12

Novel Breast Cancer Treatment by Targeting Estrogen Receptor-Alpha Stability Using Proteolysis-Targeting Chimeras (PROTACs) Technology

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Abstract: Approximately 70% of breast cancer cases are estrogen receptor-alpha-positive (ERα+). The binding of estradiol to the ligand-binding domain activates ERα. ERα can also be activated via the phosphorylation induced by growth factors. Activated ERα functions as a transcriptional regulator with a pro-tumor activity in breast cancer cells. In recent years, it has been discovered that some proteins can stabilize ERα by inhibiting its degradation via the ubiquitin-proteasome system through several mechanisms, including ERα monoubiquitination,

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deubiquitination, or phosphorylation, among others. Herein, we review the proteins associated with the inhibition of ERα degradation and discuss the role of proteolysis-targeting chimeras (PROTACs) as promising therapeutic strategies for breast cancer by inducing ERα degradation. The knowledge of the multiple mechanisms that stabilize ERα protein may be central for the development of new PROTACs for novel breast cancer treatments.

Keywords: endocrine resistance in breast cancer; estrogen receptor alpha in breast cancer; novel breast cancer treatment; PROTACs; proteolysis-targeting chimeras technology

INTRODUCTION

Breast cancer (BC) is a serious health problem and the leading cause of death in women worldwide (1–3). Estrogen receptor-alpha (ERα) is a nuclear receptor expressed in more than 70% of BC cases (1–4). ERα is localized and functions in the nucleus, cytoplasm, and plasma membrane, and the subcellular distribution of this receptor is highly regulated by several factors (5) (Figure 1). In the nucleus, ERα is a transcription factor that induces the expression of estradiol-dependent genes. In BC cells, ERα expression has been related to the upregulation of estradiol-target genes that are associated with mammary tumor development (6–8). ERα also acts as a transcriptional regulator for other transcription factors, including AP1, NF- κB, and Sp1, to modulate gene expression (6, 7). ERα can also be localized outside the nucleus, and activate the signaling pathways that contribute to BC progression and endocrine resistance (8, 9). Hence, ERα associates with diverse transmembrane receptors and cytoplasmic proteins to activate its extranuclear signaling pathways (10, 11). Moreover, ERα can be directly localized at the plasma membrane via palmitoylation to activate the signaling that influences gene expression (12, 13).

ERα contains four main domains: the activation function domain-1 (AF-1), the DNA-binding domain (DBD), the ligand-binding domain (LBD), and the activation function domain-2 (AF-2) (Figure 2). The canonical signaling pathway of estrogen hormones, such as estradiol, is initiated when the hormones are recognized by LBD (14, 15). Enhancer and promoter regions of the estradiol-target genes contain specific palindromic sequences named ERE (estrogen response elements), which bind the DBD of ERα to regulate gene expression. AF-2 recruits coregulators in the presence of E2 hormone, whereas AF-1 recruits coregulators in its absence (16–19). Furthermore, AF-1 can be modulated by phosphorylation (e.g., S118, S167) in response to growth factors (16–22). The recruitment of ERα to the enhancer and promoter sequences is also supported by pioneer factors (e.g., FOXA1 and GATA3) in BC cells (23, 24). Several studies have shown that ERα recruits coregulators, including coactivator and corepressor complexes. In BC, there is an increase in the levels of coactivators for ERα that open the chromatin to promote gene expression (25–29).

The targeted drug therapy for ERα+ BC is based on endocrine therapies that include aromatase inhibitors (AIs), selective estrogen receptor degraders (SERDs), and selective estrogen receptor modulators (SERMs). The AIs inhibit estradiol

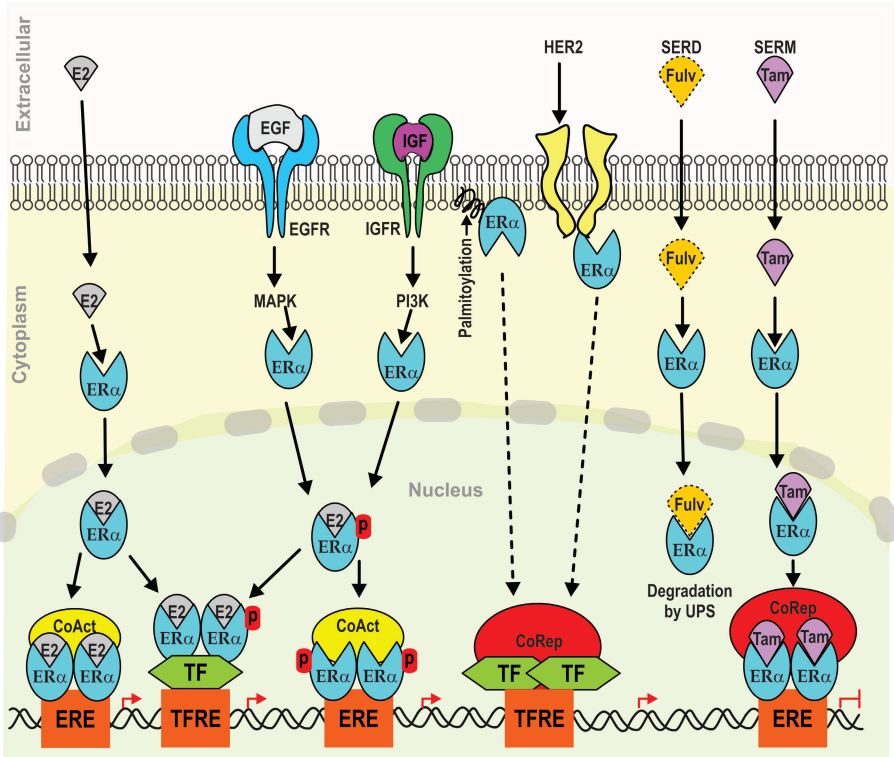

Figure 1. Signaling of ERα in breast cancer cells. ERα exhibit multiple localizations including the nucleus, cytoplasm, and membranes. In the cytoplasm, it is associated with cytoplasmic proteins, and in the membrane through palmitoylation or transmembrane receptors (e.g., HER2). Estradiol (E2) activates ERα, promoting its accumulation in the nucleus, where the ERα regulates the expression of E2-target genes. E2 and growth factors (EGF and IGF) can induce ERα phosphorylation and promote its transcriptional activity. Fulvestrant and tamoxifen are anti-estrogens that bind to ERα to mediate either its degradation or the repression of its target genes, respectively.

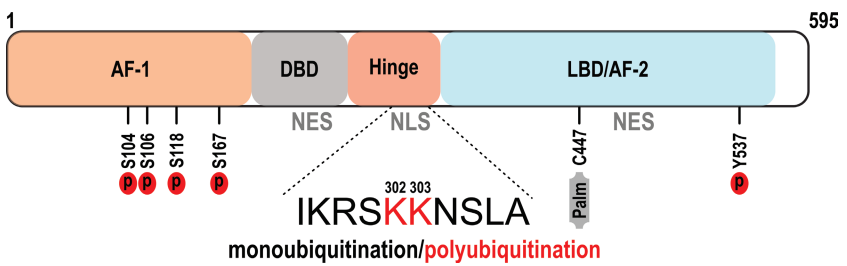

Figure 2. ERα structure. ERα contains two activation-function domains (AF-1 and AF-2) that interact with coregulators, some specialized sequences (NLS, nuclear localization signal, and two NES, nuclear export sequence) necessary for nucleo-cytoplasmic transport of ERα. ERα is a target for post-translational modifications (PTMs), including palmitoylation (Palm) and phosphorylation (P). ERα phosphorylation at sites S104, S106, S118, and S167, is promoted by E2 and growth factors (GF). K302 and K303 are involved in monoubiquitination and polyubiquitination induced by E2 or fulvestrant.

production, whereas SERMs act as antagonists for ERα and induce the recruitment of corepressors, thereby inhibiting the expression of estradiol-target genes (30–32). However, endocrine resistance, *de novo* or *acquired,* is displayed mainly for SERMs owing to mechanisms that are not completely understood (9, 31–34). In this context, SERDs appear to be more promising for BC treatment, as is discussed later.

ESR1 MUTATIONS IN BREAST CANCER

Several mutations, including Y537S, Y537N, Y537C, D538G, and E380Q, have been detected in *ESR1* gene that encodes for ERα, in both DNA from biopsies and circulating tumor DNA obtained from patients with metastatic BC, and with acquired resistance to AIs and SERMs (35, 36). These mutations are located in LBD of ERα, and are related to changes in ERα conformation that promote its estradiol-independent activity (37). Moreover, it has been reported that the gene signature modulated by ERα wild type and its mutants differ, having few common target genes. The ERα mutants can differentially interact with DNA and other proteins and modulate gene transcription, indicating their molecular complexity (36, 38–40). Moreover, in the induction of acquired endocrine resistance (by long-term E2 deprivation) in BC cells, *ESR1* mutations (Y537C and Y537S) are detected (39, 41).

ERα STABILITY AS A MECHANISM THAT PROMOTES PRO-TUMOR ACTIONS AND ENDOCRINE RESISTANCE

ERα is a target for polyubiquitination induced by estradiol, whereas its monoubiquitination is inhibited by estradiol (42). The polyubiquitination of ERα serves as a signal for its degradation by the ubiquitin-proteasome system (UPS); however, ERα monoubiquitination seems to confer receptor stability, and modulates its activity (43–45). Furthermore, both modifications are deregulated in mammary tumors. In fact, it has been reported that some proteins interact with ERα to inhibit their polyubiquitination and degradation via UPS. These ERα-polyubiquitination inhibitor proteins (EPIPs) can act by several mechanisms leading to this receptor stability in BC (Table 1) (46–64). The mechanisms that lead to the stabilization of ERα include its phosphorylation by specific kinases, association with transcription regulators, and regulation by E3-ubiquitin ligases and deubiquitinases (DUBs) (Figure 3). In recent years, new EPIPs have been discovered, demonstrating the relevance of ERα stability in pro-tumor molecular mechanisms associated with BC progression and endocrine resistance. Interestingly, several E3-ubiquitin ligases inhibit ERα degradation: RNF8, RNF31, and SHARPIN by inducing its monoubiquitination (47–49), whereas Smurf1, TRIM56, and HOIL-1 by inhibiting its K48-specific polyubiquitination (50, 51). In addition, RNF181 stabilizes ERα, inducing its K63-linked ubiquitination in BC cells (52).

Furthermore, some DUB proteins also confer stability to ERα. Nearly 100 DUBs have been identified in humans, and are classified into six families: USPs,

TABLE 1 **ERα-polyubiquitination inhibitor proteins (EPIPs)**

Protein	Name	Activity	Reference
cABL	Abelson tyrosine-protein kinase	Kinase	(53)
GSK3	Glycogen Synthase Kinase 3	Kinase	(54)
LMTK3	Lemur Tyrosine Kinase 3	Kinase	(55)
PIN1	Peptidyl-propyl cis-trans isomerase NIMA-interacting 1	Isomerase	(56)
OTUD7B	OTU Deubiquitinase 7B	Deubiquitinase	(57)
USP7	Ubiquitin-specific protease 7	Deubiquitinase	(58)
USP15	Ubiquitin Specific Peptidase 15	Deubiquitinase	(59)
USP35	Ubiquitin Specific Peptidase 35	Deubiquitinase	(60)
HOIL-1	Haem-oxidised IRP2 ubiquitin ligase-1	E3-ubiquitin ligase	(51)
RNF8	RING finger protein 8	E3-ubiquitin ligase	(47)
RNF31	RING finger protein 31	E3-ubiquitin ligase	(48)
RNF181	Ring Finger Protein 181	E3-ubiquitin ligase	(52)
SHARPIN	Shack-associated RH domain-interacting protein	E3-ubiquitin ligase	(49)
SMURF1	SMAD ubiquitination regulatory factor 1	E3-ubiquitin ligase	(61)
TRIM11	Tripartite Motif Containing 11	E3-ubiquitin ligase	(62)
TRIM56	Tripartite Motif Containing 56	E3-ubiquitin ligase	(50)
RB	Retinoblastoma	Tumor suppressor	(10)
MUC1	Mucin 1	Transcriptional regulator	(63)
ZNF213	Zinc Finger Protein 213	Transcriptional regulator	(64)

Induction of K63-linked ubiquitination: RNF181; Inhibition of K48-linked ubiquitination: HOIL-1, TRIM56, SMURF2, ZNF213; Monoubiquitination: TRIM11, RNF8, RNF31, SHARPIN.

OTUs, UCHs, MJDs, MINDYs, and JAMMs (65). The DUBs implicated in ERα stability are ubiquitin-specific proteases (USPs: USP7, USP15, and USP35), and one ovarian tumor protease (OTU: OTUD7B). In general, the proteins that stabilize ERα are upregulated in patients with mammary tumors, revealing their importance in the progression of this cancer (66). Thus, these results demonstrate the importance of understanding the mechanisms of ubiquitination (mono-and poly-ubiquitination) and de-ubiquitination in the regulation of ERα stability in BC.

SERDS and PROTACs

Fulvestrant is a SERD that induces ERα polyubiquitination and degradation via the UPS (32–34). There is a complete reduction in ERα levels in BC cells after 1 h of treatment with fulvestrant (30, 67–69). Fulvestrant has been also approved as the first-line endocrine therapy in BC and also in endocrine resistance to SERMs or AIs (70). Interestingly, endocrine resistance to SERDs, such as fulvestrant and

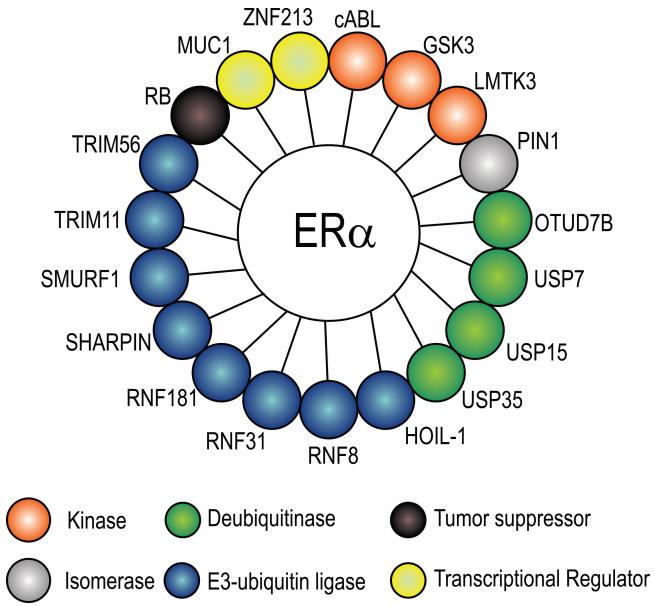

Figure 3. Modulators of ERα stability in breast cancer cells. EPIPs (ERα polyubiquitination inhibitor proteins) have been reported to inhibit ERα polyubiquitination in breast cancer cells. These proteins protect ERα from degradation via the UPS. Diverse PTMs confer stability to ERα: Monoubiquitination (produced by E3 ubiquitin ligases), the inhibition of K-48 linked polyubiquitination, and deubiquitinase (DUB) enzymes, which cut off polyubiquitination tails of ERα.

AZD9496, has not been reported for the majority of *ESR1* mutations (36, 39, 40). New SERDs that are being studied are AZD9496, bazedoxifene, RAD1901, GDC-0810, ZB716, and LSZ102. As fulvestrant exhibits poor bioavailability with intramuscular administration, the challenge is to develop a new generation of SERDs that can be orally administered and with better bioavailability (71–76).

Proteolysis-targeting chimeras (PROTACs) technology works by inducing the degradation of specific proteins. This activity is due to the bifunctional structure of PROTACs: one part of these molecules has a ligand for a targeted protein, while the other part has the ability to recruit an E3 ubiquitin ligase, thereby stimulating the targeted protein degradation via the UPS. Hence, PROTACs have emerged as a promising alternative to control the pro-tumor effect of ERα by causing its downregulation via UPS (77, 78).

PROTAC technology

PROTACs are bimodular chimeras: they contain a binding module for the target protein and another E3 ligase recognition module; a spacer or linker sequence links these two modules (Figure 4). The PROTAC binds to its target protein, carrying a signal for its ubiquitination and subsequent degradation (79). A polyubiquitinated protein is recognized by the 26S proteasome, transported to the 20S core particle, and converted into oligopeptides by a variety of enzymes that

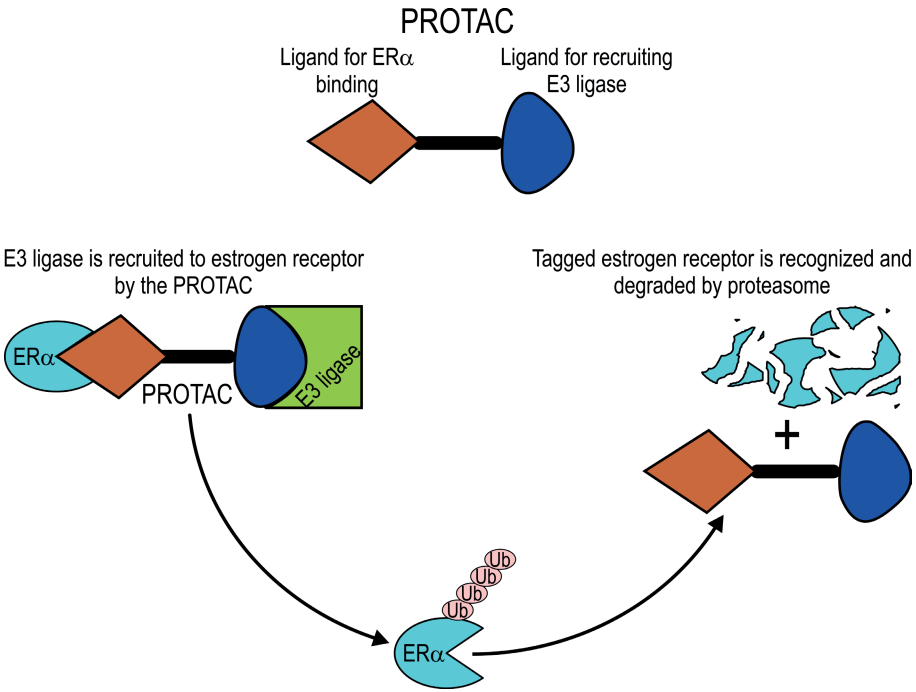

Figure 4. PROTACs are constituted of a region that recognizes its target (ERα) and a region that recruits E3-ligase (to polyubiquitinate the receptor) for its degradation via the UPS.

promote their release from the proteasome; this is followed by the recycling of ubiquitin (80, 81).

Since 2001, the technology based on PROTACs is being developed by utilizing heterobifunctional molecules (82). The first ligands for recruiting E3-ubiquitin ligases in PROTAC technology were large peptides; however, they had the disadvantage of having a low level of cellular penetration owing to their size (81). PROTAC technology was developed for the first time using a chimera containing ovalicin in one domain and the phosphopeptide IκB in another domain. Thus, this chimera had the ability to bind to methionine aminopeptidase 2 (MetAP-2) (by ovalicin) and to recruit the $SCF^{\beta\text{-TrCP}}$ E3-ubiquitin ligase complex (by phosphopeptide IκB), thereby promoting its degradation via the proteasome (81, 82). Later, other PROTACs were developed using the following E3 ubiquitin ligases: MDM2 (murine double minute 2), CRBN (cereblon), VHL (von Hippel-Lindau tumor suppressor), and IAPs (inhibitor of apoptosis proteins) (81, 83). PROTACs based on IAPs have antitumor functions; however, special care must be taken while designing them to avoid collateral effects (80, 84).

PROTACs display some disadvantages, for instance, they are generally large molecules, and their pharmacokinetic characteristics are compromised, such as bioavailability and solubility. In addition, the routes of administration of these chimeric degraders are generally intraperitoneal or subcutaneous. However, in recent years, the intravenous and oral routes are also being evaluated (79, 85, 86).

NOVEL PROTAC TECHNOLOGY FOR BREAST CANCER TREATMENT

Several PROTACs have been developed for ERα, including, SNIPER(ER)-3, PROTAC-B, PROTAC-2, compound 11, compound 24, lipophilic amino acid Boc-Trp motif, C3-linked adamantane motif, monocyclic trifluoromethyl cyclohexane motif, and ERD-148 (87). PROTACs exhibit antitumor activity and the ability to degrade ERα through the UPS in breast cancer cells (88). To date there are some limitations in the cell-penetrating and target protein degradation ability of the PROTACs designed for ERα; however, compound I-6 (a peptide-based PROTAC) contributes to cell membrane penetration. As a result, this compound can induce degradation of intracellular ERα and inhibit the ERα-dependent tumor cell proliferation and growth in *in vivo* studies, thereby supporting its potential use in breast cancer therapy (84).

Another molecule proposed for ERα + breast cancer based on PROTAC technology is PROTAC-2. This compound consists of estradiol that is covalently bound to the phosphopeptide of IκBα containing an E3 recognition domain, and thus recruits $SCF^{\beta\text{-trcp}}$, which leads to ERα ubiquitination and subsequent degradation by the 26S proteasome. The disadvantage of this PROTAC is that the IκBα peptide is susceptible to phosphatases (89). Another traditional example is PROTAC-B, which contains a pentapeptide derived from HIF-1α (hypoxia-inducible factor-1α) to be recognized by the E3-ubiquitin ligase, VHL (88, 90). Similarly, E2-octa contains a synthetic octapeptide derived from HIF-1α, which is very efficient in the degradation of ERα in human BC cells. Modifications of this PROTAC gave rise to E2-penta, which produces a reduction in ERα levels (91). It was only after 2011, when E3-ubiquitin ligase ligands were discovered, that major progress was made in the development of next-generation PROTACs. The specific and non-genetic IAP-dependent protein eraser (SNIPER), composed of a derivative of estradiol and a bestatin amide (a cIAP1 ligand), also showed a decrease in ERα levels (81, 92).

A peptide-based PROTAC design has been shown to degrade ERα through the binding of an ERα modulator peptide (TD-PERM; N-terminal aspartic acid cross-linked stabilized peptide ERα modulator) with a pentapeptide that binds to the VHL E3 ubiquitin ligase complex (77). This heterobifunctional peptide has the ability to recruit ERα and bring it to the VHL E3 ligase complex for degradation through the proteasome. Thus, this PROTAC (TD-PROTAC) induces the degradation of ERα, reduces the transcription of its target genes such as pS2, and inhibits the proliferation of cancer cells. The T47D and MCF-7 breast cancer cells that express ERα showed early apoptosis when subjected to high doses of TD-PROTAC (77).

Additionally, in a xenograft model of nude mice that received MCF-7 cells, the administration of TD-PROTAC promoted tumor regression (77). ARV-471 is another PROTAC for ERα that is well tolerated and is currently in phase 2 clinical trials. It functions by reducing the ERα levels, and ERα variants such as Y537S and D538G (80). ERD-308 is another PROTAC that displays a better ERα degradation capacity, as well as a greater cell proliferation inhibitory capacity than fulvestrant (83).

DISCUSSION

ERα stability is increased in BC cells, and it has been associated with tumor progression and the development of endocrine resistance (93). New studies are emerging, which are demonstrating that many proteins are involved in ERα stability in BC, which emphasizes the pro-tumor potential of Erα, and endocrine resistance (Table 1 and Figure 1). Therefore, the mechanisms that control ERα stability may be targeted for the development of new drugs and also the improvement of current therapies. Thereby, SERDs and PROTACs are critical therapeutic strategies for controlling breast cancer progression. Even though new SERDs are being developed, only fulvestrant has been approved thus far (32). To avoid the development of resistance to SERD treatments based on ERα degradation, it is important to consider the implications of ERα stability-associated pathways. Interestingly, although PROTACs show good promise for BC due to their abilities for causing ERα ubiquitination and degradation, the mechanisms that lead to ERα stability may also represent a limitation. This is because several USPs participate in the inhibition of polyubiquitination and degradation of ERα, and many of them are upregulated in mammary tumors. Moreover, although some E3-ubiquitin ligases can promote ERα degradation via polyubiquitination, there are others that can inhibit the degradation of this receptor by inducing its monoubiquitination (Table 1). Thus, ERα stability may also have an impact on the therapies based on the degradation of this receptor in BC cells. There is a growing need for the development of new SERDS-like molecules and PROTACs that can induce ERα degradation in BC, which can block tumor progression. Attention should be paid to the improvement of bioavailability and administration routes of SERDs and PROTACs, as well as the denominated SERD-like PROTACs (78).

PROTAC technology is a promising system that is starting to be used in patients with BC, as well as in patients with other types of cancer (80, 89). This technology shows that when suitable ligands are used to target Erα, they can promote its degradation almost entirely, and therefore, nullify its transcriptional activity and signaling, and promote the death of BC cells both *in vitro* and *in vivo* (77). Currently, some PROTACs are already in the clinical stage (80), and it is expected that they will exhibit high specificity when administered orally, with good stability and cell permeability. Thus, the PROTAC system is currently being evaluated in clinical trials conducted in patients with metastatic BC, with the interest of some pharmaceutical companies (81). At present, PROTACs are not intended to replace traditional cancer therapies, but rather, be included as co-adjuvants in such treatments. This technology could complement current cancer therapy strategies. The next step in PROTACs technology would be to improve their pharmacokinetic properties for a wider clinical application and consider the implications of the mechanisms that stabilize ERα in BC.

CONCLUSION

Several studies have suggested the existence of a wide range of proteins that confer ERα stability in BC, which is related to tumor progression and endocrine resistance. Under these conditions, the development of treatments focused on the induction

of ERα degradation is required. Both SERDs and PROTACs have the ability to downregulate ERα via degradation through the UPS with the aim of reducing its pro-tumor effects. However, further studies are required to improve these drugs for use in the treatment of BC, and also to evaluate the role of ERα stability in these treatments.

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