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Short communication

Synthesis and characterization of liquid chromatographic columns containing the immobilized ligand binding domain of the estrogen related receptor α and estrogen related receptor γ

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ABSTRACT

The ligand binding domains of the estrogen related receptors, ERRα and ERRγ were covalently immobilized onto the surface of an aminopropyl silica liquid chromatography stationary phase to create the ERRα-silica and ERRγ-silica columns and onto the surface of open tubular capillaries to create the ERRα-OT and ERRγ-OT columns. The ERR-silica and ERR-OT columns were characterized using frontal chromatographic techniques with diethylstibesterol and the binding affinities, K_d values, to the immobilized receptors were consistent with the values obtained by a radioligand binding assay. The ERRγ-silica column was also characterized using non-linear chromatographic techniques using a series of tamoxifen derivatives. The relative K_d values obtained for the derivatives were consistent with the relative ability of the compounds to inhibit the cellular proliferation of the human-derived T98G glioma cell line, expressed as IC_{50} values. The results indicate that the columns containing immobilized ERRα and ERRγ can be created and used to characterize the binding of compounds to the immobilized receptors and that the relative retention of compounds on these columns reflects the magnitude of their inhibitory activity.

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1. Introduction

In humans, most malignant brain tumors are gliomas and astrocytomas, which are extremely lethal with a median survival from diagnoses of 12–15 months [1]. Current clinical treatment of gliomas and astrocytomas is not effective and there are a number of active programs directed at the development of new drugs to treat these tumors [2,3]. One potential class of agents for the treatment of glioblastomas and astrocytomas is selective estrogen receptor modulators (SERMs), such as tamoxifen, which has displayed anti-glioma activity in both *in vitro* and *in vivo* models [4].

We have recently demonstrated that the anti-glioma effects of SERMs are due to their interactions with estrogen related receptors ERRα and ERRγ [5]. These nuclear receptors are expressed alone or in combination in brain cancers and recent data indicate that the establishment of the ERRα and ERRγ expression in a tumor can be used to tailor the therapeutic program to the properties of that tumor. However, only a few effective ERRα and ERRγ antagonists have been unidentified and these are primarily plant-derived flavanoids [6]. Thus, the objective of this project was the development of new methods to screen botanical extracts in order to identify new ERRα and ERRγ antagonists. We now report the development of immobilized ERRα and ERRγ columns for use in these screens.

We have previously demonstrated that columns which contain immobilized nuclear proteins, the estrogen receptor (ER) ligand binding domain [7] and the DNA unwinding element binding (DUE-B) protein [8], can be created, characterized and used to study ligand–protein interactions. In these studies, the ERRα and ERRγ ligand binding domains were covalently immobilized via the N-terminus onto the surface of an aminopropyl silica liquid chromatography stationary to create the ERRα-silica and ERRγ-silica columns, or on the activated surface of open tubular glass capillaries to create the ERRα-OT and ERRγ-OT columns. The results indicate that both formats can be used to study ligand–ERR interactions including the determination of binding affinities and binding sites. The results also demonstrate that the ERR-OT columns have shorter retention and wash times and, therefore, would be preferred for individual compounds characterizations, while the ERR-silica columns have significantly higher binding capacities and are the preferred format for online screening of botanical extracts.

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2. Experimental

2.1. Materials

Tamoxifen and 4-hydroxy tamoxifen were purchased from Sigma, N-desmethyl 4-hydroxytamoxifen (Endoxifen) was purchased from Toronto Research Chemicals (Toronto, Canada) and diethylstilbestrol (DES) was purchased from Fisher Scientific. His-tag fusion proteins with a 4 amino acid lysine insert with the ligand binding domain of the ERRα and γ were purchased from GenScript (Piscataway, NJ). Tricorn 5/20 glass column was purchased from Amersham Bioscience (Piscataway, NJ). BSA, ammonium acetate, gluteraldehyde, glutaric acid, glycine, pyridine (99.8%), sodium azide, and Tris buffer were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO). The water used in the study was prepared using a Milli-Q Water Purification System (Millipore Corporation, Bedford, MA). The aminopropyl silica (APS) stationary phase (12 μ 300 Å pores) was purchased from Regis Technologies (Morton Grove, IL).

2.2. Immobilization of ERRα or ERRγ via N-terminus

2.2.1. Immobilization of ERRα or ERRγ on silica stationary phase

The ERRs were immobilized based on a previously published protocol [9]. Briefly, 50 mg of APS was added to 10 ml of pyridine [10 mM, pH 6.0] in a 15 ml conical plastic tube and the mixture was rotated for 15 min, centrifuged at 1500 × *g* for 10 min, and the supernatant was discarded. The APS was suspended in 10 ml of 5% gluteraldehyde, rotated for 3 h and then centrifuged. The supernatant was discarded and the activated APS was washed three times with 10 ml portions of pyridine [10 mM, pH 6.0], a suspension of 50 μg of ERRα or γ protein in 300 μl of pyridine [10 mM, pH 6.0] was added and the mixture left for 24 h at 4 °C. After the mixture had warmed to room temperature, 5 ml of glutaric acid [1 M, pH 8.0] was added and the resulting mixture rotated for 30 min at 200 rpm in an orbital shaker, centrifuged at 1500 × *g* for 10 min and the supernatant discarded. The ERRα- or γ-silica was rinsed three times with 5 ml portions of Tris–HCl buffer [10 mM, pH 7.4] containing 150 mM NaCl, 0.1% (w/v) BSA, 1 mM EDTA, 0.1% sodium azide. The suspension containing the ERRα- or γ-silica was packed into a Tricorn 5/20 glass column (50 mm × 5 mm I.D.) and the column washed with Tris–HCl buffer [10 mM, pH 7.4] for 2 h at a flow rate 0.2 ml/min at 25 °C.

2.2.2. Immobilization of ERRα or ERRγ on the surface of open tubular capillaries

The ERRα-OT and ERRγ-OT were synthesized as described above with slight modifications. Briefly, the open tubular capillary (25 cm × 100 μm I.D.) was primed with 1 M NaOH, rinsed with water, and dried at 95 °C for 1 h. A 10% aqueous solution of aminopropyltrimethoxysilane was passed through the capillary for 5 min followed by a 30 min incubation at 95 °C and then repeated. After 18 h, a 1% gluteraldehyde aqueous solution was passed through the capillary for 1 h followed by a solution of ERRα or ERRγ (50 μg in 2 ml ammonium acetate buffer [10 mM, pH 7.4]) which was recycled through the capillary for 60 min. Both tips of the capillary were submerged into the solution for 18 h at 4 °C. After which ammonium acetate buffer [10 mM, pH 7.4] was passed through for 5 min followed by a 1 M solution of glutaric acid for 1 h.

2.3. Chromatographic system, frontal and non-linear chromatographic studies

The chromatographic system consisted of a series 1100 liquid chromatography/mass selective detector (LC/MSD) (Agilent Technologies, Palo Alto, CA) equipped with a vacuum de-gasser, a binary pump, an autosampler, a mass selective detector supplied with atmospheric pressure ionization electrospray (API-ES). The chromatographic system was interfaced to a 250 MHz Kayak XA computer (Hewlett-Packard, Palo Alto, CA) using ChemStation software (Rev B.10.00, Hewlett-Packard). The mobile phase was composed of ammonium acetate [10 mM, pH 7.4] and the experiments were carried out at a flow rate of 0.5 ml/min at ambient temperature. The parameters on the MSD were set at 10 lpm for the drying gas flow, 350 °C for the drying gas temperature, 54 psig for nebulizer pressure, and 70 V for the fragmentor. DES, 4-hydroxytamoxifen, endoxifen and tamoxifen were monitored using single ion monitoring (M−1) at *m*/*z* values 267.2 and (M+1) at *m*/*z* values of 388.1, 374.2 and 372.3 respectively.

2.3.1. Frontal chromatography studies

Serial concentrations of DES [0.05, 0.1, 0.25, 0.5, 1 μM] were prepared in ammonium acetate [10 mM, pH 7.4]:methanol (94:6, v/v). The observed retention volumes were used to calculate binding affinities (K_d values) of the studied ERRγ inhibitors using a previously described approach [10]. The data were analyzed by non-linear regression with the sigmoidal response curve using Prism 4 software (Graph Pad Software Inc., San Diego, CA).

2.3.2. Non-linear chromatography studies (NLC)

The binding kinetics of 4-hydroxytamoxifen, endoxifen and tamoxifen were determined by NLC were carried out on the ERRγ-silica column as previously described [11]. The mobile phase was composed of ammonium acetate [10 mM, pH 7.4]:methanol (80:20, v/v) delivered at a flow rate of 0.5 ml/min at room temperature. The studies utilized 20 μl injections of 4-hydroxytamoxifen [1.0, 2.5, 5.0, 10.0, and 20.0 μM], endoxifen [2.5, 5.0, 10.0, and 20.0 μM] and tamoxifen [5.0, 10.0, 20.0 and 40.0 μM]. The mathematical approach was the Impulse Input Solution [12] and the chromatographic data were analyzed using PeakFit v4.12 for Windows Software (SPSS Inc., Chicago, IL, USA) following a previously reported protocol [13].

2.4. MTS proliferation

The CellTiter 96® Aqueous cell proliferation assay was performed according to the manufacturer's protocol (Promega, Madison, WI) with slight modifications [5]. Briefly, T98G cells were seeded in 96-well plates at a density of 1750 cells/well and cultured in complete phenol red-free medium for 24 h. Test compounds dissolved in DMSO and mixed with culture medium were added to the cells in the following concentrations: tamoxifen [0.1, 1.0, 2.0, 3.0, 5.0, 7.0, 10 and 20 μM]; 4-hydroxytamoxifen [0.625, 1.25, 2.5, 3.75, 5, 7.5, 10 and 15 μM]; endoxifen [0.625, 1.25, 2.5, 3.75, 5, 7.5, 10 and 15 μM]. Control cultures were treated with DMSO. After 48 h, 20 μl of 0.5 mg/ml MTS solution was added to each well, and the cultures were further incubated for 30 min. The absorbance was measured at 490 nm with a microplate reader (Thermo Scientific, USA). Change in growth rate was calculated as follows: [Abs490 nm of treated cells/Abs490 nm of control cells]. Three wells were used for each treatment, and the experiments were repeated three times. IC_{50}s were calculated using the Graph Pad Prism Software (La Jolla, CA).

3. Results and discussion

3.1. Characterization of the ERRα-OT and ERRγ-OT columns

The ERRα-OT and ERRγ-OT columns were characterized using frontal chromatographic techniques using DES as the marker ligand. On both columns, the DES chromatographic traces contained

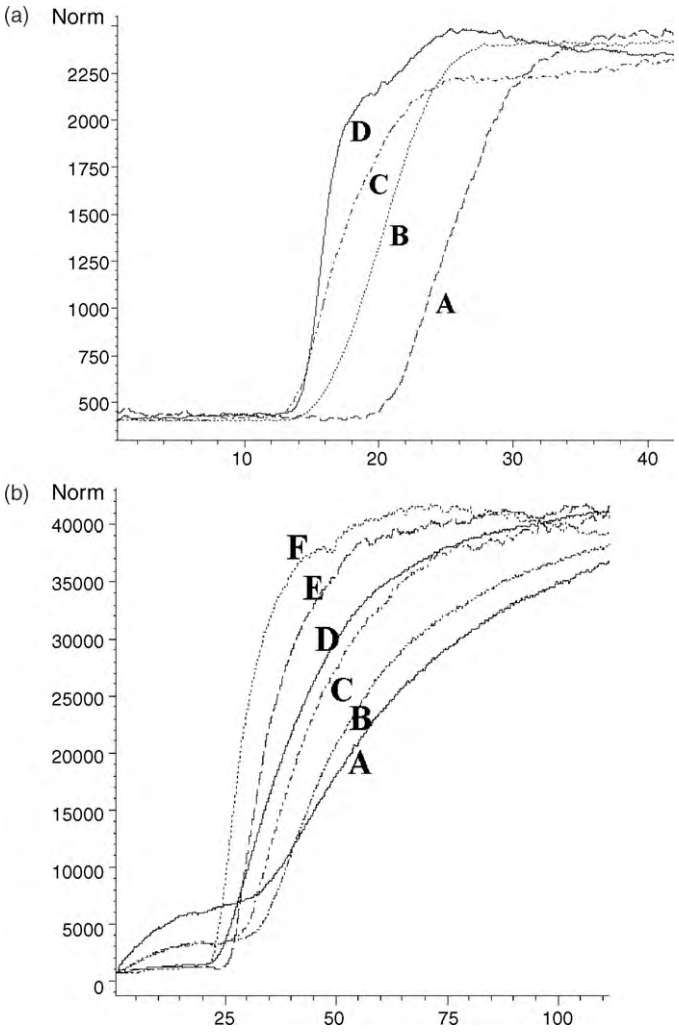

Fig. 1. (a) The effect of increasing concentration of diethylstilbesterol on its chromatographic retention on the ERRγ-OT column from right to left (0.05, 0.1, 0.25 and 0.5 μM). (b) The effect of increasing concentration of diethylstilbesterol on its chromatographic retention on the ERRγ-column from right to left (0.05, 0.1, 0.25, 0.5, 1, 2 μM).

a relatively flat initial portion representing nonspecific and specific binding of the marker to the stationary phase, followed by a vertical breakthrough representing saturation and then a plateau, c.f. Fig. 1a. Increasing mobile phase concentrations of DES produced a corresponding decrease in the breakthrough volume, Fig. 1a, and the data was used to calculate the affinity, expressed as K_d values, using previously described techniques [14]. The chromatographically determined K_d values for the binding of DES to the ERRα and ERRγ were 929 and 237 nM respectively, Table 1. The K_d value obtained for DES on the ERRγ was similar to previously reported value of 870 nM [15] and the relative order, i.e. K_d(ERRγ) < K_d(ERRα), is consistent with the previously reported IC_{50} values of 700 nM (ERRγ) [15] and 10 μM (ERRα)

Table 1
Calculated binding affinities (K_d values) for diethylstilbesterol on the ERRα-OT and the ERRγ-OT columns determine using frontal chromatographic techniques.

	K_d Obs	IC_{50}[a]
ERRα	929 nM	10 μM
ERRγ	237 nM	700 nM

[a] Reported EC_{50} values for DES [15].

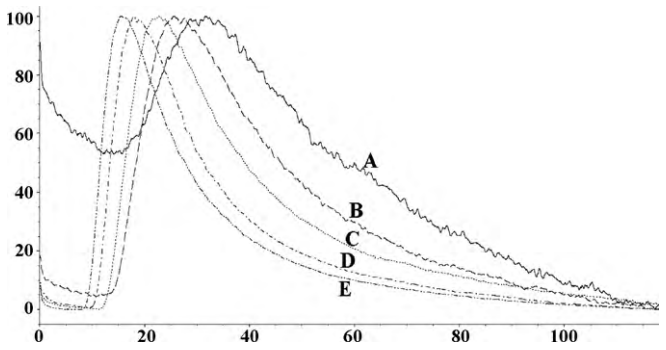

Fig. 2. Effects of increasing concentration of 4-hydroxtamoxifen from 1 to 20 μM on the ERRγ-column using non-linear chromatographic techniques.

[15]. The chromatographic data was also used to calculate the amount of active sites (B_{max}) on the OT columns which were 106 pmol on the ERRγ-OT column and 600 pmol on the ERRα-OT column.

3.2. Characterization of the ERRγ- and ERRα-silica columns

Frontal chromatographic studies were used to determine the K_d for DES on the ERRγ-silica column, Fig. 1b. The calculated K_d value, 251 nM, was the same as the K_i value, 237 nM, obtained on the ERRγ-OT column indicating that the DES–protein interactions were similar on both columns. However, the calculated B_{max} value was 4 nmol, a 40-fold increase relative to the ERRγ-OT column. The ERRα-silica column was characterized using the ERRα agonist biochanin A, and the K_d value, 64 nM, was consistent with the previously reported K_d of 45 nM [5]. It is interesting to note that unlike the ERRγ-silica column, the immobilization of ERRα on the silica support did not significantly increase the calculated B_{max} value, relative to ERRα-OT column, 700 and 600 pmol respectively.

Based upon the increased capacity of the ERRγ-silica column, this format was further characterized for use in online screening using NLC and 4-hydroxytamoxifen, endoxifen and tamoxifen. The injection of increasing concentrations of these ligands resulted in chromatographic traces containing asymmetric peaks, which are indicative of the NLC process [11], Fig. 2. The calculated K_ds were averaged over the area where a3 versus concentration were linear. Analysis of the data showed that NLC parameters can be obtained from peak profiles produced by injections of 20 μl of these solutions in concentrations ≤5 μM. Using this approach, the calculated K_d values of 4-hydroxytamoxifen, endoxifen and tamoxifen were 16.3 nM, 43.2 nM and 1.5 μM respectively, Table 2, which were consistent with previously reported K_i values of 75 nM for 4-hydroxytamoxifen and 870 nM for tamoxifen [15]. Tamoxifen, endoxifen and 4-hydroxytamoxifen were not specifically retained on the ERRα column (data not shown), which is consistent with the

Table 2
Calculated binding affinities (K_d values) of the ERRγ antagonists, 4-hydroxytamoxifen, endoxifen and tamoxifen determined using non-linear chromatographic techniques on the ERRγ column and the corresponding dose-dependent effect on cellular proliferation (IC_{50} values) determined using a 48 h MTS proliferation assay carried out using the human-derived T98G glioma cell line.

	K_d values ERRγ column (nM)	IC_{50} values T98G cell line (μM)
4-Hydroxytamoxifen	16.3	14.5
Endoxifen	43.2	3.4
Tamoxifen	1510	118

observation that these compounds have no affinity for the ERRα [15].

3.3. Relationship between chromatographically determined K_d values and inhibition of cellular proliferation IC_{50} values

The chromatographically determined K_d values for 4-hydroxytamoxifen, endoxifen and tamoxifen were compared to the effect of these compounds on the proliferation of the human-derived T98G glioblastoma cell line. Previous studies have established that T98G cells expressed ERRγ and that cellular proliferation was affected by ERRγ agonists and antagonists [5]. Treatment of T98G cells with 4-hydroxytamoxifen, endoxifen and tamoxifen for 48 h decreased cell proliferation in a dose-dependent manner and the calculated IC_{50} values were 3.4 μM (endoxifen), 14.5 μM (4-hydroxytamoxifen) and 118 μM (tamoxifen), Table 2. The results indicate that the chromatographically determined K_d values did not accurately predict the 3-fold difference in IC_{50} values between endoxifen and 4-hydroxytamoxifen, but did correctly reflect the 10-fold differences in activity between these compounds and tamoxifen. Thus, the data indicate that the ERRγ column can be used as an initial online screen for the isolation of compounds that bind to the ERRγ.

4. Conclusions

The data from this study indicate that ERRα and ERRγ have been successfully immobilized onto the silica stationary phase, as well as the surface of the open tubular capillaries, creating the ERR-silica and ERR-OT columns. The data also demonstrate that the binding affinities calculated by frontal displacement chromatography correlated with IC_{50} values obtained using cellular uptake studies, suggesting that this method can be used in place of current cellular uptake studies, which are time consuming and laborious. In addition, this technique can be used for a preliminary screen for drug candidates using the differences of their binding affinities.

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