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# Synthesis and Characterization of Liquid Chromatographic Columns Containing the Immobilized Ligand Binding Domain of the Estrogen Related Receptor $\alpha$ and Estrogen Related Receptor $\gamma$

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## Abstract

The ligand binding domains of the estrogen related receptors, ERR $\alpha$  and ERR $\gamma$  were covalently immobilized onto the surface of an aminopropyl silica liquid chromatography stationary phase to create the ERR $\alpha$ -silica and ERR $\gamma$ -silica columns and onto the surface of open tubular capillaries to create the ERR $\alpha$ -OT and ERR $\gamma$ -OT columns. The ERR-silica and ERR-OT columns were characterized using frontal chromatographic techniques with diethylstilbestrol and the binding affinities,  $K_d$  values, to the immobilized receptors were consistent with the values obtained by a radioligand binding assay. The ERR $\gamma$ -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<sub>50</sub> values. The results indicate that the columns containing immobilized ERR $\alpha$  and ERR $\gamma$  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.

## Keywords

glioblastoma; astrocytomas; estrogen related receptor; screening; biochromatography

## 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 are not effective and there are a number of active programs directed at the development of new drugs to treat these tumors. One potential class of agents for the treatment of glioblastomas and astrocytomas is selective estrogen receptor modulators (SERMs), such as tamoxifen, which has displayed antiglioma activity in both *in vitro* and *in vivo* models [4].

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We have recently demonstrated that the anti-glioma effects of SERMs are due to their interactions with estrogen-related receptors  $ERR\alpha$  and  $ERR\gamma$  [5]. These nuclear receptors are expressed alone or in combination in brain cancers and recent data indicate that the establishment of the  $ERR\alpha$  and  $ERR\gamma$  expression in a tumor can be used to tailor the therapeutic program to the properties of that tumor. However, only a few effective  $ERR\alpha$  and  $ERR\gamma$  antagonists have been indentified 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  $ERR\alpha$  and  $ERR\gamma$  antagonists. We now report the development of immobilized  $ERR\alpha$  and  $ERR\gamma$  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  $ERR\alpha$  and  $ERR\gamma$  ligand binding domains were covalently immobilized via the N-terminus onto the surface of an aminopropyl silica liquid chromatography stationary to create the  $ERR\alpha$ -silica and  $ERR\gamma$ -silica columns, or on the activated surface of open tubular glass capillaries to create the  $ERR\alpha$ -OT and  $ERR\gamma$ -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.

## 2. EXPERIMENTAL SECTION

### 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  $ERR\alpha$  and  $\gamma$  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  $\mu$  300 Å pores) was purchased from Regis Technologies (Morton Grove, Illinois).

### 2.2 Immobilization of $ERR\alpha$ or $ERR\gamma$ via N-terminus

**2.2.1. Immobilization of  $ERR\alpha$  or  $ERR\gamma$  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  $1500 \times g$  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  $\mu$ g of  $ERR\alpha$  or  $\gamma$  protein in 300  $\mu$ l 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 [1M, pH 8.0] was added and the resulting mixture rotated for 30 min at 200 rpm in an orbital shaker, centrifuged at  $1500 \times g$  for 10 min and the supernatant discarded. The  $ERR\alpha$  or  $\gamma$ -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, 1mM EDTA, 0.1% sodium azide. The suspension containing the ERR  $\alpha$  or  $\gamma$ -silica was packed into a Tricorn 5/20 glass column (50  $\times$  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 ERR $\alpha$  or ERR $\gamma$  on the surface of open tubular capillaries**—The ERR $\alpha$ -OT and ERR $\gamma$ -OT were synthesized as described above with slight modifications. Briefly, the open tubular capillary (25 cm  $\times$  100  $\mu$ m 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% glutaraldehyde aqueous solution was passed through the capillary for 1h followed by a solution of ERR $\alpha$  or ERR $\gamma$  (50  $\mu$ g in 2 ml ammonium acetate buffer [10mM, 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 [10mM, 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  $\mu$ M] 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 ERR $\gamma$  inhibitors using a previously described approach [10]. The data were analyzed by nonlinear 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 ERR $\gamma$ -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  $\mu$ l injections of 4-hydroxytamoxifen [1.0, 2.5, 5.0, 10.0, and 20.0  $\mu$ M], endoxifen [2.5, 5.0, 10.0, and 20.0  $\mu$ M] and tamoxifen [5.0, 10.0, 20.0 and 40.0  $\mu$ M]. 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  $\mu\text{M}$ ]; 4-hydroxytamoxifen [0.625, 1.25, 2.5, 3.75, 5, 7.5, 10 and 15  $\mu\text{M}$ ]; endoxifen [0.625, 1.25, 2.5, 3.75, 5, 7.5, 10 and 15  $\mu\text{M}$ ]. Control cultures were treated with DMSO. After 48 h, 20  $\mu\text{l}$  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 490nm with a microplate reader (Thermo Scientific, USA). Change in growth rate was calculated as follows: [Abs<sub>490nm</sub> of treated cells/Abs<sub>490nm</sub> of control cells]. Three wells were used for each treatment, and the experiments were repeated three times. IC<sub>50</sub>s were calculated using the Graph Pad Prism Software (La Jolla, CA).

### 3. RESULTS AND DISCUSSION

#### 3.1 Characterization of the ERR $\alpha$ -OT and ERR $\gamma$ -OT columns

The ERR $\alpha$ -OT and ERR $\gamma$ -OT columns were characterized using frontal chromatographic techniques using DES as the marker ligand. On both columns, the DES chromatographic traces contained 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 ERR $\alpha$  and ERR $\gamma$  were 929 and 237 nM respectively, Table 1. The  $K_d$  value obtained for DES on the ERR $\gamma$  was similar to previously reported value of 870 nM [15] and the relative order, i.e.  $K_d(\text{ERR}\gamma) < K_d(\text{ERR}\alpha)$ , is consistent with the previously reported IC<sub>50</sub> values of 700 nM (ERR $\gamma$ ) [15] and 10  $\mu\text{M}$  (ERR $\alpha$ ) [15]. The chromatographic data was also used to calculate the amount of active sites ( $B_{\text{max}}$ ) on the OT columns which were 106 pmoles on the ERR $\gamma$ -OT column and 600 pmoles on the ERR $\alpha$ -OT column.

#### 3.2 Characterization of the ERR $\gamma$ - and ERR $\alpha$ -silica columns

Frontal chromatographic studies were used to determine the  $K_d$  for DES on the ERR $\gamma$ -silica column, Fig 1.b. The calculated  $K_d$  value, 251 nM, was the same as the  $K_i$  value, 237 nM, obtained on the ERR $\gamma$ -OT column indicating that the DES-protein interactions were similar on both columns. However, the calculated  $B_{\text{max}}$  value was 4 nmoles, a 40-fold increase relative to the ERR $\gamma$ -OT column. The ERR $\alpha$ -silica column was characterized using the ERR $\alpha$  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 ERR $\gamma$ -silica column, the immobilization of ERR $\alpha$  on the silica support did not significantly increase the calculated  $B_{\text{max}}$  value, relative to ERR $\alpha$ -OT column, 700 pmol and 600 pmol, respectively.

Based upon the increased capacity of the ERR $\gamma$ -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  $K_d$ s were averaged over the area where  $a_3$  versus concentration were linear. Analysis of the data showed that NLC parameters can be obtained from peak profiles produced by injections of 20  $\mu\text{l}$  of these solutions in concentrations  $\leq 5 \mu\text{M}$ . Using this approach, the calculated  $K_d$  values of 4-hydroxytamoxifen, endoxifen and tamoxifen were 16.3 nM, 43.2 nM and 1.5  $\mu\text{M}$  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 ERR $\alpha$  column (data not shown), which is consistent with the observation that these compounds have no affinity for the ERR $\alpha$ [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 ERR $\gamma$  and that cellular proliferation was affected by ERR $\gamma$  agonists and antagonists [5]. Treatment of T98G cells with 4-hydroxytamoxifen, endoxifen and tamoxifen for 48 hours decreased cell proliferation in a dose-dependant manner and the calculated  $IC_{50}$  values were 3.4  $\mu$ M (endoxifen), 14.5  $\mu$ M (4-hydroxytamoxifen) and 118  $\mu$ M (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 ERR $\gamma$  column can be used as an initial online screen for the isolation of compounds that bind to the ERR $\gamma$ .

## 4. Conclusions

The data from this study indicate that ERR $\alpha$  and ERR $\gamma$  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.

## Acknowledgments

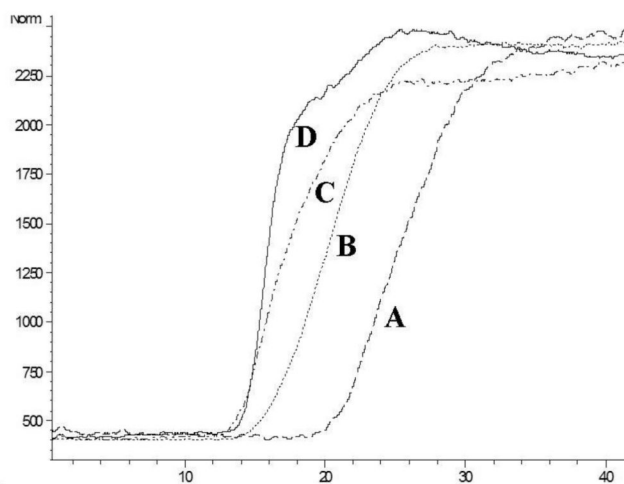
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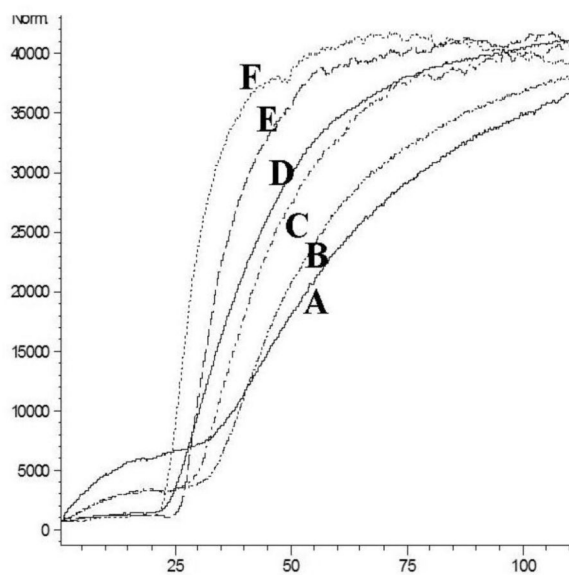
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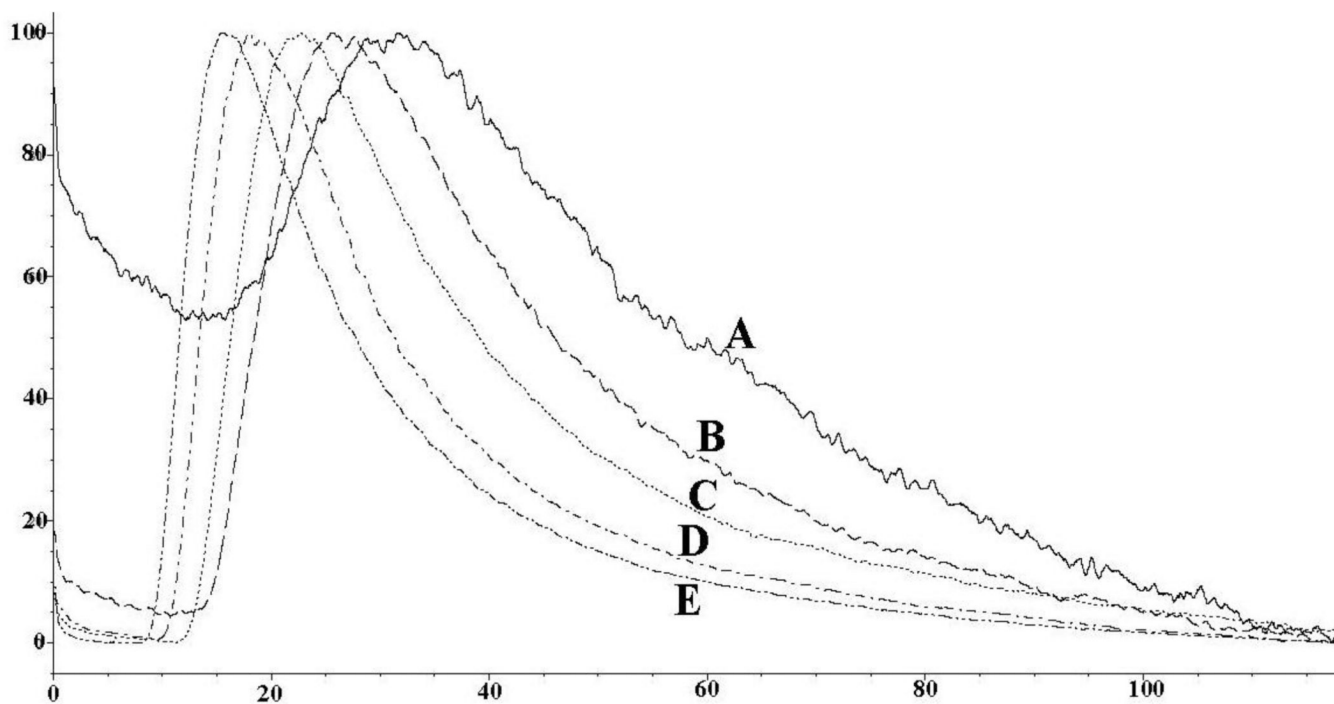
1a.



1b.

**Figure 1.**

A. The effect of increasing concentration of diethylstilbesterol on its chromatographic retention on the ERR $\gamma$ -OT column from right to left (0.05, 0.1, 0.25 and 0.5  $\mu$ M). B. The effect of increasing concentration of diethylstilbesterol on its chromatographic retention on the ERR $\gamma$ - column from right to left (0.05, 0.1, 0.25, 0.5, 1, 2  $\mu$ M).



**Figure 2.** Effects of increasing concentration of 4-hydroxymoxifen from 1  $\mu\text{M}$  to 20  $\mu\text{M}$  on the ERR $\gamma$ -column using non-linear chromatographic techniques.



**Table 1**

Calculated binding affinities ( $K_d$  values) for diethylstilbesterol on the  $ERR\alpha$ -OT and the  $ERR\gamma$ -OT columns determine using frontal chromatographic techniques.

	$K_d$ Obs	$IC_{50}$ *
<b><math>ERR\alpha</math></b>	929 nM	10 $\mu$ M
<b><math>ERR\gamma</math></b>	237 nM	700 nM

\* Reported  $EC_{50}$  values for DES (Coward et al., 2001).

**Table 2**

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

	$K_d$ values $ERR\gamma$ Column	$IC_{50}$ values T98G cell line
<b>4-Hydroxytamoxifen</b>	16.3 nM	14.5 $\mu$ M
<b>Endoxifen</b>	43.2 nM	3.4 $\mu$ M
<b>Tamoxifen</b>	1510 nM	118 $\mu$ M.