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Experimental Therapeutics for the Treatment of Triple Negative Breast Cancer

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1. Introduction

Triple negative breast cancers (TNBCs) are defined as tumors that do not express the estrogen receptor (ER), the progesterone receptor (PR) and the HER2 isoform of the epidermal growth factor receptor (EGFR)(Foulkes et al., 2010). They account for approximately 10-17% of all breast cancers (Reis-Filho & Tutt, 2008). Clinically, they are more prevalent among young African and African-American women (Reis-Filho & Tutt, 2008). TNBCs are poorly differentiated, highly malignant, more aggressive and have a poor outcome (Chen & Russo, 2009). They are also characterized by early recurrence and a high rate of visceral metastasis (Conte & Guarneri, 2009). Moreover, the peak risk of recurrence occurs within three years of diagnosis and the mortality rates are increased for five years after diagnosis (Kwan et al., 2009). The molecular changes associated with TNBCs have been characterized by various immunohistochemistry and gene expression profiling studies. Specifically, they include *p53* mutation, overexpression of Ki67 and EGFR, and dysfunction in the BRCA1 pathway (Rowe et al., 2009). It is estimated that EGFR is expressed in 60% of TNBCs (Arslan et al., 2009). In addition, TNBCs have an over expression of cytokeratins 5, 6, 14, and 17, smooth muscle actin, P-cadherin and c-kit (Irvin & Carey, 2008, Venkitaraman, 2010).

2. Naturally-derived experimental therapies

2.1 Epigallocatechin gallate

TNBCs have limited treatment options due to the lack of a specific therapeutic target, such as hormonal or antibody therapy as well as a diverse biology and treatment sensitivity (Arslan et al., 2009). Therefore, there is an urgent need for novel therapeutic agents for the management of TNBC. Accordingly, naturally-derived compounds are under investigation and provide a source of experimental drugs from which novel therapies could develop. One of these natural agents is epigallocatechin gallate (EGCG, Figure 1). It is the most abundant and active catechin obtained from green tea (*Camellia sinensis*) (Graham, 1992), as it has shown anti-tumorigenic activity in a variety of cancer models including TNBC cell lines such as MDA-MB-231 and MDA-MB-468 cells. Specifically, EGCG inhibited the proliferation of MDA-MB-468 and MDA-MB-231 cells with inhibitory concentrations (IC_{50}) of 30 and 80 µg/ml, respectively (Kavanagh et al., 2001, Masuda et al., 2002). Mechanisms for the cytotoxic effect of EGCG include induction of apoptosis as well as the modulation of various

cell signaling proteins involved in cell survival, proliferation and death. Specifically, EGCG (20-60 μg/ml) caused 20-54% of MDA-MB-468 cells to become apoptotic after 72 h (Roy et al., 2005), while 25 μM caused 12% of MDA-MB-231 cells to undergo apoptosis after 36 h (Stuart et al., 2007). Even though EGCG causes cells to undergo cell cycle arrest in the G1 phase, this is not a mechanistic driver of apoptosis in TNBC cells, as the significant increase in apoptosis precedes the increase in G1 arrest (Stuart et al, 2007). Therefore, other mechanisms drive apoptosis.

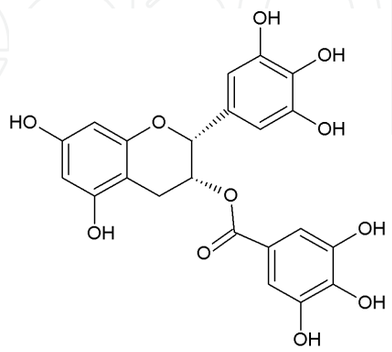


Fig. 1. Chemical structure of (-)-epigallocatechin gallate.

The cell surface epidermal growth factor receptor is over-expressed in 45-70% of TNBC cells and is associated with poor prognosis of patients (Bosch et al., 2010, Koenders et al., 1991). EGFR activation via growth fator binding causes dimerization and subsequent auto-phosphorylation of specific tyrosine residues at the intracellular C-terminal end of the receptor. Through conserved protein binding domains (SH2, SH3) that interact with the phospho-tyrosine residues of EGFR, intracellular signaling cascades such as the mitogen activated kinase (MAPK) pathway, C-Jun N-terminal kinase (JNK) pathway and the phosphoinosital-3-kinase/Akt (PI3K/Akt) pathway can be activated (Casalini et al., 2004). Of particular importance for breast cancer is the PI3K/Akt pathway which is associated with phosphatase and tensin homolog (PTEN) inactivation, commonly found to be dysregulated in cancers (DeGraffenried et al., 2004). The protein product (phosphatase) of the tumor suppressor gene PTEN is involved in cell cycle regulation, preventing cells from growing and dividing excessively (Chu & Tarnawski, 2004). In a gene expression analysis of 106 breast cancer patients it was shown that there was a 34% decrease in PTEN and a corresponding 29% increase in EGFR expression, but only in patients with triple-negative breast tumors (Andre et al., 2009). A further study with 11 TNBC patients provided evidence that low PTEN expression is associated with increased activation of Akt. Although it should be noted that the Spearman correlation between Akt and PTEN expression was 0.593, and may therefore suggest that Akt could be activated and affected by multiple mechanisms (Berrada et al., 2010). In MDA-MB-468 cells, EGCG treatment (30 μg/ml) for 72 h inhibited the phosphorylation and therefore the activation of EGFR, Akt and STAT3 in the presence and absence of TGF-α (Masuda *et al.* 2007).

Using scintillation proximity assays, EGCG inhibited all four (PI3Kα, PI3Kβ, PI3Kγ, and PI3Kδ) class I PI3K isoforms (Van Aller et al., 2011). In particular EGCG was potent towards the PI3Kα isoform with a K_i value of 380 nM. Additionally, mTOR was inhibited with a K_i of 320 nM. Further analysis showed inhibition of both PI3K and mTOR occurred via competition with ATP binding to these proteins (Van Aller et al., 2011). This was confirmed

by molecular modeling studies where the structure of PI3Kγ complexed with myricetin (PDB:1E90) was used to dock with EGCG. Myricetin, a structurally related flavonoid, is an ATP competitive inhibitor of PI3K with an IC_{50} of 1.8 μM (Walker et al., 2000). Except for the chromandiol moiety of EGCG which was flipped by 180° compared to the chromone moiety of myricetin, the binding mode of EGCG is similar to that of myricetin, further supporting the notion that EGCG is a PI3K inhibitor by competing with ATP binding (Van Aller et al., 2011). Since the mTOR (C2 isoform) is one of the 3-phosphoinositide-dependent kinases responsible for the activation/phosphorylation of Akt at Ser473, the effects of EGCG on phospho-Akt was examined in MDA-MB-231 cells. EGCG inhibited Akt phosphorylation in a concentration-dependent manner with IC_{50} values below 1 μM, which was consistent with a direct inhibition of mTOR and PI3K by EGCG (Van Aller et al., 2011).

The anti-apoptotic Bcl-2 protein family is important for mitochondrial and endoplasmic reticulum membrane permeability as well as transduction and integration of apoptotic signals upon homo- and heterodimerization (Adams & Cory, 1998). Another protein from the Bcl-2 family, Bax, is classified as pro-apoptotic protein, which directs the release of cytochrome c. The expression ratio of Bax/Bcl-2 is a determining factor for apoptosis in biochemical studies (Adams & Cory, 1998). In MDA-MB-468 cells, EGCG (20-60 μg/ml) for 48 h caused a dose-dependent 1-to-3-fold increase in the expression ratio of Bax and Bcl-2 (Roy et al., 2005). This indicated that the apoptosis inducing effects of EGCG are transmitted via reductions in anti-apoptotic signals by reducing Bcl-2 protein and increasing pro-apoptotic signals mediated by Bax (Roy et al., 2005). Western blotting of other important pro-apoptotic signaling proteins provided further verification of the underlying mechanism by which EGCG induces apoptosis. It was shown that treatment of MDA-MB-468 cells with EGCG (20 μg/ml) for 48 h elevated the expression of cytochrome c (2-fold), Apaf-1 (7-fold), caspase 3 as well as poly(ADP-ribose) polymerase (PARP) (Roy et al., 2005). Similar observations were also made in MDA-MB-231 cells, which were treated with 50 or 80 μg/ml for 24 h. The protein expression ratio of Bax and Bcl-2, as visualized by Western blotting, showed a dose-dependent relationship. The full length PARP protein (116 kDa) was also degraded into the cleaved, inactive 85 kDa form by the proteolytic caspase-3, which is an integral part of mitochondrial-regulated apoptosis (Thangapazham et al., 2007a).

Another potential target for cancer chemoprevention is the ribonucleoprotein telomerase (synthesizes the cap-telomere-end, 5'-TTAGGG-3', of eukaryotic chromosomes), as it is expressed in ~85% of human cancers (75% of breast carcinoma *in situ* and 88% in ductal and lobulal breast carcinomas (Shay & Bacchetti, 1997)). In contrast, after embryonic development, telomerase is barely detectable in normal human somatic cells (Cunningham et al., 2006). The action of EGCG on telomerase activity has also been assessed in MDA-MB-231 cells and MCF10A non-cancerous breast cells. Using a conventional gel-based PCR method, the relative mRNA levels of human telomerase reverse transcriptase (*hTERT*; the key catalytic subunit of telomerase (Cunningham et al., 2006)) were measured in both cell lines after treatment with 40 μM of EGCG for 3, 6, 9 or 12 days. EGCG was found to time-dependently inhibit *hTERT* expression (~40 and ~50% decrease after 9 and 12 days, respectively) in MDA-MB-231 but not MCF10A cells (Meeran et al., 2011). Since *hTERT* is an epigenetically regulated gene (Cunningham et al., 2006), the activity of epigenetic-modulating enzymes such as DNA methyltransferases (DNMTs), histone acetyltransferases (HATs), and histone deacetylases (HDACs) was assessed in order to determine the mechanism under which EGCG influences *hTERT* mRNA levels. Specifically, in MDA-MB-231 cells EGCG (40 μM) treatment for 9 days decreased both DNMT and HAT activity by

~40%, while HDAC activity was unaffected (Meeran et al., 2011). The authors suggested that the observed effects might be due to direct binding of EGCG to the active site of DNMTs and inhibition of HATs. Additionally, treatment with EGCG (40 μM) reduced methylation to ~60%. This indicated that the inhibition of DNMT expression following EGCG treatment could be a contributing factor in the facilitation of *hTERT* promoter demethylation, which would lead to transcriptional repression of *hTERT* expression (Meeran et al., 2011).

An intricate part of tumor growth, invasion and metastasis is the process of new blood vessel formation, referred to as angiogenesis. A key mediator required for this process is vascular endothelial growth factor (VEGF), which is expressed at 34% higher levels in TNBC patients compared to hormone-sensitive tumors (Andre et al., 2009). Angiogenesis is also stimulated by other pro-angiogenic factors such as basic fibroblast growth factor and hypoxia-inducible factor (Schneider & Miller, 2005). Therefore, VEGF targeting anti-angiogenic agents such as bevacizumab have been beneficial in the treatment of TNBC patients (Carey et al., 2010). Matrix metalloproteinases (MMPs) also play an important role in the progression of invasive and metastatic breast cancer (Schneider & Miller, 2005). Targeting various markers such as MMPs that inhibit the rapid growth and metastasis has emerged as one of the strategies for treatment of highly proliferative TNBCs (Greenberg & Rugo, 2010). The inhibition of angiogenesis is yet another critical component to the plethora of effects elicited by EGCG. Specifically, EGCG (40 μg/ml) significantly decreased VEGF peptide levels by 85% and VEGF mRNA levels by 75% compared to control in MDA-MB-231 cells (Sartippour et al., 2002). Effects of the drug were also assessed on VEGF promoter activity and results showed that EGCG (40 μg/ml) reduced promoter activity by ~30% (Sartippour et al., 2002). EGCG also decreased VEGF production in MDA-MB-468 cells by 55% as well as NFκB activity by 4-fold compared to control (Masuda et al., 2007). The effect of protein kinase C has also been examined, as this protein has been shown to be a modulator of VEGF expression (Hossain et al., 2000). Using Western blotting, it was shown that protein kinase C levels decreased by ~70% upon EGCG treatment compared to control (Sartippour et al., 2002). In a Boyden chamber assay, to assess the anti-metastatic potential of EGCG using MDA-MB-231, cells it was shown that treatment with 80 μg/ml for 24 h caused a 28% reduction in cell invasiveness. Furthermore, EGCG decreased the expression of matrix metalloproteinase-9 (MMP-9) 5-fold using microarray experiments. This was confirmed using RT-PCR, which also showed a down-regulation of MMP-9 at the transcriptional level (Thangapazham et al., 2007a). Thus, EGCG suppresses angiogenesis in TNBC via a variety of mechanisms.

An important prognostic markers for breast cancer is Met, a transmembrane receptor for the hepatocyte growth factor (HGF). Importantly, it also has been assessed as one of the targets of EGCG. High levels of Met are correlated with a lower patient survival rate, which led researchers to postulate that EGCG may affect HGF signaling via Met. Using Western blotting in MDA-MB-231 cells, it was shown that 1 h pre-treatment with EGCG (0.6-30 μM) followed by 15 min of exposure to HGF (30 ng/ml), significantly blocked HGF-induced Met, AKT and ERK phosphorylation (Bigelow & Cardelli, 2006). A Boyden chamber assay using MDA-MB-231 cells showed that the observed ~7-fold increase in invading cells by HGF was decreased to ~2-fold by EGCG (5 μM) (Bigelow & Cardelli, 2006). Therefore the modulation of Met by EGCG also contributes to its anti-cancer action in TNBC cells.

The anticancer activity of EGCG is also evident *in vivo*, as isolated EGCG and/or a mixture of green tea polyphenols have suppressed TNBC growth *in vivo*. Specifically, in a MDA-MB-231 xenograft model tumor volume after 10 weeks of treatment was reduced by 45% in EGCG treated mice and by 61% in green tea polyphenol (GTP) treated mice compared to

control (Thangapazham et al., 2007b). GTP and EGCG treatment increased the number of apoptotic cells by 3.5 and 2.6-fold, respectively. This study showed that GTP was slightly more effective than pure EGCG in reducing the tumor incidence, volume and number of apoptotic cells. However, this may be related to the dose given (~3 mg/mouse GTP vs. 1 mg/mouse EGCG) (Thangapazham et al., 2007b). These findings are supported by earlier *in vivo* studies with EGCG or green tea extracts (GTE) (Kavanagh et al. 2001, Sartippour et al., 2001). Specifically, 0.62, 1.25, 2.5 g/l of GTE dose-dependently prevented tumor growth, with a 90% reduction in tumor volume in the 2.5g/l treatment group compared to control (Sartippour et al., 2001). Using immunohistochemistry it was also shown that GTE decreased the overall microvessel density by ~50% in the treated animals compared to control (Sartippour et al., 2001). Importantly, these studies and many others demonstrated that the *in vitro* anticancer effects of EGCG translate into tumor suppression *in vivo*.

2.2 Curcumin

Curcumin (Figure 2), obtained from the roots and rhizomes of the perennial plant *Curcuma longa*, is cytotoxic towards both ER positive and TNBC cells (Verma et al., 1997). For example, curcumin (10 μM) inhibited the proliferation of MDA-MB-468 and MDA-MB-231 cells, with IC_{50} values of 1 μM and 16.25 μg/ml, respectively (Squires et al., 2003). Mechanisms for the cytotoxic effect of curcumin include G2/M cell cycle arrest, induction of apoptosis as well as the modulation of various cell signaling proteins involved in cell survival, proliferation and death. Specifically, cell cycle studies demonstrated that curcumin (20 μM) treatment for 24 h increased the proportion of MDA-MB-231 cells in the G2/M phase by 164% (Chiu & Su, 2009, Fang et al., 2011). Furthermore, curcumin (20 μM) increased the proportion of MDA-MB-468 cells in the G2/M phase by 143% (Squires et al., 2003). Thus, in contrast to EGCG, G2/M phase arrest is one of the drivers of curcumin-mediated apoptosis.

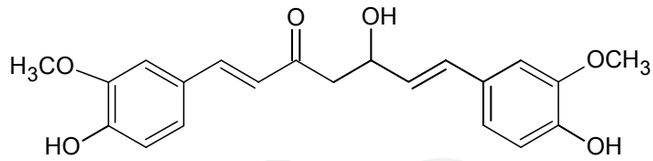


Fig. 2. Chemical structure of curcumin.

The cell cycle is promoted by activation of cyclin dependent kinases, which are positively regulated by cyclins and negatively by cyclin dependent kinase inhibitors (CDKIs) (Malumbres & Barbacid, 2009). Cyclin D1 regulates cell cycle progression through G1-phase of the cell cycle by activating CDK4 and CDK6. Cyclin D1 is a proto-oncogene which is overexpressed in ER negative breast cancers and is a predictor of poor prognosis (Umekita et al., 2002), while cyclin E along with CDK2 regulates the entry of cells from late G1 to S phase. Cyclin E overexpression is associated with poor prognosis and high proliferation in ER negative breast cancer patients (Potemski et al., 2006). CDKIs, p21 and p27 belong to Cip/Kip family of proteins and their decreased expression has been correlated with poor prognosis in breast cancer patients (Catzavelos et al., 1997, Pellikainen et al., 2003). It is reported that altered expression of proteins regulating the cell cycle makes TNBC more sensitive to cytotoxic therapy (Rouzier et al., 2005). Studies have demonstrated that curcumin modulates the expression of cyclins, CDKs and CDKIs in breast cancer cells.

Specifically, curcumin decreased the expression of cyclin D1 and increased levels of p21 expression in MDA-MB-231 cells (Liu et al., 2009) and this was followed by the induction of apoptosis (Chiu & Su, 2009).

Curcumin induces apoptosis in most, if not all, breast cancer cell lines and this occurs mainly via a mitochondrial-dependent pathway (Karunagaran et al., 2005). In most cells, curcumin induces a loss of mitochondrial membrane potential, opening of the transition pore, release of cytochrome c, caspase-9 activation, caspase-3 activation and subsequent cleavage of PARP all of which lead to DNA fragmentation and apoptosis (Aggarwal et al., 2003, Ravindran et al., 2009). Down regulation of anti-apoptotic proteins (Bcl-2 and Bcl-XL) and upregulation of pro-apoptotic proteins (Bad and Bax) also leads to curcumin induced apoptosis in many cancer cells including breast cancer (Ravindran et al., 2009). Curcumin-induced apoptosis via inhibition of reactive oxygen species (ROS) has also been shown. ROS regulate intracellular signaling pathways in various cancer cells including breast cancer (Waris & Ahsan, 2006). Higher production of ROS and glutathione depletion cause oxidative stress, loss of cell function and ultimately leads to apoptosis. Curcumin causes rapid depletion of glutathione (GSH) which results in an increase in the production of ROS and induction of apoptosis (Shehzad et al., 2010). Additionally, curcumin induced apoptosis in MDA-MB cells through the generation of ROS originating from glutathione depletion by buthioninesulfoximine thereby further sensitizing the tumor cells to curcumin (Syng-Ai et al., 2004).

The induction of apoptosis and modulation of the cell cycle result from the effect of curcumin on various intracellular pathways. Curcumin inhibits epidermal growth factor stimulated phosphorylation of EGFR and further inhibits downstream ERK1/2, JNK, and Akt activity in MDA-MB-468 cells (Squires et al., 2003). NFκB is a transcription factor that regulates various genes involved in both proliferation and apoptosis and aberrant NFκB expression is implicated in different types of breast cancers (Wu & Kral, 2005). Furthermore, it is constitutively activated in ER negative breast cancers and its inhibition suppresses cell growth and induces cell death (Biswas et al., 2001, Schlotter et al., 2008). For example, 5 μg/ml of curcumin reduced the expression of nuclear NFκB in MDA-MB-231 cells (Liu et al., 2009). Also, curcumin abolished paclitaxel induced NFκB activation in MDA-MB-435 breast cancer cells (Aggarwal et al., 2005). Modulation of the Wnt/β-catenin pathway by curcumin has been shown to play a role in the inhibition of cell proliferation and induction of apoptosis in MDA-MB-231 breast cancer cells (Prasad et al., 2009). The Wnt/β-catenin pathway is an important pathway as it is associated with worse overall survival in TNBC (Khramtsov et al., 2010).

Curcumin is an inhibitor of angiogenesis, as 50 μM suppressed the transcription levels of VEGF and b-FGF in MDA-MB-231 cells (Shao et al., 2002). Additionally, curcumin downregulated post-transcriptional levels of both HIF-1□α and HIF-1□β in MDA-MB-231 cells (Thomas et al., 2008). Curcumin also inhibited the invasive potential of MDA-MB-231 cells via down regulation of MMP-2, MMP-3 and MMP-9 and up regulation of tissue inhibitor metalloproteinase (TIMP-1, 2) which regulates tumor cell invasion (Boonrao et al., 2010, Shao et al., 2002). In another study, the anti-invasive properties of curcumin were mediated through inhibition of RON tyrosine kinase receptor (Narasimhan & Ammanamanchi, 2008). Curcumin also inhibits integrin α (6) β (4), a laminin adhesion receptor in MDA-MB-231 cells and thus inhibits cell motility and invasion (Kim et al., 2008). Recently, it was shown that curcumin induces upregulation of maspin, a serine protease inhibitor and thus inhibits invasion of MDA-MB-231 cells (Prasad et al., 2009). Furthermore, curcumin inhibited migration of MDA-MB-231 cells through the down regulation of protein

expression of the transcription factor, NFκB (Chiu & Su, 2009). Lastly, curcumin also reduced the expression of the two prometastatic cytokines, CXCL1 and -2, which in turn reduces expression of the chemotactic receptor CXCR4 along with other metastasis-promoting genes (Bachmeier et al., 2008).

The anti-metastatic effect of curcumin has also been studied in various *in vivo* models. Dietary administration of curcumin (2%) significantly decreased the incidence of breast cancer metastasis to the lung in an MDA-MB-231 xenograft model. They also observed that curcumin significantly suppressed the expression of NFκB, COX2 and MMP-9 (Aggarwal et al., 2005). In another study, curcumin decreased lung metastasis in a mouse xenograft model. MDA-MB-231 cells were inoculated into nude mice by intercardiac injection and the treatment group was fed with 1% dietary curcumin. After 5 weeks of treatment, 21% of animals from the curcumin treatment group were metastasis free compared with the control group who all had metastasis (Bachmeier et al., 2007). Many other studies have used chemical carcinogens such as dimethylbenzanthracene or diethylstilbestrol to show that curcumin inhibits mammary carcinogenesis. However, none of these models are representative of TNBC. Nevertheless, curcumin elicits a plethora of effects in TNBC and importantly, as with EGCG, anticancer activity is retained *in vivo*. Both compounds also modulated many different cell signaling pathways that cumulate in a strong apoptotic response (Figure 3).

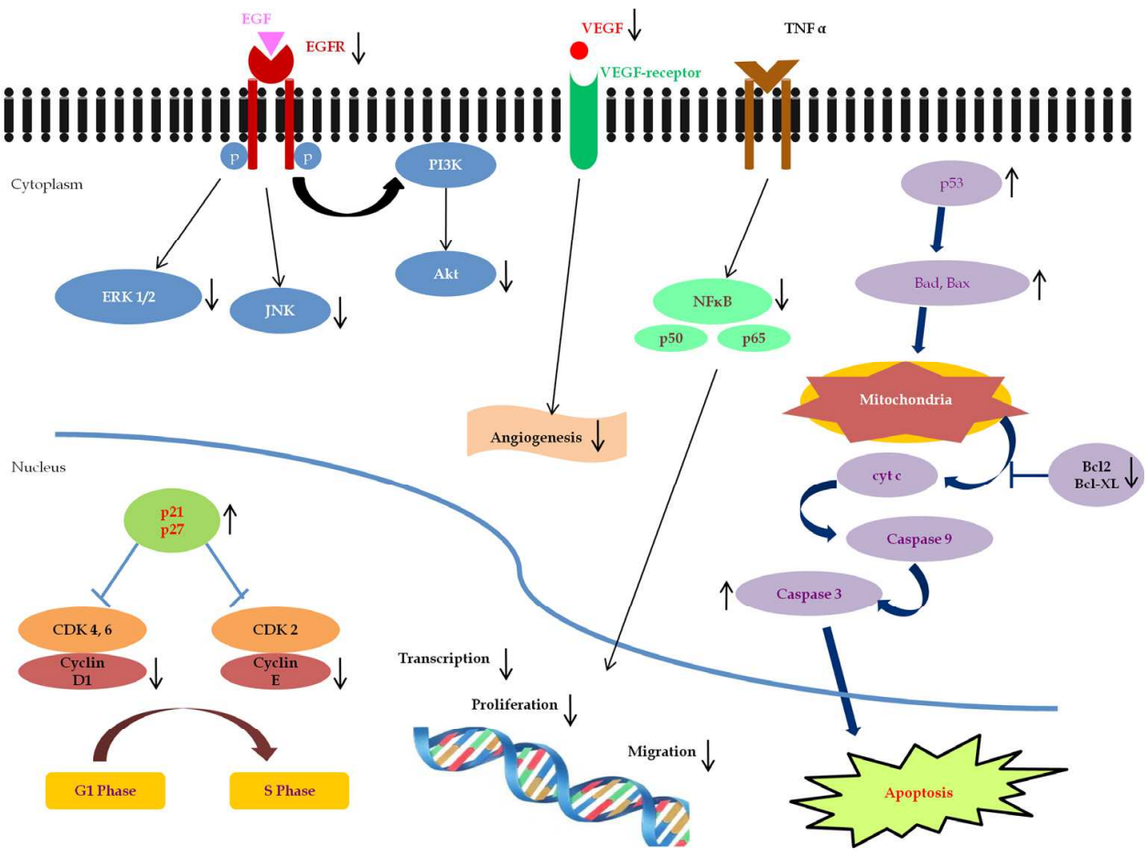


Fig. 3. Schematic diagram of intracellular cell signaling cascades activated following auto-phosphorylation of the EGFR. Arrows indicate an increase or decrease in the protein expression/activity following treatment with either EGCG or curcumin in models of TNBC.

3. Improving naturally derived compounds

3.1 Combination studies

Initial studies with EGCG or curcumin demonstrated that these natural compounds had promise as potential treatments for TNBC. However, both compounds have also been used in various combination studies in order to improve their efficacy. This was achieved in MDA-MB-231 cells where EGCG (20 μM) in combination with paclitaxel (1 μM) reduced cell viability by ~80% after 24 h compared to control (Luo et al., 2010). Treatment of just paclitaxel reduced cell viability by ~40%, whereas EGCG on its own had no effect compared to control. These results suggested that EGCG may act synergistically with paclitaxel to enhance its anti-carcinogenic effects (Luo et al., 2010). Other studies have also shown synergistic effects by using EGCG in combination with other known breast cancer treatments. One such study showed that the selective estrogen receptor modulator (SERM), tamoxifen in combination with EGCG elicited synergistic cytotoxicity as well as earlier and enhanced apoptosis in MDA-MB-231 cells (Chisholm et al., 2004, Stuart et al., 2007). This effect was further analyzed in an *in vivo* xenograft model. Specifically, mice were treated with either tamoxifen (75 μg/kg), EGCG (25 mg/kg) or a combination of the two for 10 weeks. Results showed that the tumor volume in the EGCG + tamoxifen treatment group was 75% smaller compared to vehicle or tamoxifen (Scandlyn et al., 2008). Combination treated mice also had significantly smaller tumors compared to EGCG treatment. Tumor suppression was not through the up-regulation of the ER in MDA-MB-231 cells (Scandlyn et al., 2008). Instead EGFR and its activated form were reduced by ~78% compared to control. Similar reductions in mTOR and CYP1B1 expression were also found in tumors from EGCG + tamoxifen treated mice, indicating that the reductions of EGFR, mTOR as well as CYP1B1 expression are likely to be important mechanistic contributors to the suppression of tumor growth (Scandlyn et al., 2008).

Highly interesting was the finding that EGCG at the specific concentration of 10 μM in combination with 10 ng/ml of trichostatin A (TSA; a histone deacetylase inhibitor) increased estrogen receptor-α expression 6-fold in MDA-MB-231 cells (Li et al., 2010). Using CHiP assays it was confirmed that EGCG and TSA increased histone acetyl transferase activity 5-fold compared control. Furthermore, the combination of EGCG, TSA and tamoxifen in MDA-MB-231 cells, caused a significant ~60% reduction in cell viability, which was more effective than combining tamoxifen with either EGCG or TSA (Li et al., 2010). These results showed that epigenetic modifications induced by EGCG + TSA may be useful in sensitizing ER-negative tumors to anti-hormonal therapy. However, it should be noted that the EGCG concentration required for the observed 6-fold increase in ER-α expression was exactly 10 μM, any concentration above or below did not have any effect on ER-α expression (Li et al., 2010). Additionally, a similar effect *in vivo* has not been reported.

Combining EGCG with another SERM, raloxifene, has also shown synergistic cytotoxicity. In MDA-MB-231 cells the combination of EGCG (25 μM) and raloxifene (5 μM) decreased the cell number by ~28% compared to control. Individually, both compounds at the same concentrations were not cytotoxic towards the cancer cells (Stuart & Rosengren, 2008). Additionally, the number of apoptotic cells after 48 h was 34% higher compared to control following combination treatment. The mechanism of action was further assessed by protein expression analysis and the results showed that the protein expression of EGFR, Akt, mTOR and S-6-kinase were decreased by 22, 31, 41 and 46%, respectively (Stuart et al., 2010) after 18 h of treatment. Thus these two compounds elicit a strong response in pathways downstream of the EGFR.

Interestingly, combination of the two naturally derived compounds curcumin and EGCG was effective in both *in vitro* and *in vivo* models of TNBC. In MDA-MB-231 cells these two compounds (EGCG at 25 μM and curcumin at 3 μM) increased apoptotic cells and G2 arrest 2.6-fold compared to curcumin alone (Somers-Edgar et al., 2008). This effect was only observed in TNBC cells and not in MCF-7 cells. Importantly, this *in vitro* effect translated to tumor suppression *in vivo*. Specifically, curcumin (200 mg/kg) and EGCG (25 mg/kg) significantly suppressed MDA-MB-231 xenograft tumor volume by 49% compared to vehicle control after 10 weeks of treatment (Somers-Edgar et al., 2008). This was in part driven by a 78% decrease in VEGFR-1 protein expression in tumors. Tumor suppression has also been shown in other combination studies with curcumin. Specifically, when curcumin was combined with paclitaxel in an MDA-MB-231 xenograft model, there was a significant reduction in tumor growth following combination treatment compared to either agent alone. Mechanistic studies showed that the combination decreased the expression of MMP-9 and increased apoptosis in the tumors of treated mice. Interestingly, the dose of paclitaxel was much lower (7 mg/kg) than previously reported as a single treatment (Kang et al., 2009). Success has also been shown using *in vitro* combination studies. Specifically, when curcumin was combined with piperine the two drugs worked synergistically to inhibit breast cancer stem cell self-renewal without affecting normal cells. The authors showed that this effect was mediated by the inhibition of mammosphere formation via the Wnt signaling pathway (Kakarala et al., 2010). Synergistic growth inhibition and the induction of apoptosis in MDA-MB-231 cells also occurred following the combination of curcumin and xanthorrhizol (Cheah et al., 2009). These studies all illustrate that the naturally derived compounds EGCG and curcumin can also be used in combination in order to increase the potency of these compounds and/or potentially sensitize cancer cells to the effects of other chemotherapeutic agents.

3.2 Novel drug delivery of natural compounds

Various novel drug delivery systems such as nanoparticles, liposomes, micells, adjuvants and phospholipid complexes have been developed in order to specifically target cancer cells in order to improve efficacy and bioavailability, while reducing toxicity. (Anand et al., 2008). Nanoparticles can improve the biodistribution of drugs, as they are able to act as carriers of anti-cancer drugs by selectively using the unique pathophysiology of tumors, such as their enhanced vascular permeability and extensive angiogenesis (Figure 4) (Cho et al., 2008). The term nanochemoprevention was recently introduced, combining nanotechnology with chemoprevention using EGCG as an encapsulated agent in polylactic acid–polyethylene glycol nanoparticles. The safety and improved efficacy of such gelantin nanoparticles has resulted in increased accumulation within the tumor and prolonged *in vivo* circulation (Vlerken et al., 2007). This is very important, as a downfall of both EGCG and curcumin is their low bioavailability (Siddiqui et al., 2009). To ensure that EGCG contained in such nanoparticles was functionally similar to free EGCG, it was essential to first test these nanoparticles *in vitro*. To accomplish this, MDA-MB-231 cells were treated with empty gelatin nanoparticles (5 μM), nanoparticles with EGCG (1 and 5 μM), or free EGCG (5 μM) for 30 min or 5 h. This was followed by the addition of HGF (30 ng/ml) and lysate preparation for Western blotting. The analysis showed that 5 μM free EGCG was able to block HGF-induced Met, AKT and ERK activation. On the other hand both the nanoparticles with and without EGCG did not inhibit HGF-induced signalling after pre-incubation of 30

min. However, at the 5 h time-point the gelatin coated nanoparticle containing EGCG blocked HGF-induced signalling, therefore demonstrating that EGCG activity was retained but released slowly (Shutava et al., 2009).

Recently, curcumin nanoparticles have shown enhanced bioavailability and greater cytotoxicity against breast cancer cells. For example, silk fibroin-derived curcumin nanoparticles (< 100 nm) exhibited higher uptake, intracellular residence time and efficacy against HER2 positive MDA-MB-453 breast cancer cells (Gupta et al., 2009). In another study, curcumin-PLGA nanoparticle formulation elicited an enhanced inhibitory effect on the growth of MDA-MB-231 cells compared with curcumin alone (Yallapu et al., 2010). Furthermore, curcumin-PLGA-PEG nanoparticles showed a concentration-dependent anti-proliferative effect toward MDA-MB-231 cells. It was observed that curcumin nanoparticle formulation had a higher bioavailability and longer half-life in rats compared to curcumin. Specifically, after intravenous administration of curcumin or curcumin-nanoparticle (2.5 mg/kg), the serum levels of curcumin were almost twice as high in the curcumin-nanoparticle treated rats (Anand et al., 2010). Curcumin (Cur-OEG) nanoparticles have also been studied for their anticancer effect in both *in vitro* and *in vivo* models of breast cancer. Curc-OEG nanoparticles showed broad *in vitro* antitumor activity toward several human cancer cells with an IC_{50} value of 1.4 µg/ml in MDA-MB-468 cells. These particles showed safety and efficacy *in vivo*, as a single 25 mg/kg intravenous injection of Curc-OEG nanoparticles was non-toxic to the mice and exhibited improved bioavailability and significant tumor suppression after 48 h in an MDA-MB-468 xenograft model.

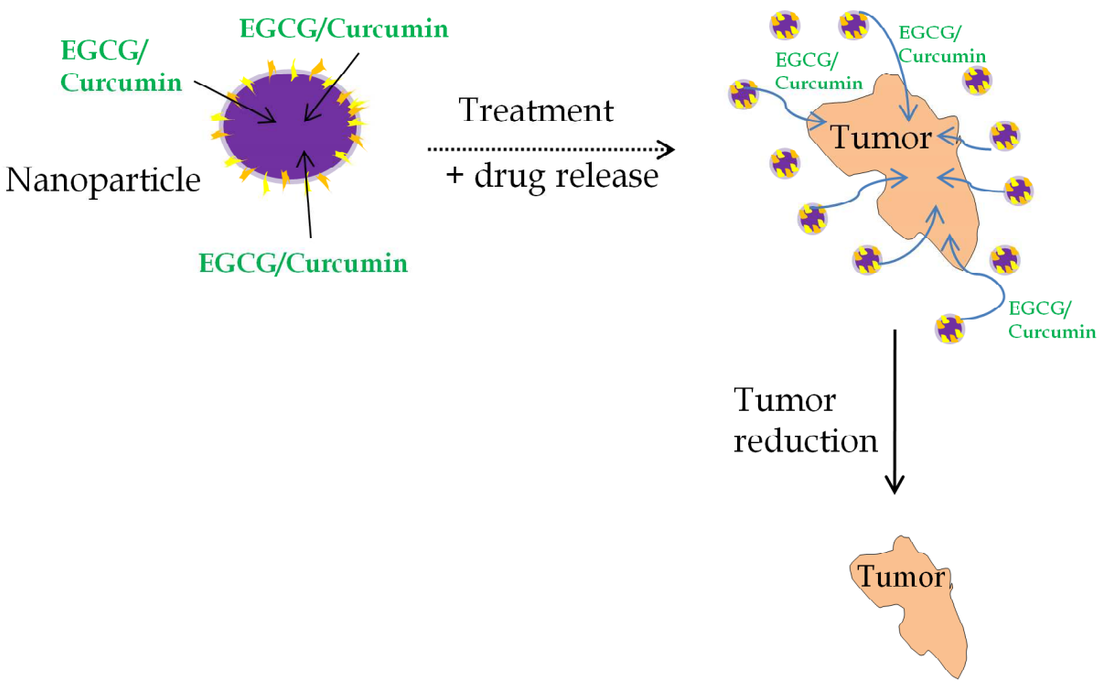


Fig. 4. Schematic of the principles of nanoparticle drug delivery. Nanoparticles, commonly consist of various different polymers and polyelectrolytes, encapsulate the drug of interest and following administration show enhanced bioavailability and accumulation within the tumor. Subsequently the drug is released (blue errors) from the nanoparticle and taken up by the tumor.

Injectable sustained release poly(D,L-lacctide-co-glycolide (PLGA)-microparticles of curcumin for breast cancer chemoprevention have also been formulated. These PLGA-microparticles exhibited enhanced bioavailability compared to curcumin in mice. Specifically, a single dose of subcutaneously injected PLGA-microparticles sustained curcumin levels in the blood for a month whereas single or multiple i.p. injections of curcumin resulted in a shorter half-life (Shahani et al., 2010). In addition, the curcumin concentration was 10–30-fold higher in the lungs and brain than in the blood. Furthermore, curcumin inhibited the growth of tumors in an MDA-MB-231 mouse xenograft model by 49% compared to the mice treated with blank PLGA-microparticles (Shahani et al., 2010). Mechanisms for this effect were attributed to the down regulation of the markers of angiogenesis, metastasis and proliferation. Specifically, the curcumin PLGA-microparticles treated group showed smaller and less well developed CD31 positive microvessels compared to curcumin alone. Furthermore, treatment with curcumin PLGA-microparticles decreased the relative VEGF expression in tumors by 78%, compared with control (Shahani et al., 2010). Additionally, the relative expression of MMP-9 in tumors from the curcumin PLGA-microparticle treated group was decreased 57% compared to control, while Ki-67 and cyclin D1 were decreased by 45% and 52%, respectively. There was also a 2.5-fold increase in the number of apoptotic cells compared to blank PLGA-microparticle treatment (Shahani et al., 2010). Since repeated systemic dosing of curcumin had no effect on tumor cell proliferation, apoptosis, or the relative cyclin D1 expression, the study concluded that sustained release microparticles of curcumin are more effective than repeated systemic injections of curcumin for breast cancer chemoprevention. Thus, significant improvement in the selectivity and potency of both EGCG and curcumin can be achieved through the use of nanomedicine.

3.3 Prodrugs, analogues and synthetic derivatives

Even though preclinical research shows promising results *in vitro* and *in vivo* with natural polyphenolic compounds such as EGCG and curcumin, clinical trials have shown limited success commonly due to inefficient delivery and bioavailability of these agents (Siddiqui et al., 2009). Thus, bioavailability is one of the major downfalls of these compounds. Additional strategies for improving these compounds include the synthesis of prodrugs, analogues and synthetic derivatives. All of these techniques aim to produce a compound with greater stability, bioavailability and ultimately efficacy.

Various studies have shown that biotransformation reactions, in particular methylation but potentially also glucuronidation and sulfate conjugation, modify the hydroxyl groups of EGCG, resulting in reduced biological activities (Landis Piwowar et al., 2007, Okushio et al., 1999). To prevent this, one group has synthesized novel fluoro-substituted EGCG pro-drug analogues by eliminating the reactive hydroxyl groups and replacing them with either peracetate groups (Pro-EGCG) or one or two fluorine(s) at the meta-position (Pro-F-EGCG2) or the meta- and para-positions on the phenyl ring (Pro-F-EGCG4) (Figure 5)(Yang et al., 2010). These analogues (50 mg/kg) were given daily via subcutaneous injections for 31 days to mice bearing MDA-MB-231 xenografts. Tumor growth was suppressed by ~63% by Pro-EGCG compared to control, whereas Pro-F- EGCG2 and Pro-F-EGCG4 were slightly more effective as tumor growth was reduced by ~67% and ~70%, respectively, compared to control (Yang et al., 2010). As an indicator of apoptosis, PARP cleavage (65 kDa) was found to a greater extent in tumors from mice treated with the fluorosubstituted analogues. Furthermore, the TUNEL assay showed apoptotic cells in the tumors of the animals treated with Pro-F-EGCG2 or Pro-F-EGCG4 but not in untreated control group. Cells with apoptotic

nuclei were also shown in the treated animals. The proteasomal chymotrypsin-like activity was reduced by 33% and 42% in animals treated with Pro-F-EGCG2 or Pro-F-EGCG4, respectively, compared to control. Additionally, it was shown using Western blotting, that the proteasome substrates p27 and Bax were increased in EGCG-analogue treated animals indicating that the proteasome activation may be a cellular target of the EGCG analogues (Yang et al., 2010). Therefore, prodrugs of EGCG are emerging as an experimental therapy with potential for clinical translation.

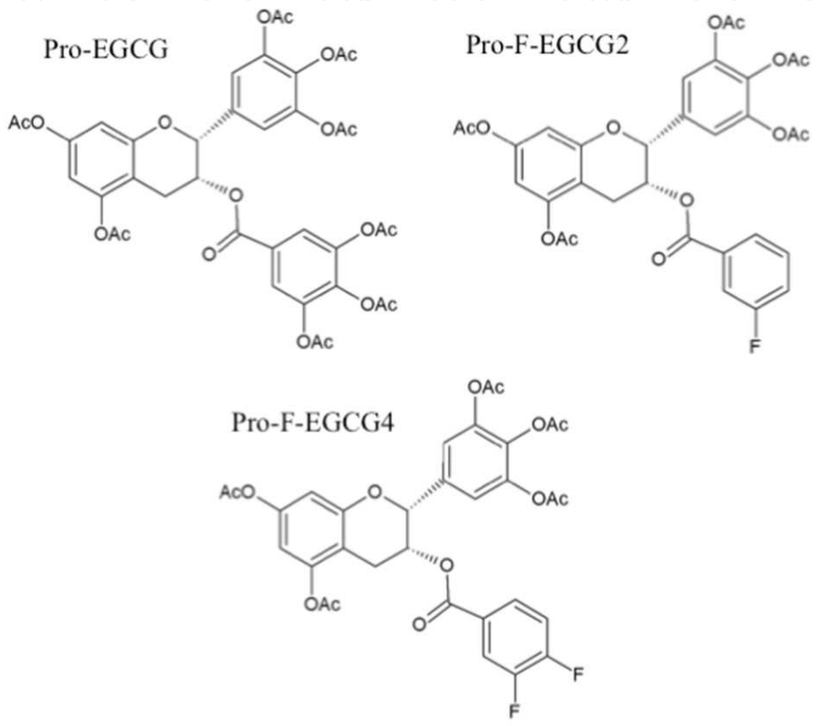


Fig. 5. Chemical structures of EGCG analogues (Yang et al., 2010).

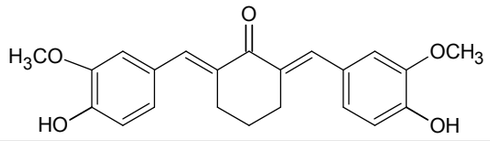
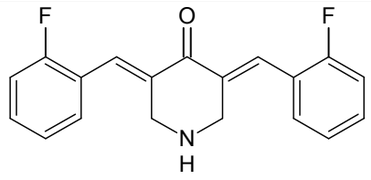
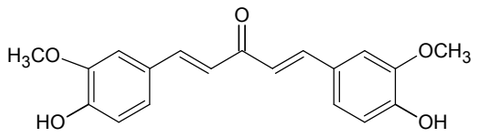
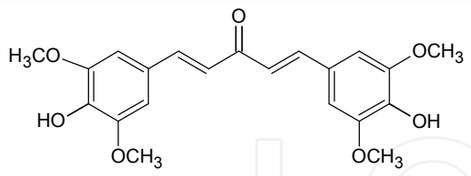
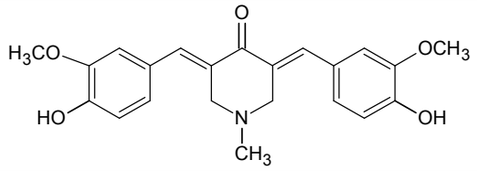
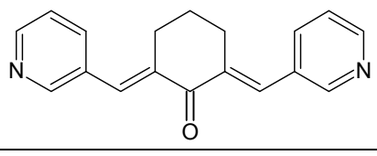
Development of curcumin analogues has developed as a strategy to enhance bioavailability and selectivity towards cancer cells. Modification of the aromatic rings and β-diketone moiety of curcumin has led to different curcumin analogues with improved activities (Table 1)(Mosley et al., 2007). The first-generation curcumin derivatives were the cyclohexanones, which exhibited enhanced activity and stability in biological medium compared to curcumin. For example, the cyclohexanone-containing curcumin derivative 2,6-bis ((3-methoxy-4-hydroxyphenyl) methylene)-cyclohexanone (BMHPC) was cytotoxic toward ER-negative breast cancer cells (IC50 of 5.0 μM) and displayed anti-angiogenic properties in human and murine endothelial cell lines (Adams et al., 2004). These results led the authors to further synthesize several fluorinated derivatives, one of which (EF-24)(Table 1) exhibited potent cytotoxicity toward MDA-MB-231 cells (IC50 of 0.8 μM) (Adams et al., 2005). Moreover, EF-24 induced breast tumor regression in athymic nude mice. Specifically, tumor weight following 20 mg/kg was decreased by ~30% compared to control, whereas 100 mg/kg decreased tumor weight by 55%. Interestingly, no toxicity was observed at a dose of 100 mg/kg, which was well below maximum tolerated dose (MTD) of 200 mg/kg (Adams et al., 2005). Mechanistic studies were also performed in MDA-MB-231 cells. Specifically, EF-24

(10 μM) inhibited cell proliferation by 70-80% and arrested cells in the G2/M phase of the cell cycle. Additionally, EF24 (20 μM) increased the percentage of early apoptotic cells to 25.3 % after 72 h. Similarly, the cell population in late apoptosis increased to 45.6%. In addition, EF-24 increased intracellular ROS levels by 55% at 48 h (Adams et al., 2005). Further mechanistic studies demonstrated that EF-24 inhibited the pro-angiogenic transcription factor, HIF-1□α □at the posttranscriptional level by a VHL-dependent but proteasome-independent mechanism in MDA-MB-231 cells (Thomas et al., 2008). The therapeutic potential of EF-24 by using coagulation factor VIIa (fVIIa) as a carrier for targeted delivery of EF-24 to the tissue factor (TF) expressed in tumor cells and vascular endothelial cells and thus showed its anti-angiogenic and anti-cancer activity in breast cancer cells. They demonstrated that EF-24-FFRck-fVIIa conjugate significantly decreased the viability of the TF-expressing MDA-MB-231 and HUVEC cells in a concentration dependent manner. Furthermore, the administration of 5 intravenous injections of the EF-24-FFRck-fVIIa conjugate (containing 50 μM of EF-24) for two weeks significantly reduced the tumor size in MDA-MB-231 breast cancer xenografts. Moreover, the tumor cells showed activation of caspase 3 as a marker of apoptosis (Shoji et al., 2008).

Another set of curcumin analogues (FLLL 11 and FLLL 12)(Table 1) produced by exchanging the β-diketone moiety for an α β unsaturated ketone, exhibited more potent antitumor activity than curcumin in various ER positive and ER negative human breast cancer cells (Lin et al., 2009). The IC_{50} values for FLLL11 and FLLL12 ranged from 9 to 48 fold lower than curcumin. Furthermore, both the analogues at 10 μM inhibited STAT3, Akt and HER2/Neu pathways and induced apoptosis. The apoptosis was mediated via activation of cleaved PARP and caspase 3. These analogues were also effective in combination with doxorubicin as they exhibited a synergistic anti-proliferative effect in MDA-MB-231 breast cancer cells. In addition, the compounds inhibited anchorage independent growth and cell migration in MDA-MB-231 cells (Lin et al., 2009). In another study, Li et al, synthesized two series of monoketone curcumin analogues namely, heptadienone and pentadienone series and investigated their anti-cancer properties *in vitro*. Among the 24 compounds that were synthesized, compound 23 (Table 1) was the most potent analogue with IC_{50} values in sub-micromolar range in MDA-MB-231 cells (Fuchs et al., 2009). Various 1,5-diarylpentadienon containing curcumin analogues with an alkoxy substitution on aromatic rings at each of the positions 3 and 5 have also been synthesized (Ohori et al., 2006). One of the analogues, GO-YO30 (Table 1) showed substantially higher cytotoxicity and anchorage independency compared to curcumin in MDA-MB-231 cells. Furthermore, it also inhibited STAT activity in a dose-dependent manner. Interestingly, GO-YO30 induced apoptosis in MDA-MB-231 at concentrations far lower than those required to elicit a comparable effect following curcumin treatment (Hutzen et al., 2009).

Monoketo curcumin analogues with a piperidone ring impart a rigid confirmation that has led to a broad spectrum of antitumor activity. Compound, 8 and 18 (Table 1) bearing the n-alkyl piperidone group showed potent cytotoxic activity towards various breast cancer cells (Youssef & El-Sherbeny, 2005). This structure was recently further modified by replacing the methylene groups and the two carbonyl groups in curcumin by N-methyl-4 piperidone. The resulting compound 5-bis (4-hydroxy-3- methoxybenzylidene)- N - methyl-4-piperidone (PAC) (Table 1) was 5 times more effective than curcumin in inducing apoptosis in ER negative breast cancer cells (MDA-MB-231, BEC114) (Al-Hujaily et al., 2010). Also, it's pro-apoptotic effect was 10 times higher against ER negative breast cancer cells than against ER positive cells (MCF-7, T-47D). Cell cycle analysis revealed that PAC (10 μM) treatment of

MDA-MB-231 cells increased the proportion of cells undergoing G2/M phase arrest by 185%. Furthermore, at 10 μM, PAC induced apoptosis in 55% of MDA-MB-231 cells (Al-Hujaily et al., 2010). PAC exhibited its cytotoxic effect by down regulating the expression of NFκB, survivin and its downstream effectors cyclin D1 and Bcl-2 and subsequently showed up-regulation of $p21^{WAF1}$ expression both *in vitro* and *in vivo*. Interestingly, PAC (100 mg/kg/day) suppressed the growth of MDA-MB-231 xenografts (Al-Hujaily et al., 2010). Importantly, the solubility of PAC was 27-fold higher than curcumin and 1 h after the injection, the levels of ^{18}F-PAC in the blood was 5-fold higher than the levels ^{18}F-curcumin. These studies suggested better pharmacokinetics and tissue bio-distribution of PAC compared to curcumin in mice (Al-Hujaily et al., 2010).

Compound Name	Structure	Cell line	IC 50 (μM)
BMHPC		MDA-MB-231	5
EF-24		MDA-MB-231	0.8
FLLL11		MCF-7 MDA-MB-231 MDA-MB-468 MDA-MB-453 SKBr3	2.4 2.8 0.3 4.7 5.7
FLLL12		MCF-7 MDA-MB-231 MDA-MB-468 MDA-MB-453 SKBr3	1.7 2.7 0.3 1.3 3.8
GO-YO30		MDA-MB-231	1.2
PAC		Not known	
RL90		MDA-MB-231 SKBr3	1.54 0.51

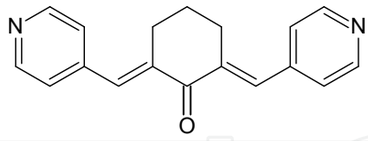
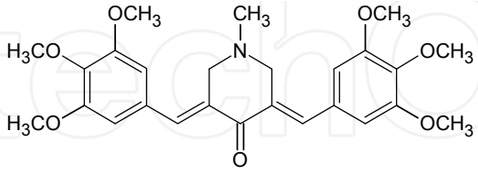
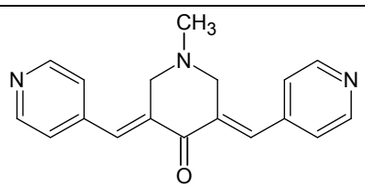
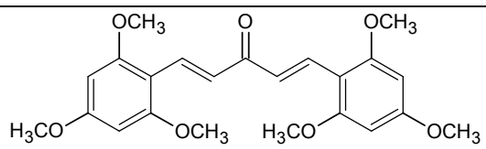
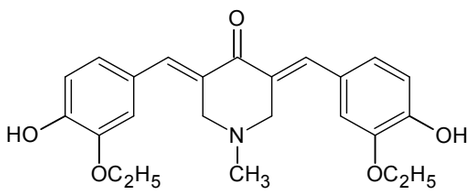
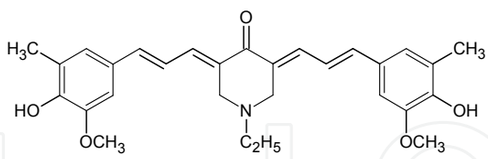
Compound Name	Structure	Cell line	IC 50 (μM)
RL91		MDA-MB-231 SKBr3	1.10 0.23
B10		MDA-MB-231 MDA-MB-468 SKBr3	0.3 0.3 0.4
B1		MDA-MB-231 MDA-MB-468 SKBr3	0.8 0.5 0.6
Compound 23		MCF-7 MDA-MB-231	0.4 0.6
Compound 8		MCF-7 MDA-MB-31 MDA-MB-435 HS-578T BT-549 T-47D	2.3 17.9 6.8 5.4 32.8 15.1
Compound 18		MCF-7 MDA-MB-31 MDA-MB-435 HS-578T BT-549 T-47D	3.3 2.8 3.7 3.8 2.6 2.7

Table 1. Chemical structures of curcumin derivatives and their relative *in vitro* potency.

Second generation curcumin analogues have been synthesized by replacing the phenyl group of cyclohexanone curcumin derivatives with heterocyclic rings. Two analogues, 2,6-bis(pyridin-3-ylmethylene)-cyclohexanone (RL90) and 2,6-bis(pyridin-4-ylmethylene)-cyclohexanone (RL91)(Table 1) showed potent cytotoxic towards ER negative breast cancer cells (MDA-MB-231, SKBr3) and modulated the expression of variety of cell signaling proteins such as EGFR, Akt, HER2, β-catenin and NFκB. Treatment with RL90 and RL91 also showed activation of stress kinases, as evidenced by phosphorylation of both JNK1/2 and p38 MAPK. Furthermore, RL90 and RL91 produced cell cycle arrest at G2/M phase in MDA-MB-231 and SKBr3 cells. Specifically, treatment of MDA-MB-231 cells with RL90 (3 μM) or RL91 (2.5 μM) significantly increased the proportion of cells in G2/M phase by 52 and 49% compared to control, respectively. RL90 and RL91 also increased the proportion of

apoptotic cells by 164% and 406% of control, respectively (Somers-Edgar et al., 2011). Thus, these second-generation curcumin derivatives are more potent *in vitro* than first generation derivatives such as BMHPC.

Another set of cyclohexanone analogues of curcumin included modification resulting in N-methylpiperidone, tropinone or cyclopentanone core groups. The aromatic substitutions on these compounds included pyrrole, imidazole, indole flouro-pyridines as well as trimethoxyphenyl and dimethoxyphenyl groups. The resulting compounds were screened for their cytotoxicity in TNBC cells. Among 18 analogues examined, 3,5-bis (pyridine-4-yl)-1-methylpiperidin-4-one (B1) and 3,5-bis (3,4,5-trimethoxybenzylidene)-1-methylpiperidin-4-one (B10) (Table 1) showed potent cytotoxicity towards MBA-MB-231 and MDA-MB-468 cells with IC_{50} values below 1 μM (Yadav et al., 2010). Furthermore, B1 and B10 induced apoptosis in MDA-MB-231 cells. Specifically, B1 (2 μM) significantly increased the proportion of apoptotic cells by 4-fold compared to control at 12 h whereas B 10 (1 μM) was more potent as there was a 10-fold increase in the proportion of apoptotic MDA-MB-231 cells after 24 h (Yadav et al., 2010).

The last set of compounds involves a series of mono-carbonyl analogues of curcumin using three different 5-carbon linkers, namely cyclopentanone, acetone, and cyclohexanone with various substituents on aryl rings. They reported that all the analogues had enhanced stability *in vitro* and improved pharmacokinetic profile *in vivo*. In this study, 500 mg/kg of compound B02 and B33 (Table 1) were orally administered to male Sprague-Dawley rats. The peak plasma concentrations of B02 and B33 were increased to 0.82 μg/ml and 4.1 μg/ml, respectively, compared to curcumin (0.091 μg/ml). This correlated with a decrease in the plasma clearance of both drugs (B02 was 125.4 l/kg/h and B33 38.98 l/kg/h) compared to curcumin (835.2 l/Kg/h). Furthermore, the half-life of B02 was increased 2-fold greater than curcumin and absorption of B33 was rapid. In addition, the analogues with acetone or cyclohexanone spacer groups showed increased cytotoxicity against several tumor cell lines. Interestingly, the analogues in which the benzene ring was replaced by a hetero aromatic ring enhanced the cytotoxic activity of these mono-carbonyl analogues (Liang et al., 2009). Overall, these studies all demonstrated that significant improvement occurs with each successive generation of synthetic analogues.

4. Conclusion

There is a large body of evidence demonstrating that the natural compounds EGCG and curcumin have potent actions in both *in vitro* and *in vivo* models of triple negative breast cancer. While these compounds both have many beneficial actions, they are hindered by poor bioavailability. However, their knowledge base of mechanistic actions have allowed them to be improved by various methods such as; 1) use in combination therapies, 2) imported via specific targeting via nanomedine and 3) improved via chemical modification into prodrugs or new structural analogues. Therefore, it is likely that a novel therapy for triple negative breast cancer will emerge as a synthetic derivative of one of these natural compounds that may ultimately be delivered to the tumor via nanomedicine.

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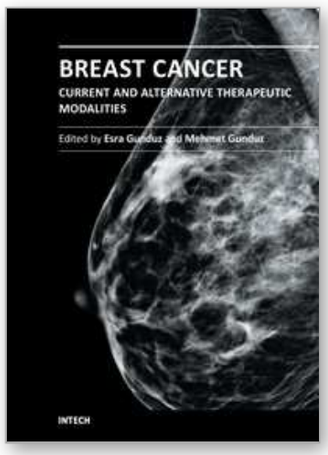

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Cancer is the leading cause of death in most countries and its consequences result in huge economic, social and psychological burden. Breast cancer is the most frequently diagnosed cancer type and the leading cause of cancer death among females. In this book, we discussed various therapeutic modalities from signaling pathways through various anti-tumor compounds as well as herbal medicine for this deadly cancer. We hope that this book will contribute to the development of novel diagnostic as well as therapeutic approaches.

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