Combining chemo-, hormonal and targeted therapies to treat breast cancer (Review)

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Abstract. Breast cancer ranks as the second most common cause of cancer death among women in the United States. Anticancer agents are an important component of breast cancer therapy. Drugs frequently used to treat breast cancer include methotrexate, 5-fluorouracil (5-FU), cyclophosphamide, anthracyclines, taxanes, trastuzumab, tamoxifen and aromatase inhibitors. These agents inhibit breast cancer progression by a variety of different mechanisms. Mutations may occur in cancer cells, which result in the elevated expression or constitutive activation of various growth factor receptors. The Raf/MEK/ERK and PI3K/Akt pathways are often activated by mutations in these growth factor receptors. These pathways are regulated by upstream Ras, which is mutated in 20-30% of human cancers. Downstream B-Raf and PI3K are also activated by mutation. Many of the events elicited by the Raf/MEK/ERK and PI3K/Akt pathways have direct effects on survival and the proliferative pathways. Aberrant regulation of the Raf/MEK/ERK and PI3K/Akt pathways can contribute to uncontrolled cell growth and lead to malignant transformation. Effective targeting of these pathways may result in the suppression of cell growth and the death of malignant cells. This review focuses on the targeting of the Raf/MEK/ERK and PI3K/Akt pathways with small molecule inhibitors, as well as on the effects of conventional chemo- and hormonal therapies in the treatment of breast cancer.

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1. Introduction

Over 210,000 new cases of breast cancer are diagnosed in the United States each year (1). Accordingly, approximately 1 in 7 women in the United States will be diagnosed with breast cancer during their lifetime (2). It is the cause of death of over 40,000 women in the United States each year, ranking it as the second most common cause of cancer death among women, and is a leading cause of cancer death in developed countries worldwide. There is consequently an urgent need to improve breast cancer therapy. Many drugs have been demonstrated to extend survival in breast cancer patients. The anticancer agents commonly used to treat the disease include methotrexate, 5-fluorouracil (5-FU), cyclophosphamide, anthracyclines,
taxanes, trastuzumab, tamoxifen and aromatase inhibitors. The mechanisms by which these agents inhibit breast cancer progression vary from drug to drug.

2. Methotrexate and 5-fluorouracil

DNA synthesis requires thymidine 5'-triphosphate (TTP), which is synthesized from thymidine 5'-monophosphate (TMP). Thymidylate synthetase generates TMP by catalyzing the transfer of a methyl group from N5,N10-methylene tetrahydrofolate to 2'-deoxyuridine 5'-monophosphate (dUMP). Methotrexate and 5-FU treatment each prevent TMP synthesis. Thymidylate synthetase is irreversibly inhibited by 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP), which is produced by 5-FU. In contrast, methotrexate treatment blocks TMP synthesis by preventing the synthesis of N5,N10-methylene tetrahydrofolate.

Methotrexate contains a single glutamic acid residue. Folyopoly-γ-glutamate synthetase (FPGS) catalyzes the addition of one or more glutamic acid moieties to methotrexate. Methotrexate and its polyglutamylated derivatives inhibit dihydrofolate (DHF) reductase (DHFR). DHFR reduces folate to DHF and DHF to tetrahydrofolate (THF). Serine hydroxy-methyltransferase converts THF to N5,N10-methylene tetrahydrofolate. Methotrexate treatment reduces TMP production by eliminating a source of N5,N10-methylene tetrahydrofolate synthesis. Reduction of TMP levels by treatment with methotrexate or 5-FU inhibits TTP production. This blocks cell proliferation by preventing DNA synthesis.

3. Cyclophosphamide

The hepatic metabolism converts cyclophosphamide to 4-hydroxycyclophosphamide, the tautomerization of which yields aldophosphamide. Acrolein and N,N-bis-(2-chloroethyl) phosphorodiamidate are produced by the spontaneous cleavage of aldophosphamide. DNA is alkylated by N,N-bis-(2-chloroethyl) phosphorodiamidate at multiple sites. The N7 position of guanine is a site that is particularly susceptible to alkylation by N,N-bis-(2-chloroethyl) phosphorodiamidate. Alkylation of the N7 position of guanine caused by cyclophosphamide treatment stabilizes the enol tautomer of guanine, which causes guanine to pair with thymine instead of cytosine. DNA damage caused by cyclophosphamide treatment induces apoptotic cell death (3,4).

4. Anthracyclines

Anthracyclines frequently used to treat breast cancer include doxorubicin (Adriamycin) and epirubicin. Anthracyclines disrupt DNA structure by intercalating between adjacent DNA base pairs. Disruption of the DNA structure by anthracyclines inhibits the synthesis of both DNA and RNA (5,6). The intercalation of anthracyclines within DNA also induces DNA cleavage mediated by topoisomerase II isoforms (7,8), including topoisomerase Iα, topoisomerase Iα-1 and topoisomerase IIB-2 (9-12). This DNA cleavage likely makes an important contribution to the cytotoxicity of anthracyclines, because less expression of topoisomerase Iα is correlated with decreased anthracycline sensitivity (13). These anthracyclines induce apoptotic cell death.

5. Taxanes

The mitotic spindle of a dividing cell functions to distribute chromatids to each daughter cell. Mitotic spindles are composed of microtubules. Cellular control of microtubule polymerization and depolymerization is essential for proper spindle function. Taxanes disrupt mitotic spindle function by stabilizing the microtubules (14-16), which are assembled from tubulin heterodimers. Tubulin heterodimers are composed of α-tubulin and β-tubulin. Taxanes stabilize microtubules by binding to β-tubulin. Disruption of mitotic spindle function by taxanes prevents cell division. Taxanes frequently used to treat breast cancer include paclitaxel and docetaxel.

6. Trastuzumab

Trastuzumab (Herceptin) is a humanized mouse monoclonal immunoglobulin G1 (IgG1) κ antibody that binds to the extracellular domain of HER2 (17,18). HER2 is amplified and overexpressed in 20-30% of breast cancers and is associated with a poor prognosis. Endocytic degradation of HER2 is accelerated by binding to trastuzumab. The induction of HER2 degradation by trastuzumab decreases the activity of the signal transduction cascades downstream of HER2 that promote cell cycle progression and inhibit apoptosis. These signal transduction cascades include the Raf/MEK/ERK and PI3K/PDK/Akt pathways. Their deactivation by trastuzumab treatment, which is most effective for breast cancers with either high HER2 protein levels or amplification of the gene encoding HER2 (19), prevents cell proliferation.

7. Tamoxifen

Tamoxifen and its metabolites inhibit the proliferation of breast cancer cells by binding to estrogen receptors (ERs) (20). ER isoforms include ERα and ERβ (21-27), which bind together in both homodimeric and heterodimeric combinations (28-31). ERs are transcription factors that induce the expression of the proteins that promote cell cycle progression and inhibit apoptosis. These proteins include cyclin D1, which promotes cell cycle progression, and Bcl-2, which prevents apoptosis (32,33). The binding of ERs to estrogens stimulates the transcription of these genes. Tamoxifen and its metabolites compete with estrogens for the same ERα and ERβ ligand binding sites and, upon binding, prevent the estrogens from promoting cell proliferation. This is because the transactivation potential of ERs bound to tamoxifen is less than that of ERs bound to estrogens. The treatment of MCF-7 cells with tamoxifen decreased the levels of Bcl-2 mRNA, as well as of Bcl-2 protein (34).

The transactivation potential of ERs bound to tamoxifen or its metabolites is dependent on whether ERα or ERβ isoforms are present. Tamoxifen is a partial agonist for ERα homodimers, but is a pure antagonist for ERβ homodimers (35). The hepatic metabolism converts tamoxifen to 4-hydroxytamoxifen (4-HT), similar to tamoxifen in that it is a partial agonist for ERα homodimers yet a pure antagonist for ERβ homodimers. The estrogenic effects of tamoxifen treatment are likely responsible for its stabilization of bone mineral density (BMD), as well as its association with increased frequencies of endometrial cancer and thromboembolic disease (36-38).
Tumor biopsy specimens from breast cancer patients are analyzed for ERα expression by immunohistochemistry to determine whether tamoxifen treatment is appropriate (39). Tamoxifen is only administered to breast cancer patients with ERα positive (ER+) tumors, as its therapeutic benefit is substantially higher in breast cancer patients with ER+ tumors than in those with ER negative (ER-) tumors (40,41). Coadministration of tamoxifen together with chemotherapeutic drugs is more effective than the administration of the same chemotherapeutic drugs without tamoxifen for the treatment of breast cancer patients with ER+ tumors, but not for those with ER- tumors (42).

8. Aromatase inhibitors

Estrogen biosynthesis is dependent on aromatase. Aromatase inhibitors prevent the proliferation of breast cancer cells by blocking estrogen production. There are two classes of aromatase inhibitors, which differ in chemical structure and mechanism of action. Non-steroidal aromatase inhibitors, such as anastrozole and letrozole, bind reversibly to aromatase. In contrast, steroidal aromatase inhibitors, such as exemestane, bind irreversibly to aromatase. Anastrozole and letrozole have each been reported to be superior to tamoxifen in first-line therapy for post-menopausal patients with hormone receptor-positive advanced breast cancer (43-46).

9. Overview of the Ras/Raf/MEK/ERK pathway

The Ras/Raf/MEK/ERK pathway is activated by many growth factors and cytokines important in driving proliferation and preventing the apoptosis of breast cells (47-51). An overview of the effects of the Ras/Raf/MEK/ERK pathway on the downstream signaling pathways leading to the growth and prevention of apoptosis is presented in Fig. 1. After receptor ligation, Shc, an Src homology (SH)-2 (SH2)-domain containing protein, becomes associated with the c-terminus of the growth factor receptor (52-54) and recruits the GTP-exchange complex Grb2/Sos, resulting in the loading of membrane-bound Ras with GTP (55,56). Ras:GTP then recruits Raf to the membrane and is activated, likely by an Src-family tyrosine (Y) kinase (57-59). Raf is responsible for the phosphorylation of mitogen associated/extracellular regulated kinase-1 (MEK1) (60-62). MEK1 phosphorylates extracellular regulated kinases 1 and 2 (ERKs 1 and 2) on specific threonine (T) and Y residues (60-62). Activated ERK1 and ERK2 serine (S)/T kinases phosphorylate and activate a variety of substrates including p90Rsk1 (63-70), which can activate the cyclic-AMP response element binding protein (CREB) transcription factor (65). Moreover, ERK can translocate to the nucleus and phosphorylate additional transcription factors, such as Elk1, CREB and Fos, which bind the promoters of many genes, including the growth factor and cytokine genes important in stimulating the...
growth and survival of multiple cell types, including breast cells (63,71-81). The Raf/MEK/ERK pathway can also modulate the activity of many proteins involved in apoptosis, including Bcl-2, Bad, Bim, Mcl-1, caspase 9 and survivin (82-91). Raf-1 has many roles, independent of MEK and ERK, that are involved in the prevention of apoptosis (51). These non-MEK/ERK effects are not sensitive to inhibition by MEK inhibitors, but may be sensitive to Raf inhibitors if they are dependent on Raf kinase activity.

Recently, Raf-1 was shown to interact with mammalian sterile 20-like kinase (MST-2) and prevent its dimerization and activation (92). MST-2 is activated by pro-apoptotic agents, such as staurosporine and Fas ligand. Raf-1, but not B-Raf, binds MST-2. Depletion of MST-2 from Raf-1-/- cells abrogated sensitivity to apoptosis, while overexpression of MST-2 increased sensitivity to apoptosis. It was proposed that Raf-1 might control MST-2 by sequestering it into an inactive complex. This complex of Raf-1:MST-2 is independent of MEK and downstream ERK. Raf-1 can also interact with the apoptotic signal kinase (ASK1) to inhibit apoptosis (51,93).

The Raf/MEK/ERK pathway can phosphorylate the BH3-only domain protein Bim (89,109). When Bim is phosphorylated by ERK, it is targeted for ubiquitination and degradation in the proteosome (91). In contrast, JNK can phosphorylate Bim at S65, which enhances its ability to induce Bax activation and stimulates apoptosis (104). Mcl-1 can also bind pro-apoptotic Bak (107). This Mcl-1:Bak interaction can be disrupted by the binding of the BH3-only domain Noxa protein, which results in Mcl-1 being ubiquitinated and degraded in the proteosome (103). Bak can then form active dimers and induce apoptosis. Unlike Bcl-2 and Bcl-XL, the half-life of the Mcl-1 protein is short due to the amino terminal PEST sequence, and its expression is regulated by both transcriptional and post-translational mechanisms (110). Certain chemotherapeutic drugs, such as taxol, will induce Mcl-1 phosphorylation at sites other than those phosphorylated by ERK (T163) (88). Oxidative stress can activate JNK, which induces the phosphorylation of Mcl-1 on S121 and T163 (111). Cytokine deprivation of certain cells induces GSK-3ß, which in turn induces the phosphorylation of Mcl-1 at S159, resulting in its ubiquitination and degradation (112).

The expression of the BH3-only domain Puma and Noxa proteins is under the control of the p53 and PI3K/Akt pathways (113). Noxa interacts specifically with Mcl-1, but not with Bcl-2 or Bcl-XL (107). Bak associates with Mcl-1 and Bcl-XL, but not Bcl-2. Upon the induction of Puma and Noxa by p53, Puma and Noxa displace Mcl-1 from Bak, which is then able to oligomerize and induce apoptosis. This may lead to Mcl-1...
degradation and apoptosis. The Raf/MEK/ERK pathway increases Mcl-1 protein levels and stability, which may lead to an increase in Mcl-1 associated with Noxa and Puma, and a decrease in free Bak levels and less apoptosis. Targeting Raf/MEK/ERK could stimulate apoptosis by decreasing Mcl-1 levels and altering its phosphorylation state.

Human caspase 9 was originally thought to be phosphorylated by Akt, but the murine caspase 9 lacks the Akt consensus phosphorylation site (66). Caspase 9 is phosphorylated by the Raf/MEK/ERK pathway at T125, which inhibits the activation of the caspase cascade (69). Mcl-1 is a substrate for activated caspase 3, thus decreased caspase 9 activation by ERK phosphorylation will reduce caspase 3 activation and Mcl-1 cleavage, and apoptosis will be suppressed. Targeting Raf/MEK/ERK could increase caspase 9 activation and increase the apoptosis of breast cancer cells.

11. Role of the Ras/Raf/MEK/ERK pathway in neoplasia

Effective targeting of signal transduction pathways activated by mutations and gene amplification may be an effective means of limiting cancer growth and metastasis. The Raf/MEK/ERK pathway can be activated by mutations/amplifications of upstream growth factor receptors. An illustration of some of the receptors, kinases and phosphatases mutated/amplified in human cancer, and how they may impact the Raf/MEK/ERK cascade, is presented in Fig. 3.

Mutations that lead to the expression of constitutively-active Ras proteins have been observed in ~20-30% of human cancers (114,115). These are often point mutations which alter key residues affecting Ras activity, although amplification of Ras is also detected in some tumors. Mutations that result in increased Ras activity also perturb the Raf/MEK/ERK kinase cascade.

B-Raf has been reported to be mutated in ~7% of all cancers (116). However, this frequency may change as increasing numbers of diverse tumors are examined for B-Raf mutation. Recent studies have indicated the presence of mutated alleles of Raf-1 in therapy-induced acute myelogenous leukemia (t-AML) (117), arising after the chemotherapeutic treatment of breast cancer patients. The mutated Raf-1 genes detected were transmitted in the germ line, and were thus not a spontaneous mutation in the leukemia, but rather may have been associated with the susceptibility to induction of t-AML in the Austrian breast cancer patients.

For many years, the Raf oncogenes were not thought to be frequently mutated in human cancer; more attention to the susceptibility to induction of t-AML in the Austrian breast cancer patients. The mutated alleles of Raf-1 have been found in melanoma and thyroid cancer. It has been proposed that B-Raf mutations may occur in certain cells that express high levels of B-Raf due to hormonal stimulation. Certain hormonal signaling events will elevate intracellular cAMP levels and result in B-Raf activation, leading to proliferation. Melanocytes and thyrocytes are two such cell types, which have elevated B-Raf expression as they are often stimulated by the appropriate hormones (122). Moreover, it is now thought that B-Raf is the most important kinase in the Raf/MEK/ERK cascade (116), with mutation at B-Raf activating downstream MEK and ERK. In some models, wild-type and mutant B-Raf activate Raf-1, which in turn activates MEK and ERK (116,123,124). Multiple pharmaceutical/biotechnological companies are attempting to develop inhibitors which specifically target mutant B-Raf alleles, but do not inhibit WT B-Raf.
In some cells, B-Raf mutations are believed to be initiating events, though not sufficient for full-blown neoplastic transformation (125,126). Moreover, there appear to be cases in which certain B-Raf (V600E) and Ras mutations are not permitted in the transformation process, as they might result in the hyperactivation of Raf/MEK/ERK signaling and expression and lead to cell cycle arrest (118). In contrast, there are other situations that depend on the particular B-Raf mutation and require both B-Raf and Ras mutations for transformation. The B-Raf mutations in these cases result in weaker levels of B-Raf activity (118,126).

Different B-Raf mutations have been mapped to various regions of the B-Raf protein. Some of the other B-Raf mutations are believed to result in B-Raf molecules with impaired B-Raf activity, which must signal through Raf-1 (116,123). Heterodimerization between B-Raf and Raf-1 may allow the impaired B-Raf to activate Raf-1. Other mutations, such as D593V, may activate alternative signal transduction pathways (116).

12. Overview of the PI3K/Akt pathway

Growth factor/cytokine receptor ligation also leads to rapid activation of phosphatidylinositol 3-kinase (PI3K) (127). PI3K consists of an 85-kDa regulatory subunit, containing SH2 and SH3 domains, and a 110-kDa catalytic subunit (127,128,130). Cytokine stimulation often creates a PI3K binding site on the cytokine receptor, which the p85 subunit SH2 domain associates with (127-129). The p85 subunit is then phosphorylated, leading to the activation of the p110 catalytic subunit. Activated PI3K phosphorylates the membrane lipid phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P2] to phosphatidylinositol (3,4,5)-tris-phosphate [PtdIns(3,4,5)P3], which activates PI3K-dependent kinase (PDK1). PDK1 then phosphorylates Akt at threonine 308 (T308), and a second kinase phosphorylates Akt on serine 473 (S473) (131-135).

Akt can transduce an anti-apoptotic signal by phosphorylating the downstream target proteins involved in the regulation of cell growth [e.g., glycogen synthase kinase-3β (GSK-3β), ASK1, Bim, Bad, MDM-2, p21Cip1], X-linked inhibitor of apoptosis (XIAP) and the Foxo3α transcription factor] (108,131,136-145). Phosphorylated Foxo3α loses its ability to induce Fas, p27Kip1, Bim, Noxa and Puma gene transcription (146,147). Akt also phosphorylates IκB, which subsequently phosphorylates IκB, resulting in its ubiquitination and subsequent degradation in proteosomes (148-160). The disassociation of IκB from NF-κB enables NF-κB to translocate into the nucleus to promote gene expression. The PI3K/Akt pathway can also phosphorylate and activate CREB, which regulates the transcription of anti-apoptotic genes, including Mcl-1 and Bcl-2 (96,161,162). The PI3K pathway also results in the activation of the mammalian target of rapamycin (mTOR) and ribosomal protein kinases such as p70S6K (163-170). It is worth noting that Akt can cause the activation of specific substrates (e.g., IκBα and CREB) or may mediate the inactivation of other proteins [e.g., Raf-1, B-Raf (by the Akt related kinase SIK), p21Cip1, Bim, Bad, procaspase 9, Foxo3α and GSK-3β].

The PI3K pathway is negatively regulated by phosphatases. PTEN (phosphatase and tensin homologue deleted on chromosome 10) is primarily a lipid phosphatase that removes the 3-phosphate from the PI3K lipid product PtdIns(3,4,5)P3 to produce PtdIns(4,5)P2, which prevents Akt activation (129,171-175). PTEN is also a protein phosphatase (174,176,177). Two other phosphatases, SHIP-1 and SHIP-2, remove the 5-phosphate from PtdIns(3,4,5)P2 to produce PtdIns(3,4)P2 (178-182). Mutations in these phosphatases, which eliminate their activity, can lead to tumor progression. Consequently, the genes encoding these phosphatases are referred to as anti-oncogenes or tumor suppressor genes.

13. Interactions between the PI3K/Akt and Raf/MEK/ERK pathways which regulate apoptosis

Akt can phosphorylate Raf-1 and B-Raf and lead to their inactivation (183-186). Akt can also activate Raf-1 through a Ras-independent, but protein kinase C (PKC)-dependent, mechanism, which results in the suppression of apoptosis (187). The suppression of apoptosis in some cells by Raf and MEK requires PI3K-dependent signals (188-192).

Both the PI3K/Akt and Raf/MEK/ERK pathways contribute to the transcriptional regulation of Bcl-2 family members, as they can regulate CREB phosphorylation. CREB binds the Mcl-1 and Bcl-2 promoter regions (94,96,98). Moreover, both pathways phosphorylate pro-apoptotic BH3-only domain protein Bad, which prevents its apoptotic effects and leads it to become cytoplasmically localized (95,97,99,100). Another MAP kinase, JNK, phosphorylates 14-3-3 proteins and results in their disassociation from cytoplasmically-localized Bad, which then translocates to the mitochondrion (101). When Bad associates with Bcl-2 or Bcl-XL, it promotes apoptosis by preventing Bcl-2 or Bcl-XL from interacting with Bax (102-108). Bad is phosphorylated in most AML specimens, suggesting that the inhibition of Bad phosphorylation may be therapeutically important in AML (193). In contrast, the anti-apoptotic Mcl-1 protein is not reported to interact with Bad (107).

Both the Raf/MEK/ERK and PI3K/Akt pathways can phosphorylate the BH3-only domain protein Bim (89,108). When Bim is phosphorylated by ERK and Akt, it is targeted for ubiquitination and degradation in the proteosome (91). Mcl-1 can bind Bim, which prevents the activation and mitochondrial translocation of Bax (88,91). In contrast, JNK can phosphorylate Bim at S65, which enhances its ability to induce Bax activation and hence stimulates apoptosis (104). Mcl-1 can also bind pro-apoptotic Bak (107). The Mcl-1:Bak interaction can be disrupted by the binding of the BH3-only domain Noxa protein, resulting in Mcl-1 being ubiquitinated and degraded in the proteosome (108). Bak can then form active dimers and induce apoptosis. Unlike Bcl-2 and Bcl-XL, the half-life of the Mcl-1 protein is short due to the amino terminal PEST sequence, and its expression is regulated by both transcriptional and post-translational mechanisms (110).

14. Roles of the PI3K/Akt pathway in neoplasia

Some Ras mutations can result in PI3K/Akt activation (194-200). Mutations at the p85 subunit of PI3K have been detected in Hodgkin's lymphoma cells (201), and the p110 subunit of PI3K is frequently mutated (~25%) in breast and...
some other cancers, but not in leukemia (202-206). Mutations and hemizygous deletions of PTEN have been detected in AML and NHL (207-213). Increased Akt expression is linked to tumor progression and drug/hormonal resistance (214-217). SHIP mutations have been detected in AML (218,219). Thus, many possible mechanisms could lead to elevated Akt levels.

The relationship between dysregulated PI3K activity and the onset of cancer is well documented. PI3K is the predominant growth factor-activated pathway in LNCaP human prostate carcinoma cells (220,221). Other reports directly implicate PI3K activity in a variety of human tumors, including breast cancer (222), lung cancer (223), melanomas (224) and leukemia (225), among others. Activated Akt can affect the expression and regulation of the responses of hormone receptors, and can lead to the ineffectiveness of hormone ablation therapies (226-228).

Activated Akt has been reported to be detected in over 50% of primary AML samples, and is associated with a poor prognosis (229). Furthermore, the Akt pathway has been shown to be involved in the regulation of multidrug resistance protein-1 (MRP-1) and drug resistance in AML (230-233). Taken together, these data endorse the substantial role that PI3K signaling plays in oncogenesis and drug resistance. Moreover, targeted inhibition of the central components of this pathway appears to be an excellent choice for future therapeutic approaches. It has been observed that overexpression of both the Raf/MEK/ERK and PI3K/Akt pathways in AML is associated with a worse prognosis than the overexpression of a single pathway (229). Thus, the development of inhibitors which target both pathways, or the formulation of combinations of inhibitors, may prove effective in the treatment of certain cancers.

15. Signaling pathways and breast cancer

Breast cancer is among the most common form of cancer. Over 210,000 new cases are diagnosed in the USA each year and, this year alone, ~40,000 women will die from the disease. It affects about 1 in 7 women in the USA, and is the second most frequent cause of cancer death (1). Although much progress has been made in breast cancer treatment, metastatic breast cancer remains a generally incurable and fatal disease as 50% of patients die from it. Cytotoxic drug treatment is an important weapon against cancer. However, cancerous cells frequently develop drug resistance to these agents.

Breast cancer originates from genetic causes. Mutated or amplified genes are either inherited or occur sporadically. Hereditary breast cancer only accounts for about 10% of all breast cancer cases, and generally results from the lack of a tumor suppressor gene, as opposed to the gain of an oncogene. Approximately 45% of hereditary breast cancer is attributable to mutations in breast cancer-associated gene-1 (BRCA1), and an additional 45% is attributable to mutations in BRCA2 (1). Other tumor suppressor genes implicated in hereditary breast cancer include p53 and PTEN (174). The p53 tumor suppressor is a transcription factor involved in cell cycle regulation and DNA damage repair. Germline p53 mutation is present in ~50% of patients with Li-Fraumeni syndrome (LFS), which is a multicancer familial syndrome that includes adrenocortical carcinoma, brain tumors, leukemia and osteosarcomas, in addition to early onset breast cancer. Breast cancer attributable to germline p53 mutation in the absence of LFS is rare. Germline PTEN mutation is present in ~80% of patients with Cowden syndrome (174,234). This disease, also known as multiple hamartoma syndrome, is another familial syndrome that includes many different types of cancer conditions, including early onset breast cancer. Mutations have been reported to occur at PTEN in breast cancer at varying frequencies (5-21%) (235-239). Loss of heterozygosity is probably more common (30%) (236). PTEN promoter methylation leads to low PTEN expression. In one study, 26% of primary breast cancers had low PTEN levels, correlated with lymph node metastases and poor prognoses (236,238,240,241). Mutations at certain residues of PTEN, which are associated with Cowden's disease, affect the ubiquitination of PTEN and prevent nuclear translocation. These mutations leave phosphatase activity intact (242). Inhibition of PTEN activity leads to centromere breakage and chromosome instability (243). PTEN therefore has diverse activities, and the disruption of PTEN activity by various genetic mechanisms could have far-reaching effects on different processes affecting the sensitivity of breast cancers to various therapeutic approaches.

Sporadic breast cancer accounts for the remaining 90% of all breast cancer cases. The PI3K p110 catalytic subunit is mutated in ~25% of breast cancer specimens, with the mutations frequently resulting in the activation of its kinase activity (202-205,239,244). Somatic mutation of p53 is associated with many cancers, and is present in ~20% of sporadic breast cancer cases. In contrast, somatic mutation of BCRA1 or BCRA2 is rare in breast cancer patients. Another important cause of sporadic breast cancer is the amplification/overexpression of HER2, which occurs in ~20-30% of breast cancer cases. This gene encodes human epidermal growth factor (EGF) receptor-2 (HER2 a.k.a., c-ErbB-2), which is a receptor tyrosine kinase. The expression and activity of downstream signal transduction cascades, such as the Raf/MEK/ERK pathway, frequently result in the activation of its kinase activity (HER2). Akt and PKC are frequently activated in breast cancer specimens (239,245). Consequently, the Raf/MEK/ERK and PI3K/Akt pathways are therapeutic targets in breast cancer.

The association of the genes that regulate signal transduction pathways with breast cancer implies that the pathways play an important role in the disease. Perhaps the best example of this is the association of HER2 gene amplification with breast cancer. While a normal breast cell possesses 20,000-50,000 HER2 molecules, amplification of this gene can increase levels of HER2 to up to 2,000,000 molecules per cell (129,246). The overexpression of HER2 in breast cancer is linked to cometo forms of ductal carcinoma in situ and occurs in ~90% of these cases. HER2 overexpression will lead to increased expression of both the Raf/MEK/ERK and PI3K/Akt pathways.

We have observed that ERK is activated after tamoxifen treatment of the MCF-7 breast cancer cell line. This is important with regards to the sensitivity of breast cancer cells to MEK inhibitors. ERK can phosphorylate and contribute to the inactivation of the tuberous sclerosis complex (TSC-2). Akt can also phosphorylate TSC-2 at a different residue, which leads to its inactivation. This leads to mTOR activation.
Inhibition of TSC-2 phosphorylation by Raf/MEK and PI3K/Akt inhibitors may make cells more sensitive to chemo- and hormonal therapy.

Activated Akt is furthermore often upregulated in breast cancer cells, and its overexpression is associated with a poor prognosis. However, this may actually render the breast cancer cells sensitive to Akt, as well as to downstream mTOR inhibitors. The formation of the rapamycin-sensitive mTORC1 complex [consisting of mTOR, regulatory-associated protein of mTOR (Raptor) and mLST8] in drug-resistant breast cancer cells that overexpress activated Akt may be different than in drug sensitive breast cells that do not overexpress activated Akt. In cells which express activated Akt, the Akt should phosphorylate TSC-2 and result in its inactivation. The mTORC1 complex is formed and downstream p70S6K and 4E-BP1 are phosphorylated, allowing the disassociation of eIF-4E, ribosome biogenesis and protein synthesis. In contrast, in the absence of Akt and ERK activation, this complex should not be formed. Rapamycin targets this complex, hence breast cancer cells that constitutively express activated Akt are more sensitive to rapamycin than those which do not. In breast cells that do not, this complex should be transiently assembled after growth factor treatment. In contrast, the assembly of the rapamycin-insensitive mTORC2 complex [consisting of the rapamycin-insensitive companion of mTOR (Rictor), mTOR, mLST8] should be lower in cells that constitutively express activated Akt than in those that do not, as there is equilibrium between the mTORC1 and mTORC2 complexes. The significance of these complex biochemical signaling events is that drug-resistant breast cancer cells which overexpress activated Akt or lack PTEN expression have an Achilles heel with regards to therapeutic intervention, as they are highly sensitive to rapamycin treatment. Drug-resistant breast cancer may also be hypersensitive to Raf/MEK inhibitors, as ERK plays a critical role in the phosphorylation of TSC-2 and p70S6K.

16. Aberrant regulation of apoptosis may contribute to breast cancer and subsequent drug resistance

Cell death following cytotoxic drug treatment is generally apoptotic as opposed to necrotic. Many chemotherapeutic drugs induce apoptosis by activating the intrinsic cell death pathway, which involves cytochrome c release and the activation of the apoptosome-catalyzed caspase cascade. During apoptosis, activation of caspase family cysteine proteases occurs. Although, as discussed earlier, the various cytotoxic drugs differ in their mechanisms of action, each ultimately relies upon built-in apoptotic machinery to elicit cell death (130,166,174,234,247,248). Caspase family cysteine proteases are responsible for the proteolytic cleavage of the carboxyl terminal of cellular proteins to aspartate residues.

In a study involving 46 breast cancer patients, 75% lacked caspase 3 mRNA transcripts and protein expression (249). The MCF-7 cell line has a mutation in caspase 3 and is deficient in certain aspects of apoptosis. In this respect, MCF-7 cells are a stringent model for the investigation of breast cancer apoptosis (250). Caspase 9 can be activated in MCF-7 cells, which can result in the sequential activation of caspases 7 and 6 (251). Caspase 7 is activated by the apoptosome complex and forms a XIAP-caspase 7 complex. This XIAP-caspase 7 complex is more stable in MCF-7 cells due to the absence of functional caspase 3. ERK activity maintains XIAP levels; however, the mechanism by which this occurs is unknown. Resistance to chemotherapeutic drugs induced by the Raf/MEK/ERK pathway may be due in part to prolonged XIAP and caspase 9 expression, which prevents caspase 7 from exerting its effects on apoptosis (145,252). ERK phosphorylates caspase 9, inhibiting its activation. The negative regulation of the caspases by ERK represents a mechanism by which Raf/MEK/ERK pathway activation prevents apoptosis. Raf/MEK inhibitors may affect caspase 9 activation and XIAP levels, and promote the apoptosis of cancer cells.

17. Therapeutic targeting of the Raf/MEK/ERK pathway

Small molecule inhibitors, such as Imatinib, have proven effective in the treatment of CML and certain other cancers which proliferate in response to BCR-ABL (e.g., some ALLs) and for cancers which proliferate in response to mutant platelet-derived growth factor receptor (PDGF-R) and c-Kit genes (49,234,239,253-255), such as gastro-intestinal stromal tumors. Lung carcinomas which have mutations in EGFR are sensitive to EGFR inhibitors (51,246,256-260). Raf and MEK inhibitors have been developed, and some are in clinical trials (174,234,239,254,261). We have determined that a consequence of doxorubicin treatment in breast, hematopoietic and prostate cancer cell lines is the induction of ERK (51). Eliminating the deleterious side-effect of these therapies with Raf and MEK inhibitors may enhance their ability to kill drug-resistant cancer. PI3K, PDK, Akt and mTOR inhibitors have been developed, mTOR inhibitors have been used for many years as immunosuppressive drugs in kidney transplant patients, but have as a side effect the inhibition of a negative feedback pathway, which can result in Akt activation (262). Bcl-2 inhibitors have been developed which suppress Bcl-2 and Bcl-XL, but not Mcl-1 (263). MDM-2 inhibitors have been developed which enhance WT p53 stability and activity (264). These inhibitors may augment the effects of chemo-, radio- and hormonal-based breast cancer therapies. A diagram illustrating the sites of action and other inhibitors is presented in Fig. 4.

Raf inhibitors have been developed as well, and some are being evaluated in clinical trials. A few ‘Raf’ inhibitors (Sorafenib) are approved for the treatment of certain cancers (e.g., renal cell carcinoma) (265-269). Certain Raf inhibitors have been developed that are small molecule competitive inhibitors of the ATP-binding site of Raf protein. These (e.g., L-779,450, ZM 336372 or Bay 43-9006, a.k.a Sorafenib) bind the Raf kinase domain and therefore prevent its activity. Some may affect a single Raf isoform (e.g., Raf-1), others may affect similar Raf proteins (Raf-1 and A-Raf), while other pan-Raf inhibitors may affect all three Raf proteins (Raf-1, A-Raf and B-Raf). We have observed that the L-779,450 inhibitor suppresses the effects of A-Raf and Raf-1 more than the effects of B-Raf (269). Like many Raf inhibitors, L-779,450 is not specific for Raf; it also inhibits the closely-related p38MAPK. Likewise, Sorafenib inhibits other kinases besides Raf (e.g., VEGF-II Receptor, PDGF-R, Kit, FLT-3 and Fms) and is more appropriately referred to as a multi-kinase inhibitor. Knowledge of the particular Raf gene mutated or overexpressed in certain
tumors may provide critical information regarding how to treat the patient, as some cancers which overexpress a particular Raf gene may be more sensitive to inhibition by agents which target that particular Raf protein. The inhibition of some Raf proteins might prove beneficial, while the suppression of others might, under certain circumstances, prove detrimental. Thus, the development of unique and broad-spectrum Raf inhibitors may prove useful in human cancer therapy.

Chaperonin proteins, such as 14-3-3 and Hsp90, regulate Raf activity (268), which is regulated by dimerization. These biochemical properties result in Raf activity being sensitive to drugs which block protein:protein interactions, such as geldanamycin (270). Geldanamycin and its 17-allylamino-17-demethoxygeldanamycin (17-AAG) analogue are non-specific Raf inhibitors as they also affect the activity of many proteins which are stabilized by interaction with Hsp90. They are currently in clinical trials (271). We often think of a single Raf protein carrying out its biochemical activity. However, Raf isoforms dimerize with themselves and other Raf isoforms to become active. Drugs such as coumerycin, which inhibit Raf dimerization, and others such as geldanamycin, which prevent the interaction of Raf with Hsp90 and 14-3-3 proteins, suppress Raf activity. Geldanamycin has also been shown to be effective in suppressing the growth of non-small cell lung carcinoma (NSCLC) cells, which are gefitinib and erlotinib (both are EGFR inhibitors) resistant due to a second mutation in the EGFR (272). Furthermore, 17-AAG potentiated the effects of paclitaxel in ovarian breast cancer lines that expressed high levels of activated Akt (273).

An alternative approach to targeting Raf is to prevent Raf activation by targeting the kinases (e.g., Src, PKC, PKA, or Akt) and phosphatases (e.g., PP2A) involved in Raf activation. It can be predicted that some Src kinase inhibitors, such as Dasatinib, would inhibit Raf activation by suppressing Raf-1 and A-Raf, but not B-Raf, activation by Src. It is worth noting that some of these kinases normally inhibit Raf activation (Akt and PKA). A major limitation of this approach would be that these kinases and phosphatases could result in the activation or inactivation of other proteins, and would thus have other effects on cell physiology.

Currently, it is believed that MEK1 is not frequently mutated in human cancer. There was recently a report that MEK1 and MEK2, as well as B-Raf, are mutated in some patients with cardio-facio cutaneous syndrome (274). Aberrant expression of MEK1 is observed in many different cancers due to the activation of the Raf/MEK/ERK pathway by upstream kinases (e.g., BCR-ABL) and growth factor receptors (e.g., EGFR, Fms, Flt-3, PDGFR), as well as by other unknown mechanisms. Specific inhibitors to MEK have been developed (PD98059, U0126, PD184352 (a.k.a., CI1040), PD-0325901, Array MEK inhibitors (ARRY-142886 and others)) (261). Their successful development may be due to the relatively small number of phosphorylation sites on MEK involved in activation/inactivation. MEK inhibitors differ from most other kinase inhibitors as they do not compete with ATP binding. This confers a very high specificity (275). MEK inhibitors are very specific and do not inhibit many different protein kinases, including p38MAPK and JNK (276). The crystal structures of MEK1 and MEK2 have been determined as ternary complexes with ATP and PD184352, and have revealed that both MEK1 and MEK2 have unique inhibitor binding sites located on a hydrophobic pocket adjacent to, but not overlapping, the ATP

Figure 4. Targeting signal transduction pathways important in breast cancer. Potential sites of action of small molecular weight inhibitors and cytotoxic antibodies are indicated. In some cases, inhibitors will suppress growth, apoptotic and cell cycle regulatory pathways. This diagram serves to illustrate the concept that targeting Raf/MEK/ERK and PI3K/Akt pathways can have dramatic effects on many growth regulatory molecules. Proteins inactivated by S/T phosphorylation induced by the PI3K/Akt pathway are indicated with a white P in a black circle.
resulted in enhanced G1 arrest. Similar experiments have shown that the effects were observed (284). The effects of EGFR and MEK inhibitors on cell cycle progression in the induction of G1 arrest, and the narrow signaling pathways that can be blocked by the inhibition of a single kinase (MEK). MEK inhibitors, such as ARRY-142886 (AZD6244), are also being evaluated to treat hematopoietic malignancies, such as multiple myeloma (278-280).

To the best of our knowledge, no small molecular weight MEK inhibitors have been developed yet; however, inhibitors to ERK could prove very useful, as ERK can phosphorylate many targets (Rsk, c-Myc, Elk and at least 150 more). There are at least 2 ERK molecules regulated by the Raf/MEK/ERK cascade, ERK1 and ERK2. Little is known about their different in vivo targets; however, it has been postulated that ERK2 has pro-proliferative effects, while ERK1 may have anti-proliferative effects (281). The development of specific inhibitors to ERK1 and ERK2 might eventually prove useful in the treatment of certain diseases.

18. Combination therapies to enhance toxicity

An approach that we have been investigating recently is to determine whether the inhibition of two signal transduction pathways is a more effective means to induce apoptosis than the inhibition of a single one. We have observed that the inhibition of the Raf/MEK/ERK and PI3K/Akt pathways is usually a more effective means, and that synergy between the two inhibitors is often observed. Many transformed cells have elevated Raf/MEK/ERK and/or PI3K/Akt signaling. These two pathways play prominent roles in the promotion of growth and the prevention of apoptosis. The PI3K/Akt pathway may be inhibited with PI3K (LY294002, PX-866), PDK1 (OSU-03012, Celecoxib), Akt (A-443654) inhibitors or downstream mTOR inhibitors, such as rapamycin and modified rapamycins (CCI-779, RAD001). Initially, mTOR inhibitors showed much promise, as PTEN is often deleted in various tumors. However, it has recently been determined that the mTOR pathway has a complicated feedback loop that actually involves the suppression of Akt, hence it can be predicted that mTOR inhibitors would activate Akt in some cells. Recent evidence has highlighted that mTOR can also be activated by Raf/MEK/ERK (233,282). This may well be another relevant cross-talk between the Ras/Raf/MEK/ERK and the PI3K/Akt pathways, and might offer a further rationale for treatments combining drugs which inhibit both signaling networks. The effects of the combination of mTOR and Sorafenib are being evaluated in clinical trials to treat melanoma (283). The effects of combining EGFR and mTOR or mTOR and MEK inhibitors on cell cycle progression in the induction of apoptosis in kidney cells were examined, and synergistic effects were observed (284). The effects of EGFR and MEK inhibitors were enhanced by the addition of rapamycin, which resulted in enhanced G1 arrest. Similar experiments have been performed on NSCLC with gefitinib and either the MEK inhibitor U0126 or the farnesyl transferase inhibitor SCH66336 (285).

In some cases, the precise gene responsible for driving the proliferation of the malignant cell is known (e.g., BCR-ABL in CML, EGFR in some cases of NSCLC, FLT-3 in some AMLs and B-Raf in melanoma). However, in many cancers there may be additional genes which are also critical to malignant transformation. Treatment of some of these diseases with specific kinase inhibitors is often effective; however, resistance to the inhibitors may develop due to further mutations in aberrant kinases, which often prevent the signal transduction inhibitor from inhibiting the altered kinase. In these novel ‘drug-resistant’ cases, additional therapeutic approaches are necessary. In some of these cases, it may be possible to inhibit the drug-resistant cells with novel inhibitors that will suppress the resistant oncproteins or combinations of the MEK and PI3K/Akt inhibitors. We have observed that Imatinib-resistant hematopoietic cells (which have mutated BCR-ABL kinase) are sensitive to MEK inhibitors, a result which is not surprising as an Src inhibitor (Dasatinib) is used to inhibit them. These cells often have overexpression of an activated Src family kinase, such as Lyn, which likely acts by inducing the Raf/MEK/ERK cascade.

Classic chemotherapy often remains the most used anti-cancer therapy for many different types of cancer treatment. Drugs such as doxorubicin and taxol are effective in the treatment of many cancers, although in some cases drug resistance does develop after prolonged treatment. Doxorubicin and taxol target cellular events, such as DNA replication and cell division, which are downstream of the targets of signal transduction pathway inhibitors. Thus, by combining classic chemotheraphy with targeted therapy, it may be possible to enhance toxicity while lowering the effective concentrations of classic chemotherapeutics necessary for the complete elimination of a particular tumor.

We have investigated the effects of combining classic chemotherapy or hormonal therapy with signal transduction inhibitors in suppressing the growth of breast cancer cells. Treatment of breast cancer cells with MEK or mTOR inhibitors and either doxorubicin or tamoxifen resulted in a synergistic response documenting the effectiveness of classic chemotherapy with targeted therapy.

19. Combining signal transduction inhibitors with antibody, hormonal and chemotherapeutic-based therapies

Recent studies have indicated that the effectiveness of certain antibody-based therapies (e.g., Herceptin, which targets HER2) may be greatly enhanced by the inclusion of mTOR inhibitors. These observations have been seen in preclinical studies performed in tissue culture and in xenograft models, and are being further evaluated in Phase II clinical trials (286). The cytotoxic effects of Herceptin can also be improved by the addition of an inhibitor such as Lapatinib, which targets both EGFR and HER2 (287).

The effectiveness of combining PI3K and mTOR inhibitors with the chemotherapeutic drug fludarabine has been examined in human leukemia cell lines (288). The combination of fludarabine and either PI3K or mTOR inhibitors resulted in...
increased apoptosis compared to what was observed following fludarabine treatment alone.

Rapamycin exerted synergistic effects when combined with paclitaxel, carboplatin and vinorelbine in certain responsive breast cancer lines in vitro (289). Rapamycin combined with paclitaxel resulted in a significant reduction in tumor volume in xenograft models when rapamycin-sensitive tumors were examined. mTOR inhibitors also increased the chemosensitivity of cervical cancer cells to paclitaxel (290). The effects of rapamycin on sensitivity to paclitaxel are dependent on functional glycogen synthase kinase 3β (GSK-3β), as rapamycin induced toxicity in GSK-3β WT cells but not in GSK-3β null cells (291).

Combinations of rapamycin and the cell cycle checkpoint kinase (Chk1) inhibitor UCN-01 also resulted in a synergistic induction of apoptosis in human leukemic cells, regulated by the Raf/MEK/ERK, Akt and JNK signal transduction pathways (292). Co-administration of UCN-01 and rapamycin reduced the levels of Mcl-1, Bcl-xL, cyclin D1 and p34^cdki. Similar studies were performed with the farnesyl-transferase inhibitor L744832 and UCN-01, which also revealed a synergistic interaction in terms of the induction of apoptosis and the interruption of both Akt and MEK/ERK pathways and the activation of SEK1/JNK (293). L744832 blocked the induction of ERK normally stimulated by UCN-01.

Novel PI3K inhibitors have been developed. PWT-458 is a novel pegylated-17-hydroxywortmannin which inhibits PI3K and has been shown to suppress glioma, NSCLC and renal cell carcinoma in xenograft models (294). PWT-458 augmented the anticancer effects of paclitaxel and pegylated rapamycin in certain xenograft models.

The PI3K inhibitor LY294002 has been shown to block drug export from drug-resistant colon carcinoma cells over-expressing MRP-1 (295). Furthermore, combining the PI3K inhibitor with doxorubicin resulted in enhanced apoptosis, while combining doxorubicin with the MEK inhibitor did not.

Perifosine is an oral bioactive novel alkylphospholipid that inhibits Akt. Perifosine enhanced dexamethasone, doxorubicin and melphanal, and bortezomib induced multiple myeloma cytotoxicity (296). Moreover, the cytotoxic effects of combinations of MEK inhibitors and paclitaxel may be specific for cells of certain origins and may depend on the levels of endogenous activated MEK/ERK present in those cells. Some studies with NSCLC cells that constitutively expressed activated MEK/ERK revealed no increase in paclitaxel-induced apoptosis upon treatment with a MEK inhibitor (305). In contrast, the addition of a dominant negative MEK gene to these cells potentiated paclitaxel-induced apoptosis.

MEK inhibitors have also been observed to affect cisplatin resistance in squamous cell carcinoma, implicating the Raf/MEK/ERK pathway in their drug resistance (306). In neuroblastoma cells, cisplatin-induced apoptosis was associated with an increase in p53 and Bax proteins. Activated ERK1,2 levels were also increased earlier in these cells with cisplatin treatment. Culture of these cells with MEK inhibitors blocked apoptotic cell death, which prevented the cisplatin-induced accumulation of p53 and Bax (307).

MEK inhibitors have also been observed to synergize with arsenic trioxide (ATO) to induce apoptosis in acute promyelocytic leukemia (APL) and AML cells (308,309). The p53-related gene p73 is a molecular target of the combined therapy. ATO modulates the expression of the p73 gene by inducing the pro-apoptotic and anti-proliferative 73 isoforms. p53 requires p63 and p73 for the induction of apoptosis in response to DNA-damaging drugs. p73 exists as a multiple transactivation competent (TA) of pro-apoptotic and anti-

20. Enhancing the effects of Ras/Raf/MEK/ERK pathway inhibitors by combination therapy

Although the precise targets of farnesyltransferase inhibitors remain controversial, the farnesyltransferase inhibitor R115777 (Zarnestra) was shown to result in disease stabilization in 64% of multiple myeloma patients in a Phase II clinical trial (301). Furthermore, R115777 was found to synergize with paclitaxel and docetaxel, but not with doxorubicin, 5-fluorouracil, cisplatin, melphanal, mitoxantrone and dexamethasone.

A side-effect of some chemotherapeutic drugs, such as taxol, is the induction of the Raf/MEK/ERK pathway. Activation of this pathway can, under certain circumstances, promote proliferation and prevent apoptosis. Combining taxol treatment with MEK inhibitors has been observed to synergistically enhance apoptosis and inhibit tumor growth (302,303). The synergistic effects of paclitaxel and MEK inhibitors are complex and not fully elucidated, but may be mediated in part by the inhibition of Bad phosphorylation at S112 by ERK (304).

Moreover, the cytotoxic effects of combinations of MEK inhibitors and paclitaxel may be specific for cells of certain origins and may depend on the levels of endogenous activated MEK/ERK present in those cells. Some studies with NSCLC cells that constitutively expressed activated MEK/ERK revealed no increase in paclitaxel-induced apoptosis upon treatment with a MEK inhibitor (305). In contrast, the addition of a dominant negative MEK gene to these cells potentiated paclitaxel-induced apoptosis.
proliferative p73 COOH-terminal splicing isofrom (α, β, γ, δ, ε, ζ), of which the two major forms are p73α and p73β. Dominant-negative (ΔN) p73 variants are expressed from a second promoter. These DN ΔNp73 variants lack the amino-terminal transactivation domain, act as transrepressors of p53- and p73-dependent transcription and have anti-apoptotic and pro-proliferative potential. Treatment of APL cells with the PD184352 MEK inhibitor reduced the level of ΔNp73 and decreased the ATO-mediated upregulation of ΔNp73, thus causing an increase in the TA/ΔNp73 ratio of dual-treated cells. High doses of ATO induced p53 accumulation in 11 of 21 patients. Combined treatment resulted in the induction of the pro-apoptotic p53/p73 target gene p53AIPI (p53-regulated apoptosis-inducing protein 1) and greatly enhanced the apoptosis of treated cells (309). This study consequently documented the effectiveness of combining ATO with MEK inhibitors in the treatment of APL, and identified the molecular mechanism responsible for the observed synergism.

MEK inhibitors have been observed to synergize with UCN-01 and induce apoptosis in multiple myeloma cells (310). Part of the synergy may be due to UCN-01 inducing ERK activation, which is suppressed by the MEK inhibitor.

It should be pointed out that the combination of MEK inhibitors and a chemotherapeutic drug may not always result in a positive interaction; in some cases combination therapy results in an antagonistic response. For example, combining MEK inhibitors with betulinic acid, a drug lethal to melanoma cells, antagonized the effects that betulinic acid normally has on apoptosis (311). Furthermore, the precise timing of the addition of two drugs is important, as they may differentially affect cell cycle progression. Therefore, one drug may need to be added before the other for a synergistic response to be observed, and perhaps to prevent an antagonistic one (297).

21. Role of the Raf/MEK/ERK pathway in drug resistance to reactive oxygen intermediate-inducing cancer treatments

Many cancer therapies induce the generation of oxygen radicals within cells. These therapies include treatments such as chemotherapeutic drugs and irradiation, and newer treatments such as photodynamic therapy (PDT). Doxorubicin, one of the chemotherapeutic drugs most effective against a wide range of cancers, works via two main mechanisms to exert anti-tumor effects and toxicity. It intercalates in the DNA and interferes with DNA polymerase by disrupting helicase activity (312). It also induces the production of free radicals and oxidative stress, which are involved in its anti-tumor effects (313,314). The generation of oxygen radicals is important for the therapeutic effectiveness of doxorubicin, because scavenging reactive oxygen intermediates results in decreased cell killing by this drug (315).

The initial reactive oxygen species generated as a consequence of ionization radiation is OH·, which is short-lived and only diffuses about 4 nm before reacting. Secondary reactive oxygen species, produced in response to ionizing radiation, include O2· and H2O2. Studies with fluorescent dyes have demonstrated the generation of reactive oxygen species within cells within 15 min after irradiation (316). Similar to doxorubicin, the generation of oxygen radicals is important for the therapeutic effectiveness of radiation therapy because scavenging reactive oxygen intermediates results in decreased cell killing in response to radiation (317).

PDT is a three-component treatment used in cancer cases (318) that requires a photosensitizer, molecular oxygen and a laser of a wavelength matching the absorption spectrum of the photosensitizer (porphyrins and porphyrin-related compounds). When a porphyrin molecule absorbs light, it can transform an oxygen molecule to an activated state. Similar to doxorubicin and irradiation, PDT also requires the production of oxygen radicals to mediate some of its anti-tumor effects (319). Thus, three well-known cancer treatments result in the generation of reactive oxygen intermediates. These same three treatments have also been shown to lead to the activation of ERK1/2 (320-323).

The Raf/MEK/ERK signaling pathway can play an adaptive role in protecting cells from oxidative stress (324). In a non-malignant murine alveolar epithelial cell line, blocking MEK activation using the MEK inhibitor U0126 prevents hypoxia-induced Nrf2 upregulation (324). Deletion of ASK1 protects cells from oxidant-induced cell death, but not from death receptor-induced apoptosis (325). Conversely, hydrogen peroxide is capable of inducing apoptosis in cardiomyocytes, which can be increased in MEKK1 negative cells (326). The deletion of ASK1 protects against hydrogen peroxide-induced apoptosis in fibroblasts and also prevents prolonged p38 activation, suggesting an apoptotic role for p38 in response to oxidative stress (327). Ras activation and subsequent signaling via Rho can also activate this pathway, as does ligation of the TNF receptor (328-331). Redox activation of ERK5/BMK1 exhibits an anti-apoptotic effect (332). U0126 and PD98059 are also reported to inhibit the activity of MEK5, the MAPKK involved in ERK5/BMK1 activation (332-335). These inhibitors decreased PC12 cell viability in response to hydrogen peroxide treatment. This decrease in cell viability occurred when the ERK5/BMK1 protein was completely downregulated using siRNA, suggesting that the effects of U0126 and PD98059 were mediated in part via the ERK5/BMK1 pathway (332). These data indicate the potential for both the ERK1/2 and ERK5/BMK pathways to promote treatment resistance to currently-used reactive oxygen intermediate-inducing cancer treatments.

22. Conclusions

A variety of anticancer agents have been observed to extend survival in breast cancer patients. Novel drugs for the treatment of breast cancer patients will undoubtedly become available in the near future. Additional therapeutic options include radiation and surgery. The large number of choices available underscore the need to identify the optimal treatment for each individual breast cancer patient. It is likely that the selection of breast cancer therapy will increasingly depend on molecular features. Expression of ERα and HER2 are but two of the many characteristics that may impact breast cancer treatment decisions in the future.

Over the past 25 years, there has been much progress in elucidating the involvement of the Ras/Raf/MEK/ERK cascade in promoting normal cell growth and regulating apoptosis, and in understanding the etiology of human neoplasia and the induction of chemotherapeutic drug resistance. From initial
several seminal studies which shed light on the oncogenes present in avian and murine oncogenes, we learned that ErbB, Fms, Ras, Src, Abl, Raf, Fos, Ets and NF-xB (Rel) were originally cellular genes which were captured by retroviruses. Biochemical studies continue to elucidate the roles that these cellular and viral oncogenes have in cellular transformation. We have learned that many of these oncogenes are connected to the Ras/Raf/MEK/ERK pathway, and either feed into this pathway (e.g., BCR-ABL, ErbB, Fms) or are downstream targets that regulate gene expression (e.g., Fos, Ets and NF-xB).

The Ras/Raf/MEK/ERK pathway has what often appears to be conflicting roles in cellular proliferation, differentiation and the prevention of apoptosis. Classical studies indicated that Ras/Raf/MEK/ERK can promote proliferation and malignant transformation, in part due to the stimulation of cell growth, and at the same time prevent apoptosis. Furthermore, recent, another overlooked aspect of Raf/MEK/ERK is its effects on cytokine and growth factor gene transcription, which can stimulate proliferation. The latest 'hot' area of the Ras/Raf/MEK/ERK pathway is the discovery of B-Raf mutations in human cancer, which can promote proliferation and transformation (116). The development and characterization of B-Raf inhibitors is a key research area in the pipeline for many pharmaceutical companies.

It was initially thought that Raf-1 was the most important Raf isoform. It was certainly the earliest-studied one, with homologous genes present in both murine and avian transforming retroviruses. Originally, it was shown that Raf-1 was ubiquitously expressed, indicating a more general and important role for it, while B-Raf and A-Raf had more limited patterns of expression. However, it is now believed that B-Raf is the more important activator of the Raf/MEK/ERK cascade and that, in some cases, Raf-1 activation may require B-Raf. However, Raf-1 has reared its head again in the field of cancer, thanks to the recent discovery that there are mutant Raf-1 alleles in certain breast cancer therapy-induced t-AMLs that are transmitted in a Mendelian fashion (117). The role of A-Raf remains poorly defined, yet it is an interesting isoform. It is the weakest Raf kinase, yet it can stimulate cell cycle progression and proliferation without the negative effects on cell proliferation that B-Raf and Raf-1 can exert. It should be remembered that, under certain conditions, the hyperactivation of B-Raf and Raf-1 can promote cell cycle arrest (51). Thus, fine-tuning these mutations will probably influence whether cell cycle arrest or malignant transformation occurs.

The activation of the Raf proteins is very complex, as there are many phosphorylation sites on Raf. Phosphorylation at different sites can lead to either activation or inactivation. Clearly, there are many kinases and phosphatases which regulate Raf activity, and the state of phosphorylation determines whether Raf is active or inactive. The Raf kinases involved in the regulation of the Raf/MEK/ERK pathway have been extensively studied, there is but very limited knowledge of the specific phosphatases involved in these regulatory events.

Raf-1 has many roles which are apparently independent of downstream MEK/ERK. Some of these functions occur at the mitochondria and are intimately associated with the prevention of apoptosis. Raf-1 may function as a scaffolding molecule to inhibit the activity of kinases, which promote apoptosis. Thus, the development of Raf inhibitors may prove useful in the suppression of some of these non-MEK/ERK mediated events.

The Raf/MEK/ERK pathway is both positively [Hsp90, kinase suppressor of Ras (KSR), MEK partner-1 (MP-1)] and negatively [RKIP, 14-3-3] regulated by its association with scaffolding proteins. The expression of some of the scaffolding proteins is altered in some human cancers (e.g., RKIP). Some of these scaffolding proteins (e.g., Hsp90) are being evaluated as potential therapeutic targets (geldanamycin). The potential roles of Hsp90 in stabilizing activated forms of Raf are intriguing, and may allow the evolution of activated mutant forms of Raf.

The Raf/MEK/ERK pathway is intimately linked to the PI3K/PTEN/Akt pathway, both of which can be regulated by Ras. Furthermore, in some cell types Raf activity is negatively regulated by Akt, indicating a cross-talk between the two pathways. Both pathways may result in the phosphorylation of many downstream targets, and impose themselves on the regulation of cell survival and proliferation. Thus, the development of strategies to inhibit the pathways may be clinically important. These pathways phosphorylate many key proteins involved in apoptosis (e.g., Bad, Bim, Mcl-1, caspase 9, ASK-1 and others), which serves to alter their activities and subcellular localization. The phosphorylation events mediated by the Raf/MEK/ERK and PI3K/Akt pathways are associated with the prevention of apoptosis. In contrast JNK, which is another MAPK, also phosphorylates many of these molecules; these phosphorylation events often have effects opposite to those elicited by the Raf/MEK/ERK and PI3K/Akt pathways.

Ras and Raf mutations may not always have similar outcomes. For example, it could be predicted that a Ras mutation would activate both the Raf/MEK/ERK and PI3K/Akt pathways, with activation of PI3K/Akt resulting in the suppression of Raf/MEK/ERK. However, mutation at either B-Raf or Raf-1 results only in the activation of Raf/MEK/ERK. Thus, depending on the particular cancer and biological effect targeted, it is appropriate to develop Ras, Raf and MEK inhibitors.

Although we often think of the phosphorylation of these molecules as being associated with the prevention of apoptosis and the induction of gene transcription, this view is oversimplified. For example, in certain situations the Raf/MEK/ERK pathway may be inhibited. In such cases, the phosphorylation of Bad and CREB, normally mediated by the Raf/MEK/ERK cascade, which is associated with the prevention of apoptosis, is suppressed. Likewise, it is important to remember that, at some protein residues, phosphorylation results in enhanced activity, whereas at others it results in decreased activity. For example, the phosphorylation of Bim by JNK is associated with the promotion of apoptosis, while the phosphorylation of Bim by the Raf/MEK/ERK or PI3K/Akt pathways is associated with the prevention of apoptosis.

A consequence of diverse cancer therapies (e.g., chemotherapy, radiation therapy, photodynamic therapy) is the induction of the Raf/MEK/ERK pathway, which may in some cases provide a survival function. The mechanism of induction of these pathways may in part be in response to ROS generated by the different therapies. Thus, in some cases it may be appropriate to combine these conventional therapies with...
small molecular weight inhibitors which target the Raf/MEK/ERK pathway.

Although it has been known for many years that the Raf/MEK/ERK pathway can affect cell cycle arrest, differentiation and senescence, these are probably some of the least studied research areas in the field. This is due to the often cell lineage-specific effects that must be evaluated in each cell type. An intriguing aspect of human cancer therapy is that, in some cases, stimulation of the Raf/MEK/ERK pathway may be required to promote terminal differentiation, while in other types of malignant cancer cells, which proliferate in response to Raf/MEK/ERK activity, inhibition of the Raf/MEK/ERK pathway may be required to suppress proliferation. Thus, we must be flexible in dealing with the Raf/MEK/ERK pathway and, as we learn more, our conceptions will continue to change.

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