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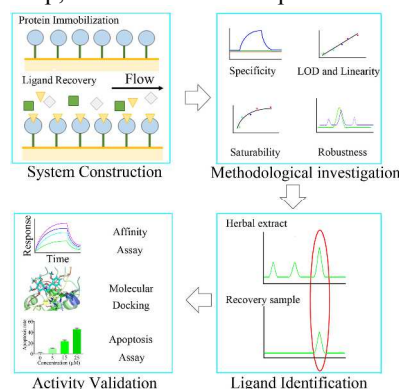
Biosensor-based Active Ingredients Recognition System for Screening STAT3 Ligands from Medical Herbs

Langdong Chen^{1,†}, Diya Lv^{1,†}, Xiaofei Chen^{1,†}, Mingdong Liu[‡], Dongyao Wang[†], Yue Liu[†], Zhanying Hong[†], Zhenyu Zhu[†], Xiaoxia Hu[†], Yan Cao^{*,†}, Jianmin Yang^{*,‡}, Yifeng Chai^{*,†}

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ABSTRACT: A surface plasmon resonance (SPR) biosensor-based active ingredients recognition system (SPR-AIRS) was developed, validated, and applied to screen signal transducer and activator of transcription 3 (STAT3) ligands. First, features of the screening system were investigated in four aspects: (1) specificity of the STAT3-immobilized chip, it shows that the chip could be applied to screen STAT3 ligands from complex mixture; (2) linearity and limit of detection (LOD) of the system, the minimum recovery cycle number was determined as 5 cycles; (3) saturability of the chip, the results indicate that it is necessary to select a proper concentration based on the compound's K_d value; (4) robustness of the system, it indicates that inactive compounds in the matrix could not interfere with active compounds in the process of screening. Next, SPR-AIRS was applied to screen STAT3 ligands from medicinal herbs. 9 candidate compounds were fished out. Then SPR assay and molecular docking were performed to verify the interplay between STAT3 and candidate compounds. Apoptosis assay and luciferase report assay were performed to investigate the drug effect of candidate compounds on STAT3 activity. Western blot results indicated that neobaicalein and polydatin could inhibit the phosphorylation of STAT3. As far as we know, this is the first time that neobaicalein and polydatin are reported as effective STAT3 ligands. In a conclusion, we have systemically demonstrated the feasibility of SPR biosensor-based screening method applying to complex drug systems, and our findings suggest that SPR-AIRS could be a sensitive and effective solution for the discovery of active compounds from complex matrix.



Medicinal herbs have not only been used in the treatment of various diseases in China, but also become one of the most important resources for screening lead compounds¹. The classical strategy for active compound screening starts with the isolation of purified compounds from herbal extracts, and is followed by bioassays of each purified compound one by one. A handful of elegant studies have applied this approach to identify active compounds from herbal medicines²⁻⁴. The advantage of this strategy is that it could provide a comprehensive analysis of herbal extract's activity. However, in the herbal extract, only a part of chemical constituents have pharmacological activities⁵. Inactive compounds will consume a lot of reagents and intensive work of researchers, which is a major limitation of this strategy.

Currently, many screening methods have been developed based on different detection principles. Some approaches are based on the detection of the binding between proteins and active compounds, such as cell membrane chromatography (CMC)^{6,7} and ultrafiltration LC/MS approach^{8,9}. CMC is a convenient and effective method to screen active compounds binding to membrane proteins¹⁰, which could directly screen active compounds from complex matrix. Ultrafiltration LC/MS is also an effective method which could assess the binding of candidate molecules to target proteins, because it would not destroy the native protein-ligand interactions in

solution¹¹. Some approaches are based on on-line pharmacological testing technology, such as microfluidics^{12,13} and on-line HPLC biochemical assays^{14,15}. Microfluidics could detect the activities of thousands compounds simultaneously¹⁶. On-line HPLC biochemical assays could find and isolate compounds with good activity against the specific disease marker. Other approaches are based on molecular docking technology, including high throughput virtual screening strategy¹⁷ and network pharmacology^{18,19}. These strategies could predict the interaction between active compounds and target proteins by docking algorithms. Nevertheless, all these methods mentioned above have their limitations. For example, CMC could not detect those active compounds with low abundance in medicinal herbs²⁰. On-line HPLC biochemical assays would consume a large number of chemical reagents and proteins. The requirement of existing protein crystal structure is a difficult problem of virtual screening strategy. More seriously, these methods could not provide information about the interplay between active compound and protein directly and precisely. Hence, a novel active ingredients screening method should be introduced to tackle these problems.

Surface plasmon resonance (SPR) biosensor is emerging as a powerful tool for biomedical analysis, which could real-time monitoring the interplay between biomolecules²¹. SPR has been widely used in the detection of bio-macromolecular in-

teraction, including protein-protein interaction^{22,23}, nucleic acid detection^{24,25} and virus detection²⁶⁻²⁸. In recent years, the advantage of SPR in the discovery and characterization of small molecular ligands specifically bound to target proteins has been recognized^{29,30}. Compared to the methods mentioned previously, SPR could provide kinetic information of the candidate compounds³¹. This feature makes SPR unique in the application of drug screening. In term of screening lead compounds from medicinal herbs, the greatest advantage of SPR is that it could detect active molecules in complex matrix directly^{32,33}, which reduces the requirement for sample separation and isolation. Some researchers have established SPR methods to screen active compounds from complex matrix. Zhang *et al.* have developed a novel SPR-high performance liquid chromatography–tandem mass spectrometry (SPR-HPLC–MS/MS) system for screening human serum albumin ligands from herbal medicine³⁴. Totally, twenty active compounds have been screened in the extract of *Radix Astragali*. Kuo *et al.* have developed a platform to screen target molecules based on SPR and atomic force microscopy³⁵. The platform was used to confirm the interaction between epidermal growth factor receptor (EGFR) and 3 EGFR ligands. We have also developed a SPR-based method to screen active compounds from medicinal plants³⁶. This method could enrich and identify active compound with low abundance in herbal extract. However, these studies were preliminary and the method was not investigated systematically. There were no corresponding experimental results which could provide reference to the selection of parameters in screening, for example, how to choose a proper sample concentration for SPR screening and how many cycles is enough to perform MS identification. Hence, the purpose of the study was to investigate the method's performance and provide guidance of screening active compounds from herbal extracts.

In this study, a SPR-based active ingredients recognition system (SPR-AIRS) was established and applied to screen signal transducer and activator of transcription 3 (STAT3). STAT3 is a typical drug target based on the following reasons: first, STAT3 has biological significance, the dysregulation of STAT3 is highly related to the genesis of many malignancies^{37,38}; then the expression and purification of STAT3 is feasible and could be used in SPR assay³⁹; finally,

the crystal structure of STAT3 has been determined, which makes it possible for further validation⁴⁰. Our experimental workflow is illustrated schematically in Fig.1. First, the characteristics of the screening system were investigated in order to provide scientific evidences to apply this system to herbal samples. (1) the specificity of the STAT3-immobilized chip was verified, and it shows that the chip could be used to screen STAT3 ligand from complex compounds mixture; (2) the linearity and limit of detection (LOD) of the system were investigated, and it reveals that 5 cycles are needed to ensure precise identification of active compounds in recovered samples and the recovery method has good repeatability and stability from 5 to 100 cycles; (3) the saturability of the chip was demonstrated, and it reveals that the amount of recovered compound will be saturated with the increasing sample's concentration and it is better to select a proper concentration based on the compound's K_d value; (4) the robustness of the recognition system was investigated in a simple background model and a complex background model, and it indicates that inactive compounds in the matrix could not interfere with active compounds. These methodology implements demonstrated the feasibility of SPR-based screening system applying to complex drug system and could provide guidance to perform screening ligands from herbal samples.

Next, this system was applied to screen ligands from herbal extracts. Thirty-two medicinal herbs were screened and nine herbs were found to have high response signal with STAT3. Nine candidate compounds were identified. Through the validation of SPR assay and molecular docking, five compounds, including neobaicalein, cryptotanshinone, polydatin, evodiamine and curcumin, were identified as STAT3 ligands. These compounds could significantly induce the apoptosis of HepG2 and MCF-7 cell lines and inhibit STAT3-driven transcriptional activity. Neobaicalein and polydatin could inhibit the phosphorylation of STAT3. This is the first time that neobaicalein and polydatin are reported as potent STAT3 ligands. These results validated that the proposed SPR-AIRS could be an effective method to screen target active compounds acting on specific protein from complex systems. It also provides a guidance to apply SPR in screening active ligands from medicinal herbs and other complex drug systems.

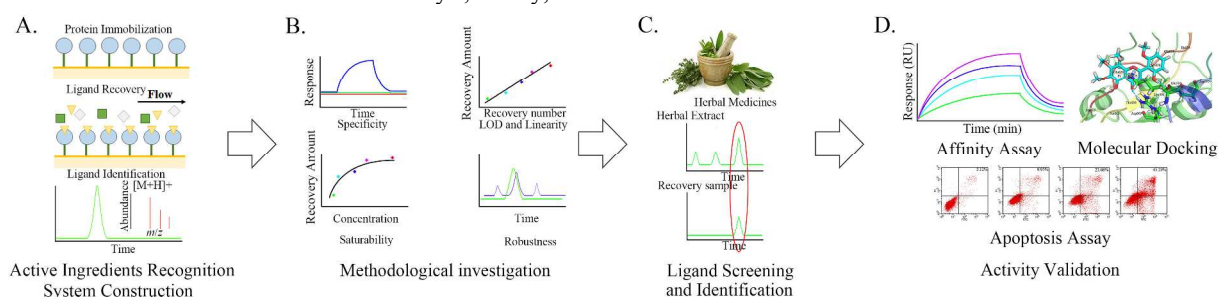


Figure 1. Workflow of SPR-AIRS. (A), methodological investigation (B), application in herbal extracts (C) and results validation (D). SPR-AIRS was successfully constructed. Active compounds which could bind to the protein-immobilized sensor chip could be recovered and analyzed by SPR-AIRS. After the construction of SPR-AIRS, the features of this system were investigated, including specificity, LOD, linearity, saturability and robustness. Then the system was applied to screen STAT3 ligands from herbal extracts. The affinities between candidate compounds and STAT3 were tested by SPR assays. The action modes between ligands and SH2 domain were calculated by DS 3.0. The apoptosis effects induced by STAT3 ligands were detected by flow cytometry.

EXPERIMENTAL SECTION

Materials and reagents. Herbal medicines were purchased from Leiyunshang Pharmacy (Shanghai, China). All these medicinal herbs were authenticated by Lianna Sun (Department of Pharmacognosy, School of Pharmacy, Second Military Medical University, Shanghai, China). Standard compounds, including neobaicalein, curcumin, saikosaponin D, cryptotanshinone, solamarine, parthenolide, evodiamine, polydatin, statin and mln8237 were purchased from Efebio (Shanghai, China). The purity of all the standard chemicals was over 99.9 %. HPLC-grade acetonitrile, methanol and formic acid were purchased from Sigma Aldrich (Missouri, United States). CM5 chips, EDC, NHS and ethanolamine were purchased from GE Healthcare (Shanghai, China). Recombinant STAT3 protein was purchased from Abcam (Cambridge, United Kingdom). Annexin V-FITC apoptosis detection kits were purchased from Donjindo (Kumamoto, Japan). Recombinant human interleukin-6 proteins were purchased from R&D system (Minnesota, United States). Dual Luciferase Reporter Assay kits were purchased from Promega (Beijing, China). Plasmids (pSTAT3-TA-luc and pRL-SV40) were purchased from Beyotime (Shanghai, China).

Immobilization of STAT3 on SPR sensor. SPR assays were performed on Biacore T200 system (GE Healthcare, Sweden). Flow cell 1 was reference cell while flow cell 2, 3 and 4 were detection cells. Recombinant STAT3 was diluted in 10 mM sodium acetate (pH = 5.0, 50 $\mu\text{g/mL}$) and then immobilized on detection cells by applying EDC/NHS cross-linking reaction⁴¹.

Recovery of STAT3-bound ingredients. The protocol of recovering STAT3-bound ingredients was referred to our previous study³⁶. A Biacore T200 system was applied to perform the recovery experiment. The process of recovery experiment is as follows: (1) sample passed through the surface of protein-immobilized chips and active components associated with protein, (2) the system was washed to remove the retained sample solution, (3) the recovery buffer was injected and ingredients bound to the protein dissociated from chips. Finally, (4) the recovery buffer containing recovered compounds was drawn back. Herbal extract was injected over the sensor surface for 180 s at 5 $\mu\text{L/min}$. Phosphate buffer saline (PBS) with 5% DMSO was selected as running buffer. The flow system was washed with 0.5% formic acid. A small volume of 2 μL sample was injected into the flow cells and incubated for 20 s. Then, the flow direction over the sensor surface was reversed and the sample containing STAT3-bound ingredients was deposited in 10 μL ammonium bicarbonate (50 mM). In a cycle, the recovery procedure was replicated for 5 times.

Experimental condition for UPLC-QTOF-MS analysis. Herbal extracts and SPR-recovered samples were analyzed on an Agilent 1290 series UPLC system (Agilent Corp., USA). Chromatographic separation was carried out on a Waters XSelect® HSS T3 2.5 μM column (2.1 $\times$ 100 mm) at 25 $^{\circ}\text{C}$. The injection volume is 5 μL . The mobile phase consisted of 0.1 % aqueous formic acid (v/v) (A) and acetonitrile (B), using a gradient elution. Elution procedure is 5 % B at 0-2 min, 5-95 % B at 2-13 min, and 95 % B at 13-15 min for herbal extracts and SPR-recovered samples. The flow rate was kept at 3.5 mL/min, and a post-column split was used to maintain a flow rate of 0.4 mL/min into the mass spectrometer source to obtain good nebulization efficiency. Detection was performed by an Agilent 6538 UHD Accurate-Mass QTOF/MS (Agilent Corp., USA). The analysis was performed using full-scan mode and

mass range was set at m/z 100-1000 in positive ion mode. The conditions of ESI source were as follows: drying gas (N_2) flow rate, 10 L/min; drying gas temperature, 350 $^{\circ}\text{C}$; nebulizer, 35 psig; capillary voltage, 4000 V; fragmentor voltage, 120 V; skimmer voltage, 60 V; octopole RF, 250 V. All the data were processed by Agilent MassHunter Software ver. B.02.00 (Agilent Technologies). Tuning mix (G1969-85000, Agilent Corp., USA) was used for lock mass calibration in the assay.

Determination of the STAT3-immobilized chip's specificity. Statin solution (20 μM), mln8237 solution (20 μM) and running buffer were injected into SPR system to detect their response signal with STAT3, respectively. Subsequently, the affinity of statin was tested according to the protocol provided by GE Healthcare. Statin was diluted in 5% DMSO PBS at concentrations ranging from 0.5 to 64 μM . Middle concentration was duplicated at the end of sequence. Analytes were injected at a flow rate of 30 mL/min. The association and dissociation times were both 120 seconds. The affinity fitting was performed by using a steady-state affinity model (1: 1) to obtain the affinity constant.

Limit of detection and Linearity. STAT3-bound compound recovery was repeated for 1, 2, 10, 20, 50 and 100 cycles. Recovery samples and standard solution (20 μM) were dried by nitrogen gas and dissolved in methanol. The samples were centrifuged at 5000 g for 3 mins and then injected into UPLC-QTOF-MS system for analysis. Signal-to-noise ratio (S/N) was calculated by MassHunter. Linearity relationship between chromatogram peak area and number of cycles was calculated.

Saturability of the biosensor. Statin solutions at different concentrations (5, 10, 20, 50 and 100 μM) were recovered. Each concentration was recovered repeatedly for three times. Recovered samples were dried by nitrogen gas and dissolved in 100 μL methanol. The samples were centrifuged at 5000 g for 3 mins and the supernatant was injected into UPLC-QTOF-MS system for analysis.

Robustness of the active ingredients recognition system. Simple background model: statin and mln8237 solution (20 mM) were diluted in methanol and injected into UPLC-QTOF-MS system separately. Then statin and mln8237 were mixed up to a concentration of 20 μM and injected into SPR system. Recovery experiment was performed for 10 cycles. Recovered samples were dried by nitrogen gas and dissolved in 100 μL methanol. The samples were centrifuged at 5000 g for 3 mins and the supernatant was injected into UPLC-QTOF-MS system for analysis.

Complex background model: statin solution (20 μM) and the extract solution of *Rhizoma Corydalis* (*R. Corydalis*, 100 ng/mL) were mixed in PBS. The samples were centrifuged at 5000 g for 3 mins and then injected into SPR for compound capture. Recovery experiment was performed for 10 cycles. Recovered samples were dried by nitrogen gas and dissolved in 100 μL methanol. The samples were centrifuged at 5000 g for 3 mins and the supernatant was injected into UPLC-QTOF-MS system for analysis.

Preparation of sample extracts. The crude drugs were smashed into powders and then sieved through a 40-mesh sieve. Powder samples in 1 g were extracted by supersonic extraction with 10 mL ethanol: H_2O (80: 20) for 30 mins. Subsequently, the extracts were centrifuged and filtered through a 0.22- μm filter. The samples were stored at room temperature.

Small molecular ligands screening from herbal extracts.

Pre-screening: herbal extract was diluted in PBS (100 ng/mL) and centrifuged under 5000 g for 3 mins before injection. Then the extract was injected into SPR system. The associate time was 60 s. PBS was injected between two herbal samples in order to clear remaining compounds in the system.

Ligands recovery and identification: extracts of the nine herbs were recovered for 10 cycles. Recovered samples were dried by nitrogen gas and dissolved in methanol. Recovery samples were centrifuged at 5000 g for 3 mins and then injected into UPLC-QTOF-MS system for analysis. Herbal extracts were diluted in methanol and then injected into UPLC-QTOF-MS. Chemical information about constituents in herbal medicines were collected from Traditional Chinese Medicine integrative database (TCMID)⁴². According to the parameters provided by the Agilent Qualitative Analysis software, such as mass accuracy, isotope abundance match, spacing match and double bond equivalent index, components were confirmed due to the fine matching of these parameters.

Affinity validation. Standard compounds were diluted in 5% DMSO PBS at concentrations ranging from 0.5 to 256 μ M. Analytes were injected at a flow rate of 30 μ L/min. The association and dissociation times were both 120 s. The affinity fitting was performed with Biacore T200 evaluation software by global fitting using a steady-state affinity model to obtain the affinity constant.

Molecular docking. The crystal structure of STAT3 was downloaded from Protein Data Bank (PDB ID: 1BG1)⁴³. The DNA chain and water molecules were removed from the complex. All chemical structures were designed and optimized using Discovery Studio v. 3.0 (DS 3.0, Accelrys Inc., San Diego, CA, USA). Binding site was defined at Src homology 2 (SH2) domain of STAT3, with a 10 Å radius from the center of the binding pocket⁴⁴. Molecular docking was performed by employing the CDOCKER module in the DS 3.0 package. STA-21 and candidate compounds screened from herbal extracts were docked into the defined catalytic site. Top hits was set as 100 and poster cluster radius was set as 0.5. The other options were set as default settings. CDOCKER energy was calculated to evaluate the best pose. The binding results were visualized in pymol.

Cell culture. HepG2 and MCF-7 cell lines were purchased from Cell Bank of Shanghai Branch of Chinese Academy of Sciences. Cells were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μ g/mL streptomycin. The cells were cultured in a humidified atmosphere of 5% CO₂ at 37 °C.

Apoptosis assay. Apoptotic cells were determined using Annexin V-FITC apoptosis detection kits by flow cytometry. Cells (2×10^5) were plated on each well of a six-well plate and treated with drugs at different concentrations for 48 h. Floating and sticking cells were harvested and washed twice in PBS. Cells were resuspended in PBS and stained with Annexin V/FITC and PI. Stained cells were analyzed using a FACS Calibur instrument (Becton Dickinson, Mountain View, CA, USA).

Transient transfection and STAT3 luciferase reporter assay. HepG2 and MCF-7 cells were seeded at a density of 1×10^6 in 10 cm culture plates. On the following day, cells were transfected with 27 μ g pSTAT3-TA-luc and 9 μ g pRL-SV40 (Beyotime) using JetPEI (Polyplus, Illkirch, France). After 6 h

of transfection, cells were trypsinized and seeded onto black-bottom 96-well plates at a density of 1×10^4 cells per well. Cells were treated with either test compounds or 0.1% DMSO for 24 h. Subsequently, IL-6 (20 ng/mL) was added into wells for another 24 h to stimulate STAT3 expression. After treatment, cells were harvested in 20 μ L of passive lysis buffer and luciferase activity was evaluated by the Dual Luciferase Reporter Assay kit on Synergy 4 microplate Reader (Biotek). The results were normalized relative to *Renilla* luciferase activity values. All luciferase experiments were repeated three times.

Western blot. Neobaicalein-treated cell extracts were lysed in lysis buffer. Lysates were then centrifuged at 12,000 g for 20 min at 4 °C to remove insoluble material and resolved on a 10% SDS-PAGE gel. After electrophoresis, the proteins were electrotransferred onto a nitrocellulose membrane, blocked in 5% non-fat milk powder-TBST at room temperature and incubated at 4°C on a shaker overnight with primary antibodies against total STAT3 (1:1000), STAT3 p705 (1:1000), poly (ADP-ribose) polymerase (PARP) (1:1000), BAX (1:1000), β -actin (1:5000). The blot was washed and incubated for 2 h in the room temperature with HRP conjugated Goat anti-Rabbit IgG (H + L)(1:10000). The blots were analyzed by scanning densitometry using an Odyssey infrared Imaging System (Licor, United States). β -actin was used as an interval control.

RESULTS AND DISCUSSION

Specificity of the STAT3-immobilized sensor chip. Recombinant STAT3 protein was first immobilized on three flow cells of the sensor chip with immobilization levels of 12564.5, 19132.0 and 16718.2 response units (RU), respectively. In order to validate the specificity of the chip, stattic was selected as a positive compound, which is a direct inhibitor for STAT3⁴⁵, and mln8237 was selected as a negative compound, which is an Aurora A selective inhibitor^{46,47}. Stattic and mln8237 standard solution were injected into the sensor chip. The response signal of stattic was approximately 10 RU, while the response signal of mln8237 was nearly 0, which was similar to the response signal of running buffer (Fig.2A). The result demonstrates the specificity of STAT3-immobilized chip. Furthermore, the activity of the STAT3-immobilized chip was characterized by testing the affinity of stattic to STAT3. As a result, stattic could bind to the sensor surface in a concentration-dependent manner and the K_d of stattic was 26.97 μ M (Fig.2B). These results collectively validate the selectivity and activity of STAT3-immobilized SPR sensor chip. According to literatures and our practices in drug discovery, a concentration range from 10 to 50 μ M is desirable for preliminary screening of lead compounds⁴⁸⁻⁵⁰. Hence, using stattic as a positive molecule could simulate the interplay of active compounds in herbal medicines and facilitate the understanding of SPR-AIRS.

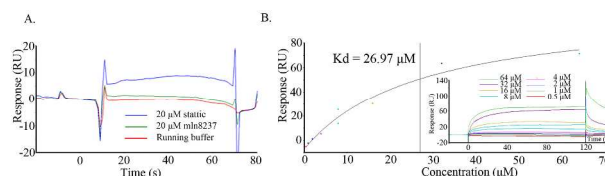


Figure 2. The specificity of the STAT3-immobilized chip was validated (A). Stattic solution had a strong response signal with STAT3, while the response signal of mln8237 was at the same level with running buffer. Subsequently, chip's activity was characterized. Sensorgrams of stattic at different concen-

trations and the fitting curve (B) are shown. The K_d of static is 26.97 μM .

Table 1 Chromatographic information of recovery samples with different recovery cycles		
Number of recovery cycles	Peak area	S/N
1	4081.65	0.9
2	8963.09	2.8
5	10483.25	3.0
10	14580.19	4.6
20	33601.24	12.9
50	67705.74	20.2
100	131252.55	38.0

Linearity of the amount of recovered sample. The concentrations of some active compounds are quite low in herbal extracts. Though these compounds could bind to protein-immobilized chips, they could not be accurately detected by UPLC-MS if the recovery replicated cycle is inadequate. Hence, it is important to verify the LOD of the system, which could help researchers determine the appropriate replicated times. Static was recovered for 1, 2, 5, 10, 20, 50 and 100 cycles, respectively. All the samples were dried, redissolved and injected into UPLC-QTOF-MS system to determine the peak area and calculate S/N. Results are shown in Table 1. S/N of 1 cycle sample (Fig.3A) and 2 cycles sample (Fig.3B) are 0.9 and 2.8, respectively. The noise of chromatogram was extremely high, which made it impossible for precise identification of compound. S/N of 5 cycles sample was just 3 (Fig.3C). In addition, the S/N was always greater than 3 when the cycle number was over 5 (Fig.3D and S1). Hence, at least 5 cycles are needed to ensure precise identification of static in recovered samples. In this study, a recovery replicated number of 10 was used to ensure ideal signal detection. A good linear relationship ($R^2 = 0.9917$) between peak area and number of cycles is shown in Fig.3E, which demonstrates that the recovery method has good repeatability and stability from 5 to 100 cycles.

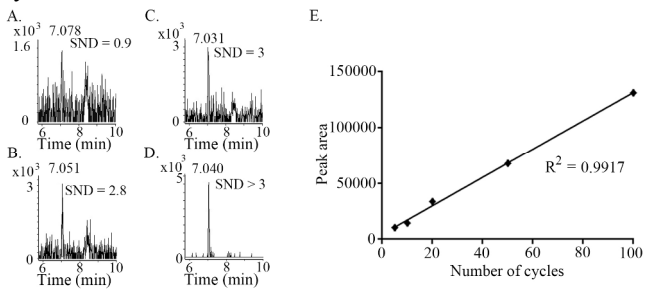


Figure 3. Extract ion chromatograms (EIC) of recovery samples with different recovery cycles: 1 (A), 2 (B), 5 (C) and 10 cycles (D). Linearity between peak area and cycle number is shown (E).

Saturability of the biosensor. In the screening process, it is important to choose proper concentrations for herbal samples. If the concentration is too high, it may induce damage to the protein and even flow cell blockage. Conversely, if the concentration is too low, it may need more time and reagents to identify the potential ligands. Hence, in order to find the relationship between the sample's concentration and the amount of recovered compound and provide reference to the selection of appropriate concentration, static solutions at different concentrations (5, 10, 20, 50 and 100 μM) were recovered (Fig.4A). According to the results, the amount of recovered static rose with the increase of static's concentration and

arrived at a plateau when static's concentration overtook its K_d value. The increase ratio of the static's peak area was less than the increase ratio of the static's concentration when the concentration surpassed 20 μM (Fig.4B). Based on the experimental results, the saturability of the biosensor was validated. This result prompts us that the concentration of sample is not as high as better in the "inject and recover" procedure. It is better to select a proper concentration based on the compound's K_d value, which could promote the efficiency of recovery. As a common criterion in the screening of lead compounds, 10 to 50 μM were desired concentrations with considerable affinity and activity^{51,52}, so it is suitable to choose the similar concentration range when performing screening process.

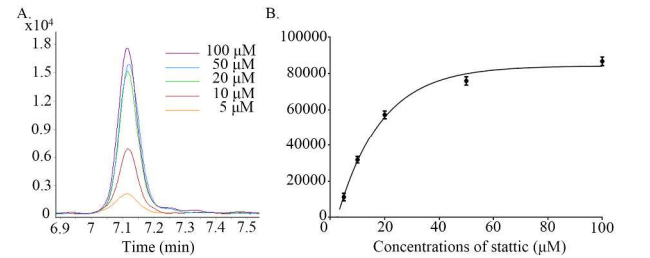


Figure 4. (A) EIC of recovered static at different sample concentrations (5, 10, 20, 50 and 100 μM). (B) Fitting curve of static's recovered static's peak areas and concentrations.

Robustness of the active ingredients recognition system. In general, there are hundreds of compounds in herbal extracts. Only small amounts of compounds have pharmacological activities. It is necessary to clarify whether inactive compounds could affect active compounds binding to the target proteins during screening process. In order to investigate the robustness of the developed system, we designed a simple background model in which static was regarded as a marker ligand and mln8237 was regarded as an inactive background compound. Mln8237 and static were first injected into UPLC-QTOF-MS system respectively, and retention times of the two compounds were 9.731 min (Fig.5A) and 7.221 min (Fig.5B). Subsequently, mln8237 and static were mixed to a final concentration of 20 μM each. The mixed solution was conducted for compound recovery. In the recovered sample, the chromatographic peak of mln8237 was not detected (Fig. 5C), while there was a chromatographic peak of static (Fig. 5D). The result suggests that inactive compound in mixture could not be detected in the recovery sample and would not interfere with active compound identification.

Furthermore, we mixed static with herbal extract (100 ng/mL *R. Corydalis*, Fig. 5E) to form a complex background model and performed recovery experiment. This model was more similar to the practical samples in screening. After recovery process, the recovered static's amount of the mixed solution was at the same level with simple background mixed solution (Fig. 5D and F). This model further demonstrates that the tremendous inactive compounds in herbal extract could not disturb the identification of potential ligands. Only compounds that really interact with protein can bind to chip surface and be recovered through the SPR system. The chip could be used to screen STAT3 ligands from complex compounds mixture.

Table 2 RT and m/z of 9 candidate compounds in different samples

Compound name	Chemical formula	Origin	<i>m/z</i>		
			herbal extract	Recovery sample	Standard sample
Neobaicalein	C ₁₉ H ₁₈ O ₈	<i>Radix Scutellariae</i>	375.1081([M+H] ⁺)	375.1069([M+H] ⁺)	375.1068([M+H] ⁺)
Cryptotanshinone	C ₁₉ H ₂₀ O ₃	<i>Radix Salviae</i>	297.1484([M+H] ⁺)	297.1483([M+H] ⁺)	297.1486([M+H] ⁺)
Parthenolide	C ₁₅ H ₂₀ O ₃	<i>Tanacetum parthenium</i>	249.1482([M+H] ⁺)	249.1479([M+H] ⁺)	249.1482([M+H] ⁺)
Polydatin	C ₂₀ H ₂₂ O ₈	<i>Rhizoma Polygoni cuspidati</i>	413.1204([M+Na] ⁺)	413.1187([M+Na] ⁺)	413.1206([M+Na] ⁺)
Curcumin	C ₂₁ H ₂₀ O ₆	<i>Rhizoma Curcuma longae</i>	369.1330([M+H] ⁺)	369.1330([M+H] ⁺)	369.1333([M+H] ⁺)
Solamarine	C ₄₅ H ₇₃ NO ₁₆	<i>Herba Solani nigri</i>	884.5000([M+H] ⁺)	884.4984([M+H] ⁺)	884.5007([M+H] ⁺)
Cucurbitacin B	C ₃₂ H ₄₆ O ₈	<i>Radix Trichosanthis</i>	581.3084([M+Na] ⁺)	581.3085([M+Na] ⁺)	581.3058([M+Na] ⁺)
Saikosaponin D	C ₄₂ H ₆₈ O ₁₃	<i>Radix Bupleuri</i>	803.4552([M+Na] ⁺)	803.4548([M+Na] ⁺)	803.4548([M+Na] ⁺)
Evodiamine	C ₁₉ H ₁₇ N ₃ O	<i>Fructus Evodiae</i>	304.1442([M+H] ⁺)	304.1444([M+H] ⁺)	304.1443([M+H] ⁺)

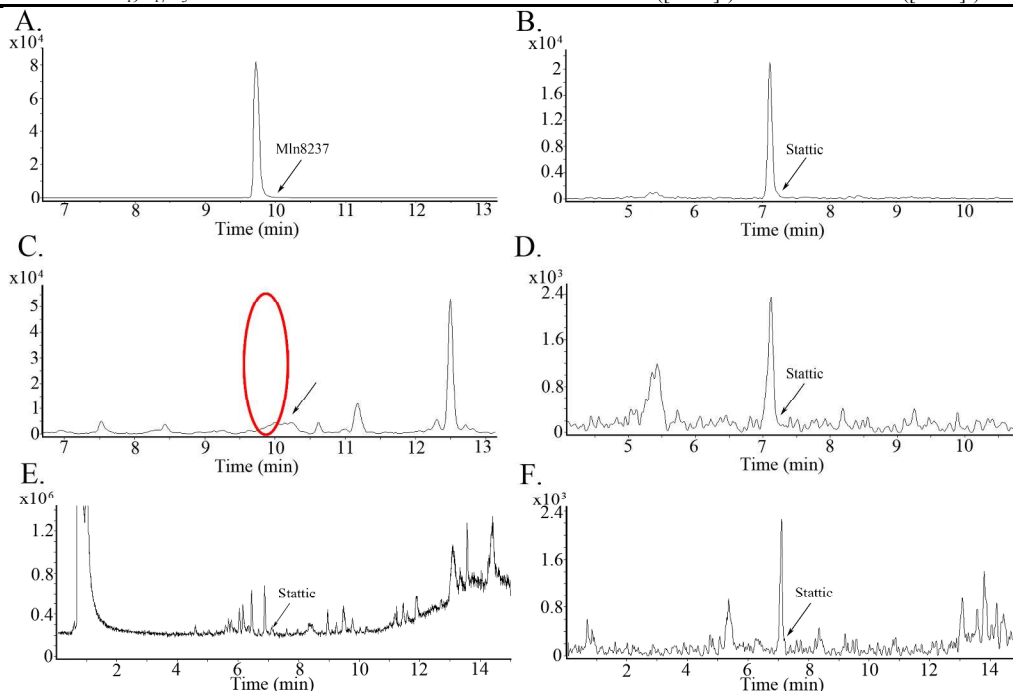


Figure 5. Robustness of the screening system was investigated. First, a simple background model was constructed. EIC of recovered mln8237 (A) and static (B) in simple background model was shown. In recovery samples, no chromatographic peak was detected for mln8237 (C), while the chromatographic peak of static (D) was found in the same position with standard sample. Subsequently, a complex background model was constructed. Static was spiked with *R. Corydalis* extract (E) and then recovered. The recovered static's amount of herbal extract mixed solution was at the same level with mln8237 mixed solution (F).

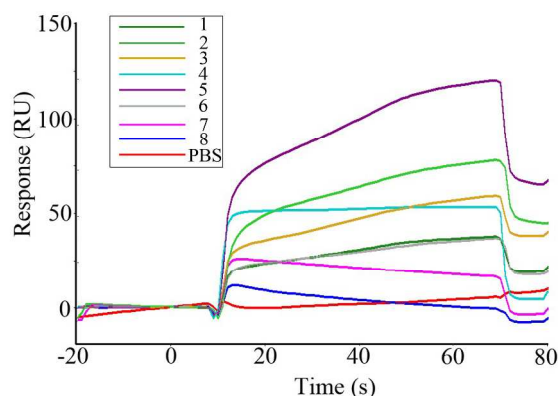


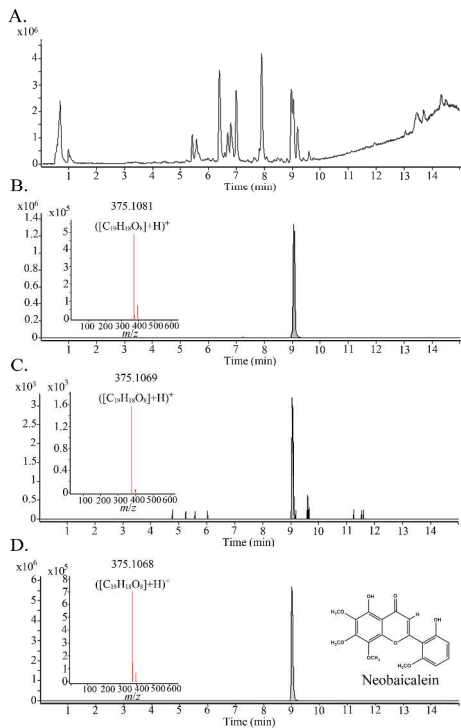
Figure 6. The pre-screening results of 8 representative herbs.

Screening of active ingredients from herbal extracts.

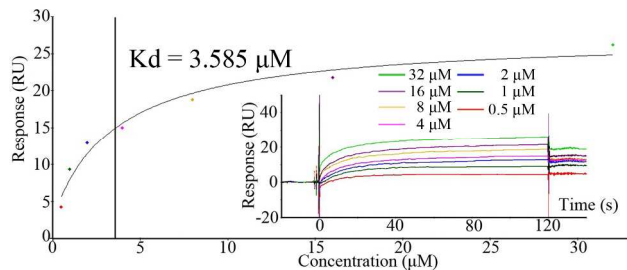
Next, this system was applied to screen STAT3 ligands from herbal extract. After querying STAT3 related medicinal herbs in PubMed and China Knowledge Network database (CNKI), thirty-two medicinal herbs were collected and screened (Table S1). The extraction samples (100 ng/mL) were injected into SPR system, respectively. The results of 8 representative herbs

are shown in Fig. 6. Among the 8 herbs, the response signal of *Rhizoma Polygoni cuspidate* was the highest, which was over 120 RU. In addition, *Radix Scutellariae* (RS) ranked the second with over 60 RU. The results of other 24 herbs could be found in Figure S2. Based on the pre-screening results, the top nine herbs were selected for ligand recovery and identification. The total ion chromatogram (TIC) of RS extract is shown in Fig. 7A. An ion signal was identified both in RS extract sample (RT= 9.056 min, *m/z* =375.1081([M+H]⁺)) and RS recovery sample (RT=9.063, *m/z* =375.1069 ([M+H]⁺)) (Fig. 7B and C). Record in database suggested that this compound might be neobaicalein. Subsequently, we injected the standard solution of neobaicalein into UPLC-QTOF-MS under the same condition. The RT of neobaicalein was 9.020 min and *m/z* was 375.1068 ([M+H]⁺) (Fig. 7D), which is the same with the ion signal identified in RS extract sample and RS recovery sample. So neobaicalein was identified as a candidate compound from RS extract sample. Totally, nine candidate compounds were identified from the nine medicinal herbs in the same way. RT and *m/z* of the nine candidate compounds in herbal extracts, recovery samples and standard solution are listed in Table 2. The chromatograms and mass spectrometry of the other eight compounds are shown in Fig.S3-10.

1 **STAT3 is a direct binding target of seven compounds.** In
2 order to validate the candidate compounds screened from
3 herbal medicines, SPR affinity assays were performed. The
4 SPR assay results are listed in Table 3. Compounds were sort-
5 ed according to the affinity with STAT3. The sensorgrams and
6 fitting curve of neobaicalein are shown in Figure 8. Results of
7 the other compounds are shown in Figure S11. According to
8 general criteria⁵³, compounds exceeding 2 times the Rmax
9 should be non-specific ligands. Saikosaponin D was suspected
10 as a non-specific ligand because its Rmax was over 2000. The
11 affinity between cucurbitacin B and STAT3 is quite weak (Kd
12 = 133.60 μ M). According to the experimental results, the other
13 seven compounds had good affinities with STAT3 and were
14 conducted for further validation.



37 **Figure 7.** (A) The TIC of *Radix Scutellariae* extract. EIC and
38 mass spectrometries of neobaicalein in *Radix Scutellariae*
39 extract sample (B), *Radix Scutellariae* recovery sample (C)
40 and neobaicalein standard solution sample (D).



47 **Figure 8.** SPR analysis of the binding between neobaicalein
48 and STAT3.

52 **Table 3 The affinities of 9 candidate compounds with STAT3**

Compound	Kd (μ M)	Rmax (RU)	chi ²
Neobaicalein	3.58	24.67	2.45
Polydatin	7.20	7.94	0.196
Cryptotanshinone	11.39	14.10	0.185
Parthenolide	17.27	40.72	2.56
Curcumin	27.56	9.79	0.0458

Evodiamine	28.96	40.41	3.33
Solamarine	56.00	98.20	7.60
Cucurbitacin B	133.60	7.41	0.0530
Saikosaponin D	15.12	2809	5.59×10^4

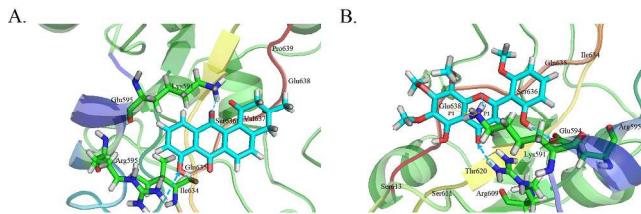


Figure 9. Action modes of STA-21 (A) and neobaicalein (B) with
STAT3 SH2 domain.

SH2 domain is active site of five compounds. According
to the theory of drug-protein interaction, a compound binding
to STAT3 does not mean that it is a direct STAT3 inhibitor,
owing to non-specific binding of the compound to non-
functional sites of STAT3⁵⁴. To reasonably evaluate the bind-
ing of compounds to the active site of STAT3, molecular
docking was performed to calculate the interplay between
seven candidate compounds and STAT3 SH2 domain by
CDOCKER in DS 3.0. SH2 domain is an important domain
which plays key role in the dimerization of STAT3, which
induce translocation of STAT3 into cell nuclear and activation
of anti-apoptotic gene, matrix metalloproteinases and proteins
that regulate cell proliferation and angiogenesis⁵⁵. SH2
domain is the most attractive functional domain of STAT3 and SH2-
targeting compounds constitute the largest class of direct
STAT3 inhibitor⁴⁴. STA-21 was selected as a positive ligand
to ensure the reliability of docking⁵⁶. The docking result of
STA-21 is shown in Fig. 9A. STA-21 could form hydrogen
bond with LYS591, ARG595 and ILE634 with a CDOCKER
energy of -25.305 kJ. The docking results of 7 candidate com-
pounds are shown in Table 4. According to the results, the
CDOCKER energy of parthenolide and solamarine were posi-
tive, which suggested that they could not specifically interact
with the SH2 domain of STAT3. Five active compounds with
negative CDOCKER energy, including neobaicalein (Fig. 9B),
curcumin, cryptotanshinone, polydatin and evodiamine, were
conducted for further biological evaluation.

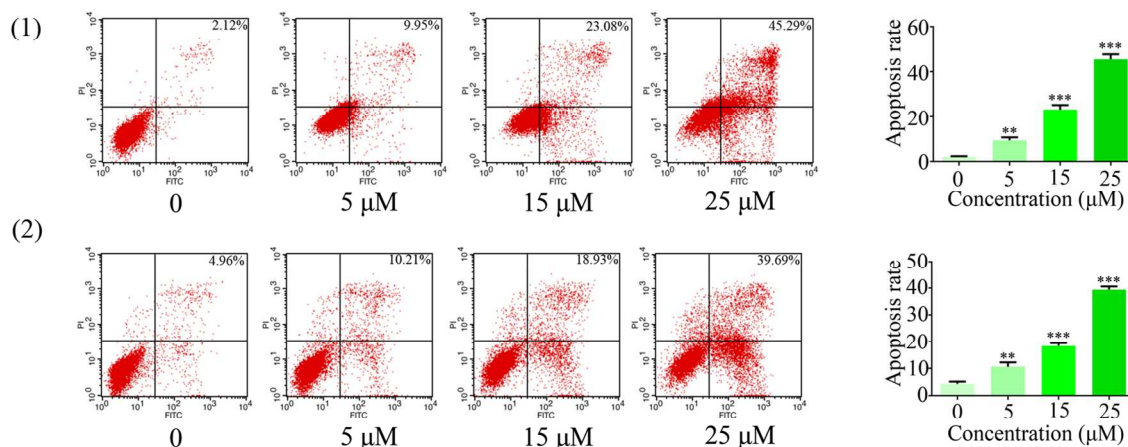
Table 4 CDOCKER energy of STA-21 and 7 candidate compounds

Compound	CDOCKER energy (kJ)
STA-21	-25.305
Curcumin	-35.066
Cryptotanshinone	-17.715
Neobaicalein	-13.537
Polydatin	-12.907
Evodiamine	-11.687
Parthenolide	>0
Solamarine	>0

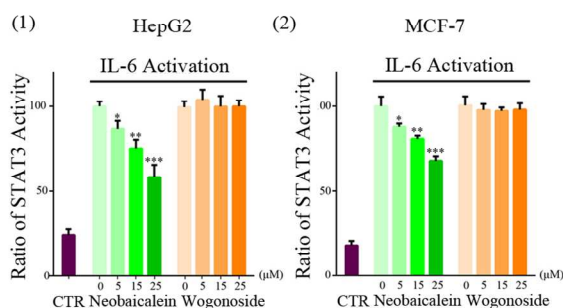
Five compounds could induce apoptosis in cancer cells.

Based on the previous experimental results, five compounds
were identified as STAT3 ligands and could bind to the SH2
domain with good affinities. Next, biological experiments
were conducted to evaluate the activity of potential ligands.
STAT3 is an important transcription factor involved in cell
growth and apoptosis⁵⁷. Inhibition of STAT3 could inhibit the
expression of anti-apoptotic protein and inducing apoptosis of
cancer cells⁵⁸. HepG2 and MCF-7 cell lines were selected for
in vitro activity evaluation of five active compounds.

A.



B.



C.

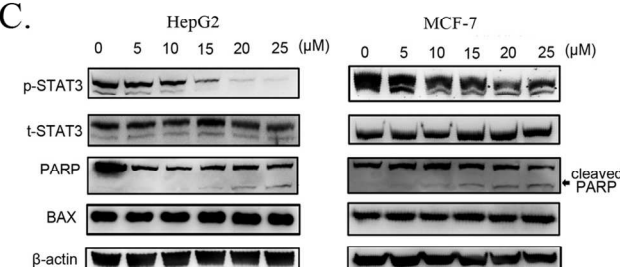


Figure 10. (A) Apoptotic effects induced by neobaicalein on HepG2 cell lines (1) and MCF-7 cell lines (2) (B) Effect of neobaicalein and wogonoside on STAT3-driven transcription activity in HepG2 (1) and MCF-7 (2) cell lines. (C) Neobaicalein inhibit the phosphorylation of STAT3 while do not affect the expression of total STAT3 in HepG2 (1) and MCF-7 (2) cells. Besides, the expression of cleaved PARP and BAX, protein markers of apoptosis, were upregulated following neobaicalein treatment. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ treatment group versus control group)

The apoptosis effect induced by neobaicalein on HepG2 cells and MCF-7 cells are shown in Fig. 10A. After exposure to neobaicalein, at different doses (5, 15 and 25 μM) for 48 hours, the percentage of apoptotic cells of drug-treated groups significantly increased compared with the control group. With the increasing in drug concentration, the percentage of apoptotic cells rises. The results of other four ligands could be found in Fig.S12 and 13.

Among the five ingredients, evodiamine has the best activity and can induce the two cell lines apoptosis effectively. Neobaicalein shows a good activity towards HepG2 cell lines and cryptotanshinone shows a good activity towards MCF-7 cell lines. Compared with the activity towards MCF-7 cell lines, curcumin has a better activity towards HepG2 cell lines. Although the affinity of polydatin is good (12.51 μM), the activity of polydatin is the worst in the five ingredients.

Five compounds could inhibit STAT3-driven transcriptional activity.

Next, we investigated the abilities of candidate compounds to antagonize STAT3-driven transcriptional activity in living cells. Transfected HepG2 and MCF-7 cells were incubated with candidate compounds and activated with IL-6 (20 ng/mL) for 24 h. According to the experiment results (Fig. 10B), neobaicalein attenuated STAT3-driven transcriptional activity in a dose-dependent manner as revealed by a reduction in the ratio of firefly to *Renilla* luciferase activity both in HepG2 and MCF-7 cell lines while the other four candidate compounds have the similar effects. (Fig S14). Besides, wogonoside,

which is a major component in *Radix Scutellariae*, was tested as a negative control compound, it could be revealed that wogonoside has little influence on STAT3-driven transcriptional activity. The luciferase report assay results indicated that five candidate compounds could effectively inhibit STAT3-driven transcriptional activity. Meanwhile, it demonstrates that our developed method could screen active STAT3 ligands from herbal medicines.

Neobaicalein and Polydatin could inhibit the phosphorylation of STAT3.

Recent reports have suggested that cryptotanshinone⁵⁹, curcumin⁶⁰ and evodiamine⁶¹ are potent STAT3 ligands, which are consistent with our research and could validate the effectiveness of our developed system. Interestingly, this is the first time that neobaicalein and polydatin are reported to be potential STAT3 ligands. Hence, we investigated the molecular mechanism of neobaicalein and polydatin on STAT3. The expression levels of pY705-STAT3 and total-STAT3 were detected (Fig. 10C). The results revealed a dose-dependent decrease in IL-6-induced STAT3 phosphorylation in HepG2 and MCF-7 cells with increasing compound concentration, while the expression of total STAT3 had very limited change. Polydatin has similar effect on inhibiting STAT3 phosphorylation compared with neobaicalein (Fig. S15).

To further confirm the induction of apoptosis in HepG2 cells by neobaicalein, apoptosis-related proteins after treatment was determined. We found that the protein level of cleaved PARP⁶² and BAX⁶³ in HepG2 and MCF-7 cells were

increased after neobaicalein treatment, compared with those of the control group. Taken together, these results strongly suggest that neobaicalein promoted the apoptosis of HepG2 and MCF-7 cells. Polydatin has similar effect on apoptosis-related proteins (Fig. S15). These results demonstrate that neobaicalein and polydatin could inhibit the phosphorylation of STAT3 and induce the apoptosis of HepG2 and MCF-7 cells.

CONCLUSIONS

In this research, we reported an active ingredients recognition system to screen small molecular ligands from Chinese medicinal herbs. Firstly, the performance of SPR-AIRS was validated in terms of 4 aspects, including: (1) the specificity of the protein-immobilized chips; (2) LOD and linearity of this system; (3) the saturability of the chip; (4) the robustness of the system. The specificity of the STAT3-immobilized chip was validated. SPR-AIRS's LOD was determined by analyzing recovery samples of different cycles. The linearity was also investigated, which shows good repeatability and stability. Furthermore, we have demonstrated the amount of recovered compound will be saturated with the increasing sample's concentration, which provides us a reference to the determination of sample concentration. It was validated that inactive compounds in the solution, especially the complex background of herbal extracts, could not interfere with the identification of active compounds. These results indicate that the system is highly sensitive and specific in the application of screening active compounds from herbal extracts.

Subsequently, SPR-AIRS was applied to screen STAT3 ligands from medicinal herbs. Nine candidate compounds were screened from complex extracts. SPR assays were performed and seven candidate compounds showed good affinity with STAT3. The action modes of candidate compounds with STAT3 were calculated by CDOCKER. Five candidate compounds which could bind to STAT3 SH2 domain were identified as potential STAT3 ligands. Experimental results reveal that the 5 potential ligands could induce apoptosis of HepG2 cell lines and MCF-7 cell lines. Furthermore, these five compounds could inhibit STAT3-driven transcriptional activity. Neobaicalein and polydatin could inhibit the phosphorylation of STAT3. To the best of our knowledge, this is the first time that neobaicalein and polydatin are reported to be effective STAT3 ligands. To sum up, our study showed that SPR-AIRS is effective and feasible for screening active ingredients from herbal extracts. This research may prove to be an effective solution to analyze active components of Chinese medicinal herbs and other complex drug systems.

ASSOCIATED CONTENT

Supporting Information

Experimental details including materials, experimental condition for UPLC-QTOF-MS analysis, limit of detection and linearity, pre-screening of herbal extracts, small molecular ligands screening from herbal extracts, SPR affinity assay, molecular docking, cell culture, apoptosis assay, luciferase reports assay and western blot. Supporting figures S1-13 including limit of detection and linearity, pre-screening results of 24 herb extracts, chromatograms and mass spectrometries of 8 candidate compounds, SPR affinity assay results, molecular docking results of 4 potential ligands, apoptosis assay results, luciferase reports assay results and western blot results. The Supporting Information is available free of charge on the ACS Publications website.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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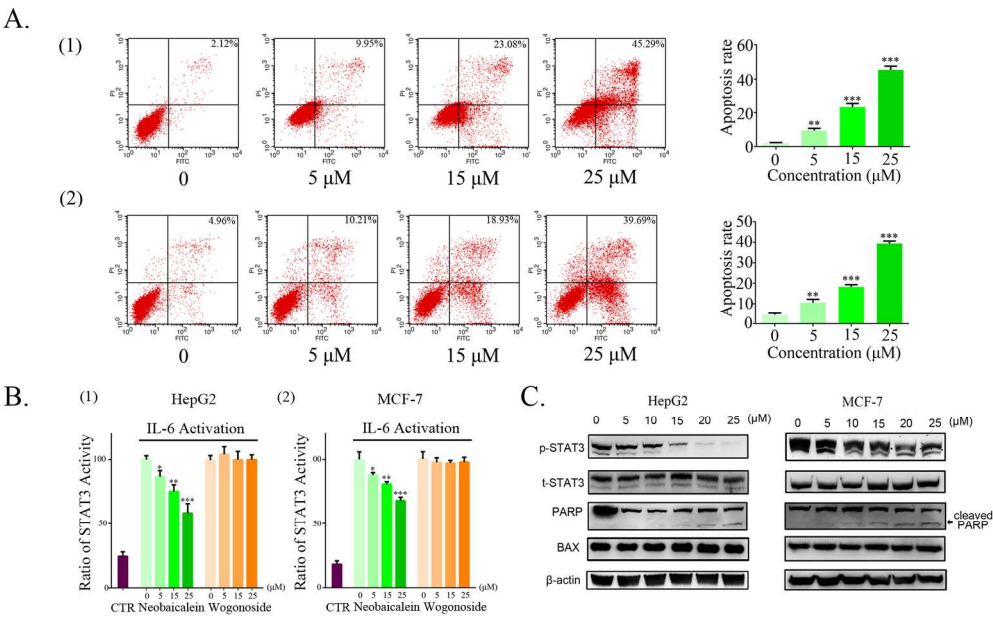


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