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The mTOR Inhibitor RAD001 Sensitizes Tumor Cells to DNA-Damaged Induced Apoptosis through Inhibition of p21 Translation

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Summary

Although DNA damaging agents have revolutionized chemotherapy against solid tumors, a narrow therapeutic window combined with severe side effects has limited their broader use. Here we show that RAD001 (everolimus), a rapamycin derivative, dramatically enhances cisplatin-induced apoptosis in wild-type p53, but not mutant p53 tumor cells. The use of isogenic tumor cell lines expressing either wild-type mTOR cDNA or a mutant that does not bind RAD001 demonstrates that the effects of RAD001 are through inhibition of mTOR function. We further show that RAD001 sensitizes cells to cisplatin by inhibiting p53-induced p21 expression. Unexpectedly, this effect is attributed to a small but significant inhibition of p21 translation combined with its short half-life. These findings provide the molecular rationale for combining DNA damaging agents with RAD001, showing that a general effect on a major anabolic process may dramatically enhance the efficacy of an established drug protocol in the treatment of cancer patients with solid tumors.

Introduction

Metazoans have evolved elaborate mechanisms to monitor genomic stability and to maintain the genetic integrity of the organism (Khanna and Jackson, 2001; Vousden and Lu, 2002). Amongst these are those that rid the organism of genetically damaged cells, which could give rise to neoplastic lesions (Khanna and Jackson, 2001; Vousden and Lu, 2002). The key molecular component, which acts in response to DNA damage, is the tumor suppressor p53 (Woods and Vousden, 2001). DNA damage leads to the stabilization of p53 and the activation of pathways which either arrest cell cycle progression, allowing DNA repair if the damage is not severe, or apoptosis if the damage is irreversible (El-Deiry, 2003; Vousden and Lu, 2002). Importantly, many tumor cells appear to be highly prone to apoptosis (Weiss, 2003), which has been exploited clinically through the use of DNA-damaging agents, such as cisplatin. Cisplatin triggers apoptosis, especially in p53 wild-type tumor cells (Siddik, 2003), which represent approximately half of all cancers (Soussi et al., 2000). However, the difficulty with such agents is general toxicity combined with a narrow therapeutic window: too low a dose has no effect, whereas too high a dose affects all cells (El-Deiry, 2003; Khanna and Jackson, 2001; Weiss, 2003). Such factors contribute to the under-dosing of patients and failure to blunt disease. Thus, drugs which would sensitize DNA-damaging agents toward apoptosis could increase their efficacy in the clinic.

Rapamycin has been suggested as a potential chemotherapeutic sensitizer (Shi et al., 1995). Rapamycin is a fungicide produced by *Streptomyces hygroscopicus* and forms an inhibitory complex with the immunophilin FKBP12, which binds to and inhibits the mammalian target of rapamycin (mTOR; Hay and Sonenberg, 2004). mTOR belongs to the phosphatidylinositide-3OH kinase (PI3K) related family of protein kinases, including ataxia telangiectasia mutated protein (ATM), ATM and RAD3 related protein (ATR) and DNA-dependent protein kinase (DNA-PK), which are involved in the DNA damage response (Keith and Schreiber, 1995; Khanna and Jackson, 2001). In contrast to the other family members, mTOR appears to act by integrating nutrient/energy signaling with that of growth factor signaling (Dennis et al., 2001; Hay and Sonenberg, 2004). Growth factors modulate mTOR activity through PI3K, which mediates protein kinase B (PKB) activation and the phosphorylation and inactivation of the tumor suppressor complex made up of tuberous sclerosis complex proteins 1 and 2 (TSC1/TSC2; Jaeschke et al., 2002; Marygold and Leevers, 2002). TSC2 acts as a GTPase activating protein (GAP) toward the Ras homolog, *R*as *h*omologue *e*nriched in *b*rain (Rheb; Garami et al., 2003; Zhang et al., 2003). It is thought that Rheb-GTP either acts on mTOR or influences mTOR's signaling downstream to such effectors as S6 ribosomal protein kinases (S6K1 and S6K2) or the eukaryotic initiation factor 4E binding proteins (4E-BP1 through 3), leading to inhibition of translation (Hay and Sonenberg, 2004).

Given the sensitivity of tumor cells to nutrients and energy, we suggested that tumor cells might be more sensitive than normal cells to rapamycin treatment (Jaeschke et al., 2004). Indeed, early clinical trial results in advanced cancer patients with the two rapamycin derivatives, RAD001 and CCI-779, have demonstrated promising antitumor activity with relatively minor toxicity (Huang et al., 2003a; Panwalkar et al., 2004). More-

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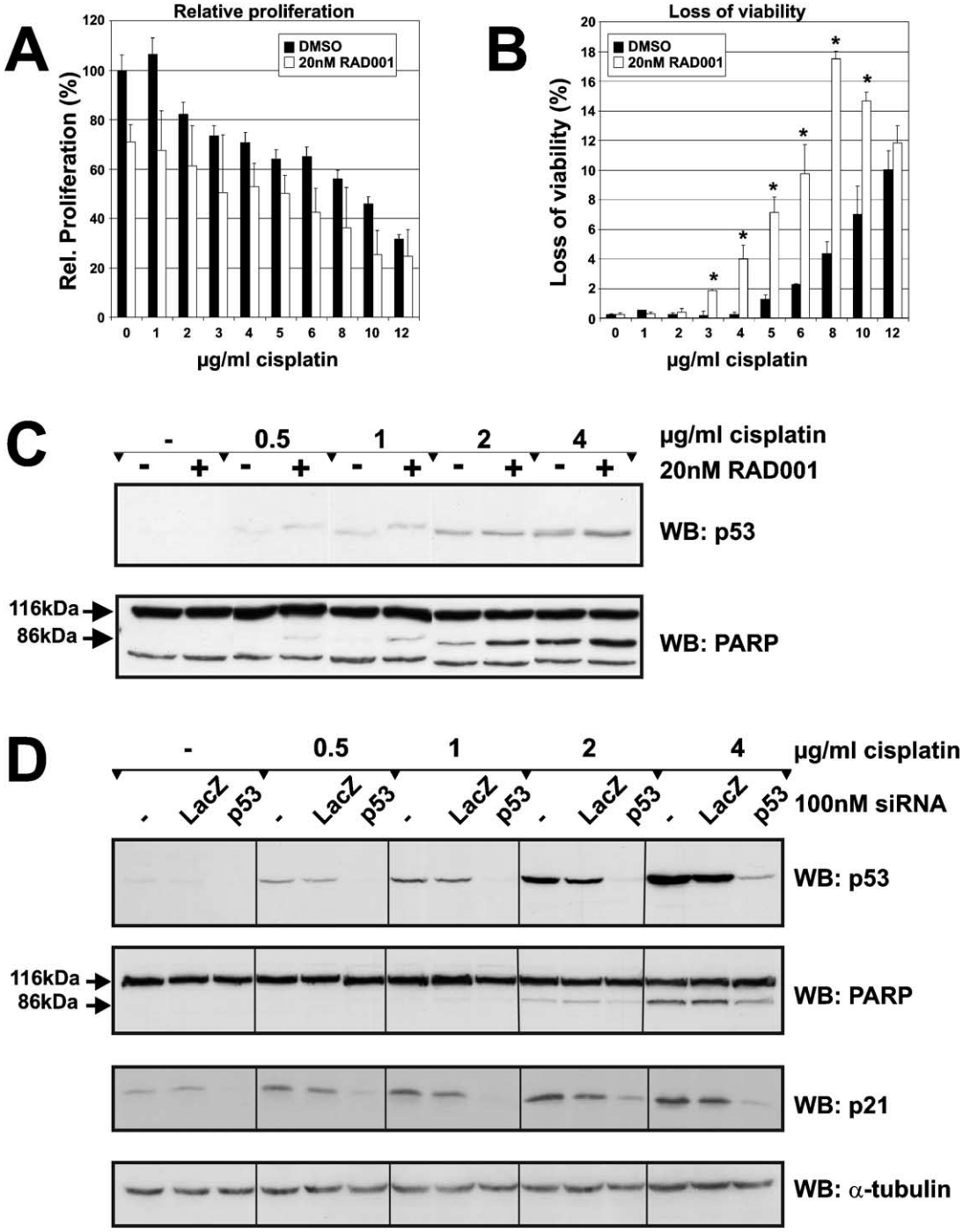

Figure 1. RAD001-Enhanced Cisplatin-Induced Apoptosis Is p53 Dependent

(A and B) A549 cells were treated for 24 hr with either DMSO or 20 nM RAD001 in combination with cisplatin. Proliferation rates and loss of cell viability were measured using the YO-PRO assay (Experimental Procedures). Data represent the mean ± standard deviation of three independent experiments (* = significant fold induction with $p < 0.05$; t tests and two-way ANOVA indicate that the interaction between RAD001 and cisplatin was highly significant [$p < 0.001$]).

(C) A549 cells were treated for 24 hr with either DMSO or 20 nM RAD001 in combination with the indicated concentrations of cisplatin. The expression levels of p53 and PARP were assessed by immunoblotting with indicated antibodies.

(D) A549 cells were untransfected or transfected with LacZ or p53 siRNAs (Experimental Procedures) and treated with the indicated doses of cisplatin. Protein levels of p53, PARP, p21, and α-tubulin were assessed by Western blotting.

over, rapamycins are well tolerated in transplantation, where they are chronically employed (Dutcher, 2004). Although under certain conditions rapamycins induce apoptosis, such as the extended withdrawal of serum from cultured cells, they generally act as cytostatic agents (Huang et al., 2001). These observations raised

the possibility that rapamycins may serve as sensitizers for DNA-damaging agents.

Here, we investigated whether RAD001 enhances the effect of DNA-damaging agents on loss of tumor cell viability and if p53 plays a role in this process. Next, we generated isogenic tumor cell lines expressing a mutant of mTOR that no longer binds RAD001 to determine if the observed effects are mediated through inhibition of mTOR. Finally, we used these tools with RNA interference to identify the downstream target and mechanism by which RAD001 enhances the effect of cisplatin on apoptosis.

Results

RAD001 Enhances Cisplatin-Induced Apoptosis

An earlier report showed that rapamycin alone has little effect on apoptosis but suggested it may enhance the effects of cisplatin (Shi et al., 1995). To test this, the effect of RAD001 and cisplatin on inhibition of cell proliferation as well as loss of cell viability was examined in A549 human nonsmall cell lung carcinoma cells. Consistent with recent findings (Boulay et al., 2004), at a concentration in 10-fold excess of its IC_{50} RAD001 caused a 30% inhibition of cell proliferation within 24 hr of treatment (Figure 1A), with cells accumulating in G1 (data not shown). Similar inhibitory effects with cisplatin alone required concentrations of 4 to 8 μg/ml, with the two drugs together having an additive inhibitory effect within the relatively short exposure time (Figure 1A). Despite the pronounced effect of RAD001 on cell proliferation, it had little effect on cell viability (Figure 1B), although it dramatically enhanced the effects of increasing doses of cisplatin ($p < 0.05$) with a more than 10-fold difference observed at 4 μg/ml cisplatin, a concentration where cisplatin alone had no detectable effect (Figure 1B). Notably, the increased sensitivity caused by RAD001 was abruptly lost at higher concentrations of cisplatin, falling to levels observed with cisplatin alone (Figure 1B). Thus, the two drugs induce distinct responses, with RAD001 having a striking effect on the loss of cell viability at suboptimal concentrations of cisplatin.

Given that A549 cells are wild-type (wt) for p53, it was reasoned that the apoptotic response was due to p53 activation. Consistent with this hypothesis, cisplatin alone induced p53 and (ADP-ribose) polymerase (PARP) cleavage, as measured by the appearance of the PARP M_r 86,000 caspase 3 cleavage product. This effect, unlike the induction of p53, was clearly enhanced by RAD001 (Figure 1C). In contrast, RAD001 alone had no effect on either p53 induction or PARP cleavage (Figure 1C), consistent with RAD001 having no effect on loss of cell viability (Figure 1B). Similar results were obtained with the DNA-damaging agent gemcitabine (data not shown). That these effects were elicited through p53 was shown by the fact that lowering p53 protein levels with a specific siRNA was paralleled by inhibition of cisplatin-induced PARP cleavage (Figure 1D), cell proliferation, and loss of cell viability (see Figure S1A in the Supplemental Data available with this article online). Moreover, this was not due to an siRNA off target effect, as the same result was obtained with two additional p53 siRNAs (Figure S1B). In contrast, a lactose permease (LacZ) siRNA had no effect on any of the three responses, including the induction of the cell cycle inhibitor p21 (Figure 1D and Figure S1A), a p53 transcriptional target (el-Deiry et al., 1993). The requirement for wt p53 was confirmed in studies with a matched set of $p53^{+/+}$ and $p53^{-/-}$ MEFs where RAD001 only enhances cisplatin-induced apoptosis in the $p53^{+/+}$ MEFs (Figure S2A). This conclusion was also supported by studies in MCF7, PC3M, and DU145 tumor cell lines, which are $p53^{+/+}$, $p53^{-/-}$, or p53 mutant, respectively. Only in MCF7 tumor cells did RAD001 enhance cisplatin-induced loss of cell viability (Figure S2B). Taken together, the ability of RAD001 to potentiate the loss of cell viability caused by DNA damaging agents is p53 dependent.

RAD001-Resistant Cell Lines

The sensitizing effects of RAD001 on loss of cell viability are presumed to be through inactivation of the mTOR pathway. To test this, A549 isogenic cell lines were generated that were either sensitive or resistant to RAD001. Cells were stably transformed with a retrovirus vector containing either a hemagglutinin (HA)-tagged wt or rapamycin-resistant (RR) mTOR cDNA, S2035T. The latter has no effect on mTOR-kinase activity but prevents FKBP12-rapamycin from binding to mTOR (Hay and Sonenberg, 2004). Two wt (wt15 and wt28) and two RAD001-resistant mutant (RR11 and RR52) clones were selected, each expressing approximately equal levels of HA-tagged mTOR (Figures 2B and 2C). All four cell lines expressed slightly higher levels of total mTOR as compared to either the retroviral noninfected A549 parental cells or A549 cells infected with the empty retroviral vector (Figures 2A–2C). To test the inhibitory effect of RAD001 on the different cell lines, the activity of mTOR was measured by assessing the phosphorylation of S6K1 threonine 389 (S6K1 T-389), with a phosphospecific antibody, or 4E-BP1 phosphorylation, by its electrophoretic mobility (Figures 2A–2C). The results show that RAD001 inhibits S6K1 T-389 phosphorylation in a concentration-dependent manner in the cell lines expressing endogenous wt mTOR (Figure 2A) or the ectopic wt cDNA (Figure 2B), whereas it was completely protected in the two RR cell lines (Figure 2C). Similarly, 4E-BP1 phosphorylation decreased, as judged by its increased electrophoretic mobility as a function of increasing concentrations of RAD001 in cells expressing either endogenous mTOR or the ectopic wt cDNA (Figures 2A and 2B), but not in cells expressing the RR cDNA (Figure 2C). Thus, the S2035T mutation confers RAD001 resistance, as judged by S6K1 and 4E-BP1 phosphorylation.

mTOR Required for Enhanced Cisplatin-Induced Apoptosis

To determine whether the antiproliferative effects of RAD001 on A549 cells were through inactivation of mTOR, the effect of the drug was tested on the wt and RR mTOR cell lines. The two wt mTOR lines exhibited similar sensitivity to RAD001 as the parental or A549 cells infected with the empty retroviral vector, despite their slightly elevated mTOR expression levels. In contrast, RAD001 had no significant effect on the proliferation rate of the RR cell lines (Figure 3A), even at con-

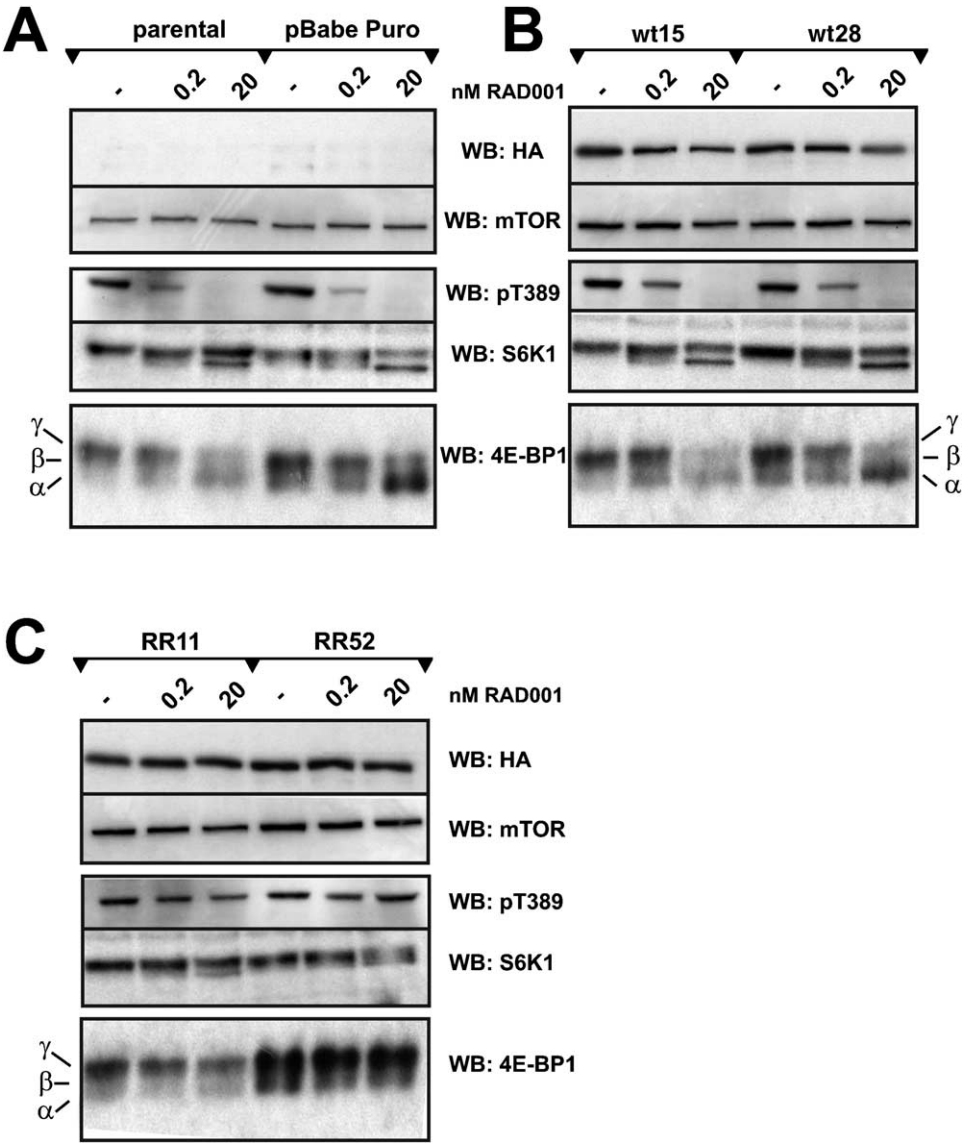

Figure 2. Rapamycin-Resistant mTOR Protects A549 Cells from RAD001

(A) A549 cells infected with the empty retrovirus (pBabe Puro) or (B) a retrovirus encoding either hemagglutinin-tagged wild-type mTOR (wt) or (C) HA-tagged rapamycin resistant mTOR (RR).

(A) A549 parental, pBabe Puro, (B) HA-mTOR wt clone 15 and 28, as well as (C) the RR clones 11 and 52 were exposed for 24 hr to DMSO, 0.2 nM or 20 nM RAD001. Cell lysates were analyzed by Western blot analysis.

centrations up to 20 μM (Figure 3B). These findings support the argument that the sole antiproliferative target of RAD001 in A549 cells is mTOR, as previously demonstrated in rhabdomyosarcoma cells (Huang et al., 2001). Importantly, RAD001 alone had little effect on cell viability of wt lines but strongly enhanced the effect of cisplatin (Figure 3C). In contrast, the RR cell lines were resistant to RAD001 enhanced loss of cell viability induced by cisplatin (Figure 3D). Thus, RAD001 sensitization of p53 wild-type cells to DNA-damaging agents appears to be through inhibition of mTOR function.

RAD001 Inhibits Cisplatin-Induced p21 Induction

The question that arises from these studies is the mechanism by which RAD001 sensitizes p53 wt tumors to DNA-damaging agents. A major target of the p53 antiapoptotic branch of the DNA damage response is p21 (Gartel and Tyner, 2002). Induction of p21 by p53 leads to cell cycle arrest, allowing the potential for DNA damage repair. Analysis of p21 protein levels in A549 cells following cisplatin treatment shows that the increase in p21 is strongly inhibited by RAD001 (Figure 4A). Similar results were also obtained for MCF7 cells (Figure 4B). At the concentrations of cisplatin used, both cell lines displayed no change in the levels of the p53-induced proapoptotic protein Bax (Figures 4A and 4B), possibly reflecting the low doses of cisplatin applied (see below). These findings suggest that RAD001 sensitizes tumor cells to DNA-damaging agents by blocking the upregulation of p21 through inhibition of

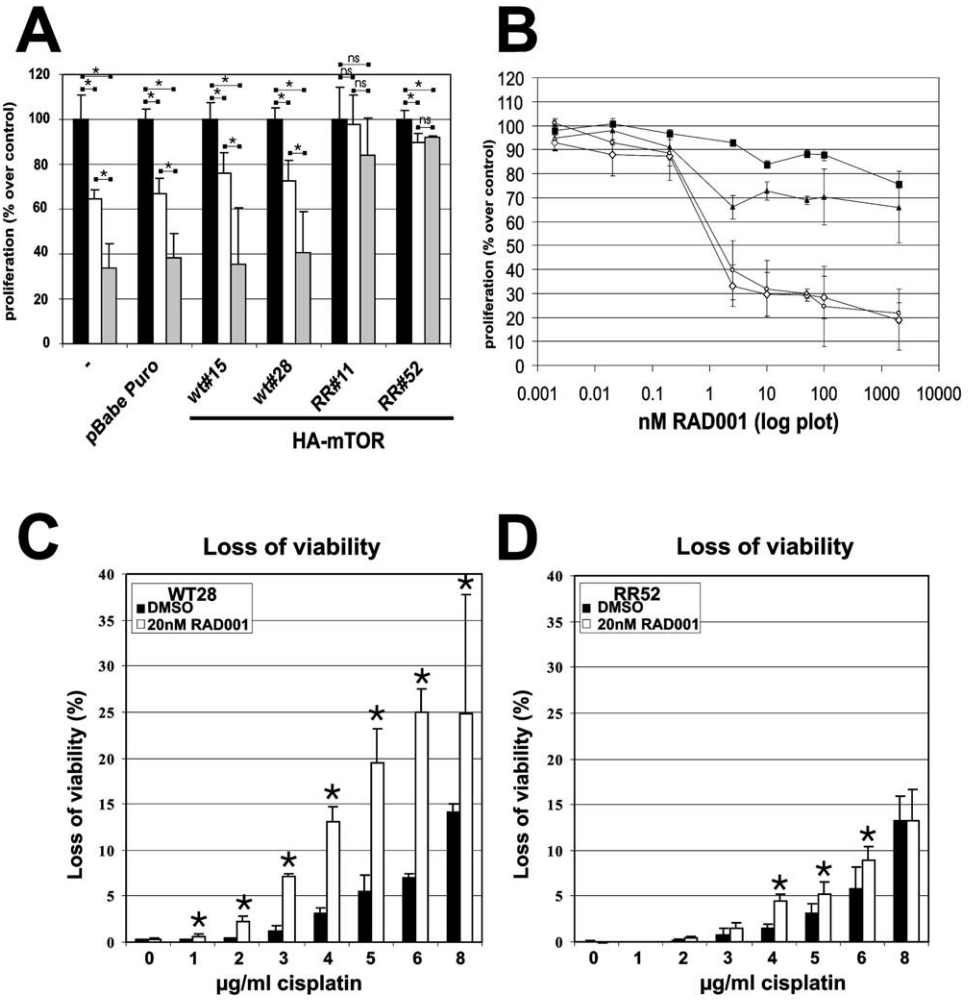

Figure 3. RAD001 Mediates Enhanced Sensitivity to Cisplatin through mTOR

(A) A549 parental, pBabe Puro, HA-mTOR wt (clone 15/28), and HA-mTOR RR (clone 11/52) cells were treated with either DMSO (black bars), 0.2 nM RAD001 (white bars), or 20 nM RAD001 (gray bars). After 4 days, cells were counted, and the data represent the mean ± standard deviation of three independent experiments (* = significant inhibition with $p < 0.05$, one-way ANOVA).

(B) A549 HA-mTOR wt clones 15 (open circle)/28 (open diamond) and RR clones 11 (filled triangle)/52 (filled square) were treated with the indicated concentration of RAD001. After 3 days, cell proliferation rates were assessed using the YO-PRO assay (Experimental Procedures) and plotted as % of DMSO control-treated cells. Data represent the mean ± standard deviation of three independent experiments.

(C) A549 HA-mTOR wt28 cells and (D) A549 HA-mTOR RR52 cells were treated with the indicated concentrations of cisplatin in the presence of DMSO (black bars) or 20 nM RAD001 (white bars). After 24 hr, cell viability was assessed using the YO-PRO-method. Data represent the mean ± standard deviation of three independent experiments (* indicates statistically significant inhibition at $p < 0.05$, t tests and two-way ANOVA indicates that the interaction between RAD001 and cisplatin was highly significant [$p < 0.001$]).

mTOR. Consistent with this, cisplatin induces PARP cleavage and increased p21 protein expression in wt28 and RR52 A549 cell lines (Figure 5); however, low doses of cisplatin have no affect on PARP cleavage but clearly induce p21 expression (Figures 5A and 5B). More striking, in the presence of RAD001, the wt28 cell line exhibited enhanced cisplatin-induced PARP cleavage and a reduction in p21 protein levels, effects completely blunted in the mutant RR52 cell line (Figures 5A and 5B). These findings are consistent with analysis of loss of cell viability (Figures 3C and 3D). These observations favor inhibition of mTOR signaling as being responsible for the reduced levels of p21 protein. Thus, the effects of RAD001 on cell viability, PARP cleavage, and repression of p21 protein levels appear to be regulated through mTOR.

Enhanced Cisplatin-Induced Cell Death Is Due to the Reduction of p21 Protein

The findings above argue that RAD001 enhances the ability of cisplatin to induce cell death by inhibiting p53-induced p21 expression, shifting the equilibrium of the p53 response from cell repair toward apoptosis. Despite this, the effect of RAD001 is lost at higher concentrations of cisplatin (Figure 1B), suggesting that if inhibition of p21 is responsible for advancing the apoptotic program, then this response should be altered at higher concentrations of cisplatin. To test this possibility, A549 cells were treated with either 1 μg/ml or 15 μg/ml of cisplatin for increasing times, and the induction of p53, p21, and PARP cleavage was monitored. At low cisplatin concentrations p53 was induced, followed by p21, with no detectable PARP cleavage (Figure 6A). In

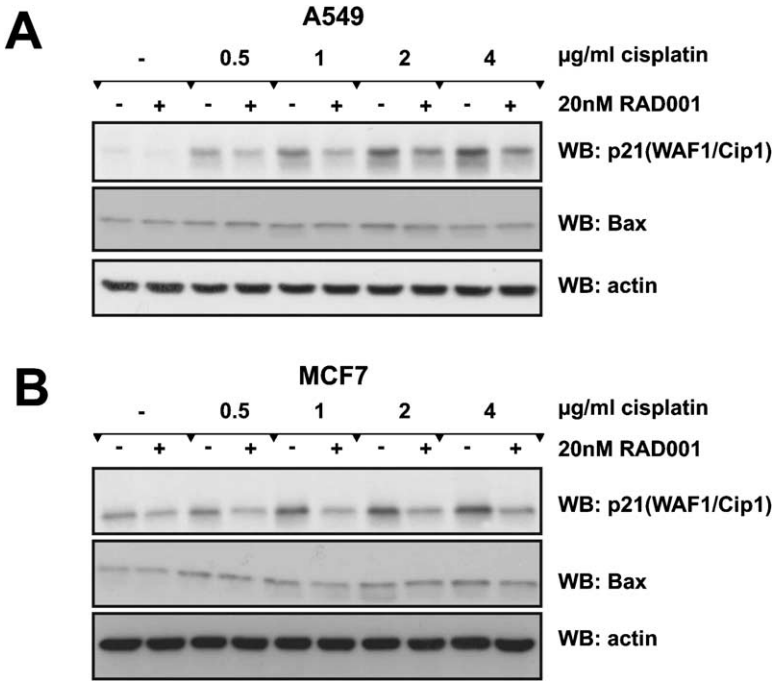

Figure 4. RAD001 Inhibits Cisplatin-Induced p21 Expression in A549 and MCF7 Cells

(A) A549 and (B) MCF7 cells were treated for 24 and 30 hr, respectively, with indicated concentrations of cisplatin in the presence of DMSO or 20 nM RAD001. Protein levels of p21, Bax, and actin were assessed by Western blotting.

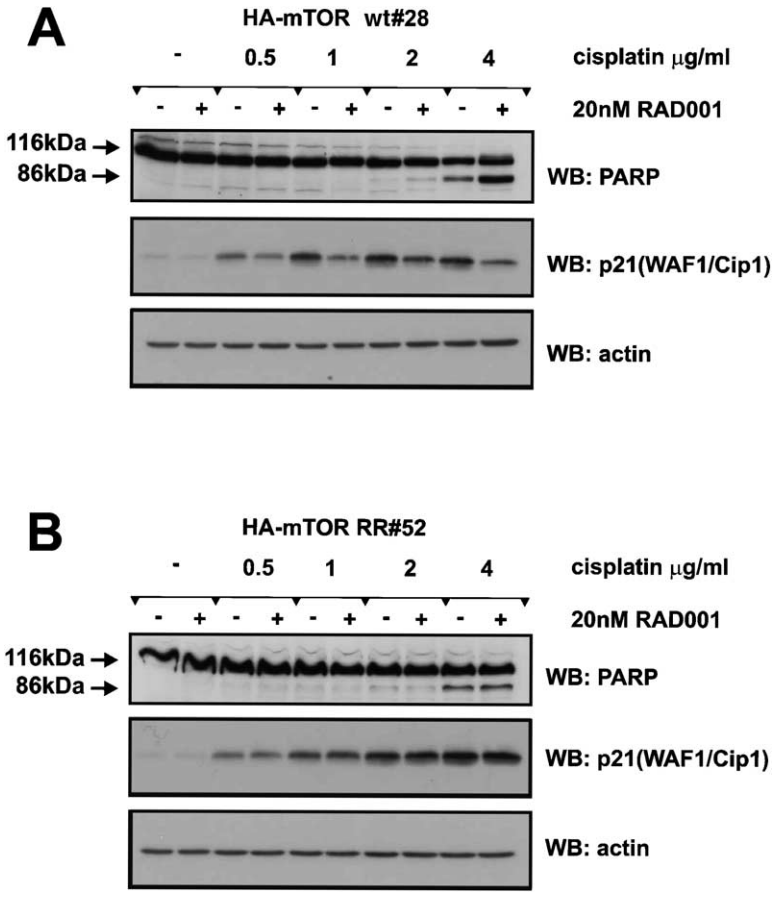

Figure 5. RR mTOR Protects Cisplatin-Induced p21 Downregulation by RAD001

(A) A549 HA-mTOR wt28 cells or (B) A549 HA-mTOR RR52 cells were treated for 24 hr with indicated concentrations of cisplatin in the presence of DMSO or 20 nM RAD001. PARP, p21, and actin protein levels were assessed by Western blotting.

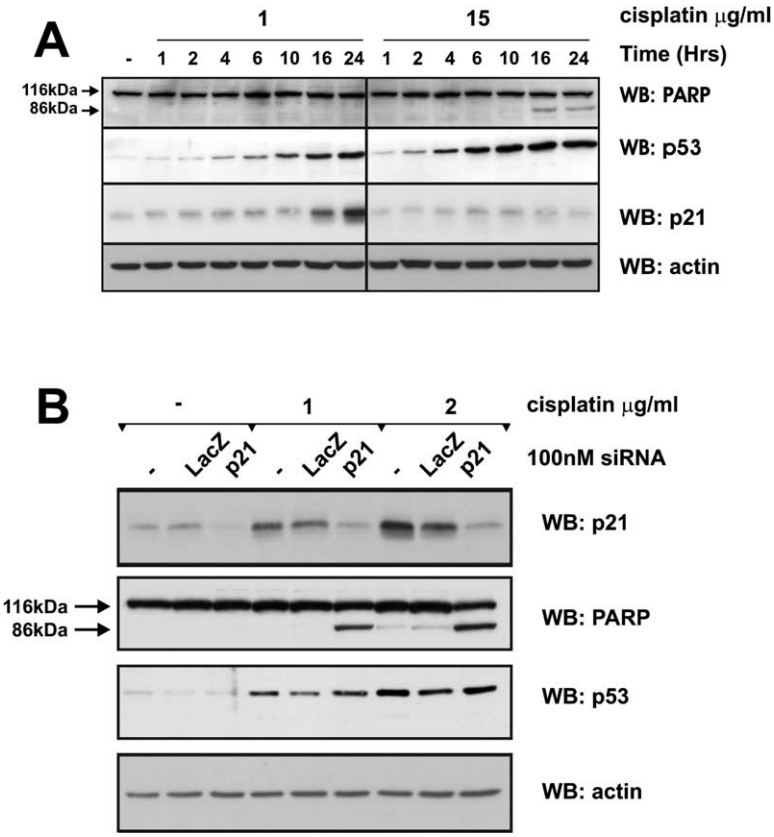

Figure 6. Activation of p53-Dependent Anti- and Proapoptotic Programs

(A) A549 cells treated with 1 or 15 μg/ml cisplatin were incubated for the indicated times. PARP, p53, p21, and actin protein levels were assessed by Western blotting.

(B) A549 cells were untransfected or transfected with 100 nM LacZ or p21siRNAs. After 30 hr, cells were treated for 24 hr with the indicated concentrations of cisplatin. PARP, p53, p21, and actin protein levels were assessed by Western blotting.

contrast, at the higher concentration, p53 induction was more rapid and robust, with PARP cleavage being detected at later time points (Figure 6A). However, despite the strong induction of p53, there was no induction of p21. This is consistent with more severe DNA damage caused by high cisplatin concentrations ablating the p53-induced p21-antiapoptotic response. That loss of p21 protein is responsible for RAD001-enhanced cell death was shown by knocking down p21 protein with a targeted siRNA and inducing PARP cleavage in A549 cells treated with low doses of cisplatin (Figure 6B). Such treatment had no effect on p53 or actin protein levels (Figure 6B), nor did LacZ siRNA have an effect on the level of any of the three proteins (Figure 6B). Moreover, the siRNA knockdown of basal p21 protein had no effect on PARP cleavage (Figure 6B). Thus, lowering p21 levels shifts the p53 response at low concentrations of cisplatin from cell repair to cell death.

RAD001 Reduces p21 Expression by Inhibiting Global Translation

A question that arises from these studies is the mechanism by which RAD001 blocks p21 protein expression. As p21 protein has a short half-life (Bloom et al., 2003), we first tested whether RAD001 accelerated this process in cells treated with 0.5 μg/ml of cisplatin by blocking nascent translation with cycloheximide. Although initial p21 protein levels in RAD001-treated cells were reduced (Figure 7A, left panel), there was no apparent difference in the half-life of p21 following cycloheximide treatment (Figure 7A, right panel). In contrast, neither RAD001 nor cycloheximide had an effect on levels of actin protein, an abundant, stable cytoskeletal component (Figure 7A). Analysis of p21 mRNA by Northern blot analysis revealed that its levels, as well as those of β-actin, were unaffected by RAD001 (Figure 7B). However, at higher concentrations of cisplatin, total p21 mRNA levels are diminished (see the Discussion). Recently, others suggested that rapamycin selectively inhibits mitogen-induced p21 expression at the translational level (Gaben et al., 2004), as previously reported for 5′ oligopyrimidine (5′ TOP) tract mRNAs, such as eukaryotic elongation factor 1α (eEF-1α) (Fumagalli and Thomas, 2000). To test this, the effect of RAD001 on the distribution of eEF-1α and β-actin mRNAs was compared to that of p21 mRNA on sucrose gradients from A549 tumor cells treated for 24 hr with cisplatin. Under these conditions, eEF-1α mRNA relocates from polysomes to nonpolysome fractions (Figure 7C), as previously shown for other cell types (Fumagalli and Thomas, 2000; Pende et al., 2004). Although β-actin mRNA transcripts are normally not affected by short-term rapamycin treatment (Fumagalli and Thomas, 2000; Pende et al., 2004), these longer exposure times led to a significant portion relocating to the nonpolysome fractions (Figure 7C). Unexpectedly, the effect of RAD001 on p21 mRNA distribution was less dramatic, as only a minor portion appeared to shift to smaller

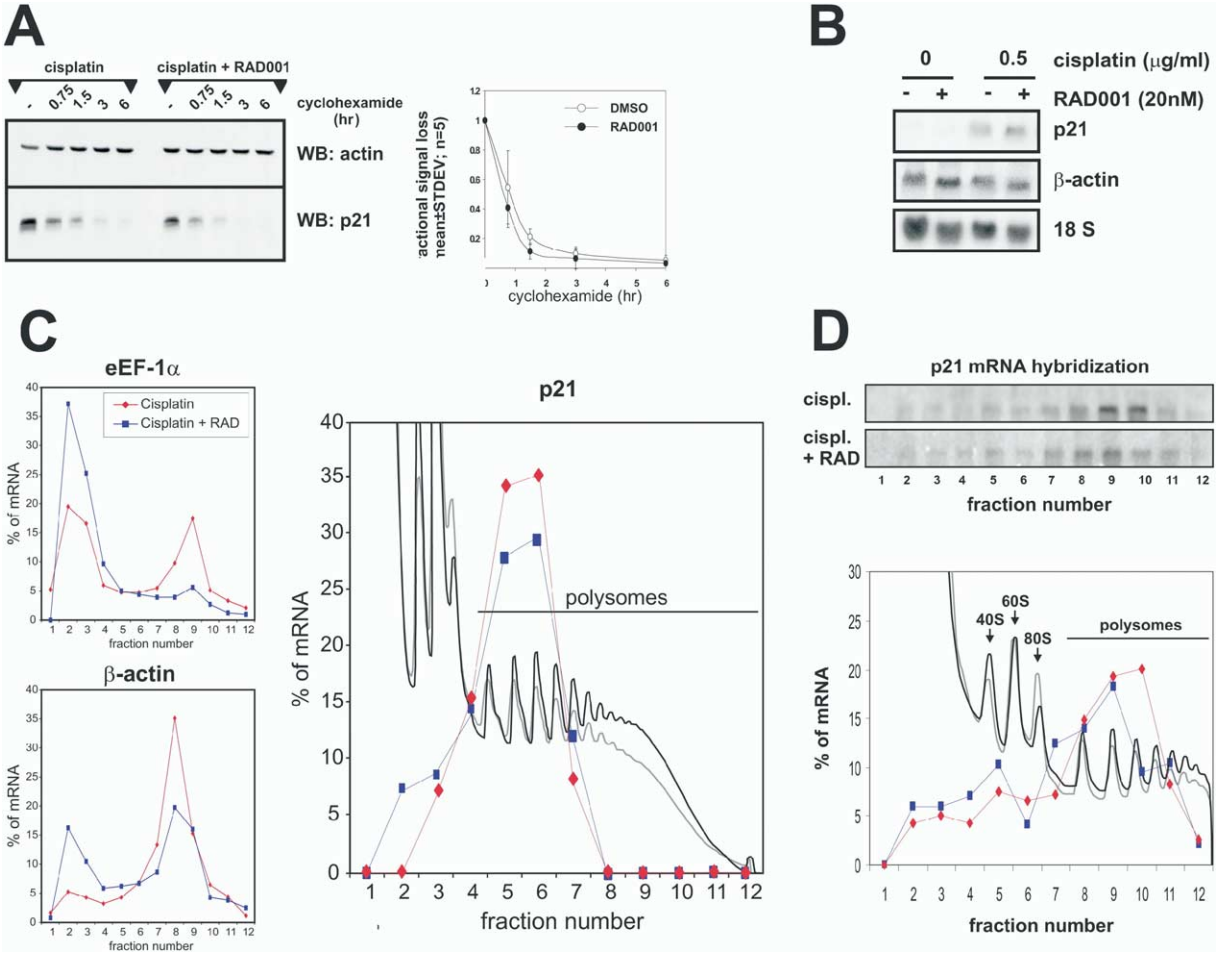

Figure 7. RAD001 Blocks p21 Expression by Inhibition of Global Translation

(A) A549 cells were treated for 24 hr with 0.5 μg/ml cisplatin in the presence of DMSO or 20 nM RAD001, followed by cycloheximide for the indicated times. Left panel, a representative experiment, in which actin and p21 protein levels were assessed by Western blotting. Right panel, the fractional signal loss of p21 protein was determined using ImageQuant. The data represents the mean ± standard deviation of five independent experiments. p21 half-life ($t_{1/2}$) was calculated for each individual sample (n = 5) using nonlinear regression (one phase exponential decay). The differences between the $t_{1/2}$ of DMSO- and RAD001-treated cells, 0.7 ± 0.1 hr and 0.5 ± 0.07 hr, respectively, was not significant (p = 0.336; t test).

(B) After 24 hr, total RNA was isolated and p21, β-actin, and 18S RNA levels were assessed by Northern blot analysis with the indicated probes from A549 cells treated with or without 0.5 μg/ml cisplatin in the presence of DMSO or 20 nM RAD001.

(C) A549 cells were treated for 24 hr with either 0.5 μg/ml cisplatin alone or in combination with 20 nM RAD001. Cell extracts were fractionated on sucrose gradients and mRNAs encoding eEF-1α (top left panel), β-actin (bottom left panel), and p21 (right panel) located by Northern blot analysis with the indicated probe.

(D) Northern blot and analysis of p21 mRNA located on polysome profiles displaying a more shallow gradient. ([C], right panel, and [D]) Gradient analysis of the polysome profiles of A549 cells treated 24 hr with 0.5 μg/ml cisplatin alone (black lines) or in combination with 20 nM RAD001 (gray lines).

(C and D) polysomes and (D) 40S subunits, 60S subunits, and 80S ribosomes are indicated.

polysomes, an effect which roughly paralleled the general decrease in mean polysome size and the proportion of ribosomes engaged in translation (Figure 7C). Note that the first polysome peak represents a disome with two 80S ribosomes bound to a single mRNA. The shift to nonpolysomes was more difficult to access for p21 mRNA as the mean polysome size is ~3 versus ~8 to 12 for eEF-1α and β-actin mRNAs (Figure 7C). However, this shift was more evident, as was the decrease in the mean polysome size of p21 transcripts from ~3 to ~2, when the analysis was performed using a shallower sucrose gradient (Figure 7D). Moreover, this effect is not specific to cisplatin-induced p21, as we observe a similar reduction of p21 protein and a shift of p21 mRNA onto smaller polysomes in MCF7 cells treated with RAD001 alone (Figure 4 and data not shown). The results indicate that RAD001-induced decrease in p21 protein levels is not due to a selective translational effect, as previously predicted (Gaben et al., 2004), but to a small but significant decrease in global translation, combined with the short half-life and low abundance of p21. Consequently, unlike highly expressed and stable housekeeping proteins, such as actin, p21 protein decreases in the presence of RAD001

facilitating an apoptotic response. This observation is in line with the effects of other translational inhibitors, e.g., cycloheximide, which have pronounced effects on proteins with a short half-life, an effect that could translate into a large therapeutic advantage.

Discussion

DNA-damaging agents, such as cisplatin, have had a major impact on the treatment of a wide range of tumor types (Siddik, 2003), although cisplatin's use is limited by cytotoxic side effects (Weiss, 2003). However, the key concern is its narrow therapeutic window: too high a dose is cytotoxic and too low a dose allows for DNA repair (Weiss, 2003). Here, we provide evidence that the use of RAD001 may increase this therapeutic window by acting as a sensitizer for cisplatin-induced apoptosis due to its ability to block p53-induced p21 expression (Figures 4, 5, and 7). Consistent with these findings, there is a positive correlation between wt p53 status and sensitivity to cisplatin (Fan et al., 1994; Segal-Bendirdjian et al., 1998), a finding in line with the significantly higher 5-year survival rate in cisplatin-treated patients with wt p53 tumors (Siddik, 2003). The capacity of cisplatin to trigger p53 expression is thought to be primarily due to its ability to form DNA-protein and DNA-DNA intrastrand crosslinks, which act as apoptotic signals, triggering the activation of DNA damage surveillance mechanisms (Siddik, 2003). Whereas minimal DNA damage results in the initiation of a DNA repair program by induction of cell cycle progression inhibitors such as p21 and GADD45α, extensive DNA damage results in the induction of a proapoptotic program, involving mediators such as PUMA, NOXA, BAX, and PIG3 (Vousden and Lu, 2002). Consistent with this, we find that at low cisplatin concentrations p53 induces p21 with no apparent effect on cell viability or PARP cleavage, whereas at high cisplatin concentrations p53 induction of p21 is suppressed (Figures 1 and 6). Myc induction is one mechanism by which the p53 DNA damage response favors apoptosis (Nilsson and Cleveland, 2003; Vousden, 2002), with Myc being selectively recruited to the p21 promoter and selectively suppressing p53-mediated p21 transcription (Seoane et al., 2002; Wu et al., 2003). Cleavage of p21 by caspase 3 has also been shown to tilt the DNA damage response toward apoptosis (Gervais et al., 1998; Zhang et al., 1999). Whether similar mechanisms are exploited by p53 as a function of the extent of DNA damage caused by cisplatin has yet to be described.

The rapamycin derivatives RAD001 and CCI-779 are currently being assessed in the treatment of advanced cancer patients. Preclinical studies indicate that they are potent inhibitors of tumor cell proliferation in vitro and in animal models of cancer (Boulay et al., 2004; Huang and Houghton, 2003; Majumder et al., 2004; Wendel et al., 2004). Furthermore, such data show that they are well tolerated (Dutcher, 2004), consistent with the fact that in general rapamycins are cytostatic (Figures 1, 3, and 5), leading to the accumulation of cells in G1 (Decker et al., 2003; Luan et al., 2002; Owa et al., 2001). However, it has been reported that rapamycin alone will induce apoptosis in p53-deficient cells stressed by extended serum depletion (Huang et al., 2001). In this case, rapamycin induces sustained activation of apoptosis regulated protein kinase 1 (ASK-1), resulting in hyper-phosphorylation of c-Jun and apoptosis. This effect is abrogated by ectopic overexpression of p21, with p21 binding to ASK-1 and blocking JNK activation (Huang et al., 2003b). It is speculated that these effects are triggered by rapamycin-induced 4E-BP1 dephosphorylation, leading to either the activation or suppression of an ASK-1 phosphatase. This represents a unique case of cellular stress quite distinct from DNA damage, as underlined by the requirement of wt p53, rather than its absence or mutation, in sensitizing tumor cells to cisplatin-induced apoptosis (Figures 1, S2A, and S2B). Although both paradigms involve the loss of p21, the observations presented here underscore the importance of the genetic makeup of the tumor in establishing strategies for combination therapies.

That rapamycin treatment leads to G1 accumulation of cells is consistent with the fact that the drug inhibits global translation and ribosome biogenesis, in part through blocking the phosphorylation of S6K1 and 4E-BP1 (Hay and Sonenberg, 2004). That these effects are through inhibition of mTOR is demonstrated by altering the rapamycin binding site, which protects S6K1 and 4E-BP1 from dephosphorylation as well as protects cells from the antiproliferative effects of RAD001 and its potentiation of apoptosis induced by cisplatin (Figures 2 and 3). The studies presented here also suggest that these effects are mediated through inhibiting p21 protein expression, an effect that was first described in T cells stimulated by IL2 (Nourse et al., 1994) and more recently in mouse fibroblasts stimulated by insulin and IGF-1 (Gaben et al., 2004). The induction of p21 in these settings, unlike that for DNA damage, is required to facilitate the assembly of active cyclin D1/CDK4 complexes (Cheng et al., 1999; LaBaer et al., 1997). In contrast, the upregulation of p53-induced p21 expression in response to cisplatin (Figures 1 and 4–7) inhibits the activity of G1 cyclin/CDK complexes, leading to cell cycle arrest (Bartek and Lukas, 2001). p21 has also been shown to attenuate cell cycle progression by binding and sequestering PCNA, an essential component of the DNA replication machinery (Li et al., 1994). By arresting cell cycle progression, p21 prevents the onset of the apoptotic program. In addition, p21 also acts to inhibit proapoptotic components, such as procaspase 3, caspase 8, and ASK-1 (Gartel and Tyner, 2002; Huang et al., 2003b). For example, p21 binds to the amino terminus of procaspase 3, protecting it from proteolytic cleavage (Suzuki et al., 1998). Consistent with this, we show that reduction of p21 in cells treated with low concentrations of cisplatin, by either RAD001 or siRNAs specific for p21, enhances PARP cleavage (Figures 5 and 6). These findings are supported by studies showing that cells lacking p21 or treated with p21 antisense display enhanced sensitivity toward apoptosis induced by DNA-damaging agents (Fan et al., 1997; Tian et al., 2000), whereas tumors expressing high levels of p21 are prone to be resistant to DNA-damaging agents (Kralj et al., 2003; Liu et al., 2004). Thus, induction of p21 by low concentrations of cisplatin prevents apoptosis by arresting cell cycle progression and inhibiting proapoptotic factors.

That rapamycin had no effect on mitogen-induced p21 mRNA levels or half-life led Gaben et al. (2004) to hypothesize that the drug lowers p21 levels through selective inhibition of its translation (Gaben et al., 2004), as has been observed for 5′ TOP mRNAs (Fumagalli and Thomas, 2000; Pende et al., 2004). Here we show at 0.5 μg of cisplatin that RAD001 does not affect the half-life, transcription, or translation of p21 in a selective manner (Figure 7). Importantly, this does not appear to be particular to cisplatin-induced p21, as we observe a similar reduction of p21 protein and a small but significant shift of p21 mRNA onto smaller polysomes in nutrient-replete MCF7 cells treated with RAD001 alone (Figure 4 and data not shown). Thus, the general inhibition of translation and ribosome biogenesis over this extended period leads to a small but important shift of p21 transcripts to the nonpolysome fraction and to polysomes of smaller mean size (Figure 7). This combined with the fact that p21 protein does not accumulate to high amounts and has a short half-life triggers an initial decrease in p21 protein levels shifting the equilibrium toward apoptosis. This observation is consistent with the inherent definition of proteins with short-half lives, i.e., inhibition of general protein synthesis leads to their selective and rapid decrease. However, it should be noted that as we raise the concentration of cisplatin and the apoptotic response ensues, we find that p21 mRNA levels decrease (data not shown), which may be due to the selective inhibition of p21 transcription as cells commit to apoptosis (Seoane et al., 2002). It should also be noted that the effect of RAD001 on global translation appears to be at the level of initiation, not elongation, as there is no increase in the mean polysome size (Figure 7C), nor do we observe a difference in elongation factor 2 phosphorylation (data not shown), a known rapamycin-induced response reported to inhibit elongation (Proud, 2004). That a decrease in initiation of translation has a larger impact on proteins with short half lives, such as p21, versus those of high copy number is most evident in comparing the distribution of actin transcripts and actin protein. Whereas a large portion of actin mRNA relocates to nonpolysomes in the presence of RAD001 (Figure 7C), there is no measurable change in actin protein levels (Figure 7A) due to its abundance and long half-life. Recent studies have demonstrated the importance of translational control in the cancer setting (Rajasekhar et al., 2003), effects which were largely assumed to be selective (McCormick, 2004; Prendergast, 2003). However, the changes in p21 protein levels reported here would not have been detected by analysis of the transcriptome or mRNAs associated with polysomes (Rajasekhar et al., 2003), underscoring the importance of global translation and the proteome.

The development of agents, which lower p21 protein levels, has been championed as an attractive approach to sensitize tumor cells to chemotherapeutic agents (Weiss, 2003). However, with the exception of antisense oligodeoxynucleotides (Weiss et al., 2003), no pharmacological agents have arisen which directly attenuate p21 protein expression (Weiss et al., 2003). The data presented here demonstrate that RAD001-induced downregulation of p21 results in enhanced apoptosis in the presence of suboptimal cisplatin concentrations. Moreover, p21 is only upregulated at low concentrations of the DNA-damaging agent (Figure 6A), in part explaining the gradual loss of enhancement when RAD001 is combined with higher doses (Figure 1B). These results may explain the molecular basis of a recent study showing that rapamycin synergizes with doxorubicin to induce apoptosis in a mouse lymphoma model driven by ectopic expression of an activated PKB cDNA (Wendel et al., 2004). In this system, the effect of rapamycin on apoptosis is bypassed by overexpressing initiation factor 4E (eIF4E). This is consistent with the authors' assumption that activated PKB drives mTOR activation, which in turn phosphorylates 4E-BP1, relieving its inhibitory effect on eIF4E (Hay and Sonenberg, 2004) and protecting tumors from apoptosis. From our data, we would predict that overexpression of eIF4E in their system drives global translation, increasing p21 levels and overriding the inhibitory effects of rapamycin on global translation (Figure 7). Despite these potential similarities, these systems are quite distinct. Wendel et al. (2004) were examining a defined set of tumors (Wendel et al., 2004) that are protected from apoptosis by the ectopic expression of activated PKB. In contrast, we set out to identify a mechanism by which RAD001 sensitizes tumor cells harboring multiple genetic aberrations to low concentrations of a DNA-damaging agent. Indeed, p21 is induced at low concentrations of cisplatin where we observe no measurable effect on apoptosis or activation of PKB (data not shown). Taken together, the preliminary findings in preclinical mouse models combined with the results presented here indicate that in the clinic, targeting mTOR with agents like RAD001 may offer the opportunity to treat p53 wild-type tumors with much lower doses of DNA-damaging agents, thereby reducing side effects while maintaining antitumor efficacy.

Experimental Procedures

Cell Culture and Pharmacological Inhibitors

A549 (CCL-185), Bing (CRL-1154), MCF7 (HTB-22), HCT-15 (CCL-225), and DU145 (HTB-81) were from ATCC, Rockville, Maryland. PC3M cells were from J. Fidler. A549, Bing, and DU145 cells were cultured in RPMI 1640 with 10% v/v fetal calf serum (FCS), 2 mM glutamine, and 100 μg/ml penicillin/streptomycin. MCF7, *P53*$^{+/+}$ MEFS, and *p53*$^{-/-}$ MEFS were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% v/v FCS, 0.8 μg/ml bovine insulin, and 2 mM glutamine. PC3M cells were cultured in MEM-EBS with 10% v/v FCS, 2 mM glutamine, 1% nonessential amino acids, 1% v/v sodium pyruvate, and 2% v/v MEM vitamines. RAD001 (Everolimus, Novartis) was prepared as a 20 mM stock solution dissolved in DMSO and stored at −20°C. Cisplatin (Platinol, Bristol-Myers Squibb) and Gemcitabine (Gemzar, Eli Lilly) were dissolved in DMSO as 10 mM stock solutions and stored at −20°C.

Stable Cell Lines

wt and RR HA-mTOR cDNAs were obtained by digestion of pRK5/HA-mTOR with NruI/HindIII. Purified fragments were blunt-ended and cloned into the SnaBI site of the pBabe Puro retroviral vector. Retroviruses were obtained by transient transfection of the packaging Bing cells with the indicated retroviral vectors using the calcium phosphate precipitation. In the first round, cells were infected with virus encoding the ecotropic receptor and selected in 0.75 mg/ml neomycin. In the second round, cell pools expressing the ecotropic receptor were infected with pBabe Puro, HA-mTOR wt, or HA-mTOR RR followed by selection in 0.5 μg/ml puromycin.

YO-PRO and Proliferation Assays

For the YO-PRO assay (Idziorek, 1995 1742), cells were seeded at 2×10^3 to 10×10^3 cells/100 μl in 96-well plates and incubated for 24 hr with the indicated concentrations of gemcitabine, cisplatin, RAD001, or the vehicle (DMSO). Cell proliferation and viability were assessed as indicated. A549 clones were plated in triplicate at a density of 3.8×10^4/60 mm and grown for 24 hr, before the medium was replaced with either the vehicle (DMSO) alone or RAD001 and cells were grown for an additional 96 hr. Cell numbers were normalized to the vehicle. For IC_{50} values, cells were seeded in triplicate at 1.5×10^3/100 μl in 96-well plates. After 24 hr, the medium was replaced with medium containing vehicle alone or RAD001, and the cells were incubated for an additional 72 hr.

siRNA

A549 cells were plated at 0.1×10^6/60 mm plate. Five microliters of twenty micromolar siRNA targeting either human p21 (Accession number, or AN: NM000389; sequence: 5′-GTG GAC AGC GAG CAG CTG A-3′), human p53 (AN: NM000546; sequence: 5′-GCA TCT TAT CCG AGT GGA A-3′) or LacZ (AN: M55068; sequence: 5′-GCG GCT GCC GGA ATT TAC CTT-3′) were mixed with 175 μl Optimem (Gibco, Cat. No.: 51985-026). In parallel, 8 μl Oligofectamine (Invitrogen, Cat. No.: 12252-011) was mixed with 12 μl Optimem. After 10 min incubation, the two solutions were mixed, incubated for 20 min, and 200 μl was added to cells, which had been washed with Optimem and then covered with an additional 1 ml of Optimem. After 5 hr, the transfection mix was replaced with 5 ml RPMI 1640 containing 10% FCS. Cells were incubated for an additional 25 hr, cisplatin added for 24 hr, and cell lysates prepared as below.

Biochemical Analysis of Apoptosis

Cells were seeded at $0.1–0.4 \times 10^6$/60 mm, incubated for 24 hr, and treated as indicated in the text. Both floating and adherent cells were collected by centrifugation, the supernatant removed, and 5 ml ice-cold phosphate-buffered saline (PBS) containing 0.1 mM PMSF was added. After washing the cells a second time under the same conditions, the cell pellet was resuspended in 60 μl extraction buffer containing 120 mM NaCl, 50 mM Tris (pH 8), 20 mM NaF, 1 mM benzamidine, 1 mM EDTA, 1 mM EGTA, 15 mM tetrasodiumdiphosphate-decahydrate, 30 mM 4-nitrophenylphosphate disoudium salt hexahydrate, 1 mM PMSF, 1 mM DTT, and 1% NP-40. Cell lysates were prepared by pipetting the extract up and down several times, cleared by centrifugation, snap frozen in liquid nitrogen, and stored at –80°C.

p21 Half-Life Determination

After 24 hr, A549 cells seeded at 0.5×10^6/10cm were treated with either 0.5 μg/ml cisplatin alone or together with 20 nM RAD001, then incubated for an additional 24 hr followed by treatment with 10 μg/ml of cycloheximide for the indicated times. Cell extracts were analyzed on Western blots.

Immunological Techniques

Western blots were performed as described (Boulay et al., 2004), with the following antibodies: anti-mTOR (2972), -phospho-S6K1 Thr389 (9205), -phospho-S6 Ribosomal Protein Ser240/244 (2215), -PARP (9542) obtained from Cell Signaling Technology, -p53 (FL-393) (sc-6243, Santa Cruz Biotechnology, Santa Cruz, California), -Bax (554104, Pharmingen), -p21 (EA10) (OP64, Oncogene), -S6 (Novartis), -4E-BP1 (N. Sonenberg, McGill University), -pan actin (MAB1501, Chemicon International, Temecula, California).

Analysis of mRNA

Preparation of cell extracts, gradient centrifugation, fractionation of polysome profiles, and analysis by Northern blot were done as described (Pende et al., 2004). To obtain polysome profiles with a more shallow gradient, 500 μg of extract was applied to a 5.1%–41% sucrose gradient and centrifuged in a SW41 rotor at 40,000 rpm for 2 hr at 4°C. Analysis and fractionation of polysome profiles were done using an UA-6 detector from ISCO.

Radioactive Labeling of Probes

The p53, p21, eEF-1α, β-actin, and 18S rRNA oligonucleotide probes were complementary to nucleotides: 1 to 1181 of the human p53 cDNA (AN: NM000546), 568 to 724 of the human p21cDNA (AN: NM000389), 51 to 112 of the mouse eEF-1α cDNA (AN: X13661), 121 to 179 of the mouse β-actin cDNA (AN: X03765), and 4656 to 4685 of the mouse 45 rRNA (AN: BK000964). Probes were labeled as previously described (Pende et al., 2004).

Satistical Analyses

Where possible, data are presented as means ±1 standard deviation. Statistical evaluations were carried out using SigmaStat 2.03 (SPSS, San Rafael, California), and curve-fitting utilized GraphPad Prism 3.02 (GraphPad Software Inc., San Diego, California). For all tests, the level of statistical significance was set at $p < 0.05$. When needed, data were transformed to achieve a normal distribution. For the cell proliferation and YO-PRO assays, t tests or Rank sum tests were used. Two-way ANOVA was used to determine the statistical significance of any possible interaction of cisplatin and RAD001. One-way ANOVA was used to analyze dose effects of RAD001. Determination of the half-life of p21 decay utilized nonlinear regression assuming one-phase exponential decay. Applicability of this regression was assured by Goodnesss of Fit and Runs tests.

Supplemental Data

Supplemental Data include two figures and can be found with this article online at http://www.cell.com/cgi/content/full/120/6/747/DC1/.

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