p28-Mediated Activation of p53 in G2–M Phase of the Cell Cycle Enhances the Efficacy of DNA Damaging and Antimitotic Chemotherapy

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Abstract

p28 is an anionic cell-penetrating peptide of 28 amino acids that activates wild-type and mutated p53, leading subsequently to selective inhibition of CDK2 and cyclin A expression and G2–M cell-cycle arrest. In this study, we investigated the cytotoxic effects of p28 treatment alone and in combination with DNA-damaging and antimitotic agents on human cancer cells. p28 enhanced the cytotoxic activity of lower concentrations (IC20-50) of DNA-damaging drugs (doxorubicin, dacarbazine, temozolomide) or antimitotic drugs (paclitaxel and docetaxel) in a variety of cancer cells expressing wild-type or mutated p53. Mechanistic investigations revealed that p28 induced a post-translational increase in the expression of wild-type or mutant p53 and p21, resulting in cell-cycle inhibition at the G2–M phase. The enhanced activity of these anticancer agents in combination with p28 was facilitated through the p53/p21/CDK2 pathway. Taken together, these results highlight a new approach to maximize the efficacy of chemotherapeutic agents while reducing dose-related toxicity. Cancer Res; 76(8); 2354–65. ©2016 AACR.

Introduction

Current cytotoxic, chemotherapeutics include DNA-damaging and antimitotic agents acting singly or in combination. DNA-damaging agents, such as doxorubicin, intercalate with DNA, inducing double strand breaks that induce ataxia-telangiectasia mutated (ATM)-dependent nuclear accumulation of p53 (1). Additional targets for DNA-damaging agents include the apoptotic pathway via Bcl-2/Bax and the caspase cascade, the necrotic pathway through Toll-like receptors, etc. Unfortunately, similar damage to normal cells results in adverse effects and possibly life-threatening toxicity (e.g., cardiotoxicity) to major organs. In contrast to intercalating agents, the DNA alkylating agent dacarbazine (DTIC) and its derivative temozolomide (TMZ) inhibit cancer growth by methylating guanine nucleotides, preventing DNA replication, and inducing G2–M arrest via p53, respectively (2). This suggests that the integrity of the G2–M cell-cycle checkpoint may be important in the cytotoxicity of these agents and a site for intervention (3). However, the multiple roles of p53 in regulating DNA-repair enzymes may be dependent on tumor type, and inherent and acquired resistance to alkylating agents present major obstacles to successful treatment (4).

Taxanes, mitotic inhibitors such as paclitaxel and docetaxel, used to treat a variety of malignant tumors, bind to the beta-tubulin subunits of microtubules. This induces a prolonged activation of the mitotic spindle checkpoint and mitotic arrest followed by mitotic slippage and induction of apoptosis (5). Although considered cytotoxic, they are in fact cytostatic, with the arrest triggering cell death (6). Taxanes also induce post-transcriptional acetylation and phosphorylation of p53. This increases intracellular p53, upregulating its target proteins p21 and Bax, inhibiting the cell cycle, and increasing apoptosis (5). Unfortunately, cancer cells have weak spindle checkpoints and activate various prosurvival signals that lead to clinical resistance toward taxanes. In addition to resistance, taxanes, like DNA-damaging agents, can also induce significant toxicity that may force treatment to become dose limiting.

Increasing the concentration of DNA-damaging and antimitotic agents or combining them to increase cytotoxicity generally enhances antitumor activity by activating multiple pathways (7). For example, the cyclin D1–CDK4/6-retinoblastoma protein (RB)–INK4 axis is universally disrupted, facilitating cancer cell proliferation and prompting the targeting of CDK4/6 as an anticancer strategy (8). The dependence of subsets of melanomas and neuroblastomas on CDK2 (9) suggests that the CDK2 pathway may also significantly affect proliferation. For example, inhibitors of CDK2 predispose cells to DNA damage and activate p53 (10), suggesting the G2 checkpoint and DNA-damage pathways may be linked (11). As direct CDK inhibitors abrogate DNA damage–induced checkpoint and repair pathways, they provide an opportunity to potentiate the response to DNA-damaging agents (12) and potentially antimotics. Specific, non-toxic cell-cycle inhibitors that maintain or increase efficacy while reducing the toxicity of either DNA-damaging or antimitic agents, alone or in combination, would clearly be welcome.

This suggests adding a CDK inhibitor to either a taxane or DNA-damaging agent would potentially decrease chemoresistance and increase efficacy (13). However, because many CDK inhibitors are not selective, their use in concert with DNA-damaging agents can be complicated by a direct cell-cycle arrest superimposed on the...
modulation of upstream components of the checkpoint and repair pathways (12). Selective (14) and pan-CDK inhibitors, such as flavopiridol (15), inhibit CDKs 1, 2, 4, 6, and 9, and induce cell-cycle arrest at G1, and G2 through competitive inhibition of the ATP-binding site, they also have a synergistic effect when combined with a DNA-damaging agent (11) or taxane (13). This effect is complicated as direct inhibitors of CDK1 and CDK2 arrest the cell cycle in G1 and G2, and may also inhibit the effect of proliferation-dependent agents (11). The absence of pharmacodynamic endpoints for pan-CDK inhibitors complicates confirmation of target inhibition, cytotoxicity is not limited to cycling cells, and appears independent from wild-type or mutated p53 or Rb (16).

Although exposure to a direct cell-cycle inhibitor in combination with chemotherapeutic agents may increase chemosensitivity, evidence remains limited for enhanced activity of either a DNA-damaging or antimitic agent via a p53-regulated, CDK-mediated pathway, independent of cell type or agent (17).

p28, amino acids 50 to 77 of azurin, a cupredoxin secreted by Pseudomonas aeruginosa, is a non-toxic, amphipathic, anionic cell-penetrating peptide that preferentially enters a wide variety of cancer cells (18, 19). Upon entry, p28 binds to a mutational "cold spot" (20) within the DNA-binding domain (DBD) of p53wt,mut where it blocks the binding of the E3 ligase Cop1, inducing a significant post-translational increase in the level and activity of wild-type and mutant p53 (21–26). This increase in p53wt,mut levels and activity upregulates p21 and p27, inducing a significant decrease in the intracellular level of the CDK2–cyclin A complex, essential for progression through the mitotic process (27), as well as FoxO3, a transcription factor for G2–M progression (24, 26). As a targeted cytostatic therapy, p28 exhibits no significant adverse effects or toxicity (28), exerts a prolonged block at G2–M that leads to a significant apoptotic cell death in breast cancer cells (26) and prevents the carcinogen-induced transformation of mouse mammary epithelial ducts (29). Chemopreventive agents such as the antimitic Vinca alkaloids that inhibit cellular proliferation also indirectly lead to apoptosis (6, 30). As such, p28 appears as a true cell-cycle–specific cytostatic agent that induces cytotoxicity indirectly via a p53wt,mut-mediated block at G2–M (18, 26).

In this study, we assessed the effects of p28 alone and in combination with IC20 and IC50 concentrations of standard DNA-damaging and antimitic agents on the degree of cytotoxic effect in p53wt,mut, null-matched human prostate, breast cancer, glioblastoma, melanoma, and neuroblastoma cell lines as a function of cell proliferation and p53 status. The results suggest that a sustained increase in p53wt,mut in the presence of lower doses of either DNA-damaging or antimitic agents is a promising avenue to reduce the overall toxicity of chemotherapeutic agents while maximizing their efficacy.

Materials and Methods

Peptide synthesis

p28 (Leu30–Asp77 LSTAADMQMCGVTDGASMGLDKDYKLKD, 2914 Da) was synthesized by CS Bio, Inc. at 95% purity and mass balance.

Cell culture

Temozolomide (>98%) and docetaxel (>97%) were obtained from Sigma-Aldrich, dissolved in DMSO and diluted in MEM-E (Invitrogen; final conc of DMSO <0.5% and <0.1%, respectively). Clinical grade of doxorubicin hydrochloride and paclitaxel was obtained from Ben Venue Laboratories and Teva Pharmaceuticals, respectively. DTIC was obtained from APP Pharmaceuticals, dissolved in sterile water and diluted in MEM-E.

Human breast fibroblast, mammary MCF-10A cell line, and human cancer cell lines were obtained from American Type Culture Collection (authenticated using morphology, karyotyping, PCR, and STR profile): prostate cancer, LNCaP (p53wt, AR+); DU145 (p53mut, AR-), and PC-3 (p53null, AR-); neuroblastoma, IMR-32 (p53wt), and SK-N-BE2 (p53mut); breast cancer, ZR-75 (p53wt) and MDA-MB-231 (p53mut); and glioblastoma, U87 (p53wt), and LN229 (p53null). Human melanoma, Mel-29 (p53wt), and Mel-23 (p53mut) were established and characterized in our laboratory (authenticated using morphologic and karyotyping; ref. 31). Cells were cultured in MEM supplemented with 10% heat-inactivated FBS (Atlanta Biological, Inc.) at 37°C in 5% CO2, (18, 24, 25).

Cell proliferation assays

Human cancer cells were cultured in MEM-E containing 2 mmol/L L-glutamine, 0.1 mmol/L essential amino acids supplemented with 10% heat-inactivated fetal bovine serum (18). The doubling time of each cancer cell line (LNCaP, DU145, IMR-32, SK-N-BE2, Mel-29, Mel-23, U87, LN229, ZR-75, and MDA-MB-231) was determined as follows. Cells were seeded (triclicate) at 20,000 cells/well and cultured in MEM-E for 72 hours in the absence of cytotoxic agent or p28 changed daily as described previously (18, 26, 32, 33). Proliferative rate was expressed as doubling time in hours. The doubling time of each cancer cell line was determined from semi-logarithmic plots of initial cell number versus cell counts at 24, 48, and 72 hours. The relationship between doubling time and cytotoxic activity or difference in cytotoxic activity between agents alone and in combination was analyzed by nonlinear regression. Curves were fitted using Graph-Pad PRISM (version 5.0) as a function of a nonlinear one-phase decay (maximum iterations: 1,000, CI: 95%).

We initially determined the concentration of p28 (~100 μM/1) that inhibited cell proliferation 20% to 50% (IC20–50) across the p53wt,mut cell lines (18, 24–26) over 72 hours by direct count, then established the IC20–50 for cytotoxicity using a standard MTT assay (ref.18; Table 1). Each cell line was then exposed to a cytotoxic agent or p28 alone or p28 in combination with IC20–50 dose of doxorubicin, DTIC, TMZ, paclitaxel, or docetaxel to establish whether cytotoxicity was enhanced. Culture medium was replaced with fresh media containing p28 (quadruplicate), chemotherapeutic agent, or an equal volume of medium (control; 8 replicates) daily for 72 hours. Results were compared by ANOVA (Newman–Keuls multiple comparisons; GraphPad InStat ver. 3.0). Methods for Western blotting and pull-down assay are described in Supplementary Methods.

Xenograft tumor models

Human breast cancer MDA-MB-231 and melanoma UISO-Mel-23 were injected s.c. on the right flank of 5- to 6-week-old female and male athymic mice, respectively. When tumors reached 3 mm in diameter, animals were randomized into control and treatment groups that received: PBS i.p. (control); paclitaxel 12.8 mg/kg (15 μmol/kg) i.p. on days 10, 14, 21, and 25 after tumor development; DTIC (dacarbazine), 4 mg/kg (22 μmol/kg), 3×/week; or p28 10 mg/kg (3.4 μmol/kg) i.p. daily for 30 days. Tumor volume was determined 3× weekly and normalized to body weight. Mice were necropsied on either days 16 or 31 (24 hours). At necropsy, any sign of tumor growth was determined. Tumor volume was determined 3× weekly and normalized to body weight. Mice were necropsied on either days 16 or 31 (24 hours). At necropsy, any sign of tumor growth was determined.
Effect of p28 treatment on the growth of human cancer cells

p28 inhibits the proliferation of a wide variety of p53\(^\text{wt}\) human tumor cells in vitro (18, 26) and in vivo (25, 35). The cytostatic effect on prostate cancer cell lines LNCaP (p53\(^\text{wt}\), AR\(^+\)) and DU145 (heterozygous p53\(^\text{mut}\), P223L/V274F, AR\(^+\)) was time- and dose-dependent, decreasing the proliferation of LNCaP and DU145 cell lines at 72 hours with 18% and 22% (P < 0.001) at 100 mmol/L, respectively (Fig. 1). p28 was not active against the p53\(^\text{mut}\) cell line PC3 (AR\(^+\)), confirming our earlier report (24). Exposure to 100 mmol/L p28 for 72 hours decreased the proliferation of neuroblastosma IMR-32 (p53\(^\text{wt}\)) and SK-N-BE2 (p53\(^\text{mut}\) C135F) cells (Fig. 3B and F). However, p28 did induce significant cytotoxicity in response to p28 alone was inversely correlated with the degree of cytotoxicity following exposure to p28 alone was inversely correlated with the degree of cytotoxicity on the same set of cancer cell lines exposed to either p28 alone (Fig. 1) or in combination with either DNA-damaging or antimotic agents.

p28 was not cytotoxic against LNCaP (p53\(^\text{wt}\), AR\(^+\)) and DU145 (p53\(^\text{mut}\), AR\(^+\) or PC3 (p53\(^\text{mut}\), AR\(^+\)) prostate cancer cells (Fig. 3A, G and H) after 72-hour exposure, with marginal effect on IMR-32 and SK-N-BE2 neuroblastosma and ZR-75, MDA-MB-231 breast cancer cells (Fig. 3B and F). However, p28 did induce significant cytotoxicity (25%–30%) in U87 and LN229 glioblastoma cells and p53\(^\text{wt}\) Mel-29 melanoma after a 72-hour exposure without significantly affecting p53\(^\text{mut}\) Mel-23 melanoma cells (Fig. 3C–E).

Additional MTT assays demonstrated that exposure to p28 at 100 mmol/L for 72 hours did not induce cytotoxicity in fibroblasts (control vs. p28, P = 0.12) and MCF-10A breast cells (P = 0.48) as previously reported (24). Collectively, the data demonstrate that p28 is an effective cytostatic agent against p53\(^\text{wt}\) and p53\(^\text{mut}\) cancer cell lines that do not harbor DNA contact mutations or a completely unfolded DNA binding domain (DBD; refs. 24, 25). These results are also consistent with suggestions that a potential gain-of-function associated with mutations in p53 is not a universal phenomenon for p53 mutants (24, 25, 34).

Similar results were observed in MDA-MB-231 and Mel-23 xenograft tumors (Fig. 2A and D), where 10 mg/kg p28 inhibited tumor growth equal to or better than the IC\(_{50}\) dose of paclitaxel in MDA-MB-231 cells and induced a dose-related inhibition of Mel-23 proliferation comparable with an IC\(_{20}\) dose of DTIC. The p28-induced inhibition of tumor proliferation (24, 25) was clearly associated with an increase in tumor p53 and p21 levels (Fig. 2B, C, E and F). In an additional study, p28 4 mg/kg alone (20%–60% less than the optimum dose; Fig. 2A and D; ref. 34) i.p daily inhibited the growth of p53\(^\text{mut}\) human melanoma (UISO-Mel-2) xenografts (Fig. 2G). Tumor growth in animals treated with p28 in combination with DTIC (4 mg/kg each) i.p. was significantly decreased from DTIC alone and p28 alone after 14 days of exposure (Fig. 2G) without any loss in body weight (control group: 25.7 ± 0.5; DTIC: 25.4 ± 0.6, combination: 25.9 ± 0.8; p28: 26.9 ± 0.9 g) or alteration in behavior (data not shown).

Table 1. IC\(_{20,50}\) values for DNA-damaging and antimotic agents determined by the MTT assay

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Prostate cancer</th>
<th>Glioblastoma</th>
<th>Melanoma</th>
<th>Neuroblastoma</th>
<th>Breast cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 status</td>
<td>LNCaP</td>
<td>DU145</td>
<td>U87</td>
<td>LN229</td>
<td>Mel-29</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>p53(^{\text{wt}})</td>
<td>8, 83</td>
<td>7, 60</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>DTIC</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.1, 0.8</td>
<td>0.5, 1.5</td>
<td>N.D.</td>
</tr>
<tr>
<td>TMZ</td>
<td>N.D.</td>
<td>N.D.</td>
<td>1.8, &gt;2</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.8, 5</td>
<td>2.5</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>0.5, 1</td>
<td>1.8, 7.5</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Abbreviations: N.D., not determined; DTIC, dacarbazine; TMZ, temozolomide.

\(^a\)Yamada et al. (25)
shorter doubling time was again correlated with the increase in cytotoxic activity observed with the combination versus the agent alone. There was no apparent relationship \((P = 0.26)\) between the increase in cytotoxicity and p53 mutation status as they did not include DNA contact mutations or mutations that produce a complete unfolding of the p53 molecule (Fig. 4D).

Although all DNA-damaging and antimitotic agents induced a cytotoxic effect in all five \(p53^{wt}, p53^{mut}, p53^{null}\) cell lines treated (Table 1 and Fig. 3), there were significant differences in the concentration of doxorubicin and p28 to effect a similar inhibitory response between \(p53^{wt}, p53^{mut}, \) and \(p53^{null}\) cell lines of the same type (Table 1 and Fig. 3A). In contrast, the IC\(_{20-50}\) concentrations of DTIC and TMZ were similar across cell lines and p53 status. In general, concentrations of the antimitotic paclitaxel and docetaxel that achieved an IC\(_{20-50}\) were also cell line dependent, but not as dependent on p53 status as doxorubicin (Table 1; Fig. 3A, F–H).

Exposure to p28 significantly increased cytotoxicity of doxorubicin on \(p53^{wt}, \) AR+ LNCaP, and \(p53^{mut}, \) AR− DU145 prostate cancer cells, but did not increase the cytotoxicity from either agent in \(p53^{null}, \) AR− PC3 cells (Fig. 3A, G and H). A similar effect

Figure 1.
Growth-inhibitory effect of p28. The effect of p28 on cell growth was determined by direct cell counting. Prostate (A, LNCaP, DU145, and PC-3), neuroblastoma (B, IMR-32 and SK-N-BE2), melanoma (C, Mel29 and Mel23), glioblastoma (D, U87 and LN229), and breast (E, ZR-75 and MDA-MB-231) cancer cells were incubated with increasing concentrations of p28 (gray bar, 50 \(\mu\)mol/L; white bar, 100 \(\mu\)mol/L; black bar, control) at \(37^\circ C\) for 72 hours. Cells were then counted in a Beckman Coulter (Z1 coulter particle counter). Cell numbers in control wells were considered as 100%. Mean ± SE. Compared with control; \(*, P < 0.05; \)**, \(P < 0.01; \)***, \(P < 0.001.\) Compared between p28 doses; #, \(P < 0.05; \)##, \(P < 0.01.\)
**Figure 2.**
Effect of p28 as a single agent on xenograft growth. A, p53mut MDA-MB-231 human breast cancer cells. Tumor volume was determined weekly and normalized to body weight for 30 days. Control (n = 9), paclitaxel (n = 6), p28 (n = 6). B, p53 and p21 expression. Tumor lysates were subjected to Western analysis (three randomly selected tumors/group). C, treatment levels are expressed as a percentage of control. Mean ± SE; *, P < 0.05. D, p53mut Mel-23 human melanoma cells. Tumor volume was determined weekly for 30 days and normalized to body weight. Control (n = 19), DTIC (n = 10), p28 (n = 10). E, expression of p53 and p21 in DTIC- and p28-treated mice. Lysates of excised tumors were subjected to Western analysis (three randomly selected tumors/group). F, each experimental level is expressed as a percentage of control. *, P < 0.05. G, treatment groups injected i.p. with DTIC, 4 mg/kg, 3x/week (n = 7); p28, 4 mg/kg i.p. daily (n = 7); or p28 in combination with DTIC under an identical dosage (n = 7) for 16 days. Control group received PBS i.p. (n = 13). Statistical comparisons were performed by ANOVA (control vs. each treatment group; *, P < 0.05, combination vs. each treatment group; #, P < 0.05).
was observed for p28 in combination with doxorubicin in p53wt IMR-32 and p53mut SK-N-BE2 neuroblastoma cells (Fig. 3B). The increase in cytotoxicity induced by p28 was not limited to the DNA-intercalating agent doxorubicin. When p53wt,mut glioblastoma and human melanoma cells were exposed to combinations of p28 and the methylating agents DTIC and TMZ, cytotoxicity was also significantly enhanced over DTIC and TMZ alone (Fig. 3C–E), suggesting that the effect is not limited to either a single type of DNA-damaging agent, p53 status, or the embryonic origin of a cell line.

While exposure to p28 did not increase the cytotoxic effect of paclitaxel on normal MCF-10A breast cells (paclitaxel vs. combination; P = 0.18) and fibroblasts (paclitaxel vs. combination; P = 0.98), exposure of p53wt ZR-75 and p53mut MDA-MB-231 breast cancer cells, and p53wt LNCaP and p53mut DU145 prostate cancer cells to p28 in combination with paclitaxel significantly increased the cytotoxicity (Fig. 3F and G). A similar response was observed for p28 in combination with docetaxel (Fig. 3H). This again suggests that a p28-induced increase in the expression of p53 mediates the increase in cytotoxic effect irrespective of the type of agent it is combined with.

p53 and downstream targets

Exposure of p53wt LNCaP and p53mut DU145 cells (but not p53mut PC3 cells) to doxorubicin increased p53, p21, and FoxM1, but not CDK2, levels relative to control. p28 alone also elevated p53 and p21 levels, but, in contrast to doxorubicin, decreased FoxM1 and CDK2 levels (Fig. 5A) and had no effect on p53mut PC3 cells. The increase in p53 in LNCaP and DU145 cells treated with p28 combined with doxorubicin was similar (LNCaP 242% vs. DU145 223%), as was the relative increase above doxorubicin alone. A similar increase in p21 levels was observed over doxorubicin, although the relative value compared with control was higher in DU145 cells (Fig. 5A), suggesting that either DNA-damaging agents or p28 may be more effective in increasing p21 in AR+ prostate cancer cells. Doxorubicin also increased FoxM1 levels significantly over control in LNCaP and DU145 cells (Fig. 5A). FoxM1, an essential transcription factor for G2–M progression in LNCaP and DU145 cells, is reportedly stabilized through the checkpoint pathway by DNA damage-sensing kinases (e.g., ATM) and downstream signaling effectors, including checkpoint kinase 2, in response to DNA damage (37). An increase in FoxM1 suggests that the G2–M transition is enhanced. As the expression level of CDK2 reportedly correlates with FoxM1 expression (38), the increase in CDK2 in DU145 cells treated with doxorubicin was associated with the increase in FoxM1 (Fig. 5A).

In contrast to doxorubicin, the p28-induced increase in p53 reduced the levels of FoxM1 in LNCaP and DU145 cells (Fig. 5A) as previously reported (25). This is not surprising as p53 negatively regulates FoxM1 expression independently of the checkpoint pathway (39). Exposure of LNCaP and DU145 cells to p28 alone or in combination with doxorubicin reduced CDK2 levels below that of control or doxorubicin alone, respectively, in parallel with FoxM1.

In contrast to doxorubicin, DTIC did not significantly increase the level of p53, p21, or FoxM1 in p53mut U87 glioblastoma cells relative to control, but did reduce CDK2 levels. However, unlike p53mut M21-23 melanoma cells (Fig. 2E and F; 3C) and p53wt U87 glioblastoma (Fig. 3D), DTIC did increase p53mut and p21 significantly in p53mut LN229 cells (Fig. 5B) without altering either FoxM1 or CDK2. p28 alone also increased the levels of p53 and p21 in p53wt U87 and p53mut LN229 cells while decreasing the levels of FoxM1 and CDK2 in both lines. Exposure to p28 in combination with DTIC increased the levels of p53 and p21 above that of either control or DTIC alone without altering the levels of FoxM1 and decreasing CDK2 in both cell lines, mirroring the increase in efficacy (Fig. 3D). A similar pattern was observed in the responses of p53, p21, FoxM1, and CDK2 for TMZ in U87 and LN229 cells. The addition of p28 produced a pattern of expression similar to that of DTIC that paralleled the activity of the combination in both lines. In sum, the increased activity of DNA-damaging agents (Fig. 5A–C) in combination with p28 appeared to result from the activation of the p53/p21 axis, significantly reducing CDK2 levels relative to each agent alone and enhancing the cytotoxicity of lower concentrations of these agents.

Exposure of p53wt LNCaP cells to the antimiotic paclitaxel increased the levels of p53 and p21, but did not alter FoxM1 or CDK2 (Fig. 5D). The increase in p53 and p21 levels in p53mut LNCaP cells treated with p28 in combination with paclitaxel was qualitatively similar to cells treated with paclitaxel alone (Fig. 5D). Although the level of FoxM1 was not altered by p28 and paclitaxel in LNCaP cells, CDK2 was significantly reduced to levels below those observed with p28 alone. The paclitaxel-induced cytotoxic effect in p53mut DU145 cells (Fig. 3D) was accompanied by a decrease in p53mut relative to control, while slightly increasing p21 and significantly elevating FoxM1 and CDK2 above control levels (Fig. 5D). The increase in FoxM1 in p53mut DU145 cells treated with paclitaxel alone (Fig. 5D) reportedly occurs in response to the paclitaxel-induced decrease in p53, a paclitaxel-induced early activation of p53/p21 pathway (40), or activation via a p53-independent pathway (41). As p28 in combination with paclitaxel increased p53 and p21 while significantly decreasing FoxM1 and CDK2 levels below those observed with paclitaxel alone and control, it suggests that the mutations in p53 in this cell line do not prevent p28 from enhancing the generalized cytotoxicity observed with paclitaxel alone (Figs. 2B, 3F and G; refs. 5, 42).

The increased activity of paclitaxel in combination with p28, coupled with the increase in p53 and p21 and decrease in FoxM1 and CDK2 compared with that of paclitaxel alone, was likely due to the post-translational increase in p53 and p21 induced by p28 (Fig. 5D) that enhanced the cytotoxic activity of paclitaxel in this cell line.

Unlike paclitaxel, docetaxel, the taxane prescribed for advanced AR+ prostate cancer, increased p53 and FoxM1 without significantly altering either p21 or CDK2 levels in LNCaP cells (Fig. 5E). Here again, p28 in combination with docetaxel further increased the level of p53 and p21 while inhibiting FoxM1 and CDK2 levels. Docetaxel, unlike paclitaxel, did not significantly alter either p53 or p21 levels in DU145 cells, but did increase FoxM1 while leaving CDK2 unchanged, suggesting subtle differences in the mechanism of action of these two taxanes. However, p28 in combination with docetaxel increased p53 and p21 levels while significantly decreasing the expression of FoxM1 and CDK2 below that in docetaxel-treated and control DU145 cells (Fig. 5E), a pattern similar to that observed with paclitaxel (Fig. 5D).

Chemotherapeutic agents do not alter the interaction of p28 with p53

Administration of cytotoxic drugs can have far-reaching secondary consequences that may contribute to overall cytotoxic...
activity. For example, doxorubicin in combination with a recombinant human IL1α induces a significant synergistic antiproliferative effect in human melanoma cells by increasing IL1α binding to the receptor on melanoma (43). Genotoxic drugs can also trigger multiple molecular events, including activation of p53-independent checkpoints, partially protecting cancer cells during chemotherapy (44). The effect can be avoided with agents targeted specifically at enhancing p53 activity. Treatment of tumor cells with doxorubicin causes phosphorylation and acetylation of p53, transcriptional upregulation of p21 and other target genes, and growth arrest (45). These observations, coupled with the ATP-dependent, nuclear pore-mediated transport of the doxorubicin-proteasome complex, suggest the possibility that doxorubicin could directly or indirectly alter the affinity of p53 for p28. We explored this possibility with competitive pull-down assays of p28 binding to p53 in lysates from cells previously treated with either doxorubicin or paclitaxel. GST coupled to p28 (GST-p28) pulled down endogenous p53 from p53wt LNCaP and p53mut DU145 cells; GST alone did not (Fig. 6). The L1 (aa 112–124) and S7–S8 (aa 214–236) loops and T140, P142, Q144, W146, R282, and L289 of the p53DBD have been identified as potential sites for p28 binding (24). Although DU145 is heterozygous for mutations in p53 (P223L, V274F), p28 pulled down p53mut from DU145 more effectively than did p53wt in LNCaP (Fig. 6, lanes 2 and 6, top). This confirms our earlier results showing that the heterozygous mutant p53 (P223L, V274F) had a higher affinity for p28 (Δ–59 kJ/mol) than wt p53 (25). Neither paclitaxel nor doxorubicin altered the interaction of p28 with p53 in LNCaP and

Figure 3.
The effect of combination of p28 and chemotherapeutic agents on cancer cells was determined by the MTT assay. Chemotherapeutic agent p28 or p28 in combination was added daily for 72 hours. Prostate (A, G, H), neuroblastoma (B), melanoma (C), glioblastoma (D and E), and breast (F) cancer cells were incubated with chemotherapeutic agent, p28 or p28 in combination for 72 hours. Concentrations are shown above each bar. MTT reagent was added to each well, and the percentage of change in absorbance at 570 nm in treated cells relative to untreated controls was determined. Values, mean ± SE. Combination versus each single agent: a, P < 0.05; a, P < 0.01; A, P < 0.001. Compared with control: b, P < 0.01; B, P < 0.001.

Figure 4.
Relationship between proliferative rate, drug sensitivity, and p53 status. All cell lines were cultured for 72 hours in complete media in the absence of cytotoxic agent or p28 and their doubling time determined. A, percent increase in the cytotoxic effect in p28-treated cells relative to untreated controls plotted as a function of the respective doubling time for each cell line (control considered as 100%). Mean values were calculated from independent replicates in the same cell lines exposed to p28 at 50 μmol/L (e.g., Fig. 3A, G, H and D, E). B, percent increase in cytotoxic activity as a function of the respective doubling time for each cell line in agent-treated cells relative to untreated controls. C, percent increase in cytotoxic activity determined by the MTT assay (percent decrease of p28/agent combination subtracted from percent decrease of the agent alone) plotted against doubling time (hours). D, scatter dot plots of percent increase in cytotoxic activity and p53 status (wt vs. mut) in cancer cell lines tested.
Figure 5. Effect of the combination of p28 and chemotherapeutic agents on the p53 pathway. Prostate cancer cells (LNCaP, DU145, and PC-3) and glioblastoma (U87 and LN229) were treated with chemotherapeutic agent p28 or combination added daily for 72 hours. Concentrations used as follows: A, p28 (LNCaP: 50 μmol/L, DU145 and PC-3: 100 μmol/L), doxorubicin (LNCaP: 1 nmol/L, DU145: 5 nmol/L, and PC-3: 100 nmol/L), or combination. B, p28 (U87 and LN229: 50 μmol/L), DTIC (U87 and LN229: 1 mmol/L), or combination. C, p28 (U87 and LN229: 50 μmol/L), TMZ (U87 and LN229: 100 μmol/L), or combination. D, p28 (LNCaP and DU145: 50 μmol/L, PC-3: 100 μmol/L), paclitaxel (LNCaP and DU145: 1 nmol/L, PC-3: 2 nmol/L), or combination. E, p28 (LNCaP and DU145: 50 μmol/L, docetaxel (LNCaP: 0.1 nmol/L and DU145: 1 nmol/L), or combination. Whole-cell lysates were subjected to Western analysis. Each band was normalized by calculating the ratio of p53, p21, FoxM1, or CDK2 over actin. The histograms are representative of at least two independent experiments (relative intensity to control expressed as 100%).
Discussion

The majority of expressed p53 mutations reportedly exhibit gain-of-function properties actively promoting prosurvival signals and tumorigenesis, independent of the loss of wild-type p53 function (46). Although gain-of-function effects are complex (46), an apoptosis-resistance feature appears to be directly linked to high expression of mutant p53 (47). Tumors that depend on an inactivated, e.g., mutated, p53 for continued proliferation are inhibited if p53 is reactivated. Because suppression of at least certain types of mutant p53 renders cancer cells highly susceptible to apoptosis, it affords the opportunity to combine restoration of p53 activity with lower doses of DNA-damaging and antimitotic agents to apoptosis, it affords the opportunity to combine restoration of certain types of mutant p53 renders cancer cells highly susceptible to high expression of mutant p53 (47). Tumors that depend on an inactivated, e.g., mutated, p53 for continued proliferation are inhibited if p53 is reactivated. Because suppression of at least certain types of mutant p53 renders cancer cells highly susceptible to apoptosis, it affords the opportunity to combine restoration of p53 activity with lower doses of DNA-damaging and antimitotic therapy to further increase efficacy and reduce toxicity. This approach would appear to be most effective in high-grade tumors with rapid doubling times, as an increase in the overall effectiveness of multiple combinations of p28 with DNA-damaging or antimitic agents appears dependent on a rapid proliferative rate (Fig. 4). DNA-damaging agents induce apoptosis in cancer cells by activating p53 through the ATM pathway (48). Taxanes also induce p53 via post-transcriptional modifications that increase both its level and activity (5). To date, strategies directed at activation of checkpoint and repair pathways (53). The inhibition of this DNA repair pathway in p53wt U87 cells in response to DTIC or TMZ treatment fails to alter p53 levels (Fig. 5B and C). However, p21 expression was increased following exposure of U87 cells to TMZ, but not DTIC, suggesting a difference in the mechanism of action of these two similar agents. This was not the case with either agent in p53mut LN229 cells (Fig. 5B and C; Table 1), where DTIC and TMZ increased p53 and p21. p53 affects both the duration of G2–M arrest and the fate of TMZ-treated human glioblastoma cells (3). If resistance to TMZ is promoted by enhanced DNA repair activity in the G2–M transition, a CDK inhibitor could suppress this activity, leading to potentiation of TMZ action on glioma cells (11). This is essentially what we observed in tumors (Fig. 3D and E; 5B and C), p28 alone increased p53 levels in U87 and LN229 cells (Fig. 5B and C), in turn decreasing the level of FoxM1 in U87 and LN229 cells. As a cell-cycle–specific inhibitor at G2–M, p28 exhibits antiproliferative activity independent of the DNA repair pathway. Because p28 in combination with DTIC or TMZ increases the levels of p53 and p21 in U87 and LN229 cells, it suggests that the increase in cytotoxicity induced by p28 in combination versus the agent alone extends to a variety of DNA-damaging agents and may also be independent of cancer cell phenotype (5A–E; 5A–C).

The repeated, significant decrease in CDK2 in response to an increase in p53 and p21, irrespective of cancer cell type, is important as CDKs are also involved in activation of DNA-damage checkpoint signaling and the initiation of DNA repair. Primary resistance to DNA-damaging agents is partly due to the activation of checkpoint and repair pathways (53). The inhibition of CDKs may prevent the activation of DNA-damage–induced checkpoint and repair pathways. It has been suggested that CDK1 (cdc2) and CDK2 may be the most attractive family members of CDK to target in combination with DNA-damaging agents.

Figure 6.
Complex formation between p28 and p53 in prostate cancer cells, LNCaP and DU145. GST alone (lanes 1 and 5, top) was used as a control. Each control and experimental band was normalized by calculating the ratio of p53 in pull-down/llysates. Each experimental level was then expressed as the (relative) percentage of control (control expressed as 100%).

Table 6

<table>
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<tr>
<th>Agent</th>
<th>LNCaP</th>
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<td>Paclitaxel</td>
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<tr>
<td>Doxorubicin</td>
<td>GST</td>
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<td>Lysates</td>
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Example, p28 increased the level of p53 and p21 in p53wt LNCaP and p53mut DU145 prostate cancer and p53mut U87 and p53mut LN229 glioblastoma cells, but decreased the p53 negatively regulated FoxM1 and CDK2 proteins (5A–C). In contrast to p28, doxorubicin significantly increased FoxM1 levels in LNCaP and DU145 prostate cancer cells (Fig. 5A). Because several DNA mismatch repair proteins (MLH1, PMS1, and PMS2) are inactivated in DU145 cells (50), the level of FoxM1 in response to DNA damage was higher than that in LNCaP cells (Fig. 5A). FoxM1 is also transcriptionally regulated by E2F1, which induces the accumulation of FoxM1 in response to DNA damage, suggesting that the DNA-damage–response pathway also regulates FoxM1 independent of p53 (51). CDK2 expression was not altered by doxorubicin alone in either cell line, but significantly reduced when combined with p28, reflecting the increase in levels of p53 and p21 above either agent alone (Fig. 5A). The levels of CDK2 in cancer cells treated with DNA-damaging agents parallel those of FoxM1, as CDK2 expression transcriptionally correlates with FoxM1 expression, supporting a role for CDK2 during FoxM1-induced cell-cycle progression (38). These data clearly suggest that the enhanced cytotoxicity with p28 and doxorubicin in combination in prostate cancer and neuroblastoma (Fig. 3A and B) is a direct result of a p28-induced increase in p53.

Expression of DNA repair proteins also plays a decisive role in protecting cells against the effect of alkylating agents. The DNA repair enzyme O6-alkylguanine DNA alkyltransferase (AT) repairs O6-methylguanine in DNA by transferring the methyl group to a cysteine acceptor site on the protein itself (32). Expression of AT is directly regulated by E2F1, which induces the expression of CDK2 during FoxM1-dependent cell-cycle progression (38). These data clearly suggest that the enhanced cytotoxicity with p28 and doxorubicin in combination in prostate cancer and neuroblastoma (Fig. 3A and B) is a direct result of a p28-induced increase in p53.
because abrogation of individual CDK1 and CDK2 activity results in defective DNA-damage–response pathways in several types of human cancer cells (12). However, because many inhibitors of CDK inhibit multiple CDK family members that carry a similar ATP-binding site, their use in combination with DNA-damaging agents is complicated by a cell-cycle arrest that may be superimposed on the checkpoint and repair pathways.

Unlike competitive small-molecule inhibitors of the ATP-binding pocket on CDKs, p28 specifically inhibits the cancer cell cycle at G2–M by inducing the endogenous CDK inhibitors, p21 and p27. This leads to lower levels of CDK2 and cyclin A, and a higher level of phosphorylated CDK1 (Thr14/Thr15; inactive form) in a p53-dependent manner (24–26). The minimal response of CDK1 following exposure to p28 is also accompanied by a significant increase in the level of cyclin B1, indicating p28 inhibits the cancer cell cycle at G2–M (26), as degradation of these two proteins is required to exit from mitosis (54). Specific inhibition of CDK2 by p28 avoids the activation of checkpoint and repair pathways observed following DNA-damaging agents.

It is also likely that a p53/p21-mediated inhibition of CDK2 plays a significant role in the enhanced effect when p28 is used in combination with anti-mitotic agents such as paclitaxel (55) and docetaxel in p53-positive cell lines irrespective of p53 status (Fig. 3F–H). The increase in activity in p53−/− LNCaP cells correlated with a paclitaxel or docetaxel induced increase in p53 and p21 and decrease in FoxM1 and CDK2. This was not the case for DU145 cells, particularly with paclitaxel, which significantly decreased p53 levels (Fig. 3D and E). This was reversed with the addition of p28, which increased p53 and p21 and significantly reduced FoxM1 and CDK2 levels when combined with either paclitaxel or docetaxel. Although the potential for taxane resistance exists when cancer cells are exposed to taxanes in combination with CDK2 inhibitors, the addition of p28 to a taxane regimen would avoid such resistance because CDK2 activity appears unrelated to paclitaxel sensitivity (56). Moreover, as p28 increases the level of phosphorylated cdc2 (CDK1) leaving the level of cdc2 virtually unchanged (26), the cytotoxic activity of a taxane is enhanced, an obvious benefit.

What is also clear is that the increase in cytotoxic activity following exposure of cancer cells to p28 in combination with either a DNA-damaging or anti-mitotic agent does not result from either class of agents increasing (or decreasing) the affinity of p53 for p28 (Fig. 6). The DNA-damaging agent doxorubicin reportedly induces significant acetylation of p53 C-terminal domain (CTD; refs. 45, 57). Although such post-translational modifications within the p53CTD induce conformational changes of the p53DBD (57), doxorubicin did not alter the p28/p53 interaction irrespective of p53 mutation status (Fig. 6). This suggests the multiple molecular events, including activation of p53-independent checkpoints triggered by exposure to genotoxic drugs that partially protect cancer cells during chemotherapy can be mitigated with p28, which directly enhances p53 activity (44).

In sum, p28, in combination with DNA-damaging and anti-mitotic agents, increased their cytotoxicity by activating p53wt,mut, which subsequently induced the endogenous CDK inhibitor p21. The increase in p21 significantly enhanced their cytotoxic activity, independent of cancer cell type. Our results demonstrate the potential therapeutic value in targeting the p53/p21/CDK2 pathway in combination with lower doses of chemotherapeutic agents to improve anticancer efficacy while reducing toxicity.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: T. Yamada, T K. Das Gupta, C W. Beattie
Development of methodology: C W. Beattie
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Yamada
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T. Yamada, C W. Beattie
Writing, review, and/or revision of the manuscript: T. Yamada, T K. Das Gupta, C W. Beattie
Study supervision: T. Yamada, C W. Beattie

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Development of methodology: C W. Beattie
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Yamada
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Effect of p53 Activation on Chemotherapeutic Agents


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