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The Non-genotropic Synthetic Ligand Estren (4-estren-3α17β-diol) is a High Affinity Genotropic Androgen Receptor Agonist

V. Krishnan[*‡], H. A. Bullock[‡], Benjamin C. Yaden, M. Liu, R. J. Barr, C. Montrose-Rafizadeh, K. Chen, Jeffrey A. Dodge, and H. U. Bryant. Musculoskeletal Research (VK, HAB, BCY, ML, KC, & HUB), Lead Optimization Biology & Business Operations (RJB, CMR), Discovery Chemistry Research (JAD), Lilly Research Laboratories, Indianapolis, Eli Lilly and Company , IN, 46225 USA.

‡ *These authors contributed equally to the manuscript.*

*Corresponding author

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Running Title: The Non-genotropic Ligand Estren is a Genotropic AR Agonist

*Corresponding author

Venkatesh Krishnan*

355 East Merrill Street, DC 0434

Lilly Research Labs, Indianapolis, IN 46225

Email: Krishnan_Gary@lilly.com

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Non-Standard Abbreviations: **ARE**= androgen response element, **AR**= androgen receptor, **ER**=estrogen receptor, **ChIP**= Chromatin Immunoprecipitation, **PSA**=Prostate Specific Antigen, **ERK**= extracellular signal-regulated kinases, **PI3K**= Phosphoinositol 3-kinase, **SERM**= Selective Estrogen receptor Modulator, **U0126**= ERK inhibitor, **R1881**= methyl trienolone a synthetic stable androgen

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Abstract:

The non-genotropic ligand estren (Kousteni, S. et al. *Science*. 298, 843-6, 2002) was evaluated for its transcriptional activity mediated by the human androgen receptor (AR). Our results show that estren can bind, translocate, transactivate, and regulate 2 known target genes of AR in androgen responsive cell lines. Estren binds recombinant AR with 10 fold higher affinity than either ERα or ERβ. Estren bound AR can translocate AR to the nucleus and stimulate the ARE-luciferase reporter activity with an efficacy similar to androgen. Estren also increased the expression of PSA in a dose-dependent manner in human LnCAP cells. Using ChIP analysis we show that the estren-bound AR co-immunoprecipitates with a region of the PSA gene promoter. Accordingly, co-treatment with an AR antagonist, bicalutamide, blocked the estren-induced increase in PSA expression. In contrast, PI3K inhibitor Wortmannin, or ERK inhibitor U0126, and ER antagonist ICI-182780 failed to block the effects of estren. *In vivo* analysis of estren's action on male orchidectomized ICR mice, revealed estren's AR agonist actions on the levator ani and seminal vesicle target tissues. Collectively, our results reveal the hitherto unidentified genotropic action of estren mediated by AR in androgen responsive cells and tissues.

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Introduction:

The synthetic ligand estren activates the estrogen receptor (ERα and ERβ)/androgen receptor (AR) via non-genotropic pathways, relying primarily on protein-protein interactions with members of the ERK (Kousteni et al., 2001; Kousteni et al., 2002b; Kousteni et al., 2003) and PI3K (Kousteni et al., 2003; Simoncini et al., 2000) enzyme complex. These studies provided an exciting opportunity to obviate the risk associated with the uterotrophic and mammary gland stimulating activities typically associated with the genotropic pathways induced by ERα (Frasor et al., 2003; Harris et al., 2002) while continuing to protect against ovariectomy induced bone loss. Thus, the prototypical molecule estren displays a profile that is unique from SERMs and estrogen because, it harbors no uterotrophic activity *in vivo* and reverses ovariectomy-induced bone loss. Interestingly, the effect of estren on bone mineral density is distinct from both estrogen and SERMs when evaluating the cortical bone changes induced these treatments (Kousteni et al., 2002a). This prompted us to examine the potential interactions between estren and AR, because androgens have been reported to possess cortical bone formation enhancing activity *in vivo* (Turner et al., 1990). Hence, we evaluated the role of AR in mediating the biological action of estren. In this study we show that estren can bind, translocate, transactivate and induce endogenous target genes of AR in 2 distinct androgen responsive cell types, using the conventional genotropic signaling. These findings add a layer of complexity to this ligand previously claimed to exclusively work via non-genotropic pathways (Kousteni et al., 2003).

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Materials & Methods:

All chemicals used were purchased from Sigma-Aldrich, (St. Louis, MO), except estren (Steraloids Inc., Newport, RI) or as otherwise indicated.

Receptor Binding and co-transfection assay

K_i values were determined using competitive binding assays. ^{3}H-17β-estradiol and baculovirus expressed ERα or ERβ receptors were used for binding to ER receptor. For AR binding assays ^{3}H-methyltrienolone (R1881 or androgen) and extracts from 293 cells over-expressing hAR receptor were used.

C2C12 cells (50,000 cells/well) were transiently transfected with a plasmid expressing hAR (1.6ug/ml)as well as a plasmid with 3X ARE-luciferase (5ug/ml). Results are expressed as relative efficacy compared to 100 nM R1881,

Immunofluorescence assay

LA20 cells were treated for various times with 10 nM estren (E) or 10 nM androgen (A), fixed (4% paraformaldehyde) and permeabilized (1% triton x-100), followed by incubation with an anti-AR rabbit polyclonal Ab (PG-21) 1:100 dilution, or IgG 1:100 dilution, followed by a secondary FITC-labeled anti-rabbit Ab (1:200 dilution). Cells were visualized using confocal microscopy (Confocal system-BioRAD MRC 1024-UV, microscope Nikon Diaphot 200; 20X magnification) and nuclei were counterstained using DAPI. The increase in nuclear AR signal for each time point after addition of

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ligand (standardized to DAPI) as measured by quantitative confocal microscopy (acquisition software LaserSharp 2000™) is shown in the above Table as % Vehicle control.

LnCAP PSA ELISA assay

LnCAP cells were seeded into 96-well plates overnight at 4 X 10^4/well. Cells were treated for 48 hours with various concentrations of androgen or estren. . Later, 10 µl of the media from each well was assayed for PSA using the Total PSA kit, Diagnostic Systems Labs, Webster, TX.

Chromatin Immunoprecipitation (ChIP) assay

The assay was performed with slight modifications to the protocol described in Shang et al., 2002. LNCaP cells were seeded in DMEM/F-12 media supplemented with 5% charcoal-dextran-stripped fetal bovine serum. Cells were cultivated for two days and stimulated twice with the appropriate ligands (once the night before, and once two hours before crosslinking). Cross-linking was performed using 1% formaldehyde followed by incubation at 37 for 10 min. Cells were washed twice with 1X DPBS (Gibco) and then collected in 5ml of DPBS. Pellets were resuspended in 0.1ml of lysis buffer (SDS lysis buffer (Upstate Biotechnology, Waltham, MA) and 1x protease inhibitor cocktail (Roche, Fishers, IN) incubated, diluted properly and sonicated three times for 20s a set. Supernatants were collected and diluted. Anti-AR (upstate) was added to a final dilution of 1:100 and rotated overnight at 4 degrees. Immunoclearing was carried out with .05ml of sheared salmon sperm DNA, and protein Sepharose A (upstate) for 1hr. Sepharose

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beads were then washed sequentially for 3 min each with the various salts provided by the ChiP kit (Upstate). Beads were finally washed with an elution buffer (.1M NaHCo3, 10% SDS) for 30 min at RT. Supernatants were transferred and de-crosslinked by a 1hr incubation at 65 degrees using the decrosslinking buffer containing 5M NaCl, 0.5M EDTA, 1M TrisCl, and Proteinase K {Invitrogen}. A standard DNA precipitation method was utilized and pellets were resuspended in .050 ml of water. PCR parameters and primers were used according to the Sheng et al., 2002 paper.

IGF-1 bDNA mRNA analysis

Rat LA20 cells were seeded at 8,000 cells/ 96-well in DMEM/F-12 (Invitrogen, Cat.# 21041-025) with 10% charcoal stripped FBS. Compound in 0.1% DMSO were dosed after 24 h, dissolved in 10% charcoal stripped DMEM/F-12 media. After 72 h the Quantigene (bDNA) assay was performed according to manufacturer's protocol (Genospectra, Inc., Fremont, CA). Briefly, 50ul Quantigene lysis mixture™ is added to cells with culture media at the end of the incubation period, followed by transfer of 100 µl of this mix to capture plates containing the universal probes. The lysis mixture contains the CE and LE probes. Mixture is incubated overnight (16 h) at 56°C, followed by addition of branch label (BL) and luminescence substrate. Later, the luminescence from each well is read using a Dynex Tech MLX luminometer. Results are plotted as RLU/8000 cells for each treatment group.

Probe list can be suppressed for publication or shown as supplemental online data

Probe List (CE= capture extender, LE= label extender, BL= branched label)

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Probe	Function	Sequence
rIGF001	CE	ccgaatgctggagccatagctttttctcttggaaagaaagt
rIGF002	CE	cgagctgactttgtaggcttcatttttctcttggaaagaaagt
rIGF003	CE	gctgggcccggatggaatttttctcttggaaagaaagt
rIGF004	CE	tttctgttcctcgggaggctttttctcttggaaagaaagt
rIGF005	CE	caggtgttccgatgttttgcatttttctcttggaaagaaagt
rIGF006	CE	ggtattgaactcattattgatatttggtttttctcttggaaagaaagt
rIGF007	LE	tctggtccggctgtggctttttaggcataggacccgtgtct
rIGF008	LE	cagccccgcaaagggtcttttttaggcataggacccgtgtct
rIGF009	LE	gcccgtctgtggtgccctttttttaggcataggacccgtgtct
rIGF010	LE	gcggagcacagtacatctccatttttaggcataggacccgtgtct
rIGF011	LE	ggttcctcaagcagcaaaggatttttttaggcataggacccgtgtct
rIGF012	BL	ctgaagagcgtccaccagct
rIGF013	BL	ccccttggtccacacacgaa
rIGF014	BL	ctgtgggcttgttgaagtaaaag
rIGF015	BL	ggaagcaacactcatccacaat
rIGF016	BL	gcctcctcagatcacagctcc
rIGF017	BL	ttgggcatgtcagtgtggc
rIGF018	BL	tcttcaagtgtacttccttctgagtc
rIGF019	BL	ttcctgcacttcctctacttgtgt
rIGF020	BL	tcctcctacattctgtaggtcttgt
rIGF021	BL	cttgcggtgacgtggcat
rIGF022	BL	gggaaatgcccatctctgaaat
rIGF023	BL	ggaatgtttacttgtgtatttcattga
rIGF024	BL	ttaacaaacactcctaaagacaatgtc

In vivo assay

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All protocols pertaining to animal use and storage for experimentation were approved by the Lilly Animal Care protocol. Twelve-wk old male ICR mice were bilaterally orchidectomized (ORX) or sham-operated. At 6 wks of post-surgery, they received treatment with and 10 mg/kg/d Estren for 14 days (s.c. n=8). TE (Testosterone Enanthate 2 mg/kg/d) was used as positive control. One group of ORX and Sham control received vehicle alone. At the end of the treatment period, the levator ani muscle and seminal vesicles were removed and weighed. Statistical analysis was performed using Dunnett's test.

Results & Discussion:

Using recombinant human AR and radiolabeled androgen (^{3}H-methyl trienolone) in a competitive binding assay, we measured direct binding of estren to AR. Our results show that estren binds to AR (K_i =27 nM) and transactivates (EC_{50}= 1.5 nM) a luciferase reporter linked to an androgen response element (ARE) in C2C12 cells (Figure 1A). Estren's binding affinity to recombinant human AR is approximately 10 fold higher than that to recombinant human ERα (K_i = 242 nM) or ERβ (K_i =272 nM) (*Inset* 1A). Furthermore, the relative efficacy (101%) of estren compared to androgen in the ARE-dependent reporter assay shows that it is a full AR agonist. Similar results were obtained with a human prostate cancer cell line PC-3 co-transfection assay (data not shown).

The hallmark of conventional Type II genotropic ligands is the translocation of the receptor from the cytoplasm to the nucleus (Jenster et al., 1993). Hence, to visualize the translocation of AR protein from its cytoplasmic apo-receptor location to the estren-bound location, we performed immuno-fluorescent analysis using an AR specific

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antibody. Our results (Figure 1B) clearly demonstrate the nuclear translocation and accumulation of AR after 15 minutes of treatment with 10 nM estren. This translocation of AR-bound estren is further evident after 6 hr and is similar to that of androgen. In contrast, treatment with E2 failed to elicit this effect on AR translocation (data not shown). Furthermore, after 24 hr of treatment with estren or androgen we observe an increase (3 fold) in nuclear AR levels using quantitative confocal microscopy (Figure 1B). Collectively, these results show that estren behaves like a classical genotropic ligand of AR.

To extend these observations to endogenous target genes of genotropic androgen signaling, we utilized the LnCAP androgen responsive human prostate cancer cell line. We measured prostate specific antigen (PSA) in the medium of cells treated with various concentrations of estren for 48 hr. PSA is a genotropic target of AR activity and its promoter elements responding to liganded-AR have been extensively studied (Cleutjens et al., 1996; Luke and Coffey, 1994). Results from these experiments (Figure 2A) clearly show the potent and efficacious AR agonist activity of estren relative to androgen (Relative Efficacy = 90%, EC_{50} = 0.4 nM). Furthermore, this increase in PSA levels was completely blocked by the AR antagonist, bicalutamide (Figures 2C). Reversal of estren action by AR antagonist is also evident in the ARE reporter assay (data not shown). However, co-treatment with the ERK inhibitor (U0126) (Figure 2C) or PI3K inhibitor Wortmannin (Figure 3C), failed to reduce this activity. The ERK and PI3K pathways were reported as prominent mediators of estren's non-genotropic activity (Kousteni et al., 2003). In addition, co-treatment with ICI,182780 the "pure" ERα/ERβ antagonist also failed to affect the estren-induced increase in PSA (Figure 2C). Hence, the lack of

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reduction after co-treatment with either the ERK, or PI3K inhibitor, on estren-induced PSA levels, is indicative of the absence of non-genotropic signaling of estren in stimulating PSA levels in LnCAP cells. To ascertain that the estren-induced expression of PSA is indeed mediated by a genotropic mechanism we used the previously described ChIP assay (Shang et al., 2002). The clear recruitment of estren-bound AR to the promoter of the PSA gene is comparable to R1881 bound AR and is indicative of a classical transcriptional regulation of PSA gene expression by AR (Figure 2D) and confirms the genotropic action of estren on a target gene of AR.

We extended these observations to another target gene of AR, by evaluating changes in IGF-1 mRNA after treatment of LA20 muscle cells with estren and androgen. IGF-1 is a reliable target gene of AR in the LA20 cell line (Chen, K., Moore, T, unpublished results). The LA20 cell line is derived from the rat levator ani (LA), and is a highly responsive target tissue of androgen (Nnodim, 2001). Our results show that estren can induce the expression of IGF-1 mRNA in a dose-dependent manner in LA20 cells with a relative efficacy comparable to that of androgen (Figure 2B),. To study the role of non-genotropic signaling in estren-induced IGF-1 stimulation, we utilized the identical set of ERK and PI3K inhibitors along with estren in LA20 cells. As observed in the LnCAP PSA assay, the inhibitors had no effect on estren-induced IGF-1 stimulation thus ruling out the proposed non-genotropic signaling on this target gene (data not shown).

Finally, to evaluate the role of AR in mediating some of estren's *in vivo* activity, we induced levator ani (LA) muscle atrophy (Krieg, 1976; Nnodim, 2001) by orchidectomy in weight-matched male ICR mice. The LA muscle and the seminal

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vesicles are primary targets of AR-mediated genotropic signaling in male mice. (Krieg, 1976). Orchidectomy-induced atrophy of the LA muscle was reversed by testosterone enanthate (TE) or estren after 2 weeks (Figure 3A). Similar results were seen with changes in seminal vesicles in these animals after 2 weeks of treatment with estren (Figure 3B). Collectively, these results point to potent and efficacious androgenic activity *in vivo* in male ICR mice, that is consistent with the genotropic AR activity *in vitro*.

In addition, treatment of ovariectomized female SD rats with estren, results in a suppression of the luteinizing hormone (LH) levels that are greatly induced as a result of ovariectomy (data not shown). We also found that in the previously reported morphine withdrawal induced hot-flush model (Simpkins JW et al., 1983) estren fails to produce any of the hot-flush ameliorating effects of estrogen, in spite of lowering LH levels at these doses (data not shown). Earlier reports have clearly demonstrated that genotropic AR ligands such as dihydrotestosterone (DHT) fail to ameliorate the hot-flush end point in this model in spite of lowering LH levels (Panidis et al., 1994) (Merchenthaler et al., 1998). Hence we believe that this unique profile of estren in both these in vivo experiments using female SD rats is primarily attributed to its androgen-like properties, found in the previous male ICR mice experiment.

In conclusion, estren was found to be a potent AR ligand using recombinant human AR and labeled androgen in competitive AR binding assays. In addition, estren was found to translocate AR to the nucleus and possess full AR agonist activity as observed in 2 distinct androgen-responsive cell based assays. Recently, it was reported

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that the conversion of estren to 19-nortestosterone was responsible for some of the androgenic effects of estren (Centrella et al., 2004). However, in our binding assays we utilize purified human androgen receptor to quantitate the binding affinity of the compound and we hypothesize that in addition to metabolism to 19-nortestosterone in certain cells, estren can directly bind AR and elicit a genotropic effect. We predict that the genotropic action of estren on AR is responsible for mediating the *in vivo* and *in vitro* effects of estren in both female SD rats and male ICR mice. Future use of this class of ligands for therapeutic intervention in treatment of osteoporosis via its non-genotropic action on ERα/ERβ must factor the potential involvement of the genotropic AR function described in this study.

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The authors wish to thank Drs. Armen Tashjian (Harvard Univ.) and Andrew Geiser (Lilly Research Labs) for critical review of this manuscript. [1]

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Figure Legends:

Figure 1) Receptor activation (EC50) was determined in C2C12 cells transiently transfected with a plasmid expressing AR as well as a plasmid with ARE-luciferase. In addition, % efficacy was determined versus activity of 100 nM methyltrienolone. 1A Inset: Ki values were determined in competitive binding assays. 3H-17b-estradiol and baculovirus expressed ERα or ERβ receptors were used for binding to ER receptor. For AR binding assays 3H-methyltrienolone and extracts from 293 cells overexpressing AR receptor were used.

Figure 2) LA20 cells were treated for various times with 10 nM Estren (E) or 10 nM R1881 (T) and subjected to immunofluorescence analysis using a rabbit polyclonal Ab (PG-21) 1:100 dilution, or IgG 1:100 dilution, followed by a secondary FITC-labeled anti-rabbit Ab (1:200 dilution). Cells were visualized using confocal microscopy and nuclei were counterstained using DAPI. The increase in AR signal (standardized to DAPI) as measured by quantitative confocal is shown in the above Table as % Vehicle control.

Figure 3A & 3B) LnCAP (A) or LA20 (B) cells were seeded into 96-well plates overnight at 4 X 104/well. Cells were treated for 48 hours with Estren or R1881 at various doses. Ten μl of the media from each well was assayed for PSA by ELISA (Total PSA kit, Diagnostic Systems Labs, Webster, TX). 100 ml of the LA20 lysate was used in the bDNA assay *$p<0.05$ significantly lower than Estren 10 nM control as determined by ANOVA Fisher's post-hocs test)

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Figure 3C) LnCAP cells were seeded into 96-well plates overnight at 4 X 104/well. Cells were co-treated for 48 hours with 1) DMSO 0.2%, 2) R1881 1 nM, 3) Estren 10 nM + 0.5 nM Wortmannin, 4) Estren 10 nM + Wortmannin 5 nM, 5) Estren 10 nM + Wortmannin 50 nM, and 6)Estren 7.5 nM + 0.1 uM ICI,182780, 7) Estren 7.5 nM + 1.0 uM ICI,182780, 8) Estren 7.5 nM + 10 uM ICI,182780, 9) Estren 7.5 nM + 0.1 uM U0126, 10) Estren 7.5 nM + 1.0 uM U0126, 11) Estren 7.5 nM + 10 uM U0126 12) Estren 7.5 nM + 1 uM Bicalutamide, 13) Estren 7.5 nM + 5 uM Bicalutamide 14) Estren 7.5 nM + 10 uM Bicalutamide. 5 ul of the media from each well was assayed for PSA by ELISA (Total PSA kit, Diagnostic Systems Labs, Webster, TX). * $p<0.05$ significantly lower than estren control (ANOVA Fisher's post-hocs test).

Figure 3D) ChIP assays of AR occupancy of the promoter and enhancer regions of the PSA gene. LnCaP cells were treated with 10 nM of R1881, Estren, and a equivalent of vehicle alone. Soluble chromatin was prepared and formaldehyde- crosslinked. Antibodies raised against AR were utilized to immunoprecipitate protein-bound DNA fragments. Fragments shown were amplified using methods of PCR.

Figure 4) Eight-wk old male ICR mice were bilaterally orchidectomized (ORX) or sham-operated. At 6 wks of post-surgery, they received treatment with and 10 mg/kg/d Estren for 14 days (s.c. n=8). TE (Testosterone Enanthate 2 mg/kg/d) was used as positive control. One group of ORX and Sham control received vehicle alone. At the end of the treatment period, blood samples were collected and the A) levator ani muscle and B) seminal vesicles were removed and weighed. *=$p<0.05$ compared to ORX control (Dunnett's test).

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Figure 1

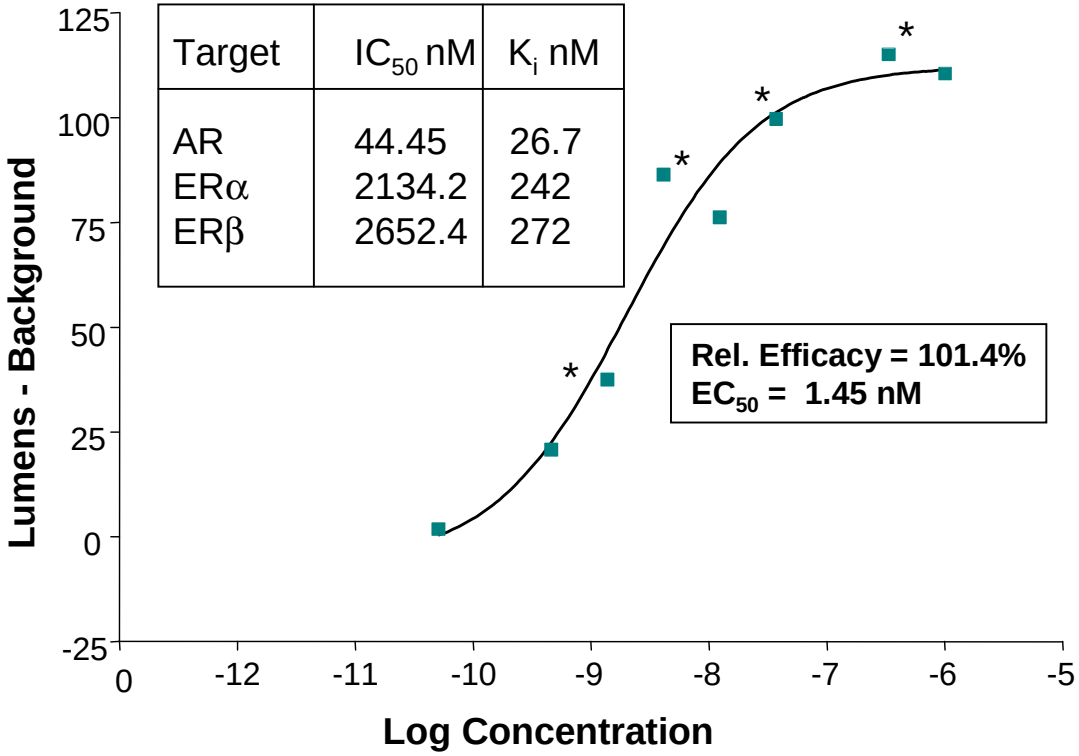

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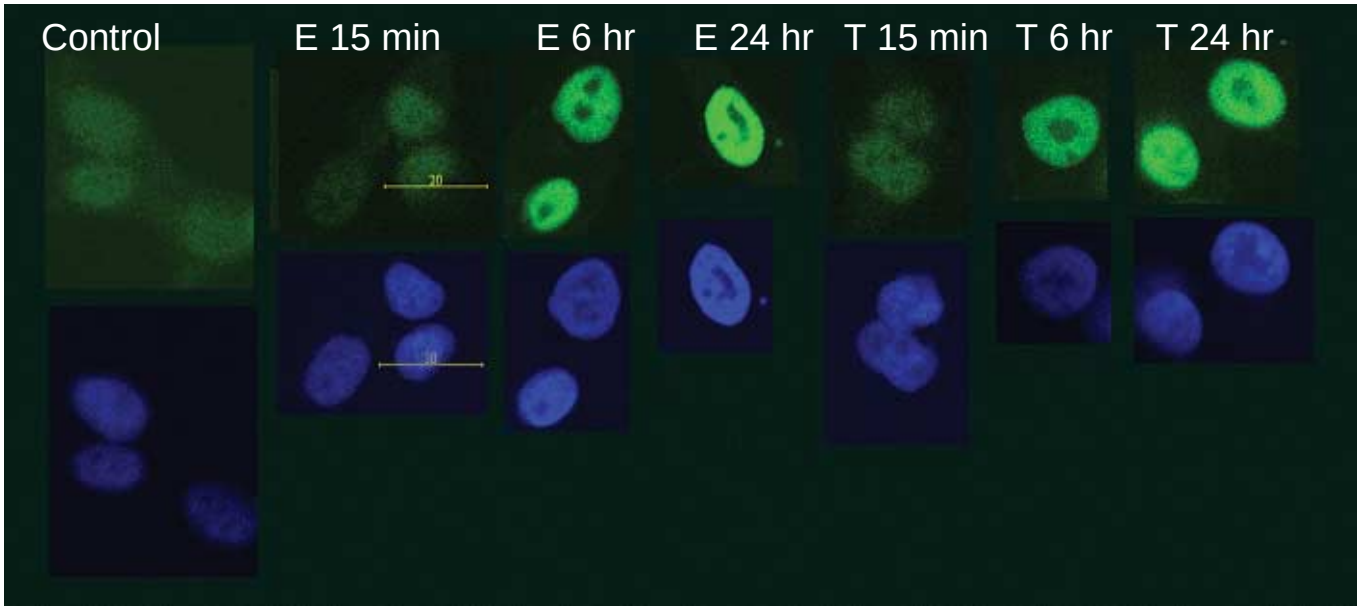
Figure 2



Time	Estren	T
15 min	104%	106%
6 hr	202%	220%
24 hr	320%	295%

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Figure 3A

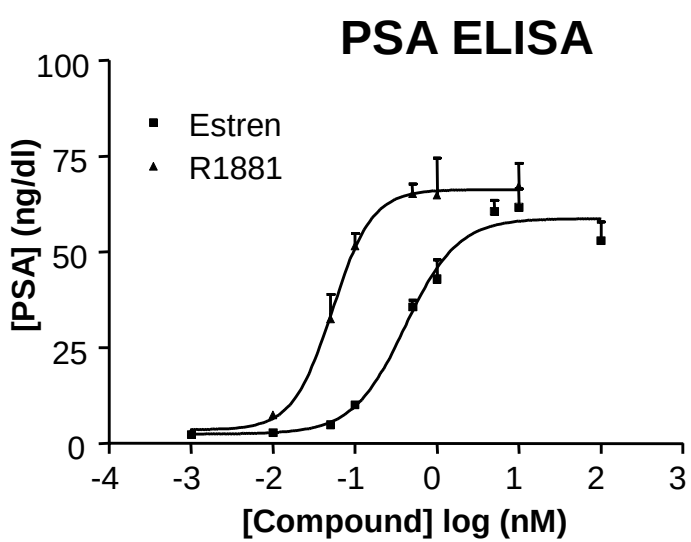

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Figure 3B

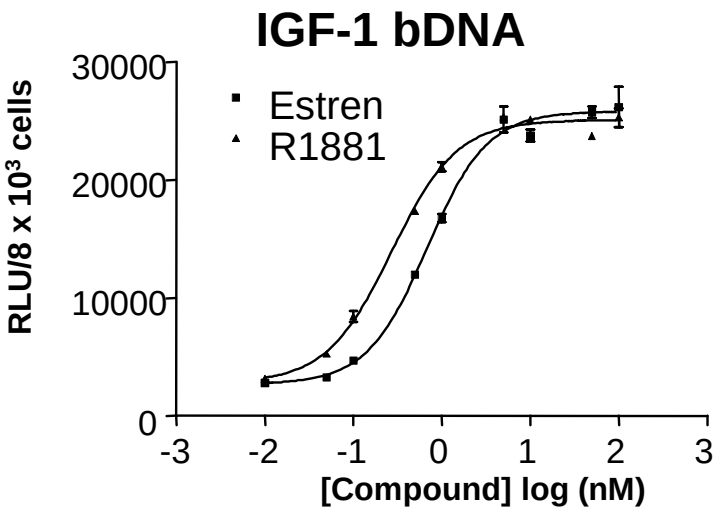

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Figure 3C

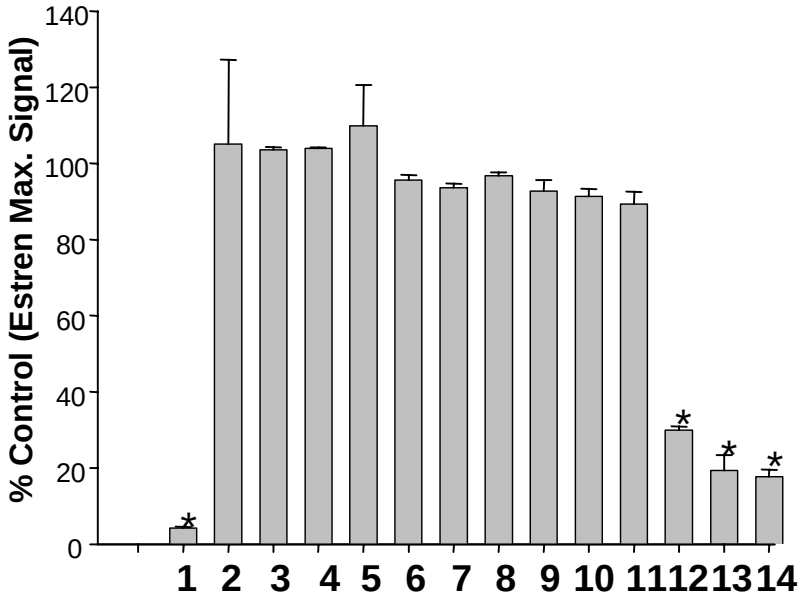

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Figure 3D

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Figure 4A

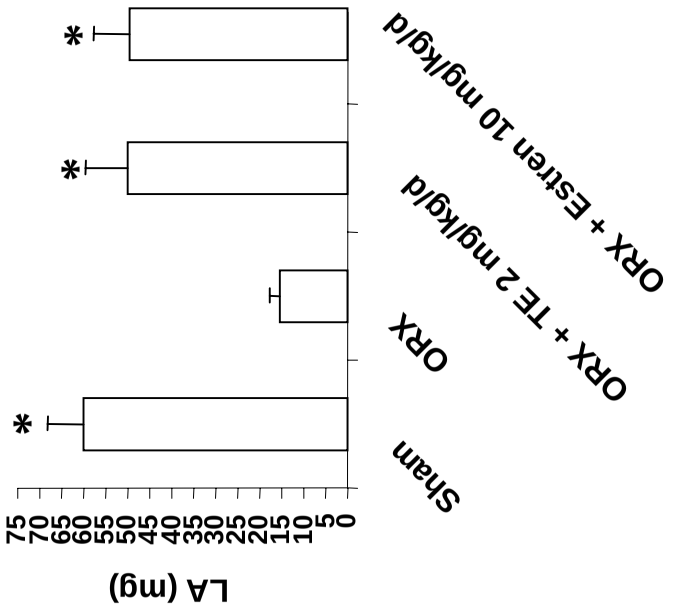

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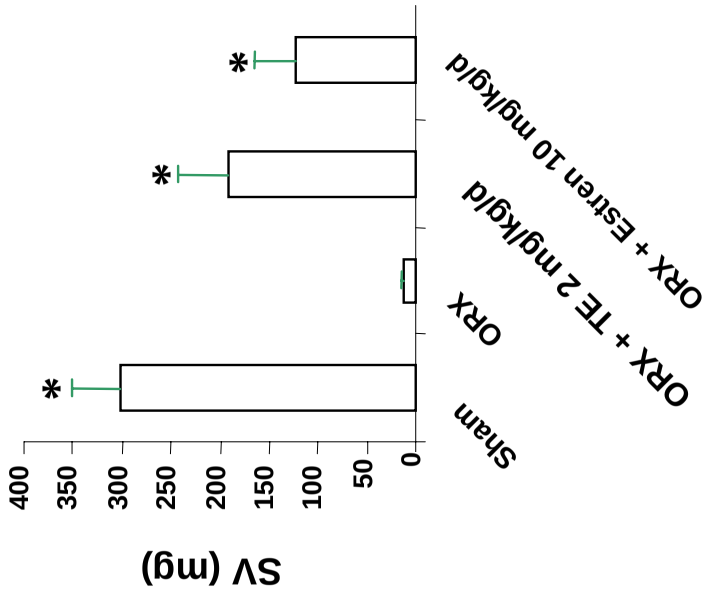
Figure 4B



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