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SHORT COMMUNICATION

Efavirenz directly modulates the oestrogen receptor and induces breast cancer cell growth

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Objectives

Efavirenz-based HIV therapy is associated with breast hypertrophy and gynaecomastia. Here, we tested the hypothesis that efavirenz induces gynaecomastia through direct binding and modulation of the oestrogen receptor (ER).

Methods

To determine the effect of efavirenz on growth, the oestrogen-dependent, ER-positive breast cancer cell lines MCF-7, T47D and ZR-75-1 were treated with efavirenz under oestrogen-free conditions in the presence or absence of the anti-oestrogen ICI 182,780. Cells treated with 17β-oestradiol in the absence or presence of ICI 182,780 served as positive and negative controls, respectively. Cellular growth was assayed using the crystal violet staining method and an *in vitro* receptor binding assay was used to measure the ER binding affinity of efavirenz.

Results

Efavirenz induced growth in MCF-7 cells with an estimated effective concentration for half-maximal growth (EC_{50}) of 15.7 μM. This growth was reversed by ICI 182,780. Further, efavirenz binds directly to the ER [inhibitory concentration for half maximal binding (IC_{50}) of $\sim$ 52 μM] at a roughly 1000-fold higher concentration than observed with 17β-oestradiol.

Conclusions

Our data suggest that efavirenz-induced gynaecomastia may be caused, at least in part, by drug-induced ER activation in breast tissues.

Keywords: efavirenz, gynaecomastia, highly active antiretroviral therapy, oestrogen receptor, oestrogens

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Introduction

The introduction of highly active antiretroviral therapy (HAART) multi-drug combination regimens has considerably improved the prognosis of patients infected with HIV by reducing AIDS-related morbidity and mortality [1]. However, chronic treatment with these regimens is associated with multiple adverse effects, nonadherence and eventually therapy failure [2]. Treatment regimens containing the nonnucleoside reverse transcriptase inhibitor efavirenz are preferred in treatment-naïve patients and are widely used in other settings [3]. While efavirenz is generally well tolerated, concentration-dependent side effects that impact drug adherence and promote resistance have been documented [4]. Common adverse effects of efavirenz include central nervous system symptoms, occurring in up to 50% of patients [5], but other less common adverse effects have also been reported. An increasing number of reports suggest that the use of HAART, in particular efavirenz-based therapy, is associated with breast hypertrophy or gynaecomastia [6–11]. While mechanisms underlying efavirenz-induced gynaecomastia are not well understood, a number of hypotheses exist, including a direct oestrogenic effect, induction of an immune response, or altered steroid hormone metabolism by cytochrome P450 enzymes. To our knowledge, none of

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these hypotheses has been tested directly. In this study, we tested whether efavirenz can induce breast cancer cell growth by binding and modulating oestrogen receptor (ER) activity. We examined the ability of efavirenz to (a) induce the growth of the oestrogen-dependent, ER-positive breast cancer cell lines MCF-7, T47D and ZR-75-1, in the presence or absence of the pure anti-oestrogen ICI 182,780; and (b) directly bind the ER using an *in vitro* fluorescence polarization-based receptor binding assay.

Materials and methods

Cell lines and culture conditions

17β-oestradiol (E2) was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Efavirenz and ICI 182,780 were purchased from Toronto Research Chemical (Toronto, Ontario, Canada). The ER-positive, oestrogen-dependent breast cancer cell lines MCF-7, T47D and ZR-75-1 were obtained from the Tissue Culture Shared Resource at the Lombardi Comprehensive Cancer Center at Georgetown University (Washington, DC). These cell lines are widely and routinely used for examinations of the activities of oestrogens and anti-oestrogens [12,19]. Cells were routinely cultured in modified Improved Minimum Essential Medium (IMEM) (Biosource International Inc., Camarillo, CA, USA) with 10% foetal bovine serum (Valley Biomedical Inc., Winchester, VA, USA), at 37 °C in a humidified 5% CO_2 atmosphere. For growth assays in oestrogen-free conditions, cells were repeatedly washed and grown in steroid-depleted medium (phenol red-free IMEM supplemented with 5% charcoal stripped calf bovine serum) as previously described [20]. Cells were plated in steroid-depleted medium at 2×10^3 cells/well in 96-well plates (Falcon, Lincoln Park, NJ, USA) and allowed to attach overnight before treatment with test drugs. Cells in oestrogen-free conditions were treated with vehicle (0.1% ethanol), E2 or efavirenz in the presence or absence of the anti-oestrogen ICI 182,780. The relative cell number after 4–6 days of growth was determined using crystal violet staining and WST cell proliferation staining (Roche Applied Science, Indianapolis, IN, USA) as described previously [21].

Receptor binding assay

Fluorescence polarization-based competitive binding assays were performed to measure the relative binding affinity of efavirenz for ER-α using a commercially available kit (P2698; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's specifications. We have previously described the use of this assay to evaluate the relative affinity of ligands for ER-α [19]. Reactions (100 μL) were carried out in black-wall, low-volume 96-well plates (6006270; PerkinElmer, Waltham, MA, USA). Following 2 hours of incubation at room temperature, fluorescence polarization values were obtained using a BMG PolarStar Omega plate reader (BMG Labtech, Durham, NC, USA).

Statistical analyses and curve fitting

Student's *t*-tests were used to compare treatments with respective controls (SIGMASTAT Version 3.5; Systat Software Inc., San Jose, CA, USA). Curve fitting and effective concentration for half-maximal growth (EC_{50}) or binding (IC_{50}) were determined using GRAPHPAD PRISM Version 4.03 (GraphPad Software, San Diego, CA, USA).

Results

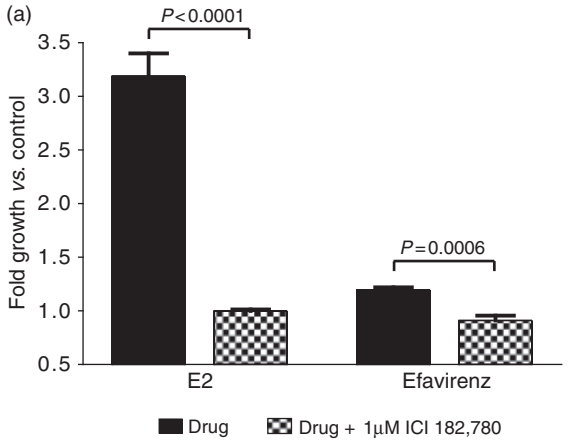
Efavirenz induces breast cancer cell growth

Efavirenz (10 μM) induced growth of MCF-7 cells that was ~1.2-fold greater than that induced by vehicle treatment (Fig. 1a; right, solid bar). This effect was blocked by the anti-oestrogen ICI 182,780 (Fig. 1a; right, chequered bar). As expected, E2 (10 nM) maximally stimulated growth (~3.2-fold) *versus* the vehicle treatment (Fig. 1a; left, solid bar). ICI 182,780 completely blocked E2-induced growth (Fig. 1a; left, chequered bar). Efavirenz induced a similar amount of growth in ZR-75-1 cells following 4 days of treatment (Fig. 1b), and this growth was blocked by ICI 182,780 (data not shown). However, efavirenz did not stimulate the growth of T47D cells following 6 days of treatment (Fig. 1b).

The concentration–effect curve for efavirenz-induced growth in MCF-7 cells is shown in Fig. 1c. Efavirenz-induced cellular growth was concentration-dependent up to 10 μM. Growth induced at any concentration was completely blocked by 1 μM ICI 182,780 (data not shown). Higher efavirenz concentrations (50 or 100 μM) were growth inhibitory to MCF-7, T47D and ZR-75-1 cells; this effect could not be blocked by ICI 182,780 (data not shown). Although this growth inhibition at high concentrations prevented full characterization of the concentration–effect relationship, we estimated an EC_{50} of approximately 15.7 μM using the data obtained for lower concentrations (1–10 μM).

Efavirenz directly binds ER-α

The affinity of efavirenz binding to the ER relative to that of E2 was determined using a competitive binding assay as described in 'Materials and methods' section. Efavirenz bound ER-α at a >1000-fold higher concentration (IC_{50} of ~52 μM) than E2 (IC_{50} of ~16 nM) under these experimental conditions (Fig. 2).



(b)

	MCF-7	T47D	ZR-75-1
Treatment time	4 days	6 days	4 days
Fold *vs.* control ± SD	1.20 ± 0.02	1.06 ± 0.03	1.15 ± 0.07
***P*-value**	0.0006	0.16	0.0068

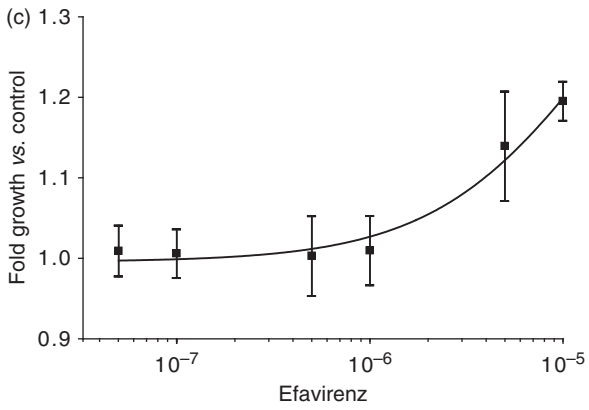


Fig. 1 MCF-7 cells were grown in oestrogen-free conditions as described in 'Materials and methods'. (a) 17β-oestradiol (E2) was added to a final concentration of 10 nM, and efavirenz was added to a final concentration of 10 μM. Cells were treated in the absence (solid bars) or presence (chequered bars) of ICI 182,780 at a final concentration of 1 μM. Bars represent mean 4-day growth ± standard deviation (SD) *versus* the vehicle-treated control for experiments in triplicate. (b) Growth induced by 10 μM efavirenz in breast cancer cell lines. *P*-values were determined for efavirenz-treated cells *versus* vehicle control. (c) Efavirenz was added at increasing concentrations from 50 nM to 10 μM. Points represent mean 4-day growth ± SD *versus* the vehicle-treated control for experiments in triplicate.

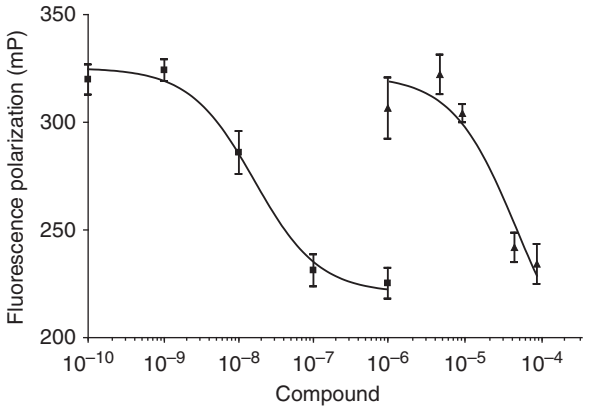


Fig. 2 Fluorescence polarization-based oestrogen receptor (ER)-α binding assays were performed as described in 'Materials and methods'. Decreasing polarization (*y*-axis) represents increased receptor occupancy by the test compound, 17β-estradiol (■) or efavirenz (▲). Points represent mean polarization ± standard deviation for experiments in triplicate.

Discussion

Reports show that 1.8–8.4% of male patients develop gynaecomastia with efavirenz treatment [6–11]. However, the precise mechanism of this adverse effect remains unknown. Our data suggest that efavirenz-induced gynaecomastia may be attributable to direct oestrogenic effects in breast tissues. We demonstrated that efavirenz induced the growth of the oestrogen-dependent, ER-positive breast cancer cell lines MCF-7 and ZR-75-1 and that this effect was completely reversed by the anti-oestrogen ICI 182,780. We have also provided evidence that efavirenz binds directly to ER-α. These data provide the first evidence that efavirenz-induced breast hypertrophy and gynaecomastia may be attributable in part to the ability of the drug to directly activate the ER.

Our data are the first to directly demonstrate that efavirenz binds to ER-α and that it induces cell growth in an E2-dependent breast cancer model. While efavirenz induced growth at $\sim 10^5$-fold greater concentrations than E2, it bound ER-α *in vitro* at much lower concentrations (only 10^3-fold greater concentration than E2), consistent with the hypothesis that efavirenz acts as a weak agonist of the ER. Further, although efavirenz was much less potent than E2 in inducing growth (EC_{50} values of 15.7 μM *vs.* 5 pM [12]), our findings may be clinically important, because efavirenz concentrations that induce growth in our cell model are within the therapeutic plasma concentration range achieved after daily oral administration of 600 mg daily (mean steady-state minimum and maximum concentrations of 5.6 and 12.9 μM, respectively, with inter-patient variability ranging from 0.4 to 48 μM) [4,13]. In addition, given the lipophilicity of efavirenz and thus the very large volume of distribution, it is likely that the concentration in breast tissues is much higher than in plasma. Efavirenz steady-state plasma concentrations in HIV-infected patients exhibit wide inter-subject variability because of the effects of genetic polymorphisms and drug interactions [4,13]. Given the concentration-dependent

ER-α binding and MCF-7 growth induction observed in our study, and that patients with higher efavirenz exposure are at increased risk for adverse effects [4,13], it is possible that patients achieving higher plasma concentrations of efavirenz are more likely to experience breast hypertrophy and gynaecomastia.

The fact that efavirenz induces growth in MCF-7 and ZR-75-1 cells, but not T47D cells, suggests that the efavirenz-induced growth may be dependent on the expression of specific ER transcription cofactors. Unique nuclear receptor cofactor expression is known to play a role in the transcriptional activity of other clinically used agents, particularly the selective ER modulator tamoxifen, which has differing oestrogenic and anti-oestrogenic activities in different target tissues [14].

We were unable to study the effect of efavirenz at high concentrations ($> 10\,\mu M$), because of nonspecific cytotoxicity or cytostatic effects. However, the fact that high-dose efavirenz-induced growth inhibition was not blocked by the ICI 182,780 suggests that this is unrelated to its oestrogenic activity. Interestingly, we found that high concentrations of efavirenz (1–10 μM) could antagonize growth induced by 5 pM E2, providing additional evidence that efavirenz indeed acts as a weak or partial agonist of ER-α (data not shown). However, we could not confirm that this growth antagonism was specifically attributable to competition for binding to ER-α with E2.

Our data may have implications beyond the potential role of efavirenz in gynaecomastia. Evidence exists for an increased incidence of AIDS-defining and certain non-AIDS-defining cancers, including breast cancer, in HIV-infected patients. Generally, HAART use has been shown to be protective for AIDS-defining cancers, although the extent of this protection for non-AIDS-defining cancers seems limited. A recent meta-analysis of the incidence of non-AIDS-defining cancers in HIV-infected patients suggests that the incidence of breast cancer in these patients has significantly increased since the implementation of HAART as standard therapy [15]). Further epidemiological studies comparing efavirenz-based and non-efavirenz-based therapies will be needed to rule out the possibility that the oestrogenic activity of efavirenz may promote breast cancer. It also remains to be seen whether efavirenz interferes with endocrine treatment of breast cancer and contributes to drug resistance.

This study demonstrates that efavirenz directly binds and activates the ER, providing a plausible mechanistic explanation for efavirenz-induced gynaecomastia in HIV-infected patients. Additional indirect support for this suggestion has been provided by Kegg and Lau [16], who reported a case of efavirenz-induced gynaecomastia that was successfully reversed using 20 mg daily tamoxifen. Tamoxifen has been widely used for the treatment and prophylaxis of anti-androgen-induced gynaecomastia in prostate cancer patients with high efficacy and low toxicity [17,18] in addition to its widespread use as a front-line therapy for the treatment and prevention of breast cancer. As multiple antiretroviral drugs are currently available to treat HIV infection, switching from efavirenz to alternative antiretroviral drugs may be one potential strategy to alleviate this adverse effect. However, multiple factors need to be considered before switching to an alternative therapy. Based on our *in vitro* data and evidence from the literature, tamoxifen and other anti-oestrogens may be useful in the treatment of efavirenz-induced gynaecomastia. Importantly, before considering the addition of an anti-oestrogen to a patient's treatment regimen, other potential causes of gynaecomastia should be assessed. A randomized control trial would be necessary to fully evaluate the utility, and more importantly tolerability, of anti-oestrogens as a treatment for efavirenz-induced gynaecomastia.

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