RESEARCH ARTICLE

Effect of single mutagenesis on the binding pocket of canine estrogen receptor alpha: Structure and binding affinity

Waraphan Toniti1*, Aekkapot Chamkasem2, Panpanga Sangsuriya1, Pranom Puchadapirom3

1 Department of Pre-clinic and Applied Animal Sciences, Faculty of Veterinary Science, Mahidol University, Nakhon Pathom, Thailand
2 Faculty of Veterinary Science, Mahidol University, Nakhon Pathom, Thailand
3 Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok, Thailand

Abstract: Hormone-related mammary gland tumors are among the most commonly diagnosed neoplasms in female dogs. Estrogen enacts its biological roles through specific receptors known as estrogen receptors (ER). In human medicine, anti-estrogen therapy has become the gold standard in ER-positive breast tumors’ therapeutic regimen. The binding pocket of the canine estrogen receptor alpha (cERα) ligand binding domain comprises of three key amino acid residues including E354, G522 and L526, which stabilize the cERα-E2 interaction via hydrogen bonding. The side chain of E354 shares hydrogen bond interaction with the A ring of its natural ligand E2, whereas the main chain of G522 and L526 interact with the E2-D ring. The single mutation of the E354 aberrant, along with the hydrogen bond interaction between cERα and both ligands, leads to a variety of binding affinities. According to this in silico model, it may be concluded that E354 plays a role in the cERα activities. The effects of single mutants might need to be studied further in vitro and in vivo.

Keywords: estrogen receptor alpha; canine; mutagenesis; binding affinity


*Correspondence to: Toniti Waraphan, Department of Pre-clinic and Applied Animal Sciences, Faculty of Veterinary Science, Mahidol University, Nakhon Pathom, Thailand; waraphan.ton@mahidol.edu

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Introduction

Hormone-related mammary neoplasms are among the most common tumors diagnosed in female dogs and also represent a remarkably heterogeneous group in terms of morphology and biological behavior[1,2]. Exposure to endogenous ovarian hormones early in life is an important source and cause of mammary tumor development in dogs[3]. The risk of mammary tumor development increases dramatically during the first few estrus cycles. Compared to intact dogs, the risk of malignant tumors in spayed dogs before the first estrus is 0.5%. However, it increases to 8% if the dog is spayed after the first estrus cycle, and it rises to 26% if spayed after the second estrus cycle[4].

In a retrospective study by Sorenmo and co-researchers, it was found that spayed dogs with two years of developed malignant tumor showed a survival advantage of two years for intact dogs over spayed dogs before mastectomy[5]. However, Morris and colleagues reported that ovariohysterectomy (OVH) had no obvious benefit on tumor-related[6] or overall death rate. Recently, Kristiansen et al. showed that undergoing OVH at the time of mammary tumor excision reduced the risk of new tumors by about 50% among dogs with nonmalignant mammary tumor; however, there was no significant effect on overall or tumor-specific survival rate[7].

The first ‘International Histological Classification of Tumours of Domestic Animals’ was published by the World Health Organization (WHO) in 1974, which in-
cluded tumors and dysplasia of the mammary gland. The latest histological classification, which included dysplasia, as well as benign and malignant neoplasms, was proposed in 2010[1]. Recently, gene expression profiling has redefined breast cancer taxonomy and identified five distinct subtypes of carcinomas: luminal A (ER+/HER2−), luminal B (ER+/HER2+), HER2 overexpressing (ER−/HER2+), basal-like (ER−/HER2−) and normal breast-like[5,6].

In 2010, Sassi and colleagues[11] categorized mammary carcinomas based on the modified classification by Yang et al.[12] as luminal-like A (ER+ and/or PR+, ERBB2−, any CK5/6 or CK14), luminal-like B (ER+ and/or PR+, ERBB2+, any CK5/6 or CK14), ERBB2-expressing (ER−, PR−, ERBB2+, any CK5/6 or CK14), basal-like (ER−, and PR−, ERBB2−, any CK5/6+ or CK14+), and unclassified or normal-like (negative for all markers). These molecular subtypes not only reflect the heterogeneity of breast carcinomas and the possible different cell lineage pathways in breast carcinogenesis, but also influence the difference in clinical outcome, with basal-like subtype associated with a more aggressive behavior[9,11,13-16]. However, conventional histologic subtype, WHO stage and tumor size remain as important prognostic factors in canine mammary gland tumors[1].

The biological role of estrogen is mediated through a high-affinity binding to the estrogen receptor (ER). Breast tumors that express ERs are stimulated by estrogen, and anti-estrogen therapy has thus become an established treatment for ER-positive breast cancer[15]. At the end of the 19th century, Beatson first reported that the growth of human breast carcinomas may be associated with ovarian function and anti-hormonal therapy has been evolving ever since[16]. A number of triphenylethylene derivatives have been developed as first-generation Selective Estrogen Receptor Modulators (SERM), e.g., tamoxifen and toremifene[15,17].

Tamoxifen is an estrogen antagonist in breast tissue; however, in certain other tissues, it acts as an estrogen agonist. Tamoxifen has been the gold standard of anti-estrogen therapy for the past 30 years despite several adverse effects. The second-generation SERMs were developed in the early 1980s. Raloxifene was developed as LY156758. As a fixed-ring anti-estrogen, LY156758 showed greater affinity for ER compared to tamoxifen[15]. Meanwhile, Selective Estrogen Receptor Down-regulators (SERD) are pure anti-estrogen with no agonist activity but increase antagonist potency. Fulvestrant, a novel ER antagonist, down-regulates ER and it is not associated with tamoxifen-like agonist effects. Therefore, fulvestrant offers a potential clinical advantage over the triphenylethylene and ‘fixed-ring’ SERMs[18,17].

SERDs are distinct from tamoxifen and other SERMs, both pharmacologically and in terms of their molecular activity. Tamoxifen binds to ER, thus leading to an inactive AF-2. However, AF-1 is still active, which allows a selective gene expression to occur. On the other hand, fulvestrant inactivates both AF-1 and AF-2, leading to a complete suppression of estrogen-dependent gene expression[17]. As there is no crystal structure of canine estrogen receptor alpha (cERα), this study aims to predict the cERα structure via a single point mutagenesis at a key amino acid residue (E354), and to estimate the binding affinities of the mutants in silico.

Materials and methods

Protein sequencing and homology modeling

Canine estrogen receptor alpha was downloaded from Universal Protein Resource, UniProt (http://www.uniprot.org/) as a sequence of F6V0I8. The selected sequence was a simulated 3D structure by the ModWeb server (http://salilab.org/modweb). In brief, the FASTA format of the amino acid sequence was first deposited. Next, the ModWeb server calculated and evaluated the models using ModPipe, based on the best available templates from the UniprotKB database. Finally, ModWeb proposed the final model based on model assessment[18,19]. This study focused on the ligand binding domain of cERα.

Single point mutation and molecular docking

The input files for molecular docking consisted of the predicted cERα as a target macromolecule and 17β-estradiol (E2) as a natural ligand. Bazedoxifene represented an example of SERM and was used as a ligand. E2 is available at Protein Data Bank and it can be downloaded and saved as a structure-data file (.sdf). Meanwhile, bazedoxifene is available at DrugBank and it can also be downloaded and saved as an SDF file.

The predicted cERα model was tested using its natural ligand (E2) and bazedoxifene using AutoDock4, AutoDockTools4 and Python Prescription[20,21], which are distributed as open source software (available at: http://autodock.scripps.edu, http://mglttools.scripps.edu/ and http://pyrx.sourceforge.net/). In a comparative study, 3uudA was selected as a template for molecular docking and mutagenesis[22]. The grid-based approach calculated a special map for the covalent ligand’s site of attachment.

Results

The amino acid sequence of cERα (belonging to Canis lupus familiaris) was downloaded from UniProt as a sequence of F6V0I8. cERα is composed of 596 amino acid
residues, with a molecular weight of 66,238 Da. The crystal structure of cERα is currently not available. According to its sequence alignment, the amino acid sequence of cERα has 537 identical positions to the human estrogen receptor alpha (hERα) protein, with a further 41 positions that are similar to those of hERα. In comparison to hERα, the transactivation of cERα’s AF-1 is located at the 1–185 amino acid residues whereas the residues 312–396 harbor the AF-2 (Figure 1). Residues 312–596 are known as the ligand binding domain (LBD).

The binding pocket of the cERα ligand binding domain comprises of three key amino acid residues, which include E354, G522 and L526 (Figure 2B). The side chain of E354 shares a hydrogen bond interaction with the A ring of its natural ligand E2, whereas the main chain of G522 and L526 interacts with the E2-D ring (Figure 2C). The interaction between the ligand binding domain of cERα and these three amino acid residues stabilizes the cERα-E2 interaction via hydrogen bonding. The present study predicted the binding affinity of the mutants for the natural ligand (E2), tamoxifen, raloxifene, bazedoxifene, and fulvestrant (Table 1). Most mutants were bound weakly to their ligands as demonstrated by the lower binding affinities. Nonetheless, it should be noted that some mutants bound more tightly to tamoxifen.

The E354 residue is located in the AF-2 transactivation domain and is also part of the hydrogen bond interaction in the third generation of SERMs, i.e., bazedoxifene (BZA) (Figure 3). Bazedoxifene is folded in the binding

![Figure 1](image1.png)

**Figure 1.** Amino acid sequence of cERα (belonging to *Canis lupus familiaris*). Ligand-dependent transactivation (AF-1) is labeled in blue, while ligand-independent transactivation (AF-2) is labeled in red. "**" identical, ":" conserved substitution, ":" semi-conserved substitution.
Figure 2. Predicted cERα using the ModWeb server. (A) The ligand binding domain (LBD) of cERα interacts with its natural ligand (17β-estradiol). (B) The cERα binding pocket shows an interaction between E2 and E354 (cyan), G522 (magenta) and L526 (green). (C) The 2D pattern of the cERα-E2 interaction demonstrates a hydrogen bond interaction with the main chain (green dash) and side chain (blue dash). The arrow is directed towards the electron donor.

Figure 3. The interaction between the ligand binding domain of cERα and SERMs. (A) The ligand binding domain (LBD) of cERα interacts with the third generation of SERMs, bazedoxifene (BZA). (B) The cERα binding pocket shows an interaction between BZA and E354 (cyan), G522 (magenta) and L526 (green). (C) The 2D pattern of the cERα-E2 interaction demonstrates a hydrogen bond interaction with the main chain (green dash) and side chain (blue dash). The arrow is directed towards the electron donor.

Table 1. The binding affinities between the single point mutation of cERα-E354 binding and E2 (17β-estradiol), tamoxifen, raloxifene, bazedoxifene, as well as fulvestrant.

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<th>Raloxifene</th>
<th>Bazedoxifene</th>
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Discussion

In comparison to the crystal structure of hERα (1GWR), it was predicted that cERα also forms three hydrogen bonds with E2. However, the difference is in the amino acid residues and the site of interaction. These indicated that the shapes of the hERα and cERα binding pockets are different between the two species [23,24]. Among the different key amino acid residues, E354 formed hydrogen bonding with both E2 and bazedoxifene. Thus, the binding affinities affected by the single mutation at the E354 residue were calculated. The binding affinities implied that both SERMs and SERDs were weakly bound to the wild-type (WT) cERs. On the other hand, they were good cER inhibitors. Even though the pocket but the A ring is still directed at E354 (Figure 3B). Moreover, the R395 residue is located close to the A ring and also shares a hydrogen bond interaction with the same E354 site (Figure 3C). The complex structure of bazedoxifene is different from E2, and thus its binding pocket shape and key amino acid residues are also different.
mutagenesis affected binding affinities, there was no statistical significance among the mutants. It should be kept in mind that different algorithms could give rise to different structural prediction and possibly different binding affinities.

The acquired SERM resistance in breast cancer cells has been studied, especially with respect to long-term treatment. Interestingly, ER expression is maintained in the acquired resistant tumors. Recently, the conformational change of ERα induced by bazedoxifene has been reported. Consequently, ERα was targeted for proteasome degradation. In the near future, anti-estrogen therapeutic regimen may become an alternative treatment, alongside surgical removal, for companion animals that suffer from ER-positive mammary gland tumors.

**Conclusion**

This mutagenesis study provided information on the structural changes of the predicted cERα’s ligand binding domain and illustrated a small part of the interaction between the steroid receptor and its ligands. The single mutation of the selected key amino acid residue aberrant, along with the hydrogen bond interaction between cERα and both ligands, leads to a variety of binding affinities. According to this in silico model, it can be concluded that E354 plays an important role in the activities of cERα. The effects of single mutants should be studied further in vitro and in vivo.

**Author contributions**

The in silico model was created by Aekkapot Chamkasem and Waraphan Toniti. Panpanga Sangsuriya, Aekkapot Chamkasem and Pranom Puchadapirom performed the statistical analysis. Waraphan Toniti designed the research and prepared the manuscript.

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**Conflict of interest**

The authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

**References**


