Review
Molecular targeted therapies for breast cancer treatment

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Abstract

Targeting the oestrogen receptor, HER2 (human epidermal growth factor receptor 2) and vascular endothelial growth factor has markedly improved breast cancer therapy. New targeted therapeutic approaches to induction of apoptosis or inhibition of anti-apoptosis, cell cycle progression, signal transduction and angiogenesis are described. The molecular pathways and their inhibitory or repair mechanisms are discussed in the preclinical and clinical settings.

Introduction

Treatment of early-stage breast cancer requires a multi-modality approach to eradicate residual cancer and prevent recurrent disease. Targeting the pathways that promote or sustain growth and invasion of carcinoma cells is critical to effective treatment of breast cancer [1,2].

Targeting the oestrogen receptor (ER) is the oldest molecular targeted therapy approach, and widespread use of the selective ER modulator tamoxifen in breast cancer is responsible for major improvements in cure rates, quality of life and disease prevention during the past 25 years. Targeting both HER2 (human epidermal growth factor receptor 2) with trastuzumab and the vascular endothelial growth factor (VEGF) with bevacizumab in combination with chemotherapy has become a further milestone of molecular targeted therapy [3-5]. However, intrinsic and acquired resistance to endocrine and/or cytostatic treatments is still a common feature that limits the benefits of these novel therapeutic strategies. Therefore, clinical trials of endocrine or cytotoxic therapies combined with growth factor pathway

inhibitors or their downstream signalling elements are warranted; such approaches may allow us to improve upon the current standard of care for breast cancer patients [6]. Unfortunately, despite encouraging preclinical data, some of these combinations have yielded disappointing results in the clinical setting [7].

This review describes and critically discusses targeted therapies for induction of apoptosis or inhibition of anti-apoptosis, cell cycle progression, signal transduction and angiogenesis (Fig. 1). Table 1 summarizes both finished and ongoing studies in this area.

Induction of apoptosis and inhibition of anti-apoptosis

Apoptosis is a precisely regulated and evolutionarily conserved programme of cell suicide, which plays important roles during embryogenesis and immunology. Disturbances in the physiological programme of apoptosis prolong the life of cells and thereby promote carcinogenesis. Consequently, apoptosis is frequently diminished in cancer cells, supposedly caused by a dominance of anti-apoptotic proteins in malignant tumours. Regulation of apoptosis is complex, but two distinct pathways can be identified: the intrinsic apoptotic pathway, also referred to as p53-mitochondrial pathway; and the extrinsic pathway, which is activated through 'death receptors' and their corresponding ligands (for example, the death-inducing cytokine TRAIL [tumour necrosis factor-related apoptosis inducing ligand]). TRAIL is a transmembrane protein that is cleaved by proteases to release a soluble form. Although it is constitutively expressed in normal


AP = activator protein; CDK = cyclin-dependent kinase; DCIS = ductal carcinoma *in situ*; EGFR = epidermal growth factor receptor; ER = oestrogen receptor; HDACi = histone deacetylase inhibitor; HER = human epidermal growth factor receptor; IAP = inhibitor of apoptosis proteins; IκB = inhibitor of NF-κB; Jd-1 = inhibitor of differentiation and DNA binding-1; MAPK = mitogen-activated protein kinase; MDR = multidrug resistance; mTOR = mammalian target of rapamycin; NF-κB = nuclear factor-κB; pCR = pathological complete response; PI3K = phosphatidyl-inositol 3-kinase; PST = primary systemic chemotherapy; TAM = tumour-associated macrophage; TKI = tyrosine kinase inhibitor; TRAIL = tumour necrosis factor-related apoptosis inducing ligand; VEGF = vascular endothelial growth factor.

Table 1

Clinical studies of targeted therapy with anti-sense nucleotides, antibodies, kinase inhibitors and other agents in breast cancer

Cellular target	Agent	Application	Clinical study	References
TRAIL receptors	TRAIL	BC, gynaecologic malignancies	Phase I	[9,14]
26S proteasome	Bortezomib	Metastatic BC	Phase II	[22]
	Bortezomib	Metastatic BC	Phase II	[23]
	Bortezomib/trastuzumab	Metastatic BC	Phase I	[24]
	Bortezomib/capecitabine	Metastatic BC	Phase I/II	[25]
mTOR	Everolimus (RAD-001)	Primary BC, neoadjuvant	Phase III, GEPARquinto, GBG 44	[88]
	Everolimus (RAD-001)	Metastatic BC, bone metastases	Phase II, GBG 41	[89]
p53	Ad5CMV-p53 and docetaxel/doxorubicin	Primary BC, neoadjuvant	Phase II	[42]
EGFR	Cetuximab and paclitaxel	Advanced BC	Phase I	[44]
	Erlotinib	Primary BC, neoadjuvant	Phase I	[50]
	Trastuzumab and capecitabine versus capecitabine	BC, beyond progression	Phase II, GBG 26,	[90]
EGFR/HER2	Lapatinib and capecitabine versus capecitabine	Advanced BC	Phase III	[60,61]
	Lapatinib and paclitaxel	Inflammatory BC, neoadjuvant	Phase II	[62]
	Lapatinib and paclitaxel/trastuzumab	Primary BC, HER2^{+}, neoadjuvant	Phase III, GBG 47, NeoAltto	[91]
	Lapatinib and trastuzumab	BC, HER2^{+}	Phase III, GBG 46, ALTTO	[92]
Ras, farnesyl transferase	Tipifarnib and gemcitabine	Metastatic BC	Phase II	[63]
	Tipifarnib and letrozole	Advanced BC	Phase II	[64]
	Lonafarnib and anastrozole	Metastatic BC	Phase II	[63]
COX-2	Celecoxib	BC adjuvant	Phase III, GBG 27	[93]
VEGF	Bevacizumab	Metastatic BC	Phase I/II	[68]
	Bevacizumab	Metastatic BC	Phase II	[69]
	Bevacizumab and vinorelbine	Metastatic BC	Phase II	[70]
	Bevacizumab and vinorelbine	Metastatic BC	Phase II	[71]
	Bevacizumab, docetaxel	Metastatic BC	Phase II	[72]
	Bevacizumab/trastuzumab, carboplatin/nab-paclitaxel versus trastuzumab carboplatin/nab-paclitaxel	HER-2 positive metastatic BC	Phase II	[73]
	Bevacizumab, docetaxel	Neo-adjuvant, nonmetastatic, metastatic BC	Phase II	[75]
	Bevacizumab doxorubicin/docetaxel	Neo-adjuvant, inflammatory, locally advanced	Phase II	[76]
	Bevacizumab and capecitabine versus capecitabine	Advanced BC	Phase III	[74]
	Bevacizumab and paclitaxel versus paclitaxel	Advanced BC	Phase III	[5]
	Bevacizumab and trastuzumab	HER2[+], metastatic BC	Phase II	[79]
	Bevacizumab and docetaxel/trastuzumab versus docetaxol/trastuzumab	HER2[+], recurrent or metastatic BC	Phase III	[80]
	Bevacizumab and letrozole versus letrozole	BC, advanced and metastatic	Phase III, GEICAM/GBG 51	[94]
	Bevacizumab and erlotinib	Metastatic BC	Phase II	[81]
	Bevacizumab and everolimus	Advanced solid tumours	Phase I	[82]

BC, breast cancer; COX, cyclo-oxygenase; EGFR, epidermal growth factor receptor; HER, human epidermal growth factor receptor; mTOR, mammalian target of rapamycin; TRAIL, tumour necrosis factor-related apoptosis inducing ligand; VEGF, vascular endothelial growth factor.

Figure 1
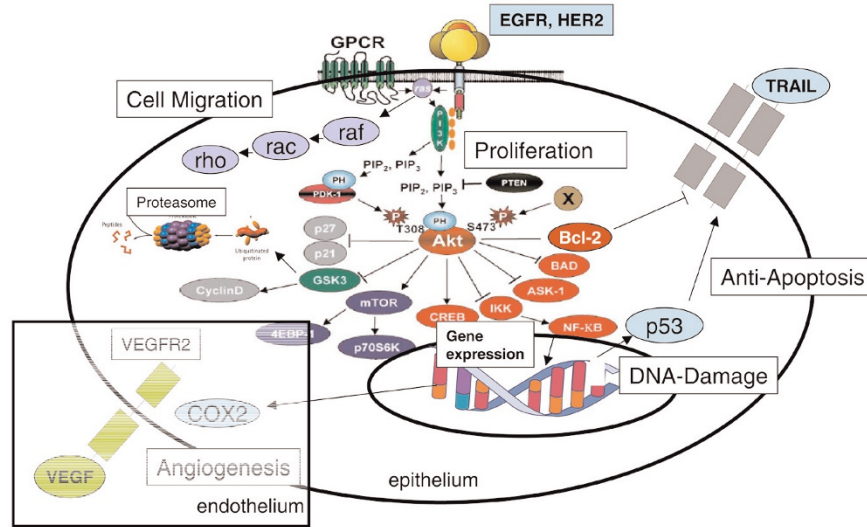
Cell signalling pathways: targets for breast cancer treatment. EGFR, epidermal growth factor receptor; GPCR, G-protein-coupled receptors; HER, human epidermal growth factor receptor; IKK, inhibitor of NF-κB kinase; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; TRAIL, tumour necrosis factor-related apoptosis inducing ligand; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

tissue, TRAIL preferentially induces apoptosis, with minimal adverse effects on normal cells. Therefore, targeting TRAIL and raising agonistic monoclonal antibodies directed against TRAIL receptor 1 or 2 have emerged as promising therapeutic approaches in cancer [8].

TRAIL receptor activating agents have been found to exhibit favourable *in vitro* and *in vivo* activity in treatment of several malignancies, including breast and gynaecological cancers. Preclinical and early phase I studies have provided some support to the assumption that these novel agents are safe, with increased target specificity for malignant cells. When these targeted agents are combined with conventional chemotherapy drugs or radiotherapy, they appear to increase cell death over single agent modalities [9] (Table 1).

Mitochondria-mediated apoptosis is regulated through anti-apoptotic (bcl-2) and pro-apoptotic (bax and bad) proteins of the bcl-2 family. Over-expression of bcl-2 occurs in 40% to 80% of human breast cancers. Most bcl-2 positive breast cancers express ER and/or progesterone receptor. This positive association of bcl-2 with hormone receptors in breast cancer may explain its apparent correlation with response to hormonal therapy. However, diminished apoptotic response caused by bcl-2 over-expression is associated with resistance of tumour cells to cytotoxic drugs. Downregulation of bcl-2 by antisense oligonucleotides has been shown to improve the efficacy of chemotherapy in experimental models. Phase I randomized clinical trials are ongoing in patients with solid tumours [10] using G3139 (oblimersen sodium), a

phosphorothioate antisense oligodeoxynucleotide that targets bcl-2 mRNA and downregulates bcl-2 protein translation. Further studies should examine the molecular effect of the regimen as well as clinical responses of cancers in which taxanes are indicated to be beneficial [11].

Inhibitors of apoptosis proteins

Negative regulators called 'inhibitors of apoptosis proteins' (IAPs) prevent uncontrolled and excessive cell death in the final course of apoptotic signalling. An important member of this group is survivin, which can be detected in approximately 90% of breast tumours. Increased survivin levels are significantly associated with high nuclear grade, negative hormone receptor status, HER2 over-expression, VEGF expression, and high urokinase-type plasminogen activator and plasminogen activator inhibitor-1 levels. Therefore, patients with elevated levels of survivin have significantly worse disease-free survival and overall survival than do patients with lower levels of survivin. In a multivariate analysis the prognostic value of survivin in terms of disease-free and overall survival was found to be independent of both TNM stage and molecular parameters.

Survivin may be a suitable target in future therapeutic strategies [12]. Although anti-cancer drugs have been designed to inhibit the growth of tumour cells, chemotherapy frequently fails because of the development of multidrug resistance (MDR). Over-expression of survivin in MCF-7 cells due to transfection with survivin expression vectors results in decreasing sensitivity to cytotoxic drugs and activation of P-

glycoprotein (MDR1), which exports drugs out of cells. Downregulation of survivin expression in MCF-7/adriamycin-resistant cells by RNA interference directed against survivin increased drug accumulation in cells because of inhibtion of P-glycoprotein. Downregulation of P-glycoprotein expression with the specific inhibitor verapamil markedly suppressed survivin mRNA expression, whereas no opposite effect of upregulated P-glycoprotein has been reported. These findings suggest that survivin may play a key role in MDR in the presence of P-glycoprotein, and this may represent a novel strategy for modulating MDR in cancer cells [13]. LY 2181308 is an antisense molecule directed against survivin that is already being studied in phase I clinical trials [14,15].

Nuclear transcription factor (nuclear factor-κB)
The nuclear transcription factor nuclear factor-κB (NF-κB) has an anti-apoptotic effect, activating IAPs. NF-κB may also work in a pro-apoptotic way by regulating 'interferon-regulated factor-1', the oncogene c-myc, p53 and caspase-1. A preclinical investigation into inhibition of NF-κB via the synthetic inhibitor PS-1.145 is underway [14]. It is already known that constitutive activation of NF-κB supports progression of breast cancer to hormone-independent growth [16] Furthermore, NF-κB inhibits extracellular signal-regulated kinase (ERK) activation to enhance cell survival during the development of tumour adaptive radioresistance [17].

Gemcitabine is a nucleoside analogue that is applied in the treatment of several solid tumours, including breast carcinoma. Despite its cytotoxic effect, clinical efficacy is impaired by the development of resistance. Immunohistochemical analysis of clinical samples of breast carcinoma validated the belief that neoadjuvant gemcitabine treatment induces NF-κB expression and downregulation of inhibitor of NF-κB (IκB). Gene expression patterns and findings of *in vitro* functional studies and analyses of tissue samples are in agreement that NF-κB plays a role in the induction of resistance to chemotherapy. These data give support to clinical strategies that combine gemcitabine with NF-κB inhibitors in breast cancer [18]. Increasing evidence supports a protective role for inhibitor of differentiation and DNA binding-1 (Jd-1) against anti-cancer drug induced apoptosis. Jd-1 expression results in increased number of viable MCF-7 breast cancer cells, reduced bax expression and enhanced bcl-2 expression, but no change in bcl-xL expression. Expression of NF-κB is augmented, whereas expressions of p53 and IκB are reduced. Finally, Jd-1 plays a protective role against taxol-induced apoptosis in breast cancer cells. Inactivation of Jd-1 is a potential therapeutic strategy that may inhibit breast cancer progression and anti-cancer drug resistance [19].

Ubiquitine-proteasome system
The ubiquitine-proteasome system regulates the cell cycle regulator p53, cyclins and cyclin-dependent kinases (CDKs), as well as proteins of the bcl family. Inhibitors of the proteasome system are responsible for the accumulation of pro-apoptotic proteins (for instance, BAX), delivering cytochrome from mitochondria and thereby activating intrinsic apoptotic signal transduction.

Inactivation of NF-κB by proteasome inhibition contributes to increased apoptosis induced by histone deacetylase inhibitors (HDACis). The HDACis constitute a novel class of anti-cancer agents that cause growth arrest, differentiation and/or apoptosis in many tumour cells, and they regulate the activity of the anti-apoptotic transcription factor NF-κB. The proteosome inhibitor MG-132 strongly reduces the activity of NF-κB. Moreover, MG-132 potentiates HDACi-induced cell death. Induction of the stress-related kinase JNK (c-Jun amino terminal kinase) and p38, and upregulation of p21 and p27 are also observed after co-treatment of cells with HDACi and MG-132. Thus, combined treatment with HDACi and proteasome inhibitors potentiates apoptosis in breast cancer cells, representing a novel potential therapeutic strategy in breast cancer [20].

Signalling pathways that converge on two different transcription factor complexes, NF-κB and activator protein (AP)-1, have been identified in ER-positive breast cancers that are resistant to the anti-oestrogen tamoxifen. The model cell lines (MCF-7/HER2 and BT 474) were treated with the IκB kinase inhibitor parthenolide or the proteasome inhibitor bortezomib (PS 341), alone and in combination with tamoxifen. Furthermore, expression microarray data available from UCSF node-negative ER-positive breast cancer patients with known clinical outcome were used to search for potential genes that signify upregulated NF-κB and AP-1 transcriptional activity in association with tamoxifen resistance. The association of these genes with patient outcome was further evaluated using node-negative ER-positive breast cancer patients identified from three other published datasets (Rotterdam, Amsterdam and Basel). Doses of parthenolide and bortezomib that were capable of sensitizing the two endocrine-resistant breast cancer models to tamoxifen can suppress NF-κB and AP-1 regulated gene expression in combination with tamoxifen. Transcript profiles from the UCSF breast cancer cases revealed three NF-κB and AP-1 upregulated genes (cyclin D_1, urokinase-type plasminogen activator and VEGF) that can dichotomize node-negative ER-positive cases into early and late relapsing subsets, despite adjuvant tamoxifen therapy; they had the greatest ability to predict outcome in younger patients. Across the four independent sets of node-negative ER-positive breast cancer cases (UCSF, Rotterdam, Amsterdam and Basel [21]), high expression of all three NF-κB and AP-1 upregulated genes was associated with earliest metastatic relapse. These findings suggest that agents that can prevent activation of NF-κB and AP-1 genes may prove useful in restoring the endocrine responsiveness of high-risk ER-positive breast cancers [21].

Bortezomib is also a potent inhibitor of the 26S proteasome, with broad anti-tumour activity (Table 1). Twelve patients with

metastatic breast cancer were treated with bortezomib in a phase II study [22]. No objective responses were observed, one patient had stable disease and 11 others experienced disease progression. The median survival time was 4.3 months. The most common grade 3 or 4 toxicities included fatigue (58%) and skin rash (33%). Although bortezomib was well tolerated, it exhibited only limited clinical activity against metastatic breast cancer when used as a single agent. The future development of this drug for the treatment of breast cancer should be guided by *in vivo* models that optimize activity in combination with other anti-tumour agents. Similar clinical experience was reported by Engel and coworkers [23], who conducted a single institution, phase II study of bortezomib in the treatment of patients with metastatic breast cancer. There were no observed objective responses in any of the 12 patients who received treatment with bortezomib. Furthermore, all 12 patients progressed while receiving therapy with bortezomib. The study was terminated after first stage because of lack of any objective response.

The two agents bortezomib and trastuzumab prevent NF-κB activation and induce nuclear accumulation of the CDK inhibitor p27^{kip1}, suggesting that combining bortezomib with trastuzumab could increase the efficacy of trastuzumab. Bortezomib induced apoptosis in HER2-positive (SKBR-3, MDA-MB-453, HER2 transfected MCF-7) and HER2-negative breast cancer cells (MCF-7) in a dose-dependent and time-dependent manner. Sequential treatment (trastuzumab then bortezomib) induced either necrosis or apoptosis, depending on the trastuzumab pre-incubation time. The addition of bortezomib to trastuzumab increases the effect of trastuzumab in HER2-positive cell lines in a synergistic way. This effect probably results from the ability of these two drugs to target the NF-κB and p27 pathways. The potential clinical application of this drug combination is currently under evaluation in a phase I clinical trial [24].

A phase I/II including 35 patients [25] was recently conducted to evaluate the combination of capecitabine and bortezomib in anthracycline-pretreated and/or taxane-pretreated patients with metastatic breast cancer. The treatment was generally well tolerated and associated with toxic effects that were consistent with the known side effects of the individual agents. The intent-to-treat overall response rate was 15%, and an additional 27% of patients had stable disease. Median time to progression and overall survival were 3.5 months and 7.5 months, respectively. The median duration of response was 4.4 months. Although bortezomib and capecitabine are well tolerated, the combination had only moderate anti-tumour activity in heavily pretreated patients.

Phosphatidyl-inositol 3-kinase
Phosphatidyl-inositol 3-kinase (PI3K) plays an important role in survival, proliferation, motility and neoangiogenesis in cancer cells. As a consequence of dysregulation of the cell cycle, PI3K is over-expressed and activates Akt, which -

through phosphorylation of NF-κB, bad and caspase 9 - has anti-apoptotic influences. Also, epidermal growth factor receptor (EGFR), as well as HER2, activate PI3K and enhance cell growth via Akt.

Preclinical data identified a possible role for the PI3K/Akt pathway in docetaxel-induced apoptosis. Specifically, inhibition of one of the three isoforms of Akt, namely Akt 2, by the PI3K inhibitor LY294002 counteracted the activity of fibronectin to protect cells from apoptosis induced by docetaxel. Further investigations showed that Akt 2 activation protects against docetaxel-induced apoptosis by regulating survivin levels in a PI3K-dependent manner. Targeting the PI3K/Akt 2 pathway might be a promising strategy for enhancing sensitivity to docetaxel in breast cancer [26]. Inhibition of the PI3K/Akt pathway improves response of long-term oestrogen-deprived breast cancer xenografts to anti-oestrogens. The combination of wortmannin with tamoxifen or fulvestrant inhibited tumour growth of long-term oestrogen-deprived aromatase-transfected human ER-positive breast cancer cells (UMB-1Ca) better than did either drug alone. The combination of wortmannin plus fulvestrant was the most effective treatment, maintaining tumour regression for a prolonged period. These data suggest that blocking both ER and growth factor receptor pathways could provide effective control over tumour growth in long-term oestrogen-deprived human breast cancers [27]. Further data reveal that loss of the PTEN gene (which encodes a phosphatidylinositol phosphatase, and therefore is a negative regulator of PI3K function) results in constitutive activation of the PI3K pathway and poor patient outcome [28]. The study indicates that aberrant PI3K pathway signalling is strongly associated with metastasis and poor survival, supporting the contention that inhibition of this pathway could improve prognosis.

The protein mammalian target of rapamycin (mTOR) is activated downstream by phosphorylated Akt. Thereby, mTOR promotes cancer cell proliferation, maintains blood and nutrient supply by neoangiogenesis, promotes tumour spread and metastasis, and inhibits cancer cell apoptosis. Inhibiting mTOR with the new drug CCI-779 (temsirolimus), which negatively influences these functions, is already being investigated in clinical studies. HER2 and PI3K pathway inhibitors were tested for their ability to inhibit breast cancer cell growth and tumour development in 20 human breast cancer lines [29]. In that study, rapamycin combined with trastuzumab was found to increase anti-tumour efficacy significantly in comparison with trastuzumab alone in HER2-over-expressing breast cancer cells. Rapamycin and trastuzumab significantly reduced levels of cyclins D_1 and D_3 and increased the cleavage of caspase 3, suggesting increased apoptosis. These findings suggest that rapamycin combined with trastuzumab has an enhanced anti-cancer effect, and this combination could be developed as an improved therapeutic regimen in breast cancer.

The German Breast Group is conducting a phase III study in primary breast cancer integrating mTOR antagonist everolimus (RAD001), bevacizumab and lapatinib in the neo-adjuvant setting (GeparQuinto), and a randomized discontinuation phase II study to determine the efficacy of everolimus (RAD001) in breast cancer patients with bone metastases (RADAR; Table 1).

Cell cycle inhibition and cyclin-dependent kinase inhibitors

Interactions of cyclins with CDKs play an important role in regulating the cell cycle. CDKs promote phosphorylation of their target proteins, initiating progression of the cell cycle. Because cells begin to undergo cell division through mitogenic stimuli, there is induction of synthesis of cyclin D_1, which is associated with the kinases CDK4 as well as CDK6. Cyclin D_1 is essential for HER2-induced cell growth and is induced by growth factors through Ras-dependent and Ras-independent signalling pathways.

In a preclinical study [30], the CDK inhibitor flavopiridol was combined with several transduction inhibitors (cyclo-oxyge-nase-2 inhibitor SC236, protein kinase C kinase inhibitor, and PI3K inhibitor LY294002). In two breast cancer cell lines, one HER2 low expressing and another HER2 high expressing (MCF/neo and MCF/18, respectively), enhanced growth inhibition was observed predominantly in the high HER2 expressing cell line. These data suggest that combinations of flavopiridol and signal transduction inhibitors warrant further studies as treatments for breast tumours, and that HER2 expression may influence the choice of inhibitor to combine with flavopiridol.

Exposure of MCF-7 cells to adriamycin, taxol, or UVB results in fourfold to fivefold increased expression of survivin. Inhibition of survivin phosphorylation by flavopiridol resulted in loss of survivin expression, and nonphos-phorylatable survivin exhibited accelerated clearance [31]. Treatment with flavopiridol caused accumulation in the G_1 phase of the cell cycle and induced apoptosis in breast cancer cell lines (SKBR-3 and MB-468). This was associated with downregulation of the levels of cyclins D_1 and B_1, and with inhibition of CDK1, CDK2 and CDK4. In MB-468 cells exhibiting over-expression of bcl-2, apoptosis was inhibited. Sequential treatment with a nontaxane tubuline polymerizing agent epothilone (Epo B) followed by flavopiridol induced significantly more apoptosis of MB-468 cells than treatment with the reverse sequence or treatment with either agent alone [32]. These findings suggest that the superior sequence-dependent anti-breast cancer activity of EpoB followed by flavopiridol may be due to flavopiridol-induced bax conformational change and downregulation of the anti-apoptotic IAP bcl-xL and Mcl-1 proteins, but this treatment may not overcome the resistance to apoptosis of breast cancer cells conferred by over-expression of bcl-2.

Human breast cancer cell lines, which express high levels of endogenous HER2 receptor, were treated with trastuzumab together with flavopiridol [33]. The combination syner-gistically inhibited DNA synthesis, cellular proliferation and contact-dependent growth. In SKBR3 cells, the combination of trastuzumab and flavopiridol inhibited the Ras/mitogen-activated protein kinase (MAPK)/Akt pathway, and decreased cyclin D_1 abundance and kinase activity to a greater extent than either drug alone. Compared with single-agent treatment, combination treatment selectively inhibited Akt and RB1 phosphorylation. Cyclin D_1 over-expression reverses drug treatment induced cell cycle arrest. Inhibition of Akt may prove to be a useful therapeutic strategy in combination with flavopiridol for HER2-positive tumours.

A mRNA expression signature including EGFR was found to predict response to the combination of trastuzumab and flavopiridol in array analysis [34]. Three lines of evidence support the contention that EGFR is a potential target of flavopiridol-trastuzumab synergy: EGFR protein was rapidly and completely lost after combination treatment; a cell line that expresses amplified levels of both HER2 and the EGFR was resistant to the combined drugs; and treatment with EGF prevented any therapeutic effects of flavopiridol and trastuzumab, either singly or in combination. The data clearly demonstrate that the interaction between HER2 and EGFR is not sufficiently addressed in targeted and combination breast cancer therapies [2] and should be studied extensively. In recent phase I clinical trials, flavopiridol has shown some promise in the treatment of a variety of human tumours. However, because of the severe toxicity observed, the use of less toxic doses in combination with other antiproliferative agents, as reported above from *in vitro* experiments, would be desirable. Consequently, phase I studies are underway to determine the toxicities and characterize the pharmaco-kinetics of docetaxel and flavopiridol in patients with meta-static breast cancer [35].

p53: a target for gene therapy

The p53 gene acts as a regulator of cell growth and DNA repair in normal cells; inactivation of the gene appears to lead to cancer. It is the most frequently mutated gene in human cancers; 35% of breast tumours have p53 mutations, of which 88% are located within exons 5 to 8 [36]. Mutations in the p53 gene occur more frequently in ER-negative, basal-like and HER2-amplified tumours than in luminal, ER-positive breast tumours [37]. p53 mutations occur in 24.5% of the axillary node-negative breast carcinomas and more frequently in cancers with HER2 amplification (38.9% versus 20.9% in those without HER2 amplification). Elevated risks for disease recurrence and mortality were identified in patients with both p53 mutation and HER2 amplification [38].

p53 mutations may help to identify a subset of very high risk breast cancer patients with worse prognosis [39]. To investigate the effect of specific inhibition of mutated p53

gene, breast cancer cells harbouring an inactivating p53 mutation were transfected with antisense RNA using a cationic liposome-mediated method. Forty-eight hours after transfection, the antisense RNA (Asp53 exon 8'RNA) had a significant retarding effect on p53-related proliferation inhibition, along with a decrease in p53 protein expression. Such an approach may be used as a therapeutic option in human malignancy [40]. Cells expressing p53 mutations are either more sensitive to cisplatin and melphelan or more resistant than untransfected cells, depending on the mutation. However, there is no difference in response to daunorubicin treatment. These data also suggest that the nature of the p53 mutation influences sensitivity to cytotoxic drugs [41].

Intratumoural administration of a nonreplicating adenoviral vector (Ad5) that contains the human wild-type p53 (Ad5CMV-p53) combined with chemotherapy could increase the efficacy of primary systemic chemotherapy (PST) as measured by pathological complete response (pCR) in the management of patients with locally advanced breast cancer (Table 1). In a prospective, open label, phase II trial [42], 13 patients with locally advanced breast cancer were treated with six 3-week cycles of PST, which consisted of intra-tumoural injections of Ad5CMV-p53 for 2 consecutive days plus docetaxel and doxorubicin followed by surgery. The trial was terminated early because none of the patients achieved pCR. Eight patients (73%) had a p53 mutation; serial biopsies showed increases in p53 mRNA and p21 (WAF1/Cip1 mRNA). All 12 evaluable patients achieved an objective clinical response. Surgical specimens revealed scattered tumour cells with extensive tumour infiltrate leucocytes, predominantly T lymphocytes. There was no increase in systemic toxicity. Ad5CMV-p53 combined with PST was safe, active and associated with local immune modulatory effects. The promising clinical activity of this combination deserves further investigation in randomized studies.

Monoclonal antibodies and tyrosine kinase inhibitors for EGFR and HER2

EGFR, HER2, HER3 and HER4 are members of the ErbB family of receptor thyrosine kinases. EGFR and HER2 are over-expressed in a variety of human tumours, and over-expression generally correlates with poor prognosis and decreased survival. Three of the four receptors comprise an extracellular amino-terminal domain to bind corresponding ligands (for example, EGF to EGFR; ligands to HER2 are not known) and an intracellular carboxyl-terminal domain lodging a tyrosine kinase with the exception of HER3. Binding of ligands induces dimerization of the receptor and activation of the kinase through autophosphorylation, stimulating PI3K/Akt and/or MAPK signalling, various transcription factors (such as STAT-3, c-fos and ELK-1) and enhanced production of VEGF. Proliferation, migration, adhesion and angiogenesis are favoured, whereas apoptosis is inhibited [43].

From 15% to 30% of breast cancers have been shown to express high levels of EGFR and HER2. Moreover, hormone-resistant disease is associated with an inreased expression of both EGFR and EGFR ligands. Therefore, EGFR and its downstream signalling pathways are promising anti-tumour targets.

Cetuximab, an IgG_1 chimeric monoclonal antibody, competes with ligand binding to the EGFR ectodomain, resulting in an efficient blockade of the tumour-promoting downstream signalling pathway. Preclinical studies have indicated a synergistic effect for the combination of anti-EGFR therapy plus paclitaxel in breast cancer models (Table 1). The feasibility of this combination was evaluated in a dose-escalation phase I trial using cetuximab/paclitaxel in patients with metastatic breast cancer [44]. Treatment consisted of weekly cetuximab therapy and paclitaxel given every 3 weeks, with dose escalation of cetuximab until the maximum tolerated dose was reached. Twelve patients were enrolled into three treatment cohorts. Two out of six patients in the second cohort (cetuximab 100 mg/m^2) developed dose-limiting toxicities, presenting as grade 3 rash. In the third cohort (cetuximab 100 mg/m^2 and paclitaxel in a weekly schedule), one out of three patients developed grade 3 skin toxicity. Of 10 patients evaluable for response, two experienced stable disease and eight had disease progression. Because of prohibitive dermatological toxicity and disappointing preliminary efficacy, this combination of paclitaxel/cetuximab was not considered promising.

ZD 1839 (gefitinib) is an example of an orally active, selective EGFR tyrosine kinase inhibitor, which has shown extensive preclinical activity. Gefitinib has exhibited *in vitro* activity as both monotherapy and in combination with other agents such as paclitaxel and doxorubicin, inhibiting the growth of breast cancer cells that are resistant to endocrine agents such as tamoxifen [45].

To test whether continous or pulsatile inhibition of EGFR signalling is effective in inhibiting tumour proliferation, combinations of paclitaxel and gefitinib, using either intermittent or continous dosing schedules, were compared in mice [46]. In combination with paclitaxel, pulsatile gefitinib was significantly superior to continous dosing. When gefitinib was administered for 1 or 2 consecutive days before paclitaxel, much higher doses could be given safely. Two days of gefitinib treatment before paclitaxel was most effective, causing significantly greater mean tumour regression and higher percentage of complete responses than other schedules.

Preclinical studies conducted in human ductal carcinoma *in situ* (DCIS) xenografts in nude mice suggest a potential role for EGFR inhibitors. Gefitinib has been shown to produce a response in DCIS through a decrease in epithelial prolifera-tion. These findings indicate that tyrosine kinase inhibitor

(TKI) blockade of EGFR has potential in the treatment and chemoprevention of DCIS [47] (Table 1).

The spontaneous pulmonary metastasis mouse model was applied to evaluate the ability of the EGFR TKI erlotinib (OSI-774) to prevent pulmonary metastasis in curatively resected breast carcinoma [48,49]. The expression levels of EGF and EGFR were significantly higher in pulmonary metastatic nodules than in primary breast cancer tissue. Treatment of erlotinib given to mastectomized mice inhibited the incidence of pulmonary metastases. The number of metastatic pulmonary nodules was significantly reduced in the erlotinib-treated group compared with the control. The effects of treatment with erlotinib for 6 to 14 days was evaluated in a presurgical study conducted in 41 patients with breast cancer of stage I to III [50]. Grade 2 rash and diarrhoea were the main toxicities. Erlotinib inhibited tumour cell proliferation (Ki-67), and phosphorylation of EGFR and HER2. Treatment was associated with significant reductions in phosphorylated MAPK and Akt in ER-positive cancers. Inhibition of proliferation occurred in ER-positive but not in HER2-positive or triple-negative carcinomas.

Trastuzumab, a humanized monoclonal antibody that targets HER2, is of special importance in breast cancers that over-express HER2. When combined with standard cytotoxic chemotherapy, trastuzumab improves the outcome and survival in patients with metastatic disease [3]. Furthermore, over the past 3 years, studies incorporating trastuzumab in sequence or concurrently with taxane-based chemotherapy in the adjuvant setting have demonstrated considerable benefit in this subset of patients, and longer term findings regarding outcomes and late toxicity are expected during the next few years [51-55] (Table 1). Based on these impressive results, trastuzumab represents the standard of care in the adjuvant treatment of HER2 over-expressing breast cancers. The role of trastuzumab in the neoadjuvant setting is very promising but must be further evaluated in larger prospective randomized trials [56].

However, there is still a large proportion of patients over-expressing HER2 who do not respond to trastuzumab. Results from a gene expression analysis indicated that lower expression of genes involved in CD40 signalling is associated with a greater risk for residual cancer after trastuzumab-containing preoperative chemotherapy [57]. The optimal combination of trastuzumab with other agents requires further evaluation this patient cohort. The role of the novel antibody pertuzumab remains to be defined in breast cancer lacking HER2 amplification [58].

Heterodimerization, compensatory crosstalk and redundancy exist in the HER2/EGFR network, which provides a sound scientific rationale for dual inhibition of EGFR and HER2. Trials of approved agents in combination, for example trastuzumab and cetuximab, are underway. Preclinical studies

conducted in cell lines (MCF-7/HER2 and BT474) showed that combined treatment with gefitinib, trastuzumab and pertuzumab in order to block signals from all HER homodimers and heterodimers inhibits growth of HER2 over-expressing xenografts significantly better than single agents and dual combinations [59]. There is also a new generation of small-molecule TKIs and monoclonal antibodies that target two or more ErbB receptors (Table 1). Lapatinib, a TKI of EGFR and HER2, has shown clinical benefit in trastuzumab-refractory breast cancer and is now approved by the US Food and Drug Administration [60]. A phase III trial demonstrated that lapatinib plus capecitabine is superior to capecitabine alone in women with HER2-positive advanced breast cancer that progressed after prior therapy including trastuzumab [61]. The addition of lapatinib prolonged time to progression significantly and resulted in a trend toward improved overall survival and fewer patients with central nervous system involvement at first progression. However, extracellular domain HER2 (HER2 ECD) baseline serum did not predict benefit from lapatinib. Paclitaxel monotherapy in combination with lapatinib could be a successful approach to managing inflammatory advanced breast cancers patients, because this type of breast cancer most commonly expresses EGFR and/or HER2 [62]. In a neoadjuvant phase II study, a clinical response rate of 77% (complete and partial responses) and a pCR of 17% were achieved in HER2-positive tumours. Eighty per cent of EGFR-positive patients exhibited a response.

Farnesyltransferase inhibitors

The Ras protein regulates genes that are involved with transcription, translation, cell growth, cell survival and cellular interactions, as well as development of the cytoskeleton. Because the gene is mutated in 30%, the regulation of Ras appears to be a further rational anti-tumour strategy. Activation of Ras is induced by farnesyl transferase ('farnesylation'). In preclinical studies inhibition of breast cancer cell growth through some farnesyl transferase inhibitors has been confirmed. The efficacy of the oral inhibitors tipifarnib and lonafarnib is currently being examined in phase II studies [63]. However, preliminary findings from randomized controlled studies are rather disappointing. Tipifarnib combined with letrozole did not show any benefit compared with letrozole alone [64].

Targeting tumour neoangiogenesis: VEGF and VEGF receptor

Tumour-associated macrophages (TAMs) are associated with tumour progression and metastasis. Pro-angiogenic factors were detected as being released by TAMs such as transforming growth factor-β, tumour necrosis factor-α, matrix metalloproteinase-9 and VEGF [65]. Besides, legumain (a member of the asparginyl endopeptidase family) is over-expressed by TAMs and provides a therapeutic target molecule. In murine models of metastatic breast, colon and non-small-cell lung cancers, legumain-based DNA vaccine

induced a CD8+ T lymphocyte response against TAMs, which dramatically reduced their density in tumour tissue and resulted in a marked decrease in angiogenesis.

VEGF has emerged as a key target in the treatment of cancer. As the ligand to the VEGF receptor, it plays a central role in promoting tumour angiogenesis. Over-expression of VEGF leads to poor outcomes in patients with breast cancer and other tumours. Preclinical studies have shown that the humanized monoclonal antibody to VEGF bevacizumab can reduce tumour angiogenesis and inhibit the growth of solid tumours, either alone or in combination with chemotherapy [66,67]. A phase I/II dose escalating study in previously treated metastatic breast cancer [68] identified an overall response rate of 6.7% and a median duration of response of 5.5 months. Four of the 75 patients included experienced adverse events such as hypertensive encephalopathy, nephrotic syndrome and headache combined with nausea and vomiting, and therefore discontinued therapy. In a randomized phase II trial with single-agent bevacizumab at two dosages in 35 patients with pretreated metastatic breast cancer, one patient in the high dose arm (10 mg/kg) was shown to have a complete response [69]. In combination with established chemotherapies, such as vinorelbine [70,71], docetaxel [72], carboplatin and nab-paclitaxel [73], use of bevacizumab has yielded more encouraging results in phase II clinical trials in patients with refractory metastatic breast cancer.

In a randomized phase III trial conducted in heavily pretreated breast cancer patients [74], the addition of bevacizumab to capecitabine resulted in a significant increase in response rates (19.8% versus 9.1%) and was well tolerated. However, this did not translate into improved progression-free survival (4.86 months versus 4.17 months) or overall survival (15.1 months versus 14.5 months). However, in an open-label, randomized phase III trial [5], initial therapy for metastatic breast cancer with paclitaxel plus bevacizumab significantly prolonged progression-free survival (11.8 months versus 5.9 months), but not overall survival (26.7 months versus 25.2 months), as compared with paclitaxel alone. Unfortunately, adverse events were more frequent in patients receiving paclitaxel plus bevacizumab. However, bevacizumab as first-line therapy in metastatic breast cancer is under investigation in a confirmational phase III trial that is currently taking place in Europe (AVADO trial).

In the neoadjuvant setting bevacizumab has already been evaluated. In a first study 34 patients with nonmetastatic and metastatic unresectable breast tumours exhibited five complete clinical responses and 24 partial responses [75]. In another neoadjuvant trial [76] patients with inflammatory and locally advanced breast cancer received bevacizumab alone for the first cycle followed by six cycles of bevacizumab with doxorubicin and docetaxel. After completion of preoperative chemotherapy, eight out of 13 patients exhibited confirmed partial responses [76].

Konecny and coworkers [77] measured HER2 and VEGF levels in primary breast tumour tissue from 611 patients and found a positive association between their expression levels and a correlation between increased HER2/VEGF levels and worse clinical outcomes. In a phase I study initiated by Pegram and colleagues [78], they assessed the optimal dose schedule and safety of bevacizumab in combination with trastuzumab; this was the first trial to test a combination of multiple monoclonal antibodies in humans. Pharmacokinetic data from this study suggested that the combination did not alter the pharmacokinetics of either agent. The response rates and the tolerable safety profile of this study led to a phase II extension to evaluate this combination as first-line treatment for HER2-positive metastatic breast cancer [79]. Studies to test this combination are currently being performed, including a randomized, open-label, phase III study with trastuzumab plus a taxane with or without bevacizumab in HER2-positive locally recurrent or metastatic breast cancer [80]. Further investigations are evaluating the combination of bevacizumab and erlotinib [81] and combination therapy with bevacizumab and the mTOR inhibitor everolimus [82].

Future trials should not just focus on the combination partner; the timing of administration is also of major importance. Because tumour angiogenesis occurs in a very early stage of carcinogenesis, there is a strong rationale for earlier use of bevacizumab in the adjuvant setting [83].

Conclusion

Treatments of breast cancer continue to evolve rapidly. New scientific and clinical achievements are constantly changing the standard of care and have already led to substantial reductions in breast cancer mortality. Despite encouraging preclinical data, some of the molecular targeted agents ('biologicals') have yielded low response rates in the clinical setting [7]. A further issue is the successful management of side effects of these agents, such as acneform rash with anti-EGFR therapy, cardiac insufficiency with trastuzumab treatment, and hypertension and cerebrovascular ischaemia with bevacizumab therapy. New opportunities to select the right patient for a benefical therapy has resulted from knowledge of most of the genes in the human genome and the development of whole-genome gene expression analysis by array technology. Many studies have been performed and are still underway whose aim is to achieve improved matching of effective drug(s) to the molecular characteristics of the individual cancer patient [84-86]. Further studies must provide much needed data on predicting response to targeted therapies, revealing the mechanisms of resistance to such therapies and maximizing the patient's benefit [87].

Competing interests

The authors declare that they have no competing interests.

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