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Trends in Cell Biology

Review

Unfolding Protein Response: Promising Therapy to Breast Cancer

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Keywords: UPR, Breast Cancer, Combination Therapy, Single Therapy, Current Advancement

The Unfolded Protein Response (UPR) is a complex adaptive mechanism regulating proteostasis in Endoplasmic Reticulum. Under normal conditions, UPR remains inactivated. UPR activation happens during ER stress, induced by cellular accumulation of misfolded proteins especially in diseases like breast cancer (BC). Recent studies elucidated UPR to influence BC carcinogenesis. Throughout history, the race for anti-BC therapy never ceases, in search of an effective and less invasive approach. Developed UPR-targeting drugs exhibit promising BC treatment: combined therapy to prevent tumor relapses in resistant BC cells or as a single therapy. This review will discuss the various exploited UPR-targeting drug mechanisms focusing on targeting IRE1 and PERK in favor of anti-BC therapy while exploring the contributions of UPR on BC survival.

Highlights

Demanding the need for an effective approach to breast cancer treatment that is able to withstand post-treatment relapse due to chemotherapy resistance.

UPR pathways, specifically IRE1 and PERK are tightly linked to breast cancer carcinogenesis, dormancy, and apoptosis.

The development of UPR-targeting drugs in breast cancer therapy can work in synergy with existing chemotherapy drug agents as combined breast cancer therapy, sensitising resistant breast cancer cells and improving their efficacy.

UPR-targeting drug have the potential to be administered as a single breast cancer therapy. However, further research and clinical trials are still required to elucidate its potency and effectiveness *in vivo*.

The prevalence of Breast Cancer

Breast cancer (BC) is a malignant disease of the mammary glands leading to the accumulation of uncontrollable and undifferentiated BC cells compared to healthy ones, forming cell lumps, able to metastasise to other parts of the body via the blood or lymphatic system (Figure 1A) [1]. BC has been a widely studied life-threatening disease throughout history (Box 1). In 2020, 2.3 million women were BC-diagnosed with 685,000 global mortality cases, establishing itself as the world's most prevalent cancer [2]. Based on its pathology, most BC is categorised as carcinoma, the minority is sarcoma [1]. Multiple studies on recurrence patterns and the presence of molecular markers involving Hormone receptors (Estrogen (EsR+) and Progesterone (PR+)) alongside Human Growth Factor Receptor 2 (HER2) further classifies BC into four subtypes: (i). Luminal A: EsR+/PR+/HER2-; (ii). Luminal B: EsR+/PR+/HER2+; (iii). HER2+; and (iv). Triple Negative Breast Cancer (TNBC) with EsR-/PR-/HER2- expressions [3-4]. The lack of receptors as therapeutic targets makes TNBC cells more challenging to cure, more aggressive, and more prone to relapses (see glossary) than other BC subtypes. Like other cancers, BC has cancer hallmarks to satisfy for its survival (Figure 1B). To maintain its cancerous

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hallmark, BC requires the mediation of various cellular signaling pathways, including the Unfolded Protein Response (UPR) [5].

Box 1 – Brief History of BC: Occurrence and Immediate Treatment

The first incidence of BC was recorded in ancient Egyptians and Greece approximately 3000-2500 B.C.E, accurately coinciding with Edwin Smith and George Ebers's papyri: describing BC as an incurable protruding tumor of the breast [60]. In 400 B.C.E, Hippocrates, also known as the father of medicines, elucidated BC as a humoral disease with progressive stages, which became the first BC causes hypothesis [61]. The human body contains four humors: phlegm, blood, yellow bile, and black bile. He explained that excess black bile was produced in an untreated hard and black tumor. Then, in 200 A.D. Galen proved that BC is a systemic disease with invasive and metastatic properties, as evidenced by the black bile accumulation in the blood [60-61]. He proposed that some BC are more dangerous than others. 'Karkinos' was the name he used for cancer (Greek name after a crab) which describes the tentacle-like tumors of BC. Between 476 and 1500 A.D. religious beliefs favor faith healing more than surgical treatments [60]. Thus, surgical therapy was not practiced. Until the surgical armamentarium was introduced by Henri de Mondeville ('father' of French surgery, 13th century), Guy de Chauliac (France, 14th century), and Albucasis. This procedure uses caustic pastes to exterminate the tumor prior to surgery. The golden age of BC treatment via surgery began only in the 16th to 18th century when the anatomic of the human body, breast ligaments, and subareolar lymphatics plexus were elucidated by Andreas Vesalius (Belgium, 16th century), Cooper (England, 18th century), and Sappey (France, 18th century) respectively. Collectively, John Hunter, also known as the Scottish father of investigative surgery, rejected 'black bile' and suggested lymph as a cause of BC. In the midst of finding the truth underlying BC, multiple surgeries were carried out from simple lumpectomies (surgical lump removal where cancer has not spread) to breast amputations as an attempt to treat BC due to its emerging cases recorded.

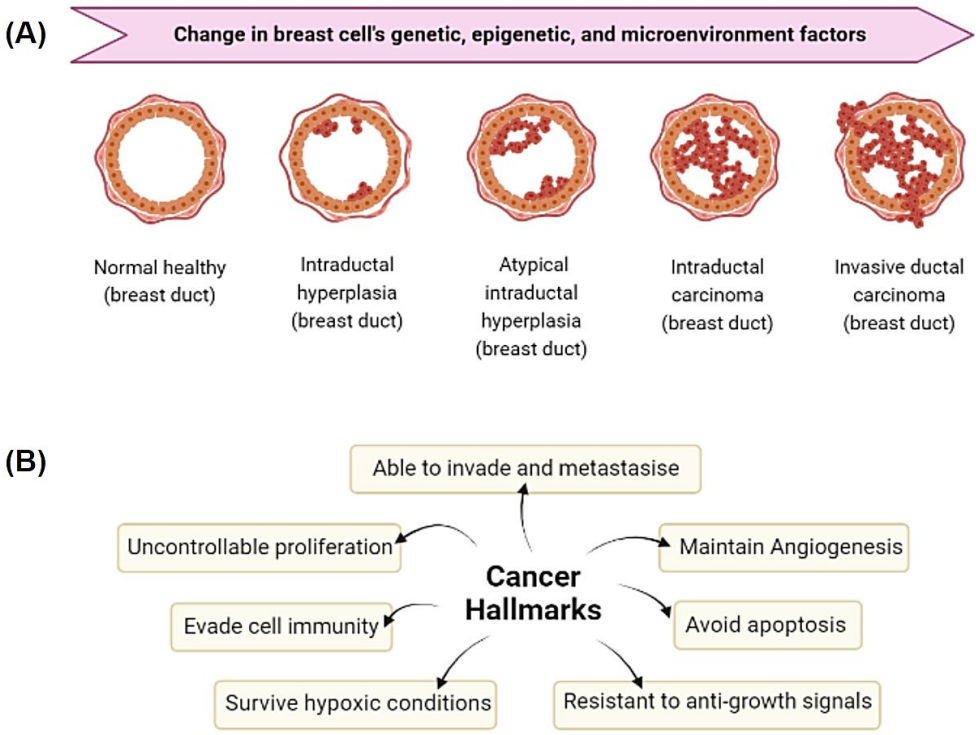

Figure 1. Breast cancer development

(A). Progression of breast cancer cells from its normal state into its invasive carcinoma state ready to metastasise due to genetic (increasing oncogene expressions or loss of tumor suppressing genes), epigenetic (alteration in DNA structure), and microenvironment factors (loss of immune response, etc). Tumor remains 'in situ' until intraductal carcinoma stage. Tumor invasion only starts from invasive ductal carcinoma stage. Once tumor spreads via blood or lymphatic system, it is called "metastatic cancers". **(B).** Breast cancer cell hallmarks, essential for its survival, invasion, and metastasis.

Glossary

Adjuvant: A drug that is administered pre or post-chemotherapy drugs to enhance its efficacy or potency.

Efficacy: the measure or extent of the drug to treat a disease, measuring its effectiveness in vivo.

Hypoxia: a condition where oxygen is insufficient to sustain cellular homeostasis.

Paraptosis: Is a programmed cell death that is accompanied by vacuolation of the ER and/or mitochondria, making it morphologically different from apoptosis.

Pharmacodynamics: the effects of a drug and its therapeutic mechanism of action on the body as well as the response of the body towards the drug.

Prognostic: An indication of the likely course of a medical condition.

Relapses: A condition where diseases are likely to return causing the health of the patient to worsen.

UPR pathways

UPR is essential to mitigate normal proteostasis by relieving Endoplasmic reticulum (ER) stress that arises from the accumulation of unfolded or misfolded proteins in the ER [5-7]. UPR activation balances protein synthesis demand and folding capacity, stimulating more chaperone production, protein degradation, or inhibiting translation which involves three stress sensors: IRE1α, PERK, and ATF6 (Figure 2A-C).

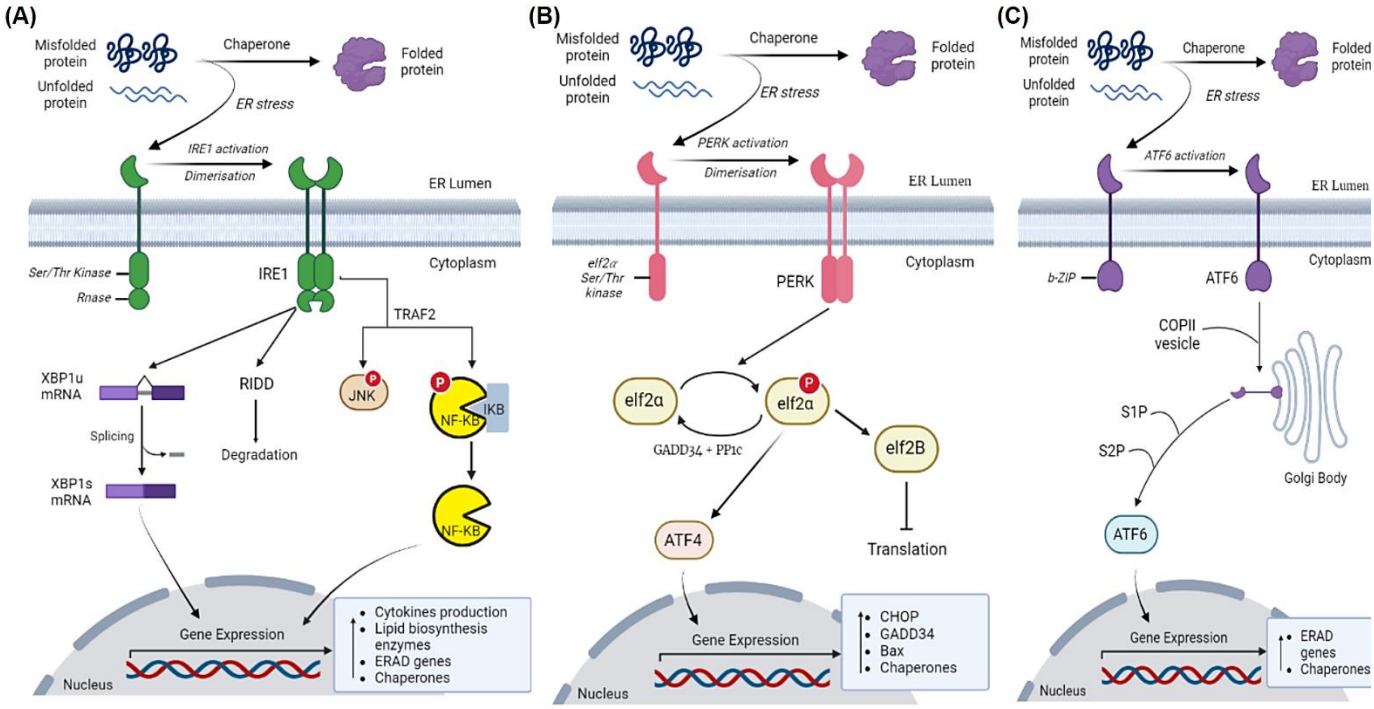

Figure 2. UPR pathways activation

(A). Illustrates the IRE1 activation pathway. Upon ER stress, active IRE1 dimer exhibits RNase and Kinase function. RNase activity permits splicing of unspliced X-box binding protein 1 (XBP1u) mRNA into spliced XBP1 (XBP1s) mRNA which translocates to the nucleus, expressing both chaperones and lipid biosynthesis enzymes. Moreover, it increases RIDD process. Kinase activity phosphorylates both c-Jun N-terminal Kinase (JNK) and NF-kB by TRAF2. Activated NF-kB translocates to the nucleus to express genes increasing cytokine production. **(B).** Illustrates the PERK activation pathway. Upon ER stress, active PERK dimer phosphorylates elf2α (eukaryotic initiation factor 2α) leading to ATF4 expression which will later translocate to the nucleus to express genes in response to ER stress: chaperones (increase folding capacity), CHOP and Bax (stimulate apoptosis) or GADD34. The increase in elf2α levels inhibits further translation (red inhibitory arrow). **(C).** Illustrates the ATF6 activation pathway. Activated ATF6 translocate to the Golgi body forming a transcriptionally active ATF6 to the nucleus, aided with S1P (site-1-protease) and S2P (site-2-protease), increasing chaperone and ERAD genes production.

IRE1

IRE1 is a type I transmembrane protein comprising an endoribonuclease and a serine/threonine kinase domain in its C-terminus (Figure 2A) [5-7]. IRE1 oligomerises in response to ER stress, triggering trans-autophosphorylation and activating its RNase domain [5][8]. IRE1 activation can either stimulate cell survival or apoptosis. Its RNase activity permits both functions by a spliceosome-independent cleavage of a 26-nucleotide unspliced XBox binding Protein 1 (XBP1u) mRNA intron yielding spliced XBP1 (XBP1s) mRNA [6-7].

XBP1s mRNA promotes cell survival by permitting more chaperone and lipid biosynthesis enzyme expressions while inducing apoptosis by increasing ER-associated protein degradation (ERAD) component expressions. Furthermore, its RNase-induced apoptosis stimulates IRE1-dependent decay (RIDD) which targets ribosomal RNAs, micro RNAs, and ER localised RNAs [6][8]. Meanwhile, IRE1 kinase activity only stimulates apoptosis by recruiting TRAF2 which later either activates c-Jun N-terminal kinase (JNK) or translocates NF-kB to the nucleus, expressing inflammatory cytokines as pro-tumorigenic factors [5-6][9]. Downstream, these cytokines can induce the production of pro-angiogenic factors like Vascular Endothelial Growth Factor (VEGF) [10].

PERK

Like IRE1, PERK is also a type I transmembrane protein, with a serine/threonine kinase domain (Figure 2B) [7-8]. During ER stress, PERK gets activated and oligomerises, phosphorylating serine-51 residue of eukaryotic translation initiation factor 2α (elf2α) [7]. Phosphorylated elf2α (elf2α-p) inhibits protein translation, favoring an increase in ATF4 translation [8]. ATF4 is a transcription factor enhancing gene expressions for improved ER folding capacity and autophagy. In relation to autophagy, ATF4 upregulates CCAAT/Enhancer-Binding Protein Homologous Protein (CHOP), increasing pro-apoptotic proteins like Bcl-2-associated X protein (Bax) expressions to induce cell death. ATF4 also upregulates GADD34, restoring protein synthesis by dephosphorylating elf2α-p. This flexibility in elf2α phosphorylation allows PERK to respond in both acute and chronic ER stress, managing tumour microenvironments (TME) like hypoxia (see glossary) and modifications in cell cycle checkpoints components: Cyclin-Dependent Kinases (CDKs)4/6 and Cyclin-D1 and Bcl-2, which are critical in cancers to evade cell death. [6].

UPR is critical in cellular progression and signaling transduction, especially in cancers. This proposed the idea of exploiting UPR in BC therapy. Understanding the role of UPR in BC is important to develop anti-BC drugs with high efficacy (see glossary) and low relapse properties. Hence, this review aims to investigate the effects of activating or suppressing IRE1 and PERK pathways in existing resistance-prone BC therapies. Additionally, discuss potential UPR-targeting inhibitor as an alternative single therapy to existing multiple BC treatments (Box 2).

Box 2 - Multiple approaches to BC Therapy

BC research milestones began with the first radical mastectomy performed by William Halsted in 1882, practiced globally until the 20th century on more than 90% of BC patients in the US [62]. Halsted's radical mastectomy was carried out by surgical large incisions and extensive tissue removals such as the mammary gland, the entire axillary lymphatic tissue, and both pectoral muscles. However, this procedure generated important morbidity and complications.

Thereafter, a modified radical mastectomy was proposed by Patey and Dyson in 1948 [61]. This procedure involved keeping both pectoral muscles by having an elliptical incision surrounding the breast. This results in reduced post-operative complications with a less traumatic experience and a

better survival rate. In the same year, McWirther performed a simple mastectomy in conjunction with radiotherapy. Post-treatment results obtained a similar outcome with radiotherapy having better patient experiences. Hence, breast conservation therapy, an approach to limit the removal of excess breast tissue was initiated by utilising radiotherapy as an alternative BC therapy, evading the extensive use of invasive mastectomy procedures.

The use of chemical-based therapy in cancer also known as "chemotherapy", started during the Second World War between 1950-1960 [63]. To ensure successful treatment, chemotherapy was usually administered together with either surgical or radiotherapy procedures, a common approach is seen today. In 1974, Doxorubicin by Pfizer was FDA-approved and used until today. Soon after, in 1977, Tamoxifen, the first anti-estrogen drug was introduced. Tamoxifen is proven to be effective as a post-surgical therapy, preventive measure, and adjuvant (see glossary). In 1998, the first BC immunotherapy, Trastuzumab (Herceptin) was introduced. Following this, multiple anti-cancer drugs catering to different BC types or prognoses were developed in subsequent years: Paclitaxel (2002), Bortezomib (2003), etc.

How does UPR influence BC?

Tumorigenesis

The formation of tumors leads to the enhancement of unhindered normal breast cell division, perturbing ER homeostasis, and stimulates UPR activation [6]. ER stress can be triggered by cytokine-induced inflammation, which is critical in BC carcinogenesis involving tumorigenesis, proliferation, metastasis, and angiogenesis [11-12]. Inflammation induces an immune response towards pathogens but also triggers DNA damage which increases the tendency for mutations to occur in BCs. A study in TNBC cells observed constitutive IRE1 RNase activity increasing cytokine productions as pro-tumorigenic factors (e.g., IL-6, IL-8, CXCL1, and GM-CSF), elevating tumor growth, proving critical in BC tumorigenesis [13]. For instance, the binding of IL-6 to its receptor activates Akt/mTOR pathways which is an important signaling transduction regulator for cell proliferation, angiogenesis, metastasis, and apoptosis [14-15]. IL-8, a crucial cytokine in BC carcinogenesis tends to be referred to as a pro-angiogenic factor with autocrine and paracrine functions. IL-8 has the ability to modulate the TME by increasing VEGF and VEGF receptor expression, enhancing angiogenesis, to favor BC survival [10]. Therefore, the correlation between BC development and IRE1-induced cytokines production shows a negative prognostic (see glossary) marker, suggesting IRE1's positive contribution to BC cell tumorigenesis.

As BC proliferation rate increases, malignant BC cells in late BC carcinogenesis can either survive in hypoxic conditions to stimulate angiogenesis, metastasis via Extracellular Matrix (ECM) remodeling, and immunosuppression or experience either dormancy or apoptosis [16].

Angiogenesis

Angiogenesis is a physiological process of forming new blood vessels from existing ones. In BC cells, angiogenesis is extensively triggered by hypoxic conditions. To stimulate vascular growth, tumors overcome hypoxia via UPR

activation to increase VEGF secretion as observed using a cDNA microarray assay [17]. For example, an XBP1 silencing assay of TNBC patients using CD31 immunostaining displayed lesser intratumoral blood vessels [18]. Similarly, *in vitro* PERK pathway silencing experiment in MCF7 (EsR+) BC cell line showed a decrease in angiogenesis mediators [17]. Taken *in vivo*, PERK inhibition was established to decrease both tumor proliferation rate and blood vessel density. These findings suggest hindered angiogenesis in IRE1 and PERK inhibition and highlight both importance of IRE1 and PERK activation to increase pro-angiogenic factors and decrease angiogenic inhibitors to favor BC angiogenesis.

Metastasis

Metastatic tumors with invasive ability are characterised by a loss in cell-to-cell interaction, activating abnormal epithelial-to-mesenchymal transition (EMT) [6]. EMT is induced by UPR activation. Non-cancerous EMT cells are required for new EMT synthesis & migration in wound healing. However, malignant cancerous EMT cells have an enhanced ER stress sensitivity via PERK activation [19]. A study showed an increase in ECM secretion (critical for migration) which enhanced ER protein-folding alongside ER to cis-Golgi vesicular transport.

Chemoresistance

BC patients experience chemoresistance post-therapy. Initially, tumor regression implicates positive recovery. However, multiple factors may negatively impact drug efficacy, causing a relapse in tumor growth. Drug resistance can arise from either intrinsic or acquired chemoresistance [20]. Acquired chemoresistance includes utilising the IRE1 pathway [21]. Multiple studies on Tamoxifen resistance in (EsR)+ BC showed a high XBP1s level correlation in endocrine-resistant BC cells, stimulating chemoresistance to anti-estrogen chemotherapy drugs like Tamoxifen [22-24]. Hence, showing that XBP1 increase contributes to BC chemoresistance and cell viability.

PI3K/AKT/mTOR importance in UPR-induced BC carcinogenesis

PI3K/AKT/mTOR pathways, an upstream UPR signaling process has been shown to affect BC survival. PI3K/AKT/mTOR induced by hypoxia stimulates ER stress, activating UPR to induce chemoresistance, tumor cell growth, and metastasis [15]. In 2011, Pi3K mutational assay claimed that PI3K/AKT/mTOR pathway is overexpressed only in HER2+ and EsR+ BC cell lines [25]. However, a novel study in 2019 showed that PI3K/AKT/mTOR pathway is also associated with TNBC cells impacting 10-21% of BC cell proliferation stage [26]. This highlights its role in BC dormancy stage.

Dormancy

Dormancy is a temporary suspension of cellular development. The build-up of tumor dissemination can induce dormancy via ER stress-stimulating UPR. Initially, tumor cells form unstable interactions and degrade the ECM, decreasing Extracellular Regulated protein Kinases that promote cell survival via DNA damage repair stimulation and increasing MAPK p38 [21][27]. Subsequently, activating the PERK pathway to halt tumor growth in the G0-G1 cell cycle, inducing metastatic dormancy. The G1 stage regulates cellular contents and is prone to signals from anti-growth and anti-mitogenesis signals, forcing cell entry into G0, a quiescent stage [28]. Experiments on MDA-MB-231 and HER2 BC cell lines showed Hyaluronan-induced G0-G1 arrest [29-30]. Further work established a decrease in the levels of cyclin D1, an allosteric regulator of cyclin-dependent kinases (CDKs)4/6 in G1-S phase transition by phosphorylating & inactivating retinoblastoma suppression on E2F transcription factor to transcribe proliferation genes (Figure 3) [31-32]. The decrease in cyclin D1 levels indicates BC disability to proceed into the S phase. Specific to MDA-MB-231, Hyaluronan upregulates P21$^{\wedge CIP1}$ (a CDK inhibitor) which decreases cyclin D1 contributing to BC dormancy. However, this dormancy effect was not seen in HER2+ BC cells. Altogether, BC dormancy via PERK activation is cell-cycle specific: growth arrest provides BC the opportunity to evade apoptosis while awaiting a suitable TME to replicate or in inevitable situations, apoptosis.

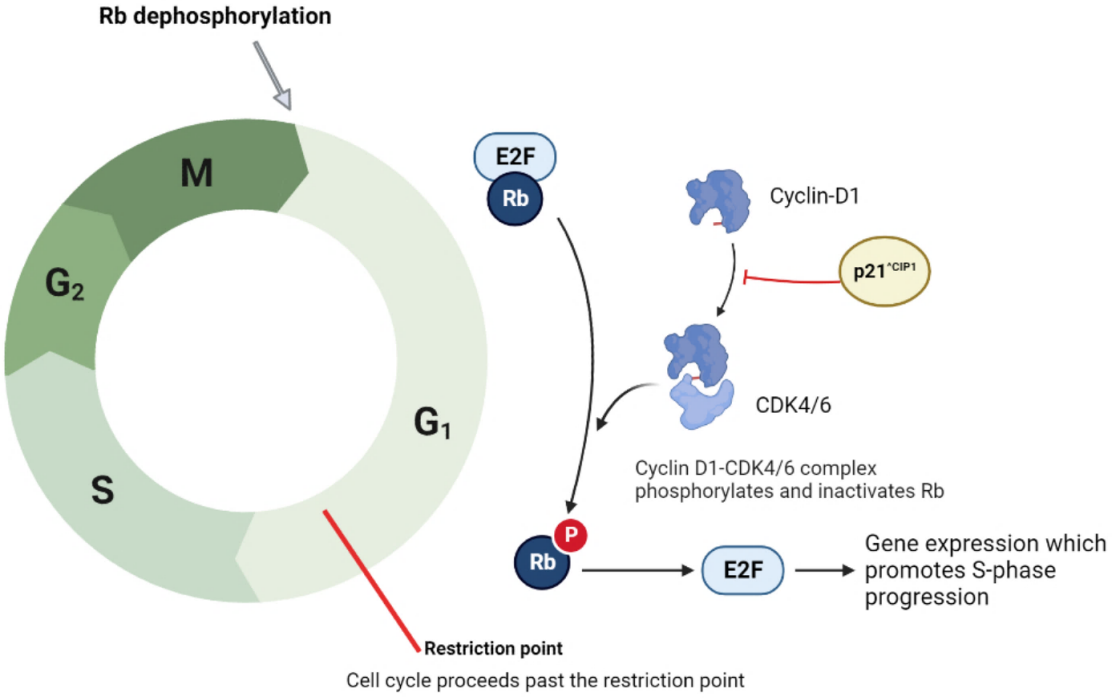

Figure 3. Cell cycle control by Cyclin D1-CDK(4/6)

This figure illustrates cell cycle control by cyclin-dependent kinases (CDK4/6) and their allosteric regulator, Cyclin-D1 in G1 phase. Retinoblastoma (Rb) exerts its inhibitory function on E2F transcription factor at the start of G1 phase. Cyclin D1-CDK4/6 complex phosphorylates Rb to release E2F, permitting gene expressions to enable cellular progression to S phase. The inhibitory function of P21$^{\wedge CIP1}$ on Cyclin-D1 is symbolised with a red inhibitory arrow.

Apoptosis is a programmed cell death inducible by UPR activation as an attempt to reduce tumor survival rate [21][33-34]. Aforementioned, PERK activation increases ATF4 expression. Thus, a study on a knockdown and mutated elf2α-p (heterozygous S51A) assay was investigated in mice expressing HER2 oncogenes. Results showed that BC progressed faster in mutated elf2α-p for approximately 112 to 120 days on average compared to 140 days in wild-type elf2α control, indicating a substantial increase in BC progression rate [35]. However, upon ER stress-induced PERK activation, the increase in ATF4 levels stimulated P21$^{\wedge CIP1}$ (a CDK inhibitor) expression, leading to apoptosis.

Apart from UPR downstream components, caspase-3-induced apoptosis in relation to PARP cleavage is an apoptosis indicator. Specifically, caspase-3 overexpression was correlated with a potent anti-BC drug-induced apoptosis. However, novel studies suggested caspase-3 levels enhancement triggers ER stress to stimulate carcinogenesis and is associated with poor survival, particularly in HER2 and Hormone receptors BC patients [36-38]. Rendering caspase-3's paradoxical role as an invalid BC prognostic marker.

Can UPR-targeting drugs enhance the potency of existing breast cancer chemotherapy drugs, sensitising resistant tumor cells?

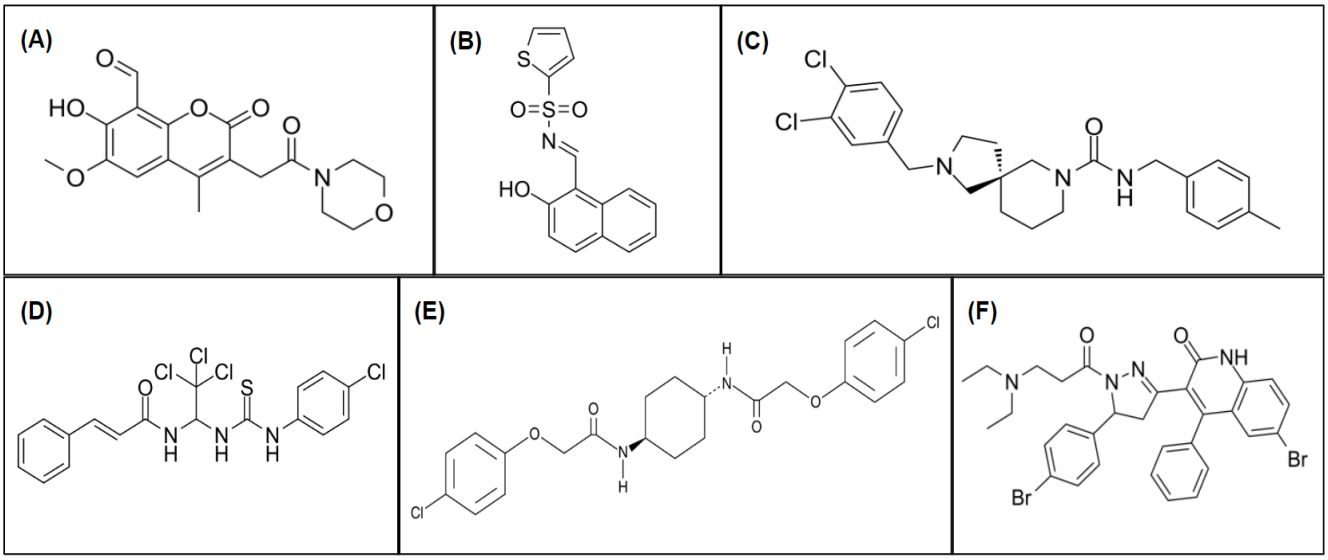

Figure 4. Chemical structures of UPR-targeting drugs
Illustrating chemical structures of UPR-targeting drugs effective in breast cancer treatment. (A) MKC 8866, (B) STF-083010, (C) GSK2850163, (D).SAL003, (E) ISRIB, (F). CCT020312. (A-C) shows UPR inhibitor structures targeting IRE1α/XBP1s pathway. (D-F) shows UPR inhibitor structures targeting PERK/elf2α/ATF4 pathway

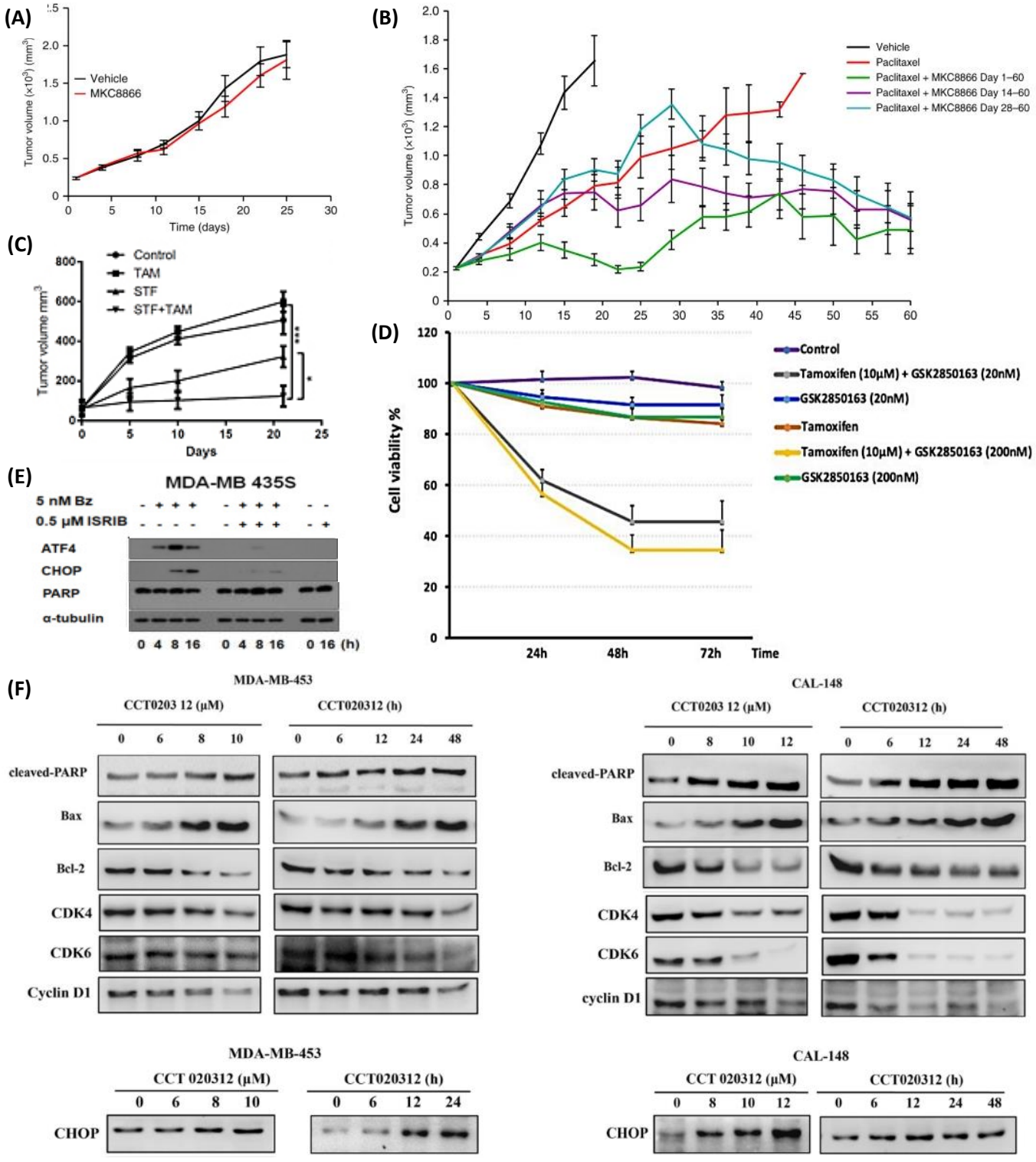

Figure 5. Experimental results of UPR-targeting drugs tested.

(A). Mice xenografts injected with 5x106 MDA-MB-231 cells (vehicle as negative control) against effects of single 300 mg/kg MKC8866 treatment on tumor growth analysed in 25 days. Adapted from [13] **(B).** 10 mg/kg Paclitaxel treatment by intravenous injection in single and co-treatment with 300 mg/kg MKC8866 treatment set in assays for multiple days: 1-60, 14-60, and 28-60. Tumor growth was analysed every 2-3 days by caliper measurement. Adapted from [23] **(C).** Tumor growth curve of in vivo STF-080310 studies in nude mice comprising: control (negative),

Tamoxifen-treated (TAM), STF-080310-treated (STF), and co-treatment of STF-080310 and Tamoxifen (STF + TAM) groups. Tumor growth was measured in days: 5, 10, and 21 post MCF7-TAMR cells injection. Adapted from [22] **(D).** Cell viability assay (WST-1) on the effects of GSK2850163 on Tamoxifen sensitivity tested in MCF-7(R). Multiple assays for 72 hours on different doses for each experiments are presented in the graph. Adapted from [35] **(E).** Western blot analysis of MDA-MB-435 cells treated with 5 nM Bortezomib (Bz) and 0.5 μM ISRIB for 16 hours. α-tubulin (loading control). Adapted from [53] **(F).** Western blot analysis of CCT020312 study on both MDA-MB-453 and CAL-148 BC cell lines. The analysis was conducted on varying CCT020312 concentrations: 0-10 μM (MDA-MB-453 cell line) and 0-12 μM (CAL-148 cell lines) with 0 μM acting as a negative control. This was analysed up to 24 and 48 hours. Adapted from [54].

MKC8866

MKC8866 is a salicylaldehyde analogue (Figure 4A) of an IRE1 RNase-specific inhibitor (Table 1, Key Table) [13]. Cell cycle assay via 5-ethynyl-2'-deoxyuridine (EdU) incorporation showed that MKC8866 inhibits all BC cell lines from entering the S phase, reducing BC proliferation but did not induce apoptosis or dormancy. Despite lacking the ability to stimulate apoptosis, MKC8866 was investigated on MDA-MB-231 cells as an attempt at an alternative anti-TNBC therapy to current adjuvant chemotherapies like paclitaxel. Paclitaxel increases IRE1 RNase-dependent pro-tumorigenic cytokine production and interferes with microtubule dynamics in mitosis, inducing apoptosis [39]. However, paclitaxel's paradoxical effects enable tumor relapse post-paclitaxel treatment [40-41].

MDA-MB-231 cells were MKC8866-treated against control for 48 hours on a cytokine assay investigating MKC8866's effects on 102 different pro-inflammatory factors [13]. Results further confirmed by ELISA and Q-PCR showed a decrease in IL-6, IL-8, GM-CSF, and CXCL1 cytokines secretion in MKC8866-treated MDA-MB-231 cells. Compared to other TNBC cell lines: MDA-MB-468, BT-549, and HCC1806, only CXCL1 exhibits a significant reduction. This suggests that IRE1 RNase activity in TNBC cells is critical in CXCL1 production. Aforementioned, IRE1 RNase inhibition prevents BC tumorigenesis by hindering cytokines production. Further *in vivo* experiments of MDA-MB-231 tumor xenografts in athymic nude female mice of (225-250 mm^3) size were carried out. Initially, 300 mg/kg MKC8866 single treatment reduces IRE1 RNase activity but did not decrease tumor growth (Figure 5A), rendering MKC8866 single anti-TNBC therapy ineffective. More assays with 10 mg/kg Paclitaxel alone and in co-treatments with MKC8866 with multiple-day injections were investigated (Figure 5B). Collective results deduced that MKC8866 enhanced paclitaxel's efficacy, decreasing tumor volume. After 28 days and before tumor relapse occur, MKC8866 systemic concentrations were measured at ~110 μg/ml with no toxicity or body weight changes. Although promising, paclitaxel-MKC8866 co-treatment in TNBC cells recorded tumor relapse in 8 out of 10 mice.

STF-083010 and GSK2850163

Tamoxifen, an anti-EsR+ therapy is an antagonist of EsRα66, a competitive inhibitor for E2's receptor site, inhibiting E2 effects in BC [42-43]. However, Tamoxifen is also an agonist, binding to DNA and stimulating carcinogenesis to induce tamoxifen resistance in EsR+ BC cells. Hence, multiple research on IRE1 inhibitors to overcome Tamoxifen resistance were investigated on MCF7 with Tamoxifen-resistant properties (MCF-7(R)) cells [22-23].

The first studied compound, STF-083010 (Figure 4B) increases MCF-7(R)'s sensitivity to Tamoxifen [23]. STF-083010 selectively inhibits IRE1 RNase activity (Table 1). To study STF-080310's efficacy in ER-induced stress of MCF-7(R) cells, Thapsigargin was used. Thapsigargin is an ER stress inducer, stimulating IRE1-induced XBP1 splicing [44]. Co-treatment between STF-080310 and Tamoxifen exhibits a significant reduction in MCF-7(R) cell viability, sensitising MCF-7(R) to Tamoxifen [23]. By contrast, separate treatments were ineffective with unhindered cell viability. Further *in vivo* experiments on xenograft female mice (150 mm^3) groups were treated separately for 3 weeks, each group comprising 7 mice: control (DMSO), Tamoxifen, STF-080310, and co-treatment of Tamoxifen and STF-080310. Mice treated with both Tamoxifen and STF-080310 exhibit smaller tumor volume (Figure 5C), weight, and slower tumor progression rate. An immunohistochemistry assay on this group showed a reduced XBP1 expression. However, H&E staining observed no significant difference in MCF-7(R) proliferation stage. This suggests other undetermined mechanisms involved, affecting xenografts tumorigenesis. Further assay on Tamoxifen and STF-080310 co-treatment recorded the highest caspase3-positive staining. Aforementioned, caspase-3 expression in BC is a defective apoptosis indicator. Hence, apoptosis can not be deduced. Overall, *in vivo* STF-080310 administration significantly restores Tamoxifen sensitivity in MCF7-TAMR cells and is used in combined therapy with Tamoxifen to inhibit BC progression.

Recently, a second compound GSK2850163 (Figure 4C) was examined [22]. GSK2850163 is both an IRE1 RNase and Kinase inhibitor (Table 1). Like the STF-080310 study, Thapsigargin was also used to induce ER stress pre-GSK2850163 treatment. After GSK2850163 administration, Thapsigargin-induced ER stress effects were reversed at both 20 nM and 200 nM doses, decreasing MCF-7(R) cell viability (Figure 5D). As expected, GSK2850163 is more potent at 200 nM, reducing MCF-7(R) proliferation rate. A greater reduction in tumor growth was seen in co-treatment with Tamoxifen. This combined therapy managed to sensitise MCF-7(R) cells and reduce tumorigenesis and metastasis potential more than single GSK2850163 therapy.

SAL003

HER2 BC patients show overexpression in HER2 receptors, often indicated by poor clinical outcomes. In 1998, Trastuzumab exhibited a promising HER2 therapy. Trastuzumab is an antibody targeting HER2 receptors, inhibiting HER2-mediated signaling [45]. However, clinical reports showed two-thirds of HER2 patients were Trastuzumab unresponsive, leading to Trastuzumab-resistant HER2 cells. Hence, multiple alternatives were explored including using a salubrinal derivative, SAL003 (Figure 4D) [35]. SAL003 inhibits elf2α-specific phosphatase and permits continuous PERK activation and elf2α expression (Table 1). SAL003 study in HER2 Trastuzumab-resistant cell, (BT474-(R)) showed an increase in elf2α-p, ATF4, and P21$^{\wedge CIP1}$ levels. In a separate assay, immunohistochemistry staining of BT474-(R) biopsies revealed that Trastuzumab therapy increases elf2a-p levels. Upon co-treatments of SAL003 with Trastuzumab, elf2α-p, ATF4, and P21$^{\wedge CIP1}$ levels were further recorded to rise, exhibiting strong anti-proliferative abilities. Aforementioned, enhancement in ATF4 production decreases cell viability by triggering G1-S cell-cycle arrest. Specific to HER2 cells, PERK activation ensures continuous elf2α-p, inducing P21$^{\wedge CIP1}$ expression, blocking CDK enzyme activity, and stimulate apoptosis.

ISRIB

For a long time, Bortezomib was an effective anti-endocrine therapy until Bortezomib-resistant BCs arose [46]. Bortezomib is a proteasome inhibitor, preventing pro-apoptotic factors degradation [46-47]. Consequently, unfolded proteins accumulate, activating caspase-induced apoptosis. Since Bortezomib is not a hormonal therapy, it encourages the possibility of administering Bortezomib in multiple BC cell lines including TNBCs. However, BCs are prone to Bortezomib resistance. Therapeutic advances to overcome Bortezomib resistance were difficult as its resistance mechanism is unknown [46]. That is until ISRIB was developed (Figure 4E). ISRIB is a PERK inhibitor creating allosteric antagonising inhibition of elf2α-p on eIF2B (Table 1) [48-49]. *In vitro* binding of either ISRIB or elf2B, discourages the other to bind to elf2α-p, altering elf2α's structure. Hence, ISRIB's efficacy depends on elf2α-p's structure [50]. ISRIB's ability to inhibit the PERK pathway correlates with elf2α-p level: affecting only toxic chronic PERK activation with low elf2α-p levels without perturbing strong acute PERK activity with high elf2α-p levels [51]. At high elf2α-p levels, active eIF2B gets sequestered, abolishing ISRIB's anti-BC effect. *In vivo*, ISRIB has no open side effects [52]. This suggests ISRIB as a promising therapeutic candidate for BC therapy.

A study of ISRIB's efficacy on MCF7 (EsR+) and MDA-MB-453 (TNBC) BC cell lines was examined [53]. This study selected insensitive BC cells to Bortezomib based on their morphology and <40% decrease in BC cell viability on 5 nM and 100 nM post-Bortezomib treatment respectively. The SUnSET assay revealed

that PERK activation is a common BC response to Bortezomib therapy despite its insensitivity. Upon combinational therapy with ISRIB, both PERK and elf2α phosphorylation levels were increased. Interestingly, western blot analysis on MDA-MB-435 cells showed that PARP was not cleaved (Figure 5E). However, both ATF4 and CHOP levels were diminished. By contrast, dose-dependent ISRIB + Bortezomib-treated BC cells exhibit enhanced cell death. Aforementioned, caspase-3-induced apoptosis by PARP cleavage can not be concluded. Furthermore, western blot analysis was not carried out on MCF7 cells. Hence, similar deductions on PARP, ATF4, and CHOP levels can not be made for MCF7 cells. Further fluorescence assay using YFP-ER and MitoTracker-Red (MTR) staining of mitochondria with confocal microscopy, suggests enhanced paraptosis (see glossary) in ISRIB-Bortezomib therapy, explaining enhanced cell death observed. This study showed that ISRIB sensitises Bortezomib-resistant cells to Bortezomib's cytotoxic effects by increasing translation to enhance proteotoxic stress and inducing paraptosis.

Can UPR-targeting drugs be administered in single breast cancer treatment?

CCT020312

Currently, given its lack of responses to both hormonal and targeted therapies, chemotherapy has always been administered to TNBCs [4]. However, a novel study on CCT020312 revealed a promising single-TNBC treatment via PERK-targeting. CCT020312 (Figure 4F), a selective PERK activator was shown to induce G1 cell-cycle arrest and apoptosis of both TNBC cell lines: MDA-MB-453 and CAL-148 in a dose-dependent manner (Table 1) [54]. PERK activation is closely linked to cell cycle stage progression [21][27]. As previously mentioned, PERK activation leads to dormancy in TNBC cells as indicated by the decrease in CDK4/6, cyclin-D1, and Bcl-2 levels upon CCT020312 treatment analysed by western blot (Figure 5F) [54]. Interestingly, CCT020312 treatment also showed increases in apoptosis indicators: CHOP, PARP cleavage, and Bax levels. The underlying reasons for this shift in cell fate remain obscure. Furthermore, CCT020312 managed to hinder the AKT/mTOR pathway which plays a critical role in tumor progression upon PERK activation, indicating a negative correlation [54-57]. Therefore, synergistic effects of both PERK activation and AKT/mTOR pathway inhibition further decrease tumor growth, increasing the anti-BC potency of CCT020312 treatment.

Although convincing, CCT020312 experimental anti-TNBC effects were only compared between 'Triple Negative A' TNBC subtypes: MDA-MB-231 and CAL-148 cells [58]. This ensures similar outcomes, preventing accurate

Outstanding Questions

In MDA-MB-231 cells an increase in $P21^{\wedge CIP1}$, decreases Cyclin-D1 which induced BC dormancy. However, the CCT020312 study showed induced apoptosis in MDA-MB-453 cells. Do different TNBC subtypes result in a different cell fate upon PERK activation to favor BC anti-carcinogenesis effect? If yes, what components are critical? Can future PERK-targeting drugs be developed to increase their specificity by targeting these components?

Does GSK2850163 have higher efficacy than STF-080310?

UPR is a complex mechanism with multiple downstream pathways, affecting various cellular signaling transduction. Thus, the development of UPR-targeting inhibitors in BC therapy is dangerous if left unregulated. Are there any precautions or procedures pre or post-UPR drug administration to ensure that anti-BC UPR drug efficacy only lasts during breast cancer treatment? What measures can be taken to ensure UPR-targeting drugs in commercial use are highly specific to highly proliferating BC cells, unaffecting normal healthy breast cells?

Combination therapies using UPR-targeting drugs are used to counter the chemoresistance effect of existing chemotherapies. In worse BC cases with high tendencies of relapse cases to emerge upon existing chemotherapies administration, can PERK-targeting drugs be used synergistically with IRE1-targeting drugs to enhance their anti-BC carcinogenesis functions? Will this cause an overdose or have better efficacy?

deductions to be made on CCT020312 anti-BC drug efficacy on all TNBC cell lines.

UPR inhibitors	Pharmacological Target	Administration in Breast Cancer Therapy
MKC8866	IRE1 inhibitor (IRE1 RNase activity) [13]	Combined Therapy with Paclitaxel [13]
STF-080310	IRE1 inhibitor (IRE1 RNase activity) [23]	Combined Therapy with Tamoxifen [23]
GSK2850163	IRE1 inhibitor (IRE1 RNase + Kinase activities) [22]	Combined Therapy with Tamoxifen [22]
SAL003	PERK activator (prevents elf2α-p dephosphorylation) [35]	Combined Therapy with Trastuzumab [35]
ISRIB	PERK inhibitor (prevents elf2α-p inhibition on translation) [53]	Combined Therapy with Bortezomib [53]
CCT020312	PERK activator (enhance apoptosis) [54]	Single Therapy [54]

Table 1. Summary of UPR-targeting drugs with their therapeutic target and administration in Breast Cancer.

Concluding Remarks

BC, a widely researched disease with a high mortality rate and prevalence has urged the need for a highly potent and efficacy anti-BC therapy [59]. Existing chemotherapies have developed tumor resistance, providing complications as relapses arise from poor pharmacodynamics (see glossary). Thus, multiple inhibitors targeting signaling pathways including UPR-targeting inhibitors were developed to overcome tumor relapses or an alternative to BC single therapy. UPR is a highly complex pathway, closely linked to cell proliferation and fate. UPR modulation effects vary in different diseases, having either pro or anti-carcinogenesis functions. Hence, UPR-targeting drugs should have high specificity with known pharmacodynamics.

Specific to BC, UPR modulation by PERK-targeting drugs exhibits a more promising BC therapy than IRE1-targeting drugs. PERK-targeting drugs showed higher BC therapeutic coverage, effectively treating multiple BC cell lines. Moreover, PERK-targeting drugs were seen to induce cell death and have better efficacy than IRE1-targeting drugs: lesser relapses were recorded in CCT020312 than in MKC8866 compounds, both treating TNBC cells. Thus, it is essential to have further studies on IRE1-targeting drug relapse mechanisms in drug development prior to its administration in BC therapy.

Currently, it remains unclear why certain PERK-targeting drug like CCT020312 is more potent and can be administered as a single BC therapy, unlike ISRIB and SAL003. In the future, it is interesting to uncover the mechanisms behind their pharmacodynamics to improve the potency of anti-BC drugs. More clinical trials should be carried out involving UPR-targeting drugs in multiple BC cell lines and subtypes. For example, more CCT020312 testing should cover other TNBC subtypes 'see Outstanding Questions'. Compared to TNBC and EsR+, studies on UPR-targeting HER2 BCs tend to be discounted. It is unknown if ISRIB can be administered in HER2 therapy like TNBC and EsR+ therapies. Further ISRIB research, depending on its efficacy in anti-HER2 therapy, may mark an outstanding achievement in the race to discover a highly potent anti-BC drug effectively targeting all BC cell lines while counteracting chemoresistance.

Acknowledgement

I would like to thank Dr. Anastasia Zhuravleva for her guidance.

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