Bioorganic & Medicinal Chemistry Letters 28 (2018) 2827–2836

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Digest

Recent advances in peptidomimetics antagonists targeting estrogen receptor α-coactivator interaction in cancer therapy

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ARTICLE INFO

Keywords:
Breast cancer
Estrogen receptor
Protein-protein interaction
Peptidomimetics
Inhibitor

ABSTRACT

Estrogen receptor α (ERα) is a crucial target for ERα positive breast cancer treatment. Previous drug discovery efforts were focused on developing inhibitors that targeted the canonical ligand binding pockets of the ligand binding domain (LBD) of ERα. However, significant percentage of patients developed cancer relapse with drug-resistance. ERα peptidomimetic modulators have been considered as promising treatments for drug resistant breast cancers as they are targeting ERα-coactivator interacting interface instead of the ligand binding pocket of ERα. Herein, we reviewed the recent development of ERα peptidomimetics antagonists.

Nuclear Receptors (NRs) are multi-domain transcription factors involved in regulation of cellular phenotypes, embryonic development, proliferation and metabolism. Abnormal regulation of NR signaling may cause diseases such as obesity, diabetes and cancer.[1] NRs are modulated by their ligands binding to their ligand binding pockets, which cause subsequent conformational changes of the NRs and regulate transcription by the recruitment of different coregulator proteins.[2,3] Estrogen receptors (ERs) belong to the nuclear receptor (NR) superfamily and contain two members, ERα and ERβ.[4] ERα and ERβ regulate the transcription of different genes by attaching to different sites on genome of cell and by subsequent recruitment of different co-factor proteins.[5] Selective agonists of ERβ may not stimulate the proliferation of breast tissues, while activation of ERα is associated with proliferation of breast and uterine tissues.[6,7] ERα is overexpressed in more than 70% breast cancers and promotes development of ER positive breast cancer.[8] Tamoxifen is an endocrine drug that has been broadly utilized to treat ERα-positive breast cancers.[9–11] It could target the ligand binding pocket of ERα and competitively inhibit the binding of native ER ligand 17β-estradiol (E2). However, many ER-positive breast cancer patients relapse after treatment with tamoxifen that targets the ligand binding pocket of ERα.[11–13] Besides, recently reported ERα mutants that are constitutively activated and involved in cancer metastases highlight the needs of developing new inhibitors targeting other than the ligand binding pocket of ERα. Targeting ERα-coactivator interaction might be a potential therapeutic strategy for intractable ERα positive breast cancers[14–17]

The transcriptional activation of ERα needs the recruitment of steroid receptor coactivator (SRC) protein. The interaction is mediated by an α-helical peptide of the coactivator protein with a conserved LXXLL motif (L means leucine and X means any amino acid), termed the NR box.[18,19] The LXXLL motif is involved in the binding to ERs while its neighboring residues confer selectivity/specificity among the nuclear receptor superfamily proteins.[19–22] The large, shallow or incontinuous interfaces in the protein-protein interactions (PPIs) may be "undruggable" targets for conventional small molecules.[23] Stabilized peptidomimetics with improved potency and stability compared to native peptides, might inhibit protein-protein interactions and become potential therapeutic agents.[24–27] Many synthetic methods have been applied to constrain peptides into stabilized structures aiming to disrupt different PPIs.[28–33] To this end, several constrained peptidomimetics containing the LXXLL motif had been developed to inhibit the therapeutic important ERα–coactivator interactions.[34–39] Several synthetic small molecules that mimic the secondary structure of the LXXLL peptide motif were also developed to disrupt this PPIs.[40–50] In this review, recently developed small molecules and stabilized peptides targeting the intractable ERα–coactivator interaction will be discussed (Fig. 1).

Small molecule ER-coactivator binding inhibitors

In 2004, Rodriguez et al. reported the design of first pyrimidine-based ER-coactivator binding inhibitor (CBI).[40] To mimic the structure of coactivator peptide containing LXXLL motif, they designed molecule containing a central core of triangle dimension. Various hydrophobic substituents were added to the central core to mimic the structure of the

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https://doi.org/10.1016/j.bmcl.2018.05.062
Received 31 March 2018; Received in revised form 25 May 2018; Accepted 30 May 2018
Available online 31 May 2018
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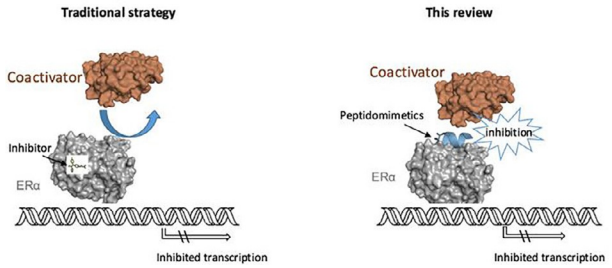


Fig. 1. Scheme of the binding mode of traditional inhibitor targeting the ligand binding pocket of ERα (left) and peptidomimetics targeting ERα-coactivator interaction discussed in this review (right).

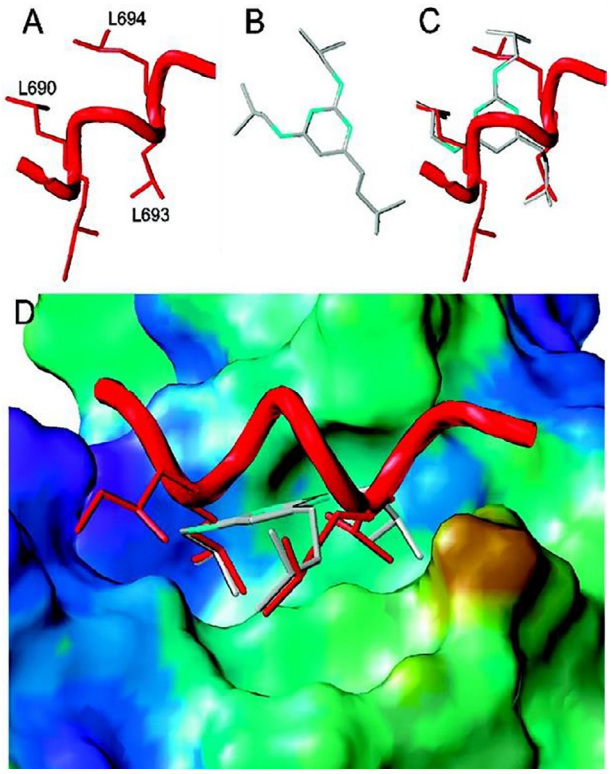


Fig. 2. Structure-based design of small molecules containing pyrimidine core based on the structure of ERα LBD in complex with coactivator peptide (PDB code: 3ERD). (A) Structure of SRC-2 peptide containing LXXLL motif from 3ERD crystal structure showed in cartoon. Leucine residues are labeled. (B) Structure of CBI 2,4-diisobutylamino-6-isopentylpyrimidine (compound **1**). The structural formula of **1** is shown in Fig. 3. (C) Overlay of CBI with the SRC-2 peptide. (D) Side view of the overlay of CBI with the SRC-2 peptide in coactivator binding groove of ERα. (Reprinted with permission from Ref. 41. Copyright (2008) American Chemical Society.)

three vital leucine residues of the coactivator SRC-2 peptide (Fig. 2). Different polar functionalities were added in the molecule to interact with the pivotal binding "charge clamp" residues Glu542 and Lys362 of ERα LBD. Small molecules bearing different cores were chosen based on the molecular modeling results and their effects on disrupting the ERα-coactivator interaction. The most promising CBIs were found in the pyrimidine family and compound **1** (2,4-diisobutylamino-6-isopentylpyrimidine), showed the best binding affinity with K_i values of about 30 μM.[40] Then, structure-activity relationships had been examined using different substituents on the pyrimidine core to improve the specific binding affinity. Both time-resolved fluorescence resonance energy transfer (TR-FRET) and cell-based reporter gene assays confirmed that the most potent member compound **2** (Fig. 3), which contained branched hydrophobic substituents that mimic the key leucine

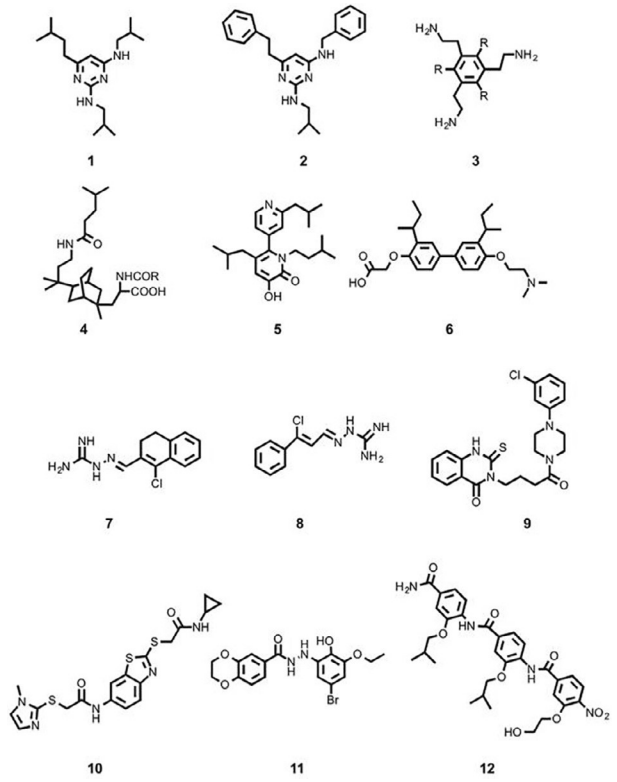
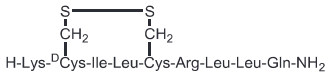
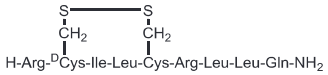
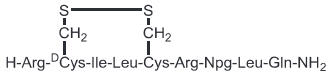
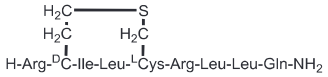
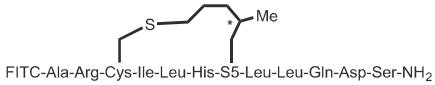
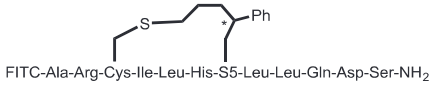
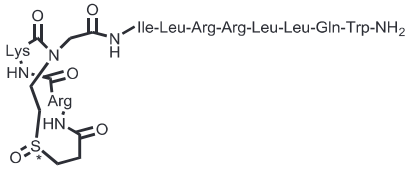
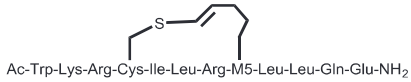
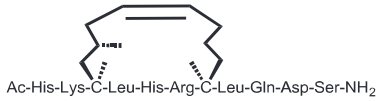
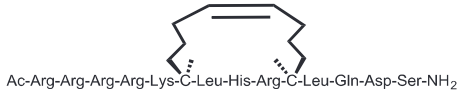
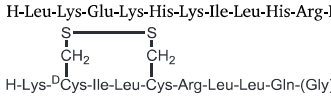
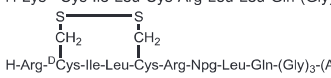
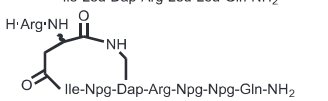


Fig. 3. Molecular structures of several small molecule CBIs that could target ERα-coactivator interaction.

residues, inhibited ERα-coactivator interaction with K_i of about 2 μM.[41] These compounds were identified as non-LBP antagonists since increased concentration of estradiol didn't affect their inhibition effects of ERα-coactivator interaction. In addition, most CBIs displayed better binding affinity towards ERα over ERβ based on the TR-FRET assay. Similar approach was used in the design of the amphipathic benzenes (compound **3** in Fig. 3), which aimed to disrupt ERα-coactivator interaction. These inhibitors were designed based on an amphipathic benzene scaffold that also mimic the α-helical LXXLL motif of SRCs that interacted with the shallow binding groove of ERα.[42] TR-FRET assays and cellular reporter gene assays demonstrated that amphipathic benzenes were also non-LBP antagonists. Other CBIs such as bicycle2.2.2octane (compound **4**),[43] pyridylpyridone (compound **5**),[44] biphenyl proteomimetic (compound **6**),[45] ERI-5 (compound **7**),[46] guanylhydrazone (compound **8**),[47] and so on, which mimic the structure of the LXXLL binding motif were also developed. In 2011, Katzenellenbogen et al. reported two novel structure CBIs (compound **9** and **10**) by high-throughput screening (HTS).[48] They investigated many analogs of the two compounds and some molecules showed low micromolar potencies in inhibiting ERα-coactivator interaction in cell-based reporter gene assay.

In 2015, Singh et al. reported carbohydrazide chemical class as a lead inhibitor of ERα by in silico virtual screening. They reported that compound **11** of the carbohydrazide chemical class could significantly downregulated ERα transcriptional activity, and selectively inhibited the proliferation of ERα-positive breast cancer cells. More importantly, it reduced mRNA and protein levels of downstream targets of ERα.[49] However, all these small molecule CBIs had only moderate potencies, limiting their potential clinic applications. In 2017, Raj et al. reported a small molecule compound **12**, which was the most well-characterized small molecule for inhibiting ERα-coactivator interaction.[50] Compound **12** is a tri-benzamide that could inhibit the proliferation of several different ERα-positive breast cancer cells. Notably, it could regress the growth of ERα-positive breast cancer xenograft in vivo. **12** was

Table 1
Sequences and binding affinities of the peptides.

Name	Peptide sequence	Binding Affinity ERα (nM)	Binding Affinity ERβ (nM)
NR-2 peptide	H-Cys-Leu-Thr-Glu-Arg-His-Lys-Ile-Leu-His-Arg-Leu-Leu-Gln-Glu-NH_2	900	N.D
PXLXXLLXXP$_{13}$	H-His-Pro-Leu-Leu-Met-Arg-Leu-Leu-His-His-Pro-Ser-NH_2	1541	3640
PXLXXLLXXP$_{22}$	H-His-Pro-Leu-Leu-Met-Arg-Leu-Leu-Leu-Ser-Pro-NH_2	185	25
PERM1	 H-Lys-^{D}Cys-Ile-Leu-Cys-Arg-Leu-Leu-Gln-NH_2	25	390
PERM2	 H-Arg-^{D}Cys-Ile-Leu-Cys-Arg-Leu-Leu-Gln-NH_2	11	77
PERM3	 H-Arg-^{D}Cys-Ile-Leu-Cys-Arg-Npg-Leu-Gln-NH_2	0.07	1.2
Peptide 3	 H-Arg-^{D}C-Ile-Leu-^{L}Cys-Arg-Leu-Leu-Gln-NH_2	6.9	64
ER-1b	 FITC-Ala-Arg-Cys-Ile-Leu-His-S5-Leu-Leu-Gln-Asp-Ser-NH_2	1	N.D
ER-2b	 FITC-Ala-Arg-Cys-Ile-Leu-His-S5-Leu-Leu-Gln-Asp-Ser-NH_2	69	N.D
Peptide 7A/7B		7A: 183.5 7B: 386.2	N.D
Peptide 1h	Ac-Trp-Lys-Arg-Ala-Ile-Leu-Arg-Ala-Leu-Leu-Gln-Glu-NH_2	89.3	179.3
Peptide 2h	 Ac-Trp-Lys-Arg-Cys-Ile-Leu-Arg-M5-Leu-Leu-Gln-Glu-NH_2	100.6	177.8
SRC2-SP3	 Ac-His-Lys-C-Leu-His-Arg-C-Leu-Gln-Asp-Ser-NH_2	89	N.D
SRC2-wt	Ac-His-Lys-Ile-Leu-His-Arg-Leu-Leu-Gln-Asp-Ser-NH_2	2600	N.D
R4K1	 Ac-Arg-Arg-Arg-Arg-Lys-C-Leu-His-Arg-C-Leu-Gln-Asp-Ser-NH_2	19	N.D
Tif-2 Box2-R9-PEG-biotin	H-Leu-Lys-Glu-Lys-His-Lys-Ile-Leu-His-Arg-Leu-Leu-Gln-Asp-Ser-Ser-Ser-Pro-Val-$(Arg)_9$-PEG-biotin	9590	N.D
PERM-1-R7	 H-Lys-^{D}Cys-Ile-Leu-Cys-Arg-Leu-Leu-Gln-$(Gly)_3$-$(Arg)_7$-NH_2	749	N.D
PERM-3-R7	 H-Arg-^{D}Cys-Ile-Leu-Cys-Arg-Npg-Leu-Gln-$(Gly)_3$-$(Arg)_7$-NH_2	94	N.D
12a	 Ile-Leu-Dap-Arg-Leu-Leu-Gln-NH_2	85	N.D
6 w	 Ile-Npg-Dap-Arg-Npg-Npg-Gln-NH_2	67	297
TD-PRTOAC	 Ile-Leu-Dap-Arg-Leu-Leu-Gln-AHX-Leu-Ala-Pro(OH)-Tyr-Ile-NH_2	30	N.D

(N.D, not determined.)

designed to bind to the coactivator binding groove of ERα, but the mode of action and precise binding site were not fully elucidated yet.

Peptide based ER-coactivator binding inhibitors

The powerful recognition selectivity and binding potency of peptides can be explained by various functional groups, facile construction and easy acquisition of structural information. There has been much interest in developing peptide or peptidomimetics to disrupt protein-protein interactions. Phage display studies showed that the α-helical peptide of the coactivator protein containing the conserved LXXLL motif could target ER-coactivator interactions to block the transcriptional activity of ERα.[20] However, linear peptides have limitations in cell penetration and stability, precluding their further applications.

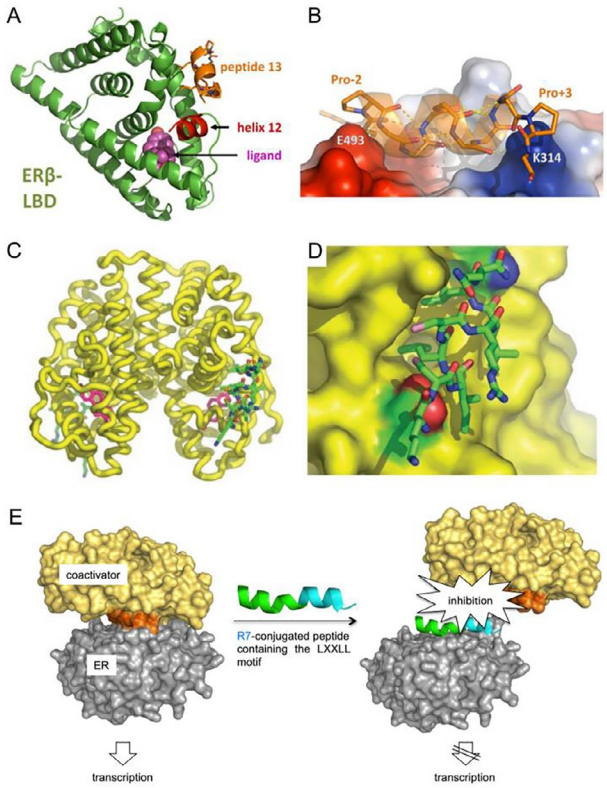


Fig. 4. (A) General view of co-crystal structure of peptide **PXLXXLLXXP$_{13}$** (HPLLMRLLHHPS, orange), 17β-estradiol (E2, purple), critical helix 12 (red) in the ERβ-ligand binding domain (ERβ-LBD) (green). (PDB code: 4J26) (B) The crystal structure of peptide **PXLXXLLXXP$_{13}$** bound to ERβ LBD showed in electric static surface, the Pro-2 and Pro + 3 are marked for delineating α-helix length. The peptide is showed in stick and carbon atoms colored orange. (Reprinted with permission from Ref. 21. Copyright (2013) American Chemical Society.) (PDB code: 4J26) (C) Peptide **PERM1** (green) bound to ERα-LBD (yellow) in the presence of estradiol (red). (D) Close-up view of **PERM1** (green) interacted with ERα-LBD. The "charge clamps" of ERα-LBD were colored in red and blue, which play vital role of the orientation of peptide anchored. (Reprinted with permission from Ref. 36. Copyright (2003) The National Academy of Sciences.) (E) Scheme of the mechanism of the R7-conjugated peptides inhibiting ER-coactivator interactions. (Reprinted with permission from Ref. 61. Copyright (2015) Elsevier Ltd.

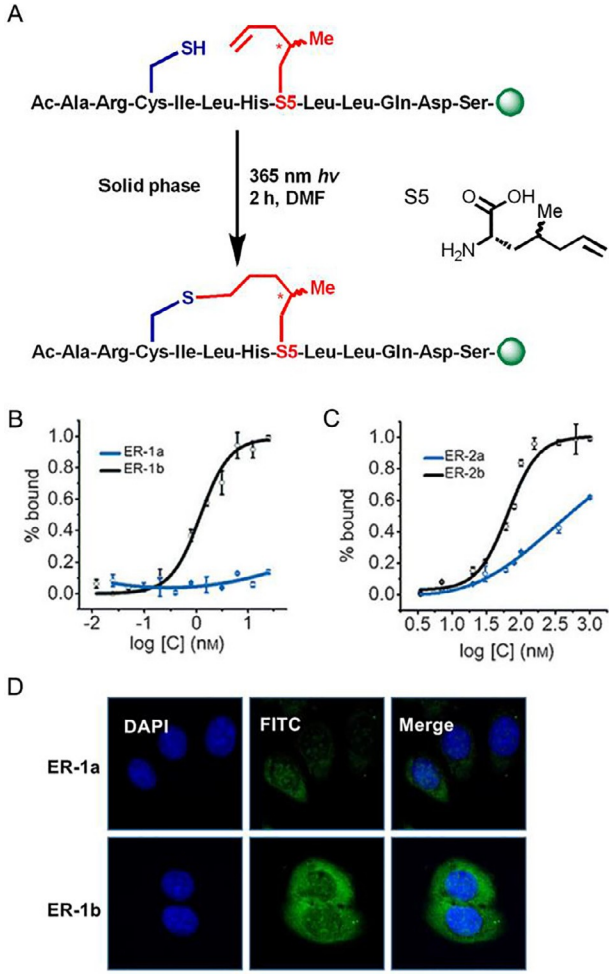


Fig. 5. (A) Synthesis scheme of constrained peptide with chiral center positions. (B and C) Binding affinity of peptides **ER-1a/1b** and **ER-2a/2b** to ERα measured by fluorescence polarization assays (FP) respectively. (D) Fluorescent confocal microscopy images of peptides **ER-1a/1b** labeled with FITC in MCF-7 cells (37 °C, 2 h).

Stabilization of linear helical peptides by chemical methods would enhance the biophysical properties and improve their stabilities and potencies. To this end, many stabilizing methods have been developed including N-cap helix nucleation and side-chain crosslink. The N-cap helix nucleation methods are based on "capping box effects" and helix-coil transition theory.[51,52] Li et al. also reported a new N-cap helix nucleation method based on tethered terminal iso-aspartic acid strategy.[53,54] Side chain crosslink strategies usually utilize covalent linkage to tether amino acids at position i and i + 3, i + 4 or i + 7 of one or two turns of a helix. Many side chain crosslinking methods have been applied for peptides that could target ERα such as disulfide bond stabilized strategy,[36] macrolactam linkage,[34,35] all-hydrocarbon stapled peptide linkage,[39] Thiol-ether linkage,[30,55] Thiol-yne linkage.[32] Herein, peptides and peptidomimetics targeting ERα by different stabilizing methods will be discussed. (All peptides in this review were showed in Table 1.)

Linear peptides development

Linear peptides

In 1998, Rosenfeld et al. found that LXXLL-containing motifs might be the basics for nuclear receptors to recruit the coactivator complexes, and the LXXLL box might contribute to specific receptor and ligand assembly of the coactivator complexes.[56] McDonnell and coworkers proposed that the C-terminal AF-2 domain of ER might provide a surface to recruit coactivator proteins, so that it may act as a molecular switch for receptor to distinguish from agonists and antagonists.[57] Then, they screened peptides that interact selectivity with either estradiol- or tamoxifen-bound ERα by phage display and reported several peptides containing LXXLL motif that could inhibit the activity of ERα.[1,20,58] In 1999, Katzenellenbogen et al. demonstrated that **NR-2 peptide** could stabilize agonist ligands in ER by fluorescence assay and increase the half-time of the ER-agonist complex. But **NR-2 peptide** had nearly little effect on the dissociation rate of antiestrogens such as Faslodex (ICI182780). These results showed that **NR-2 peptide** could specifically bind to agonist bound ER and stabilize the complex.[59]

In 2013, Brunsveld and coworkers reported a highly conserved and potent recognition motif peptide containing sequence of PXLXXLLXXP to ER by ribosome display. They demonstrated that the flanking prolines could prime the secondary structure of the ER binding helical peptides by crystal structure and molecular modeling studies and improve the binding affinities of the peptides with ER (Fig. 4A,B).[21] They tested the binding affinity of these selected peptides to ERs and **PXLXXLLXXP$_{22}$** showed the binding affinity with K_i of 185 nM to ERα-LBD.

However, the linear peptides are often with poor stability and cell permeability under physiological conditions, limiting their potential

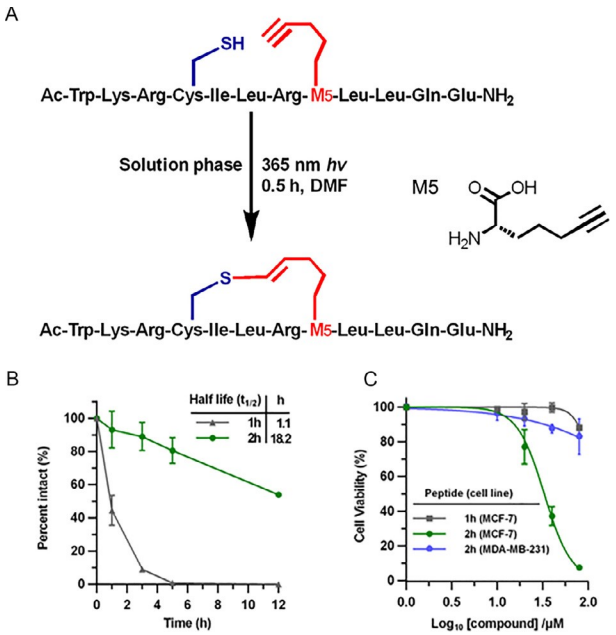


Fig. 6. (A) Schematic diagram of intramolecular thiol–yne hydrothiolation to form constrained peptide via the photo-induced technique. (B) Serum stability of peptide **1h** and **2h**. Constrained peptide **2h** was more stable in fresh mouse serum compared to the linear analog **1h**. (C) MTT assay result of the peptides in MCF-7 cells and MDA-MB-231 cells. Constrained peptide **2h** showed high selective inhibition of the growth of MCF-7 cells.

therapeutic application. To tackle this problem, additional cell-penetrating sequence was widely applied to the peptide design.

Linear peptides linked with cell penetrating sequences

In 2008, Cardoso's research group identified that L-R9 and L-R10 peptides containing rich arginine residues possessed good penetration ability in various mammalian cell lines.[60] In 2009, Brunsveld et al. reported synthetic cell-permeable LXXLL peptide probes with nona-arginine tag could bind to cellular ERα and compete with the binding of coactivators.[22] They demonstrated that peptide **Tif-2 Box2-R9-PEG-biotin** could suppress ER-mediated transcription and down-regulate the mRNA of pS2 gene in ER positive MCF-7 cells.

Due to the flexible conformation and instability of the linear peptides, development of stabilized peptide to improve the binding affinity towards ER-coactivator interaction is highly demanded.

Constrained peptides

Disulfide-bridged peptides

Wemmer et al. reported constructing constraint peptides by disulfide bond stabilized strategy in 1990.[62] To design short cyclic peptides binding potently and selectively to ERα, Spatola et al. firstly reported an (*i, i + 3*) linked disulfide bond stabilized peptide analog (**PERM1**) containing the LXXLL motif. The K_i of **PERM1** was about 25 nM, measured by time-resolved florescence-based coactivator interaction assay.[36] They also reported the X-ray co-crystal structure of **PERM1** in complex with the ligand binding domain of ERα. The structure showed that this stabilized peptide bound at the coactivator binding groove of ERα, as showed in Fig. 4C and D. In 2005, Spatola

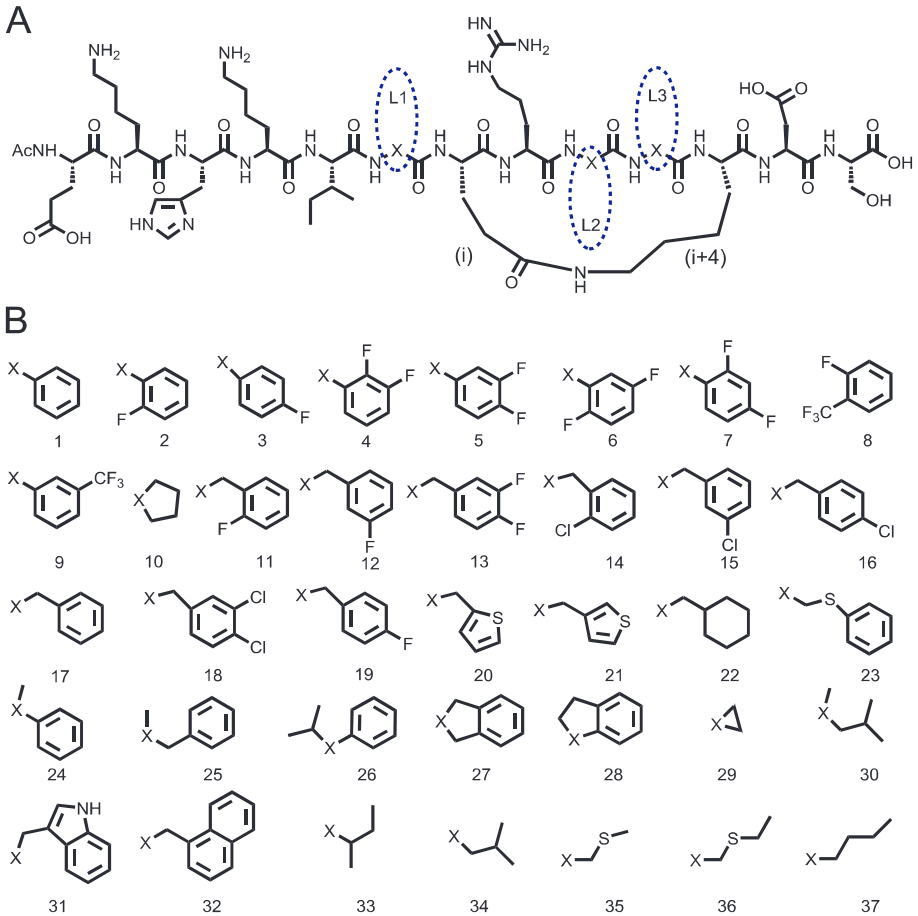


Fig. 7. Synthesis proteomimetics analogs of **SRC2-2**. (A) The scaffold sequence for constructing series of peptides of **SRC2-2**. (B) The substituent groups superimposing the X on the scaffold. The replaced leucine at positions L^1, L^2, and L^3 respectively with non-natural amino acids.

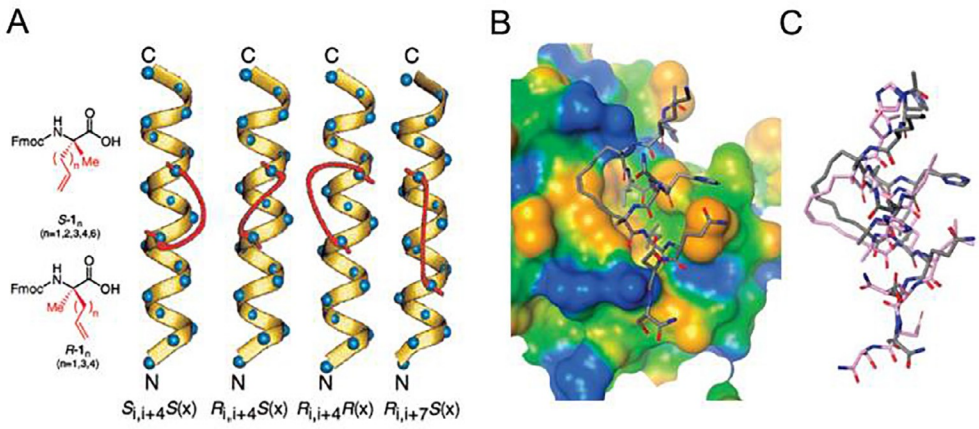


Fig. 8. Scheme for stabilizing helical peptides by all-hydrocarbon cross-linking strategy. (A) The key amino acids of the system are *S-1*$_n$ or *R-1*$_n$ containing olefinic side-chains. It could be linked by olefin metathesis to cross-link one or two turns with the un-natural amino acids located at position of *i* and either *i* + *4* or *i* + *7* on peptide, respectively. (Reprinted with permission from Ref. 66. Copyright (2000) American Chemical Society.) (B) Crystal structure of peptide **SP2** (gray) bound to ERα-LBD. (PDB code: 2YJA) (C) Overlap image of the crystal structure of peptide **SP2** (gray) with that of peptide **SP1** (pink). (D) Binding affinities and kinetics of the peptides measured by surface plasmon resonance (SPR) technology. (The position of Leu in the NR box are highlighted). (Reprinted with permission from Ref. 39. Copyright (2011) American Chemical Society.)

D

Peptide Sequence	ERα			ERβ		
	k_a (x10^{-5})	k_d	K_D	k_a (x10^{-5})	k_d	K_D
N: Ac-HKILHRLLQDS-NH_2	1.39	0.347	2.5	0.792	0.376	5.0
SP1: Ac-HS5ILHS5LLQDS-NH_2	3.80	0.256	0.674	2.55	0.509	1.99
SP4: Ac-HKILHS5LLQS5S-NH_2	n.d.	n.d.	>15	n.d.	n.d.	>15
SP3: Ac-S5KEKS5KILHQLLQDS-NH_2	n.d.	n.d.	8.0	n.d.	n.d.	8.3
SP5: Ac-HKILHQLLQS5SSSS5V-NH_2	n.d.	n.d.	4.0	n.d.	n.d.	2.8
SP2: Ac-HKS5LHQS5LQDS-NH_2	5.48	0.193	0.352	5.36	0.339	0.632
SP6: Ac-EKHKILS5RLLS5DS-NH_2	17.8	0.129	0.075	7.14	0.155	0.155

[a] Abbreviations: k_a, on-rate constant (in M^{-1} s^{-1}); k_d, off-rate constant (in s^{-1}); K_D, dissociation constant (in μM); n.d., not determined.

and coworkers examined the inhibitory effects on ER- coactivators interaction by utilizing a number of cyclic and linear peptides containing homocysteine, penicillamine, tertiary leucine and neopentylglycine respectively.[37] They found the most effective disulfide-bridged peptide **PERM3** targeting with ERα (K_i = 70 pM) contained a neopentylglycine substitution of leucine in the NR box. It also showed binding selectivity over ERβ (K_i = 1200 pM). **PERM1** peptide was the first reported stabilized peptide that could potently bind ERα and further studies targeting ER-coactivator interactions were inspired by the effective **PERM1** sequence reported by Spatola and his colleagues.

In 2014, Kurihara and coworkers utilized the R7-conjugated **PERM1** and **PERM3** peptides to study the cell penetration efficiency in ERα-positive T47D breast cancer cells (Fig. 4E). They demonstrated that the R7-conjugated fragment did not disrupt the peptides' helical structures in solution while **PERM-3-R7** peptide could remarkably disturb ER-mediated transcriptions and down-regulate pS2 mRNA expression.[61,63]

Thioether-bridged peptides

Spatola and his coworkers reported cyclic peptides stabilized by (*i*, *i* + *3*) spaced thioether-bridged amino acids could considerably enhance the binding affinity of constrained peptides. They demonstrated that as for the cystine disulfide, cystathionine was a good redox-stable, isosteric replacement. They found **peptide 3** (showed in Table 1) containing cystathionine showed higher helical content and could potently inhibit ERα-coactivators (CoAs) interactions (K_i, 6.9 nM), in comparison to the spaced cystine analogs (**PERM2**).[55]

In 2016, we reported a constrained peptide containing a precisely positioned chiral center in the side chain of the peptide (Fig. 5A). We demonstrated that the absolute configuration *R* of the chiral center could improve the helical conformation of the stabilized peptide.[30] Peptide **ER-1b** (K_d is about 1 nM) and **ER-2b** (K_d is about 1 nM), with *R* configuration, showed higher binding affinity with ERα compared to their conformational isomer **ER-1a** and **ER-2a** (K_d > 600 nM), with *S* configuration (Fig. 5B,C). Cellular uptake experiments demonstrated that peptide **ER-1b** (*R* configuration) showed higher cell penetrating efficiency than its epimer (Fig. 5D). It might be caused by the methyl group at the stereo center which formed additional interaction with ERα, suggesting that the chiral center might be further modified to improve the peptide's activity.

This work successfully applied chiral center strategy in the development of ERα peptidomimetic inhibitors with enhanced binding affinity and permeability. We also designed peptides (**peptide 7A/7B**) targeting ERα with an in-tether chiral sulfoxide center on the N-terminal and proved that the in-tether chiral center might modulate the peptides' binding efficacy to ERα with improved stability.[64]

Vinyl sulfide stapled peptides

In 2016, We developed a facile stapling technique of photo-induced

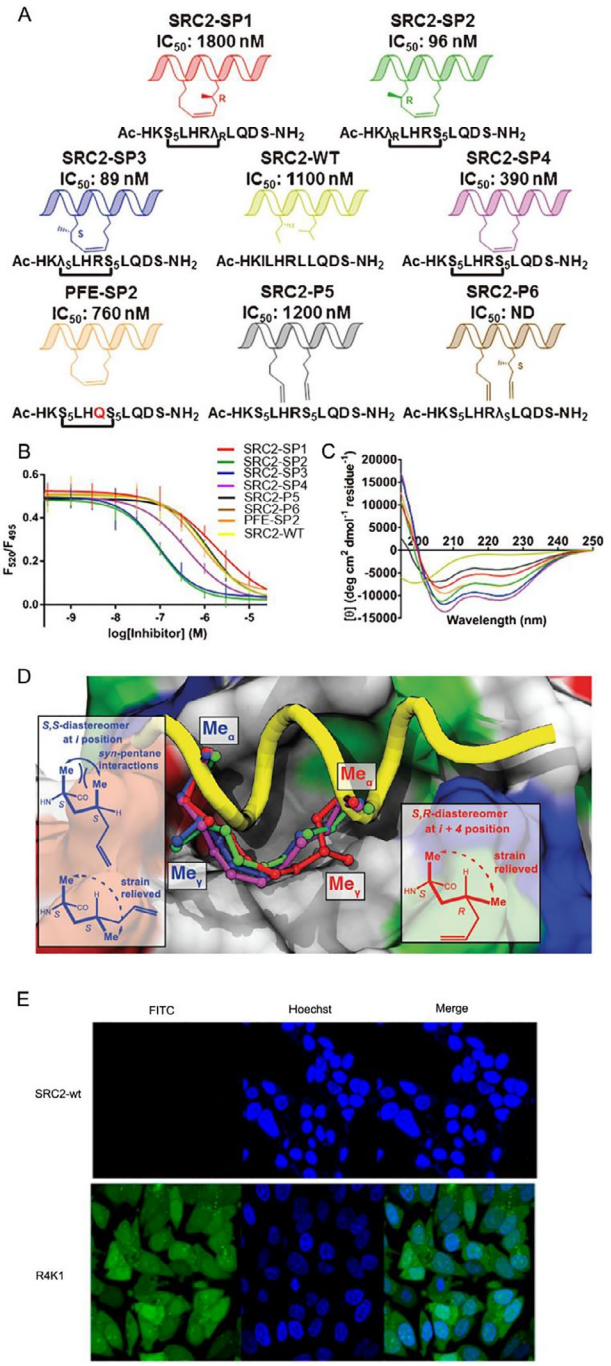


Fig. 9. (A) The sequences of peptides. (B) The competitive binding curves of respective peptides to disrupt ERα/SRC3 interaction by time-resolved fluorescence resonance energy transfer (TR-FRET) assay. (C) CD spectra of the corresponding peptides. (D) The superimposed X-ray co-crystal structures of **SRC2-SP1** (red, PDB code: 5DXB), **SRC2-SP2** (green, PDB code: 5HYR), **SRC2-SP3** (blue, PDB code: 5DX3), **SRC2-SP4** (magenta, PDB code: 5DXE) and **SRC2-WT** (yellow, PDB code: 3ERD). The methyl groups adopt conformations of relieved strain in peptide **SRC2-SP1**, **SRC2-SP2**, and **SRC2-SP3**. (Reprinted with permission from Ref. 38. Copyright (2016) John Wiley and Sons.) (E) The peptide **R4K1** showed good cell penetration capability in MCF-7 cells. (Reprinted with permission from Ref. 68. Copyright (2018) American Chemical Society.)

intramolecular thiol-yne macrocyclization without metal catalyst to construct stapled peptides targeting ER-coactivator interactions.[32] The vinyl-sulfide stapled peptide **2h** showed high binding affinity to ERα, enhanced serum stability, cellular uptake and anti-proliferative activity towards MCF-7 cells in comparison to its linear analog peptide **1h** (Fig. 6).

Macrolactam cyclization peptides

In 2003, Guy et al. designed many coactivator peptidomimetics containing the $L^1XXL^2L^3$ motif. Based on the original sequence, NH2-$_{685}$EKHKILERLLKDS$_{697}$-COOH from NR box II of steroid receptor coactivators (**SRC2-2**), they first built a library of stabilized α-helical peptides.[35] They adopted the cyclization method of (*i, i + 4*) macrolactam linkage at positions E691 and K695, and replaced leucine at amino acid site of L^1, L^2, and L^3 with unnatural amino acids respectively (Fig. 7). They applied fluorescence polarization (FP) equilibrium competition technique and successfully obtained some peptides selectively targeting human ERα. Notably, one peptide displayed over 600-fold higher affinity to human ERα than another nuclear receptor, human TRβ. These results indicated that it might be possible to design competitive inhibitors targeting specifically to one nuclear receptor, which imitated the LXXLL motif of SRC binding pockets with different unnatural amino acids. Thereafter, they performed in vitro competition assays using the same library of **SRC2-2** peptidomimetics to search for inhibitors selective for ERα and ERβ in the presence of 17β-estradiol (E2), diethylstilbesterol (DES), or genistein (Gen).[34] They found that different ligand bound to ER isoform could obtain different selective peptidomimetic inhibitors containing LXXLL NR box, demonstrating that each ligand played specific allosteric effects on the interface of SRC binding site.

All-hydrocarbon stapled peptide

Macrocyclic peptide helices synthesized by a remarkably facile RCM reaction were firstly developed by Grubbs in 1998.[65] Verdine et al. developed an all-hydrocarbon strategy with enhancements in secondary structure, stability, binding affinity, and cell permeability of peptides in 2000.[31,66] The all-hydrocarbon strategy was based on ring-closing olefin metathesis (RCM) to cross-link one or two turns at the *i* and either *i* + *4* or *i* + *7* position, respectively (Fig. 8A).[66]

In 2011, Phillips et al. synthesized stapled peptides by all-hydrocarbon strategy based on the crystal structure complex of NR-coactivator peptide bound to ERα LBD (PDB entry 2QGT).[39,67] Crystal structure of peptide **SP2** were showed in Fig. 8B and C. The sequences of stapled peptides were showed in Fig. 8D, and the unnatural amino acids S5 were linked at position (*i, i + 4*) of the peptide.[39] Peptide **SP6** showed highest binding affinity to ERα with K_D of 75 nM.

In 2016, Moore et al. synthesized (*i, i + 4*) stapled peptides containing a γ-position methyl group in amino acid S5 to mimic Ile689 and Leu693 in the $I_{689}LXXLL_{694}$ box. They demonstrated that the S-γ-methyl group could enhance the binding affinity to ERα (Fig. 9A–C). They analyzed the crystal structures of different peptide-bound ERα Y537S mutants. They found the γ-methyl group of **SRC2-SP2**/**-SP3** and Ile689 of the wild-type peptide overlaid in the same region, and **SRC2-SP3** had higher binding affinity with ERα (IC_{50} = 89 nM, Fig. 9A). In addition, the methyl groups adopt conformation of relieved strain in peptide **SRC2-SP1**, **SRC2-SP2**, and **SRC2-SP3** (Fig. 9D).[38] To enhance cell penetration capability of the stapled peptides, they applied molecular dynamic simulations to optimize the all-hydrocarbon stapled peptides that could bind to ERα with high affinity. They designed staple peptide **R4K1**, which possessed high cell permeability and cellular activity (Fig. 9E).[68] **R4K1** also exhibited high binding affinity towards ERα and disrupted the interactions between ERα and coactivator in vitro with low nanomolar potency. Besides, **R4K1** repressed the transcription of native genes mediated by cellular ERα and inhibited the proliferation of ERα positive breast cancer cells. This stapled peptide was first demonstrated as a proof of principle example in preparing cell-permeable stapled peptide inhibitors to target ERα-coactivator interaction.

N-cap helix nucleation strategies

In 2016, our group developed a powerful helix nucleating template

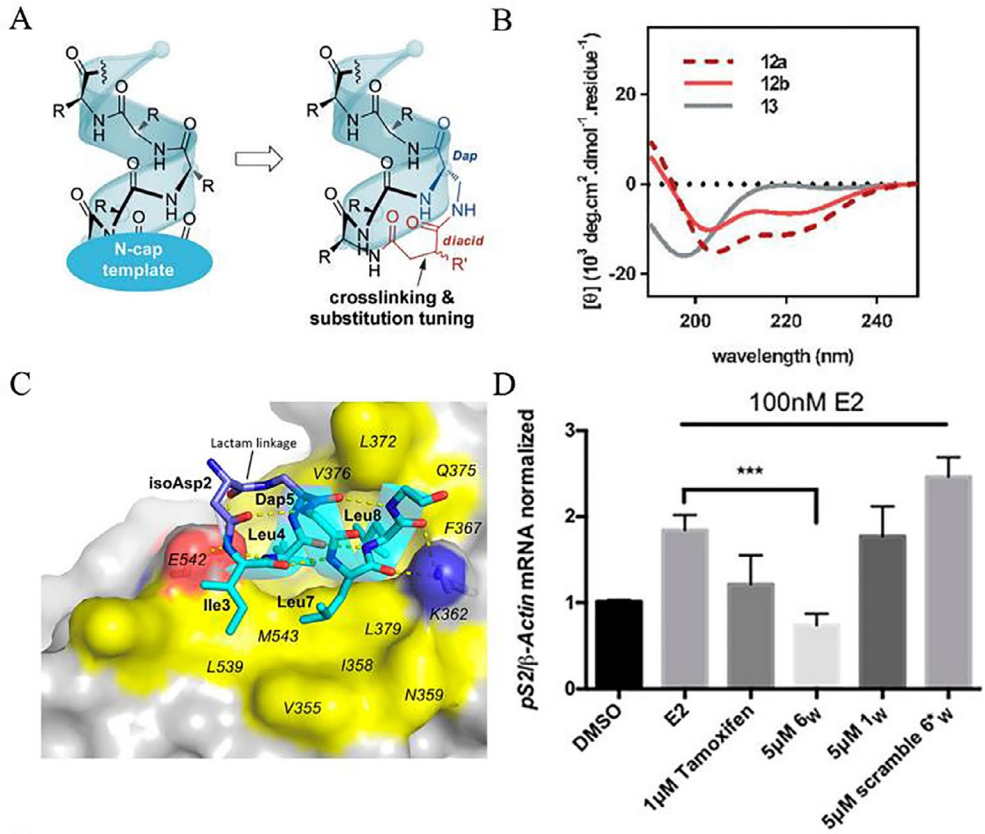


Fig. 10. (A) Scheme of N-terminus helix-nucleating template crosslinked by Dap and isoAsp amino acids. (B) Circular dichroism (CD) spectra of peptides **12a**, **12b**, and **13** in PBS (pH 7.4, 298 K, concentration normalized). (C) Crystal structure of peptide **12a** (green) bound to ERα. Peptide **12a** shows good helix conformation and isoAsp and Dap and the formed lactam linkage are marked in blue. (PDB code: 5GS4) (D) Constrained peptide $\mathbf{6_w}$ could remarkably down-regulate the mRNA expression of pS2. (E) Binding affinity of peptide $\mathbf{6_{FITC}}$ labeled with FITC to ERα, ERβ, PR (progesterone receptor), and VDR (vitamin D receptor). Peptide $\mathbf{6_w}$ showed selective binding affinity to ERα.

E

	K_D(SEM) [nM]						
	ERα	ERβ	PR	VDR	ERβ/ERα	PR/ERα	VDR/ERα
$\mathbf{6_{FITC}}$	67(6)	297(73)	248(136)	>2000	4.4	3.7	>30

based on the cross-link of terminal aspartic acid (Fig. 10A, B).[53] The unnatural aspartic acid was tethered at the N-terminus of the peptide. The advantage of this strategy was that the peptide could be further modified at the intentionally preserved N-terminal amine on the tethered aspartic acid. We utilized this strategy to designed PERMs and found peptide **12a** showed good binding affinity (K_D = 85 nM) with ERα using fluorescence polarization assay. The cell penetration, cellular activity and stability in serum were improved in the modified PERMs using this strategy in comparison to corresponding linear peptides. These peptides might offer better therapeutic outcomes, which further proved that this **TD** strategy could be used for biologically relevant helical peptides.

After that, we further studied the co-crystal structure of peptide **12a** in complex with the LBD of ERα (Fig. 10C). Based on the crystal structure and other reports, we further optimized the stabilized peptides. Peptide $\mathbf{6_w}$ containing three neopentylglycines that replace three leucines showed high selective and strong-affinity binding to ERα (K_D = 67 nM) while weak-affinity binding to ERβ (K_d = 297 nM), progesterone receptor (PR) (K_d = 248 nM) and vitamin D receptor (VDR) (K_d > 2 μM) (Fig. 10E).[54] Peptide $\mathbf{6_w}$ could remarkably down-regulate the mRNA expression of pS2 in MCF7 cells (Fig. 10D). The crystal structure of ERα LBD in complex with peptide **6** and CD spectra confirmed that peptide **6** formed an ideal α-helical conformation and competed the coactivator binding groove of ERα.

PROTAC technology

Crews et al. developed proteolysis-targeting chimera (PROTAC) technology initially.[69,70] The PROteolysis TArgeting Chimeric moleculeS (PROTACS) mediated protein degradation utilized the hydroxyproline motif in HIF1-α (H-Leu-Ala-Pro(OH)-Tyr-Ile-NH_2) that was involved in the von-Hippel-Lindau (VHL) ubiquitin ligase pathway.[71] PROTACS uses heterobifunctional molecules that strategically combine ligands targeting the target protein and the E3 ubiquitin ligase with a specific linker moiety (Fig. 11A, B).[70] So far this PROTAC technology has been widely applied for degrading target protein by ubiquitin-proteasome pathway.[72] As a proof of concept, in 2016, Kurihara et al. designed a novel peptide-based PROTAC to degrade ERα. The compound contained a previously reported peptide (**PERM3-R7**) that could target ERα and a MV1 molecule that could recruit IAP protein (Fig. 11C). They found that the compound with five β-alanine as a linker to link **PERM3-R7** with MV1 molecule could efficiently induce the degradation of ERα by the ubiquitin-proteasome pathway.[73]

Recently, our group applied PROTAC technology to design stabilized peptide based PROTACs to target cellular ERα for treating ER-positive breast cancer. We identified a fusion peptide, which contained both the previously reported peptide **12a** that could bind ERα and HIF1-α peptide that could recruit the VHL E3 ligase complex (named **TD-PROTAC**). We demonstrated that it was able to target and degrade ERα in a proteasome-dependent manner (Fig. 11D,E).[74] **TD-PROTAC** could selectively inhibit proliferations of ERα-positive cancer cells and promote cell apoptosis. Notably, **TD-PROTAC** could induce significant tumor regression compared to tamoxifen positive control without obvious toxicity in MCF7 xenograft mice model (Fig. 11F). It would be promising to use PROTAC as an alternative therapy to degrade cellular ERα and to treat intractable ERα-positive breast cancers.

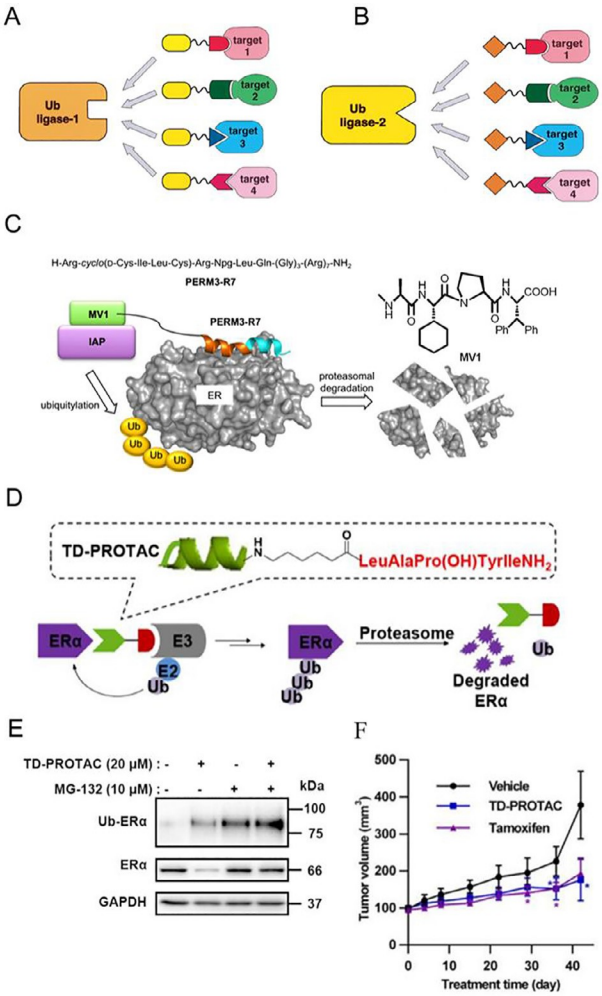


Fig. 11. (A and B) Schematic snapshots of Protacs. Illustrations of how ligand-specific ubiquitination could be utilized for targeted degradations by unique Protacs. (Reprinted with permission from Ref. 70. Copyright (2001) The National Academy of Sciences.) (C) Scheme of the peptide **PERM3-R7** and MV1 complex for the degradation of ER. (Reprinted with permission from Ref. 73. Copyright (2016) Elsevier Ltd.) (D) Illustration of stabilized peptide-based PROTACs against estrogen receptor α. (E) Western blot analysis of ERα and ubiquitinated ERα in T47D cells incubated with 20 μM peptide in the presence/absence of 10 μM MG-132 for 24 h. (F) The suppressive effect on MCF-7 xenografts after treated with peptide **TD-PROTAC** (10 mg/kg), tamoxifen (4 mg/kg), or vehicle (PBS).

Breast cancer remains one of the most prevalent malignant tumors in women with over seventy percent of cases are associated with ERα overexpression. Clinically, ERα mutants with prolonged activation are often found in metastatic breast cancers, which highlights the needs of searching for new drugs to treat ERα-positive cancers. As alternative approach, targeting ER-coactivator interactions other than the ligand biding pocket of ERα appeared to be a promising route to fight against the raising problem of drug resistance. In recent years, huge breakthrough has been seen in inhibitors development targeting non-canonical ligand binding pocket of ERα. Despite many small-molecule inhibitors mimicking the "LXXLL" binding motif involved in ERα–coactivator interactions have been developed, the moderate binding affinity of these molecules limited their applications. Currently, many peptides stabilizing methods have been applied to stabilize ERα peptide ligand into a helical conformation to inhibit ERα-coactivator interactions. The stabilized peptides often possess higher binding affinity to ERα ligand binding domain (LBD) comparing to those small molecules. Several chemical stabilized peptides have already successfully demonstrated both efficient cell penetration capabilities and transcriptional inhibitory effects to cellular ERα. Since the stabilized peptides bind to the coactivator binding site other than the ligand biding pocket of ERα, the combination of stabilized peptides with commercial endocrine therapy drugs such as tamoxifen may be a potential strategy in treatment of ERα-positive breast cancer to minimize drug resistance. In addition, stabilized peptide based PROTACs could efficiently target and degrade ERα via ubiquitination-proteasome system, and therefore suppressing the progression of ERα-positive breast cancers. Overall, these applications have significantly broadened the applicable range of PROTACs and cell permeable stabilized peptides, opening a new area of research for developing novel anti-ERα positive breast cancer drugs. More efforts need to be made in order to develop more selective, potent and safe peptidomimetics antagonists to disrupt the intractable ERα-coactivator interactions for breast cancer treatment.

Acknowledgments

This work supported by High-performance Computing Platform of Peking University. We acknowledge financial support from the Natural Science Foundation of China grants 21778009 and 81701818; MOST 2015DFA31590; the Shenzhen Science and Technology Innovation Committee, JCYJ20170412150719814 and GJHS20170310093122365.

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