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RESEARCH PAPER

Surface plasmon resonance biosensor combined with lentiviral particle stabilization strategy for rapid and specific screening of P-Glycoprotein ligands

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Abstract

A novel surface plasmon resonance–based P-gp ligand screening system (SPR-PLSS) combined with lentiviral particle (LVP) stabilization strategy was constructed to screen out potential P-gp inhibitors from natural products. Firstly, we constructed LVPs with high and low expression levels of P-gp. The LVPs can ensure the natural conformation of P-gp based on the principle that LVPs germinated from packaging cells will contain cell membrane fragments and P-gp they carry. Then the LVPs with high P-gp expression for active channel and LVPs with low P-gp expression for reference channel were immobilized on CM5 chip respectively. The affinity detection was thus carried out with the signal reduction on the two channels. The P-gp inhibitors, Valspodar (Val) and cyclosporin (CsA), as positive compounds, were detected to characterize the chip's activity, and the K_D of Val and CsA were 14.09 μM and 16.41 μM, respectively. Forty compounds from natural product library were screened using the SPR CM5 chip, and magnolol (Mag), honokiol (Hon), and resveratrol (Res) were screened out as potential P-gp ligands, showing a significant response signal. This work presented a novel P-gp ligand screening system based on LVP-immobilized biosensor to rapidly screen out P-gp ligands from natural product library. Compared with traditional cell experiments which the screening time may take up to several days, our method only takes several hours. Furthermore, this study has also provided solid evidences to support that some complicated membrane proteins would apply to the lentivirus-based SPR screening system.

Keywords P-Glycoprotein · Multidrug resistance · Lentiviral particles · Surface plasmon resonance · Screening system · Natural products

Introduction

P-Glycoprotein (P-gp; ABCB1; MDR1), a representative member protein of the ATP-binding cassette (ABC) transporter superfamily, is a vital transporter that crosses the membrane multiple times and has multiple glycosylation site [1]. P-gp shows polarized expression, existing in the plasma membranes of cells in elimination organs and multiple barriers which play an important role in drug transportation and metabolism. The main physiological role of P-gp is to act as efflux pump for a wide range of substrates. It can lead to multidrug resistance (MDR) when highly expressed in cancer cells [2]. Multiple transporters are represented by P-gp, the expression of which has increased in many resistant tumor cells compared with normal cells [3]. Unfortunately, higher level of P-gp boosts the efflux of anticancer drugs through membrane, which can lead to chemotherapy failure [4]. Therefore, co-administration of the antineoplastic drugs and various P-gp inhibitors could increase the anticancer drug concentration via inhibiting the function of P-gp. However, none of P-gp inhibitors can be used in clinic to reverse MDR in cancer treatment until now owing to unacceptable toxicities or insufficient efficacy [5]. Exploitation of new P-gp inhibitors is of great significance for improving the efficacy of anticancer drugs under the circumstance of MDR.

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Up to now, the complexity of P-gp protein and the difficulty of designing proper P-gp ligands caused by this are two major limitations that hinder the exploitation of new P-gp inhibitors. First, P-gp is a complex transmembrane protein which needs accurate lipid environment to ensure the native state of P-gp, but the binding features of P-gp substrates have not been understood fully due to a variety of substrates and substrate-binding sites (SBSs) in P-gp transmembrane domain [6]. The mechanism how the membrane proteins specifically recognize and interact with substrates is unclear, and it is still difficult to obtain purified P-gp protein. Thus, screening of potential P-gp ligands directly is challenging [7]. In addition, nature products and their synthetic intermediates have provided abundant resources for screening P-gp inhibitors with high efficacy, low toxicity, and clear mechanisms [8]. In the past few years, some studies have analyzed the existing P-gp inhibitors by three-dimensional quantitative structure-activity relationship (3D-QSAR). Most of the inhibitors have common physical and chemical properties: (1) a hydrophobic center and a certain fat solubility; (2) a cationic structure or basic center under the physiological pH range; (3) at least two hydrophobic aromatic rings and coplanar aromatic rings and as active sites; (4) a certain spaced apart hydrogen bond acceptor (O or N atom), or hydrogen bonding ligand (OH or NH group) [9–11]. Based on the above physical and chemical properties, we have established natural product structure database containing 200 small-molecule compounds, which can help quickly screen out efficient and specific P-gp potential inhibitors.

Several methods that could characterize the interactions between P-gp and test drugs have been established to screen out potential P-gp ligands in vitro, such as bidirectional transcellular transport assay, molecular docking, and cell membrane chromatography (CMC). Bidirectional transcellular transport assay is identified as the gold standard for screening out P-gp substrates in vitro [12]. The procedures of transport assay for assessing P-gp substrates and inhibitors have previously been published in their guidance by the FDA [13]. Zang et al. [14] confirmed that deoxypodophyllotoxin could solve the problem of P-gp-mediated MDR using this method. Thus, deoxypodophyllotoxin can be identified as a promising agent for overcoming the challenge of MDR cancers. However, the shortcoming of monolayer efflux assays is relatively low throughput with the problem of time-consuming and labor-intensive [15]. Molecular docking is a useful method for screening out ligands of P-gp. Klepsch et al. [16] have built on the mouse P-gp binding to the cyclic peptide QZ59-RRR and implemented a docking protocol to discriminate a large group of 1608 inhibitors and non-inhibitors of P-gp. However, there are several challenges and limitations for the flexibility inherent to P-gp and no human-crystallized P-gp has been made. Although a set of crystal structures of P-gp has been found, they only cover a small part of the conformational space of the protein, which makes the results less accurate than experimental structures [17]. Cell membrane chromatography shows a promising prospect in screening out potential active components for membrane proteins (MPs). Our group established a CMC method and aimed to screen out P-gp ligands from traditional Chinese medicine preparations, which have potential anticancer activity. However, the presence of many membrane proteins on the cell membrane makes this method less specific and not suitable for screening out P-gp ligands [18]. The existing methods cannot fully meet the demands for screening out P-gp ligands; thus, it is very urgent and valuable to establish a high-throughput screening method to fish out potential P-gp inhibitors from various candidates.

Surface plasmon resonance (SPR) biosensor could be used to detect kinetic information of the candidate ingredients, which makes it special in the application of screening out ligands [19]. Vega et al. established a lentivirus-based strategy that indirectly immobilizes native MPs (C-X-C chemokine receptor type 4 (CXCR4)) on SPR chips [20]. The principle of this strategy is that lentiviral particles (LVPs) germinated from packaging cells will contain cell membrane fragments and the proteins they carry [21]. The benefits of LVPs for label-free experiments are high receptor expression level, high activity, stability, mini-size, mono-dispersity, and fixed orientation of proteins [22]. As described above, P-gp is a complex transmembrane protein without very clear binding profiles and is more complicated than the CXCR4 we investigated before. Whether such a membrane protein could work in the lentivirus-based SPR screening system is unknown.

In this research, a novel surface plasmon resonance–based P-Glycoprotein ligand screening system (SPR-PLSS) combined with lentiviral particle stabilization strategy was established and aimed to directly screen out ligands targeting P-gp from the natural product library we established. First, P-gp was genetic engineering expressed on the surface of LVPs ($P\text{-gp}^{+}$/LVPs) and mock lentiviral particles ($P\text{-gp}^{-}$/LVPs) were also prepared as controls (Fig. 1a). Secondly, $P\text{-gp}^{+}$/LVPs and $P\text{-gp}^{-}$/LVPs were immobilized on the SPR biosensors, respectively, to construct the SPR screening system, and the activity and suitability were verified by positive and negative controls (Fig. 1b). Thirdly, forty compounds from natural products were selected as candidates and screened by the system. Three compounds showed significant response signals that could directly bind to P-gp (Fig. 1c). Lastly, the activity verification of the compounds was conducted by cell experiments (Fig. 1d). Compared with traditional cell experiments, the SPR-based method can save lots of time and improve screening efficiency. Up to now, it is the first time that the lentivirus-based SPR screening strategy was successfully used in the study of P-gp ligands. The results have further proved that the screening system has wide applicability regarding the membrane proteins, no matter how complex the protein structure is and whether the binding sites are known or

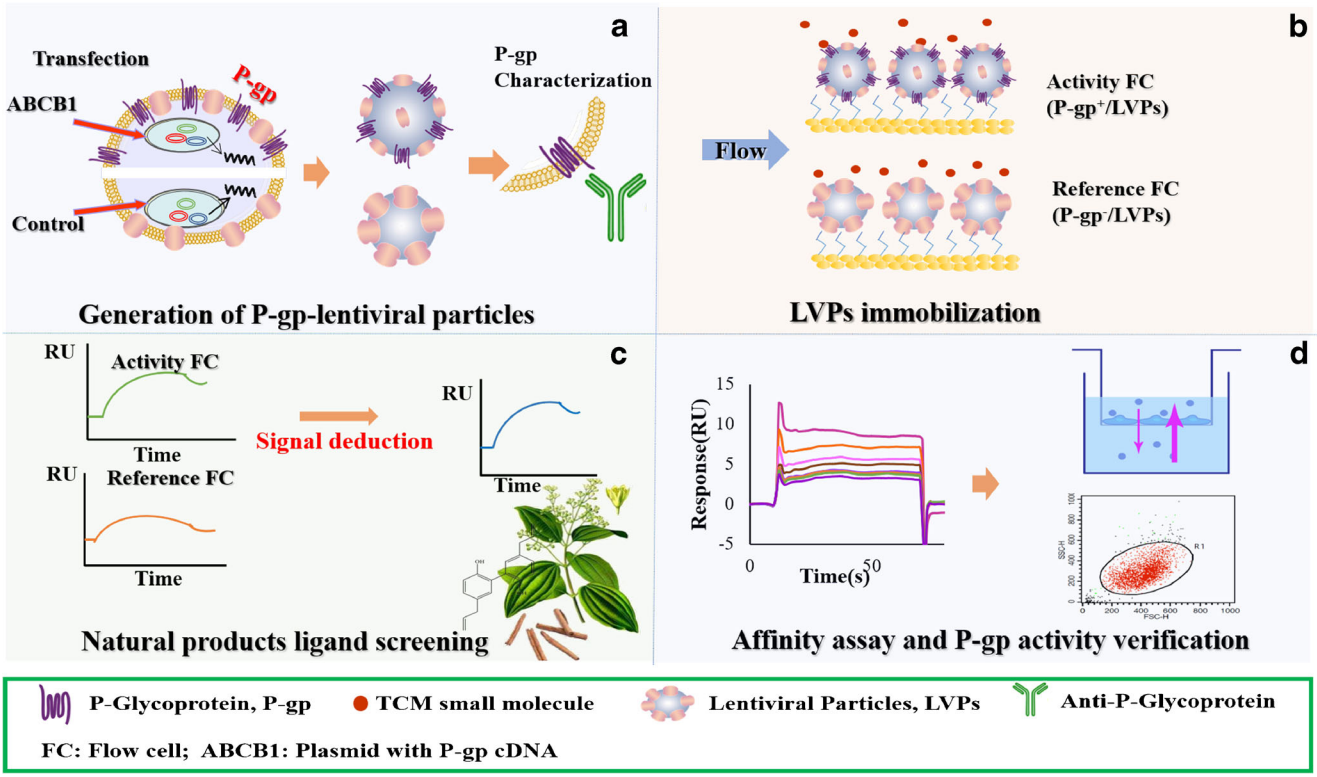

Fig. 1 The workflow for screening out P-gp small-molecule ligands from natural products based on SPR-PLSS. **a** The different expression of P-gp LVPs was produced. **b** The different expression of P-gp LVPs was immobilized on different channels of the chip. **c** The affinity between natural products and P-gp was obtained by two-channel signal deduction, and the target compounds which a significant response signal are screened out. **d** The effects of active compounds on P-gp function and expression are verified

not. As far as we know, there is no report about LVPs ensuring the correct conformation of P-gp and the detection of the interaction between P-gp and ligands based on SPR.

Experimental section

Materials and reagents

Lentiviral particle packaging plasmids were purchased from OBiO Technology Co. (Shanghai, China), including envelope vector, packaging vector, and transfer vector. Plasmid was designed and synthesized: P-gp cDNA (pSLenti-EF1-EGFP-F2A-Puro-CMV-ABCB1-6xHis-WPRE); control (pSLenti-EF1-EGFP-F2A-Puro-CMV-MCS-WPRE). Rabbit anti-P-gp antibody (ab170904) for western blot (1:1000) was purchased from Abcam, and monoclonal mouse Anti-p24 antibodies (MAB7360-SP) was purchased from R&D. Mouse monoclonal anti-P-gp (Phycoerythrin) antibody (ab93590) for flow cytometry was purchased from Abcam. P-gp rabbit pAb (A11758) used for immunofluorescence (IF) (1:50) was purchased from ABclonal (Wuhan, China). All standard compounds were purchased from Standard Technology Co. (Shanghai, China). EDC, CM5 chips, ethanolamine, and NHS were purchased from GE Healthcare (Shanghai, China). Transwell filters were purchased from Corning Costar (Cambridge, MA, USA). Rh-123 was purchased from the Sigma Chemical Co. (St Louis, MO, USA), and RPMI 1640 and fetal calf serum and 0.25% trypsin were purchased from Corning Costar (Cambridge, MA, USA). Primer pairs and probe were purchased from Sangon Co. (Shanghai, China). The purity of other chemicals was highest available.

The production and purification of lentiviral particle

P-gp over-expression was performed using a lentiviral packaging system. To construct P-gp over-expressing LVPs, full-length P-gp (NM_000927) was cloned into the expression vector pSLenti-EF1a-EGFP-P2A-Puro-CMV-MCS-WPRE (OBiO Technology, Shanghai, China). P-gp and control were transfected into HEK293T cell lines according to the manufacturer's instructions, respectively. Transfection HEK293T cells were with 32 μg plasmid. Envelope vector and packaging vector: Transfer vector plasmids were at a ratio of 1:1. Meanwhile, 293T cells were classified into two groups: control group with an empty vector in transfer vector plasmid and experimental group with P-gp cDNA plasmid. The supernatant was harvested and combined after 48 h and 72 h post-transfection of plasmids. Cell fragments were got rid of by centrifugation at a low speed with 0.22 μm filter. Then the

supernatant was ultra-speed centrifuged at 30000 rpm for 2 h at 4 °C. Resuspend the lentivirus precipitated on the bottom of the ultracentrifuge tube with PBS and stored at − 80 °C.

Characterization of P-gp on LVPs

P-gp on LVPs was characterized by western blots, flow cytometry analysis, and immunofluorescence analysis, respectively. Western blots: Protein analysis was performed by western blot with specific antibodies. LVP extracts were analyzed in western blot with anti-p24 (2 μg/mL), and anti-P-gp (1:2000) mAb. The blots were analyzed by scanning densitometry using an Odyssey infrared imaging system and the gray value is calculated by ImageJ software. Flow cytometry: LVPs were mixed with aldehyde/sulfate latex beads [23]. Then the LVP beads were incubated with PE-labeled anti-human P-gp mAb based on manufacturer's instruction, and the non-specific labeling was corrected by its isotype control. Finally, samples were injected into flow cytometry to measure the fluorescent intensity. Immunofluorescence analysis: LVPs were mixed with aldehyde/sulfate latex beads. After incubated with 2% bovine serum albumin for 1 h at RT, the LVP bead was incubated with primary antibodies against P-gp (1:200) overnight at 4 °C. The LVP bead was washed twice with PBS and incubated for 2 h at RT with HRP-conjugated goat anti-Rabbit IgG (H + L) (1:5000) in the dark. The sample was washed three times with PBS, and 4′,6-diamidino-2-phenylindole stain was then added dropwise in a circle, and incubated for 10 min at RT in the dark. After removing the supernatant by centrifugation, the precipitate was resuspended with anti-fluorescence quencher and added to the slides. Samples were observed under an inverted fluorescent microscope.

Immobilization of LVPs on the CM5 chip

SPR assays were performed on Biacore T200 system (GE Healthcare, Sweden). The physical absorption response signal of LVPs on CM5 chips with three different pH buffers (4.0, 4.5, and 5.0) was tested to find the optimal buffer. Then the physical absorption response signal of LVPs on CM5 chips at three different concentrations (100, 200, and 500 μg/mL, use total protein concentration of LVPs as reference) was detected to find the optimal concentration of LVPs. LVPs were immobilized on chips based on EDC/NHS cross-linking reaction [24]. For affinity detection module, flow cell (FC) 1 as reference flow channel was immobilized with LVPs with low P-gp level (P-gp$^-$/LVP) and FC2 as activity flow channel was immobilized with LVPs with high P-gp expression (P-gp$^+$/LVP).

SPR characterization of P-gp immobilized on LVPs

Valspodar (Val), cyclosporin (CsA), and dexamethasone (Dex) solution were used to detect affinity by using affinity detection module. The flow rate was set as 30 μL/min and contact time was 60 s. Then, different concentrations of Val and CsA solutions were injected at the same flow rate and contact time as above for kinetic analysis, respectively.

Kinetic analysis

The affinity detection module was applied to collect binding kinetic information. Firstly, 5% DMSO PBS was used to dilute compounds into a series of concentrations. Then, analytes were injected into the SPR machine and the flow rate was set as 30 μL/min. Sensor grams were corrected for obtaining signals in the reference flow channel, which is a control chamber with P-gp$^-$/LVP. The dissociation and association times were both set as 120 s. All experiments were conducted according to the automated robot of the system, and all stages were tracked in real time in response to changes in the signal represented by the unit (RU). The Biacore T200 evaluation software was applied for affinity fitting to obtain the affinity constant using a steady-state affinity model (1:1).

P-gp ligand screening

5% DMSO PBS was used to dilute standard compounds (64 μM) and a series of concentrations were injected into the SPR machine using affinity detection module at a 10 μL/min flow rate, followed by a 30-s injection period of running buffer alone over the surface (dissociation phase). All steps were performed using manual injection**.** Further affinity validation was performed followed above when the RU on the activity flow channel was higher than the reference flow channel. A total of 40 compounds from natural products were screened in this experiment.

Cell lines and culture conditions

MCF-7 cells and MCF-7/ADR cells were purchased from Shanghai Xinyu Biological Technology Co., Ltd. (Shanghai, China). MCF-7 and MCF-7/ADR cells were cultured in RPMI 1640 containing 10% (v/v) fetal bovine serum and 1000 μg/mL glutamine, and 1000 ng/mL Adriamycin (Adr) was added to the MCF-7/ADR cells. MDR1-transfected Medin-Darby canine kidney cell lines (MDCK-MDR1) were kindly provided by Dr. Zeng (Zhejiang University). Cells were cultured in Dulbecco's modified eagle's medium containing 10% (v/v) fetal bovine serum and two antibiotics (100 μg/mL streptomycin and 100 U/mL penicillin). All cells were cultured at 37 °C in 5% CO_2.

Rhodamine123 (Rh123) transport experiment

Twelve-well Transwell plates were used to perform the bidirectional flux studies, and 5×10^5 cells were seeded per well, and the medium was changed into fresh medium every other day until confluent monolayers were formed. When doing the experiments, the culture medium was discarded and assay buffer was used to wash the cells twice. The experiments were performed by replacing the transport medium in the apical side for absorption transport (0.5 mL) or basolateral side for efflux transport (1.5 mL) with the potential compounds and Val (5 μM) for 30 min preincubation. Rh123 (10 μM) bidirectional transports with or without the presence of potential compounds were carried out. Samples (100 μL) were collected from the receiver compartment at 15, 60, 90, 120, and 180 min and replaced with drug-free assay buffer. In addition to a short sampling time, the Transwell plate was incubated on an orbital oscillator at 37 °C for the entire experimental time. The fluorescence was measured at 485 nm (excitation wavelength) and 528 nm (emission wavelength) on a Cary Eclipse Fluorescence spectrophotometer (Varian Inc., Palo Alto, CA, USA). The apparent permeability (P_{app}) for the directional transport was calculated using the following equation [25]. The permeability was:

$$P_{app}\ (\mathrm{cm/s}) : P_{app} = \{dQ/dt\} \times \{1/(A \times C_0)\}$$

In this equation, Q means the accumulation quantity of the test compound in the receiver side (μM), dQ/dt means the linear appearance rate of the compound in the receiver side (μM/s), C_0 means the initial concentration in the donor side (μM), and A means the surface area of the membrane insert (cm^2).

$$\text{Efflux Rate (ER)} = P_{app}\ (\mathrm{BL{-}AP})/P_{app}\ (\mathrm{AP{-}BL})$$

BL-AP means from basolateral to apical, and AP-BL means from apical to basolateral.

Cell viability assay

To detect the cytotoxicity of compounds, 5×10^3 cells per well were seeded into 96-well plates and CCK8 reagent was used as previous description [26]. After incubated for 48 h with 5 μM compounds, the cell viability was detected. The cytotoxicity toward MCF-7/ADR induced by Adr was also detected by CCK8 assay after incubation of cells with Adr with or without pretreatment with different compounds.

Determination of P-gp expression

Western blot analysis: MCF-7/ADR cells were treated for 48 h with 5 μM compounds in cell growth medium and were then incubated in ice-cold lysis buffer for 20 min and collected by centrifugation (12,500g) at 4 °C for 10 min. The protein concentration of supernatants was determined by Bradford assay, and the P-gp expression was analyzed by western blot with P-gp mAb and GAPDH mAb. Flow cytometry analysis: MCF-7/ADR cells were treated for 48 h with 5 μM compounds in cell growth medium and trypsinized from the confluent monolayer of MCF-7/ADR cells, and the cell pellet was washed twice with ice-cold PBS. Cells were incubated with PE-labeled anti-human P-gp mAb based on manufacturer's instruction, and the fluorescent intensity was measured by flow cytometry.

Results and discussion

Lentiviral particle characterization

First, western blot assays were performed to analyze the influence of the plasmid designed on P-gp expression. 293T cells were lysed and proteins were extracted 24 h after plasmid transfection. The result was shown in Fig. 2a, indicating that P-gp plasmid could effectively enhance the expression of P-gp on 293T cells, and the empty vector plasmid did not have a significant influence on the expression of P-gp on 293T cells. Then, we evaluated the P-gp expression levels on two groups of LVPs by western blot, flow cytometry, and immunofluorescence with specific antibodies. As shown in Fig. 2b, the expression of P-gp on the P-gp$^+$/LVP was significantly higher than the P-gp$^-$/LVP. The results demonstrated that the transfection of P-gp plasmid could remarkably enhance the expression of P-gp on 293T cells. Combining with results from flow cytometry (Fig. 2c), the expression of P-gp$^+$/LVP was relatively 1.5 times higher compared with its counterpart on P-gp$^-$/LVP. P-gp$^+$/LVP were stained with anti-P-gp mAb and analyzed by immunofluorescence (Fig. 2d), which further demonstrated the higher expression of P-gp on P-gp$^+$/LVPs. These results indicated that LVPs with different P-gp level between the two groups were produced successfully. Cells in treated group transfected with P-gp plasmid were used to produce LVPs with high P-gp expression level (P-gp$^+$/LVP), while cells in the control group transfected with empty vector plasmid were used to produced LVPs with low P-gp expression level (P-gp$^-$/LVP). Then, these P-gp-LVPs were immobilized on CM5 chips to screen out active drugs.

LVP immobilization and system validation

Next, we coupled the lentiviral particles to the SPR chip to construct the SPR-PLSS. In order to improve the immobilization of LVPs on the chips with high activity and response, we had optimized the conditions for LVP immobilization. At last, the condition of pH 4.0 buffer and 500 μg/mL (total protein concentration of LVPs) of LVP concentration was regarded as the optimal, as shown in Fig. 3. P-gp$^-$/LVP and P-gp$^+$/LVP

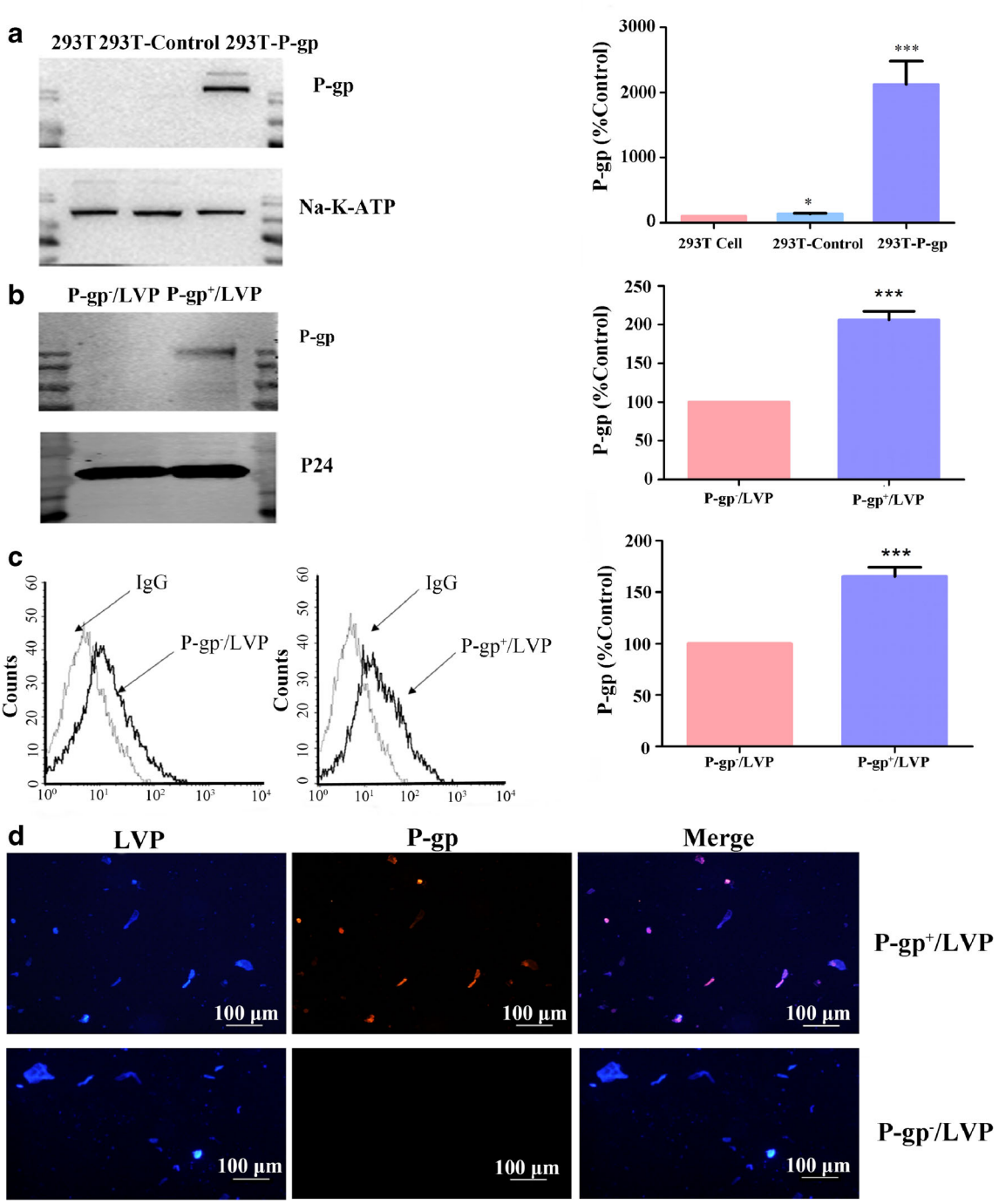

Fig. 2 Characterization of the P-gp expression levels on the surface of LVPs. **a** P-gp expression on 293T cells with different treatments was analyzed by western blot, and Na-K-ATP was used as an internal control (***$p < 0.001$, 293T-P-gp versus 293T cells). **b** P-gp expression on P-gp$^+$/LVP and P-gp$^-$/LVP was analyzed by western blot, and p24 was internal control (***$p < 0.001$, P-gp$^+$/LVP versus P-gp$^-$/LVP). **c** P-gp expression on P-gp$^+$/LVP and P-gp$^-$/LVP was analyzed by flow cytometry. (***$p < 0.001$, P-gp$^+$/LVP versus P-gp$^-$/LVP). **d** Immunofluorescence staining was used to detect the expression of P-gp on P-gp$^+$/LVP and P-gp$^-$/LVP. Blue fluorescence indicates nuclei of the LVPs, and red fluorescence indicates the target protein (P-gp)

were immobilized on FC1 and FC2 (about 8000 RU) of a CM5 chip, respectively.

To verify the specificity of the system, CsA and Val, which are known P-gp ligands, were analyzed and Dex was set as negative compound for verification. CsA and its analogs Val are inhibitors of P-gp and part of the binding site is in the extracellular segment [27]. The results indicated that CsA and Val could have response signals on the chip as shown in Fig. 4a, while Dex has no response signal on the chip. As expected, the small-molecule ligand can only bind to the

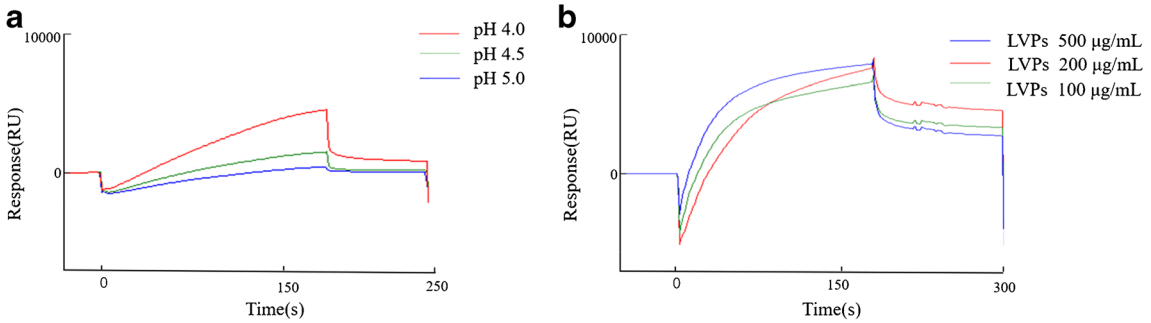

Fig. 3 Immobilization conditions scouting, including buffer pH (**a**) and LVP concentration (**b**)

extracellular segment of the membrane protein. Then, we analyzed the kinetic parameters of CsA and Val. The K_D of CsA and Val were determined as 16.41 μM (Fig. 4b) and 14.09 μM (Fig. 4c), respectively. The results indicated the feasibility of the developed system on determining the affinity of small molecular ligands of P-gp; therefore, it could be used to screen out P-gp ligands.

P-gp ligand screening from natural products

Next, the system was applied for screening out P-gp ligands from natural products. Forty natural products were collected from the structure database, including flavonoids, coumarins, alkaloids, and others (see Supplementary Information (ESM) Table S1). We found magnolol (Mag), honokiol (Hon), and resveratrol (Res) have good response signals on the chip, and the list of compounds and specific experimental results is shown in the ESM (Fig. S1). The structures of the three compounds were shown in Fig. 5d. To validate the interactions between active compounds and P-gp, SPR affinity test was performed by using the affinity detection module. SPR assay revealed the active compounds could bond to P-gp with good affinity, and the K_D values of Mag, Hon, and Res were 15.88 μM, 65.44 μM, and 70.01 μM, respectively, as shown

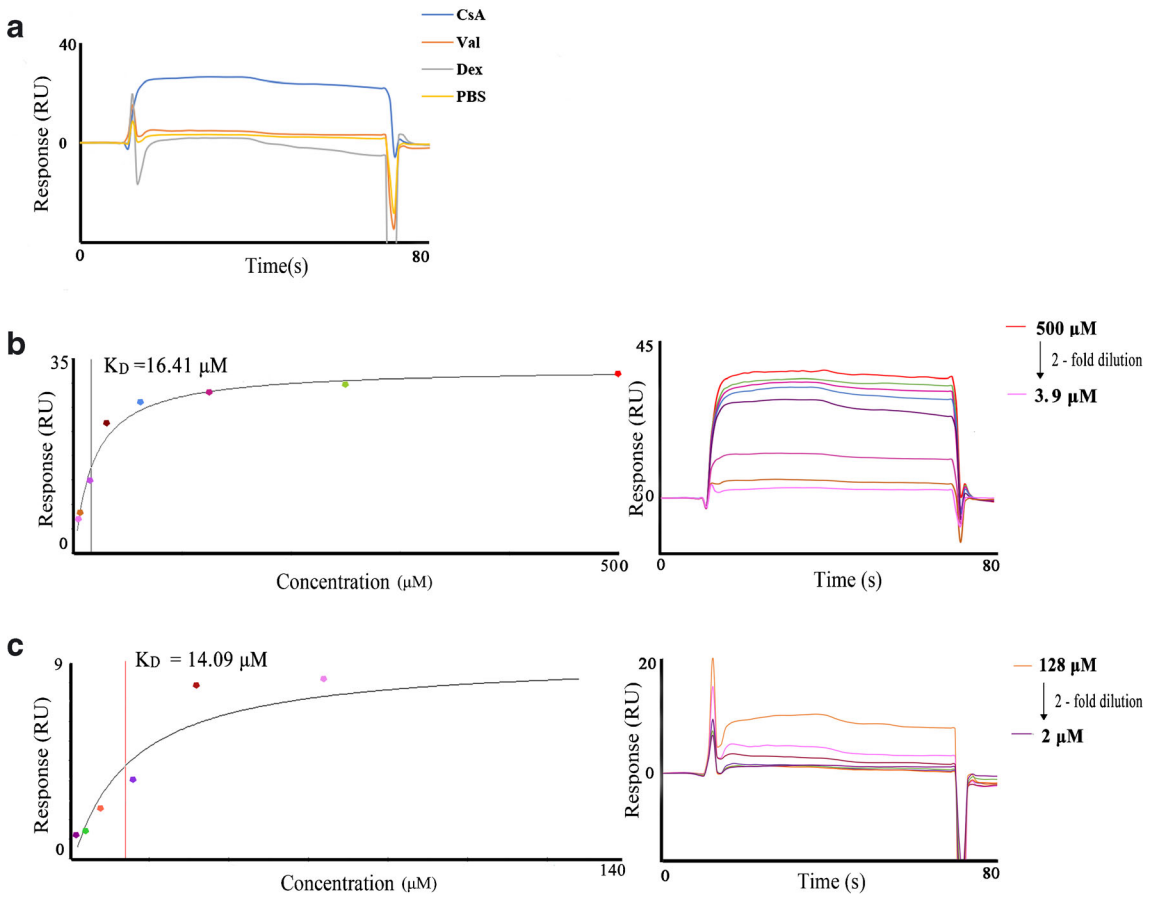

Fig. 4 The sensorgrams of different compounds on SPR sensor chip **a**. The kinetic parameters of interaction between CsA (**b**), Val (**c**) and P-gp

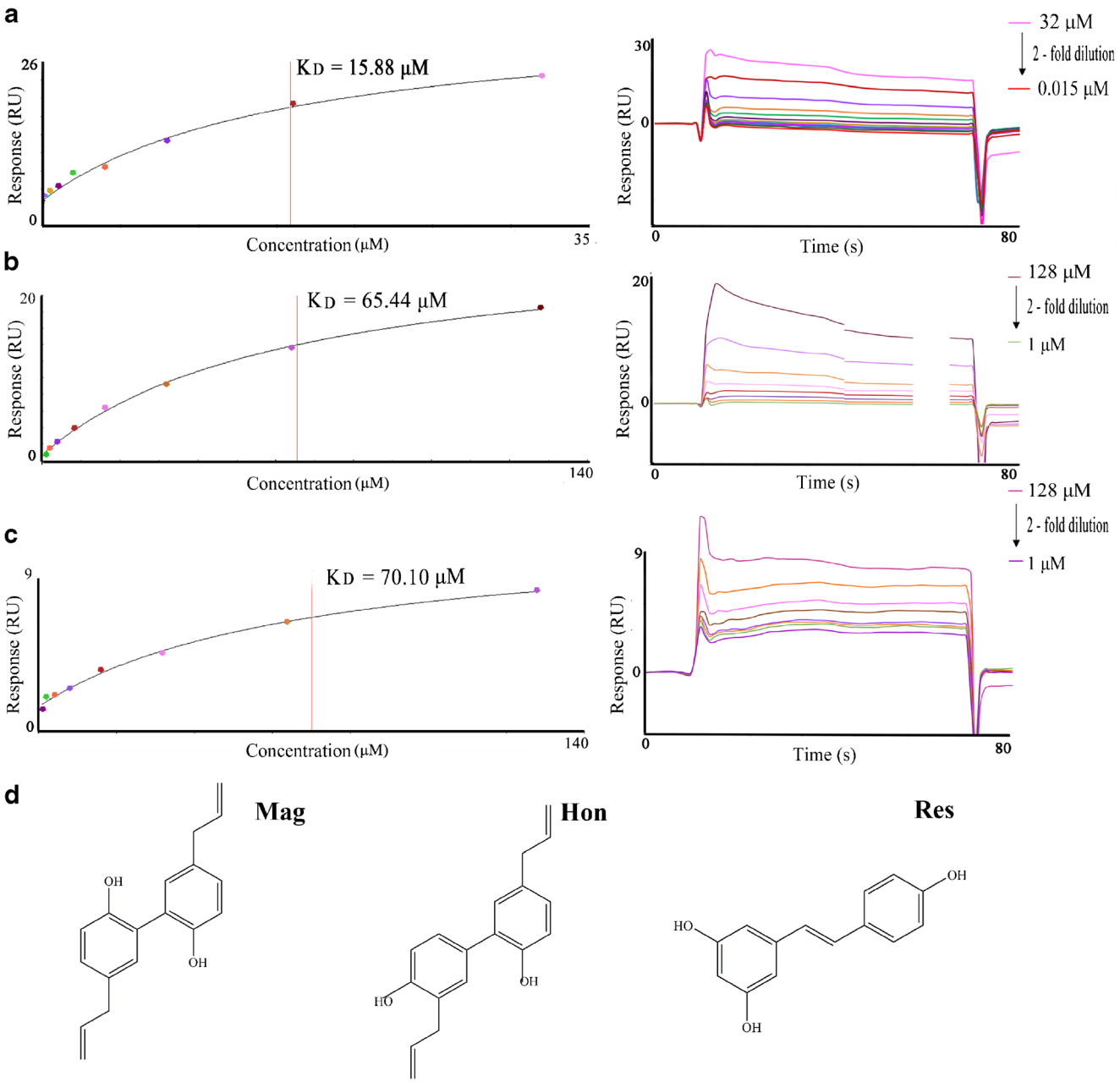

Fig. 5 The affinity between Mag (**a**), Hon (**b**), Res (**c**), and P-gp, respectively, and the structure of the active compounds which could bind to P-gp with good affinity (**d**)

in Fig. 5, and the parameters of affinity detection were listed in Table 1. The three compounds were selected as active compounds for further study.

Table 1 Parameters of affinity detection for active compounds

Compounds	K_D (μM)	R_{max} (RU)	Chi^2 (RU^2)
Mag	15.88	20.46	0.216
Hon	65.44	26.52	0.137
Res	70.01	8.16	0.030

Effects of active compounds on P-gp-mediated Rh123 transport in MDCK-MDR1

The activity of the active compounds screened needs to be verified. We firstly conducted the Rh123 transport experiment to investigate the effect of Mag, Hon, and Res on P-gp function. Rh123, a substrate for P-gp, has been widely used as an indicator for testing the activity of P-gp. Before analyzing their effects on P-gp function, the cytotoxicity of Mag, Hon, Res, and Val was performed by using the CCK8 assay. Over 95% cell viability was maintained in MDCK-MDR1 cells after 24-h exposure to these potential compounds and Val

Table 2 The effect of active compounds on the bidirectional P_{app} values and ER of Rh123 in the MDCK-MDR1 cell monolayer ($n = 3$)

Compounds	Control		Val		Mag		Hon		Res	
	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP
P_{app} ($\times 10^{-6}$)	2.38	5.28	3.92	3.91	2.59	3.97	5.55	5.08	2.38	3.67
ER	2.35		1.11*		1.72*		1.43*		1.64*	

*$p < 0.05$

was 20 μM or less based on their solubility in HBSS solution as shown in ESM (Fig. S2). The individual non-toxic concentrations for active compounds and Val were 5 μM, and the specific results are shown in the ESM. The inhibitory effects of four compounds on P-gp-mediated Rh123 transport were explored in MDCK-MDR1 cells. In our study, we found that Val, Mag, and Hon exhibited significant inhibition effect on P-gp as P_{app} (BL-AP) of Rh123 across the MDCK-MDR1 cells was decreasing and P_{app} (AP-BL) was increasing, while Res also showed inhibition on Rh123 transport in this cell model with markedly decreasing P_{app} (BL-AP) and no P_{app} (AP-BL) changing, as shown in Table 2. Val, Mag, Hon, and Res decreased the efflux rate (ER) of Rh123 markedly.

The reversal effect on MDR of MCF-7 and MCF-7/ADR cells

Then, we selected MCF-7 cells for further experiments to see whether Mag, Hon, and Res can reverse MDR. We firstly tested the cytotoxicity of Mag, Hon, Res, and Val on MCF-7 and MCF-7/ADR cell. The results indicated that all compounds at the range of 0.1~5 μM was not able to significantly inhibit the growth of MCF-7 and MCF-7/ADR cells, and the specific results were shown in the ESM (Figs. S3~S4). To assess the effects of Mag, Hon, and Res on toxicity caused by Adr, CCK8 assay was used to evaluate the Adr-induced toxicity toward MCF-7 and MCF-7/ADR with or without treatment with above four compounds. The results indicated that treatment with Hon and Res in MCF-7/ADR cell could significantly increase the toxicity toward Adr. As shown in Fig. 6, the IC_{50} of Adr toward MCF-7/ADR was 9.56 ± 0.60 μg/mL and 8.00 ± 1.60 μg/mL with treatment of Hon and Res, respectively, which were lower than that without treatment of compounds (17.54 ± 4.54 μg/ml). Therefore, these results indicated Hon and Res could reverse the MDR of MCF-7/ADR cells.

Mag and Hon downregulate level of P-gp

Rh123 transport experiment indicated the Mag, Hon, and Res could inhibit the efflux of Rh123. In MCF-7/ADR cells, Res was also able to increase the intracellular Adr concentration.

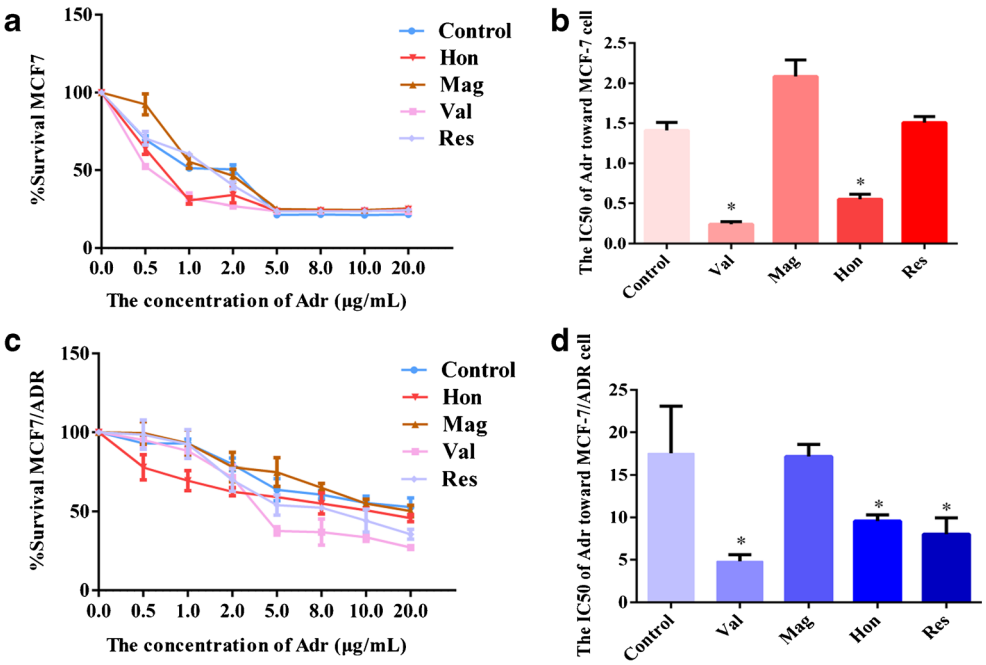

Fig. 6 Effects of Mag, Hon, Res, and Val on the sensitivities of MCF-7 (**a**, **b**), and MCF-7/ADR (**c**, **d**) toward Adr tested by a CCK8 assay. The cells were incubated with 5 μM compounds for 48 h. Data are shown as mean ± SD of three independent experiments ($n = 3$, *$p < 0.05$)

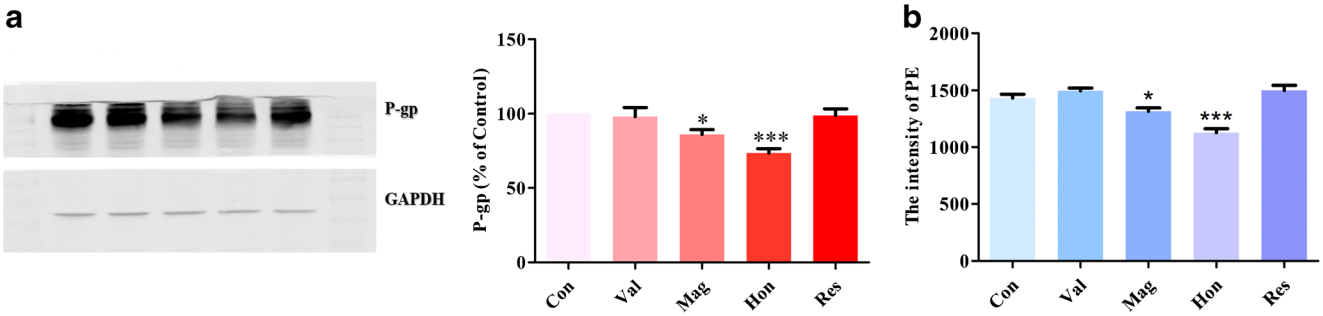

Fig. 7 The expression of P-gp in MCF-7/ADR cells when preincubation of cells with Val, Mag, Hon, and Res (5 μM) for 48 h was characterized by western blot (**a**) and flow cytometry (**b**) (Mag versus control group, $*p < 0.05$; Hon versus control group, $***p < 0.001$)

We next performed western blot assays to find out whether Mag, Hon, and Res are downregulators of the expression of P-gp. In Fig. 7a, the results showed that the level of P-gp was decreased when there is pretreatment of MCF-7/ADR cells with Hon and Mag. Then, the inhibition of P-gp expression was further investigated by flow cytometry. As shown in Fig. 7b, cells were pretreated with compounds at 5 μM for 48 h and measured for P-gp by flow cytometry. The results also indicated Hon and Mag were able to decrease P-gp level in MDR cancer cells. Hon could lead to apoptosis in human B cell chronic lymphocytic leukemia with apoptotic resistance [28]. Wang et al. found that Res could enhance the antiproliferative activity of bestatin in K562/ADR cells [29]. These researches deeply proved the feasibility of this method for screening out small-molecule ligands to P-gp.

Conclusion

In this research, we established a novel SPR-PLSS combined with LVP stabilization strategy aiming to screen out potential inhibitors of P-gp. First, we constructed P-gp differently expressed LVP models and characterized the expression of P-gp. The LVPs with different expression of P-gp were immobilized on different flow cells of CM5 chip, and the screening system was established and used to screen out ligands. The known P-gp inhibitors CsA and its derivative Val showed that the SPR-PLSS can be used to study the interaction between small-molecule ligands and the extracellular segment of P-gp. Then the system was applied in the screening out P-gp ligands from natural products. Three active compounds (magnolol, honokiol, resveratrol) were screened out by the system, and the affinity constants between three compounds and P-gp were 15.88, 65.44, and 70.01 μM, respectively. Cell experiments proved that magnolol, honokiol, and resveratrol had the inhibitive effect on P-gp, of which magnolol and honokiol could decrease the expression of P-gp in the MDR cancer cells. However, at present, the activities of compounds screened by SPR are limited. In order to improve the activities, we will perform chemical modification on the structures of compounds, and the activities of reversing the MDR in cancer should be further explored for future application in clinic when they were used as a single agent or together with other anticancer agents.

In conclusion, we have for the first time established a screening method based on LVPs to screen out small molecular compounds that could bind with the extracellular segment of P-gp in vitro. The lentiviral particles can ensure the natural conformation of P-gp and will help to discover potential P-gp inhibitors that could be used to reverse P-gp-mediated MDR in cancer during clinical tumor chemotherapy. Compared with traditional cell experiments, this method can shorten the screening time from several days to a few hours when screening 40 compounds. Furthermore, this study has also provided solid evidence to support that some complicated membrane protein of interest would apply to the lentivirus-based SPR screening system, and this system is an alternative complement to existing active screening methods by giving reliable results efficiently.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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