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PRINCIPAL INVESTIGATOR: Huey-Jing Huang, Ph.D.

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, North Carolina 27710

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Huey-Jing Huang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University Medical Center
Durham, North Carolina 27710

E-Mail: Huang022@mc.duke.edu

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13. ABSTRACT (Maximum 200 Words)

Tamoxifen, a SERM (Selective Estrogen Receptor Modulator), is the most commonly used endocrine treatment for all stages of breast cancer. However, progression from tamoxifen sensitivity to tamoxifen resistance occurs in a substantial portion of the tumors. Full antiestrogens, such as ICI 182,780, are currently used as the second line therapy after failure of long-term tamoxifen therapy. To facilitate the design and characterization of more appropriate therapeutic agents for endocrine therapy of breast cancer, it is very important to understand the functional mechanisms that distinguish full antiestrogens from SERMs. It has been shown that estrogen receptor (ER) can recruit corepressors N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid receptors) in the presence of tamoxifen, suggesting a possible role of N-CoR/SMRT in mediating the antagonist activity of tamoxifen. However, it is not clear if apo-ER or ICI 182,780-bound ER can recruit N-CoR/SMRT or other corepressors. To investigate the possible involvement of different corepressors in the actions of different antiestrogens and unliganded ER, we have constructed a focused phage display library which contains the "CoRNR box" motif, a binding site important for N-CoR/SMRT to interact with the nuclear receptors. In this report, we have shown that screening of the CoRNR box library with ER treated with no hormone or different antiestrogens led to the isolation of peptides that differentially interact with apo-ER, tamoxifen-bound ER, or ICI 182,780-bound ER. These interactions observed *in vitro* have also been confirmed *in vivo* using a mammalian two-hybrid assay. Using a series of ER mutants, we were able to show that these CoRNR box-containing peptides have different binding characteristics from the peptides that contain the coactivator LXXLL motif. These peptides can be used to probe the conformational changes of ER induced by different antiestrogens and will be valuable for the design of screens for novel ER-antagonists.

14. SUBJECT TERMS
Tamoxifen, ICI 182,780, estrogen receptor, corepressor, NCoR, SMRT, proteomics, phage display

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Key Research Accomplishments

- **Identification of distinct peptides that bind apo-ER or complexes of ER with different antiestrogens using phage display technology**

  The main goal of Task 1 is to use the peptides identified in our study to distinguish the mechanisms used by mixed agonist/antagonist (tamoxifen) and full antagonist (ICI 182,780). Since the corepressors NCoR/SMRT have been implicated in the antagonist activity of tamoxifen, a "CoRNR box" phage display library was created for this purpose. "CoRNR box" is a motif required for NCoR/SMRT to interact with TR and RAR. Enrichment of the CoRNR box motif in the library was expected to facilitate the identification of NCoR/SMRT-like peptides which otherwise have a lower probability to be identified using a random peptide library. The CoRNR box library was screened for phage expressing peptides that bound with high affinity to ERα or ERβ in the absence of hormone or in the presence of tamoxifen or ICI 182,780. After three rounds of affinity selection, significant enrichment of phage was observed using ERβ whereas minimal enrichment of ERα-interactors was observed. We believe that this is due to differences in the stability of the purified recombinant receptors since most of the peptides obtained using ERβ as the target interact in an efficient manner with ERα in a mammalian two-hybrid assay (see below). Because of the difficulty of using ERα in the screen, we used ERβ as the target for all subsequent primary screens. ELISA was used to identify the phage clones that bind ER with high affinity and ligand dependency. Of 48 random clones analyzed in each pool, 32 clones bound apo-ERβ, 17 clones bound tamoxifen-ERβ, and 31 clones bound ICI 182,780-ERβ. The amino acid sequence of the interacting peptides was deduced following DNA sequencing of the phage inserts. Table 1 shows the peptide sequences and frequency of occurrence for the phage clones obtained. The bN, bT, and bI phage clones represent phage isolated using apo-ERβ, tamoxifen-ERβ, and ICI 182,780-ERβ as targets, respectively. All of the bT and bI phage were isolated using receptor immobilized directly to plastic plates. However, enrichment of bN phage can only be obtained using receptor bound to immobilized EREs, suggesting that apo-ER may adopt a more favorable conformation for CoRNR box peptide binding when an ERE is present. Among the four unique bN clones obtained, clones bN1 and bN2 were found most frequently. Seventeen clones showed specific binding to tamoxifen-bound ERβ. However, 16 of the clones in this group share the same sequence (bT1). Importantly, bT17 has the same sequence as bN2, suggesting that ER may adopt a similar conformation at the binding site of this peptide in the absence of hormone or in the presence of tamoxifen. Twenty-four unique clones were obtained that showed specific binding to ICI 182,780 bound-ER. However, only two of the bI clones (bI2 and bI29) show specific binding to ER using the mammalian two-hybrid assay (see below), suggesting that ICI 182,780 treatment *in vitro* may have forced ER to adopt a conformation that is not normally seen *in vivo*.

- **Confirmation of ER-peptide interaction *in vivo* using mammalian two-hybrid assay**

  In Task 1, we also proposed to use mammalian two-hybrid assay to confirm that the CoRNR box-containing peptides identified *in vitro* interact with ER in the context of an intact cell. Specifically, the DNA inserts from each phage were cloned into a vector that enabled the peptide to be expressed as a Gal4-DBD-peptide fusion protein. The ability of the fusion proteins to interact with full-length VP16-ERα or VP16-ERβ occupied by different estrogens/antiestrogens in mammalian cells was determined by assaying the ability of the complex to activate a luciferase reporter construct under the control of five copies of a Gal4 upstream enhancer

element (5xGal4-TATA-Luc). Figures 1A and 1B show the results from a mammalian two-hybrid assay confirming the ER-peptide interactions in HepG2 cells. Although the CoRNR box peptides chosen for this analysis were isolated using ERβ as a target, most peptides interact with ERα also (Figure 1A). Similar results were also observed in HeLa and MCF7 cells (data not shown). Most importantly, all of these peptides display distinct binding characteristics with ERα or ERβ in the absence of hormone or in the presence of tamoxifen or ICI 182,780 and none interact with estradiol-bound ER. These peptides thus provide a useful tool to examine the different conformations adopted by ER when bound to different antiestrogens and this assay can be used to screen for new classes of agents that lack cross-resistance to standard tamoxifen therapy.

- **Determination of the region within ERα required for CoRNR box peptide binding**

A series of mutants were generated to define the region(s) within ER required for CoRNR box peptide binding. We selected ERα for a more extensive analysis in this study since its pharmacology is more clearly defined. A schematic diagram of ERα and its mutants used in the current study is shown in Figure 2. Mutations in helix 12 have been shown previously to decrease ERα transcriptional activity and abolish the interaction of the receptor with the coactivator GRIP1 (1) and with LXXLL motif-containing peptides (2). Mutations of lysine 362, leucine 372, and valine 376 were chosen because amino acid substitutions at equivalent positions in TRα or RXRα are known to decrease their ability to interact with NCoR/SMRT and to abrogate ligand-independent repression activity (3). All of these mutations were created in the background of a full-length VP16-ERα protein to enable evaluation of receptor-peptide interactions using a mammalian two-hybrid assay in HepG2 cells. The interactions of several different classes of LXXLL peptides with mutant ERs were also analyzed for comparison. Three LXXLL peptides D11, D47, and F6 with distinct receptor binding characteristics (2) were used. The GAL4-DBD-GRIP1 NR-box fusion contains the middle three copies of the LXXLL motif found in the coactivator GRIP1. As shown in Figures 3A-D, mutations in helix 12 did not affect the binding of the CoRNR box peptides to ERα but actually enhanced the binding of bT1 and bI2 peptides to the receptor. The interaction of the CoRNR box peptides and the ERα helix 12 mutants were interesting in that the exquisite hormonal specificity observed on the wild-type ERα (wtERα) was not preserved (Figures 3A-C). This suggests that the positioning of helix 12, influenced by the nature of the ligand bound, is an important regulator of CoRNR box peptide binding. Mutations at the amino acid residues Lys-362 and Val-376 within ERα decreased its ability to interact with both the CoRNR box and LXXLL peptides, suggesting that the binding surfaces for these two classes of peptides are close or overlapping. The results obtained with ER-L372R are the most interesting, as this specific mutation totally abolished the interaction between ERα and two of the CoRNR box peptides (bT1 and bI2) while having no effect on the binding of the LXXLL peptides to the receptor (Figure 3). Thus, although the binding sites on ERα for the CoRNR box and LXXLL peptides are closely linked, they can be functionally separated.

- **Expression of Gal4DBD-CoRNR box peptides in cells does not reverse the antagonist activity of antiestrogens**

The objective of Task 3 was to use the CoRNR box peptides identified in our screen to disrupt the interaction of ER and corepressor and decrease the repressional activity of ER mediated by antiestrogens. However, we found that expression of these Gal4DBD-CoRNR box peptides did not affect the trascription activity of ER in HepG2, HeLa, or MCF7 cells. It is possible that these CoRNR box peptides may not bind to the same site as the corepressors but simply interact with

the receptor at some distal point whose local structure was altered upon antiestrogen binding. Another explanation is that the interaction between NCoR/SMRT and ER may involve multiple domains of the corepressors or other protein complexes. In these cases, peptides containing only one CoRNR box motif as used in this study may not be able to effectively displace corepressors from the receptor. It is also very possible that corepressors other than NCoR/SMRT may be involved in the antagonist activity of antiestrogen. To explore this possibility, we will identify novel corepressors of ER using a proteomic approach (Task 2). Other random peptide phage display libraries will also be screened against antiestrogen-bound ER to derive a consensus of peptide sequence that is important for binding to ER in the presence of different antiestrogens.

Reportable Outcomes

- **Huang H.-J.**, Norris J.D., McDonnell D.P. (2002) Identification of a negative regulatory surface within estrogen receptor alpha provides evidence in support of a role for corepressors in regulating cellular responses to agonists and antagonists. *Molecular Endocrinology*, 16: 1778-1792.
- Poster presentation at Keystone Symposia on Nuclear Receptor Superfamily, Snowbird, UT (2002). Identification of a negative regulatory surface within estrogen receptor alpha provides evidence in support of a role for corepressors in regulating cellular responses to agonists and antagonists.

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2. Chang C-Y, Norris JD, Grøn H, Paige LA, Hamilton PT, Kenan DJ, Fowlkes D, McDonnell DP 1999 Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors alpha and beta. Mol Cell Biol 19:8226-8239

3. Hu X, Lazar MA 1999 The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors. Nature 402:93-96

Table 1. Sequences of CoRNR Box-Containing Peptides That Interact with ER *In Vitro*

H I
xxxxxxxLxxIIxxxLxxxxxxx
(N)
(L)

Peptide	Sequence	Frequency
bN1	QETIQRW**L**RG**HI**QRE**L**GTMELKD	14/32
bN2	EYHEKRW**L**EG**HI**HHR**I**KSLLENS	16/32
bN16	HSTTLTG**L**AS**II**RER**I**LTELRDE	1/32
bN18	PENFRQA**L**RA**HI**ADL**I**TNQDYRS	1/32
bT1	ELFDAFQ**L**RQ**LI**LRG**L**QDDIPYH	16/17
bT17(=bN2)	EYHEKRW**L**EG**HI**HHR**I**KSLLENS	1/17
bI1	FMNNGVL**L**AT**NI**ENL**L**RTQPGSN	1/31
bI2	EMEWMKA**L**RQ**HI**SGE**L**RRNYTEE	5/31
bI5	NAAPRTA**L**SH**HI**HSD**L**LDGPTTT	1/31
bI7	PKTPGVP**L**NP**LI**SPE**I**TSDTSML	1/31
bI8	AAYDPAA**L**NN**NI**RYA**L**VKHSQIK	3/31
bI9	NTYNTGA**L**RF**NI**VES**I**WASKKLR	1/31
bI11	VPRFTKG**L**VG**NI**PLA**I**DTNSGTV	1/31
bI12	ESERANL**L**KE**HI**KMT**L**PEERKKT	1/31
bI13	NVIAQPT**L**AS**II**PPS**L**KRQSEAR	1/31
bI15	SPFTQVT**L**KG**NI**APS**I**VGSQGMA	1/31
bI16	PIAHRVN**L**RH**NI**TED**I**TLSHRFL	1/31
bI17	IDDGHPH**L**WK**NI**WDT**L**DKPGLGA	1/31
bI18	FKPGTSS**L**DT**HI**PLG**L**NKSFHHN	1/31
bI20	NQGNTKE**L**LG**NI**NYF**L**THHTVPA	1/31
bI21	LTYPIRE**L**KM**NI**TSG**I**RLDKRVL	1/31
bI22	HKDSQLT**L**AN**NI**MGQ**L**SSTGGKH	1/31
bI23	WDVGEIR**L**RR**HI**KMP**L**SEEAIAE	1/31
bI24	SHYEKYS**L**PG**II**VRK**I**STTDWRP	1/31
bI25	QHQNRQQ**L**GS**NI**AAT**L**PGKRESV	1/31
bI26	KSQTKAE**L**PY**LI**GKQ**I**TKNQPEQ	1/31
bI27	DALDNQR**L**LG**NI**DNV**L**KVTNPNA	1/31
bI28	GQSARFA**L**SQ**HI**PSK**I**YDHPRPN	1/31
bI29	KWESLDA**L**QG**LI**SSH**L**SAMGPIP	2/31
bI30	RAVHERA**L**NP**LI**LDR**L**LHELKSE	1/31

bN peptides were isolated using ERβ immobilized on biotinylated ERE. bT peptides were isolated using ERβ treated with 4-hydroxytamoxifen (1 μM). bI peptides were isolated using ERβ treated with ICI 182,780 (1 μM). Residues that were fixed to display the desired amino acids are in bold.

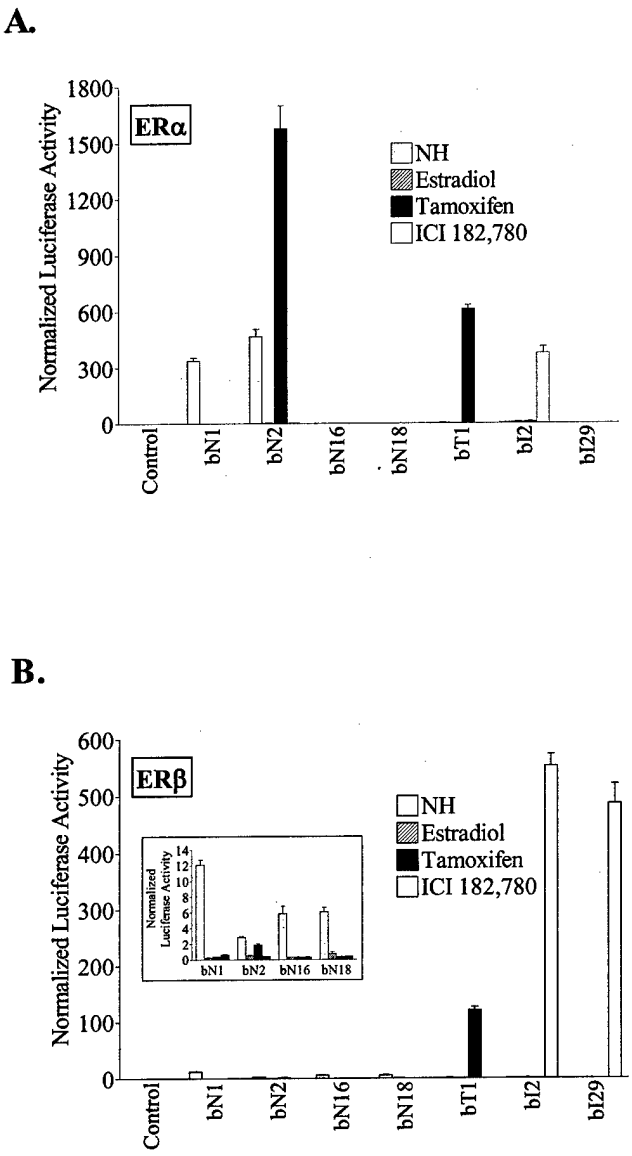

Figure 1. Evaluation of the interaction between receptor and CoRNR box-containing peptides in mammalian cells. HepG2 cells were transiently transfected with expression vectors for the Gal4-DBD-peptide fusion protein, a Gal4 responsive luciferase reporter construct (5xGal4-TATA-Luc), the β-galactosidase control plasmid, and VP16-ERα (A) or VP16-ERβ (B). Transfection of the Gal4-DBD alone is included as control. Following transfections, cells were treated with 100 nM of estrogen or antiestrogens for 24 h and assayed for luciferase and β-galactosidase activity. Data are presented as normalized luciferase activity, which was obtained by dividing the luciferase activity by β-galactosidase activity. The inset in panel B magnifies the lower part of the luciferase activity to emphasize the hormone-specific interaction between ERβ and CoRNR box-containing peptides.

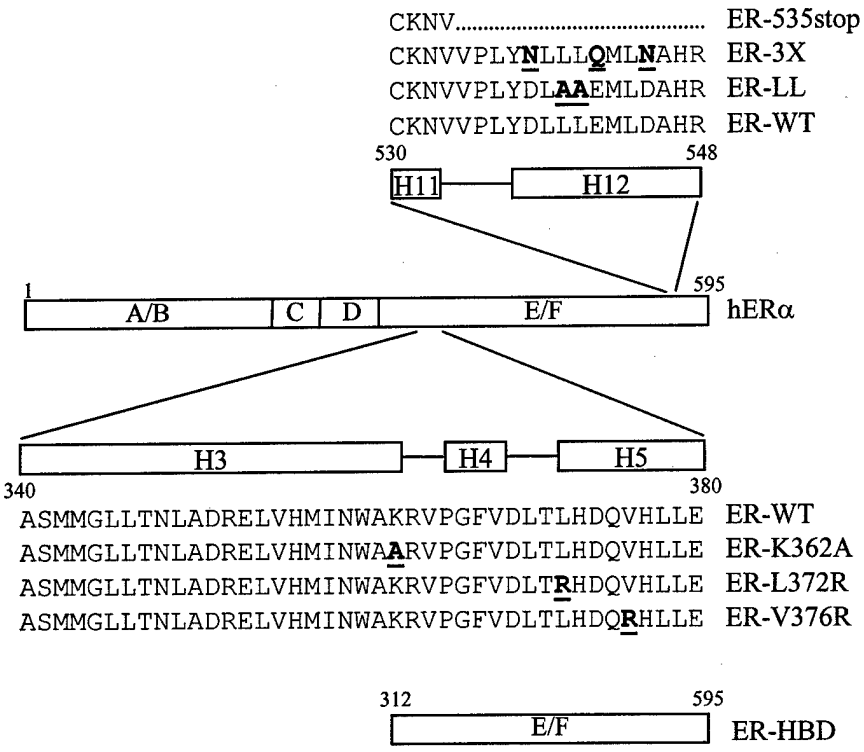

Figure 2. ERα mutants used in this study. Schematic representation of ERα showing amino acid substitutions in helices H3, H5, and H12. Residues that were mutated are in bold and underlined. Also shown is a schematic of the ER mutant receptor that contains only the F domain and part of the E domain (H2 to H12).

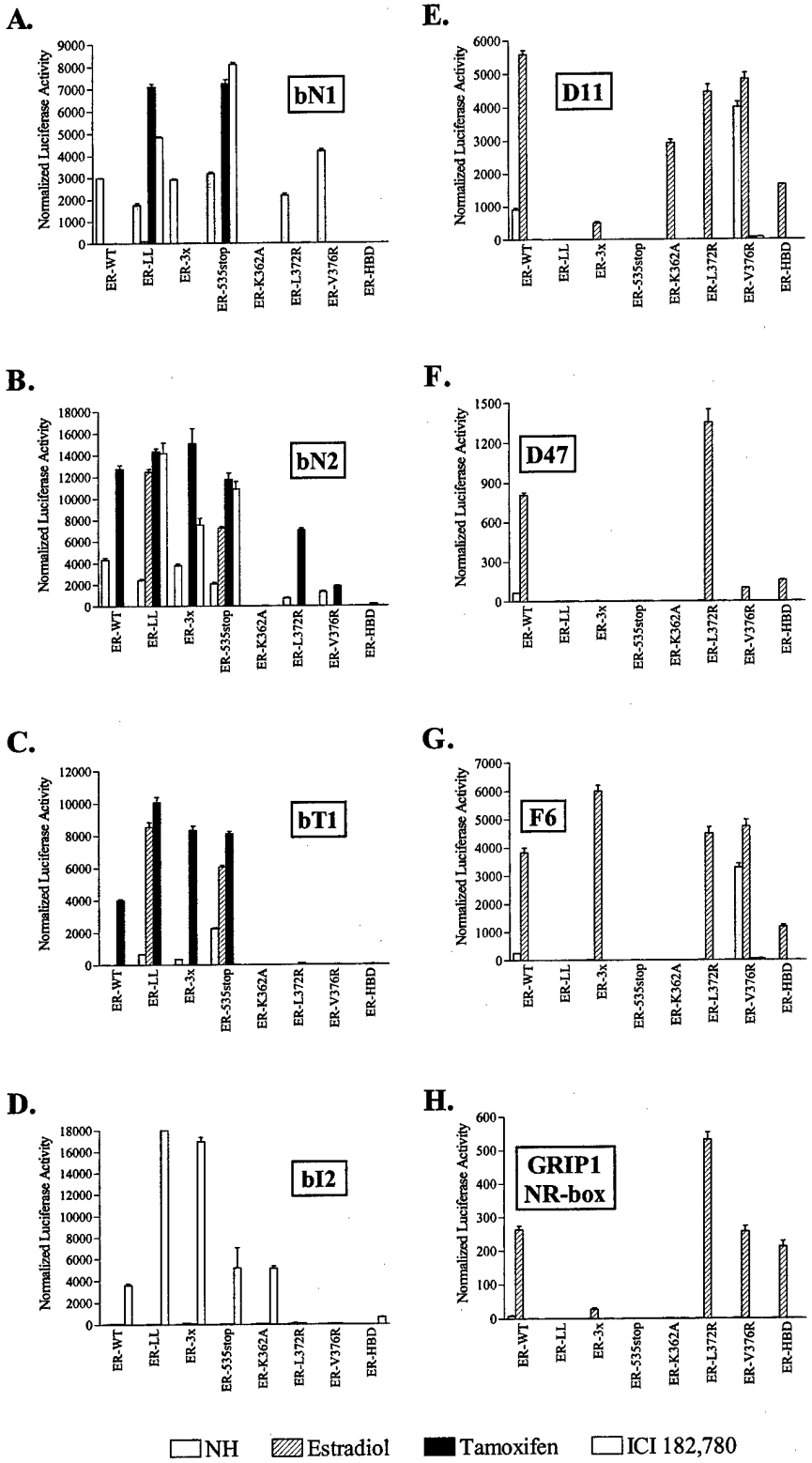

Figure 3. The binding surface for CoRNR box-containing peptides is located on helices H3 and H5 but not H12 of ERα. Mammalian two-hybrid assays were performed using wild-type or mutant VP16-ERα and Gal4-DBD-CoRNR box or Gal4-DBD-LXXLL fusion peptides. Indicated vectors were transfected into HepG2 cells with a reporter plasmid (5xGal4-TATA-Luc) and the control β-galactosidase plasmid. Following transfection, cells were treated with 100 nM of hormone for 24 h before harvested for luciferase and β-galactosidase activity. Data are presented as normalized luciferase activity, which was obtained by dividing the luciferase activity by β-galactosidase activity.