Identification of Small Molecule Activators of the Janus Kinase/Signal Transducer and Activator of Transcription Pathway Using a Cell-Based Screen

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Type I interferons (IFN-α/β) have been widely used in the treatment of many viral and malignant diseases by activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway, but the side effects of protein-based IFN therapy severely limit their clinical use. Discovering small molecules to activate the JAK/STAT pathway will greatly facilitate the development of new drugs which have similar pharmacological function to IFNs but with fewer side effects. To screen a natural products-based library, we established a cell-based screening assay using human hepatoma HepG2 cells stably transfected with a plasmid where the luciferase reporter activity is driven by interferon α-stimulated response element (ISRE), the motif specifically recognized by type I IFN-induced activation of JAK/STAT pathway. Among 1,431 natural product compounds screened, four compounds (emodin, quercetin, apigenin and luteolin) were identified as activators of the JAK/STAT pathway. Further studies demonstrated that these four compounds could increase the endogenous antiviral gene expression regulated by the IFN-activated JAK/STAT pathway. The identified small molecule activators are valuable for structural modification and warrant further investigation for use in new antiviral drugs as IFN mimics or adjuvants.

Key words interferon; Janus kinase:signal transducer and activator of transcription; emodin; quercetin; luteolin

Type I interferons (IFN-α/β) play central roles in the innate immune response against viral infection. In the case of virus invasion, type I IFNs are secreted by the infected cells and then elicit rapid activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway signaling in surrounding cells, which leads to the expression of a variety of antiviral genes to counteract the viral infection.1 In response to type I IFNs stimulation, IFN receptor (IFNAR) initiates rapid signal transduction through JAK kinases (Jak1 and Tyk2) and STATs (STAT1 and STAT2), leading to the formation of a transcription complex composed of phosphorylated STAT1, STAT2 and IRF9, which translocates into the cell nucleus and recognizes a specific interferon-α-stimulated response element (ISRE) motif in the promoter region of certain genes for transcription to perform the relative physiological functions.2

Besides IFNs, the JAK/STAT pathway is also utilized by many other cytokines such as interleukins, erythropoietin, and granulocyte colony-stimulating factor (G-CSF).3 Dysfunction of the pathway will lead to the occurrence of many pathological diseases.4 Many studies have been carried out to find small molecule inhibitors of constitutively active STAT 3 or STAT5 and JAK3, which are correlated with tumorigenesis and human autoimmune disorders, respectively.5,6 However, small molecule activators of the JAK/STAT pathway are seldom reported, which limits the discovery of new antiviral drugs considering the increase in pandemic outbreaks of viral diseases such as SARS and bird influenza in recent years. At present most of the marketed antiviral drugs are used to directly inhibit the replication of virus by targeting the viral proteins, but the drugs will gradually lose their efficacy due to the rapid mutation of viruses.7 Thus, as a major defense system in the human body to counteract diverse viruses from nature, the JAK/STAT pathway would be an appropriate target to find new antiviral lead compounds.

Currently IFNs have been widely used in the treatment of diverse viral diseases such as hepatitis B or hepatitis C, several cancers and multiple sclerosis, but the side effects of protein-based IFN therapy such as neuropsychiatric effects, hematologic toxicities and drug intolerance often severely limit their clinical uses and efficacy.8 Thus, small molecules which mimic IFN functions or activate the JAK/STAT pathway will provide a new way to treat viral diseases and obviate the side effects of IFN therapy, which has been supported by the findings of small molecule agonists of G-CSF and small molecule mimics of insulin.9,10 Recently, a peptide mimetic of human IFN-β was reported to activate heterodimeric cytokine receptors,11 and a small molecule enhancer of STAT1 transcriptional activity was found to accentuate the antiproliferative effects of IFN-γ in cancer cells.12 These findings suggest the potential to discover small molecules that will activate the JAK/STAT pathway similarly to IFNs. Such small molecule activators would be of great value for the development of new antiviral and anticancer drugs.

Natural products have long been a major resource for marketed drugs, and have shown success in dissecting biological pathways and protein functions as chemical small molecule probes.13 In recent years, natural products are gaining attention again in drug development due to their diversified scaffolds and preferred affinities for cellular proteins compared with randomly synthesized compounds. Thus, to identify small molecule activators of the JAK/STAT pathway, we set up a library consisting of 1431 compounds predominantly containing natural products and synthesized analogues. The library was screened in the well-behaved human hepatocellular carcinoma HepG2 cells, which stably express firefly luciferase reporter under the control of an ISRE consensus promoter. The screening led to the identification of four compounds (emodin, quercetin, apigenin and luteolin) which could potentially activate the JAK/STAT pathway and increase endogenous...
antiviral gene expression.

MATERIALS AND METHODS

Reagent and Compound Preparation IFN-α (recombinant human IFN-α 2a) or IFN-β (recombinant human IFN-β 1b) was purchased from ProSpec-Tany Techno Gene, Ltd. (Shanghai, China). The lyophilized protein was reconstituted in sterile water with a carrier protein (0.1% bovine serum albumin (BSA)) at the concentration of 100 µg/mL and stored at −80°C. The compound library contains 1431 diverse natural products or synthetic analogues. A stock solution of each compound was prepared in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/mL and stored at −20°C. A working solution of each compound was prepared in RPMI 1640 medium at a concentration of 10 µg/mL in the plate.

Plasmid and Cell Line pGL4.26-ISRE-Luc2 cis-reporter plasmid was constructed by inserting five direct repeats of the ISRE oligos (TAGTTTCACCTTTTCCTTTT) upstream from the basic promoter element (TATA box) through KpnI and Nhel endonuclease restriction sites to the pGL4.26 vector (Promega, Madison, WI, U.S.A.). The human hepatocellular carcinoma HepG2 cell line was obtained from ATCC, and was maintained in RPMI-1640 (Invitrogen, Carlsbad, CA, U.S.A.) medium supplemented with 10% (v/v) calf serum and antibiotics (100 µg/mL penicillin and 0.1 g/L streptomycin) at 37°C in the presence of 5% CO2. The HepG2-ISRE-Luc2 stable cell line was generated by transfecting HepG2 cells with pGL4.26-ISRE-Luc2 plasmid using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, U.S.A.) in RPMI-1640 without antibiotics. After 48 h of transfection, cells were trypsinized and replated in RPMI 1640 with 10% calf serum and 300 µg/mL Hygromycin B (Solarbio, Beijing, China). Hygromycin B-resistant clones were selected and expanded in several rounds for one month, and cells showing sensitive induction of luciferase activity by IFNs were chosen and frozen for further use.

Cell Viability Assay Cell viability was studied using the Alamar-Blue (SunBio™, Shanghai, China) Assay. HepG2-ISRE-Luc2 cells were plated 0.1 × 10⁵ cells/well in 96-well plates with 100 µL RPMI 1640 culture medium, and then treated with different concentrations of tested compounds. After 24 h, 10 µL Alamar-Blue reagent was added to the medium and incubated for another 2 to 4 h until the blue color changed to pink. The relative fluorescence intensity in each well was measured using Thermo Scientific Varioskan™ Flash (Thermo Scientific, U.S.A.).

Screen Assay The screening was performed in the 96-well format. For each plate, 16 wells were used for the negative and positive controls, and the remaining 80 wells were used as test compounds which were diluted to 10 µg/mL before using. HepG2-ISRE-Luc2 cells were plated at 0.5 × 10⁵ cells/well in 96-well plates and incubated for 24 h. Then the cells were stimulated with either the test compounds or 200 µM IFN-α as a positive control for another 24 h. The cells were lysed in Reporter Lysis Buffer and luciferase activity was measured by the Luciferase Reporter Assay System according to the manufacturer’s instructions (Promega). The luminescence intensity was measured by Thermo Scientific Varioskan™ Flash (Thermo Scientific, U.S.A.).

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total cellular RNA was isolated from the HepG2 cells treated with selected compounds using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. RNA (2 µg) was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen) with oligo(dT)₅ primers. Equal amounts (1 µL) of cDNA were subjected to real-time quantitative PCR with the fluorescent dye SYBR Green I using a Chromo4 detection system (Bio-Rad, CA, U.S.A.). Real-time PCR reaction mixtures contained 10 µL of 2 × SsoFast EvaGreen Supermix (Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.), 0.4 µL of each primer (0.2 µM), and 1 µL template cDNA. Sterile distilled water was added to a final volume of 20 µL. The following conditions were used for real-time RT-PCR: Enzyme activation at 95°C for 30 s, 40 cycles of denaturation at 95°C for 5 s, annealing and extension at 60°C for 20 s. The primer pairs used in the assay for interferon-induced double-stranded RNA-activated protein kinase (PKR), 2′-5′-oligoadenylate synthetase (2′-5′-OAS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are as follows: 5′-GTGTGCTTCTCTGGGGCTT3′ (PKR sense), 5′-GCTATTTCCTTCTCCGATC3′ (PKR antisense), 5′-AGGTTGTAAGGGGTGCTCC3′ (2′-5′-OAS sense), 5′-ACACACCGTCGCCTACAG3′ (2′-5′-OAS antisense) and 5′-TGCAACCACACCTGGTAC3′ (GAPDH sense), 5′-GGCATGACTGTCGATGAG3′ (GAPDH antisense). Standard curves were established for each primer set, and both reference and target gene reactions were performed for each sample. The relative quantity (n-Fold) of PKR and 2′-5′-OAS mRNA were calculated by the Δ(ΔCt) method using GAPDH as a reference amplified from the same sample.

Plot and Data Analysis Concentration–response data were fitted to a 4-parameter Hill equation using Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, U.S.A.), and EC₅₀ values were calculated based on three independent experiments performed in triplicate. The Z’ factor value was calculated from the following equation developed by Zhang et al.⁴¹:

$$Z' = 1 - \frac{3 \times (SD_{signal} + SD_{background})}{(M_{signal} - M_{background})}$$

Signal-to-noise (S/N) ratios were calculated as

$$S / N = \frac{M_{signal} - M_{background}}{\sqrt{(SD_{signal})^2 + (SD_{background})^2}}$$

where SDsignal is the standard deviation of the positive control signal (200 µM IFN-α), SDbackground is the standard deviation of the negative control (0.1% DMSO), Msignal is the mean of the positive control, and Mbackground is the mean of the negative control (0.1% DMSO). All experiments except the primary screening were repeated at least three times. Quantitative data were expressed as the mean ± S.D. Significant differences were evaluated by one-way analysis of variance (ANOVA) followed by Scheffé’s F test at p<0.05.

RESULTS

Establishment of a Cell-Based Assay for Screening Small Molecule Activators of the JAK/STAT Pathway Co-evolved with proteins for millions of years, natural
products are considered to contain more diverse chemical spaces and preferred affinities in binding protein targets compared with randomly synthesized chemical compounds. Thus, we first established a natural products-based library consisting of 1431 pure compounds isolated from natural products and their synthetic analogues. As illustrated in Fig. 1A, the compounds were clustered and organized based on their chemical structures. The library represents a high level of chemical diversity, covering terpenoids (20%), flavonoids (10%), steroids (7%), volatile oils (4%), phenylpropanoids (3%), lignans (3%), coumarins (3%), alkaloids (3%) and others (2%).

To monitor the activation status of JAK/STAT pathway signaling, pGL4.26-ISRE-Luc2 cis-reporter plasmid was constructed. As illustrated in Fig. 1B (upper panel), five repeated copies of ISRE consensus enhancer, which is widely used to evaluate the transcription effect of activated JAK/STAT pathway by type I IFNs, were inserted in front of the basal TATA box for the recognition and transcription of luciferase reporter by activated STAT1 complex. pGL4.26-Luc2 plasmid lacking an ISRE motif was used as a negative control (Fig. 1B, lower panel) to exclude the effect of non-specific luciferase reporter activity increase induced by IFNs or test compounds.

The stably transfected HepG2-ISRE-Luc2 cell line was generated as described in Materials and Methods. Treatment with 200U/mL of IFN-α or IFN-β caused the luciferase activity in the HepG2-ISRE-Luc2 cells to increase 6- to 10-fold, compared with the same cells receiving no IFN treatment (Fig. 1C). IFN-α or IFN-β did not stimulate luciferase expression from HepG2 cells transfected with the pGL4.26-Luc2 plasmid (Fig. 1C), indicating IFNs had no non-specific activation effect on basal luciferase expression. IFN-α also concentration-dependently activated ISRE reporter gene expression in HepG2-ISRE-Luc2 cells (Fig. 1D), indicating the transcription of the luciferase reporter was specifically controlled by the activated JAK/STAT pathway. These results demonstrated that the stably transfected HepG2-ISRE-Luc2 cell line was highly responsive to the activation of a JAK/STAT pathway, and therefore suitable for further screening.

Cell-Based Assay Optimization To determine optimal screening conditions, we examined three key assay parameters influencing the S/N ratio of the cellular reaction, including the system solvent, incubation time and variation of cell number. IFN-β was used to optimize HepG2-ISRE-Luc2 cells. The optimization was focused on getting the appropriate S/N ratio and ensuring the time effectiveness of the assay. As shown in Fig. 2A, 0.1% DMSO had no apparent cytotoxic effect on HepG2-ISRE-Luc2 cells after 24h incubation, indicating no more than 0.1% DMSO should be used during the screen-
formed in separate experiments in 96-well plates. (A) Cells were plated at 0.5 × 10⁵ RPMI-1640 for 24 h, followed by substitution with or without 100 different results compared to the control.

Medium containing 2000 U/mL IFN-β for 24 h. Cell proliferation was examined by Alamar-blue assay and the values were compared with 0.01—0.1 × 10⁵ cells/well. Considering time 36 h. Cells were plated at 0.5 × 10⁵ cells/well in 100 µL RPMI-1640 medium for 24 h, followed by substitution with compounds diluted at 0.1% DMSO concentration and incubated for 24 h to examine luciferase activity.

**Analysis and Characterization of Positive Compounds** Under the optimized conditions, the primary screening was performed in duplicate at the concentration of 10 µg/mL for pure compounds. The Z’ factor, a characteristic parameter for the quality of the assay without the intervention of test compounds, was chosen for evaluating the reproducibility and quality of overall assays because it is sensitive to data variability as well as the signal dynamic range. The overall Z’ value for each assay plate in a total of 50 plates was 0.62 ± 0.05, indicating the robustness of this assay (Fig. 3A). After the primary and confirmatory screening, the compounds that increased the firefly luciferase activity more than 300% compared with the negative control were picked and validated, resulting in four hits, including emodin, quercetin, apigenin and luteolin (Fig. 3B). Their chemical structures are illustrated in Fig. 3C. Various concentrations of identified compounds were used to treat HepG2-ISRE-luc2 cells to examine their dose response effect on JAK/STAT pathway activation. As shown in Fig. 3D, all four natural products increased ISRE reporter activity in a concentration dependent manner. The calculated EC₅₀ values of emodin, quercetin, apigenin and luteolin were 6.35 µM, 1.15 µM, 4.11 µM, 2.54 µM respectively.

**Effect of Identified Compounds on the Endogenous Antiviral Gene Expression** Two IFN-α responsive genes, 2′, 5′ oligoadenylate synthetase (2′, 5′-OAS) and interferon-induced, double stranded RNA-activated protein kinase (PKR), both of which contain ISRE consensus in the promoter region, were examined for their mRNA expression when treated by emodin, quercetin, apigenin and luteolin, respectively. As shown in Fig. 4, IFN-α induced the increased expression of 2′, 5′-OAS and PKR compared with the negative control. The identified compounds emodin, quercetin and apigenin up-regulated the expression of 2′, 5′-OAS, but had no effect on PKR, whereas luteolin increased the mRNA expression of both PKR and 2′, 5′-OAS (Fig. 4). These results indicated that emodin, quercetin, apigenin and luteolin were able to increase endogenous antiviral genes expression.

**DISCUSSION**

Some biochemical or cell-based screens for the JAK/STAT pathway have been developed to discover small molecule cytokine modulators with significant utility in the clinic. Although the activated JAK/STAT pathway by type I IFNs plays important roles in defending humans from viral infection and tumorigenesis, small molecules which activate the JAK/STAT pathway similarly to IFNs, and have potential antiviral effect, are rarely found. Thus, in this study HepG2-ISRE-luc2 cell-based screening was established to find small molecule activators from a natural products-based library. The HepG2-ISRE-luc2 cells faithfully and sensitively react to the activating state of the JAK/STAT pathway induced by type I IFNs. Two rounds of screening resulted in four hits, including emodin, quercetin, luteolin, and apigenin, which potently activated ISRE reporter activity and endogenous antiviral gene expression.
Emodin has been found to inhibit Hepatitis B virus replication in human hepatoma cells. Its analogue, aloe-emodin, was also found to increase ISRE reporter expression and showed an inhibitory effect against Japanese encephalitis virus and enterovirus 71. The finding here not only validates our screening assay, but also proposes it as a starting point for the development of a novel antiviral drug. Quercetin and apigenin also have been found to have antiviral effects and to show an additive antiproliferative effect with IFN-α, but the underlying mechanism was still unknown. Here we found that they could activate the JAK/STAT pathway and antiviral gene expression.

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expression, which may be a clue to their mechanism in inducing this effect. Quercetin is found to inhibit the expression of heat shock protein 90 (Hsp90), whereas Hsp90 is involved in the regulation of JAK/STAT activation.20) Thus, whether quercetin acts on the JAK/STAT pathway via Hsp90 needs to be further studied. Luteolin has also been found to have an antiviral effect and shows a synergistic effect with IFN-β in modulating the immune responses of peripheral blood mononuclear cells isolated from multiple sclerosis patients, thus, it is proposed as a promising adjutant for multiple sclerosis therapy.21,22) Although its mechanism in these effects was still unknown, our finding that luteolin potently activated the JAK/STAT pathway sheds new light on its preclinical study. Luteolin is found to be a protein kinase C (PKC) inhibitor, whereas PKCβ is found to negatively regulate IFN-induced JAK/STAT activation.23,24) Thus, whether luteolin activates the JAK/STAT pathway through inhibiting PKCβ needs to be further studied.

We found emodin, quercetin and apigenin up-regulated the expression of 2′,5′-OAS, but had no effect on PKR, whereas luteolin increased the expression of both PKR and 2′,5′-OAS. It has been found that the expression of endogenous IFN-responsive genes is controlled by different co-activators or co-repressors in a cell type specific manner, thus the identified compounds may have different effects on the complex JAK/STAT signaling network and lead to the differential gene expression patterns.25,26) Elucidating these will reveal new regulation mechanisms of JAK/STAT signaling and provide new insights into the understanding of the tissue and cell specific regulation of IFN response. It is observed that low levels of IFN-α/β are produced even in the absence of viral infection, so as to keep the constitutive weak IFN-α/β signal available to elicit a rapid strong cellular response against infection.27) Our findings in this study give new clues for understanding how these compounds exert their antiviral effects, and what roles they play in potentiating antiviral JAK/STAT pathway signaling. Although more structure–activity relationship studies are needed to further promote the potency of these compounds in activating the JAK/STAT pathway, the lead compounds found in this study provide new potential options for the development of novel IFN therapy adjuvants or antiviral drugs.

In conclusion, a cell-based assay was developed and validated for screening small molecule activators of the JAK/STAT pathway. Emodin, quercetin, apigenin and luteolin were all found to activate ISRE reporter expression. Further analysis revealed that these four compounds could also increase endogenous antiviral gene expression. These compounds are an ideal starting point for structural modification and warrant further investigation in the development of new antiviral drugs.

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REFERENCES


23) Shi RX, Ong CN, Shen HM. Protein kinase C inhibition and X-


