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Advances in Small Molecule Drug Discovery for Triple-Negative Breast Cancer

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ABSTRACT

Triple-negative breast cancer (TNBC) is a subtype of poor prognosis, highly invasive and difficult-to-treat breast cancers accounting for around 15% of clinical cases. Given the poor outlook and lack of sustained response to conventional therapies, TNBC has been the subject of intense studies on new therapeutic approaches in recent years. The development of targeted cancer therapies, often in combination with established chemotherapy, has been applied to a number of new clinical studies in this setting in recent years. This review will highlight recent therapeutic advances in TNBC, focusing on small molecule drugs and their associated biological mechanisms of action, and offering the possibility of improved prospects for this patient group in the near future.

KEY TERMS:

Triple negative breast cancer

Triple negative breast cancer is a heterogeneous disease that accounts for approximately 15-20% of all breast cancers. The disease is characterised by the lack of expression of oestrogen and progesterone receptors (ER/PR), and lack of human epidermal growth factor 2 (HER2) amplification. TNBC is associated with higher relapse and lower survival rates.

Cytotoxic chemotherapy

Cytotoxic chemotherapy drugs are compounds that primarily target DNA and cellular replication processes, causing cell death within proliferating cell (i.e. those progressing through active cell cycle). Although these compounds have a broad spectrum of activity in numerous malignancies, they are non-specific; consequently the major drawbacks of chemotherapy are dose limiting toxicological side effects and drug resistance.

Tyrosine kinase inhibitors

TKIs are small molecule inhibitors, which target one or more components of receptor tyrosine kinases (RTKs), normally located at the cell surface. Inhibition of RTKs is characterised by deregulation in the signal transduction pathways involved in key cellular regulatory processes, such as proliferation, differentiation, cell survival and metabolism, cell migration, and cell cycle control.

PARP inhibitors

PARP inhibitors are small molecule inhibitors of the DNA repair enzyme, poly(ADP-ribose) polymerase (PARP). Inhibition of PARP is characterised by multiple double strand DNA breaks, which cannot be repaired in tumour cells with BRCA1/2 mutations, thus resulting in efficient and selective cell death.

Heat shock protein family

Heat shock protein family are a diverse group of molecular chaperone proteins that interact with unfolded, aggregated or misfolded proteins to prevent cell damage. They are also thought to be involved many other cellular processes, including cell proliferation, and cell survival and death.

INTRODUCTION

Triple negative breast cancer (TNBC) is a subtype used to characterize invasive breast cancers that lack expression of the oestrogen and progesterone receptor (ER/PR) and HER2 [1]. Clinical surveys reveal that approximately 15% of all breast cancers are diagnosed as TNBC, occurring more frequently among younger women (<40 years old) and more common in black women compared to Caucasian women [2]. TNBC is associated with a poor disease prognosis, high risk of recurrence and a worse disease-free survival [2]. The median survival of patients with metastatic TNBC is only 13 months and virtually all women with metastatic TNBC ultimately die of their disease despite systemic therapy. TNBC tumours are associated

with a high histological grade and an increased risk of distant recurrence to develop visceral metastasis early in the course of their disease [2].

The development of gene array profiling has allowed for the classification of breast cancer into several subtypes based on distinctive gene expression signatures [3]. One such subtype includes basal-like breast cancer which shows a high expression of characteristic basal epithelial proteins which include cytokeratin 5 and 6 (CK5/6), CK14, CK17, P-cadherin, p53 mutations, epidermal growth factor receptor (EGFR) and α B-crystallin [4,5]. Although the terms “triple negative” and “basal-like” are not synonymous, most (80%) basal-like breast cancers do not express ER, PR receptors and HER2 [6]. Since the hormone receptors and HER2 are central to the biologic variance among breast cancers, clinicians tend to categorise TNBC by routine immunohistochemical staining as a surrogate profiling for the basal-like breast cancer in the clinical settings. Further gene expression analysis has identified six distinct TNBC subtypes, including two basal-like (BL1 and BL2), immunomodulatory, mesenchymal (M), mesenchymal stem like (MSL) and luminal androgen receptor (LAR) [6,7]. Different TNBC subtypes exhibit unique biology and tend to present distinct responses to a given therapy. Even so, distinguishing one TNBC subtype from another can be a challenge at clinical histologic examination and therefore it is inappropriate to treat all TNBCs as a single entity. Additionally, triple negative breast cancers are characterized by a wide spectrum of genomic alterations and instability, some of which are the result of DNA repair defects such as homologous recombination, discussed in more detail below with reference to the use of PARP inhibitors in this setting. Studies of TNBC gene signature and their different response to therapeutic intervention are an active area of study that will inform future biomarker and drug target discovery.

TNBC patients do not benefit from hormonal or trastuzumab-based therapy because of the loss of target receptors such as ER, PR and HER2 [8]. Surgery and/or cytotoxic chemotherapy remains the standard course of TNBC treatment despite the lack of long-term effectiveness [9]. These factors make treatment options for TNBC particularly problematic, and the development of new and improved therapeutics for TNBC as one of the highest priorities of current breast cancer research. Recent studies highlight the important roles of certain proteins such as EGFR (expressed by 66% of TNBC), c-Kit and α B-crystallin in the progression of TNBC and suggest potential targets for new therapeutic drugs (Figure 1).

This review provides a selective overview of recent developments in therapeutic approaches to the treatment of TNBC. The focus of the review is firmly on small molecule drugs and drug candidates; important developments in antibody-based therapies are not covered here except for passing mention as part of combination therapy. In addition, our selective coverage mainly focuses on approaches that have progressed to at least pre-clinical

development in the setting of TNBC. Early stage molecules acting on relevant targets within the *in vitro* context receive only minimal focus here.

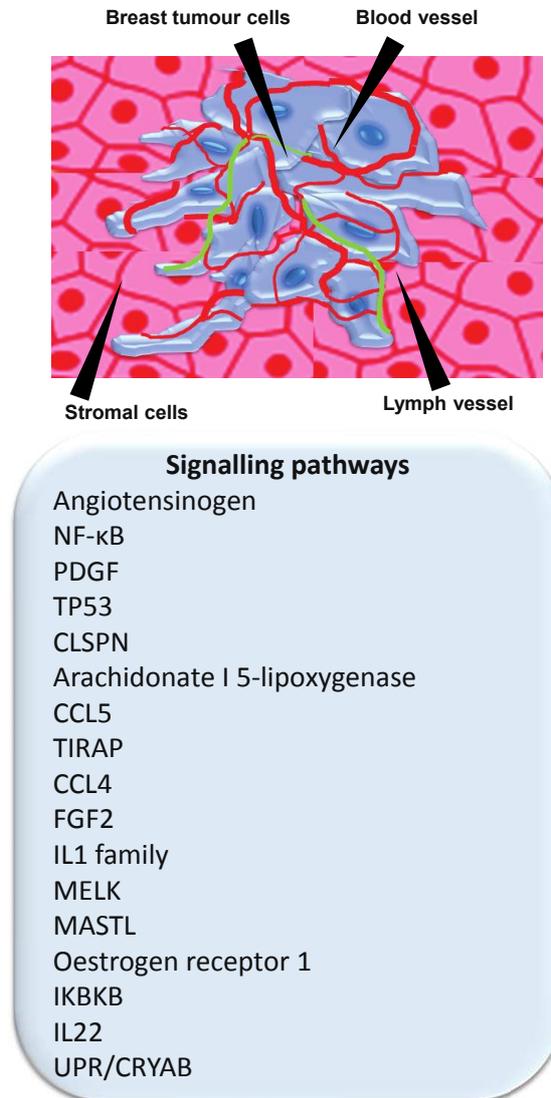


Figure 1: The importance of the TNBC microenvironment (*top*) and potential targets for new therapeutic drugs against TNBC (*bottom*).

CURRENT THERAPY AGAINST TNBC

Systemic cytotoxic chemotherapy

Therapy of TNBC is based on surgery, radiotherapy, and chemotherapy, because currently there are no targeted treatment options available [10]. Combination cytotoxic chemotherapy, administered in a dose-intensive or metronomic regimen used as an adjuvant or neoadjuvant therapy, remains the standard treatment for early-stage TNBC. The most common cytotoxic agents are a combination of taxanes, anthracyclines and cyclophosphamide. Although TNBC

has a high recurrence rate, it has a better initial response to conventional chemotherapy than breast cancers that are hormone-receptor positive [11,12].

Platinum-based chemotherapy (PBC)

Platinum agents are one of the established drug classes that are finding new applications in the treatment of TNBC. Platinum-based compounds are DNA interacting agents which lead to DNA cross-link strand breaks resulting in impairment of DNA repair/synthesis. Tumours with BRCA 1/2 mutations, including the majority of TNBC, have deficient double-stranded DNA break repair which leads to an increased sensitivity to chemotherapeutic agents that cause DNA damage [13].

Recently, in the GeparSixto trial (Phase II), the addition of neoadjuvant carboplatin to a regimen consisting of taxane-anthracycline chemotherapy and targeted therapy significantly increases pathological clinical response in patients with stage II or III triple negative breast cancer [14]. Sikov and collaborators have studied the addition of other drugs such as carboplatin and/or bevacizumab in a neoadjuvant chemotherapy regimen to sequential taxane-anthracycline in a Phase II trial [15]. The results indicate that pCR breast rates were higher with the addition of carboplatin (60% vs 44%; $P=0.018$) or bevacizumab (59% vs 48%; $P=0.0089$), whereas only carboplatin (54% vs 41%; $P=0.0029$) significantly raised pCR breast/axilla in both clinical stage II and III TNBC. Taking into account the studies mentioned above, the potential of carboplatin is evident when used as a new treatment option for patients with TNBC. Another interesting trial is the comparison of cisplatin vs carboplatin with docetaxel neoadjuvant therapy in 144 patients with TNBC [16]. The cisplatin-based regimens were superior to the carboplatin-based regimens in terms of overall and progression free survival. It was concluded that the treatment with cisplatin/docetaxel was well tolerated and a potentially effective therapy in locally advanced TNBC.

There are two Phase III trials in progress evaluating the benefit of platinum-based chemotherapy versus standard chemotherapy in TNBC patients. One is comparing carboplatin versus docetaxel (NCT00532727), and the other contrasting the use of gemcitabine/cisplatin versus gemcitabine/paclitaxel (NCT01287624) [17].

DNA Repair Pathways

DNA-repair mechanisms play a crucial role in maintaining the integrity of DNA. There are numerous different DNA repair pathways, including non-homologous end joining, homologous recombination, mismatch repair, nucleotide excision repair and base excision repair. Deregulation of DNA-repair mechanisms is associated with the development of

cancer, most notably in breast tumours with mutations BRCA1 and BRCA2 genes, a concept known as synthetic lethality

PARP inhibitors

Pharmacological inhibition of poly(ADP-ribose) polymerase (PARP), an enzyme which regulates the DNA base-excision repair pathway to repair single-strand breaks (SSBs), has emerged as an exciting therapeutic target for TNBC [18]. Mutations in the breast cancer susceptibility genes known as BRCA1 and BRCA2, that code for tumour suppressor proteins involved in DNA repair, account for around 5-10 % of all breast cancers and around 15% of ovarian cancers. Pioneering studies have demonstrated that BRCA-deficiency dramatically and selectively sensitizes tumours to the effects of PARP inhibition due to the inability to effect DNA repair in these cells, a concept known in biology as synthetic lethality [19]. It has been established that triple-negative tumours are likely to have a deficiency in BRCA1/2 and therefore be more susceptible to the targeting of DNA repair machinery through PARP inhibition.

Iniparib (BSI-201) (**1**, Table 1) was one of the first anticancer PARP inhibitors described in preclinical models. In a Phase II study it has been shown that the addition of iniparib to gemcitabine and carboplatin significantly improved all measures of clinical efficacy in metastatic TNBC, including overall survival (OS), progression-free survival (PFS), and the rate of objective complete or partial response [20]. However, in a Phase III clinical trial with the same treatment combination among 516 patients with TNBC, the results suggest that iniparib did not meet the criteria for significance for co-primary endpoints of OS and PFS [21]. Disappointingly, iniparib was discontinued due to loss of efficacy and associated toxicity in Phase III clinical trials [22,23]. Furthermore, recent studies suggest that iniparib may not actually inhibit the PARP enzyme [24,25].

The most important PARP inhibitor studied in TNBC to date is olaparib (AZD2281), a drug also registered for the treatment of ovarian cancer (**2**, Table 1). In recent years, Phase I/II clinical trials have shown that PARP inhibition by olaparib in breast cancer is confined to BRCA-mutated breast cancer, including TNBC [26,27]. In a recent Phase I trial, 28 patients (8 with TNBC) have been treated with olaparib in combination with the anti-angiogenic drug, cediranib and the results for TNBC patients showed limited clinical activity [28]. The combination of olaparib and weekly paclitaxel is being evaluated in an ongoing Phase I trial but the effectiveness of this treatment has not been determined due to a significant clinical interaction [29]. Ongoing trials are being held for olaparib combined with other chemotherapeutic agents (see Supplementary Information).

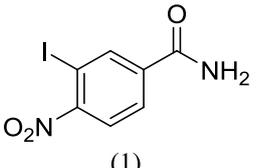
Although there are few papers supporting the PARP inhibitor veliparib (ABT-888) (**3**, Table 1) as a promising treatment in combination with standard chemotherapy in TNBC,

there are a large number of trials being carried out in the last few years. Significantly, positive data was recently observed in the ISPY-2 trial looking at the combination of veliparib and carboplatin plus standard chemotherapy in neoadjuvant settings in TNBC. The trial found that patients who received the combination of veliparib and carboplatin combination plus standard chemotherapy were more likely to attain pathologic complete response (52%) compared chemotherapy alone (26%) [30]. A Phase III clinical trial (NCT02032277) is currently recruiting participants for the above treatment combination, and there is a high probability that this study will generate successful results.

One relevant paper that evaluates *in vitro* activity of four PARP inhibitors suggests that rucaparib (**4**, Table 1) is the most cytotoxic compound in three TNBC cell lines tested. These PARP inhibitors exhibited differential antitumour activities, with the relative potencies of rucaparib > olaparib > veliparib > iniparib [31]. Comparing the efficacy of iniparib against olaparib in seven TNBC cell lines it was concluded that olaparib, in contrast to iniparib, is a strong inhibitor of breast cancer cell growth and may have efficacy in TNBC [32]. Recently, E7449 (**5**, Table 1), a novel orally bioavailable small molecule PARP inhibitor has been tested in TNBC as either a monotherapy or in combination with other anticancer therapies. E7449 inhibits both PARP 1 and PARP 2 with IC₅₀ values of 1.0 and 1.2 nM, respectively. Additionally, E7449 showed dose-dependent selective inhibition of PARP activity and a potent antitumour activity against BRCA-deficient breast cancer cell line in *in vivo* models, with no observed toxicity [33]. Furthermore, E7449 in combination with eribulin or carboplatin in several TNBC xenograft models showed a significant increase in antitumour activity in the MDA-MB-468 subtype of TNBC [34]. A Phase I/II trial of E7449 as a single agent or in combination with chemotherapy drugs in advanced solid tumours including TNBC is ongoing (Table 1).

Table 1 shows the chemical structures of PARP inhibitors under comparative clinical investigation in TNBC. Table 2 (Supplementary Information) gives further information on combination study clinical trials that are being carried out with PARP inhibitors in TNBC.

Table 1: Chemical structures and comparative study of PARP inhibitors in clinical trials for TNBC.

PARP inhibitors	Molecular Structure	Study design	Drugs	Clinical trial identifier	Ref
Iniparib (BSI-201)	 <p>(1)</p>	Phase III	Iniparib + gemcitabine and carboplatin	NCT00938652	[22,35]

Olaparib (AZD2281)	<p>(2)</p>	Phase I	Olaparib + BKM120/BYL719	NCT01623349	[29,36]
		Phase I	Olaparib	NCT02227082	
		Phase I	Olaparib + Carboplatin and/or Paclitaxel	NCT00516724	
		Phase I	Olaparib + Carboplatin	NCT01445418	
Veliparib (ABT-888)	<p>(3)</p>	N/A	Veliparib + Lapatinib	NCT02158507	[37,38]
		Phase I	Veliparib + Cisplatin and Vinorelbine ditartrate	NCT01104259	
		Phase II	Veliparib + Carboplatin and standard chemotherapy	NCT01818063	
		Phase III	Veliparib + Carboplatin and/or standard chemotherapy	NCT02032277	
		Phase II	Veliparib + Cyclophosphamide	NCT01306032	
Rucaparib (AG014699)	<p>(4)</p>	Phase II	Rucaparib + Cisplatin	NCT01074970	[39]
E7449	<p>(5)</p>	Phase I/II	E7449 alone, E7449 + Temozolomide or Carboplatin and Paclitaxel	NCT01618136	[33]

Targeting molecular pathways in TNBC

Receptor-tyrosine kinases (RTKs) are cell surface receptors, many of which are key regulators of critical cellular processes, such as cell proliferation and differentiation, cell

survival and metabolism, cell migration, and cell cycle control. Deregulation of RTKs is prevalent in many cancers. In basal-like cancers (the major subtype of TNBC), amplification of several components of RTKs have been observed including PIK3CA, KRAS, EGFR, FGFR, IGFR, MET, and PDGFRA, to name but a few. Thus, there is scope for the development of small molecule kinase inhibitors that block or attenuate RTK activity to target TNBC [40-42]. Figure 2 summarises the major kinase-based signalling pathways discussed with respect to treatment of TNBC using small molecule inhibitors.

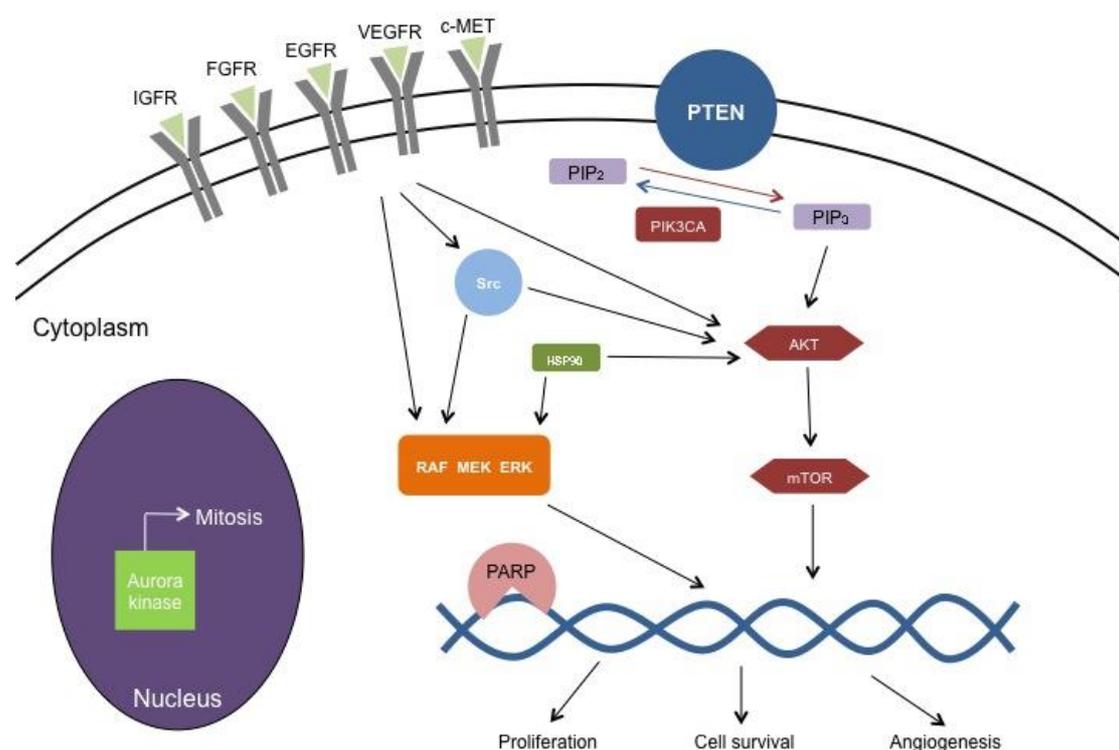


Figure 2: Major signaling pathways relevant to the development and progression of TNBC

Small molecule Tyrosine Kinase Inhibitors (TKIs)

EGFR inhibitors

The epidermal growth factor receptor (EGFR) and its downstream signalling pathway is important for regulating cell growth, survival, and apoptosis. Many cancers have been shown to convey deregulation of the EGFR-mediated signalling by distinct molecular mechanisms, such as over-expression, acquired mutations of the receptor, and activation induced by ligands [43,44]. Approximately 60% of all basal-like breast cancers, which is the major subtype of TNBC tumours over-express EGFR [42,45-49]. This high expression of EGFR has been shown to be a negative prognosis factor in TNBC, thus EGFR is considered to be a potential therapeutic target against TNBC [50]. Many EGFR inhibitors have since been clinically investigated against TNBC.

undergoing a Phase I/II trial in combination with the mTOR inhibitor, temsirolimus in patients with metastatic HER2-amplified or TNBC (NCT01111825). The results from a Phase I trial showed the combination was well tolerated with a response rate (RR) of 67% [60]. However, lapatinib showed a lack of activity in combination with paclitaxel in patients with advanced TNBC [61]. Recently, lapatinib was shown to elicit activation of nuclear factor (NF)- κ B to sensitise TNBC cell lines to proteasome inhibitors. This result suggest that a combination therapy of a proteasome inhibitor with lapatinib may be beneficial to TNBC patients [62]. In a recent preclinical study, Tao et al also showed that the combination of a dual EGFR and HER3 inhibitor, MEHD7945A with either ipatasertib (AKT inhibitor) or GDC-0941 (PI3K inhibitor) inhibited the growth of xenografts derived from TNBC patient tumours [63]. From these studies, it appears that EFGR inhibition alone is not effective in targeting TNBC, therefore a likely scenario will be a combination therapeutic strategy comprising of different components of RTK pathways.

VEGFR inhibitors

Angiogenesis is the development of new blood vessels from existing vasculature. This process is regulated by vascular endothelial growth factor (VEGF) and VEGF receptors (VEGFR), and it is essential in early stage tumourigenesis and subsequently, metastasis. TNBC is a highly vascularised disease which correlates with high levels of intratumoural VEGF. The high levels of VEGF is a negative prognostic factor in TNBC, providing the foundation for clinically evaluating VEGFR inhibitors [64].

Sunitinib (**10**, Figure 4) is a multi-targeted TKI, which potently inhibits VEGFR-1/2/3, PDGFR and KIT. It is an orally bioavailable pyrrole substituted 2-indolinone inhibitor approved by the FDA in 2006 for the treatment of renal cell carcinoma [65]. Sunitinib was evaluated in a Phase II multicentric trial in patients with MBC formerly treated with anthracyclines and taxanes. Seven patients achieved a partial response (median duration, 19 weeks), giving an ORR of 11%. Interestingly, a RR of 15% was observed in patients with metastatic TNBC [66]. Moreover, a randomised open-label Phase II study constructed to evaluate the efficacy of sunitinib monotherapy with that of single-agent standard-of-care (SOC) chemotherapy in patients with previously treated advanced TNBC, found that mean PFS with sunitinib was 2.0 months, compared with 2.7 months with SOC chemotherapy. Furthermore, the mOS was not prolonged with sunitinib compared with SOC (9.4 months compared with 10.5 months, respectively) [67]. Sunitinib has also been assessed in combination with first-and second-line chemotherapy, with docetaxel and capecitabine, respectively, in two large Phase II trials in patients with HER2-negative MBC. However, neither trial observed any benefit pertaining to the combination therapies [68]. Results are

awaited for a neoadjuvant Phase I/II trial examining the combination of sunitinib with paclitaxel and carboplatin in TNBC (NCT00887575).

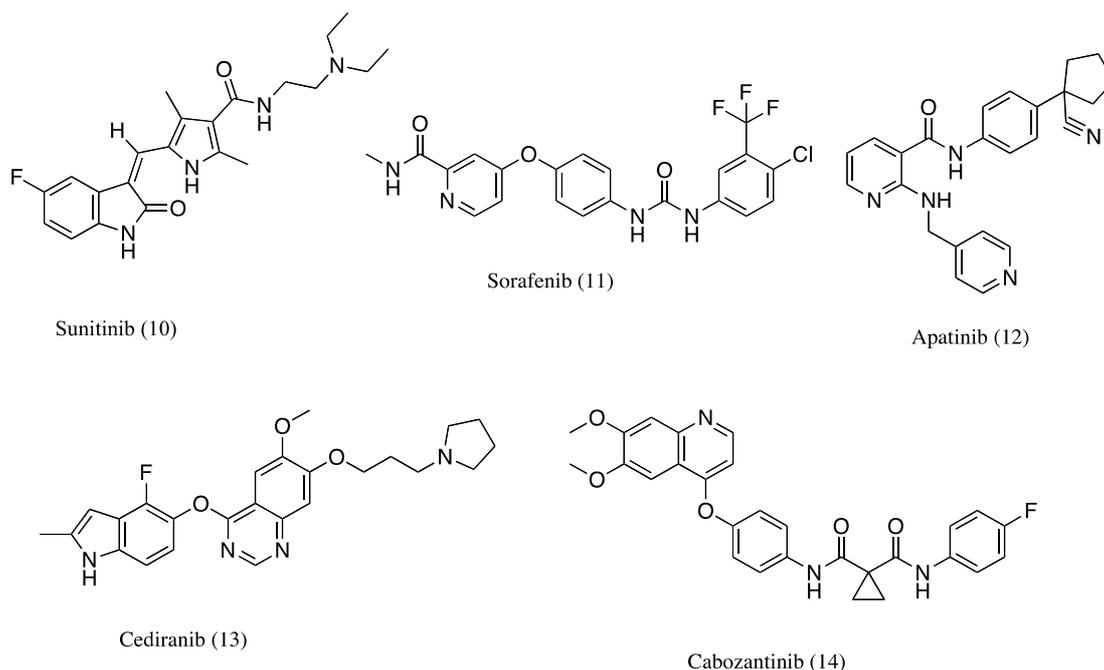


Figure 4: Chemical structures of small molecule VEGFR inhibitors tested in TNBC

Sorafenib (**11**, Figure 4) is another multi-targeted TKI, which exhibits anti-proliferative and anti-angiogenic activity by inhibiting VEGFR-1/2/3 and Raf [59]. It is an orally available biarylurea inhibitor first approved for the treatment of hepatocellular carcinoma (HCC). As monotherapy for patients with MBC, sorafenib showed modest activity, with patients demonstrating 2% RR and 13% SD at 6 months [65]. However, a series of four randomized, double-blind, placebo-controlled Phase IIb trials, known as Trials to Investigate the Efficacy of Sorafenib (TIES) evaluating the effect of the drug in patients with HER-2 negative advanced or metastatic BC, revealed the therapeutic potential of sorafenib in combination with selected chemotherapies. The studies also concluded that Phase III trials are necessary for confirmatory purposes [66,69]. Recruitment is currently underway for a neoadjuvant Phase II trial involving sorafenib in combination with cisplatin followed by paclitaxel for patients with early stage TNBC (NCT01194869).

Apatinib (**12**, Figure 4) is a highly potent, orally available TKI selective inhibitor of VEGFR2. It is currently undergoing a Phase II trial as monotherapy in patients with MTNBC (NCT01176669). Another quinazolinone derivative TKI, cediranib (**13**, Figure 4) has advanced to Phase II clinical trial in TNBC. It is being evaluated with olaparib in patients with recurrent TNBC (NCT01116648). Cabozantinib (**14**, Figure 4) is a quinoline derivative VEGFR2 and MET inhibitor. A Phase II trial to evaluating its safety and effectiveness in MTNBC is ongoing (NCT01738438).

IGFR inhibitors

The insulin-like growth factor (IGF) signalling pathway is activated in breast cancers, with one of the receptors of this pathway, IGF-IR expressed in approximately 90% of breast cancers. This was found to correlate with poor prognosis in patients with ER+ breast cancer [70,71]. There is evidence that mutations in tumour suppressor genes such as p53 and BRCA1 represses the IGF-IR promoter, leading to elevated IGF-IR levels in TNBC. This evidence established the role of IGF-IR in TNBC and provided a rationale for developing IGF-IR therapies against TNBC. Recently, Litzenburger *et al* examined the sensitivity of TNBC cell lines with IGF gene expression, by reversing the gene expression signature in three different models (cancer cell lines or xenografts) of TNBC, with different anti-IGF-IR therapies. The TNBC cell lines were particularly sensitive to the dual IGF-IR/InsR inhibitor, BMS-754807 (15, Figure 5), and sensitivity correlated to the expression of the IGF gene signature. A combination of the same inhibitor with docetaxel showed growth inhibition and tumour regression that was associated with reduced proliferation, increased apoptosis, and mitotic arrest [72]. These studies support the combination of IGF-IR/InsR and chemotherapy in TNBC patients. Results are presently pending for a Phase I study of BMS-754807 in combination with paclitaxel and carboplatin in patients with advanced or metastatic solid tumours (NCT00793897).

Since IGF-1R signalling through the PI3K pathway is a key regulator for metabolism control, a combination therapy with mTOR and IGF inhibitors has been proposed based on the results of several preclinical studies. In these studies, the combination showed a synergistic effect by disrupting IGF-1R mediated AKT activation mechanism induced by mTOR inhibition [70]. Dual inhibition of IGF-IR and mTOR has also shown improved antitumour activity in some human cancer cell lines including breast cancer [73]. The results of the following trials are expected to demonstrate the benefits of this co-targeting approach; Phase I/II trial of temsirolimus and cixutumumab (NCT00699491), and Phase I trial of everolimus IGF-1R inhibitor AMG479 for patients with advanced solid tumours (NCT01122199).

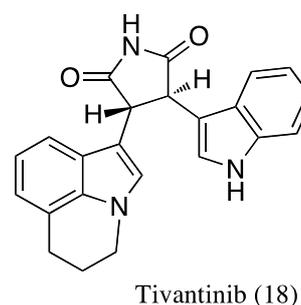
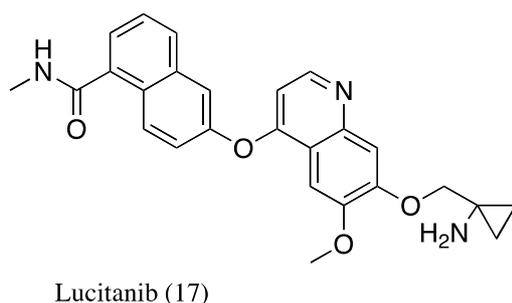
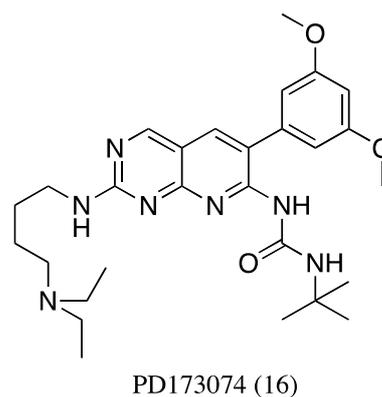
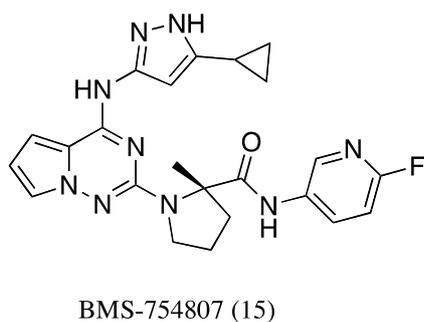


Figure 5: Chemical structures of small molecule inhibitors of IFGR, FGFR, and MET tested in TNBC

FGFR inhibitors

The rationale for targeting fibroblast growth factor receptor (FGFR) is due to the amplification of this receptor in TNBC; approximately 9% and 2-4% of TNBCs show amplification of FGFR1 and FGFR2, respectively [42,48]. In a preclinical study where 56 TNBCs were subjected to high-resolution microarray-based comparative genomic hybridisation (aCGH), cell lines with FGFR were highly sensitive to a dual FGFR/ VEGFR inhibitor PD173074 (**16**, Figure 5), and to RNAi silencing of FGFR2 [74]. A study of 31 breast cancer cell lines by Sharpe *et al.* also showed that TNBC cell lines and other FGF expressing breast cancer cells, were sensitive to PD173074, with 47% of TNBC cell lines showing significant growth reduction [75]. There are currently no selective FGFR inhibitors in clinical testing, however due to the structural similarity between FGFR and VEGFR kinase domains [76], some inhibitors of both receptors are under investigation in TNBC. Lucitanib (**17**, Figure 5) is a potent inhibitor of FGFR1/2/3, VEGFR1/2/3, and PDGFR. A Phase II trial is recruiting patients to participate in the evaluation of lucitanib monotherapy in FGF aberrant metastatic breast cancers, including TNBC (NCT02202746).

MET inhibitors

MET is a cell surface receptor of the growth and motility factor, hepatocyte growth factor/scatter factor (HGF/SF). These play a fundamental role in cancer, including uncontrolled cell survival, growth, angiogenesis and metastasis, thus, providing a clear rationale for targeting this pathway in cancer [77]. An over-expression of MET and HGF have been reported in 46% of breast cancers and it is associated with negative prognosis [70,78]. Gastaldi *et al.* recently shown that constitutive activation of MET facilitated cell commitment towards the basal lineage [79]. Since the major subtype of TNBC is basal-like, MET could potentially play a crucial role in the development of this disease therefore, MET inhibitors could be potential therapeutic targets against TNBC. In a Phase II trial, the MET inhibitor tivantinib (**18**, Figure 5) was well tolerated in patients with MTNBC, however as monotherapy, tivantinib was mainly inactive [80]. As mentioned in a previous section, cabozantinib (**9**, Figure 4), a VEGFR2 and MET inhibitor, is under evaluation in patients with MTNBC (NCT01738438). Interestingly, recent reports suggest concomitant targeting of MET and EGFR pathways could have a beneficial effect in TNBC. This hypothesis was based on preclinical studies, which showed that dual inhibition of MET and EGFR produces a synergistic effect in TNBC cell lines [78,81].

PI3K/AKT/mTOR pathway inhibitors

The phosphoinositide 3-kinase (PI3K) / protein kinase B (AKT) / mammalian target of rapamycin (mTOR) signalling pathway is associated with cell cycle regulation, survival, and proliferation [73,82-86]. This pathway is highly significant in breast cancer because it represents the most frequently mutated pathway. A growing body of evidence has shown that mutation, and/or up-regulation of this pathway affects almost all its downstream molecular components, resulting in resistance and disease progression. Across all TNBC subtypes, there is an elevated frequency of aberration in PI3K, p53, and PTEN (a protein that inhibits activation of AKT/mTOR pathway) [6,42,83,87], making this pathway a desirable target for therapies against TNBC. Furthermore, a recent study by Sohn *et al.* in patients with residual TNBC after standard anthracycline-taxane chemotherapy, showed that several PI3K pathway components were activated; the authors concluded that this pathway may present potential therapeutic targets in this disease [88]. Multiple small molecule targets of this pathway are currently under investigation in TNBC including: PI3K inhibitors, mTOR inhibitors, dual PI3K/mTOR inhibitors, and AKT inhibitors.

PI3K inhibitors

BKM120 (**19**, Figure 6) is an oral pan-PI3K inhibitor, which inhibits all forms of PI3K. A partial response was confirmed in a TNBC patient in a Phase I trial [89]. A study by Juvekar *et al.* showed that a combination of the PARP-inhibitor olaparib and BKM120 produced a

synergistic activity, resulting in a tumour doubling time of over 70 days, compared with 26 and 16 days for BKM120 and olaparib alone, respectively [82,90]. This observation has also been demonstrated in TNBC cell lines without BRCA mutations where, Ibrahim *et al.* proved that PI3K blockage resulted in impairment and sensitisation to PARP inhibition in TNBCs without BRCA mutations, providing a rationale for combined PI3K and PARP inhibitors therapies [91].

Several trials are presently ongoing to evaluate BKM120 in TNBC including; a Phase II trial where BKM120 is administered in combination with paclitaxel in patients with HER2-negative, locally advanced or metastatic BC, with or without PI3K pathway activation (BELLE-4 trial) (NCT01790932). A Phase II trial evaluating BKM120 as monotherapy in patients with MTNBC is also in progress (NCT01790932). Finally, a Phase II trial is looking at BKM120 with capecitabine for TNBC patients with brain metastases (NCT02000882) [73,92].

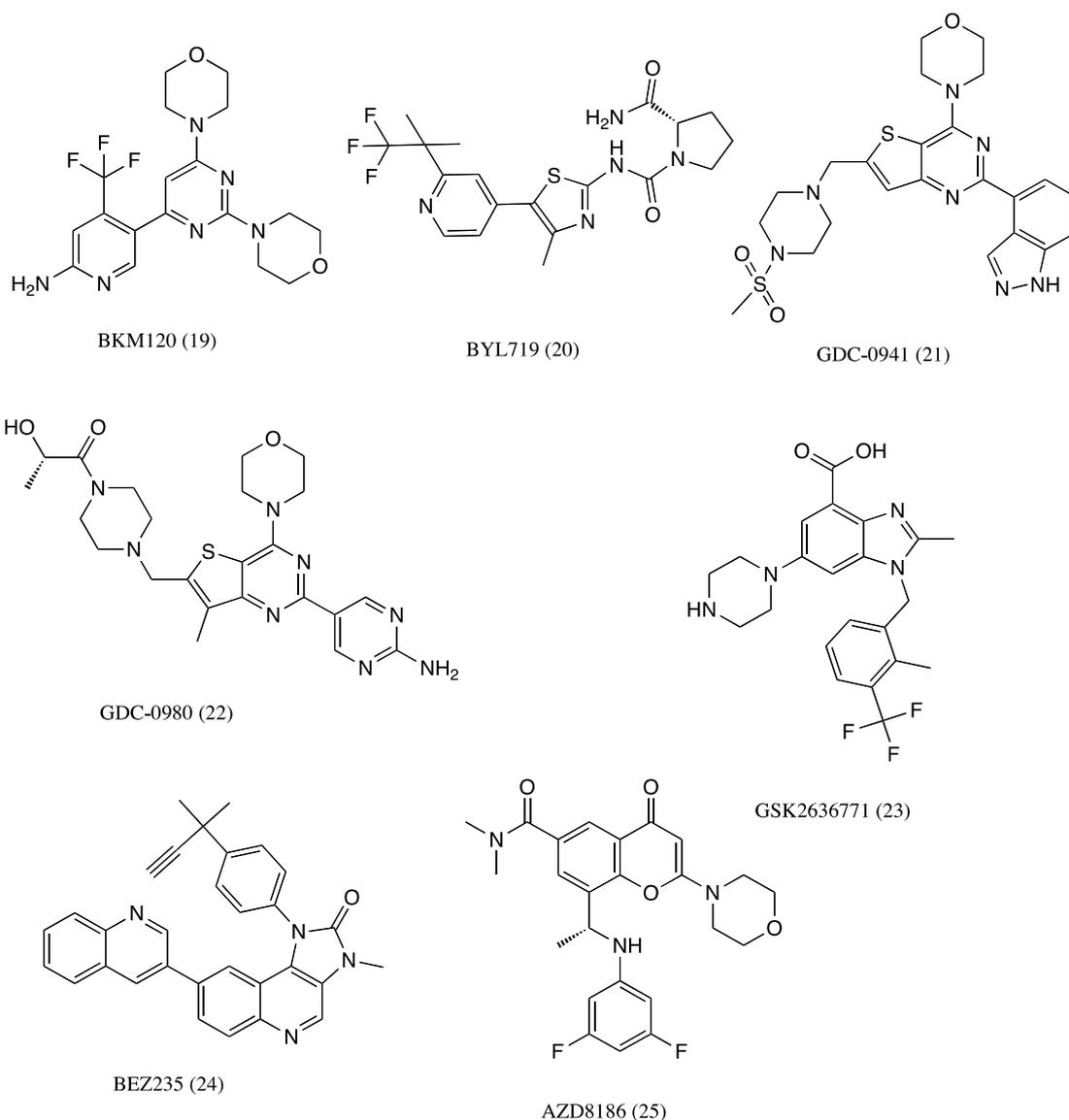


Figure 6: Chemical structures of small molecule PI3K inhibitors tested in TNBC.

BYL719 (**20**, Figure 6) is a 2-aminothiazole-substituted selective PI3K inhibitor. In preclinical studies, BYL719 shown preferential antiproliferative activity against PIK3CA-mutant and/or amplified breast cancer cell lines, and their corresponding tumour xenografts with promising results shown in a Phase I clinical trial [70]. As previously mentioned, BYL719 is under evaluation in combination with olaparib in patients with recurrent TNBC or high-grade serous ovarian cancer (NCT01623349). GDC-0941 (**21**, Figure 6) is another PI3K inhibitor being evaluated in combination with chemotherapy in TNBCs; a Phase IB trial is investigating GDC-0941 in combination with paclitaxel, with or without bevacizumab (NCT00960960). Furthermore, a Phase I/II trial looking at a combination of GDC-0941 and cisplatin in patients with androgen receptor negative TNBC is currently recruiting participants (NCT01918306). Recently, Lehmann *et al* have shown that the combination of GDC-0941 or the dual PI3K/mTOR inhibitor GDC-0980 (**22**, Figure 6) with or with the anti-androgen,

bicalutamide significantly reduced the growth and viability of androgen receptor-positive TNBC. This result provides a rationale for the pre-selection of TNBC patients with AR expression who are less likely to benefit from the current standard of care chemotherapy regimens [93].

Several preclinical studies have demonstrated that certain PTEN-deficient tumours are dependent on p110 β pathway for activation, growth and survival. These findings prompted a new clinical trial with the p110 β -selective inhibitor GSK2636771 (**23**, Figure 6) in patients with PTEN-deficient advanced solid tumours including patients with TNBC tumours (NCT01458067) [87,94]. BEZ235 (**24**, Figure 6) is a competitive dual PI3K/mTOR inhibitor. The rationale for evaluating this drug in TNBCs was based on the fact that BEZ235, exhibited significant antiproliferative and antitumour activity in cancer cells with activating mutations in PI3KCA [73,94,95]. A Phase I/II study of a combination of BEZ235 with the MEK inhibitor MEK162 in different cancer types that also included TNBC was recently concluded and results are awaited (NCT01337765). AZD8186 (**25**, Figure 6) is a novel potent small molecule TKI that selectively targets PI3K β as opposed to PI3K α . *In vivo* studies showed that AZD8186 alone or in combination with docetaxel inhibits PI3K pathway biomarkers in both prostate and TNBC tumours [96]. NCT01884285 is a Phase I clinical trial investigating AZD8186 in patients with advanced castrate-resistant prostate cancer (CRPC), squamous non-small cell lung cancer (sqNSCLC), TNBC, and known PTEN-deficient advanced solid malignancies.

AKT inhibitors

In cancer cells, the main biological consequences of the activation of AKT are survival, proliferation, and growth [84]. AKT is also thought to be involved in the development and progression of breast cancer [82]. The four main AKT inhibitors under investigation in TNBCs are shown in Figure 7.

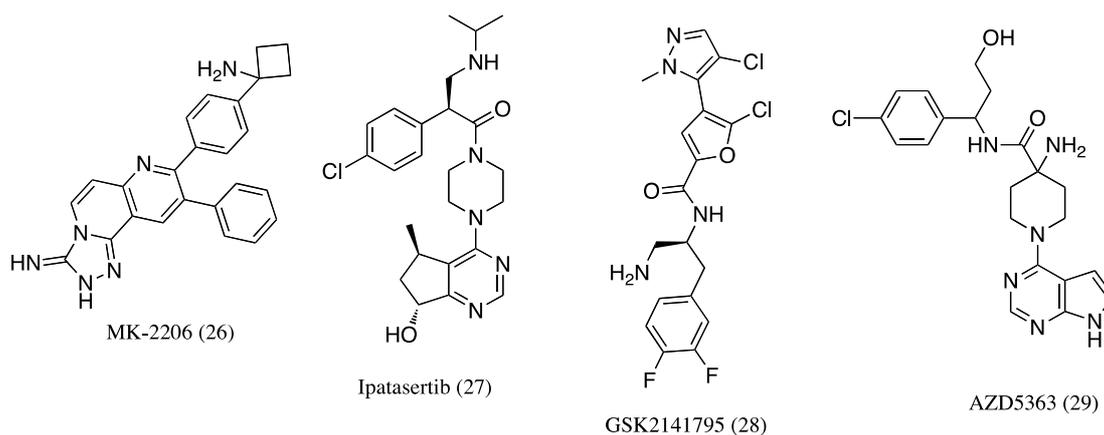


Figure 7: Chemical structures of small molecule AKT inhibitors tested in TNBC.

MK-2206 (**26**, Figure 7) is a highly selective, non-ATP competitive allosteric inhibitor of AKT1/2/3. Preclinical studies revealed MK-2206 was able to inhibit AKT signalling and cell cycle progression, and increased apoptosis in breast cancer cell lines. A significant increase in sensitivity to MK-2206 has been reported in BC cell lines with PTEN or PIK3CA mutations. Finally MK-2206 was shown to have a synergistic effect with paclitaxel, both *in vitro* (cell lines) and *in vivo* (xenograft models) [97]. MK-2206 is currently in a Phase II trial for advanced BC patients with PI3K/AKT mutation or PTEN alterations (NCT01277757). Two randomised Phase II trials are recruiting patients to estimate the efficacy of ipatasertib (**27**, Figure 7), a selective pan-AKT inhibitor, combined with paclitaxel in MTNBC patients (NCT02162719), as well as patients with early stage TNBC (NCT02301988). GSK2141795 (**28**, Figure 7) is an orally bioavailable potent and selective pan-AKT inhibitor; recruitment is ongoing for a Phase II trial in combination with trametinib (MEK inhibitor) in patients with advanced TNBC (NCT01964924).

A pyrrolopyrimidine derived AKT inhibitor, AZD5363 (**29**, Figure 7) is being developed in a Phase II trial with PARP inhibitor olaparib, and mTORC1/2 inhibitor AZD2014 in patients with recurrent endometrial, TNBC, ovarian, primary peritoneal, or fallopian tube cancer (NCT02208375). Additionally, a randomised Phase II placebo-controlled study in combination with paclitaxel in advanced or metastatic TNBC is recruiting patients.

mTOR inhibitors

The mammalian target of rapamycin (mTOR) is an effector of the PI3K pathway regulated by AKT and PTEN. Growth factors and hormones, such as insulin, signal to mTORC1 via AKT to regulate critical cellular processes such as growth, proliferation, transcription, protein synthesis, and ribosomal biogenesis [41,98]. Preclinical studies confirmed that upregulation of mTOR or aberrant PI3K/AKT pathways confer sensitivity to mTOR inhibitors. These

studies suggested that mTOR could be a good target for breast cancer therapy, especially in tumours with AKT activation or loss of PTEN function [49]. Currently, there are three mTOR inhibitors under investigations in TNBCs as either monotherapies or in combination with other drugs (Figure 8).

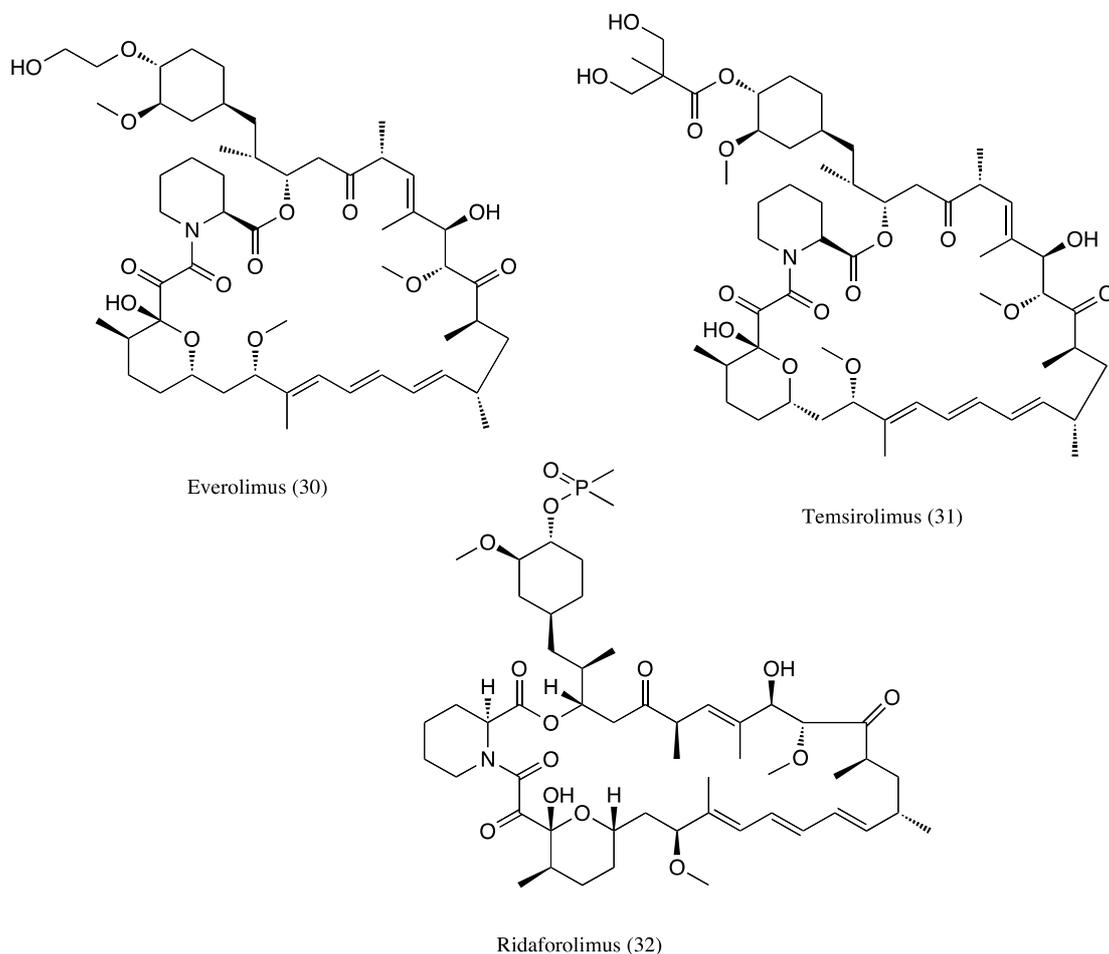


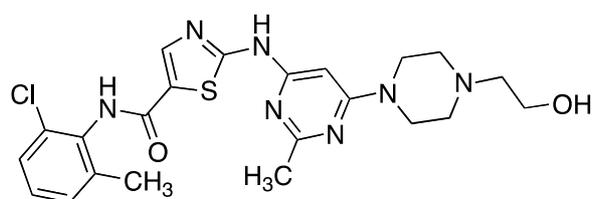
Figure 8: Chemical structures of mTOR inhibitors tested in TNBC.

Everolimus (**30**, Figure 8) is an orally bioavailable small molecule inhibitor of mTOR1. Several clinical trials have reported the effectiveness of everolimus when used in combination with trastuzumab or hormone therapy against HER2-overexpressing or hormone-receptor-overexpressing breast cancer, respectively. One trial reported a PFS of 34% PFS [73]. Yunokawa *et al* examined the effects of everolimus against nine different TNBC cell lines; five of the nine cell lines were found to decrease proliferation. This study confirmed everolimus as a promising therapeutic agent for targeting basal-like subtypes of TNBC, with CK5/6 as positive predictive markers, while cancer stem cell markers are negative predictive markers [99]. Several clinical Phase I and II trials of everolimus alone or in combination with other agents in TNBC malignancies are ongoing including: carboplatin, which was recently completed and is awaiting results (NCT01127763), neoadjuvant cisplatin and paclitaxel, which is ongoing (NCT00930930), and finally a Phase I/II trial of gemcitabine and cisplatin

with everolimus in patients with MTNBC which is currently recruiting patients (NCT01939418). Temsirolimus (**31**, Figure 8) is another inhibitor of mTOR, which is administered intravenously. Results are awaited for a Phase I study designed to determine the maximum tolerated doses of cisplatin, temsirolimus, and erlotinib in a combination treatment for TNBC patients (NCT00998036), and recruitment is in progress for a Phase I/II clinical trial of temsirolimus in combination with neratinib in MTNBC (NCT01111825). Meanwhile, results are awaited for a randomized Phase II trial of ridaforolimus (**32**, Figure 8) and dalotuzumab (NCT01234857).

Src inhibitors

Src is a non-receptor protein tyrosine kinase, an important mediator of many downstream effects of RTKs, including the EGFR family. Src also plays a significant role in several signal transduction pathways involved in cell growth, survival, motility, and angiogenesis. Numerous studies have shown Src to be overexpressed in TNBC, which correlates with metastatic disease progression. Furthermore, TNBC cells were shown to be susceptible to growth inhibition by dasatinib, a Src inhibitor, in preclinical studies [100-102]. These results supported the development of Src inhibitors against TNBCs. Dasatinib (**33**) is an oral, small molecule multi-kinase inhibitor that targets Bcr-Abl and the Src family of kinases. In a preclinical study using baseline gene expression profiling of a panel of 23 breast cancer cell lines that correlate with response to dasatinib, TNBC cell lines demonstrated greater response to dasatinib compared with other BC subgroups [103,104].



Dasatinib (**33**)

However, the results of a Phase II trial of dasatinib monotherapy in patients with MTNBC were disappointing. Dasatinib showed only modest efficacy; of the 43 response-evaluable patients, 2 had PRs lasting 14 and 58 weeks (ORR of 4.7 %), 11 patients had SD (9.3 %), and median PFS was 8.3 weeks [105]. Recently, some preclinical studies have demonstrated the ability of dasatinib to undergo synergism with chemotherapy and other RTK inhibitors [106-109], providing justification for re-evaluation of dasatinib in combination therapies.

MAPK signalling pathway inhibitors

The Raf/MEK/ERK pathway, also known as the mitogen-activated protein kinase (MAPK) pathway, is vital for normal human physiology, and it is commonly found to be dysregulated in several human cancers, including breast cancer through activation of the Ras oncoprotein. Although Ras-related genetic alterations in BC are rare, deregulation of this pathway at the gene expression level may be potentially significant in TNBC. Several preclinical studies have reported a high expression of several gene sets related to the Raf/MEK/ERK pathway in TNBC compared with other BC subtypes [110]. Recently, Bartholomeusz and co-workers have found the over-expression of ERK2 (a result of aberrant Ras function) to be a negative prognostic factor in TNBC patients [111]. This data support the clinical development of inhibitors of the MAPK pathway in TNBC.

In a preclinical study of 21 breast cancer cell lines with MEK1/2 inhibitor trametinib (**34**, Figure 9), 11 TNBC cell lines were highly sensitive to the inhibitor. A phosphatase DUSP6, that decreases pERK2 activity upon MAPK activation, was identified as a potential marker of sensitivity to the drug [112]. Currently, a clinical trial is recruiting patients to define the TNBC kinome response to treatment with trametinib in order to identify potential biomarkers (NCT01467310). Another MEK inhibitor, cobimetinib (**35**, Figure 9) is under evaluation in a Phase II trial in combination with paclitaxel in MTNBC patients (NCT02322814). Interestingly, many preclinical data have demonstrated synergism between the PI3K pathway and MAPK pathway by evaluating a combination of PI3K and MEK inhibitors [110,113]. These findings imply that a possible combination therapeutic strategy for targeting TNBC may be more efficacious. A Phase II trial is currently in development to evaluate this combination using trametinib and the AKT inhibitor, GSK2141795 (**28**) in patients with advanced TNBC (NCT01964924). Recently, El Touny and co-workers showed that concomitant MEK and Src inhibition eliminated a population of dormant tumour cells, thus this combination could also prevent BC recurrence [114].

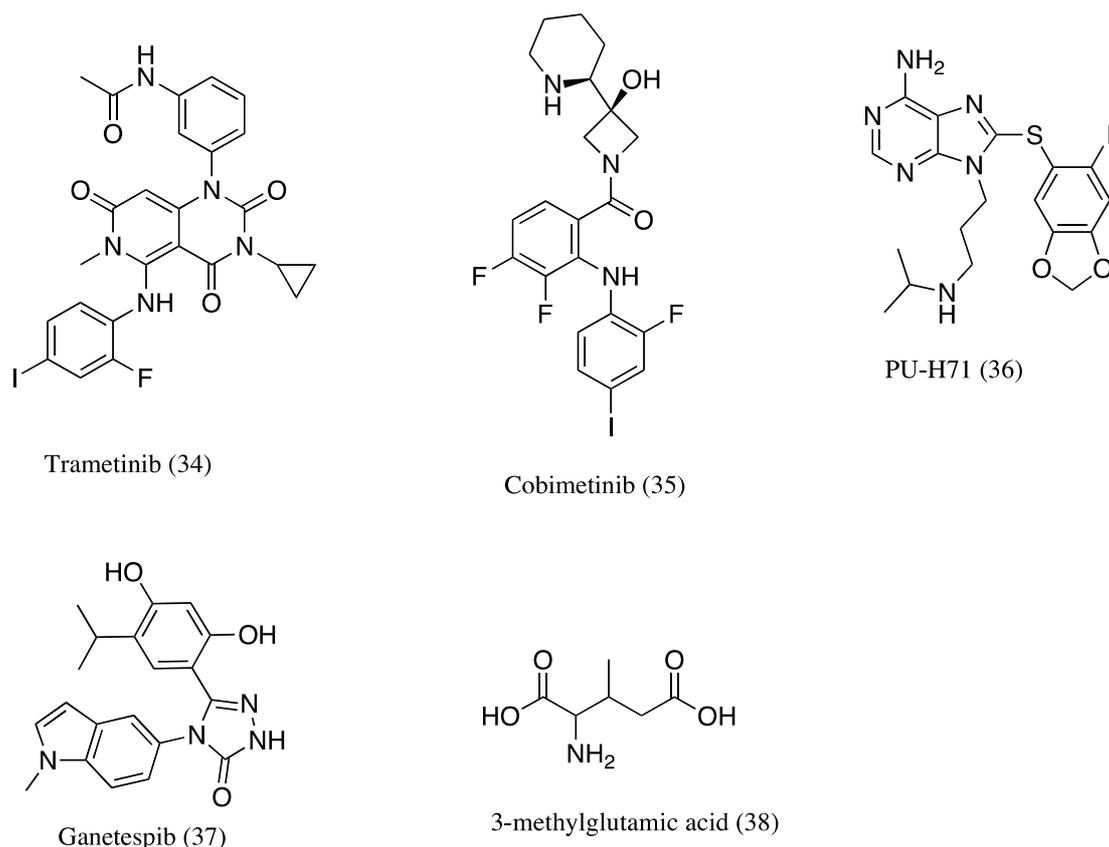


Figure 9: Chemical structures of small molecule inhibitors of MEK and HSP90 tested in TNBC.

Heat-shock protein family inhibitors

The heat-shock protein 90 (HSP90), a member of the heat-shock protein family, is a chaperone protein involved in the proper folding and conformational stability of various oncogenic signalling proteins, including AKT, HER2, EGFR, PDGF- α , and CDK4 [66,68]. Preclinical studies have shown that HSP90 is overexpressed in many human tumours, and appears to play a major role in facilitating tumour progression by chaperoning mutated and over-expressed oncogenes [115]. A HSP90 inhibitor PU-H71 (**36**, Figure 9) has since shown potent and sustained antitumour effects in TNBC xenografts, including a complete response and tumour regression, without evidence of resistance or toxicity to the host over a 5 month period [116]. This provided justification for the evaluation of Hsp90 inhibitors in TNBC patients. Recently, a triazolone derivative HSP90 inhibitor, ganetespiib (**37**, Figure 9), showed simultaneous deactivation of multiple oncogenic pathways resulting in the reduction of TNBC cell viability, and the suppression of lung metastases in experimental models [117]. Ganetespiib also potentiated the cytotoxic activity of doxorubicin through enhancement of DNA damage and mitotic arrest, conferring better efficacy to a doxorubicin–cyclophosphamide regimen in TNBC xenografts [117]. Patients are presently being recruited

for an open-label multicenter Phase II study of ganetespib in patients with HER2-positive BC and TNBC (NCT01677455).

Another member of the heat-shock protein family, α B-crystallin (CRYAB), is found to be prevalent in high frequency in basal-like breast carcinomas. CRYAB is thus used as a biomarker and corresponds with poor prognosis in TNBC [118]. The main function of CRYAB is thought to be as a chaperone to bind and correct intracellular misfolding of VEGF in tumour microenvironment [119]. Recently, Jun and co-workers identified 3-methylglutamic acid (**38**, Figure 9) as a very potent inhibitor of the interaction between CRYAB and VEGF [120]. *In vitro* studies showed an inhibitory effect of 3-methylglutamic acid on the proliferation and invasion of TNBCs. Additionally, 3-methylglutamic acid also decreased tumour growth and vasculature development in human breast cancer xenografts [120].

Aurora kinase inhibitors

The Aurora kinase family, which consists of Aurora A, B, and C, are serine/threonine kinases that are major regulators of mitosis and multiple signalling pathways. Aurora A and B are found to be over-expressed in many human cancers and are associated with tumour formation and progression. The Aurora A gene, formally known as breast tumour activated kinase (BTAK) because its mRNA is over-expressed in breast tumors, plays a crucial role in the transformation of breast tumour cells [121,122]. Recently, Aurora A was confirmed to be over-expressed in TNBC, with this effect correlating with poor overall survival ($P = 0.002$) and progression-free survival ($P = 0.012$) [123]. Aurora kinases were thus deemed potential therapeutic targets for TNBC treatment. The efficacy of Aurora kinase inhibitors has since been shown both *in vitro* and *in vivo* in TNBCs. In an *in vitro* study, human TNBC cells demonstrated higher sensitivity to AS703569 (**39**, Figure 10), an orally available competitive inhibitor of all three Aurora kinases, compared with other breast cancer cells. *In vivo*, AS703569 administered alone significantly inhibited tumour growth in 7 of 11 breast cancer xenografts. Furthermore, a combination of AS703569 and doxorubicin-cyclophosphamide resulted in significant inhibition of tumour recurrence. These findings support the use of Aurora inhibitors as either monotherapy or in combination with other anticancer agents [124]. Currently, a Phase II trial is investigating the selective Aurora kinase A and angiogenesis inhibitor ENMD-2076 (**40**, Figure 10) in advanced and metastatic TNBCs (NCT01639248).

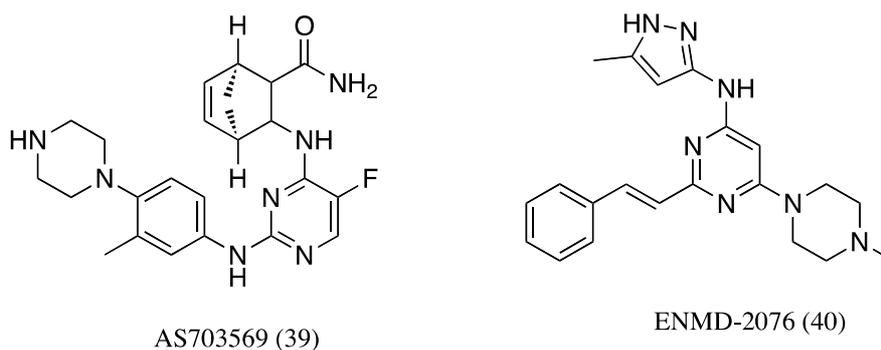


Figure 10: Chemical structures of aurora kinase inhibitors tested in TNBC.

Developmental signalling pathway targets

The biology and targeting of developmental pathways in cancer, such as those involved in tumour initiation and cancer stem cell maintenance, has been the subject of widespread study in recent years. The aberrant activation of signalling pathways such as Wnt, Notch and Hedgehog (Hh) through mutations or ligand over-expression has received particular attention. In parallel, studies on identification of small molecule pathway inhibitors have continued to flourish, exemplified by the approval in 2012 of the Hedgehog pathway inhibitor, Vismodegib (**41**, Figure 11) developed by Genentech for the treatment of basal-cell carcinoma [125]. Application of developmental pathway inhibitors to the specific setting of TNBC is currently at an early stage of development, compared to the therapies discussed in the sections above.

Generally, agents acting on these pathways have not yet entered clinical development in TNBC, with some notable exceptions mentioned below. A study using an oral inhibitor of smoothened (SMO, a transmembrane receptor required for Hh signalling) known as LDE225/erismodegib (**42**, Figure 11) in combination with docetaxel in TNBC is currently recruiting (NCT02027376) [126]. In addition the triazole-based antifungal agent itraconazole (**43**, Figure 11) has also been reported to possess anticancer properties based on inhibition of both hedgehog signalling and angiogenesis [127]; on this basis evaluation of itraconazole pharmacokinetics in patients with metastatic breast cancer is ongoing (NCT00798135). The observation that non-steroidal anti-inflammatory drugs (NSAIDs) are associated with decreased incidence of breast cancer and can inhibit the Wnt/ β -catenin pathway [128] provides further encouragement in the search for drugs targeting developmental pathways that may have value in the setting of TNBC.

An example of an agent broadly targeting developmental pathways in TNBC is provided by CDDO-Im (**44**, Figure 11), a potent synthetic triterpenoid derivative shown to induce growth inhibition in a range of cellular cancer models. Previous studies had shown that CDDO-Im inhibited tumour growth and inflammatory in breast cancer cells *in vivo* [129]. More recent studies focused on effects on tumorspheres from the triple-negative breast cancer

cell line SUM159, where the cancer stem cell subpopulation (CD24-/EpCAM+) was markedly enriched. The ability of CDDO-Im to reduce tumorsphere-forming capacity was related to down-regulation of key stem cell signalling pathway molecules, such as Notch, TGF- β /Smad, Hedgehog and Wnt [130].

Promising candidate drug molecules targeting developmental pathways such as those described above provide confidence that this area of work within TNBC therapy will continue to flourish in the near future.

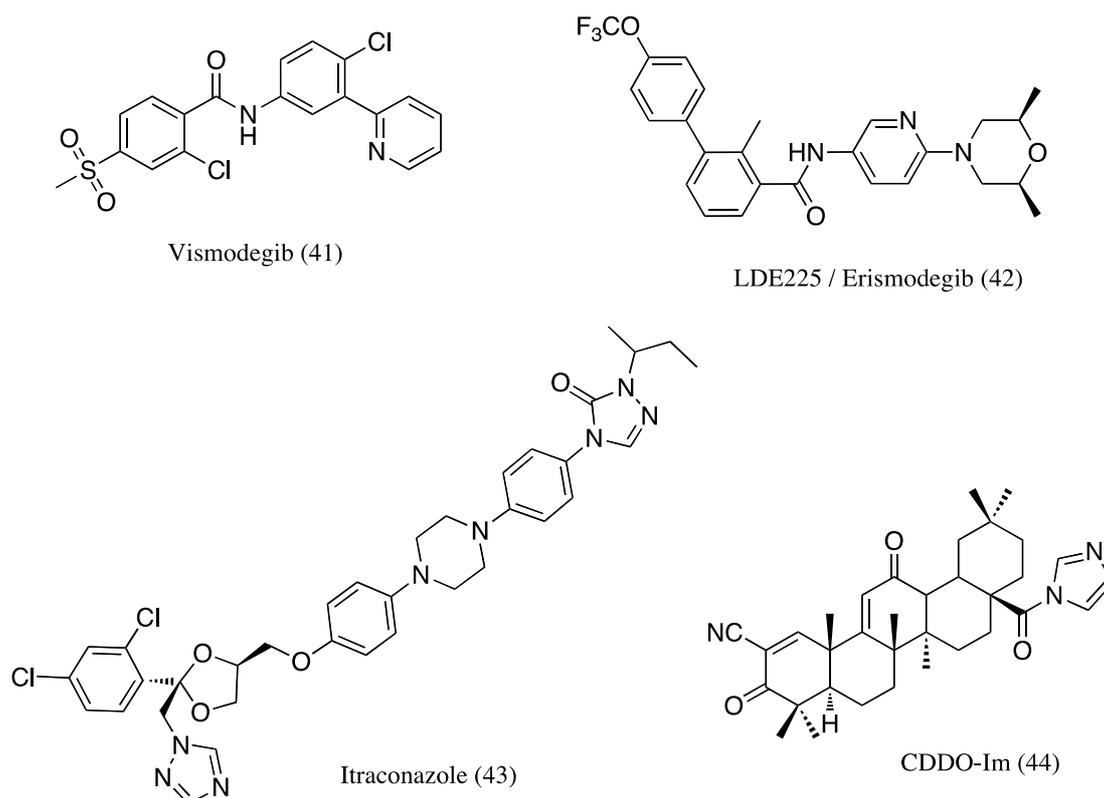


Figure 11: Chemical structures of compounds targeting developmental signalling pathways.

CONCLUSIONS

This review article focuses on the development of small molecule agents to treat triple-negative breast cancer, which represent an important and clinically challenging subset of breast cancers patients characterised by poor prognosis and long-term survival. We hope that the review helps to capture and reinforce the extensive and strenuous effects being made to improve prospects for this important patient group. This is exemplified by the wide range of ongoing clinical trials and application of molecularly targeted agents and chemotherapy, many of which are highlighted in this overview.

EXECUTIVE SUMMARY

Background

Triple-negative breast cancer (TNBC) represents around 15% of clinical breast cancer cases, characterised by a lack of expression of oestrogen receptor (ER), progesterone receptor (PR) and HER2. This disease sub-group, often referred to as basal-like breast cancer patients (with significant overlap between these designations), have a particular poor prognosis and outlook compared to other types of breast cancer.

Therapy

TNBC patients are still most commonly treated with cytotoxic chemotherapy, with poor overall survival prospects in many cases. The introduction of a range of targeted therapies into TNBC patient trials, most often alongside chemotherapy, is likely to improve prospects for this patient group in the near future, although long-term benefits will be marginal in many cases. This review provides an update on many of the targeted agents being trialled in TNBC, for example belonging to the diverse class of tyrosine kinase inhibitors and modulators of related signalling pathways.

The future

Current research efforts delineating developmental signalling pathways (e.g. Wnt/ β -catenin, Notch and Hedgehog signalling) in this setting, associated with the identification of new drug targets involved in processes such as tumour initiation and stem cell maintenance, offer great encouragement as part of future combination therapy strategies. Translation of drug discovery efforts to developmental pathway targets offer the prospect of more durable responses and improved patient outlook in the medium- to long-term.

FUTURE PERSPECTIVE

Over the next 2-5 years, current clinical trials utilising targeted therapies alongside cytotoxic chemotherapy would offer improved prospects for patient in terms of progression-free and overall survival. However these developments are only likely to yield marginal benefits for the patient within this notoriously difficult disease setting.

More promising over the next 5-10 years will be the further stratification of triple-negative patients into the six subtypes described in the introductory section, and further personalisation of medicines most appropriately matched to the patient's tumour at the individual level. For example, in future the luminal androgen receptor sub-type might be treated with a combination regimen including an approved androgen receptor antagonist. Further longer term developments that will improve patient prospects will see the translation of drugs developed against targets central to tumour initiation and stem cell maintenance

incorporated into therapeutic strategies. This is likely to offer both improved efficacy and reduced incidence of drug resistance through targeting of this tumour initiating sub-population.

The next 5-10 years will see the further development and inhibition of new molecular targets not previously exploited in the context of TNBC therapy. An increasing focus on development of new drug targets implicated in tumour initiation and stem (progenitor) cell maintenance, such as Wnt/b-catenin, Notch and Hedgehog signalling is anticipated. Alongside the development of new pathway signalling inhibitors, the development of more informative model systems for drug candidate testing is eagerly anticipated. For example, primary stem cells and progenitor cells from the breast can be enriched within mammospheres in the form of tumorspheres, to offer great potential for more sophisticated *in vitro* screening to inform further drug development. The design and development of drug candidates against newly validated targets in TNBC, alongside more informative and patient tumour representative model systems, presents a powerful combination for the identification of new drug candidates for future treatment of this difficult disease.

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