

p21^{Waf1/Cip1} as a therapeutic target in breast and other cancers

Robert H. Weiss*

Division of Nephrology, Department of Internal Medicine and Cancer Center
University of California, Davis, Davis, California 95616, and
Department of Veterans' Affairs Northern California Health Care System, Mather, California 95655
*Correspondence: rhweiss@ucdavis.edu

The cyclin kinase inhibitor p21, originally described as a universal inhibitor of cyclin-dependent kinases, has since been shown to have additional functions other than CDK inhibition. It is likely that a key role of p21 is to keep cells alive after DNA damage and subsequent p53 induction, in order for the cell to effect repairs. Thus, the increase in p21 seen in some cancers may impart these cells with a survival advantage. Here we discuss how this antiapoptotic aspect of p21 makes it an attractive target for cancer therapy; attenuation of p21 in malignant cells may subvert the normal repair process induced by DNA-damaging chemotherapeutic agents and thus make such drugs more effective.

While p21 is a member of the cip/kip family of cyclin kinase “inhibitors,” it possesses a variety of properties that make this appellation, in many ways, a confusing misnomer. The initial descriptions of p21 focused on its location in the tumor suppressor pathway downstream of p53 (el-Deiry et al., 1993), its function as an inhibitor of G1 cyclin kinases (Xiong et al., 1993; Harper et al., 1993), and its role in differentiation (Sherr and Roberts, 1999). However, more recent investigations have shown that p21 also plays roles in allowing cell cycle transit as well as preventing apoptosis; since programmed cell death is the ultimate mechanism by which cancer chemotherapeutics exert their salutary effects on tumor cells, this property of p21 has considerable untapped potential to be of fundamental importance in the therapy of human cancer.

p21 and the cell cycle

Sequential activation of cyclin/CDK pairs regulates progression of cells through the cell cycle. Due to its placement in the p53 signaling cascade downstream of this all-important tumor suppressor, the initial descriptions of p21 focused on its ability to complex with cyclin A/CDK2 and cyclin D1/CDK4 pairs (on separate binding sites of the p21 protein) and inhibit their activity *in vitro*. However, the story became more complicated when it was found that p21/cyclin A/CDK2 complexes exist in both catalytically active and inactive forms (Zhang et al., 1994), suggesting that perhaps it was a stoichiometry issue related to the number of molecules of p21 relative to the cyclin/CDK complex which dictated the ultimate response of a cell to p21. This mechanistic possibility was called into question by later work, based on the crystal structure showing that a single p21 molecule can bind to both cyclin and CDK subunits, demonstrating substantial cyclin A/CDK2 inhibition at a mere 1:1 molar ratio of p21 to the cyclin A/CDK2 complex (Hengst et al., 1998; Adkins and Lumb, 2000). In addition to the cyclin and CDK binding sites on p21, there exists a distinct PCNA binding site on p21, raising the possibility that sequestration of PCNA, which is required for DNA polymerase δ function, could be a mechanism by which p21 attenuates cell growth (Rossig et al., 2001). This possibility was supported by data showing that the PCNA interaction with p21 increases the latter protein's stability (Cayrol and Ducommun, 1998).

Regardless of the stoichiometry and PCNA binding arguments, and based on the previously mentioned finding that

p21/cyclin/CDKs can exist in active complexes, more recent work in our and other laboratories has led to the discovery that p21 is required for G1→S phase progression in at least some cell types. The role for p21 as an assembly factor for cyclin D1/CDK4 complexes (Weiss et al., 2000; LaBaer et al., 1997) helps explain the heretofore mysterious result that p21 levels are increased soon after mitogen stimulation. Data is emerging showing that, in addition to its growth inhibitory role, p21 can function in a positive fashion toward cell proliferation (Dong et al., 2003; Dupont et al., 2003; Zhang et al., 2003), a finding, of course, with practical relevance toward cancer therapy. In retrospect, this finding was not altogether unexpected based on a growing body of evidence for p21 as a target for PI3K through Akt-induced phosphorylation, since PI3K itself possesses cell cycle progression properties (in addition to its effect on apoptosis—see below). It now appears that the switch between cell cycle promotion and inhibition by p21 may occur by virtue of the subcellular localization of this protein, as cytosolic localization of p21, using a mutant that mimics phosphorylation on threonine 145, resulted in a loss of the cell cycle inhibitor property of p21 (Zhou et al., 2001). In addition, the forced cytosolic localization of p21 using an NLS-deficient construct resulted in cell cycle progression (Dong et al., 2003) as well as protection from apoptosis (Asada et al., 1999). In other related studies, p21 has been shown to mediate the pro-proliferative effect of IGF-1 (Dupont et al., 2003) and Ets-1 (Zhang et al., 2003) in a variety of cells. However, p21 also appears to play a positive role in cellular senescence, at least in HeLa cells, as its overexpression in these cells induces this phenotype while attenuation inhibits senescence (Wells et al., 2000; Goodwin and DiMaio, 2000). Furthermore, microinjection of anti-p21 antibody into senescent Hs68 human foreskin fibroblasts resulted in their reentry into the cell cycle (Ma et al., 1999).

After all is said and done, however, and despite these advances in knowledge of the molecular biology of p21, the precise mechanism by which this multifunctional protein regulates cell cycle progression remains, for the moment at least, unknown.

p21 and apoptosis

Cells with damaged DNA and consequent increased p53 activation can either have their DNA repaired or, if the damage is so

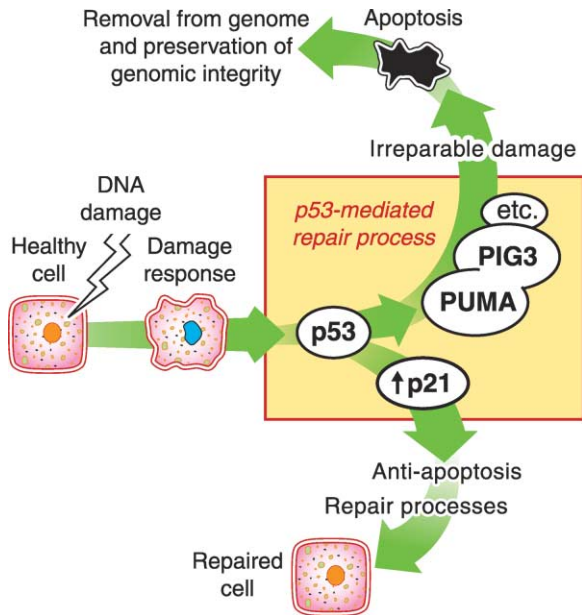


Figure 1. p21 determines the fate of DNA-damaged cells

Upon detection of as little as a single strand break, the p53 pathway is activated, leading to either apoptosis (through activation of such genes as PUMA and PIG3) or antiapoptosis (through p21 mediated by Myc and the DNA binding protein Miz-1). The subsequent increase in p21 allows the cell to survive the DNA repair process.

extensive that this is not possible, the cells will be sent to the apoptotic garbage heap, thereby preserving genomic integrity (Figure 1). The function of p21 to arrest growth after DNA damage (in response to activated p53) has been well established, yet recently a critical role of p21 to concomitantly prevent apoptosis has been uncovered. This sequence of events makes teleological sense because a repairing cell requires time; a dead cell with its DNA repaired is a waste of cellular energy.

In response to DNA damage and subsequent p53 activation, the cell has to make a decision between cell cycle arrest and apoptosis. In an important study addressing the mechanism of this phenomenon, Massague's group demonstrated a critical role of Myc and the DNA binding protein Miz-1, being recruited to the p21 promoter and influencing the outcome of the p53 response to DNA damage (Seoane et al., 2002). Another key mechanistic study showed that caspase-3 mediated cleavage of p21 during the apoptotic process sends growth-arrested cells to apoptosis (Zhang et al., 1999)

Phosphorylation of p21 by Akt, which lies downstream of the antiapoptotic signaling protein PI3K, resulted in increased p21 stability as well as enhanced glioblastoma cell survival (Li et al., 2001). Constitutively activated Akt, which lies downstream of the antiapoptotic signaling protein PI3K, results in cytosolic localization of p21 and suppression of its growth-inhibiting activity (Zhou et al., 2001). The latter study also suggested that cytosolic p21 may increase cell proliferation in Her-2/neu overexpressing cells. Paclitaxel was found to cause cytosolic localization of p21, resulting in attenuation of its antiproliferative effect in squamous carcinoma RPMI-2650 cells (Heliez et al., 2003). p21 also becomes localized to the cytoplasm during differentiation of at least some cell types (Asada et al., 1999; Tanaka et al., 2002); this localiza-

tion allows p21 to act upon targets molecules such as SAP (Shim et al., 1996) and ASK1 (Asada et al., 1999) kinases to inhibit their catalytic activities and thus prevent apoptosis. These data further suggest that subcellular localization is not only important for the antiapoptotic effect, but may be key to promoting cell proliferation, consistent with the cell cycle progression activity seen in vascular smooth muscle (VSM) cells with forced increased cytosolic p21 (Dong et al., 2003). Thus, p21 is not only a cell cycle inhibitor and cyclin/CDK assembly factor, but it also functions as an antiapoptotic and growth-promoting protein; the latter properties suggest that, at least in some contexts, this protein may be eminently exploitable in cancer.

p21 and cancer

The cancer community has been somewhat slow to embrace the possibility of using the CKIs as cancer targets, which is understandable considering the original press on this protein showing that (1) it lies downstream of the all-important guardian of the genome, p53, which, of course, is not to be tampered with; and (2) it is a "universal inhibitor" of cyclin kinases (Xiong et al., 1993). However, in light of the recent data described above that p21 also functions, under the appropriate conditions, as an antiapoptotic and growth-enhancing protein, its potential use in cancer needs to be revisited.

The reason that cancer is a relatively rare disease in humans, considering the number of cell divisions taking place, is that the organism has evolved an exquisite mechanism for ridding the body of genetically damaged cells such that they do not acquire neoplastic autonomy. These events are in large part due to p53 and its relatives, such that loss of function of this protein is indeed cataclysmic. On the other hand, many cancer cells with deregulated oncogenes are in fact treading on thin ice, as they appear to be highly sensitive to apoptosis. This property has not escaped the notice of clinical oncologists, since much of the chemotherapeutic armamentarium is designed to terminally damage DNA of cancer cells, such that p53-mediated repair is impossible.

The bane of cancer chemotherapy is its narrow therapeutic window: too low a dose will lead to complete cell repair and no effect, too high will lead to death of more cells (including non-cancerous ones) than the organism can bear. Thus, any "sensitizer" that might force cells with even mild DNA damage into an apoptotic program would have the potential to greatly enhance the efficacy, and thereby limit toxic side effects, of DNA-damaging chemotherapeutic agents. Enter p21: the executor of p53's antiapoptotic agenda.

Consistent with the previously discussed finding that p21 is increased early after mitogen stimulation, it has been found that overexpression of p21 is an early event in the development of at least some neoplasms (Biankin et al., 2001). Could these findings be related? It has also been shown that p21 is required for the effective coordination between S phase and mitosis, and, utilizing p21(-/-) cells, these investigators demonstrated an enhanced apoptotic effect of chemotherapeutic agents and γ irradiation, as compared to p21(+/-) colorectal cancer cells (Waldman et al., 1996). Subsequently, using a xenograft model, it was found that p21-deficient tumors were more sensitive to radiation as measured by both clonogenic survival and regrowth of the tumors following treatment (Wouters et al., 1997). p21-disrupted clones of HCT-116 colon cancer cells were found to have higher sensitivity to cisplatin and nitrogen mustard (Fan et al., 1997). Furthermore, paclitaxel increases p21 and causes it

to relocate in the cytoplasm where, as discussed above, it is antiapoptotic and causes less cell cycle inhibition (Heliez et al., 2003). Consistent with this data, cytosolic p21 is indeed associated with a poorer prognosis, as assessed by decreased overall as well as relapse-free survival, in breast cancer (Winters et al., 2003). These findings suggest the possibility that attenuating expression or stability of p21 may be useful as possible chemotherapy, as will be discussed below.

Therapeutic possibilities of p21 manipulation in cancer

Although much of the original experimental work has been done in p21 knockout cells, the stage has now clearly been set for attempting to attenuate p21 levels in cancer cells. Theoretically, this may ultimately be clinically useful in conjunction with DNA-damaging chemotherapeutic agents (Figure 2). The search for specific pharmaceutical agents to attenuate p21 has not yielded many players, in part because p21 attenuation has not, as yet, been intensively studied for this purpose. In general, as discussed above, standard DNA damaging chemotherapeutic agents, such as Adriamycin, increase p21 through p53-dependent and -independent pathways; these mechanisms serve the cancer cell quite well and protect these malignant cells from apoptosis (Bunz et al., 1999), an event which does not help the patient. On the other hand, triptolide, an immunosuppressive extract of the Chinese herb *Tripterygium wilfordii*, shows some promise, as it causes apoptosis by induction of p53 with concomitant inhibition of p21 expression (Chang et al., 2001). The use of peptides or small molecule inhibitors (Lam et al., 2003) to attenuate p21, or to modulate its interaction with antiapoptotic partner proteins such as Akt, has enormous potential but has not yet been described in this regard.

Thus far, the most widely reported method for attenuation of p21 which has potential clinical applicability has been the use of antisense techniques, either by transfection of an antisense oligodeoxynucleotide (ODN) or by utilizing antisense plasmid technology. We showed that transfection of a 21 bp antisense p21 ODN results in attenuation of rat VSM cell growth (Weiss et al., 2000), and another group showed that this same ODN induced apoptosis in other VSM cell lines (Zhang et al., 2003). A human ODN, homologous to the rat one above, promoted apoptosis in both T47D and MCF7 human breast cancer cell lines (Fan et al., 2003), and a different antisense p21 ODN caused irradiated HCT116 human colon cancer cells to convert from growth arrest to apoptosis (Tian et al., 2000). Stable transfectants of antisense p21 encoded plasmids resulted in sensitization of BCap37 human breast cancer cells to paclitaxel and its combination with 5-fluorouracil (Johnson and Fan, 2002); this latter study is of particular interest, since it seems that one mechanism by which cancer cells might resist paclitaxel therapy is through escape from apoptosis by means of an increase in p21 synthesis after exposure to this agent (Heliez et al., 2003). Antisense ODNs to p21 also enhanced γ irradiation-induced apoptosis and cytotoxicity in radioresistant human glioma cells (Kokunai et al., 2001). We showed in a pilot study of an allograft model of breast cancer that antisense p21 ODN, when injected subcutaneously in nude mice, results in attenuation of Met-1 breast cancer growth (Weiss et al., 2003). The few reported studies using siRNA to suppress expression of p21 (in two specialized cell lines) resulted in inhibition of apoptosis in insulin-secreting cells (Huo et al., 2003), and restoration of immortalization as well as Ras-mediated transformation in *cdk4* null cells (Zou et al., 2002). The difference in outcomes between

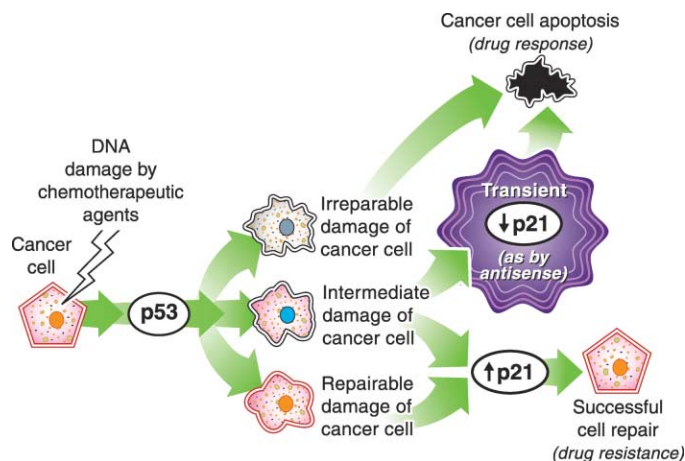


Figure 2. A scenario for the use of p21 attenuators to enhance chemotherapeutic efficacy

Depending on the severity of DNA damage, response to a chemotherapeutic agent will be either apoptosis (an effective drug) or successful repair (drug resistance). Based on the current knowledge of p21 as an antiapoptotic protein, it is conceivable that transient attenuation of p21 concomitant with DNA damaging chemotherapy, such as with antisense techniques or small molecule inhibitors (purple shading), would improve the efficacy, and thus decrease toxicity, of standard chemotherapeutic agents.

these two techniques of p21 attenuation may be due to the magnitude and/or duration of its suppression, and requires further study.

In breast cancer, both increased cytosolic p21 (Winters et al., 2003) and higher (total) p21 expression by immunostaining (Yang et al., 2003) have been linked to poorer prognosis. In the latter study, patients treated with CMF who had positive p21 expression in their tumors had significantly worse disease-free survival than those whose tumors stained negative for p21. Although both studies involved breast cancer patients, in contrast to Winters et al., Yang et al. showed a marginally significant ($p = 0.05$) association of nuclear p21 with worse disease-free survival, and no similar association with cytosolic localization; the difference between these studies may be due to the fact that all of the patients studied by Yang et al. had already received CMF, while the patients in Winters et al. received a variety of adjuvant treatments or none at all. The finding by Yang et al. that ErbB2 overexpression correlated significantly with p21 positivity in these patients suggests that an immune-mediated approach, utilizing already available tumor-targeting anti-HER2 monoclonal antibodies and concomitant specific p21-attenuating therapy (as with immunoliposomes carrying antisense ODNs [Park et al., 2002; Rodriguez et al., 2002]), may ultimately be feasible. In addition, staining of breast tumors for p21 or cytosolic-localized p21 may prove useful in stratifying patients who may respond to such p21-attenuating therapy.

While antisense techniques show promise and have been available for some time, their movement into the clinical arena is still under investigation; based on the studies described above, antisense p21 ODN or gene therapy to attenuate p21 levels in tumors and/or patients has tremendous potential as therapy, likely in combination with standard DNA-damaging agents.

p21 levels and mutations in cancer: A caution

While in general, p21 mutations are rare in cancer, there are scattered reports of mutations in several cancer types, such as oral (Ralhan et al., 2000), esophageal (Bahl et al., 2000), and breast (Balbin et al., 1996). A close examination of the several polymorphisms which have been identified in p21 in human breast cancer in small studies (Powell et al., 2002; Keshava et al., 2002) reveals one (described only in thyroid carcinomas [Shi et al., 1996]) which lies in intron 2 between exons 2 and 3, which is spliced out to create the NLS sequence and is therefore a possible site for oncogenic mutations; patients with this mutation had a slightly higher breast cancer risk. The only exon polymorphism described in the p21 NLS and PCNA binding region is in codon 149. Another report describes a mutation in one tumor (out of 36 screened) which does not lie in the PCNA binding region and yet impairs the ability of p21 to inhibit proliferation (Balbin et al., 1996). While cytosolic localization of p21 is associated with a poorer prognosis in breast cancer (Winters et al., 2003), a finding which might in the future be able to identify patients who would respond to p21 attenuating therapy, studies correlating levels of p21 with prognosis and tumor grade have been inconsistent.

Surprisingly, in light of its location downstream of p53 in the tumor suppressor pathway, p21(-/-) mice showed normal development and did not show spontaneous tumor development at 7 months in the original study (Deng et al., 1995), yet embryonic fibroblasts were deficient in their ability to arrest in response to DNA damage. In addition, p21(-/-) thymocytes showed a dramatic increase in cells undergoing apoptosis (Deng et al., 1995), and p21(-/-) keratinocytes showed an increased proliferation potential (Missero et al., 1996). A later study showed that p21(-/-) mice, when followed longer, showed an increase in spontaneous tumor development at an average age of 16 months, whereas wild-type mice are typically tumor-free beyond 2 years (Martin-Caballero et al., 2001). Furthermore, disruption of the p21 gene in mice led to an increased susceptibility to chemically induced skin carcinoma formation (Topley et al., 1999), and p21 null mice had accelerated tumor onset and multiplicity in urethane-treated animals, although this mutation had no effect on tumor growth rate in v-Ha-ras mice (Jackson et al., 2002). While these studies should inject a note of caution into translational p21 attenuation pursuits, it is necessary to keep in mind that (1) future therapies employing p21 attenuation will likely focus on the proapoptotic (rather than growth-regulatory) properties of these techniques, and (2) effective p21-attenuative therapies will result in transient and partial attenuation, in contradistinction to constitutive absence of this gene as is seen in knockout models.

Future directions

Due to the unacceptable failure rate of currently available cancer chemotherapeutics, the search for new molecular targets is in high gear. While initially touted (and investigated) as an inhibitor of cell growth, p21 has now emerged as a powerful antiapoptotic signaler under some conditions. Since many cancer cells are able to escape a chemotherapeutic death by repairing their damaged DNA by means of p21 augmentation, methods of transiently attenuating p21 in combination with standard DNA-damaging pharmaceuticals hold considerable promise to limit chemotherapy toxicity and improve patient outcomes. Future work in this area should focus on, among other methods, antisense and small molecule inhibitor techniques to transiently inhibit p21 in animal studies and subsequently in clinical trials.

Acknowledgements

We thank Drs. Kit Lam, Hsing-Jien Kung, and Benjamin Davis for critical reading of this manuscript, Claudia Graham for assistance with the figures, and members of the Weiss laboratory for their hard work. This work was supported by the grant 1R21CA 91259-01A1 from the National Cancer Institute, the Research Service of the U.S. Department of Veterans Affairs, and Dialysis Clinics, Inc.

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