Chapter 4

Cell Cycle Deregulation in Breast Cancer:
Insurmountable Chemoresistance or Achilles' Heel?

Laura Lambert and Khandan Keyomarsi*

Abstract

Deregulation of the G1 cyclin, cyclin E, has been shown to be both the most powerful predictor of prognosis in early stage breast cancer as well as a significant determinant of tumor aggressiveness.[1,2] It may also contribute to treatment failure due to chemoresistance. Because some form of cell cycle deregulation is present in all malignant cells,[3] increasing understanding of these processes is starting to provide new opportunities to overcome the cells' resistance mechanisms.

One particular form of cyclin E deregulation, the generation of hyperactive low molecular weight isoforms, is especially intriguing. Because only tumor cells contain the machinery necessary to generate these isoforms,[4] they not only provide a mechanism of targeting critical cell cycle events, but their presence may also provide both a means of increased specificity for targeting malignant cells, as well as an objective measure of response.

This review describes the mechanisms of resistance to commonly used systemic therapies for the treatment of breast cancer, with particular respect to the role of the cell cycle. The mechanisms and effects of the deregulation of cyclin E in breast cancer are reviewed and novel approaches to circumventing chemoresistance through abrogation of the malignant cell cycle are proposed.

Introduction

Tumor resistance to systemic antineoplastic therapy is the main cause of failure of breast cancer treatment. For early stage breast cancer, adjuvant endocrine and cytotoxic agents have resulted in only an 8-37% reduction in mortality.[5,6] For patients with more advanced disease the success rate is even lower. Investigation into the means by which tumor cells resist cytotoxic therapies have revealed multiple mechanisms of drug resistance and efforts to devise ways of circumventing resistance are currently underway.

The cytotoxic mechanisms of most conventional chemotherapeutic agents used in the current treatment of breast cancer (doxorubicin, cyclophospamide, 5-flourouracil, methotrexate and the taxanes) are attributable to their damaging or inhibitory effects on DNA. However, as illustrated by the high rate of resistance, this approach is limited in a number of ways. First it is highly nonspecific. Second, these agents rely upon a relative rate of cell division to establish a cytotoxic threshold to distinguish between rapidly dividing malignant cells and normal cells. Another limitation is the nonlethality of the effect of the drug on the DNA with the ultimate outcome (susceptibility versus resistance) dependent upon the status of the cell's mechanisms of DNA repair and apoptosis. Because of the redundancy of the cell salvage pathways, continuing to use conventional approaches only prolongs the inevitable occurrence of drug resistance (Table 1).

*Corresponding Author: Khandan Keyomarsi—Departments of Surgical Oncology and Experimental Radiation Oncology at University of Texas, MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas 77030, U.S.A. Email: kkeyomar@mdanderson.org

Breast Cancer Chemosensitivity, edited by Dihua Yu and Mien-Chie Hung.

Table 1. Response rates and possible mechanisms of resistance in neoadjuvant chemotherapy and endocrine regimens for breast cancer

Neoadjuvant Chemotherapy/ Endocrine Regimen	Response	Possible Mechanism(s) of Resistance	Ref.
Adriamycin (doxorubicin) and **cyclophosphamide** (AC)	Pathologic complete 10% Objective clinical 70%	**Adriamycin**: Increased cellular efflux Alterations in topoisomerases Aberrant intracellular localization **Cyclophosphomide**: Intracellular inactivation Increased conjugation	19-27, 31-42, 117
Adriamycin and **Taxol** (paclitaxel) (AT)	Pathologic complete 16% Objective clinical 89%	**Taxol**: Increased cellular efflux Impaired microtubule polymerization Microtubule instability	87-92, 117
Flourouracil, **Adriamycin**, and **cyclophosphamide** (FAC)	Pathologic - complete 24% - partial 55% Clinical - complete 18% - partial 82%	**Flourouracil**: Reduced anabolism Increased catabolism Reduced FdUMP affinity Increased thymidylate synthase Mode of administration	43,44, 50-56, 118
Taxol	Pathologic - complete 24% - partial 55% Clinical - complete 18% - partial 82%	(See above)	118
Tamoxifen	Objective clinical 17-36%	**Tamoxifen**: Her2 over-expression ER-negative tumor	87-92, 119-124
Aromatase inhibitors Letrozole Anastrozole Exemestane	Objective clinical Letrozole 30-55% Anastrozole 21-43% Exemestane 41%	**Aromatase inhibitors**: Lack of estrogen-response	114, 119-124
Trastuzumab (Herceptin)*	Objective - complete 6% - partial 20%	**Trastuzumab**: Decreased PTEN	116,125

*Used for treatment of metastatic breast cancer.

The *sine quo non* of the malignant phenotype is deregulation of the cell cycle.[3] However, while deregulation of the tightly controlled cell cycle events clearly leads to malignant transformation, it also provides intriguing targets for alternative therapeutic approaches to overcome the problem of chemoresistance. One target of particular interest for this approach is the cyclinE/cyclin-dependent kinase 2 (Cdk2) complex and the G1/S transition of the cell cycle.

The G1/S transition is regulated through the cooperation of two essential, parallel cell cycle pathways, RB and Myc, which converge on the control of the G1 cyclin-dependent kinase

- Cdk2.[7-12] Cdk2 activity in the G1/S transition is both rate-limiting and necessary for cell replication, and it is dependent upon appropriate interaction with the G1 cyclin, cyclin E.[13,14] A number of recent studies have suggested that deregulation of cyclin E plays a significant role in the aggressiveness of breast cancer and other malignancies.[1,2,15-18] In fact, a form of cyclin E deregulation caused by the generation of recently identified hyperactive low molecular weight (LMW) isoforms has been shown to be the most powerful predictor of outcome in patients with early stage breast cancer.[2] Because only tumor cells possess the machinery to generate these forms,[4] they provide both a potential means of identifying malignant versus normal cells as well as a multi-leveled target within an essential cell cycle pathway. For these reasons therapies designed to take advantage of the deregulation of cyclin E and the G1/S transition are appealing. This review describes the mechanisms of resistance to commonly used systemic therapies for the treatment of breast cancer, with particular respect to the role of the cell cycle. The mechanisms and effects of the deregulation of cyclin E in breast cancer are reviewed and novel approaches to circumventing chemoresistance through abrogation of the malignant cell cycle are proposed.

Conventional Chemotherapies of Breast Cancer

Anthracyclines

Anthracycline-based chemotherapy is the current standard of care in breast cancer treatment. Anthracyclines (doxorubicin, epirubicin) are intercalating, topoisomerase II poisons that bind to double-stranded DNA causing structural changes which interfere with DNA and RNA synthesis. Multiple forms of resistance to these drugs have been identified. Because many of these agents are natural products, resistance by cellular efflux mechanisms, such as the *mdr1*, *mrp1* and *mrp2* gene product members of the ATP-binding cassette (ABC) family, have been demonstrated.[19-21] In addition, alterations in topoisomerases, including point mutations as well as defects in phosphorylation, have been described in some drug-resistant cell lines.[22,23] Furthermore, aberrant intracellular localization (cytoplasmic) has been implicated by decreasing the potential for DNA binding.[24-27] Finally, although not yet clearly demonstrated, because these agents function by causing structural DNA damage which should ultimately lead to apoptosis, alterations in the apoptotic proteins of the cell (e.g., p53 and the Bcl-2 family), have been suggested to confer drug resistance.[28]

Alkylating Agents

The alkylating agent cyclophosphamide is frequently used in anthracycline-based chemotherapy regimens for breast cancer. A member of the nitrogen mustard family, cyclophosphamide activation requires cytochrome P450-mediated oxidation in the liver to produce 4-hydroxycyclophosphamide. Relatively nonpolar, 4-hydroxycyclophosphamide readily diffuses into target cells where its tautomer, aldophosphamide, decomposes to the active alkylating agent, phosphoramide mustard.[29] At least three mechanisms of resistance to cyclophosphamide have been identified. Because cyclophosphamide enters the cell through diffusion, it is not a known substrate for the multiple-drug-resistance (MDR) export systems.[30] Intracellular inactivation of cyclophosphamide by its natural detoxifier, aldehyde dehydrogenase, has been shown not only to protect normal cells from the cytotoxicity of this agent, but also to confer resistance in tumor cells.[31-36] In addition, increased 4-hydroxycyclophosphamide glutathione conjugation, either spontaneous or through enhanced transcription of glutathione S-transferase, has been shown to contribute to cyclophosphamide resistance.[37-41] Finally, resistance related to the cell's ability to either repair DNA interstrand cross-links or to arrest in the G2 phase of the cell cycle in response to the alkylating damage has also been demonstrated.[42]

Antimetabolites

The pyrimidine analog 5-flourouracil (5-FU) is used in the management of many epithelial malignancies, including breast cancer. Potential mechanisms of cytotoxicity caused by 5-FU include RNA incorporation,[43,44] dTTP depletion by thymidylate synthase inhibition,[45] DNA incorporation, or DNA damage due to excision of uracil or 5-FU.[46-49] Resistance to 5-FU therapy has been demonstrated in the form of reduced anabolism of the analog to the nucleotide form either through altered condensation with pyrophosphorylribose-5-PO4 (PRPP) or the pyrimidine salvage pathway.[43,44] In addition, increased catabolism of 5-FU due to elevated dihydropyrimidine dehydrogenase (DPD) activity can lead to decreased sensitivity and has been shown to be a predictor of decreased response in some tumor types.[50,51]Other mechanisms of resistance have been related to changes in thymidylate synthase (reduced affinity for FdUMP,[52] increased rate of synthesis or activity[53]), and the mode of exposure to the drug (enteral versus parenteral).[54-56]

Folate Antagonists

Another important agent in the management of breast cancer is the folate antagonist, methotrexate (MTX). MTX stoichiometrically inhibits the enzyme dihydrofolate reductase (DHFR) leading to decreased availability of thymidine, decreased DNA synthesis and ultimately cell death.[57] Resistance to MTX can be either intrinsic or acquired. A significant mechanism of intrinsic resistance to MTX is reduced formation of long-chain MTX polyglutamates due to decreased folylpolyglutamate synthetase (FPGS) activity which can lead to both decreased affinity for DHFR as well as increased cell efflux.[58-62] Other mechanisms of intrinsic resistance to MTX include impaired transport through the reduced folate carrier (RFC),[63,64] and increased DHFR levels due to increased levels of the transcription factor E2F which occur in the absence of the tumor-suppressor retinoblastoma protein.[65-67] Acquired mechanisms of resistance to MTX include increased DHFR activity due to amplification of its gene,[68-74] altered binding of MTX to DHFR due to DHFR mutations,[75-79] decreased MTX uptake secondary to decreased long-chain polyglutamate formation, and decreased influx through the RFC.[80]

Microtubule-Targeting Agents

Recently added to the breast cancer chemotherapy armamentarium are the taxanes (paclitaxel, docetaxel) which are naturally-occurring antimicrotubule agents. Taxanes have been shown to prevent depolymerization of the microtubule by binding and stabilizing the molecular conformation of the protofilament of the microtubule.[81] This stabilization causes a mitotic arrest at the metaphase/anaphase juncture.[82] The mechanisms of cell death caused by the taxanes include apoptosis through the activation of caspase 3 and 8 as well as a noncaspase activated mechanism of DNA fragmentation that causes apoptosis.[83-86] Multiple possible mechanisms of resistance to taxane therapy exist including increased expression of the *mdr1* gene and Pgp efflux pump,[87] structural alterations in the α- and β- tubulins which impair microtubule polymerization,[87-92] and dynamic instability of the microtubule caused by increased expression of the β_{III} isotype of β tubulin.[90-92]

Hormonal and Targeted Therapies

Because of the important role of estrogen in the development of breast cancer, endocrine therapy, either in the form of anti-estrogens or estrogen deprivation, plays a significant role in the medical treatment of breast cancer. With respect to the cell cycle, estrogen has been shown to have a regulatory role of the molecules involved in the G1/S phase progression, including the expression and function of c-Myc[93-95] and cyclin D1.[96,97] Furthermore, other studies have demonstrated estrogen-mediated inhibition of the generation of the cyclin-dependent kinase inhibitor (CKI) p21, resulting in increased cyclinE/Cdk2 complex activity.[96,98,99] Deregulation of any of these cell cycle regulators may contribute to increased anti-estrogen resistance.

In addition, increasing evidence suggests that breast cancer growth may also be influenced by the coordinated actions of the estrogen receptor (ER) and the HER2 growth factor receptor signaling pathway.[100] Estrogen binding of the ER induces a series of both membrane-bound (G-protein-coupled receptor activation[101]) and nuclear events (phosphorylation of the receptor, conformational alteration, receptor dimerization, receptor complex-promoter binding, and recruitment of coactivators).[102] The nuclear events ultimately lead to the transcriptional regulation of the ER target genes.[103,104] The membrane-bound events have been shown to lead to the paracrine or autocrine activation of the HER2 signaling pathway through the release of epidermal growth factor (EGF).[105] Activation of the HER2 signaling pathway initiates a kinase signaling cascade which has been shown to augment the transcriptional activation potential of ER resulting in enhanced cell proliferation and survival.[105-107] This "crosstalk" between the ER and the HER2 signaling pathway may also be one of the major mechanisms for resistance to endocrine therapy in breast cancer treatment.[105,108,109]

The current mainstay of anti-estrogen therapy, tamoxifen, is known to display partial agonist-antagonist activities in different tissues and cells, depending upon the various ER coactivators and corepressors present.[110] Like estrogen, tamoxifen also has both nuclear and membrane-bound effects.[105] In addition to preventing the binding of estrogen to the ER, under favorable conditions such as negative or very low levels of HER2, tamoxifen's effects are primarily antagonistic and nuclear. In this setting, the ER conformation induced by the binding of tamoxifen leads to the recruitment of corepressors and deacetylases which inhibit transcriptional activity. On the other hand, in the setting of abundant HER2, evidence suggests that agonist effects of tamoxifen may predominate through membrane-bound events which lead to HER2 signaling activation, tumor growth and resistance to anti-estrogen therapy.[105,110]

Options to overcome anti-estrogen therapy resistance in breast cancer patients include two currently used therapies: estrogen deprivation through aromatase inhibition and inhibition of HER2 signaling by the monoclonal antibody receptor tyrosine kinase inhibitor—trastuzumab (Herceptin). Aromatase inhibitors (AIs) are a group of agents that inhibit the steroid hydroxylations involved in the conversion of androstenedione to estrone, thereby lowering both the circulating and intratumoral amounts of estrogen available to bind the ER.[111] In theory, these agents should be able to abrogate both the membrane-bound HER2 activating ER events, as well as the nuclear steroid signaling events. In support of this theory, clinical trials have demonstrated the superiority of AIs over tamoxifen in both HER2-overexpressing breast cancers as well as ER-positive/PR-negative tumors.[112,113] Resistance to AIs is thought not to be due to failure of these agents to suppress estradiol, but rather through resistance to the hormone itself.[114]

Trastuzumab is a humanized monoclonal antibody that specifically binds to the extracellular domain of the HER2/neu tyrosine kinase receptor. Down-regulation and inactivation of the receptor by the antibody occur through multiple mechanisms including accelerated degradation, interference with the hetrodimerization of the receptor, and targeting of the immune system to HER2 overexpressing cells.[115] In addition, trastuzumab has been shown to stabilize and activate the PTEN tumor suppressor leading to down-regulation of the P13K-Akt signaling pathway and initiating cell cycle arrest.[116] Recently, Nagata et al demonstrated that when the expression of PTEN is reduced, the antitumoral effects of trastuzumab are impaired. Based on these findings, the authors predicted and confirmed that clinical resistance to trastuzumab correlated with low levels of PTEN.[116]

The Cell Cycle as a Therapeutic Target in Breast Cancer

Deregulation of G1/S Transition

Cell division is a complex and orderly process divided into four phases involving cell growth and monitoring (G1 and G2 phase), DNA synthesis (S phase), and mitosis (M phase).[126] In the settings of favorable cellular and tissue environments, cells can initiate their own division

and enter a mitogen-dependent growth phase (early G1). Upon entering the cell cycle, the order and quality of the cell cycle events are monitored and ensured by a series of checkpoints.[127] Commitment to genome replication and eventual cell division occurs late in the G1 phase at a period defined as the restriction point.[128] Recent studies have suggested that this molecular "point of no return" revolves around the activity of Cdk2 and its G1-associated cyclin, cyclin E, which is also the point of convergence of the RB (p16-Cdk4/6-cyclin D-pRb) and Myc proto-oncogene pathways.[7-12]

Cdk2 belongs to a family of serine and threonine protein kinases whose substrates include intracellular, cell cycle-regulatory proteins that control the major cell cycle events: DNA replication, mitosis and cytokinesis. One of the most important functions of Cdk2 is the mid-late G1 phase phosphorylation and inactivation of the tumor suppressor pRb which, in normal cells, is essential to cell cycle progression. Like all Cdks, Cdk2 activity is governed by an array of enzymes and proteins, the most prominent of which are cyclins. Unlike Cdk levels which normally remain constant throughout the cell cycle, cyclins, as the name implies, undergo a tightly regulated cycle of synthesis and degradation resulting in the cyclic assembly and activation of cyclin-Cdk complexes.[129,130] In each phase of the cell cycle, Cdk activity is dependent upon binding to the appropriate cyclin protein and it is this activation that propels the cell through the cell cycle. In late G1 phase, cyclin E complexes with Cdk2 to control the transition into S-phase.[131]

In normally dividing cells, the G1-synthesis and S phase-degradation of cyclin E are tightly regulated.[132] In late G1, cyclin E transcription is activated when pRb is hyperphosphorylated by cyclin D/Cdk4/Cdk6 complexes, relieving repression of the cyclin E gene. This event causes a G1 arrest allowing further accumulation of cyclin E protein. This accumulation continues to a level where cyclin E/Cdk2 itself phosphorylates pRb, relieving the repression of the S-phase cyclin, cyclin A, and Cdk1, and allowing the cell cycle to progress to mitosis.[133] Concomitant activation of cyclin E-Cdk2 kinase also occurs through the Myc proto-oncogene pathway.[12] c-Myc proto-oncogene is a mitogen-induced transcription factor of the helix-loop-helix/leucine zipper protein family whose role in cyclin E activation includes both direct mechanisms (transcriptional effects) and indirect mechanisms (sequestration or enhanced degradation of the cyclinE/Cdk2 inhibitor p27).[11,12,134-136] Deregulation of any of these cell cycle components can lead to the unscheduled expression of cyclin E that is often seen in cancer (Fig. 1).

Multiple mechanisms of malignant deregulation of cyclin E have been identified including gene amplification,[137,138] overexpression,[139,140] downregulation of inhibitory proteins such as p27,[141] faulty degradation[139,140,142] and the generation of LMW isoforms of cyclin E.[4,143] Of these cyclin E alterations, the most profound is the generation of the LMW isoforms which have been associated with poor clinical outcomes in breast cancer and other malignancies. In fact, in a retrospective study of 395 breast cancer patients, the presence of the LMW isoforms of cyclin E was found to be eight times more predictive of poor prognosis than nodal status.[2] Significant biochemical and functional differences between the full-length and LMW isoforms of cyclin E are thought to explain the correlation between this type of deregulation and increased breast cancer mortality.[144]

Six cyclin E isoforms (EL1-6) have been identified (Fig. 2).[1] The predominant, full-length (50-kDa) isoform (EL1) is the only isoform found in normal cells. The LMW isoforms (EL2-6) are generated either by alternative translation (EL4) or proteolytic processing of the full-length protein by an elastase-like protease which creates two paired-isoforms (EL2/3 and EL5/6). Only tumor cells are capable of processing cyclin E into its LMW forms which are nuclear and functionally hyperactive.[143]

Tumorigenic properties associated with the LMW cyclin E isoforms involve both aberrant control of both the cell cycle as well as many aspects of DNA replication. In normal cells, direct binding of chromatin by cyclin E initiates DNA replication and also potentially blocks rereplication.[145] Cyclin E has been shown to induce histone gene transcription at the beginning of S phase through the phosphorylation of NPAT[146,147] and control centrosome

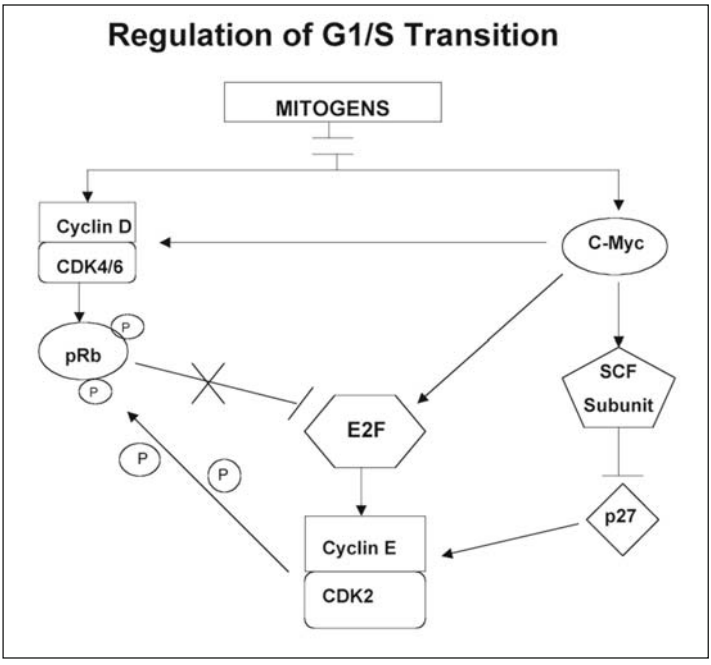

Figure 1. Regulation of the G1/S transition by the cyclin-dependent kinase (Cdk) 2 and its G1-associated cyclin, cyclin E, at the point of convergence of the RB (p16-Cdk4/6-cyclin D-pRb) and Myc proto-oncogene pathways.

duplication through the phosphorylation of nucleophosmin B23[148,149] and stabilization of the Mmps1p-like kinase.[148] Additional cyclin E substrates involved in other DNA replication processes such as transcriptional regulation (SWI/SNF),[150] pre-mRNA splicing (spliceosomal protein)[151,152] and modulation of transcription factors

(Id2, Id3)[153,154] have also been identified. Deregulation of cyclin E impacts many of these aspects of DNA replication, often conferring a growth advantage to tumor cells.

With respect to the cell cycle, the LMW forms of cyclin E have been shown to result in decreased cell doubling times, decreased cell size and loss of growth factor requirements for proliferation.[131,155] These effects are due to both the increased biochemical and biological activity of the LMW forms as compared with the full-length cyclin E. Specifically, because of the increased affinity for Cdk2 of the LMW cyclin E, there appears to be at least a two-fold increase in associated Cdk2 kinase activity and a three- to five-fold increase in resistance to the Cdk inhibitors p21 and p27 in cells with these forms.[156] Through this increased activity deregulated cyclin E has been shown to independently and sufficiently phosphorylate pRb, enough to induce aberrant cell cycle progression.[4]

Targeting the G1/S Transition Therapeutically

The central role of cyclinE/Cdk2 in the regulation of the G1/S transition makes this complex an attractive target for novel cancer therapy. First, differential expression of the tumor-specific LMW cyclin E provides a unique means of both identifying and targeting tumor cells only, potentially increasing selective lethality of the therapy. In addition the same target may also act as a more objective measure of both the degree of tumor aggressiveness as well as therapeutic response. Elucidation of the mechanisms of this differential expression have helped identify opportunities for therapeutic exploitation.

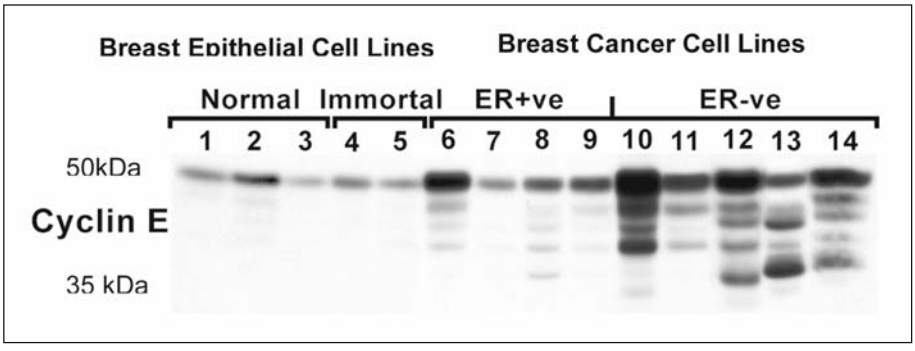

Figure 2. Western blot analysis of cyclin E in normal and immortalized breast epithelial cell lines and estrogen receptor positive (ER+ve) and negative (ER-ve) breast cancer cell lines. Deregulated cyclin E caused by the proteolytic generation of hyperactive, low molecular weight isoforms (35-50 kDa), is seen only in the breast cancer cell lines.

Proteolytic processing of the full length cyclin E has recently been identified as the mechanism responsible for the generation of the hyperactive LMW forms of cyclin E seen in some tumor cells.[4,143] Two proteolytically sensitive domains in cyclin E have been identified and four of the five LMW forms are accounted for by proteolysis at these two sites, with post-translational modification creating two closely migrating doublets—EL2/3 and EL5/6. Sequence analysis of the proteolytically cleaved regions of cyclin E have identified an elastase-like serine protease as responsible for generating these LMW forms.[4]

The differential expression of the LMW forms of cyclin E in tumor versus normal cells may be due to either increased elastase-like activity in tumor cells, increased elastase inhibitor levels in normal cells, or decreased elastase-inhibitor levels in tumor cells. Each of these possible mechanisms presents a potential target for cancer therapy. Recent studies looking at the neutrophil (elastase) inhibitor, CE-2072, demonstrated partial abrogation of some of the LMW forms of cyclin E in the breast cancer cell line MDA-MB-157, a cell line that expresses all 6 isoforms of cyclin E. In comparison, CE-2072 treatment of MCF-10A breast cancer cells, which do not express the LMW isoforms, did not affect the expression of cyclin E in these cells. In addition, treatment with CE-2027 was found to cause partial arrest in the G1 phase of the cell cycle in tumor cells, but not normal cells. These results suggest a cause and effect relationship between the disappearance of some of the LMW forms of cyclin E in tumor cells and partial growth arrest of these cells.[156] Although elastase inhibitors are not used in the clinic for the treatment of cancer at this time, some reports have suggested that the use of these agents for chemotherapy may provide a high therapeutic index. Following identification of the specific protease of the elastase class which cleaves cyclin E into the LMW forms, cyclin E-specific protease inhibitors may then be engineered.

The differential expression of the LMW forms of cyclin E in tumor versus normal cells may also occur through a relative decrease in the presence or function of an endogenous elastase inhibitor—elafin.[156] Thus an alternative approach to elastase inhibition could be to increase intracellular levels of functional elafin. Potential mechanisms for this approach include increased elafin expression through adenoviral gene therapy or by the administration of the elafin protein in target-specific, trigger-specific liposomes. While effective results through this means of drug delivery remain on the horizon,[157] it is possible that someday liposomes targeted to breast cancer-specific membrane receptors (e.g., ER, HER2) could deliver the relatively small elafin protein (9 kDa) intracellularly where a tumor-specific enzyme (elastase) could release the liposomal payload. Finally, as on-going studies better elucidate the mechanisms by which elafin is down-regulated in tumor cells, other approaches to increasing elafin expression will become available.

Another target at this nodal point in the cell cycle is Cdk2. Because of their central role in cell cycle regulation, Cdks have been targeted for both drug and small molecule therapy. The two basic schemes employed to inhibit Cdks include either direct blockade of their kinase activity or targeting of their major regulators (indirect). Over 50 direct chemical Cdk inhibitors have been described with varying degrees of Cdk specificity. Most of these compounds modulate kinase activity by interacting specifically with the ATP-binding pocket of the enzyme. Both in vitro and in vivo Cdk-specific cell cycle and anti-tumoral effects have been described for three Cdk modulators—flavopiridol, R-roscovitine, and BMS-387032—which have also recently been tested in phase I and II clinical trials.

Flavopiridol is a semisynthetic flavonoid which appears to induce cell cycle arrest by direct inhibition of all Cdks as well as through transcriptional repression of cyclin D1.[158-160] Phase I trials for flavopiridol have demonstrated tolerable toxicity with some objective responses across a spectrum of advanced solid and nonsolid tumors.[161,162] Furthermore, a Phase II trial in metastatic lung cancer showed a median overall survival consistent with both a randomized trial of four platinum-based chemotherapy regimens and with the survival observed with the approved EGFR inhibitor gefitinab (Iressa).[163-165] R-roscovitine (CYC202) is an olomoucine analogue and a potent inhibitor of Cdk1, Cdk2, and Cdk5.[166] Preclinical studies in multiple xenograft models have shown antitumoral effects in the forms of both cell cycle arrest as well as evidence of apoptosis.[167] Two phase I clinical trials of oral CYC202 have demonstrated tolerable toxicity[168,169] and both single agent and combination chemotherapy phase II clinical trials are being planned. BMS-387032 is an aminothiazole Cdk2 inhibitor with a 10-100-fold selectivity for Cdk2 over Cdk1, Cdk4 and other kinases.[170] In vitro and in vivo antiproliferative effects of this class of compounds include cell cycle arrest with loss of pRb phosphorylation and some evidence of apoptosis. Three phase I trials have shown tolerable toxicity and some objective responses.[171-173] Phase II and combination phase I trials are planned.

One nonspecific chemical Cdk modulator, UCN-01, has also been tested in clinical trials. In addition to anti-Cdk activity, UCN-01 also exhibits a number of other cell cycle and non-cell cycle molecular effects. With respect to the cell cycle, UCN-01 has been shown to abrogate both the G1[174-181] and G2 checkpoints through inappropriate cdc2 activation[182] and chk1 inhibition,[183-185] and also appears to possess increased cytotoxicity in cells with p53 mutations.[182] Important non-cell cycle effects include potent inhibition of protein kinase C isoenzymes and modulation of the PI3 kinase/Akt survival pathway.[186,187] UCN-01 has been evaluated in both phase I and II trials with tolerable toxicity and some objective responses.[188,189] Synergistic effects of UCN-01 have been observed with many chemotherapeutic agents in preclinical models[174,190-193] and clinical trials of combination chemotherapies are underway.

While the Cdk modulation approach is certainly intriguing, one major limitation of the current agents under investigation is their lack of true cytotoxicity. Although most of the agents being tested in clinical trials have shown some preclinical evidence of inducing apoptosis, a G1 or G2 cell cycle arrest is the predominant result. For this reason, results of the combination chemotherapy trials are eagerly awaited.

Another limitation shared by both these agents and other conventional chemotherapies is a lack of tumor-specificity. Once again, cell cycle deregulation in the form of LMW cyclin E isoforms may help overcome this lack of specificity. Indole-3-carbinol (I3C) is an indirect Cdk2 inhibitor which has recently been shown to induce a G1 arrest in breast cancer cells by inhibiting Cdk2 activity associated with the LMW forms of cyclin E.[194] In a study by, Garcia et al, MCF-7 breast cancer cells treated with I3C demonstrated a shift in the size distribution of the Cdk2 protein complex from an enzymatically active 90kDa protein to a larger, 200kDa protein, with reduced kinase activity. In addition, the treated cells appeared to have lost their association with the 35 kDa LMW isoform of cyclin E as compared with nontreated cells. Furthermore, I3C treatment was also associated with a subcellular cytoplasmic localization of the Cdk2-cyclin E complex. These changes were felt to be indole-specific as treatment with the I3C natural dimerization product, DIM, or the anti-estrogen, tamoxifen, did not produce similar results. No changes in CKI (p21 or p27) levels were seen with I3C treatment. While

compelling, this study is not without some limitations. Whether the effects of I3C on MCF-7 breast cancer cells are tumor-specific has not been determined as they were not compared to normal breast epithelial cells. Nor was the generalizability of the I3C treatment effects assessed in other cancer cell lines that express the proteolytic generated LMW isoforms of cyclin E (e.g., MDA-MB-157, MDA-MB-436, and Ovcar).

Other potential indirect modulators of Cdk2 activity worth considering include the CKIs p27 and p21. With respect to breast cancer, increasing the expression of p21 may provide an additional means of overcoming some anti-estrogen resistance as well as increase anti-estrogen sensitivity in ER-negative breast cancers. In a study by Chen et al,[195] after demonstrating a strong association between p21 and ER expression, the investigators proceeded to induce the ER and estrogen receptor element promoters in an estrogen responsive manner through over-expression of p21 in a p21-negative, ER-negative breast cancer cell line. These cells were sensitive to both the growth inhibitor effects of anti-estrogen treatment as well as the growth stimulatory effects of 17β-estradiol. These findings suggest that p21 may play a significant role in the estrogen-signaling pathway and raise the possibility that anti-estrogen therapy may be effective in p21-positive, ER-negative breast cancers. Furthermore, a number of commonly used breast cancer chemotherapeutic agents have also been shown to induce p21, including paclitaxel,[196,197] doxorubicin,[198] and vinorelbine,[199] raising the potential of treatment strategies that combine chemotherapy and anti-hormonal therapy in ER-negative breast cancers induced to express p21.

Other possible strategies for targeting CKIs include increased protein expression through gene therapy or administration of tumor-targeted peptidomimetics of CKIs or other peptides that inhibit CDK activity. Because both p21 and p27 are substrates for ubiquitination and proteosome-dependent degradation, strategies designed to decrease the turnover of these CKIs through inhibition of ubiquitin-mediated proteolysis by the proteosome should also be considered. In fact, induction of both p21 and p27 in MDA-MB-157 cells through inhibition of the proteosome by treatment with the HMG-CoA reductase inhibitor, lovastatin, has been demonstrated to cause a G1 arrest.[200] In this study, the mechanism of p21 and p27 accumulation was clearly shown to be due to unique inhibitory effects of the closed-ring prodrug form of lovastatin on the proteosome, and not related to the HMG-CoA reductase inhibition of the open-ring form of the drug. With respect to breast cancer, as low levels of p27 have also been correlated with poor prognosis in young breast cancer patients,[16] efforts geared towards increasing the levels of both p27 and p21, for previously described reasons, may be particularly helpful in overcoming cell cycle-related drug resistance. Currently investigations with other proteosome inhibitors such as farnesyl transferase inhibitors are also on-going.

Summary

As some facet of cell cycle deregulation is present in all tumors, it is reasonable to consider cancer a disease of the cell cycle. In addition to driving the malignant transformation of normal cells, cell cycle deregulation also contributes to the chemotherapy resistance of cancer cells, as these agents often rely on the presence of normal cell cycle checkpoints to cause cell death. However, while this cell cycle-driven resistance often seems insurmountable, it may ultimately prove to be the Achilles' heel of cancer cell survival.

As illustrated in this review, the deregulated cell cycle provides multiple opportunities for tumor targeted therapies to either break the cycle by reregulation or to target it in combination with more conventional chemotherapies in ways that result in mitotic catastrophe (e.g., DNA damage plus G1 and G2 checkpoint abrogation.) However, in order for these cell cycle-directed strategies to work, there are some basic requirements that need to be met. First, specificity through differential expression of the target in normal versus tumor cells must be present. Second, the mechanism of the differential expression needs to be understood. Finally, the mechanism needs to be exploited therapeutically. Deregulation of cyclin E through the proteolytic generation of hyperactive LMW isoforms meets these criteria and means of exploiting this potential Achilles' heel are underway.

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