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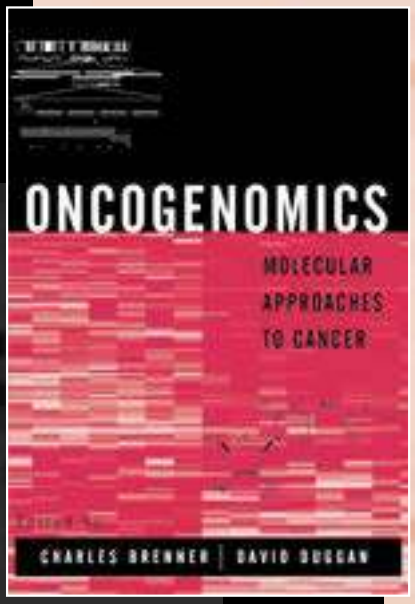

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Oncogenomics: Molecular Approaches to Cancer offers the first rigorous yet broadly accessible treatment of the impact of genomics on cancer research and care. Clearly written by world-renowned scientists in the field, and edited for overall cohesion, **Oncogenomics** progresses logically from molecular profiling to model systems, cancer pharmacology, and clinical trials.

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PROTEIN KINASES AS TARGETS IN CANCER THERAPY: VALIDATED AND EMERGING APPROACHES

Paul Nghiem, Yong-son Kim, and Stuart L. Schreiber

INTRODUCTION

The discovery of hundreds of protein kinases since the 1980s gave rise to a bewildering proposal that 1001 kinases may exist to carry out diverse functions within our cells and tissues (reviewed in (Blume-Jensen and Hunter, 2001). The fact that each of these enzymes uses adenosine triphosphate (ATP) as a substrate to phosphorylate protein targets suggests that specificity of kinase inhibitors would be a major challenge. Indeed, for many years, kinase-selective small-molecule inhibitors did not exist and the pharmacologic manipulation of kinases appeared to be an unlikely approach to successful therapy for cancer. After all, if a cell required hundreds of protein kinases for normal function, a nonselective inhibitor would be expected to have diverse unintended toxicities.

For these reasons, the approval of Gleevec (STI571/imatinib), a small-molecule inhibitor of the BCR-ABL tyrosine kinase, served as a very welcome proof of principle for this class of therapeutics. Gleevec demonstrated impressive efficacy in controlling chronic myelogenous leukemia as well as surprisingly low toxicity from a small

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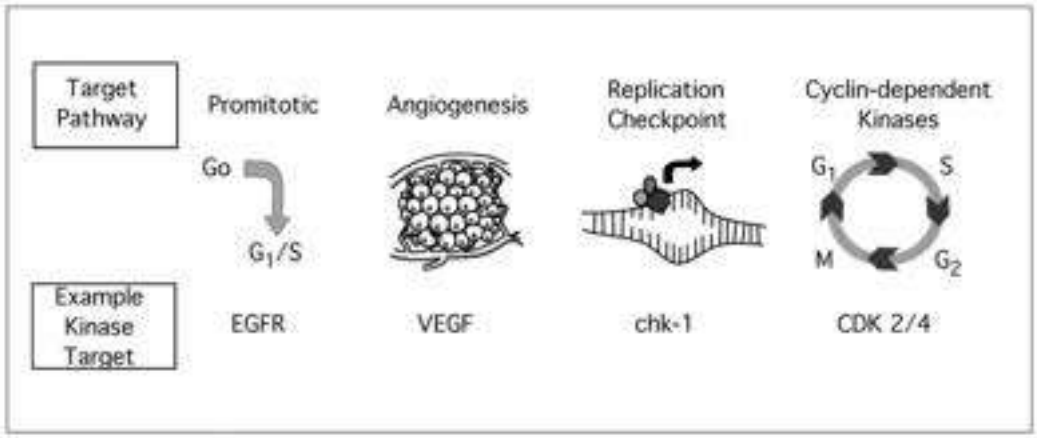

Box 13-1: Four major biological processes that are promising targets of small molecule inhibition in cancer therapy. Abbreviations: EGFR (epidermal growth factor receptor), VEGF (vascular endothelial growth factor), chk-1 (replication checkpoint kinase) and CDK (cyclin-dependent kinase).

molecule that inhibits several kinases other than the intended target, BCR-ABL. This agent, together with the approval of Herceptin (trastuzumab), an antibody that targets the Her2 tyrosine kinase, has generated an explosion of interest in kinases as therapeutic targets. These developments coincide with a greatly expanded understanding of how kinases normally function and are abnormally regulated in cancer cells. Indeed, according to the Pharmaceutical Research and Manufacturers Association, approximately 400 cancer-targeted drugs were under development or investigation in 2002, many of which target kinases (Box 13-1).

We review the biological rationale and development of four major protein kinase classes. (1) promitotic kinases (growth factor tyrosine kinases and cyclin-dependent kinases); (2) proangiogenic kinases, which are required for the increased vascular supply that tumors need to grow beyond about 2 mm; (3) DNA replication checkpoint kinases, which appear to be more important for cancer cells than normal cells; and (4) kinases involved in nutrient sensing and metabolic regulation. Throughout the chapter we have highlighted particular small molecules or agents in development because of the interesting pathways they target or their more advanced status in clinical trials. This chapter is not, however, intended to be an exhaustive review of the many kinase inhibitors in development.

PROMITOTIC KINASES

The Epidermal Growth Factor Receptor Family

The epidermal growth factor receptors (EGFRs) are a family of signaling kinases implicated in a large fraction of carcinomas (cancers of the epithelial tissues such as breast, colon, stomach, and prostate). The EGF receptor family is composed of 4 transmembrane receptor subunits and at least 11 ligands that bind and activate them in various combinations, depending on the tissue. These receptors are membrane tyrosine

kinases that bind to another family member upon ligand binding. They include the EGF receptor (ErbB1), Her2/neu (ErbB2), ErbB3, and ErbB4. Of these, ErbB2 is of special interest because it is overexpressed in many human cancers, and this overexpression is correlated with resistance to chemotherapy and poor prognosis (Harari and Yarden, 2000). Upon activation by a ligand, EGFR family members form dimers that lead to autophosphorylation of their cytoplasmic kinase domains. This in turn activates an array of downstream signaling pathways affecting cell survival and division. The exact nature of the activated downstream signaling pathways is determined by several factors including the expression pattern of receptors on the cell, the number of receptors expressed, and the amount and type of ligand that stimulates the cell.

Two-thirds of solid cancers are derived from epithelia, and EGF receptor signaling alterations are heavily implicated in a large fraction of these. Multiple mechanisms allow aberrant EGFR/ErbB signaling: Receptor mutations that constitutively activate the function of the receptor occur in glioma, non-small-cell lung cancer, prostate, ovary, breast, and stomach carcinomas; overexpression of normal ligands can induce activation of normal EGFR—for example TGF-alpha is overproduced by many tumor types and activates EGFR signaling in an autocrine loop; overexpression of wild-type EGFR family members occurs as a result of increased transcription or gene amplification and is associated with invasiveness, frequency of relapse, and prognosis in a variety of carcinomas (reviewed in Harari and Yarden, 2000).

The first agent to be approved that targets this pathway is an antibody directed against Her2 called Herceptin (trastuzumab; see Table 13-1). This antibody is approved for use in a subset of breast cancers that demonstrate ErbB2 overexpression (roughly 25–30% of breast cancers; Slamon et al., 2001). The clinical efficacy of this antibody is significant: It roughly doubles the response rate of Taxol (paclitaxel) in breast cancer as first-line therapy (from 15% for taxol to 38% with Taxol and Herceptin).

When Herceptin• was added to chemotherapy in metastatic Her2-overexpressing breast cancer, overall survival improved to 25.1 months versus 20.3 months, and time to disease progression improved (7.4 vs. 4.6 months; $p < 0.001$) relative to chemotherapy alone (Slamon et al., 2001). The major side effect from this therapy was heart disease. Specifically, Herceptin was associated with decreased left ventricular function, especially in patients who also received cardiotoxic anthracycline chemotherapy simultaneously.

In patients who received Herceptin and anthracyclines, 27% developed significant heart defects. This is a much higher number than among those receiving Taxol and Herceptin, of which 13% developed heart disease. Only 1% of Taxol-treated patients experienced this major side effect (Slamon et al., 2001). There is strong evidence that this cardiotoxicity is related to the actual mechanism of Her2-signaling inhibition by Herceptin: Transgenic animals in which Her2 was selectively deleted from the heart developed marked cardiac abnormalities (Crone et al., 2002). These mice developed thinning of the ventricular wall and decreased contractility, suggesting that ongoing Her2 expression and function is required for cardiac function.

A further point of interest about Herceptin is that it has shown marked antiangiogenic effects in a mouse model of human breast cancer, possibly by downregulating the vascular endothelial growth factor (VEGF) as well as other proangiogenic factors (Izumi et al., 2002). In summary, Herceptin, the first approved agent to target a receptor tyrosine kinase in cancer, does show significant efficacy over standard chemotherapy but also has a surprising mechanism-based cardiotoxicity as well as an additional unexpected therapeutic mechanism (angiogenesis inhibition).

TABLE 13-1. Summary of Kinase Inhibitors[a]

Name	Alternate Name	Target	Type of Inhibition	Class	Biological Mechanism
Herceptin	Trastuzumab	HER2/Neu/ErbB2	Antibody	Promitotic	Blocks signaling in HER2 overexpressing carcinomas
2 C-4		$ErbB_2$	Antibody	Promitotic	Blocks ErbB2 signaling
Cetuximab	IMC-C225	$ErbB_1$/EGFR/HER_1	Antibody	Promitotic	Binds/inhibits transmembrane receptor
Iressa	ZD 1839	EGFR	Small molecule	Angiogenic	Blocks EGFR tyrosine kinases
Gleevec	STI 571 or imatinib mesylate	bcr-abl Tyrosine kinase	Small molecule	Promitotic	Inhibits BCR-ABL, c-kit, PDGFR
Flavopiridol		Cyclin D_1/$CDK_{4/6}$	Small molecule	Promitotic	Blocks multiple cyclin-dependent kinases
SU 5416		KDR/VEGF-R_2/Flk-1	Small molecule	Angiogenic	Blocks the VEGF receptor—most important in tumor vessels
SU 6668		VEGF/PDGF/FGF receptor + c-kit	Small molecule	Angiogenic	Blocks kinase activity of VEGFR and PDGFR
Bevacizumab		pan-VEGF	Antibody-fusion protein	Angiogenic	Binds and disables VEGF
Rapamycin ester	CCI-779	FRAP/mTOR/RAFT1	Small molecule	Nutrient sensing and angiogenic	Counteracts loss of PTEN
UCN-01		Chk-1 and protein kinase C	Small molecule	Replication checkpoint	Inhibits Chk-1

[a]Presented in order discussed in chapter.

A new antibody-based inhibitor of Her2 activity, called 2C4, works by a distinct mechanism to target the recruitment of Her2 into EGFR-ligand complexes. Unlike Herceptin, 2C4 blocks signaling mediated by Her2 by interfering with dimerization of Her2 to other members of the EGF receptor family (Her2 cannot dimerize with itself or bind to a ligand directly, so it must interact with another member of this family). Importantly, when Her2 does form an active dimer with ErbB1, ErbB3, or ErbB4, it signals very potently to activate the cell and is less sensitive to downregulation than when the dimer does not contain Her2. These effects are believed to underlie the potent oncogenic activity of Her2 (Harari and Yarden, 2000). A major advantage of the mechanism of 2C4 is that it allows a broader range of efficacy among carcinomas including those of the breast and prostate that do not overexpress Her2 (Agus et al., 2002). Cetuximab (IMC-C225) is an antibody that binds ErbB1 and has also shown preliminary promise in a variety of epithelial cancers.

Several small-molecule inhibitors of the entire EGFR family are in development as cancer therapies. Iressa (ZD1839) is currently the most advanced of these drugs. Iressa works as a competitive inhibitor of ATP binding by EGFR tyrosine kinases and has shown antitumor activity in multiple tumor types in humans in a phase I trial (Herbst et al., 2002b). Toxicities in this trial were minimal and mostly involved diarrhea and an acne-like rash. Interestingly, Iressa also has antiangiogenic properties that may support its efficacy in cancer therapy via inhibition of EGFR function in vascular proliferation (Hirata et al., 2002).

BCR-ABL and Gleevec

Chronic myelogenous leukemia is rare among malignancies in that it is highly dependent on a single genetic mutation: a translocation between chromosomes 9 and 22, often resulting in a distinct cytogenetic phenotype known as the Philadelphia chromosome. It is present in over 95% of patients with chronic myelogenous leukemia (CML) and leads to production of the BCR-ABL oncogene. BCR-ABL is a tyrosine kinase with aberrant regulation of the kinase domain derived from the normal cellular version of the Abl tyrosine kinase. Expression of BCR-ABL can induce a CML-like disease in mouse models, an observation that contributed to great interest in developing inhibitors of its kinase activity.

Gleevec (imatinib, STI571) was approved by the FDA in 2001 after demonstration of marked efficacy in early and late stages of CML (Druker et al., 2001a, 2001b). Of 54 patients with CML who had been unresponsive to interferon-alpha treatment, 53 experienced a complete hematologic response with Gleevec treatment (Druker et al., 2001b). The success of this small-molecule inhibitor has provided great encouragement to the idea that selective kinase inhibitors can be effective therapy for cancer.

In the case of Gleevec, a lack of specificity (it also inhibits the c-kit and PDGF receptor tyrosine kinases) in fact has led to its utility in two other rare malignancies. Gastrointestinal stromal tumors (GISTs) and chronic myeloproliferative diseases have also been effectively treated with Gleevec. GI stromal tumors depend on overexpression of c-kit activity and are highly refractory to conventional therapies (Demetri et al., 2002). In a trial of 147 patients with advanced GIST, over half had a sustained objective response in the form of stable or diminished disease (there were, however, no complete responses; Demetri et al., 2002). For a subset of patients with myeloproliferative diseases associated with a constitutively active platelet-derived growth factor

receptor beta (PDGFB), Gleevec has proved effective in controlling their disease in four of four cases (Apperley et al., 2002). Strikingly, the side-effect profile of Gleevec is quite mild, including skin rashes and edema that rarely require discontinuation of the drug.

The major limitation of Gleevec appears to be the onset of resistance. In the case of CML, this appears to be mostly related to the emergence of mutations in the BCR-ABL kinase itself, rather than in the acquisition of efflux pumps for the drug in cancer cells (Shah et al., 2002). The mechanism of Gleevec's inhibition of BCR-ABL has been studied at the structural level. Like most kinase inhibitors, Gleevec binds the kinase in the ATP-binding pocket/catalytic domain. Most kinases are very similar in structure when they are in their active conformation, as they all must bind ATP and act as a phosphotransferase when in this active shape. In contrast, there are many mechanisms by which their activity is switched to an "off" position in which they do not bind ATP and substrate (Shah et al., 2002). The significance of this concept is that small molecules that bind to the kinase catalytic domain when it is in the active state are likely to have minimal specificity for a particular kinase. In contrast, there are more opportunities for specificity when small molecules bind to the inactive state of a kinase.

Indeed, in the case of Gleevec, elegant structural studies have demonstrated that this drug binds to the catalytic domain only in the inactive state, stabilizes this state, and thus prevents kinase function (Schindler et al., 2000). Thirty-two patients whose disease relapsed after an initial response to Gleevec were studied for mutations in the coding sequence of BCR-ABL. The 15 mutations were grouped into two classes: (1) those that alter the amino acids that directly interact with Gleevec, presumably interfering with binding to the drug; and (2) those that alter the kinase so that it cannot adopt its inactive (Gleevec-binding) state, hence locking it in an active state and causing Gleevec-resistant, constitutive activity of BCR-ABL (Shah et al., 2002). These detailed structural/molecular/clinical studies being performed on Gleevec are aiding in the development of second-generation drugs. Such modified Gleevec-like drugs will hopefully have less propensity for resistance, or could be used in a cocktail (much as HIV drugs are now administered) to minimize emergence of resistance.

Cyclin-Dependent Kinase Inhibitors

Because all dividing cells depend on the function of cyclin-dependent kinases to progress through the cell cycle, inhibition of this class of kinases would certainly be effective in controlling cancer if toxicity were acceptable. Flavopiridol is the only agent of this class that is currently in clinical trials for cancer (Zhai et al., 2002). Initially known as an inhibitor of protein kinase A and EGF receptor tyrosine kinase, flavopiridol• emerged from the anticancer drug screening program at the National Cancer Institute as a potent inhibitor of cell proliferation. Although it is a nonspecific inhibitor of multiple cyclin-dependent kinases (CDK2, CDK4, CDK6; see Fig. 13-1), it has shown activity against gastric and renal cancers in early clinical trials (Zhai et al., 2002). Future agents in this class will no doubt demonstrate some level of specificity for individual cyclin-dependent protein kinases, which will likely improve the therapeutic index.

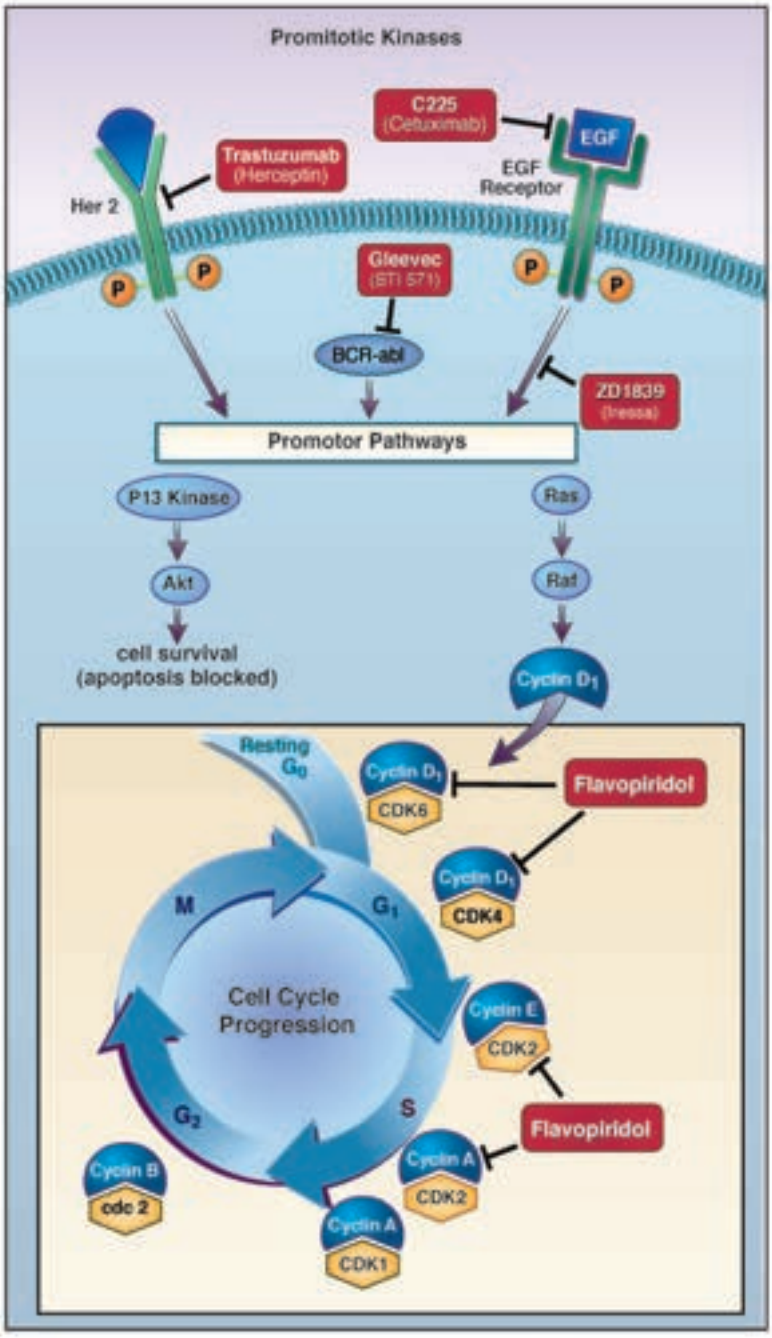

Figure 13-1. Promitotic kinases. Complex signaling networks link growth factors to survival and cellular proliferation. Tyrosine kinases are heavily represented in these pathways and their function is directly or indirectly upregulated in many cancers. These tyrosine kinases are activated when they bind ligand, dimerize, and phosphorylate each other to form an actively signaling complex. Several tyrosine kinases have been selected as targets for cancer therapy. As shown, the two recently approved protein kinase inhibitors, Herceptin and Gleevec, both target proteins in these pathways that promote cell survival and mitosis. In addition, a nonselective first-generation cyclin-dependent kinase inhibitor, flavopiridol•, targets the kinases required for progression of the cell cycle. EGF = epidermal growth factor; CDK = cyclin-dependent kinase; Her2 = ErbB2 = Neu = a member of the epidermal growth factor family that is often misregulated or upregulated in cancer.

Why Does Inhibiting Oncoprotein Signaling Often Kill Cancer Cells? The Addiction Model

On initial consideration, pharmacologic inhibition of a signaling pathway that promotes cell division might be expected to temporarily slow the progression of cancer and have few other effects. In the terminology used for antibiotics, such drugs are cytostatic rather than cytocidal. A major reason for excitement about inhibitors of oncoproteins is the surprising observation that temporarily blocking the signaling activity of these molecules can lead to death and/or loss of the malignant phenotype, rather than merely a temporary mitotic arrest. This has been observed in a variety of pathways required for cancer cell proliferation and likened to the cancer cell becoming "addicted" to the activity of the pathway (Weinstein, 2002).

Many of these observations have been made in mouse models of cancer involving hyperactivity of promitotic oncoproteins such as Myc and Ras. These studies made use of genetic switches to specifically turn on and off the expression of an oncogene temporarily (akin to having a totally specific small molecule targeting only one oncoprotein). In diverse examples, temporarily switching off the activity of the target caused the tumor to undergo extensive apoptosis, as though the cancerous "house of cards" collapsed when one key support was temporarily removed. In the case of Ras (Chin et al., 1999), the temporary loss of Ras expression caused regression of tumor vasculature and cancer cell death. In the case of osteosarcomas (bone tumors) induced by ongoing Myc overexpression, the temporary discontinuation of Myc expression in these tumors led to longstanding differentiation into mature bone tissue (Jain et al., 2002).

Using an elegant chemical genetic approach, Fan and co-workers transformed fibroblasts with a modified version of *v-erbB*. They used an engineered version of the *v-erbB* kinase that is constitutively active but also mutated so that it can be specifically inhibited by an ATP-competitive inhibitor that has no effect on other kinases. Addition of the inhibitor to cells that express this *v-erbB* kinase caused the cells to arrest. As expected, withdrawal of the inhibitor allowed *v-erbB* kinase activity to become constitutive again. However, unexpectedly the cells failed to reenter the cell cycle after temporary inhibition of this pathway (Fan et al., 2002), again suggesting that a temporary disruption of promitotic signaling can have a long-lasting effect on the malignant phenotype.

In addition to these studies of the "addiction" paradigm in mouse and in vitro models, there is now excellent evidence that human cancers behave similarly in many cases. Gleevec serves as an important example, with tumors such as chronic myelogenous leukemia addicted to BCR-ABL signaling (Druker et al., 2001b) and gastrointestinal stromal tumors addicted to c-kit signaling (Demetri et al., 2002). In each of these cases, the majority of the malignant cells are not merely arrested in mitotic activity but also depleted in their numbers by many orders of magnitude when deprived of the oncogenic kinase activity to which they have become addicted.

PROANGIOGENIC KINASES

The concept that tumors depend on angiogenesis (recruitment of blood vessels) for their growth has received extensive attention in the past few years in the scientific and lay press. This basic concept has been championed for three decades by Folkman and his colleagues, and has gained general acceptance. There is, however, much controversy

over how effective this approach will ultimately be and when it may show efficacy. Phase I human trials using the initially described peptides (endostatin and angiostatin; Herbst et al., 2002a) are not meeting the high expectations created by the prior mouse studies (O'Reilly et al., 1997) and extensive media coverage. The field is now maturing beyond initial excitement and beyond these inhibitory peptides. The most active and promising areas of antiangiogenesis research are now based on antibodies and small molecules targeted against kinases.

A key concept underlying the approach of targeting cancer by depriving its blood supply is that the blood vessels that feed malignant tumors are unstable and highly immature relative to those of normal tissues. Tumor-associated vessels appear to depend on ongoing growth factor stimulation from the tumor in a manner that is different from blood vessels elsewhere in normal mature tissues. In particular, tumor vessels express high levels of vascular endothelial growth factor (VEGF) receptors. Such high-level expression of VEGF receptors is not characteristic of mature vessels (Brown et al., 1993). In support of the functional significance of this expression, inhibition of VEGF signaling causes rapid apoptosis of tumor-associated endothelial cells but not normal endothelial cells (Laird et al., 2002). These concepts have added enthusiasm to the possibility of transiently inhibiting VEGF signaling and inducing selective toxicity to tumor tissue while sparing normal tissue.

The VEGF family of transmembrane tyrosine kinase receptors is composed of three subtypes of receptors bound in different combinations by seven ligands. VEGF-receptor function is highly similar in nature to that of other tyrosine kinase growth factor receptors in signaling and activation. As shown in Figure 13-2, VEGF/ligand binding to the receptor causes dimerization of two receptor subunits and autotransphosphorylation of defined tyrosine residues on the intracellular portion of the receptor, leading to activation of signal transduction pathways including src and ras-MAP kinases (Arbiser et al., 1997; Kroll and Waltenberger, 1997).

One VEGF receptor of particular interest is KDR (VEGF-R-2/FLK1), which is important in endothelial cell proliferation and angiogenesis, facilitating tumor progression. A small molecule called SU-5416 specifically inhibits this target (Fong et al., 1999). Relative to other VEGF-receptor subtypes, inhibition of the KDR receptor (via genetic approaches) in particular shuts down tumor growth and causes regression in animal models (Brekken et al., 2000; Yoshiji et al., 1999). These observations argue that the VEGF-R-2/KDR receptor is of particular importance as a cancer therapeutic. In addition, an anti-KDR/flk receptor antibody is being developed (Zhu et al., 1999).

Several approaches are being developed to more broadly inhibit the signaling from all VEGF receptors. SU6668 is a small molecule that targets the catalytic activity of a wide variety of tyrosine kinases including all three VEGF receptors: PDGF receptor, FGF receptor, and c-kit (see Fig. 13-2). Bevacizumab is a monoclonal antibody that binds the VEGF ligand, hence acting like a sponge to bind up VEGF and prevent it from interacting with VEGF receptor present on vascular endothelial cells. An initial trial of this antibody in metastatic renal cell carcinoma showed a decrease in average time to cancer progression among 37 patients treated with Bevacizumab (5 months to progression) compared with 38 patients who received placebo (<2 months to progression; Yang et al., 2002).

Rapamycin is a small molecule that is currently approved for prevention of rejection of transplanted kidneys via its inhibitory effects on the activation of lymphocytes. Recently, however, both rapamycin and the closely related rapamycin ester, CCI-779,

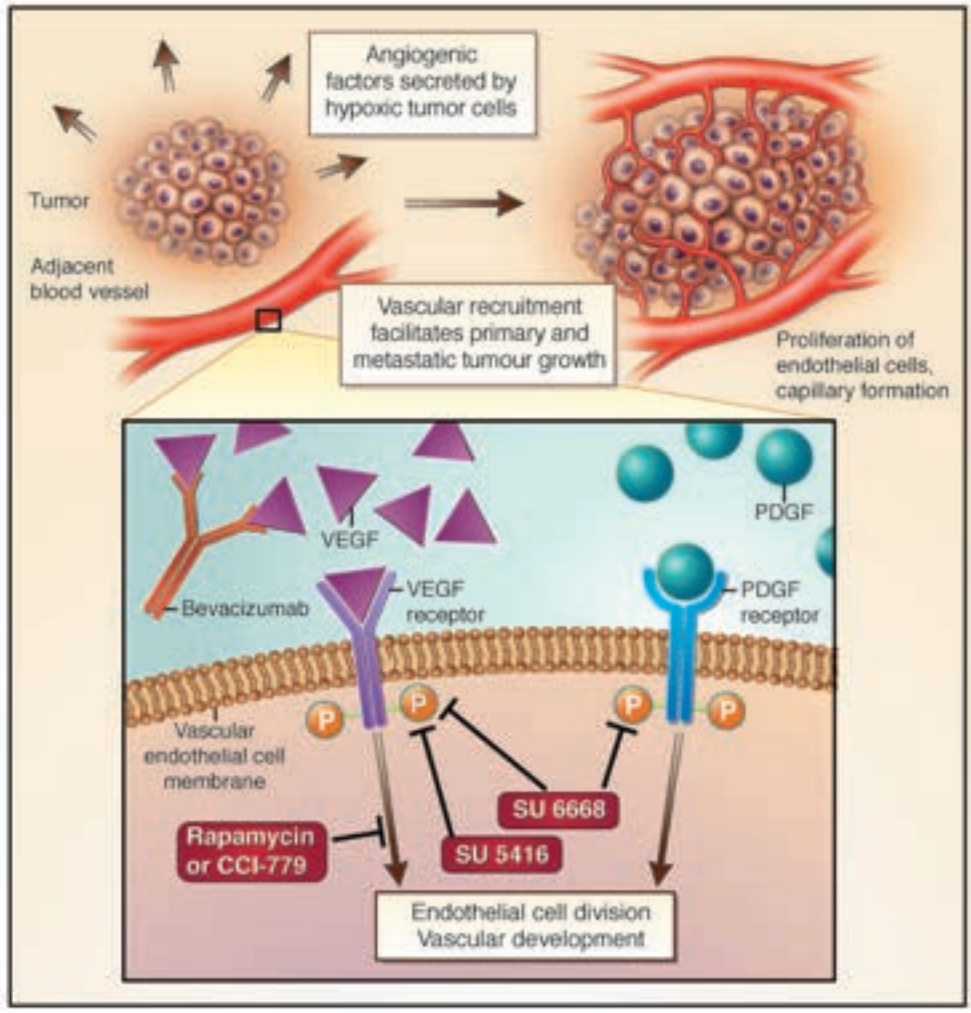

Figure 13-2. Angiogenesis-related kinases. Unlike the normal blood vessels that supply mature tissues in our bodies, the blood vessels that supply malignant tumors require ongoing growth factor stimulation to avoid apoptosis as well as to expand. For these reasons, inhibiting proangiogenic kinase pathways has shown great promise in preclinical and early clinical trials. Small molecules and antibodies that disrupt key steps in these pathways are described in the text.

have been shown to inhibit the intracellular signaling pathway leading from the VEGF receptor to proliferation in endothelial cells (Guba et al., 2002). There are also other mechanisms by which rapamycin appears to act against cancer, as described next in the section on kinases involved in metabolism and nutrient sensing.

As with many other approaches to cancer therapy, the combination of antiangiogenic treatments with other established therapies appears promising (Klement et al., 2000) and will be extensively investigated once these agents become readily available.

KINASES INVOLVED IN NUTRIENT SENSING AND METABOLIC PATHWAYS

Since the early 1990s, great progress has been made in understanding the mechanism of action of the immune suppressant and anticancer agent called rapamycin.

The relevant cellular target for rapamycin is a large protein kinase known as FRAP (mTOR/RAFT1; Brown et al., 1994; Sabatini et al., 1994). FRAP/mTOR is not directly bound by rapamycin but instead is complexed when rapamycin binds a small cellular protein called FKBP12; this FKBP12-rapamycin complex inhibits FRAP/mTOR function. The rapamycin ester CCI-779 (cell cycle inhibitor-779) has an altered stability and solubility profile but functions in the exact same manner within cells. CCI-779 is on the fast track for clinical development in renal cell carcinoma through the National Cancer Institute.

Through studies in yeast (where the FRAP/mTOR ortholog is known as the target of rapamycin or TOR) and in mammalian cells, it now appears that the normal cellular role of FRAP/mTOR is to serve as a nutrient sensor, regulating transcription and translation in order to optimally respond to the abundance or paucity of nutrients such as amino acids and glucose (Hardwick et al., 1999; Peng et al., 2002; see Fig. 13-3). To a first approximation, rapamycin's ability to inhibit lymphocyte activation is due to the signal it seems to send cells: "Nutrient supply is inadequate—do not proliferate or grow."

Given this background, it came as something of a surprise to find that rapamycin has anticancer effects as cancer cells have typically lost their sensitivity to most growth controls. Recent work has identified the molecular basis of why a major subset of cancers shows sensitivity to rapamycin. Using mouse and human cell approaches, several independent laboratories have concluded that the loss of a tumor suppressor called Pten (phosphatase and tensin homolog) is characteristic of cancer cells with rapamycin sensitivity (Mills et al., 2001; Neshat et al., 2001).

Figure 13-3 puts our knowledge of this pathway into a perspective that allows us to explain why rapamycin would have this "synthetic lethal" effect when combined with the loss of Pten function. As discussed in Chapter 10, yeast geneticists define synthetic lethality as a combination of individually nonessential mutations that lead to inviability. Similarly, a single chemical inhibitor that is tolerated by wild-type cells but not tolerated by mutants deficient in another protein target can reveal synthetic lethal relationships. Interestingly, as shown in Figure 13-3, a tumor suppressor lies on each arm of these two converging pathways. In the arm leading down from mitogens (e.g., insulin), Pten has now been established as an important tumor suppressor. People with a mutant copy of the *PTEN* tumor suppressor develop Cowden's disease, which is characterized by benign tumors called hamartomas, which often occur on the skin as hair follicle tumors called trichilemomas (Li et al., 1997). In contrast, tuberous sclerosis results when the *TSC* tumor suppressor is mutated (Cheadle et al., 2000). This tumor suppressor functions on the arm of the pathway that integrates information about nutrient status (Gao et al., 2002; Fig. 13-3). When this TSC tumor suppressor is lost, the net effect is again numerous benign hamartomas, which often manifest in skin as oil gland tumors called sebaceous adenomas. Cowden's disease and tuberous sclerosis are inherited in an autosomal-dominant pattern and behave as classic tumor suppressors according to the Knudson hypothesis: All somatic cells have lost one copy of the tumor suppressor and the loss of the other copy in a random manner leads to preneoplastic progression. For both *Pten* (Cowden's disease) and *TSC* (tuberous sclerosis), loss of the tumor suppressor leads to benign tumors and a predisposition for more serious malignancies of epithelia such as gastrointestinal and breast cancer.

It appears that a key readout of these two pathways relates to the signals for growth and cell survival that are mediated by the protein kinases Akt and p70S6

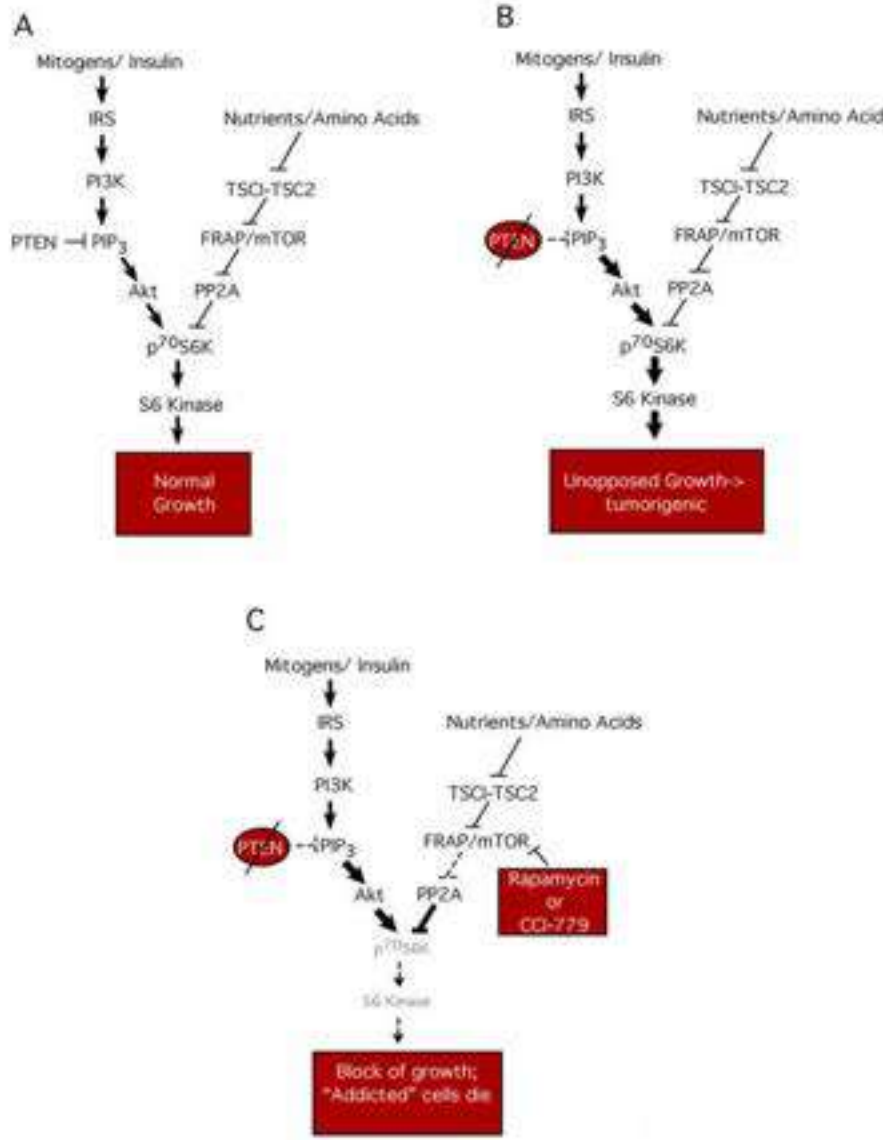

Figure 13-3. PTEN and FRAP/mTOR interactions in cancer. As shown in A (normal state), PTEN is a phosphatase that regulates the activation of the pivotal kinases, Akt and p70S6 kinase, by decreasing production of PIP_3. An opposing pathway integrates information about the nutrient status of the cell via the tuberous sclerosis tumor suppressors (TSC1 and TSC2), FRAP/mTOR, and PP2A, a phosphatase that is normally restrained by FRAP/mTOR (Peterson et al., 1999). As shown in B, a large subset of cancers have decreased PTEN activity, causing unopposed activation of Akt, p70S6 kinase, and cell growth. Panel C shows how the addition of rapamycin (or the closely related rapamycin ester, CCI-779) inhibits this pathway. Rapamycin leads to death of the cancer cells that have grown "addicted" to signaling via the PTEN pathway.

kinase. As shown in Figure 13-3, in the normal state the mitogen-activated pathway is held in check by Pten and by the inhibitory signals from the nutrient-sensing pathway involving Tsc. In cancers that have lost Pten function, there is unopposed activity of Akt and p70S6 kinase, leading to more rapid growth in cell size and protein translation (Vivanco and Sawyers, 2002). In a strong analogy to the addiction model presented in the promitotic kinase section, it appears that Pten-deficient cancer cells become highly dependent on this augmented level of Akt and p70S6 kinase activity. Indeed, when rapamycin is used to shut down the activity of p70S6 kinase (rapamycin inactivation is dominant over Akt hyperactivation for this pathway), these Pten-deficient tumor cells are far more sensitive than normal cells to this agent. We are still in the early days of investigating this type of cancer therapy, and it is likely that rapamycin (or other more promising "rapalogs" under development) will be capable of synergy with other, established cancer treatments.

Of significance to a large population of organ transplant recipients, rapamycin has allowed a dissection of the process of immune suppression from tumor promotion—that is, most immune suppressants that have been used for preventing the rejection of transplanted organs (cyclosporine and azathioprine) have shown a marked increase in the incidence of malignancies in the rapidly growing segment of our population that requires immune suppression. This has led to a hypothesis that immune suppression *per se* may lead to increased cancer incidence. A strong indication that this is not the case came in 1999 when cyclosporine was shown to promote cancer growth directly, even in mice that had no immune system (Hojo et al., 1999). In contrast to cyclosporine, at doses that prevent organ rejection, rapamycin has been associated with a *suppression* of malignant progression (Guba et al., 2002; Luan et al., 2002). Because malignancies that develop during prolonged immune suppression are now a cause of major morbidity and mortality (affecting up to half of transplant recipients by 10 years after transplant), rapamycin may become the immune suppressant of choice in this population.

REVISITING THE WARBURG EFFECT WITH MODERN MOLECULAR BIOLOGY

In the mid-1950s, Warburg believed he had solved the problem of cancer by applying principles he had discovered relating to the metabolism of sugars (Warburg, 1956). Recall that ATP can be produced by anaerobic glycolysis or aerobic respiration. In an observation that has held up quite well over time, Warburg noted that cancer cells frequently displayed an unusual metabolic process known as aerobic glycolysis, or lactic acid production from glucose, despite the presence of oxygen. Normal cells, in contrast, use oxygen when it is present to metabolize pyruvate fully to carbon dioxide and water, leading to respiration-derived ATP production.

In a jump of logic that did not turn out to be accurate, Warburg promoted the idea that it was merely the process of aerobic glycolysis (primary ATP production by an anaerobic process even under aerobic conditions) that was solely responsible for malignancy. He then reasoned that inhibiting this glycolytic process would have selective toxicity to cancer cells. Many decades later, as shown in Figure 13-4, we finally have the beginnings of a molecular understanding of the factors that regulate cellular metabolism. These factors switch cells between anaerobic and aerobic ATP production. Overactivity of the Her2 kinase promotes aerobic ATP production, whereas the myc

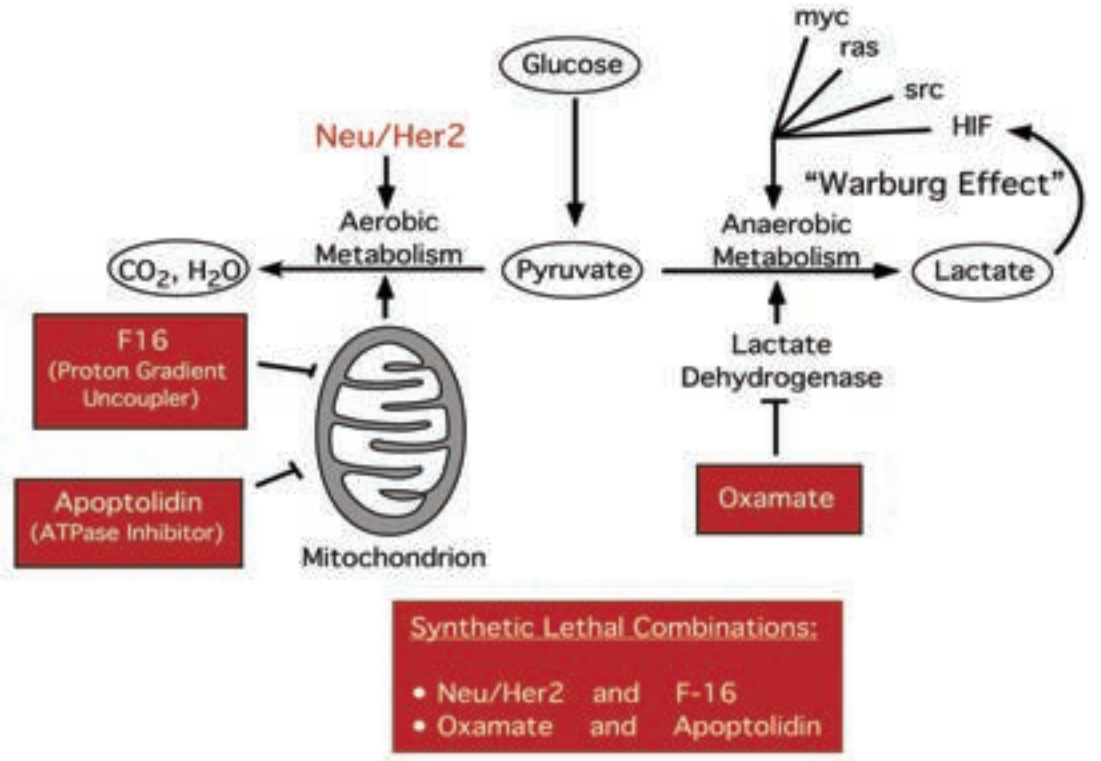

Figure 13-4. A modern model of the Warburg effect and Cancer. In the presence of oxygen, most normal cells break down glucose to pyruvate, then proceed to the left side of this figure, using mitochondrial oxidative metabolism to produce carbon dioxide and water. In 1956, Otto Warburg noted that many cancer cells instead produced lactate from pyruvate and suggested this pathway could be inhibited, leading to cell death. Recent studies have revived this notion after the observation that the Warburg effect is promoted by certain oncogenes (myc, ras, src) as well as HIF (hypoxia inducible factor; Lu et al., 2002a). In contrast, overexpression of Her2 has been shown to promote aerobic metabolism as judged by increased transmembrane potential across the mitochondrion. Synthetic lethality has been observed with agents that inhibit one of these pathways in cells that have been made to be dependent on that pathway. For example, Her2 overexpression promotes aerobic metabolism and makes cells selectively sensitive to killing by F16, a small-molecule proton gradient uncoupler. By inhibiting lactate dehydrogenase (and hence forcing cells to use aerobic metabolism), oxamate sensitizes cells to apoptolidin and other ATPase inhibitors. See text for details.

oncoprotein and HIF (hypoxia inducible factor) promote anaerobic ATP production (Lu et al., 2002a).

Several recent publications suggest that it is now time to revisit the Warburg effect to explain the antitumor activity of several small molecules. Fantin and co-workers searched for small molecules that would selectively kill cells that overexpressed the Her2 kinase but would not damage an isogenic clone of cells that did not overexpress this protein kinase (Fantin et al., 2002). This unbiased approach yielded a small molecule called F16 that killed Her2-overexpressing cells, had little effect on normal cells, and appeared to act by uncoupling the proton gradient within the mitochondria necessary for aerobic ATP production (Fantin et al., 2002).

In an analogous study, Salomon and co-workers found that a class of small molecules that act as F_0F_1 ATPase inhibitors had a striking pattern of selectivity among the 60 cancer cell lines established by the National Cancer Institute (Salomon et al., 2000), discussed in Chapter 7. A subset of the cancer cell lines were highly sensitive to inhibition of ATPase function by each of the three ATPase inhibitors used: apoptolidin, oligomycin, and ossamycin. The simplest explanation for this observation is that these cell lines were highly dependent on aerobic production of ATP through mitochondrial function such that inhibiting the mitochondrial ATPase produced toxicity in these cells.

Salomon and co-workers then found that the cell lines that were resistant to the mitochondrial F0F1 ATPase inhibitors could be made sensitive by inhibiting anaerobic ATP production using a variety of small molecules. Taken together, the data from these two distinct studies suggest that cancer cells (1) often have abnormal regulation of glucose metabolism that makes them sensitive to certain metabolic inhibitors; and (2) can be shifted in their metabolism so that they are selectively sensitive to such inhibitors.

Metabolism in cancer is only recently reemerging into the limelight, and the possibilities for characterization of the oncogenes that regulate these energy utililization pathways and the small molecules that can disable them are truly exciting. We anticipate that studies of the interactions of metabolic and oncogenic signaling networks will be a fertile area for cancer biology in the future.

DNA REPLICATION CHECKPOINT KINASES: ATR AND CHK-1

Human cells encounter major challenges in maintaining genomic fidelity as they divide in the presence of environmental and endogenous DNA-damaging agents such as chemicals, oxygen radicals, and radiation. When DNA is damaged or DNA synthesis is otherwise delayed, a cell requires additional time to ensure complete DNA replication prior to cell division. The replication checkpoint is the mechanism by which a cell ensures that DNA replication is complete prior to initiating chromatin condensation, an initial stage of mitosis. Studies in yeast and more recently mammalian cells have elucidated many of the key players in the replication checkpoint pathway. In humans, the protein kinase ATR and its downstream target, Chk-1, are required for the replication checkpoint and for survival after DNA damage (see Fig. 13-5). A major reason for the interest in therapeutically targeting this pathway is that cancer cells appear to be especially sensitive to inhibition of this pathway.

Why would cancer cells need ATR or Chk-1 more than normal cells? Many cancer cells have defects in regulation of the G0/G1 transition—that is, they tend to progress into the cell division cycle more rapidly than normal cells, especially through its early phases. This is often caused by loss of the tumor suppressors retinoblastoma (Rb) or p53 or other components of these pathways. There are many mechanisms through which p53 function is inhibited in various cancers, including overexpression of the normal cellular protein MDM2 (which inhibits p53 function), direct genetic mutation of the p53 gene, or expression of the human papilloma virus protein E6, which degrades cellular p53 (Levine, 1997). Through loss of p53 or other tumor suppressors, the net effect is that many cancer cells are defective in early cell cycle checkpoints.

We have demonstrated in vitro that many of these cancer-associated mutations make cells more sensitive to death following the combination of low-dose DNA damage and ATR inhibition (Nghiem et al., 2001; see Fig. 13-6). Similar observations

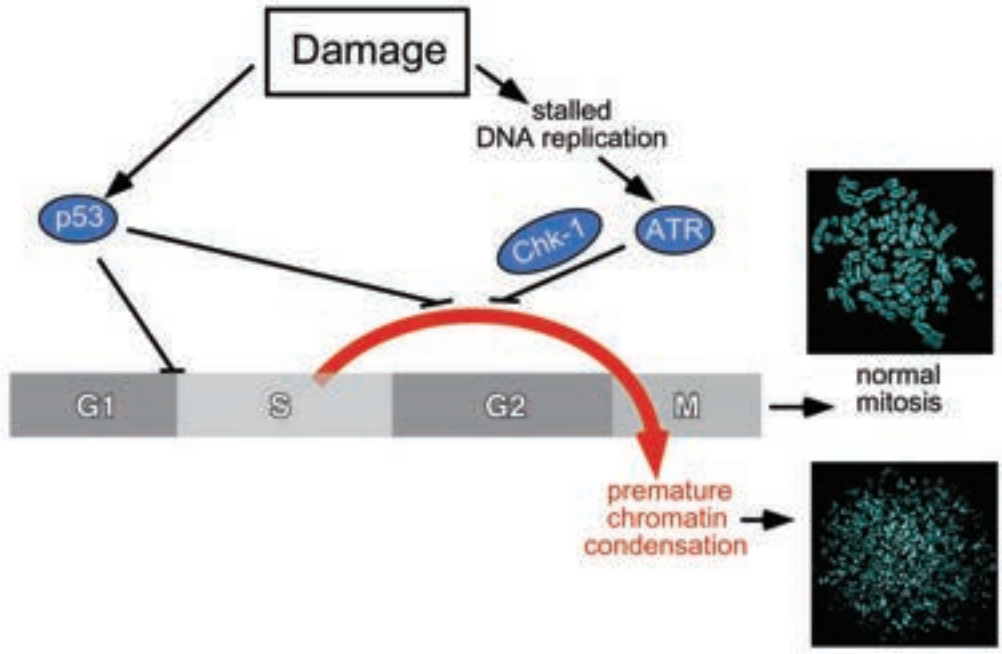

Figure 13-5. The replication checkpoint and p53 in the cell cycle. DNA damage activates p53 and causes DNA replication to stall, which in turn activates ATR. A major role for ATR is to activate Chk-1 and hence ensure that DNA replication (S phase) is complete before a cell enters mitosis (Nghiem et al., 2001). The curved arrow represents premature chromatin condensation (PCC), typically a lethal cellular event. PCC occurs when cells that have not completed DNA synthesis (S phase) undergo chromatin condensation. p53 is activated independently of ATR (Nghiem et al., 2002) and plays an important role in the replication checkpoint, synergizing with ATR and Chk-1 (Wang et al., 1996). (*Source*: Figure modified from Nghiem et al., 2002.).

of increased killing of p53-negative cells have been made for Chk-1 inhibition by UCN-01 (a staurosporine analog; Wang et al., 1996) or for methylxanthines (caffeine and pentoxyfylline) at high concentrations (Fingert et al., 1986; Powell et al., 1995). It is now known that methylxanthines inactivate ATR as a key target in sensitization of p53-negative cells to DNA damage (Nghiem et al., 2001; Sarkaria et al., 1999). The simplest explanation is that in cells that have already lost cell cycle checkpoint function, further checkpoint inhibition is especially toxic or synthetically lethal when combined with the underlying functional defect.

How might replication checkpoint inhibition be used therapeutically? Caution will be required in approaching this problem as complete loss of function of the replication checkpoint by genetic deletion of either ATR or Chk-1 is rapidly lethal to a developing embryo or to dividing cells in culture. In contrast, partial inhibition of the replication checkpoint by small-molecule inhibitors or by expression of dominant negative ATR or Chk-1 is tolerated for many cell divisions in unstressed cells. In cells that have lost other G0-G1 checkpoint pathways that are typically lost in cancer, partial inhibition of ATR by genetic or chemical approaches has been shown selectively to induce premature chromatin condensation, leading to cell death (Nghiem et al., 2001, 2002). Several studies of Chk-1 demonstrated similar effects (Wang et al., 1996; Graves et al., 2000).

Subsequent studies have shown that one of ATR's key roles is to safeguard chromosomal stability at so-called fragile sites within the genome (Casper et al., 2002).

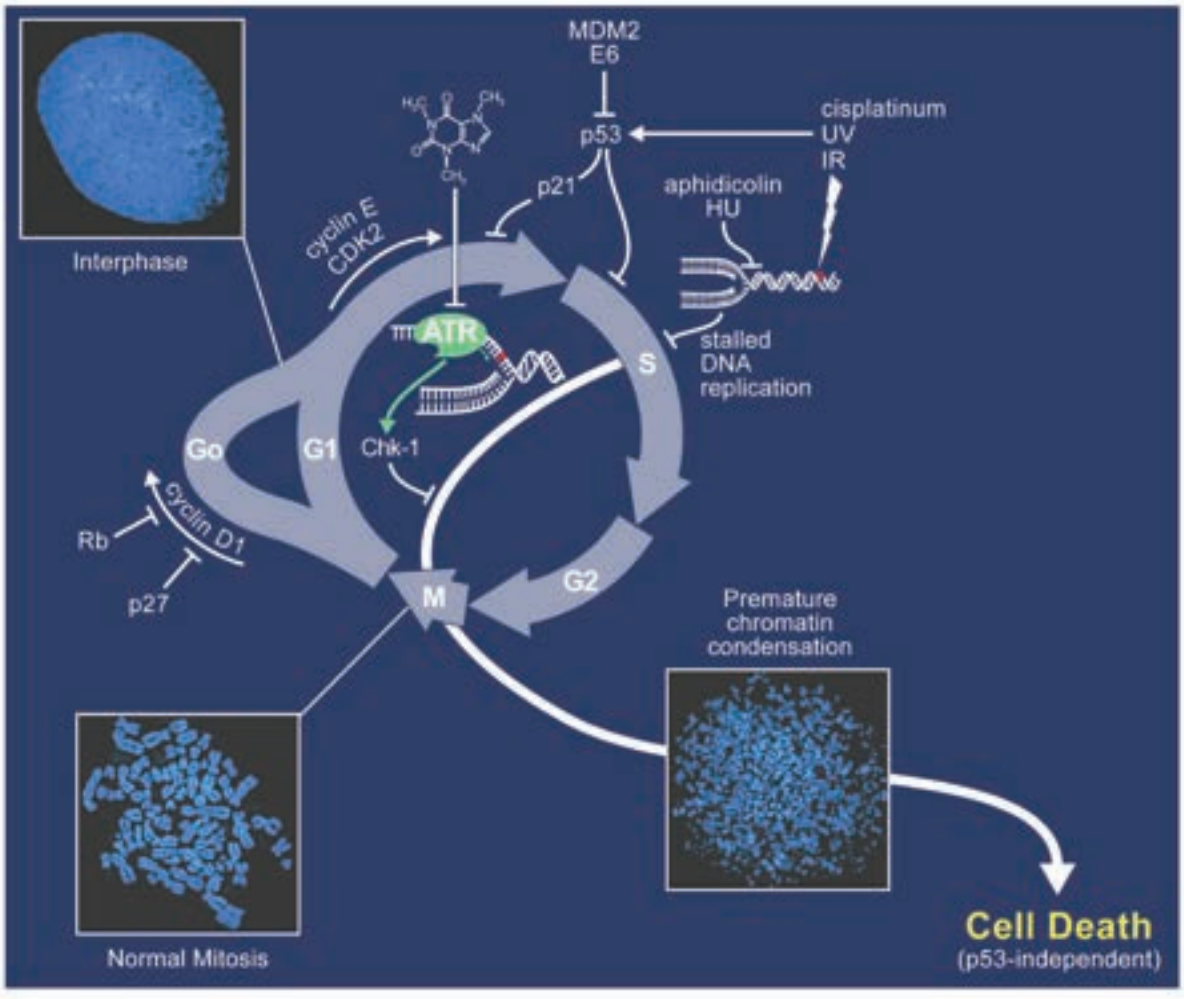

Figure 13-6. A model of ATR and Chk-1 function in the cell cycle of normal and cancer cells. DNA damage promotes cell cycle arrest via multiple mechanisms including activation of p53 and p21, but these checkpoints are defective in cancer cells (Levine, 1997). In addition, DNA-damaging agents (UV, ultraviolet; IR, ionizing radiation and cisplatinum, a DNA cross-linking agent) and DNA synthesis inhibitors (aphidicolin and hydroxyurea) increase the number of stalled replication forks (i.e., regions where DNA synthesis has halted abnormally). Naturally, such problems prolong the time required for DNA synthesis.

The signal that DNA replication is not complete is sent by ATR (bound to a stalled DNA replication fork). When ATR detects that replication is incomplete/stalled, it phosphorylates Chk-1, leading to the delay of mitosis while DNA remains incompletely replicated. The net effect is that the cell avoids a lethal error (represented by the white line that short-circuits the DNA synthesis [S]/G2 phases, leading directly to mitosis [M], premature chromatin condensation and death). Less severe defects can occur when premature chromatin condensation takes place at only a few sites in the genome, leading to gaps, breaks, and translocations that can be found in cancer cells at chromosomal fragile sites (Casper et al., 2002; Huebner and Croce, 2001). The chemical structure of a small-molecule inhibitor of ATR (caffeine) is depicted blocking ATR activity. For an interactive, animated version of this model, see www.schreiber.chem.harvard.edu/home/animation.html.

This observation is of significant interest in cancer biology because of extensive evidence that cancer cells show frequent breaks and translocations at these same fragile sites as normal cells (Huebner and Croce, 2001). This suggests that cancer cells have previously experienced sublethal replication checkpoint insufficiency: "battle scars," as it were, of premature chromatin condensation. On the one hand, it suggests that cancer

cells are already deficient in replication checkpoint function and hence would be very susceptible to inhibition of this checkpoint by small molecules. On the other hand, it is plausible that inhibiting this replication checkpoint may cause genomic instability at these sites, disrupt known tumor suppressors at these sites, and promote carcinogenesis. One likely mechanism for such an effect would be loss of the Fhit tumor suppressor that lies on chromosome 3 within the most common fragile site in the human genome (Huebner and Croce, 2001).

Several approaches will be required in order to address the feasibility of inhibiting the replication checkpoint as a therapy for cancer. First and foremost, there is a great need for selective small-molecule inhibitors of these kinases. The best-known inhibitor of ATR is caffeine (Nghiem et al., 2001; Sarkaria et al., 1999), which has been used extensively for in vitro studies of this checkpoint. Interestingly, topical application of caffeine to the skin has recently been used to markedly suppress the development of carcinomas in mouse skin that arise after ultraviolet radiation treatment (Lu et al., 2002b). Unfortunately, in order to inhibit ATR systemically, caffeine must be administered in doses that are too high to be tolerated in humans due to its unintended inhibition of phosphodiesterases and other targets, leading to seizures and cardiac arrythmias (Stewart et al., 1987). For Chk-1, the best-known inhibitor is a small molecule called UCN-01 that was initially identified due to its potent inhibition of protein kinase C. Indeed, UCN-01 is currently in clinical trials with some evidence of efficacy in phase I studies (Sausville et al., 2001).

The future of this approach of inhibiting the replication checkpoint depends on development of new small-molecule inhibitors and on studies that illuminate the effects of replication checkpoint inhibition on normal tissues. The characterization of the three-dimensional structure of the human Chk-1 kinase serves as a solid working point to determine and improve upon small-molecule inhibitors of this kinase (Chen et al., 2000). The crystal structure suggests that the amino-terminal kinase domain may be controlled by interaction with the carboxyl terminus of Chk-1, as deletion of the carboxyl terminus increases kinase activity (Chen et al., 2000). Efficacy of these agents will likely require combination with other DNA-damaging or replication inhibitors but may well provide a novel mode of selectively targeting cancer cells based on common defects in their cell cycle checkpoint regulation.

SUMMARY

The past decade has brought great excitement and promise to the concept of targeting protein kinases for the therapy of cancer. The four general classes of protein kinases were presented roughly in the order of their realization. Exemplified by Gleevec and Herceptin, inhibitors of promitotic kinases are already available in the clinic and more will soon be available. For the angiogenic kinases, rationally designed inhibitors are now in clinical trials. In addition, several kinase inhibitors that were approved based on other known mechanisms (rapamycin and Herceptin) have demonstrated significant antiangiogenic properties. FRAP/mTOR, a key kinase that regulates nutrient-sensing pathways, can be inhibited by rapamycin or the closely related CCI-779, which selectively kills cancer cells deficient in the Pten tumor suppressor. Regarding the ability to target cancer selectively via a newly acquired understanding of the Warburg effect relating to cancer cell metabolism, studies are still in the preclinical phase, but

good small-molecule leads exist. The class of replication checkpoint kinases, the least advanced class presented here, has one agent, UCN-01, in clinical trials. Many questions remain to be answered, however, regarding the risk/benefit profile of inhibiting the ability of a cell to sense whether it has completed DNA replication.

A major challenge for the future remains the discovery of novel inhibitors that demonstrate specificity toward the desired kinases and acceptable side-effect profiles. It is now clear, however, that in many cases this will be both possible and therapeutically effective.

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CHAPTER 13 PROTEIN KINASES AS TARGETS IN CANCER THERAPY: VALIDATED AND EMERGING APPROACHES

ABSTRACT

Protein kinase signaling is abnormal in a large proportion of human cancers. We discuss progress in targeting kinases that serve in each of four distinct functional classes relevant to cancer: (1) promitotic kinases (c-abl, c-kit, cyclin-dependent kinases, and epidermal growth factor receptor kinases including Her2/neu/ErbB2); (2) proangiogenic kinases (vascular endothelial growth factor and platelet-derived growth factor receptor kinases). The third and fourth classes involve emerging concepts relating to kinases involved in sensing cellular stresses: (3) DNA replication checkpoint kinases (ATR and Chk-1) that normally prevent entry into mitosis if replication is inhibited by DNA damage or insufficient nucleotides and (4) kinases involved in nutrient sensing and regulating metabolic pathways (FRAP/mTOR and Her2). In each of these four cases, the underlying biological pathways are outlined schematically, the unique features of targeting kinases in these pathways are highlighted, and the status of development of inhibitors is described.

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