn generates the second messenger cyclic adenosine monophosphate (cAMP) and activates cAMP-dependent protein kinase (PKA); the receptors that couple to Gq-protein modulate the activity of phospholipase C to generate diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) and subsequently activate protein kinase C (PKC) (Barki-Harrington *et al*., 2004). The β_2-adrenoceptor couples to Gs and activates AC, resulting in elevated cAMP levels and subsequent activation of PKA. In the respiratory system, there is great therapeutic interest in the β_2-adrenoceptor (Abraham *et al*., 2003). The β_2-adrenoceptor agonists are the most widely used agents in the treatment of asthma with their bronchodilator actions (Milic *et al*., 2006).

In recent years, a cell-based functional assay to directly monitor GPCR activation, based on a common

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GPCR regulation mechanism and reporter gene techniques, has been used in drug discovery (Goetz *et al*., 2000). In the cell model, when the agonist activates GPCR, it will couple to Gs-protein and lead to activation of adenylate cyclase cAMP formation, subsequently increasing the activity of PKA. PKA then phosphorylates intracellular cAMP response element binding (CREB) protein. This phosphorylation allows the CREB to recruit the transcriptional adapter CREB protein, and therefore mediates a reporter gene, such as green fluorescent protein (GFP) transcription (Clark *et al*., 1999). GFP is originally isolated from the bioluminescent jellyfish *Aequorea avictoria* (Shimomura *et al*., 1962), and has been used to investigate many properties and behaviors of cells. The chromophore of GFP is produced through an internal posttranslational autocatalytic cyclization that does not require any cofactors or substrates (Zimmer, 2002). GFP as a reporter that is expressed under the control of an interested promoter can be used to monitor the interested gene expression. Measuring the GFP fluorescence can directly indicate the gene expression level in living cells. Enhanced GFP (EGFP) is extremely stable. Its variant form destabilized EGFP (d_2EGFP) has a reduced half life of about 2 to 3 h. d_2EGFP has been used extensively in cell-based assays as a transcription reporter (Kain, 1999).

Chinese medicinal herbs have been used to treat many diseases (such as asthma and hypertension) and have shown effectiveness for thousands of years. In the search for new candidates for β_2-adrenoceptor agonists, the natural compounds from these herbs could provide a rich source. In this article, we describe in detail the process of assay development for β_2-adrenoceptor and its screening of the compounds.

MATERIALS AND METHODS

Cells and culture conditions

Human embryonic kidney 293 (HEK293) cells were obtained from the American Type Culture Collection, Manassas, VA (ATCC CRL-1573). Cells were cultured in 75 ml flasks (Orange, Belgium) containing Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (PAA, Austria) in 5% CO_2 and at 37 °C according to standard procedures.

Plasmid cloning and preparation

Plasmids pd_2EGFP and pCRE-Blax were from BD Clontech, Palo Alto, CA, USA. The plasmid pd_2EGFP was promoterless, whereas in the plasmid pCRE-Blax, the β-lactamase gene was controlled by a synthetic promoter consisting of four copies of CRE-binding sequences. The CRE-binding sequences were excised by *Bam*HI and *Eco*RI from the pCRE-Blax. The plasmid p4*CRE-d_2EGFP was constructed by inserting CRE-binding sequences into pd_2EGFP. The resulting product was confidential by sequencing. Full length cDNA clone encoding the human β_2-adrenoceptor was cloned by polymerase chain reaction (PCR) from human genome DNA. The primers used were 5′-CTAGCTAGCACCATGG GGCAACCCGGGAAC-3′ and 5′-CCCAAGCTT CAGCAGTGAGTCATTTGTAC-3′. The cloned β_2-adrenoceptor was inserted into the eukaryotic expression vector pcDNA3.1/Hygro(+), and the construct pcDNA3.1-h β_2-adrenoceptor was confirmed by sequencing.

Transfection and cell line generation

HEK293 cells were cotransfected with p4*CRE-d_2EGFP and pcDNA3.1-h β_2-adrenoceptor by lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfected cells were selected by hygromycin (150 μg/ml). After around 15 d, hygromycin-resistant cells were picked and diluted into a 96-well plate at a concentration of 1~2 cells per well and cultivated for 15 d. The resulting colonies were stimulated with 1 μmol/L of β_2-adrenoceptor agonist isoproterenol to monitor the d_2EGFP expression. The stably transfected cell clone with the best signal/noise (S/N) was selected for further experiments.

Confirmation of the selected cell clone

Cells were homogenized in TRIzol reagent (Life Technologies, Inc., Roskilde, Denmark), and total RNA was extracted following the manufacturer's protocol. RNA was quantified by measuring absorbency at 260 and 280 nm and the ratio was 1.8 or higher. The integrity of the RNA was checked by visual inspection of the two ribosomal RNAs on an ethidium bromide-stained agarose gel. The first strand cDNA was synthesized from the isolated RNA using reverse transcriptase with an oligo-dT primer.

The relative quantities of targets (β_2-adrenoceptor and d_2EGFP) to reference (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) were performed with an SYBR-green fluorescence (Invitrogen) using ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The increase in fluorescence was measured in real-time PCR during the extension step. The threshold cycle (*Ct*) was calculated, and the relative gene expression was calculated as described (Livak and Schmittgen, 2001). All samples were amplified in triplicate. All reaction sets included water blanks as negative controls. Primers used were listed in Table 1.

Preparation of Chinese medicinal herb extracts

One hundred and twenty herbal plants with therapeutic indications for asthma treatment based on traditional Chinese medicine (TCM) were collected from a herb market in Anguo, Northern China. A modified method by Zhang *et al*.(2007) was used. Briefly, the dry Chinese herbs were dissolved in 90% (v/v) ethanol for 12 h and then sonicated for 0.5 h. Then the remains were soaked with 50% (v/v) ethanol for 12 h and extracted for 0.5 h. Then the ethanol extract was filtered and lyophilized. The product after lyophilization was re-dissolved in 90% (v/v) 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 and dissolved in dimethylsulfoxide (DMSO) and were kept in –80 °C freezer.

Screening and quantification

Stably transfected cells were seeded into 96-well tissue culture plates (Corning, USA) at 2×10^4 cells per well, and the final volume of each well was 90 μl. The cells were cultured for 24 h before treatment. The testing compounds, 10-fold diluted in DMEM, along with the controls, were added in the medium (10 μl). The cells were cultured for an additional 16 h and then screened for d_2EGFP expression using Olympus IX70 fluorescence microscope (Olympus, Japan) equipped with a filter set (U-MWIB2, excitation BP 460~490 nm, emission LP 510 nm) according to the manufacturer's instruction. The fluorescent intensity of d_2EGFP was quantified by measuring the area and intensity of whole image, and the results were expressed as integrated optical density (IOD): *IOD*=area×intensity of fluorescence. The herbs that possessed activity (hit samples) were extracted on a larger scale and were HPLC-fractionated as described earlier (Zhang *et al*., 2007). The isolated compounds were subjected to activity verification using the HEK293-PTHR (parathyroid hormone receptor)-d_2EGFP cell line as well as parental control cells (HEK293-d_2EGFP), and the dose response curve was determined using a receptor assay cell line.

Data analysis

Data were presented as mean±*SD*. Statistical significance was evaluated with Student's *t*-test and the difference was considered statistically significant at $P<0.05$.

RESULTS

Cell line generation

Vector p4*CRE-d_2EGFP and human β_2-adrenoceptor gene were stably cotransfected into HEK293 cells. As shown in Figs.1a and 1b, from the stably transfected clones, selected were those that showed d_2EGFP expression after treatment with the β_2-adrenoceptor agonist isoproterenol. Low or high serum (0.1% (v/v) bovine serum albumin (BSA) or 10% (v/v) FBS) concentration had no influence on

Table 1 Primers used in the real-time PCR

Gene	Primer	Product length (bp)
β_2-adrenoceptor	Forward: 5′-CCATTGCCAAGTTCGAGCGTCTG-3′	292
	Reverse: 5′-ATGATCACCCGGGCCTTATTCTTG-3′	
d_2EGFP	Forward: 5′-TCATGGCCGACAAGCAGAAGAACG-3′	228
	Reverse: 5′-CGGCGGCGGTCACGAACTC-3′	
GAPDH	Forward: 5′-GACCACAGTCCATGCCATCAC-3′	210
	Reverse: 5′-AGGTCCACCACTGACACGTTG-3′	

d_2EGFP: destabilized enhanced green fluorescent protein; GAPDH: glyceraldehyde-3-phosphate dehydrogenase

d_2EGFP expression in HEK293 cells induced by isoproterenol (data not shown). The responses to agonist stimulation were in a dose-dependent manner with the half maximal effective concentration ($EC_{1/2\ max}$) value of 87 nmol/L (Fig.1c). Pretreatment of β_2-adrenoceptor antagonist propranolol for 15 min blocked the effect.

Cell clone validation

We analyzed β_2-adrenoceptor and d_2EGFP mRNA expressions in the positive clone that showed ligand-dependent fluorescence. Analysis by real-time PCR showed that this clone expressed significantly more β_2-adrenoceptors than the parent HEK293 cell (Fig.2a). Moreover, compared with the clone without treatment, stimulation of different concentrations of isoproterenol had no effect on the receptor expression. The control compound (20 μg/ml cryptotanshinone) induced significant reduction in β_2-adrenoceptor mRNA levels; the mechanism was not clear. The same clone was analyzed by real-time PCR for d_2EGFP mRNA expression (Fig.2b). A significant increase in d_2EGFP mRNA expression was observed in clones treated with isoproterenol. As expected, d_2EGFP mRNA expression was not induced by control compound.

Screening of Chinese medicinal herbs extracts

To assess the value of this cell model for searching for the β_2-adrenoceptor agonists, over 2000 HPLC-fractionated samples from the ethanol extracts of Chinese medicinal herbs were used. The d_2EGFP expression in the cell was recorded. As shown in Fig.3, fractions of six samples, *Panax japonicus*, *Veratrum nigrum*, *Phellodendron amurense*, *Fructus Aurantii Immaturus*, *Chaenomeles speciosa*, and *Dictamnus dasycarpus*, exhibited significant effects on active d_2EGFP expression, and were retested for their activities using fresh dilution from the same DMSO compound stocks. The medium control (background) and isoproterenol (1 μmol/L, positive control) were used. DMSO (1%, v/v) and medium control did not induce d_2EGFP expression. The test using parental HEK293-CRE-d_2EGFP cell line was conducted to ensure that the activation of reporter was not due to the non-specific stimulation of signaling pathways by selected hit compounds (data not shown).

Identification of hit compounds

Three herbs, *Phellodendron amurense*, *Fructus Aurantii Immaturus*, and *Chaenomeles speciosa*, which generated fractionated samples and showed the better activities in the screening, were selected for further dose-response study. Fig.4 shows HPLC chromatograms of *Phellodendron amurense*, *Fructus Aurantii Immaturus*, and *Chaenomeles speciosa*. As shown in Fig.5, the three hit compounds stimulated the d_2EGFP expression in a dose-dependent manner, with $EC_{1/2\ max}$ values of 4.2, 2.7, and 4.8 μg/ml, respectively. In addition, methyl thiazolyl tetrazolium (MTT) test showed that compound treatment did not decrease cell viability (data not shown). We used HEK293 cells cotransfected with parathyroid hormone receptor (PTHR) and p4*CRE-d_2EGFP to assess the specificities of these hits. PTHR is a GPCR, also transmits its signals to the Gs protein, subsequently causing an increase of the second messenger cAMP, and thereby mediates reporter-gene transcription. The three hit compounds showed no effects on active d_2EGFP expression in the HEK293-PTHR-d_2EGFP cell line (data not shown). All the results indicate that the three hit compounds are β_2-adrenoceptor-specific agonists.

DISCUSSION

The human β_2-adrenoceptor is a member of the 7-transmembrane family of receptors widely distributed in the respiratory tract (Johnson, 2006). It plays an important role in regulation of vascular and bronchial smooth muscle tone. It also exists in human heart where it can mediate positive inotropic and chronotropic effects (Brodde and Leineweber, 2005). The β_2-adrenoceptor agonists, including terbutaline, formoterol, salmeterol, albuterol, levalbuterol, etc., are commonly used drugs to treat asthma. These agents dilate airway by stimulating β_2-adrenoceptors that couple to the Gs proteins. Once activated by receptor binding, the α subunit of Gs protein activates AC, resulting in cAMP formation. cAMP, in turn, activates PKA, which results in cell relaxation through effects on K^+ channels, Na^+/K^+ ATPases, Ca^{2+} sequestration, Ca^{2+} sensitivity of myosin, IP_3 formation, and CREB phosphorylation at serine 133 that permits CREB protein bound to CRE to initiate gene transcription (Guyot *et al*., 1998).

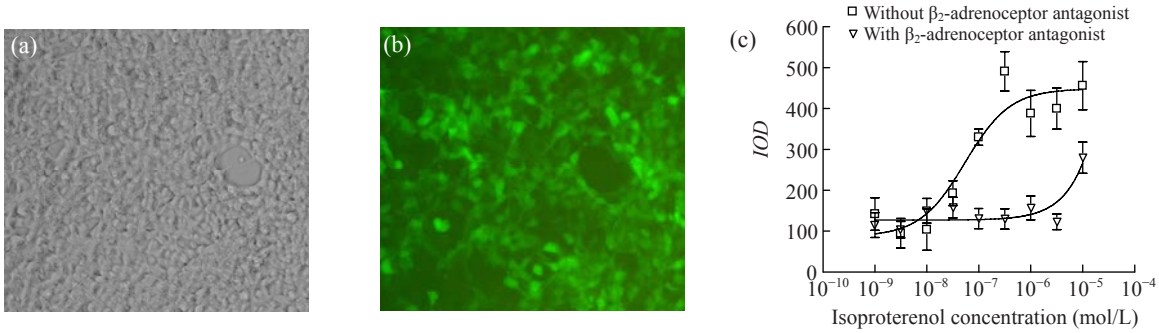

Fig.1 Effect of isoproterenol on HEK293-β_2-adrenoceptor-d_2EGFP clone. (a) Cell morphology and density under inverted microscopy (magnification 100×); (b) d_2EGFP expression after addition of isoproterenol (1 μmol/L) for 16 h (magnification 100×); (c) Dose-response curve for isoproterenol-stimulated d_2EGFP signal on positive cell clone in the presence or absence of 1 μmol/L of the β_2-adrenoceptor antagonist

Cell clone was incubated for 16 h after addition of isoproterenol. Results are shown as mean±*SD* (*n*=3)

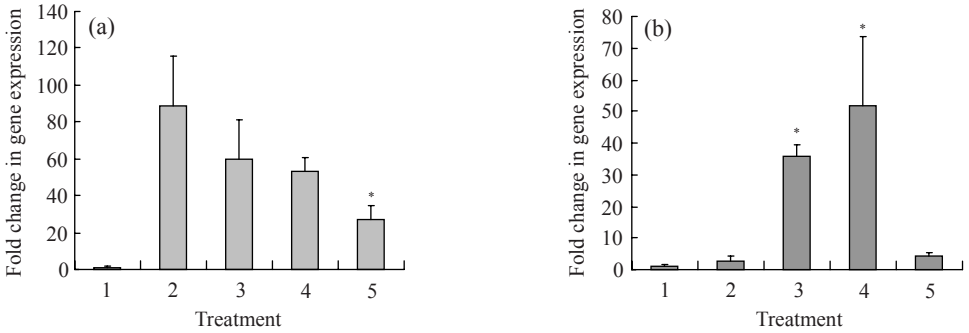

Fig.2 The mRNA expression on HEK293-β_2-adrenoceptor-d_2EGFP clone compared with HEK293 cell with or without compound treatment for 16 h. (a) β_2-adrenoceptor mRNA expression; (b) d_2EGFP mRNA expression

Treatment: 1: HEK293; 2: HEK293-β_2-adrenoceptor-d_2EGFP clone; 3: HEK293-β_2-adrenoceptor-d_2EGFP clone treated with isoproterenol 100 nmol/L; 4: HEK293-β_2-adrenoceptor-d_2EGFP clone treated with isoproterenol 1 μmol/L; 5: HEK293-β_2-adrenoceptor-d_2EGFP clone treated with compound control. Results are shown as mean±*SD* (*n*=3). $^*P<0.05$ vs HEK293-β_2-adrenoceptor-d_2EGFP clone

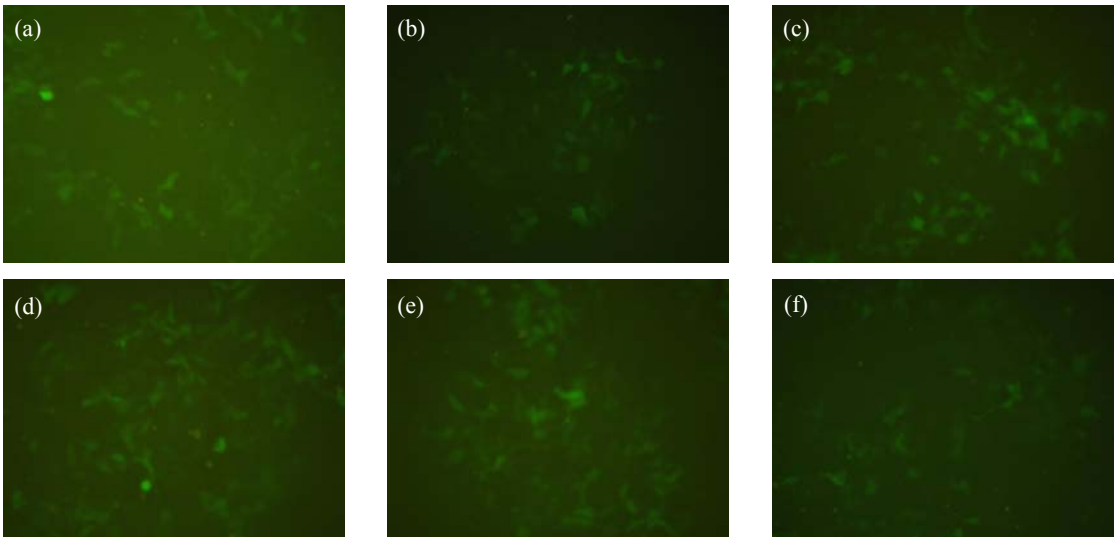

Fig.3 Effect of hit compounds on d_2EGFP expression. (a) *Panax japonicus*; (b) *Veratrum nigrum*; (c) *Phellodendron amurense*; (d) *Fructus Aurantii Immaturus*; (e) *Chaenomeles speciosa*; (f) *Dictamnus dasycarpus*

Cells stably expressing β_2-adrenoceptor and d_2EGFP were incubated with test compounds (20 μg/ml each) for 16 h and images were taken by fluorescence microscopy (magnification 100×)

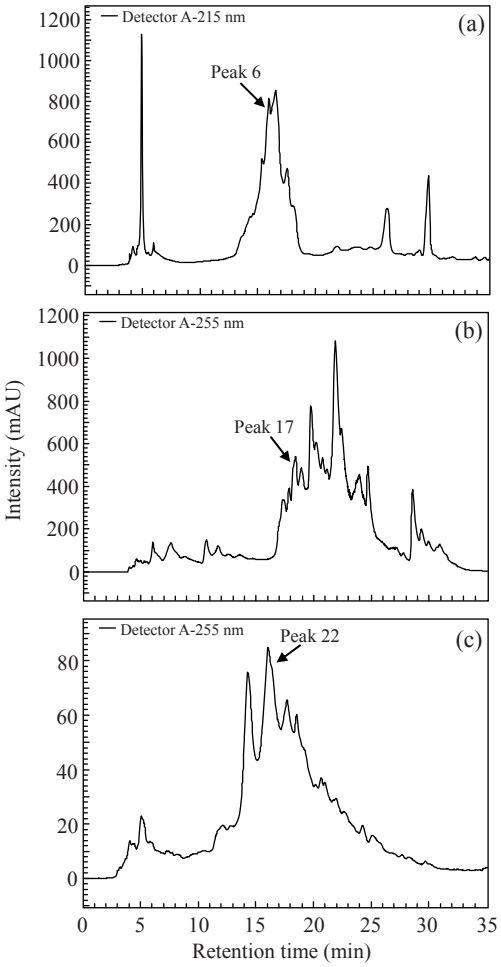

Fig.4 HPLC chromatograms. (a) *Phellodendron amurense*; (b) *Fructus Aurantii Immaturus*; (c) *Chaenomeles speciosa*

The arrows indicate the positions of active fractions on the chromatograms

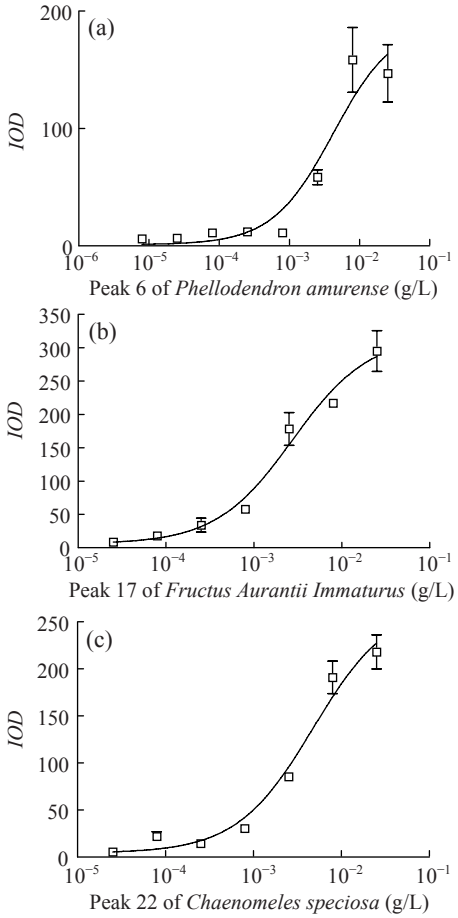

Fig.5 Dose-response curve of induced d_2EGFP expression. (a) Peak 6 of *Phellodendron amurense*; (b) Peak 17 of *Fructus Aurantii Immaturus*; (c) Peak 22 of *Chaenomeles speciosa*

Cell clone was incubated for 16 h after addition of compounds. Results are shown as mean±*SD* (n=3). The peak numbers are shown in Fig.4

In this study, the plasmid containing the reporter gene d_2EGFP under the control of CRE promoter was constructed. The resulting product p4*CRE-d_2EGFP was cotransfected with β_2-adrenoceptor to HEK293 cell to construct a cell model for screening β_2-adrenoceptor agonists. The d_2EGFP is a powerful tool for cell-based assays owing to the intrinsic fluorescence of this protein that allows real-time analysis of molecular events in living cells, while there are some limitations about GFP, including the slow formation of posttranslational chromophore, the requirement of oxygen, and the difficulty in distinguishing GFP from background fluorescence when the GFP expressed weakly. The low sensitivity of GFP is due to the fact that there is no signal amplification, because each GFP has only one chromophore, but the low sensitivity can be overcome by using a sensitive photon counting devices (Zimmer, 2002). In this assay, we used Olympus IX70 fluorescence microscope equipped with a filter set to detect the fluorescent intensity of d_2EGFP and quantified by Image-Pro Plus. The results show that the sensitivity and the specificity were sufficient for the primary screening.

In the HEK293-β_2-adrenoceptor-d_2EGFP cells, the binding of ligands to β_2-adrenoceptors resulted in the expression of d_2EGFP. The screening of large libraries in order to obtain hits for receptors of interest has been the mainstay of drug research for some time. It is increasingly being recognized that this is a relatively inefficient way to achieve this end, and the screening of libraries either designed or selected to hit particular targets is rapidly becoming the method of

choice (Crossley, 2004). Traditional Chinese medicinal herbs are effective for treatment of asthma and hypertension (Qiu and Kao, 2003; Shichinohe *et al*., 1996; Chang *et al*., 1979). We tried to develop natural products and natural compounds from various Chinese herbs to screen novel leads for new drugs. This study investigated the assay to screen hits in traditional Chinese medicinal herb library, which could activate reported gene expression by β_2-adrenoceptor/cAMP-mediated signaling. We constructed a cell model for the screening of the library and found six hits from over 2000 samples. The six hits all showed the ability to activate d_2EGFP expression in HEK293-β_2-adrenoceptor-d_2EGFP cells, while they did not activate d_2EGFP expression in HEK293-PTHR-d_2EGFP cells or parental HEK293-CRE-d_2EGFP cells (data not shown). Therefore, we speculate that they might activate β_2-adrenoceptor/cAMP-mediated signaling. In the previous studies, the extracts from *Phellodendron amurense* and *Chaenomeles speciosa* showed anti-inflammatory and other important activities (Cuéllar *et al*., 2001; Uchiyama *et al*., 1989; Chen and Wei, 2003). *Phellodendron* extracts have been investigated for their potential antitumor effect on human prostate cancer cells (Garcia *et al*., 2006). The ethanol extract of *Fructus Aurantii Immaturus* was found to activate α-adrenoceptors (Airriess *et al*., 1997). Our research shows other possible effect of those traditional Chinese medicinal herbs, such as stimulating β_2-adrenoceptor, therefore, to dilate airway.

In summary, the assay based on β_2-adrenoceptor-mediated cAMP accumulation and CRE-mediated d_2EGFP transcription was suitable for drug screening in a low-cost manner.

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