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(54) Title: PEPTIDE

(57) Abstract: The present invention relates in general to pure antiestrogens and selective estrogen receptor degraders and, in particular, to a peptide that interacts specifically with pure anti estrogen bound to estrogen receptor α (ER α) and to a method of identifying pure antiestrogens or selective estrogen receptor degraders using same.



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PEPTIDE

This application claims priority from U.S. Provisional Application No. 60/783,860, filed March 21, 2006, the entire content of which is incorporated herein by reference.

5

TECHNICAL FIELD

The present invention relates, in general, to pure antiestrogens and selective estrogen receptor degraders and, in particular, to a peptide that interacts specifically with pure antiestrogen bound to estrogen receptor α (ER α) and to a method of identifying pure antiestrogens or selective estrogen
10 receptor degraders using same.

BACKGROUND

Breast cancer is still the most prevalent cancer of women in the United States. The majority of breast cancers express estrogen receptor alpha (ER α) and display estrogen (E2)-dependent growth (Beatson, Lancet 148:162-165
15 (1896), Epstein, N. Engl. J. Med. 344:276-285 (2001)). ER α , a member of the nuclear receptor superfamily, functions as a ligand inducible transcription factor (McDonnell and Norris, Science 296:1642-1644 (2002)). Although the mechanism is not fully understood, the activity of E2 in breast cancer progression is thought to be mediated through binding and activating ER α
20 (Epstein, N. Engl. J. Med. 344:276-285 (2001)). Tamoxifen (TOT) functions as an antagonist in the breast by competing with E2, and inducing a unique conformation in ER α that inhibits the recruitment of proteins involved in activation of transcription (Brzozowski et al, Nature 389:753-758 (1997), Shiua et al, Cell 95:927-937 (1998)). Therefore, hormonal therapies such as
25 TOT are used for the treatment of breast cancer (Gradishar, The Oncologist 9:378-384 (2004), Howell et al, J. Clin. Oncology 22:1605-1613 (2004)).

TOT has tissue-selective agonistic properties, which results in activation of ER α in some tissues (Howell et al, J. Clin. Oncology 22:1605-1613 (2004)). TOT and similarly acting compounds have been named selective estrogen receptor modulators (SERMs). Unfortunately, TOT
5 treatment has been linked to endometrial cancer and thromboembolic disease. In addition, the majority of breast tumors treated with TOT become resistant to treatment (Howell et al, J. Clin. Oncology 22:1605-1613 (2004), Fisher et al, J. Natl. Cancer Inst. Monogr. 30:62-66 (2001), Chung and Carlson, Curr. Treat. Options Oncol. 4:133-140 (2003)). For these reasons, the development
10 of hormonal therapies with mechanisms of action different from TOT remains necessary. One such compound, termed a pure antiestrogen (i.e. ICI 182,780 [Faslodex], or "ICI"), has no agonist activity on the majority of genes in target cells and accelerates degradation of the receptor, which are thought to be responsible for its antiestrogenic actions (Howell et al, J. Clin. Oncology
15 22:1605-1613 (2004)). ICI (Faslodex) has recently been approved for the treatment of metastatic breast cancer (Howell et al, J. Clin. Oncology 22:1605-1613 (2004), Bross et al, Clin. Cancer Res. 9:4309-4317 (2003), Robertson et al, Eur. J. Can. 41:346-356 (2005)).

As with most hormonal therapies, many breast cancer patients on ICI
20 (Faslodex) treatment lack growth inhibition or relapse during treatment, despite tumors being ER α (+) (Gradishar, The Oncologist 9:378-384 (2004)). The cause of relapse may be linked to the proteins involved in pure antiestrogen-mediated degradation of ER α . Identifying such proteins may provide the underlying mechanism for relapse in breast cancer patients treated
25 with antiestrogens and provide markers for identifying those patients that would be expected respond to pure antiestrogen treatment. Another caveat to ICI (Faslodex) treatment is that it is not orally bioavailable, further underscoring the need for new therapeutic agents. The present invention provides a method for identifying such agents.

SUMMARY OF THE INVENTION

The present invention relates to a peptide that interacts specifically with pure antiestrogen bound to ER α and to the use of such a peptide in a method of identifying pure antiestrogens or selective estrogen receptor
5 degraders.

Objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. ELISA of T7 phage screens of HEPG2 and MCF7 cDNA
10 libraries against E2-bound ER α . HEPG2 and MCF7 pooled T7 phage from five rounds were incubated with E2-bound ER α for the ELISA. Bound phage were detected using an antibody against the T7 capsid protein conjugated to horseradish peroxidase.

Figures 2A-2C. Identification of a peptide fragment that specifically
15 binds ER α in the presence of pure antiestrogens and inhibited by estrogen. Fig. 2A, Mammalian two-hybrid assays were performed as follows: HEPG2 cells were seeded in MEM without phenol media supplemented with 10% charcoal stripped FBS. 24 hours later, the cells were transfected with a 5XGal4-TATA-luc reporter, a VP16-ER α expression plasmid and either
20 GAL4DBD alone or GAL4DBD-AEIP expression plasmid. Cells were treated with vehicle or 100 nM of the following ligands: 17 β -estradiol (E2), 4-OH Tamoxifen (4-OH TOT), GW7604, raloxifene (Ralox), EM-652, ICI 182,780, or RU 58-668 for 24-36 hours. Transcriptional activation of 5XGal4-TATA-luc and, therefore, ER α /AEIP interaction was measured by luciferase assay
25 and activity was normalized for transfection efficiency by transfected CMV- β Gal plasmid. Graph is the average of at least 3 triplicated experiments for all

ligands except raloxifene (2 triplicates) with standard errors. Fig. 2B, Mammalian two-hybrid assays were performed as previously described and cells were treated with vehicle, treated alone or in combination with ICI 182,780 or 17 β -estradiol (E2) at various concentrations from 0.1 nM to

5 100 nM. ER α /AEIP interaction was measured as in Fig. 2A. Graph is the average from 2 triplicated experiments with standard errors. Fig. 2C. Mammalian two-hybrid assays were performed as described using GAL4DBD-AEIP and VP16-ER α , -ER β long form (L), -ER β short (S), - androgen receptor (AR), - glucocorticoid receptor (GR), - progesterone
10 receptor A or B form (PRA or PRB), or retinoic acid receptor (RAR). Cells were treated with either vehicle (V), 100 nM ICI 182,780 (ICI), 17 β -estradiol (E2), casadex (cas), R1881, RU486 (486), dexamethasone (dex), RU5020, (5020) or 9-cis retinoic acid depending upon receptor transfected. GAL4DBD-AEIP interaction with various receptors was determined by
15 luciferase expression as in Fig. 2A and compared to GAL4DBD only interaction. Graph represents average of ER α , ER β L, AR, and PRA. Interaction with AEIP from 2 triplicate experiments with standard errors and the average of ER β S, GR, PRB, and RAR interaction with AEIP from 1 triplicate experiment.

20 Figures 3A-3D. Identification of the specific amino acids in AEIP necessary for the pure antiestrogen induced interaction between AEIP and ER α . Fig. 3A. Diagram of AEIP amino acid sequence and amino acid sequences of mutated peptides. AEIP: Represents the amino acids of AEIP beyond the GAL4DBD; Oligo 1 and 2: 15 and 18 amino acid peptides
25 respectively identified by NCBI blast to show similarity to AEIP. Bold amino acids are same positioned amino acids found in AEIP. Underlined amino acids represent additional amino acids in the vector that were expressed beyond the GAL4DBD protein, but were not identified from the T7 screen; NAAIRS mutants: represents the 7 regions (1, 2, 3, 4, 5, 6, 7) of the AEIP

peptide that were individually mutated to the NAAIRS amino acid sequence while keeping the other 6 regions wild type. Alanine mutants: represents the amino acids that were mutated to alanines either individually (1, 2, 3, 4, 5, 6) or together (1&2). The underlined amino acids show the specific amino acids in AEIP necessary for the pure antiestrogen induced AEIP/ER α interaction. Figs. 3B, 3C, 3D. Mammalian two-hybrid assays were performed in HEPG2 cells as previously described in Fig. 2, to determine the interaction of VP16-ER α with: Fig. 3B, Gal4DBD-Oligo 1 and Oligo 2 in the presence of vehicle or 100nM 17 β -estradiol (E2), 4-hydroxytamoxifen (4-OH TOT), or ICI 182,780 - graph represents the average interaction from 5 triplicate experiments with standard errors, Fig. 3C, various AEIP-NAAIRS mutants in the presence of vehicle or 100nM ICI 182,780 - graph represents the average interactions from 3 triplicate experiments with standard errors, or Fig. 3D, various AEIP alanine mutants in the presence of vehicle or 100 nM ICI 182,780 - graph represents the average interactions from 3 triplicate experiments with standard errors.

Figures 4A-4C. Amino acids surrounding AEIP interaction domain play a role in proper presentation of the peptide for interaction with pure antiestrogen bound ER α . Fig. 4A, Diagram of AEIP amino acid sequence and amino acid sequences of mutated peptides. AEIP: Represents the amino acids of AEIP beyond the GAL4DBD; AEIP (-add. a.a.): have the additional amino acids deleted and only expresses the T7 screen identified AEIP sequence fused to Gal4DBD; AEIP 16, 14, 12: express the previously identified AEIP interaction domain with 5, 4 or 3 amino acids respectively flanking the region previously identified by the NAAIRS mutants to be involved in the pure antiestrogen mediated ER α /AEIP interaction and fused to Gal4DBD. Figs. 4B, 4C, Mammalian two-hybrid assays were performed in HEPG2 cells as previously described in Fig. 2, to determine the interaction of VP16-ER α with: Fig. 4B, increasing amounts of Gal4DBD-AEIP (-add. a.a.) in the presence of

vehicle or 100nM ICI 182,780 - graph represents the average interaction from 1 triplicate experiment, or Fig. C, the previously identified AEIP interaction domain with 5 (AEIP 16), 4 (AEIP 14), or 3 (AEIP 12) flanking amino acids in the presence of vehicle or 100nM ICI 182,780 - graph represents the
5 average interaction from 1 triplicate experiment.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to peptides that can be used in a method of identifying pure antiestrogens and/or selective estrogen receptor degraders that induce a conformation in ER α similar to the conformation induced by
10 pure antiestrogens. Compounds so identified can be used in combating relapse or lack of response by patients to other hormonal agents used in the treatment of estrogen responsive malignancies. In a preferred embodiment, ER α degraders identified in accordance with the present method are orally administerable.

15 More specifically, the present invention relates to a peptide comprising the amino acids SPM and to a method of using same to identify compounds with pure antiestrogens and/or selective estrogen receptor degrader properties. That is, the peptide of the invention comprises the sequence X(n)SPMX(n) where X can be any amino acid and (n) represents any number of amino acids.
20 The peptide is preferably at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17 or 18 amino acids in length. Advantageously, the peptide comprises the amino acid sequence VPNSPM. The invention includes the peptide PEFPSTSLYKKAGWRRNQFSRLDPVPNSPMYVKVCGLCKGFA, and fragments thereof (advantageously at least 6 consecutive amino acids in
25 length) comprising SPM or VPNSPM. The peptide can be bound to a solid support. Further, the peptide can bear a detectable label.

The invention further relates to a nucleic acid sequence encoding the above-described peptide and to a vector (e.g., viral, plasmid, etc) comprising

such a nucleic acid. The nucleic acid can be present in the vector operably linked to a promoter.

The peptide of the invention, having conformation specific properties identified for AEIP (see Example below), can be used in the identification of pure antiestrogens and/or selective estrogen receptor degraders using, for example standard *in vitro* peptide receptor binding assays. For example, such identification can be effected using a variety of biochemical or cell-based interaction based assays. Biochemical assays, including alpha-screen, fluorescence polarization, fluorescence resonance energy transfer or homogenous time resolved fluorescence, are extremely sensitive and reproducible and require purified estrogen receptor and peptide in addition to specific fluorescent labels depending on the assay type. The mammalian 2-hybrid assay (e.g., see Dang et al, Mol. Cell. Biol. 11:954 (1991); Fearon et al, Proc. Acad. Sci. USA 89:7958 (1992)) is an example of a suitable cell-based assay that can be used in accordance with the identification method of the invention. The mammalian 2-hybrid assay detects the interaction of 2 proteins expressed as fusion proteins, the first (the SPM containing peptide) fused to the DNA binding domain of the *S. cerevisiae* transcription factor GAL4 and the second (ER α) to the transcriptional activation domain of the Herpes virus protein VP16. Plasmids expressing these fusion proteins can be transfected into cells (e.g., HepG2 cells) in the presence of a suitable reporter (e.g., a Gal4-Luciferase reporter), the ligand induced interaction between the peptide and estrogen receptor, in the presence and absence of a test compound, can be detected by measuring the activity of the reporter, e.g., via a luminescent assay. This assay can be run according to protocols standard in the art.

The majority of breast tumors express estrogen receptor alpha (ER α) and are dependent upon estrogens for growth. As indicated above, for this reason, hormonal therapies such as antiestrogens are used to treat ER α positive breast tumors. These agents work by competitively inhibiting the interaction of ER α with agonist and/or inducing degradation of the receptor.

This latter class of antiestrogens display these antiestrogenic effects in either all tissues (pure antiestrogens) or within specific tissues (selective estrogen receptor downregulators [SERDs]). Both pure antiestrogens and SERDs have shown considerable efficacy in the clinic, although the mechanism(s) underlying these unique activities are largely unknown. It was suspected that the surfaces exposed on ER α following binding of the receptor to pure antiestrogens allowed the binding of proteins involved in degradation of the receptor. Supporting this hypothesis was the discovery of a 42 amino acid peptide (AEIP) that bound specifically to ER α when liganded with pure antiestrogens. In contrast, no significant interaction was observed with apo ER α or receptor bound to agonists or selective estrogen receptor modulators (SERMs). Mutations within the ER α ligand binding domain demonstrated that AEIP appears to bind to a pure antiestrogen induced hydrophobic pocket on the receptor that is similar to the agonist induced hydrophobic pocket responsible for coactivator binding. Further analysis demonstrated that AEIP was unable to bind pure antiestrogen bound ER β . Interestingly, ER β is not subject to turnover in the presence of pure antiestrogens. Therefore, it was suspected that AEIP was binding to a surface on ER α involved in the degradation of the receptor, possibly mimicking an endogenous protein involved in this process. This was supported by experiments which showed that adenovirus mediated expression of AEIP in both MCF7 (ER α +) and Hela cells (ER α -) partially inhibited pure antiestrogen mediated turnover of both endogenous and exogenous receptor, while an adenovirus expressed scrambled peptide had no effect. Through the use of NAAIRS and Alanine scanning mutations, the AEIP motif responsible for pure antiestrogen bound ER α interaction was localized (see Example below). Furthermore, given these findings, T7 phage display protein libraries constructed from breast cancer and endometrial cancer cell cDNA were screened against DNA bound pure antiestrogen liganded ER α and resulted in the identification of possible candidate interacting proteins. These findings should enhance the

understanding of the molecular mechanisms of pure antiestrogens, and assist in elucidating the cause of tumor resistance to these drugs and in the identification of novel pure antiestrogens with improved pharmacological properties.

- 5 Certain aspects of the invention can be described in greater detail in the non-limiting Example that follows.

EXAMPLE

- Previously, nuclear receptor interacting proteins were identified through methods that were limited in both sensitivity and throughput, such as yeast
10 two-hybrid screenings. To circumvent these issues, a T7 bacteriophage viral based system was developed that (a) allowed screening for nuclear receptor interacting proteins from multiple tissues/cells simultaneously, (b) enabled the identification of interacting proteins with both high and low affinity for the receptors, and (c) permitted the use of full length receptors in these screens.
- 15 The advantage of using the T7 bacteriophage system over other viral systems for identifying nuclear receptor interacting proteins is the bacterial lytic nature of the phage. This allows for the expression of large recombinant protein fragments and out-of-frame peptides with protein interacting motifs (up to 1200 amino acids). These recombinant proteins and peptides are incorporated
20 into the viral coat of the T7 bacteriophage and are available for binding to the nuclear receptor. T7 bacteriophage libraries have been constructed using cDNA libraries from tissues such as brain, muscle, liver, kidney, ovary, adrenal gland, small intestine and the cell lines HEPG2 (human liver carcinoma), MCF7 and T47D (ER α [+] breast cancer) and LNCap (prostate
25 cancer).

Although the goal was to identify proteins that interacted with ER α bound to pure antiestrogens, the screen was initially validated and optimized using E2/ER α complexes as targets. Recombinant biologically active E2 bound ER α (Affinity Bioreagents) was screened using HEPG2 (liver

carcinoma cell line) and MCF7 (ER α [+] breast cancer cell line) T7 libraries. E2/ER α complexes were tethered to DNA in 96 well plates, incubated in the presence of T7 phage, and washed stringently. Bound phage were eluted and amplified in bacteria. A total of five rounds of screening were performed, and
5 phage ELISAs from each round were performed to test for enrichment (Fig. 1). Pooled phage were plated, and individual phage were isolated, analyzed by PCR and then sequenced. This resulted in the identification of known ER α interactors, SRC-1, AIB-1, and ASC-2, along with several novel interacting protein fragments and out-of-frame peptides with interacting motifs.

10 The length of one identified out-of-frame peptide fragment was determined to be 16 amino acids from the T7 screenings described above. As with all protein fragments and peptides identified in the T7 screens, the ligand specificity of the peptide fragment for binding to ER α was determined in mammalian two-hybrid assays. The methodology for the assays is explained
15 in the Brief Description of Fig. 2. As can be seen in Fig. 2A, when tested in mammalian two-hybrid assays, this peptide bound to ER α specifically in the presence of two known pure antiestrogens (100 nM ICI 182,780 and RU 58-668), while little to no interaction was observed when ER α was liganded with the ER α agonist 17 β - estradiol (E2) or selective estrogen receptor modulators
20 (4-hydroxy Tamoxifen [4-OH TOT], GW7604, Raloxifene [Ralox.], or EM-652. Since this peptide interacted specifically to ER α bound to pure antiestrogens, it was designated Pure Antiestrogen Interacting Peptide (AEIP). This is the first known peptide with such properties.

To further determine if this interaction between AEIP and pure
25 antiestrogen bound ER α was due to a specific pure antiestrogen induced conformation in the receptor, mammalian two-hybrid assays were used to determine if increasing amounts of estrogen (E2) could inhibit the AEIP interaction with pure antiestrogen bound ER α by competing with pure antiestrogen for binding to the receptor and thus altering the conformation. As
30 shown in Fig. 2B, increasing concentrations of E2 in the presence of ICI

182,780 inhibited the AEIP interaction, thus demonstrating that the ICI 182,780 induced conformation is responsible for the AEIP interaction with ER α . In addition, since this interaction with ER α only occurred when the receptor was bound to pure antiestrogens, a determination was made as to whether the interaction was specific to ER α or whether an interaction between AEIP and other nuclear hormone receptors in presence of their agonists and antagonists could be observed. Interestingly, as shown in Fig. 2C, AEIP only bound to pure antiestrogen bound ER α in mammalian two-hybrid assays, while no interaction was observed between either the long or short form of ER α in the presence of E2 or ICI 182,780 (ER α does not undergo degradation when bound to ICI 182,780), or other nuclear hormone receptors. This further demonstrated that the binding of AEIP to pure antiestrogen bound ER α was due to a specific conformation in the receptor induced by the binding of pure antiestrogens.

To further characterize AEIP, a decision was made to determine which amino acids in this peptide were responsible for the interaction between AEIP and pure antiestrogen bound ER α . In Fig. 3A, a diagram of the AEIP peptide sequence expressed beyond the Gal4 DNA binding domain (Gal4DBD) used in the mammalian two-hybrid assays is presented. The first 26 amino acids represent additional amino acids that are part of the Gal4DBD expression vector but, as shown in Figs. 3C and 3D and Figs. 4B and 4C, are important for the interaction between AEIP and pure antiestrogen bound ER α . Amino acids 27 through 42 represent the amino acids identified in the T7 screen. The amino acid sequence of AEIP is:

PEFPSTSLYKKAGWRRNQFSRLDPVPNSPMYVKVCGLCKGFA.

Analysis of NCBI Blast using the 16 amino AEIP peptide sequence identified in the T7 screen (NSPMYVKVCGLCKGFA) resulted in the identification of a hypothetical protein that contained two regions (corresponding to Oligo1 and Oligo 2) (Fig. 3A) with amino acid sequences similar to identified AEIP T7 peptide sequence. Thus, peptides corresponding

to Oligo 1 and Oligo 2 representing sequences from the hypothetical protein were cloned into the Gal4DBD vector. As seen in Fig. 3B, neither Oligo 1 nor Oligo 2 interacted with ER α in the presence of ICI 182,780. Thus, the minor differences, mostly at the junction between the additional amino acids and the identified T7 AEIP peptide fragment, must play a role in the AEIP/ER α interaction. To further identify which amino acids were necessary, regions within the full 42 amino acid AEIP peptide were changed sequentially (6 amino acids at a time) to the amino acid sequence NAAIRS (Asparagine, Alanine, Alanine, Isoleucine, Arginine, Serine), a sequence previously shown to be observed in both alpha helices and beta sheet protein structures and which typically does not disrupt protein structures (Fig. 3A NAAIRS mutants). As shown in Fig. 3C, the region (region 5) of AEIP containing the amino acid sequence VPNSPM, when changed to the amino acids NAAIRS, resulted in a significant decrease in the ability for AEIP to bind to pure antiestrogen bound ER α . To further define which of the amino acids from region 5 were necessary for this interaction, each amino acid was mutated separately (1, 2, 3, 4, 5, 6) to A (Alanine), or together (1&2) as shown in Fig. 3A Alanine mutants. As shown in Fig. 3D, the amino acids SPM were defined as the specific amino acids needed for the interaction between AEIP and pure antiestrogen bound ER α .

Since the amino acids SPM were determined to be the specific amino acids involved in the interaction and were located within the identified T7 fragment, the decision was made to determine if the additional amino acids found in the Gal4DBD vector were necessary for this interaction by deleting these amino acids (Fig. 4A AEIP (-add. a.a.)). As shown in Fig. 4B, increasing amounts of AEIP (-add. a.a.) did not result in an interaction with ICI 182,780 bound ER α to a level observed with the wild type AEIP containing the additional amino acids. Furthermore, a determination was made as to whether the number of amino acids flanking the identified interacting region could be decreased (Fig. 4A AEIP 16, AEIP 14, and AEIP 12). It was observed that

these shortened peptides did not interact with pure antiestrogen bound ER α in mammalian two-hybrid assays (Fig. 4C). This suggested that although the sequence VPNSPM or SPM is the specific region and amino acids necessary for the pure antiestrogen bound ER α /AEIP interaction, additional amino acids
5 found in the 42 amino acid AEIP peptide surrounding the sequence SPM are involved in properly presenting this specific region of AEIP for binding to pure antiestrogen bound ER α . While this appears to be true, the data suggest that any amino acid can serve this purpose of presenting the AEIP/ER α interacting region.

10

* * *

All documents and other information sources cited above are hereby incorporated in their entirety by reference.

WHAT IS CLAIMED IS:

1. A peptide comprising the sequence:
5 X(n)SPMX(n)
wherein X is any amino acid, and
(n) represents any number.
2. The peptide according to claim 1 wherein said peptide
10 comprises at least 6 amino acids.
3. The peptide according to claim 2 wherein said peptide
comprises at least 10 amino acids.
4. The peptide according to claim 3 wherein said peptide
15 comprises at least 14 amino acids.
5. The peptide according to claim 4 wherein said peptide
comprises at least 18 amino acids.
20
6. The peptide according to claim 1 wherein said peptide
comprises the amino acid sequence VPNSPM.
7. The peptide according to claim 6 wherein said peptide
25 comprises the amino acid sequence:
PEFPSTSLYKKAGWRRNQFSRLDPVPNSPMYVKVCGLCKGFA.
8. The peptide according to claim 1 wherein said peptide
comprises a fragment of the amino acid sequence:
30 PEFPSTSLYKKAGWRRNQFSRLDPVPNSPMYVKVCGLCKGFA

at least 6 consecutive amino acids in length.

9. The peptide according to claim 1 wherein said peptide is bound to a solid support.

5

10. The peptide according to claim 1 wherein said peptide bears a detectable label.

10

11. A nucleic acid encoding the peptide according to claim 1.

12. A vector comprising the nucleic acid according to claim 11.

13. The vector according to claim 12 wherein said nucleic acid is present in said vector in operable linkage with a promoter.

15

14. A method of identifying a pure antiestrogen comprising:

i) contacting the peptide according to claim 1 with estrogen receptor α in the presence and absence of a test compound under conditions such that said peptide can bind said receptor in the presence of a pure antiestrogen, and

20

ii) monitoring for binding of said peptide to said receptor, wherein binding of said peptide to said receptor in the presence of a test compound indicates said test compound is a pure antiestrogen.

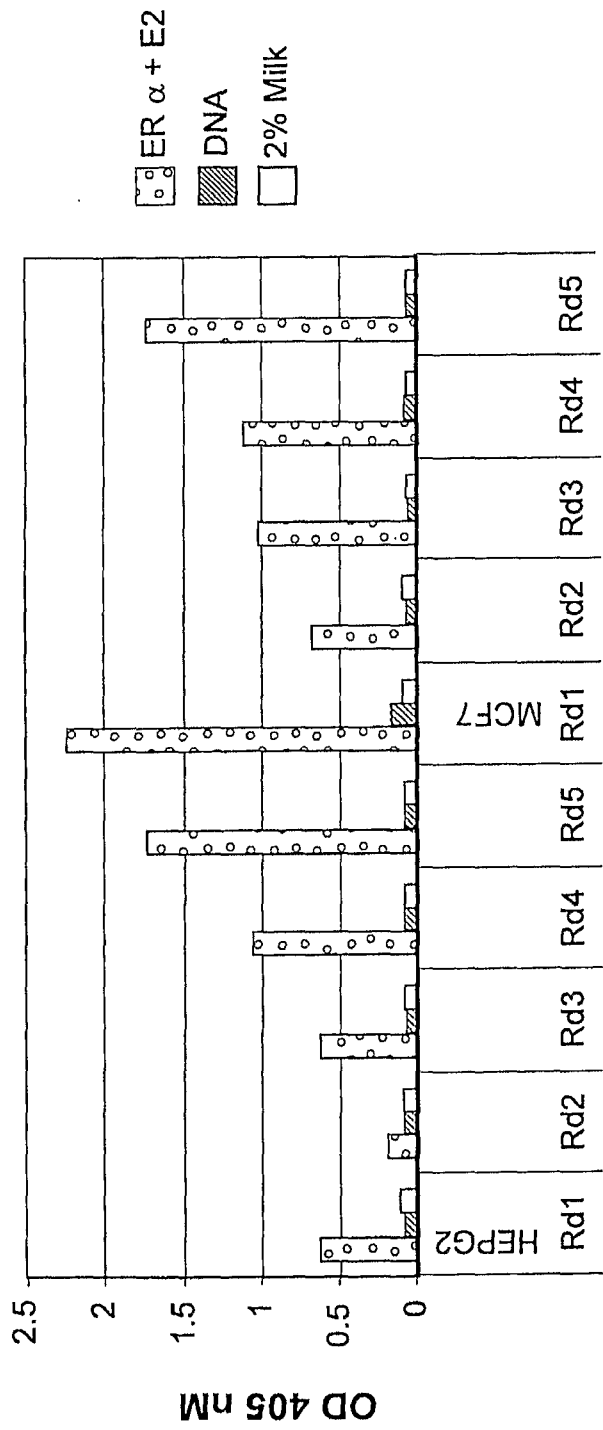


FIG. 1 ELISA of T7 phage screens of HEPG2 and MCF7 cDNA libraries against E2-bound ER α

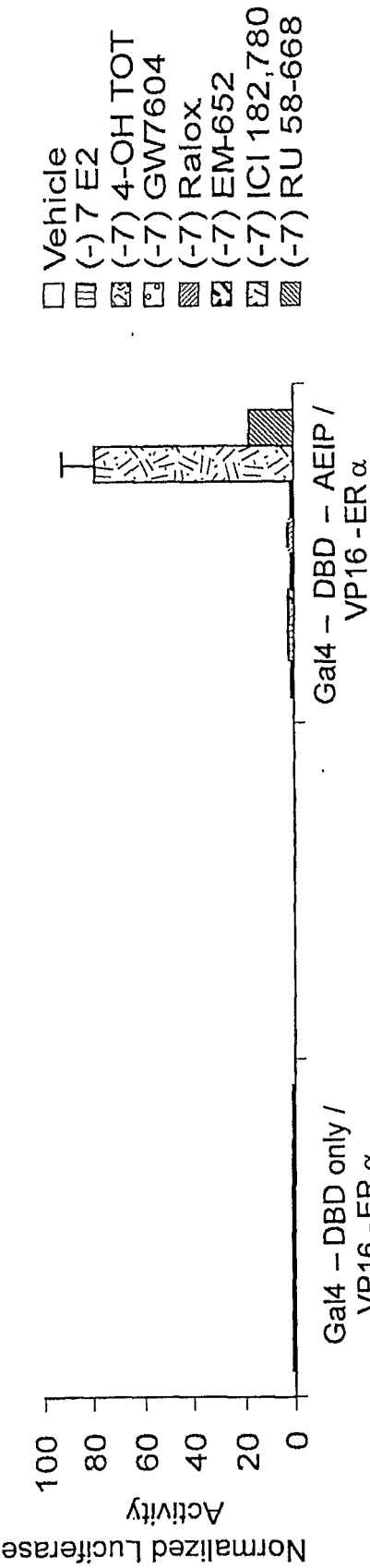


FIG. 2A

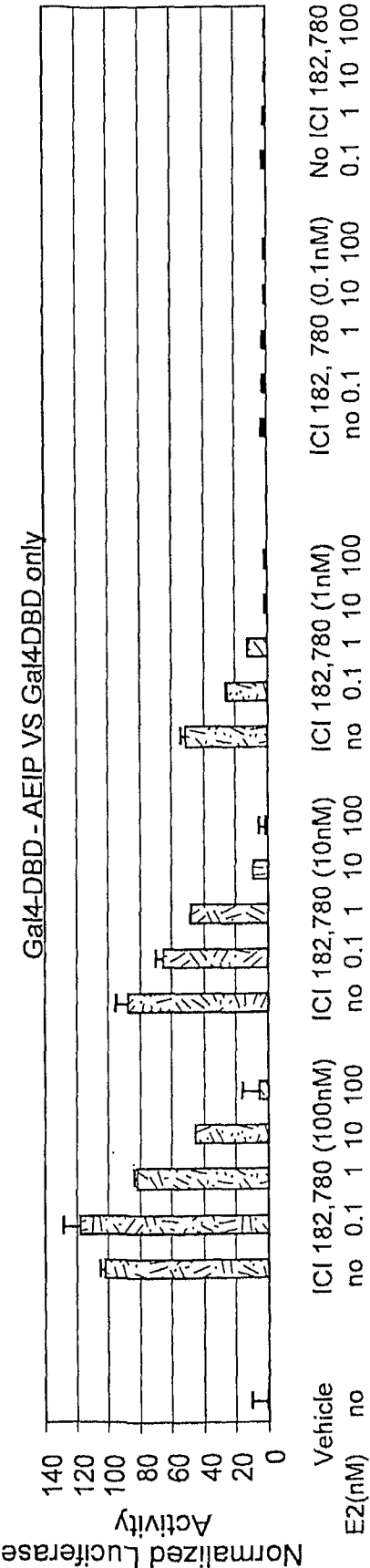


FIG. 2B

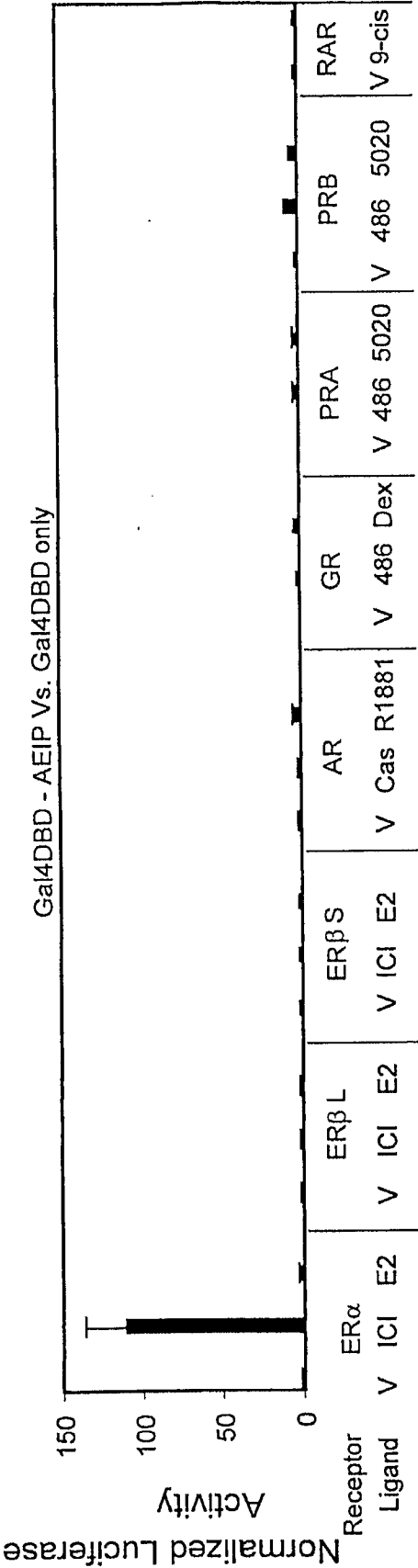
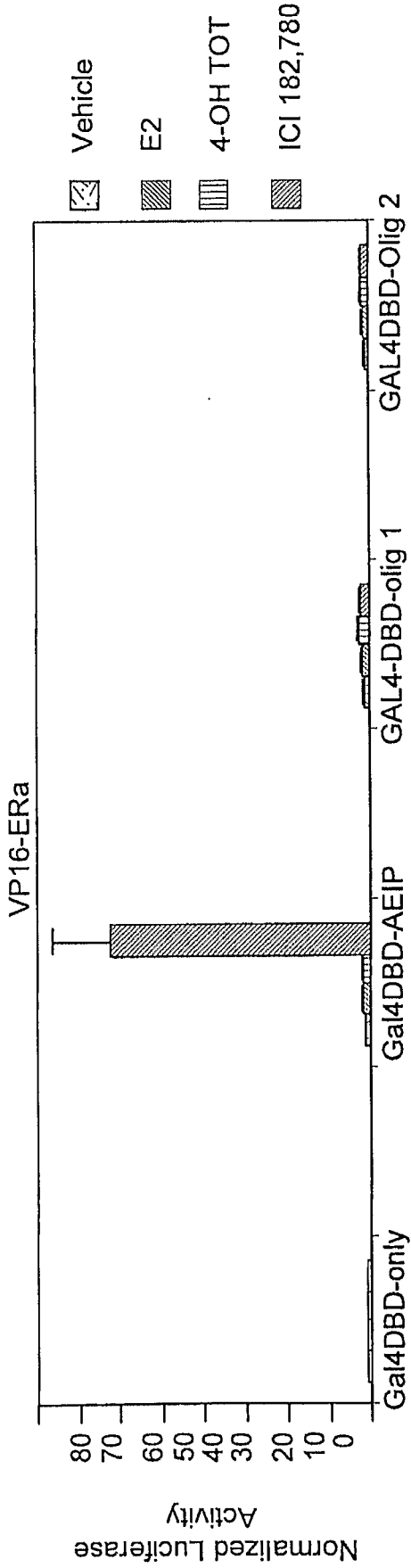


FIG. 2C

Additional amino acids in the GAL4DBD vector expressed beyond the GAL4DBD and not identified from T7 screen																									
T7 Screen Identified Sequence																									
Amino Acids:																									
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
AEIP: Gal4 DBD-P	E	F	P	S	T	S	L	Y	K	K	A	G	W	R	R	N	Q	F	S	R	L	D	P	V	P
Olig 1:Gal4 DBD-P	E	F	P	S	T	S	L	Y	K	K	A	G	W	R	R	N	Q	F	S	R	L	D	P	V	P
Olig 2 Gal4 DBD-P	E	F	P	S	T	S	L	Y	K	K	A	G	W	R	R	N	Q	F	S	R	L	D	P	V	P
NAAIRS mutants:																									
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
P	E	F	P	S	T	S	L	Y	K	K	A	G	W	R	R	N	Q	F	S	R	L	D	P	V	P
Alanine mutants:																									
P	E	F	P	S	T	S	L	Y	K	K	A	G	W	R	R	N	Q	F	S	R	L	D	P	V	P
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A

FIG. 3A



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FIG. 3B

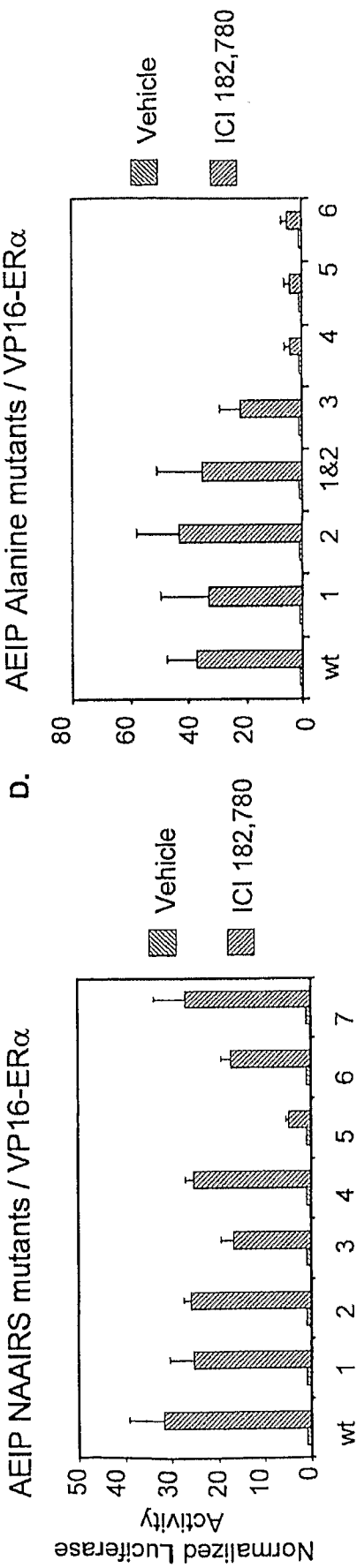


FIG. 3C

FIG. 3D

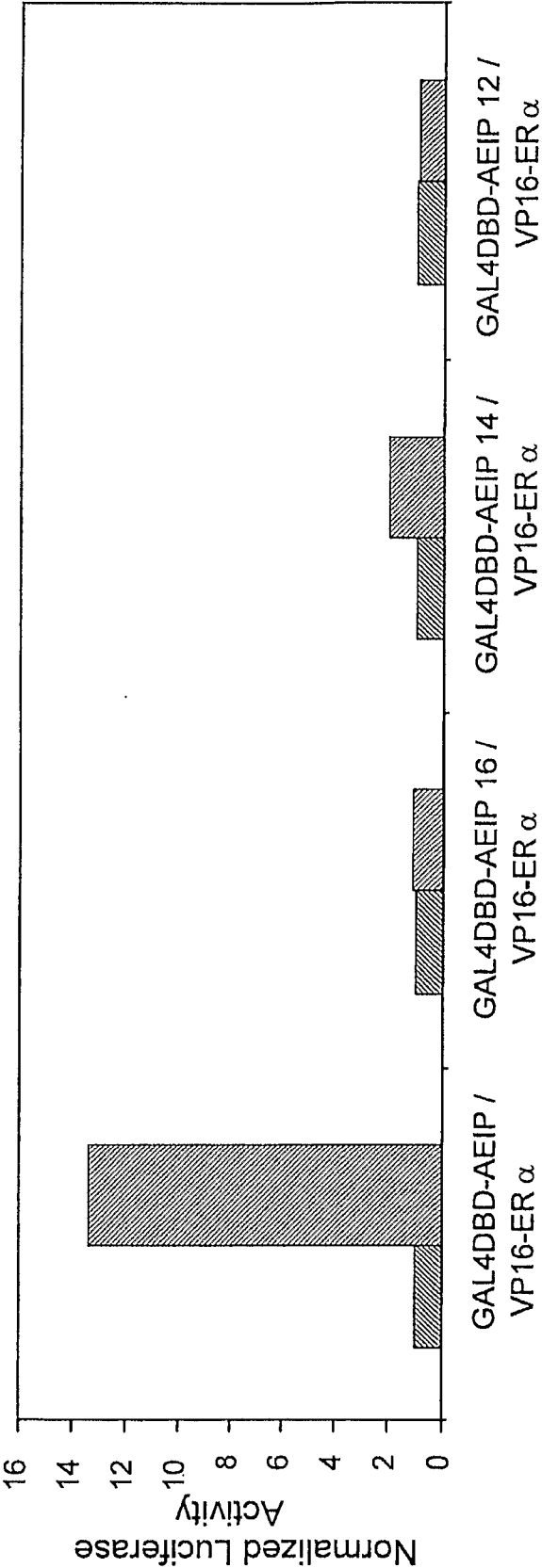


FIG. 4C