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Screening of active compounds as neuromedin U2 receptor agonist from natural products

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Abstract—Reporter cell lines are often used for high-throughput screening of natural product libraries or chemical libraries to identify new receptor ligands. Screening of neuromedin U2 receptor (NMU2R) ligands may be very useful to treat obesity for the reason that centrally administered neuromedin U affects feeding behavior, energy expenditure, and pituitary. Here, we have developed a stable cell line of neuromedin U2 receptor (NMU2R) to screen for its agonist. The experimental results demonstrate that icariin, isolated from *Herba epimedii*, was a strong agonist for NMU2R, which could selectively activate NMU2R, but not M1R, MC4R, and negative cell lines.

1. Introduction

Obesity is characterized by a chronic imbalance between energy expenditure and energy intake. While the corresponding mechanisms underlying obesity are far from being fully understood, it has become clear in the last few years that obesity is, in part, centrally regulated and that several neuropeptides play an important role in this regulation.[1] In particular, these neuropeptides that are essential in the pathophysiology of obesity are ligands of deorphanized G protein-coupled receptors (GPCRs), one of them being neuromedin-U.[2,3]

There is a growing body of evidence which suggests that neuromedin U may be involved in the hypothalamic modulation of feeding and energy balance.[4] NMU2R is expressed most abundantly in the central nervous system. Using in situ hybridization, rat NMU2R is detected in the PVN, the wall of the bird ventricle in the hypothalamus and in the CA1 region of the hippocampus.[5,6] Consistent with brain localization of NMU and its receptors, NMU also acts centrally to decrease fast-induced and overnight food intake and gastric acid secretion, with an increase in body temperature, oxygen consumption, and locomotor activity.[4] Drugs targeting, GPCRs are believed to have the potential to alleviate obesity processes; therefore, discovery and identification of NMU2R agonist are very useful from naturally occurring and synthetic chemical agents for nutritional obesity. Natural products provide a huge source to be explored for drug discovery and development.

To develop a highly potent agonist and to examine the activity in vitro, the model of high-throughput screening for NMU2R has been established in this study. Two hundred and eleven extracts of natural products were screened with this system. Combined with the programs of comparison with standard sample and identification with thin-layer chromatography and high-performance liquid chromatography, icariin was shown as a strong agonist for NMU2R. The experimental results have demonstrated that this cell system makes possible a new high-throughput screening approach for finding a novel agonist for NUM2R.

Keywords: Neuromedin U2 receptor; High-throughput screening; *Herba epimedii*; Icariin.
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2. Materials and methods

2.1. Materials

Human hypothalamic cDNA was purchased from Clon-Tech Laboratory Inc., pCR2.1-TOPO and pcDNA3.1 vector from Invitrogen, White 96-well assay plates from Corning Costar, Bright-Glo™ assay system reagent and pGL3 vector from Promega, Fugene 6 transfection reagent from Roche, and DMEM cell culture medium and fetal bovine serum (FBS) from Hyclone. G418 was from Gibco and the Standard icariin was purchased from National Institute for the Control of Pharmaceutical and Biological Products. All chemical reagents (AR) were obtained from Chongqing Chem. Ltd.

2.2. Plasmids

The reporter gene construct containing $3\times$MRE/$3\times$CRE/$3\times$SRE, followed by a luciferase gene, was made by inserting MRE, CRE, and SRE elements into a pGL3 vector, as described before.[7,8] Upon ligand binding, Gq-coupled receptor enhances calcium and protein kinase C signal, which, in turn, activates MRE and the reporter gene.[9] The human NMU2R was cloned by PCR using human hypothalamic cDNA as template. The primers used in the PCR cloning were: 5′-ATGTCA GGGATGGAAA AACTTC-3′ and 5′-TCAGGTTTTG TTAAAGTGGAAGC-3′. The PCR product was cloned into pCR2.1-TOPO vector and further subcloned into pcDNA3.1 mammalian expression vector. NMU2R clone was confirmed by DNA sequencing.

2.3. Cell line generation and luciferase assay

HEK293 cells were stably transfected with the human NMU2R, MC4R, or M1R clone and a plasmid containing MRE/CRE/SRE-LUC. Wild-type HEK293 cells were cultured at 37 °C in DMEM with 10% fetal bovine serum to be grown to 50–80% confluence. Transfection was carried out in 6-well dishes with Fugene 6 transfection reagent (Roche) using the protocol provided by the supplier. The ratio of human NMU2R receptor DNA and MRE/CRE/SRE-LUC plasmid is 1:5. Stable cell clones were picked up in the presence of 800 μg/ml G418. One day before the luciferase assay, $2–10\times10^4$ cells were placed into a 96-well plate with 100 μl regular medium. After the cells attached, 11 μl compounds at different concentrations or vehicle were added. With a continuous incubation of 6–8 h, Bright-Glo assay reagent (Promega) was added to the media at 110 μl /well, and luminescence was measured using the Reporter (Turner Design).

2.4. Samples of natural products

Two hundred and eleven samples of natural products were boiled in 75% ethanol under reflux for 2 h, cooled to room temperature and the solid residue was removed by filtration. The extraction was repeated on the retentate and the two filtrates were combined. If the sample showed the signal to activate the NMU2R selectively, based on the index of activity, it was separated with a successive solvent partition, and a further partition with Sephadex LH-20 column chromatography, and then isolated with thin-layer chromatography and high-performance liquid chromatography.

2.5. Statistical analysis

All data are presented as means ± SD. Data were analyzed by a one-way ANOVA, followed by Dunnett's test and an unpaired Student's t test. Differences among groups were considered statistically significant at $P < 0.05$.

3. Results

3.1. Luciferase assay of the human NUM2R cell line

In this study, our objective is to develop a stable cell line that could respond to both Gs and Gq-coupled NMU2R. Therefore, in the reporter gene plasmid for this screening system, three MRE, three CRE, and three SRE regulatory elements and a promoter of SV40 were inserted in front of the luciferase reporter gene to enhance the sensitivity of the reporter gene to the stimulus resulting from NMU2R agonist exposure. The basal and inducible expression levels of luciferase in NMU2R cell clones, which stably carried reporter genes, are shown in Figure 1.

Figure 1 shows that the activating level of neuromedin U alone was up to 2-fold level of control, and the activating level of the combination of neuromedin U with forskolin, a activator of CRE, was up to 5-fold level of forskolin alone. These data have demonstrated that although neuromedin U alone can activate the reporter gene, combination of the neuropeptide with forskolin would increase the response.

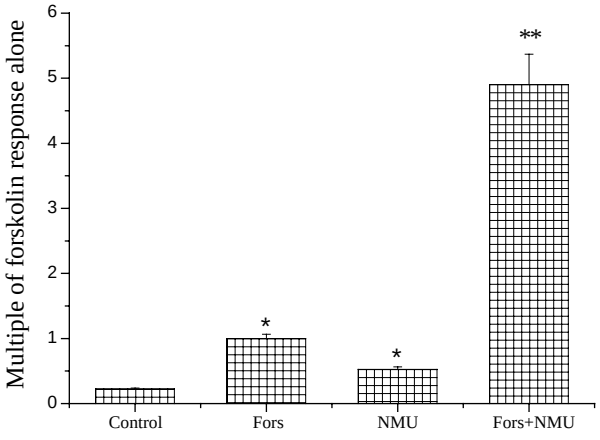

Figure 1. Basal and induced levels of luciferase expression in human NMU2R cells stably carrying the indicated reporter genes. After the cells were treated with 5 μM forskolin and/or 1 μM NMU, luciferase activity was determined using the Reporter (Turner Design) with Bright-Glo agents (Promega), treatment with equal volume vehicle (DMSO) taken as control. The results represent at least three independent experiments and are expressed as means ± SD. ($N = 6$, $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control.)

3.2. Luciferase assay of the negative cell lines

To demonstrate that this cell-based assay can be used to identify new NMU2R agonist selectively, and to study ligand specificity, the negative cell line and other receptor (such as melanocortin 4 receptor, MC4R, and muscarinic 1 receptor, M1R) cell lines have also been established. The negative cell line is stably transfected with pcDNA3.1 blank vector and 3 × MRE/3 × CRE/3 × SRE-LUC plasmid. The experimental results have shown that, although forskolin could activate the negative cell line, NMU could not activate them (Fig. 2).

3.3. Screening of the natural product compound library

As described in Materials and methods, we have established a natural product compound library using crude extracts from natural products. Using our optimized reporter gene assay, 211 extracts of natural products were screened. Based on the results of first screening, *Herba epimedii* was shown as a strong agonist for NMU2R, the activating level of *H. epimedii* being up to 12-fold of forskolin alone. To check the selectivity and specificity of *H. epimedii* on NMU2R, we took MC4R, M1R, and MRE/CRE/SRE negative cell line as control. As shown in Figure 3, the extract of *H. epimedii* did not induce responses in other receptor-transfected cells, such as M1R, MC4R and the negative control cell line (MRE/CRE/SRE). But, selective agonists of α-MSH (3 μM) and acetylcholine (1 mM) could evidently activate the MC4R and M1R separately. These results have suggested that *H. epimedii* could selectively activate the reporter gene through NMU2R.

3.4. Identification of icariin as NMU2R agonist

To look for active components, extracts of *H. epimedii* were isolated with the method of column chromatography, followed by separation of thin-layer chromatography and high-performance liquid chromatography. Combined with the program of comparison with standard sample to activate the reporter gene and identification with thin-layer chromatography and high-performance liquid chromatography, we found that icariin (about 0.3425% in *H. epimedii*) is an active component, which could activate the reporter gene through NMU2R selectively (Fig. 4). The structure of icariin is shown in Figure 5.

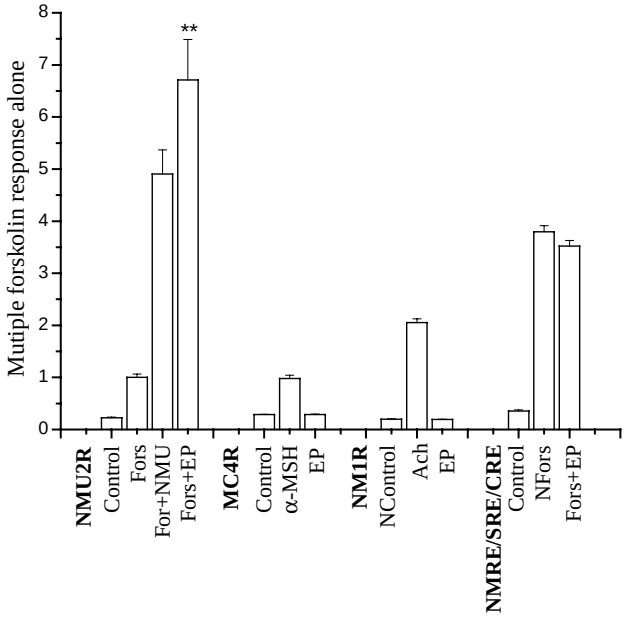

Figure 3. Response of MC4R, M1R, and negative cell line of MRE/CRE/SRE to *Herba epimedii*. The data are expressed as means ± SD from three independent experiments. ($N = 5$, $^{**}P < 0.01$ versus MRE/CRE/SRE negative cell line treated with 5 μM Forskolin.)

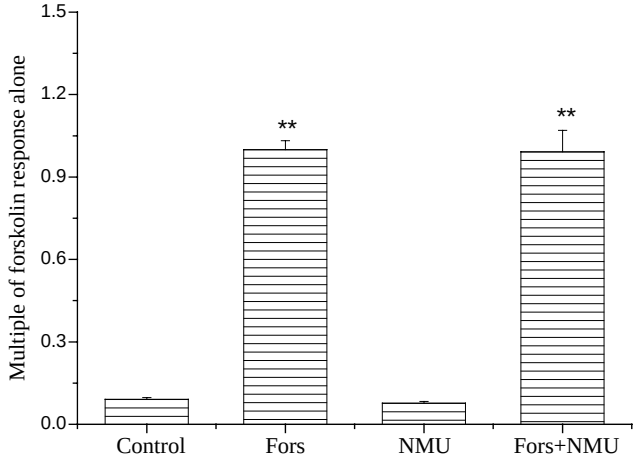

Figure 2. Effect of NMU (1 μM) and forskolin (5 μM) on the 3 × MRE/3 × CRE/3 × SRE-LUC plasmid transfected cells, a negative cell line. After the cells were treated with 5 μM forskolin and/or 1 μM NMU, and equal volume vehicle was taken as control, luciferase activity was determined, as described in Materials and methods. The results represent at least three independent experiments and are expressed as means ± SD. ($N = 5$, $^{**}P < 0.01$ versus control.)

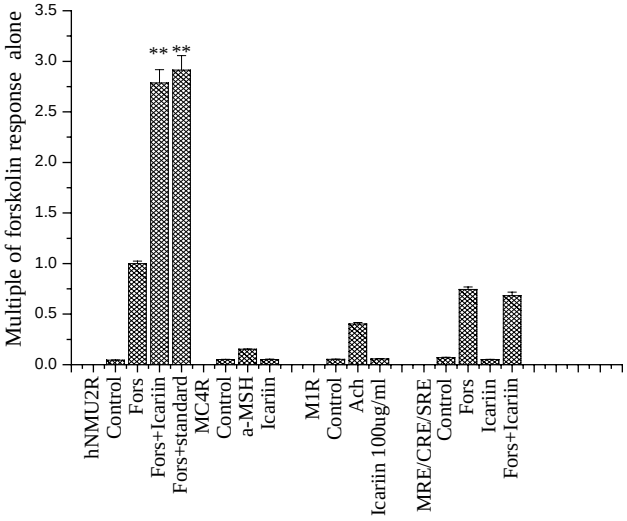

Figure 4. Response of NMU2R, MC4R, M1R, and MRE/CRE/SRE negative cell line to icariin. After the cells were treated with 100 μg/ml icariin for 6–8 h, the luciferase activity was determined as described in Materials and methods. The data are expressed as means ± SD of three independent experiments. ($N = 6$, $^{**}P < 0.01$ versus MRE/CRE/SRE negative cell line treated with 5 μM Forskolin.)

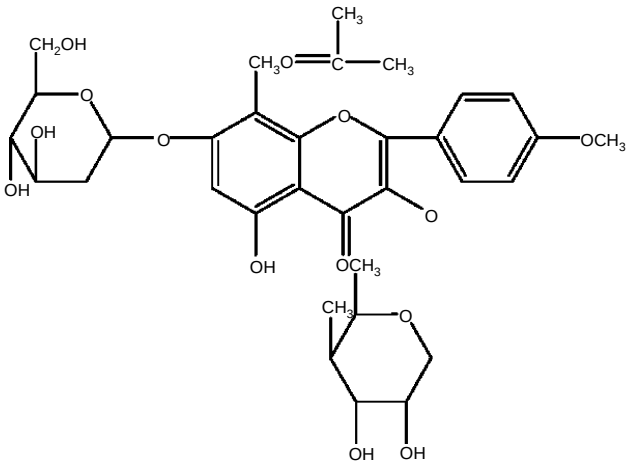

Figure 5. The structure of icariin.

As shown in Figure 4, the activating level of icariin (purity > 98%) separated from *H. epimedii* could stimulate the luciferase activity of NMU2R cells up to 3-fold of forskolin alone. Furthermore, α-MSH and acetylcholine, the selective agonists for MC4R and M1R, could activate the MC4R and M1R separately, but, icariin failed to induce responses in other receptor-transfected cells, including M1R, MC4R and the negative control cell line (MRE/CRE/SRE). These results suggest that icariin is a selective agonist for NMU2R.

3.5. Relationship between dose and active responses of icariin

We also measured the dose response of icariin on the NMU2R, the results showing that icariin is a strong agonist of NMU2R, which could activate this reporter system in a of dose dependent manner (Fig. 6). When the concentration of icariin reaches up to 10^{-4} M, the luciferase activity of icariin is stronger than that of 5 μM forklin alone.

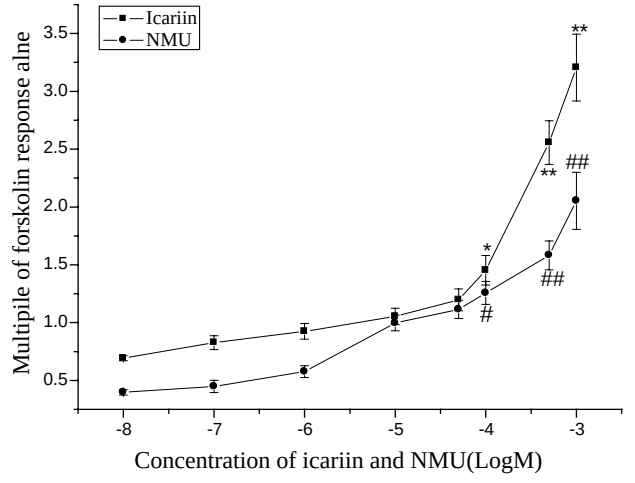

Figure 6. Dose dependency of luciferase activity of icariin and NMU in NMU2R cell line. NMU2R cell line was treated with icariin for 6–8 h. Luciferase activity was determined with the Bright-Glo agent (Promega) using the Reporter (Turner Designs). Equal volume vehicle was taken as control. The data are expressed as means ± SD of at least three independent experiments. ($N = 6$, $^{\#,*}P < 0.05$, $^{\#\#,**}P < 0.01$ versus control.)

4. Discussion

Multitudes of physiological processes are mediated by signal transduction pathways that involve the interaction of agonist with GPCRs that are increasingly targeted by the pharmaceutical industry. In this study, we report the development of a microplate assay using luciferase as the reporter that enables simple and rapid screening of natural product library to identify a new NMU2R agonist. This assay is based on HEK293 cells that are co-transfected with the human NMU2R gene and an integrated copy of a luciferase reporter gene driven by a SV40 promoter under the control of a 3 × MRE, 3 × CRE, and 3 × SRE enhancer elements. The assay measures the stimulation of GPCRs by detecting changes in the luciferase levels.

In these experiments, we have demonstrated the feasibility of using this assay to perform high-throughput screening of a small molecule library. Having initially screened 211 extracts of natural products using this assay, we found that the extract of *H. epimedii* could activate the reporter gene response evidently. Combined with the activity of standard sample to stimulate the expression of reporter gene and the program of identification with thin-layer chromatography and HPLC, it is shown that icariin is a strong agonist of NMU2R, which could selectively activate the NMU2R, but not MC4R, M1R, and negative cell line.

The increasing data show that administration of NMU suppresses food intake[9] and stimulates the HPA axis. These effects may be mediated by corticotrophin-releasing hormone (CRH). NMU stimulates the release of CRH from hypothalamic explants in vitro[10] and increases c-fos expression in the CRH-containing parvocellular regions of the PVN.[11] Furthermore, administration of NMU induces a behavioral response consisting of a marked increase in grooming and locomotion, and a decrease in sleeping which is comparable to that induced by CRH,[12] and raises plasma adrenocorticotrophic hormone and corticosterone levels. Since NMU has been demonstrated to suppress nutritional obesity in an experimental model,[4] icariin is very likely to have similar action as NMU. More importantly, as a selective agonist for NMU2R, a subtype NMU receptor, icariin may have more potential in therapeutic implication for clinical use in nutritional obesity.

High-throughput screening is an important tool for discovering new drugs that target various biomolecular sites.[13] We expect that the results presented here would serve to accelerate the application of living cells in screens for drug discovery. It has been shown that this new assay, as a cell-based functional one, could provide a platform for identifying NMU2R ligands. Such constructs hold great promise to generate ever new NMU2R ligands that will find increasing clinical utility.

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