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Capillary electrophoresis using immobilized whole cells with overexpressed endothelin receptor for specific ligand screening

A new capillary electrophoresis method using immobilized cells as the stationary phase has been developed. The power of this method is demonstrated by the separation and identification of endothelin antagonists on a capillary column coated by the transfected Chinese hamster ovary (CHO) cells with overexpressing endothelin receptors. The screening results are validated by functional assays suppressing the increase of intracellular calcium concentration induced by endothelin-1. Instead of making efforts in isolating protein receptors, the easily prepared whole-cell capillary column provides a superior tool on the basis of ligand/receptor affinity for a rapid screening of potent drug candidates from compound libraries.

Keywords: Affinity capillary electrophoresis / Endothelin receptor antagonists / High-throughput screening / Immobilized whole cells / Receptor-ligand interaction
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1 Introduction

Many biological events are triggered by ligand/receptor interactions. For example, endothelin-1 (ET-1), a 21-peptide ligand locally produced in various cell types under different physiological stimuli, has a strong affinity toward endothelin receptor A (ET_A) located on the surface of an endothelial cell membrane [1–3]. This ligand/receptor interaction is coupled with G-protein, which triggers a series of biological events to induce an increase of intracellular calcium concentration, $[Ca^{2+}]_i$ [4]. Antagonism of the endothelin vasoconstrictor is a potential approach to the treatment of a variety of human diseases including hypertension and congestive heart failure [5]. Screening of natural and synthetic compounds based on the concept of ligand/receptor recognition is an indispensable strategy to search for endothelin receptor antagonists [6–9].

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Abbreviations: ACE, affinity capillary electrophoresis; **CHO cells**, Chinese hamster ovary cells; **ET_A**, endothelin receptor A; **ET-1**, endothelin 1; **ET-1$^{(16–21)}$**, endothelin 1 fragment containing 16–21 residues; **FBS**, fetal bovine serum

Affinity capillary electrophoresis (ACE) is a powerful separation method with the advantages of high resolution, small sample requirement, rapid sample throughput, and compatibility to biological conditions [10–14]. The components in an analyte can be separated by ACE due to their different electrophoresis motilities. By coupling with various sensitive detectors, the ACE technique is widely used to separate bioactive compounds and to determine the biomolecular noncovalent interactions [15–18]. When ACE is applied to screen active ligands in an analyte solution, the target receptor is often immobilized as the stationary phase on the inner wall of a capillary column [10–20]. However, this approach may encounter a problem in isolating of the desired receptors in sufficient quantity. Also, many membrane-bound receptors are unstable in isolation, as is the case of ET_A. Another problem is that receptors may lose their active conformations upon conjugation to capillary columns. We therefore investigated the possibility of using whole cells with overexpressing receptors, in lieu of the isolated receptors, as the stationary phase in ACE for the evaluation of active ligands [21, 22].

In this study, we demonstrate that a whole-cell stationary phase consisting of ET_A-overexpressing Chinese hamster ovary (CHO) cells provides a successful ACE protocol for the screening of the ET_A-specific ligands. The peptide and non-peptide ET_A antagonists (Fig. 1) were satisfactorily resolved on ACE, in accordance to the order of their affinity and antagonist potency toward ET_A.

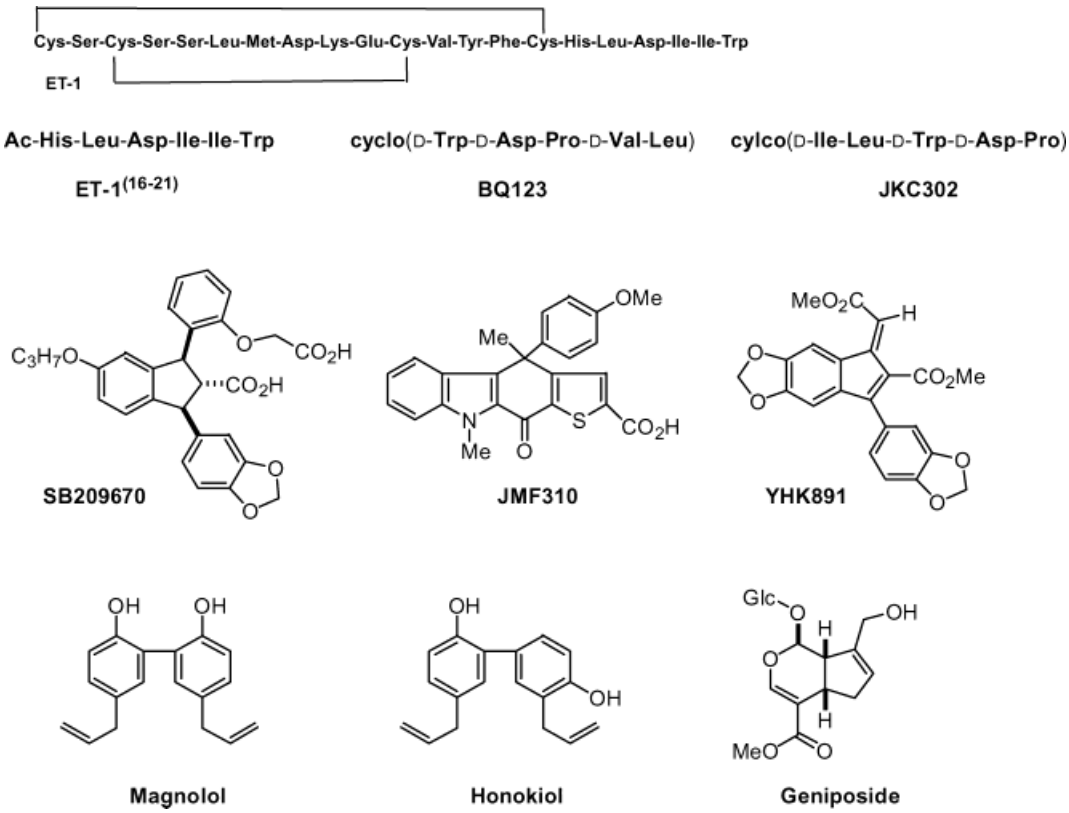

Figure 1. ET-1 and the examined substrates in this study.

2 Materials and methods

2.1 Construction of CHO-K1 cell line overexpressing ET_A and ligand-binding assay

The lipofectin-mediated transfection method described by Tseng *et al.* [23] was used to construct stable CHO cell lines overexpressing ET_A. Cells were grown to 30–40% confluence in 60 mm dishes and transfected with 1 μg of pcDNA-3 expressing plasmid harboring ET_A using lipofectin reagent for 6–8 h in serum-free medium. Cells were then returned to 5% fetal bovine serum (FBS), cultured 36 h, then replated at reduced density in 150 mm plates in the presence of 0.75 mg/mL (active) G418. G-418 resistant colonies were selected and screened for ET_A by binding of (3-[^{125}I]iodotyrosyl)endothelin-1 ([^{125}I]ET-1). Binding was conducted to cells plated in 24-well dishes at 2–3 × 10^5 cells/mL the day before the binding assay. For cell-binding assays, [^{125}I]ET-1 (10 pM) was added to HR buffer (5 mM NaCl, 4.7 mM KCl, 1 mM Na_2HPO_4, 1.28 mM $CaCl_2$, 10 mM HEPES, pH 7.4, with 0.5% bovine serum albumin, and 0.1 mg/mL soybean trypsin inhibitor). Cells were incubated to equilibrium (2 h at 37°C), then washed twice with ice-cold phosphate-buffered saline. The cells were then solubilized with 1 mL of 0.1 N NaOH and radioactivity quantified in a γ-counter. Nonspecific binding was determined in the presence of 100 nM ET-1.

2.2 Preparation of a cell-immobilized capillary column

A fused-silica capillary column (60 cm effective length × 200 μm inner diameter, ~1.88 mL whole volume) was activated by washing successively with MeOH (*ca.* 20 mL), 1 N HCl (*ca.* 20 μL), deionized water (*ca.* 20 μL), 1 N NaOH (*ca.* 20 μL), and deionized water (*ca.* 20 μL). The column was stored in the presence of 1 mM PBS buffer (containing 31.7 mg of NaH_2PO_4 and 206 mg of Na_2HPO_4 at pH 7.3 per liter). The transfected CHO cells harboring ET_A (~2.5 × 10^5 cells/mL) recovered from the culture media were fixed by treatment with formaldehyde (3.7% in water, 5 mL) for 30 min to furnish the desired cross-linkage. The fixed cells were stored at 4°C in PBS (1 mM, pH 7.3). For loading of the fixed cells onto the capillary column, the column was washed with EtOH (95%, *ca.* 40 μL), purged with air in order to dry it, charged with poly-L-lysine (15 000–

30 000 molecular weight, 0.5 mg/mL in water) for 5 min, and incubated for 30 min [24, 25]. The column was then charged again with poly-L-lysine for 5 min and incubated for 2 h. The column was then dried by airflow for 2 h, the fixed cells in the PBS buffer were purged into the poly-L-lysine-coated column. After 5 min of incubation time, another batch of fixed cells was purged into the column for 30 min of incubation. Then 1% FBS in PBS (*ca.* 20 μL) was purged to cap the exposed area of poly-L-lysine. The column immobilized with the transfected CHO cells was finally washed with PBS (*ca.* 40 μL), and stored at room temperature (~25–27°C). No apparent degradation was observed after 7 days. Capillary electrophoresis experiments were performed on an P/ACE system (Beckman Instruments, Fullerton, CA, USA) at a constant voltage of 10 kV. The sample (~1.5 μL of ~10^{-6} to 10^{-7} M solution in 1 mM PBS, pH 7.0) was introduced into the capillary column by pressure injection at 0.5 psi/3 s. The background electrolyte was PBS (1 mM, pH 7.0). Electrophoresis was monitored by an absorbance detector held at 214 nm. The low concentration of PBS (1 mM) ensured no interference with 214 nm absorbance. The temperature of the capillary column was maintained at 25°C.

2.3 Samples and reagents

ET-1, the ET-1 fragment ET-$1^{(16–21)}$ containing 16–21 residues [26, 27], and the cyclic peptide antagonists BQ123 [28] and JKC302 [29, 30] were synthesized by using an ABI 433A peptide synthesizer (ABI, Foster City, CA, USA). We also prepared the samples of non-peptide endothelin receptor antagonists SB209670 [31, 32], JMF310 [33], and YHK891 [34]. The active herbal components, Magnolol, Geniposide and Honokiol, were purchased from Wako Pure Chemical Industries (Japan). The ^{125}I-labeled ET-1 ((3-[^{125}I]iodotyrosyl)endothelin-1, 81.4 TBq/mmol) was purchased from NEN Life Science Products (Wilmington, DE, USA). The fluorescent reagent, fura-2 penta(acetoxymethyl) ester, was from Calbiochem-Novabiochem (La Jolla, CA, USA), poly-L-lysine hydrobromide and G418 antibiotic were from Sigma Chemical (St. Louis, MO, USA). All other chemicals were of analytical grade. Distilled water was used in all experiments. All buffers were filtered through 0.45 μm filters before use.

3 Results and discussion

3.1 Identification of transfected CHO cells

The cultured CHO cells were subjected to transfection with the ET_A-expression plasmid DNA using a lipofectin reagent. The efficacy of ET_A expression was demonstrated by the competitive binding assay with a synthetic sample of ET-1 and the radiolabeled [^{125}I]-ET-1 (Fig. 2). The binding affinity of the transfected cell line by the agonist ET-1 was established to have a dissociation constant of K_d = 1.52 nM. The receptor density (B_{max}) of 6.3×10^5 sites/cell was estimated from a Scatchard plot [38, 39]. This result indicated that ET_A receptors were successfully overexpressed in the CHO cells. The CHO cells harboring ET_A were fixed by treatment with formaldehyde. A fused-silica capillary column (200 μm inner diameter) was first charged with poly-L-lysine, and then purged with the cross-linked CHO cells. After capping the exposed portion of poly-L-lysine with FBS, the cell-immobilized capillary column was furnished and ready for ACE. It was estimated that about 1–3 cells were attached to the capillary cross-section. Among several possible candidates of base material, poly-L-lysine turned out to exhibit a superb adhesive property for the immobilization of cells on the capillary column. The whole-cell immobilized capillary column could be easily prepared, and no obvious decomposition was found on storage with PBS buffer at room temperature for seven days.

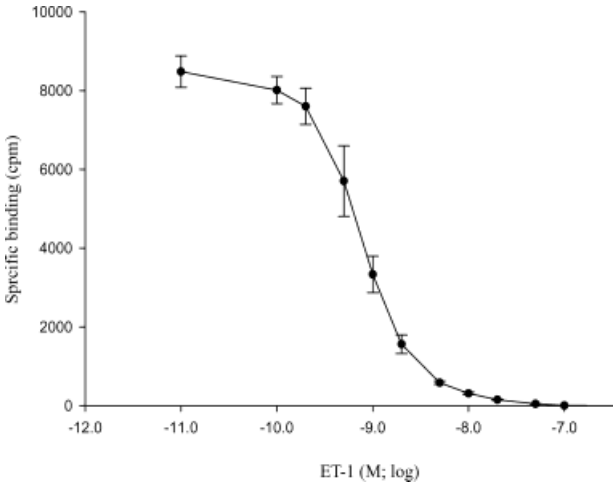

Figure 2. [^{125}I]-Endothelin-1 binding assay was performed with a synthetic endothelin-1 (ET-1). The binding affinity of transfected CHO cell-line was established with K_d = 1.52 nM, and B_{max} was estimated at 6.3×10^5 sites/cell.

3.2 Validation of peptide ligands

In order to validate our ACE method, we first analyzed three known peptide ligands of the ET_A receptor: an ET-1 *C*-terminal fragment ET-$1^{(16–21)}$ containing six amino acid residues [26, 27] and two cyclic pentapeptides, BQ123 [28] and JKC302 [29, 30]. The behavior of these ligands on three different capillary columns was examined, *i.e.*, an uncoated column, a column coated with poly-L-lysine,

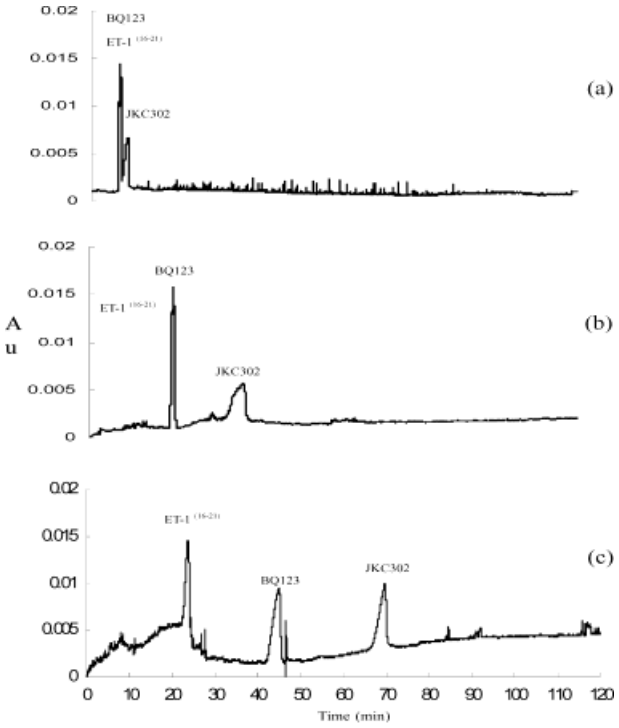

Figure 3. ACE of peptides JKC 302, BQ123, and ET-1$^{(16–21)}$ on a column (a) uncoated; (b) coated with poly-L-lysine; (c) coated with fixed ET_A-overexpressing CHO cells. Background electrolyte, 1 mM PBS; absorbance detector at 214 nm. Au, arbitrary unit.

and a column coated with fixed ET_A-overexpressing CHO cells. The first set of electropherograms indicated that these three compounds were poorly resolved on an uncoated column, as one would expect. The eluting order of three peptides on the poly-L-lysine-coated column differs from that on the cell-coated column, presumably due to the random electrostatic interactions exerted by poly-L-lysine on the peptide analytes. Complete separation of the mixture of ET-1$^{(16–21)}$, BQ123, and JKC302 was realized on a capillary column with the stationary phase of immobilized ET_A-overexpressing CHO cells (Fig. 3). The cyclopeptide JKC302 with the longest retention time on the cell-coated column should exhibit the highest affinity toward ET_A, whereas the hexapeptide ET-1$^{(16–21)}$ with the shortest retention time should have the least affinity.

The speculation of relative affinity JKC302 > BQ123 > ET-1$^{(16–21)}$, as deduced from the ACE experiment, was further supported by the functional assay of their antagonistic potency against ET-1. It is well known that the binding of ET-1 with ET_A will trigger an increase of intracellular calcium concentration [4]. A control experiment (Fig. 4a) was performed by treatment of ET-1 in 10^{-7} M to the ET_A overexpressing CHO cells, which were first incubated with a calcium-chelating agent fura-2 applied as its penta(acetoxymethyl) ester [39, 40]. The $[Ca^{2+}]_i$ change was monitored by a ratiometric method using dual excitations at 340 and 380 nm wavelengths, and the fluorescence

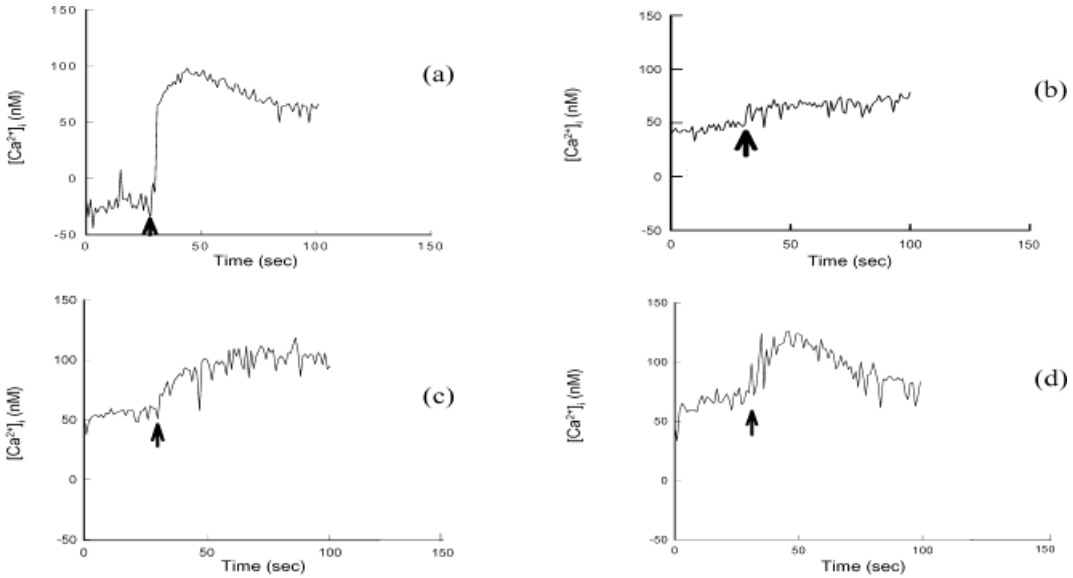

Figure 4. Fluorescence measurements of intracellular calcium concentration ($[Ca^{2+}]_i$) by addition of tested samples (as shown by the arrow) to the ET_A-overexpressing CHO cells in the presence of fura-2. The induced $[Ca^{2+}]_i$ change is taken as a measure of antagonist potency against ET-1: (a) control experiment with addition of ET-1 (10^{-7} M), (b) treatment with a mixture of JKC302 (10^{-6} M) and ET-1 (10^{-7} M), (c) treatment with a mixture of BQ123 (10^{-6} M) and ET-1 (10^{-7} M), and (d) treatment with a mixture of ET-1$^{(16–21)}$ (10^{-6} M) and ET-1 (10^{-7} M).

emission at 505 nm was measured. The treatment with ET-1 induced a great degree of $[Ca^{2+}]_i$ (Fig. 4a). When a mixture of JKC302 (10^{-6} M) and ET-1 (10^{-7} M) was used in such a functional assay, the ET-1 induced $[Ca^{2+}]_i$ change was entirely suppressed (Fig. 4b). The degree of inhibition against the ET-1 induced $[Ca^{2+}]_i$ can serve as a measure of the potency of an antagonist. By comparison of the transient $[Ca^{2+}]_i$ assays (Figs. 4b–d), the relative potency JKC302 > BQ123 > ET-$1^{(16–21)}$ in ET_A antagonism is in good agreement with that derived by the eluting order on the capillary column coated with whole cells.

3.3 Validation of nonpeptide ligands

Our present whole-cell coating ACE method is not limited to peptide antagonists; it is also applicable for screening nonpeptide antagonist molecules. For example, a 1,3-diarylindane-2-carboxylic acid SB209670 is a potent ET_A antagonist against ET-1 [31]. The molecular computations together with bioassay of a series of derivatives indicated that SB209670 bears a carboxyl group at the 2-position to mimic the carboxylic terminal of ET-1, and two aryl groups at 4- and 9-positions to mimic the aromatic residues of Tyr-13 and Phe-14. On the basis of this structural protocol, a carbazolothiophene-2-carboxylic acid JMF310 [33] was designed as a possible ET_A antagonist, and an indenecarboxylate ester YHK891 [34] was also examined for comparison. Indeed, SB209670 that strongly inhibited the ET-1 induced $[Ca^{2+}]_i$ (Fig. 6b) also showed a very long retention time on the whole-cell immobilized capillary column (Fig. 5), as a consequence of its high affinity toward

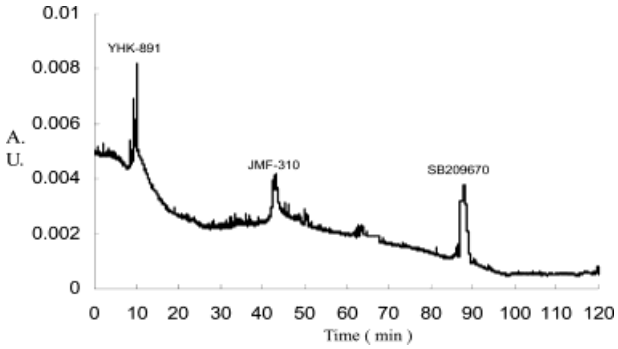

Figure 5. ACE of a mixture of SB209670, JMF310, and YHK891 on a capillary column coated with fixed ET_A-overexpressing CHO cells. Background electrolyte, 1 mM PBS; absorbance detector at 214 nm. A.U., arbitrary unit.

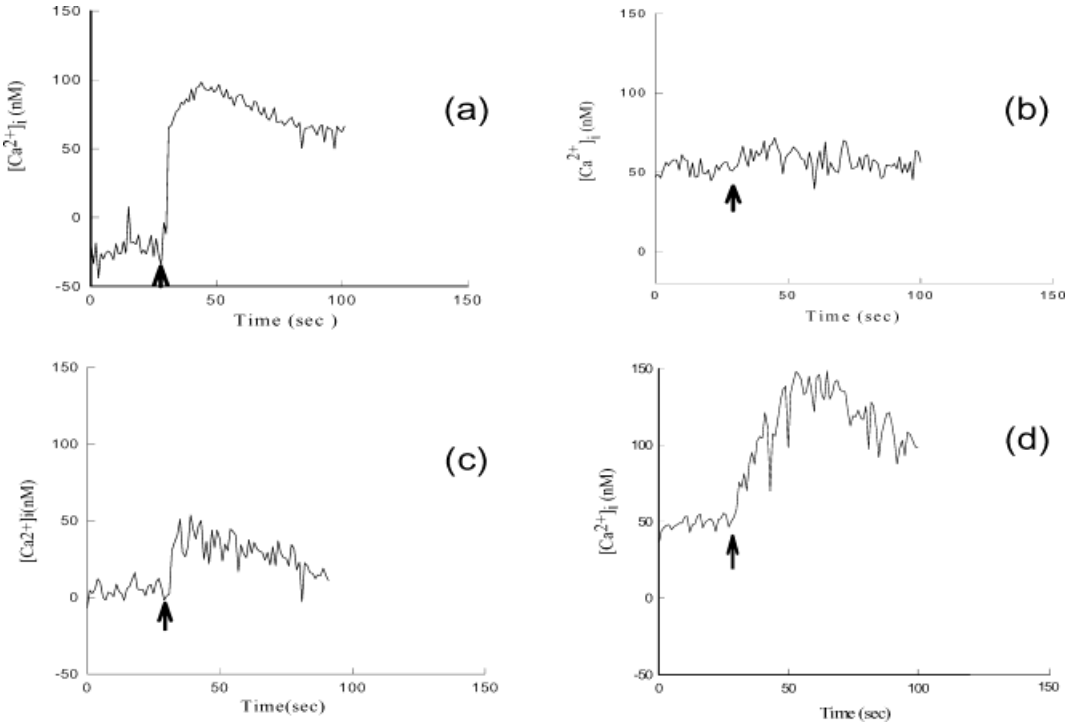

Figure 6. Fluorescence measurements of intracellular calcium concentration ($[Ca^{2+}]_i$) by addition of tested samples (as shown by the arrow) to the ET_A-overexpressing CHO cells in the presence of fura-2. The induced $[Ca^{2+}]_i$ change is taken as a measure of antagonist potency against ET-1: (a) control experiment with addition of ET-1 (10^{-7} M), (b) treatment with a mixture of SB209670 (10^{-6} M) and ET-1 (10^{-7} M), (c) treatment with a mixture of JMF310 (10^{-6} M) and ET-1 (10^{-7} M), and (d) treatment with a mixture of YHK891 (10^{-6} M) and ET-1 (10^{-7} M).

the surface endothelin receptors of the transfected CHO cells. The modest antagonist potency of JMF310 (Fig. 6c) was also reflected in the ACE elution profile. On the contrary, the YHK891 sample having only a marginal inhibitory effect against ET-1 (Fig. 6d) was rapidly eluted out from the whole-cell coating column.

3.4 Validation of active herbal components

In addition to the rationally designed molecules, the random screening of active components can also be achieved by the whole-cell ACE method. We have selected several active herbal components that are known to possess bioactivities related to vascular dilation or signal transduction. By ACE we found that an antiplatelet agent, Magnolol [42–44], might function as a new lead compound against ET-1 in ET_A receptor binding. Magnolol is the 2,2'-dimer of 4-allylphenol. As shown in the profiles of ACE analysis (Fig. 7) and $[Ca^{2+}]_i$ assay (Fig. 8), Magnolol is a stronger ET_A antagonist than its structural isomer, Honokiol [45]. Geniposide [46], an iridoid glucoside that exhibits neuritogenic effect on PC12h cells and enhanced responses of cells to carbacol in terms of cytoplasmic free-calcium concentration, turned out to have a temperate binding affinity and modest antagonistic activity against ET-1.

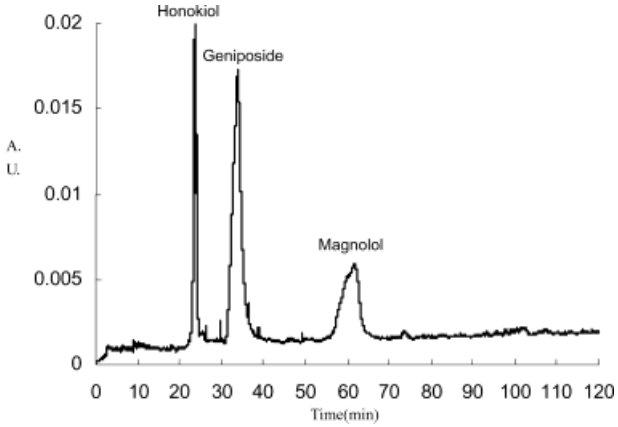

Figure 7. Screening of the Chinese herbal active components Magnolol, Honokiol, and Geniposide by ACE on a capillary column coated with fixed ET_A-overexpressing CHO cells. Background electrolyte, 1 mM PBS; absorbance detector at 214 nm. A.U., arbitrary unit.

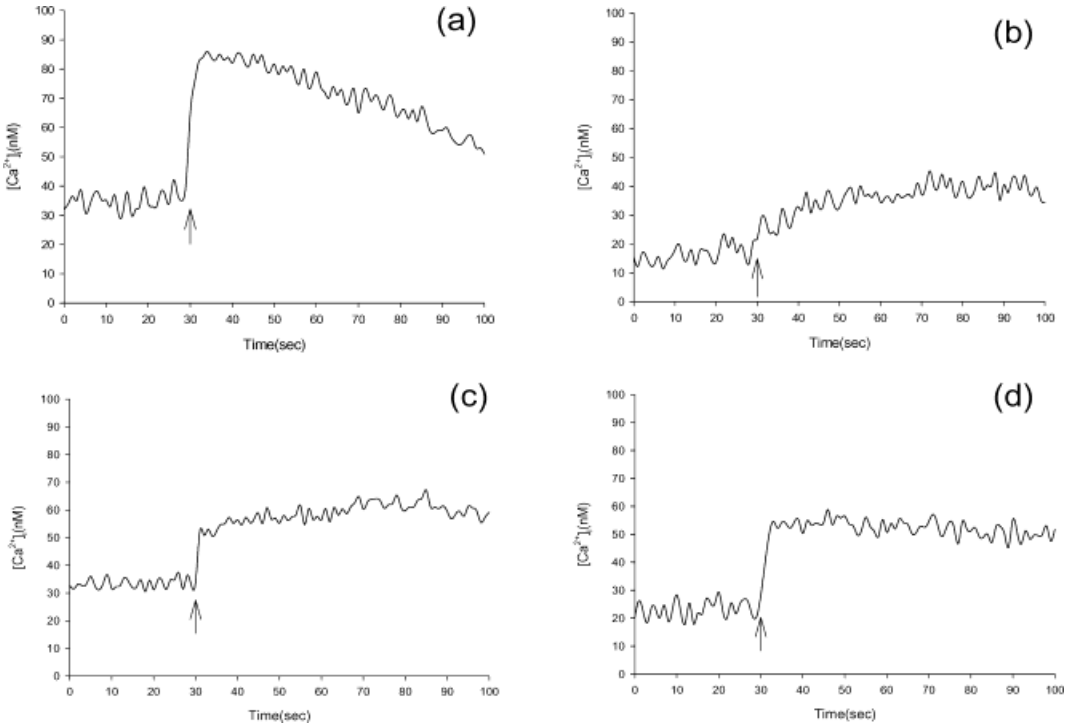

Figure 8. Fluorescence measurements of intracellular calcium concentration ($[Ca^{2+}]_i$) by addition of tested samples (as shown by the arrow) to the ET_A-overexpressing CHO cells in the presence of fura-2. The induced $[Ca^{2+}]_i$ change is taken as a measure of antagonist potency against ET-1: (a) control experiment with addition of ET-1 (10^{-7} M), (b) treatment with a mixture of Magnolol (10^{-6} M) and ET-1 (10^{-7} M), (c) treatment with a mixture of Geniposide (10^{-6} M) and ET-1 (10^{-7} M), and (d) treatment with a mixture of Honokiol (10^{-6} M) and ET-1 (10^{-7} M).

4 Concluding remarks

There are several distinct advantageous features for using whole cells as the stationary phase of ACE (i) isolation of (unstable) receptors is not needed, (ii) the stability of whole cells is improved by immobilization, and (iii) whole cells are easily coated on a fused-silica capillary column *via* the guidance of poly-L-lysine template. We have demonstrated this concept of whole-cell immobilized ACE by a protocol using the capillary column coated with the CHO cells harboring overexpressing endothelin receptor A. The capillary column prepared as such exhibits excellent affinity for separation and identification of endothelin receptor A antagonists (Fig. 9, Table 1). There is a good correlation between the relative inhibition of the ET-1 induced $[Ca^{2+}]_i$ and the retention time of the examined compound on the capillary column coated with ET_A-overexpressing CHO cells. This ACE method only requires a small quantity of sample, and offers a reliable assessment of a library of compounds in a relatively short period. The ACE with immobilized whole cells could be developed as a high-throughput screening method based on specific receptor/ligand interactions.

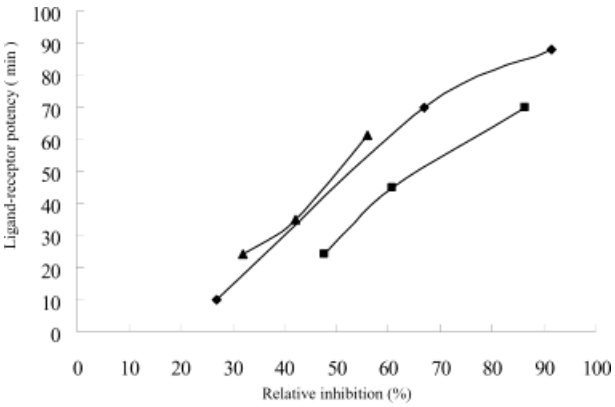

Figure 9. Correlation between the retention time of the examined compound of the capillary column coated with ET_A-overexpressing CHO cells and the relative inhibition of the ET-1 induced increase of intracellular calcium ion concentration. In each line, the stronger affinity of a compound toward ET_A shows a longer retention time and more potent inhibitory effect. (■) From top to bottom are JKC302, BQ123, and ET-$1^{(16–21)}$; (◆) from top to bottom are SB209670, JMF310, and YHK891; (▲) from top to bottom are Magnolol, Geniposide, and Honokiol.

Table 1. Relative inhibition of the ET-1 induced $[Ca^{2+}]_i$ and retention time of the examined compound on the capillary column coated with ET_A-overexpressing CHO cells

Compound	Relative inhibition (%)	Retention time (min)
JKC302	86	69.7
BQ123	61	45.0
ET-$1^{(16–21)}$	48	24.2
SB209670	91	87.8
JMF310	67	69.7
YHK891	27	10.0
Magnolol	56	61.4
Geniposide	42	34.7
Honokiol	32	24.1

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