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Antisense attenuation of p21 sensitizes kidney cancer to apoptosis in response to conventional DNA-damaging chemotherapy associated with enhancement of phospho-p53

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Abstract

Purpose—Kidney cancer is notoriously difficult to treat when metastatic due to its resistance to conventional chemotherapy. Thus, the 5-year survival rate of patients with metastatic renal cell carcinoma (RCC) is less than 10% and, novel approaches to treatment are needed. p21 is a cyclin kinase inhibitor which generally conveys an anti-apoptotic function through its induction by the DNA damage responsive p53 pathway. In this study, we capitalize on this function of p21 and utilize an antisense approach to sensitize p53-wt RCC cells to chemotherapy-induced apoptosis by attenuation of p21 protein levels.

Materials and Methods—Human RCC cell lines ACHN and SN12C cells were transfected with antisense and control oligodeoxynucleotides (ODNs), and assessment of p21 and apoptosis-relevant protein levels as well as apoptosis was performed using standard techniques.

Results—Pre-incubation of ACHN and SN12C cells with phosphorothioate antisense p21 ODN markedly attenuates p21 and sensitizes cells to apoptosis induced by doxorubicin and cisplatin, such that an order of magnitude less doxorubicin or cisplatin can be used in the presence of antisense to achieve equivalent or greater cell death. In addition, the mechanism of ACHN cell death associated with p21 attenuation involves decreases in the levels of anti-apoptotic proteins as well as an increase in the active form of the pro-apoptotic protein p53.

Conclusions—Since phosphorothioate antisense ODNs accumulate to a higher degree in kidney and liver than in any other organ, our findings suggest a re-evaluation of conventional chemotherapy in kidney cancer in association with antisense p21 ODN.

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INTRODUCTION

Kidney cancer, while relatively rare when compared to other human malignancies, is associated with a striking lack of effective treatment options once the disease is metastatic¹. Notwithstanding the newer targeted therapeutics which are just now moving into the mainstream, the prognosis for metastatic renal cell carcinoma (RCC) at this time remains abysmal: approximately 13,000 people per year die of this disease in the US, a number which is steadily increasing over time. While novel molecularly-targeted therapeutics, such as VEGF and other kinase inhibitors, may ultimately improve these survival statistics, new approaches to sensitization of tumors to pharmaceuticals that are already available may result in earlier and more substantial clinical benefit.

Targeted therapies, as well as the mTOR inhibitors, have shown the most promise in this disease, but “standard” non-surgical treatment of RCC has for years relied on immunomodulating agents, such as interferon and interleukin-2¹. While the experience with conventional DNA-damaging chemotherapeutic agents, when used alone in this disease, has been largely disappointing, there is evidence that, at least in other malignancies, these and other drugs can be sensitized using additional agents; this strategy applied to RCC may result in re-examination of such agents in this disease. Sensitization, by whatever means (but not limited to combination chemotherapy), often allows for lower doses of drug to be employed, thereby reducing adverse effects and increasing efficacy. For example, in the case of cisplatin, an agent with minimal effects at low doses (at least in an alveolar epithelial cell line examined), apoptosis is markedly enhanced with the addition of the mTOR inhibitor RAD001², or with the siRNA-induced attenuation of PAX2³. In addition, gemcitabine combined with 5-fluorouracil may have better efficacy in metastatic RCC than either agent alone, and the efficacy of immunomodulating agents in this disease is enhanced by the addition of a targeted therapy. Thus, revisiting of conventional agents in RCC is appropriate in light of novel available combination therapies including the antisense approach to cyclin kinase inhibitor attenuation described here.

The cyclin kinase inhibitor p21, while originally described as an inhibitor of the cell cycle through cyclinD/cdk4, has since been shown to have multiple and pleiotropic effects in a variety of cancer and non-cancer derived cell lines. Such effects include cell cycle promotion through cyclin/cdk assembly, and both promotion and inhibition of anti-apoptosis. In most cancer cell lines which have been examined thus far, the anti-apoptotic outcome seems to predominate, such that attenuation of p21 leads to the progression of apoptosis, as in breast cancer cells⁴. Since p21 is increased in DNA-damaged cells, likely in an attempt to repair said damage, its anti-apoptotic property can be exploited in chemotherapy-resistant cancers. For example, there are many instances in which down-regulation of p21 increases apoptosis, and we have shown that antisense p21, when injected subcutaneously in mice, decreases tumor growth⁵. Given the finding that the sensitization of cisplatin-induced apoptosis requires inhibition of p21 translation², we asked whether attenuation of p21 using antisense techniques results in sensitization of the DNA-damaging chemotherapeutics cisplatin and doxorubicin to apoptosis. We now show that there is at least an order of magnitude increase in sensitivity of a p53-wt RCC cell line as a result of antisense p21 transfection, and we further demonstrate that apoptosis as a result of p21 attenuation is associated with decrease in specific anti-apoptotic proteins and an increase in the pro-apoptotic and pro-senescence activated form of p53. Due to the fact that antisense ODNs are highly localized in the kidney when administered both intravenously and intraperitoneally, our work may lead to novel chemotherapy sensitizing approach to RCC utilizing antisense techniques.

MATERIALS AND METHODS

Materials

Cisplatin and doxorubicin were purchased from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal anti-recombinant full-length p21^{Waf1/Cip1} antibody was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). p57 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-human PARP purified antibody and apoptosis detection kit was obtained from BD Biosciences (San Diego, CA). Phospho-p53 antibody (pSer15) was obtained from Calbiochem (Darmstadt, Germany). Goat anti-mouse and goat anti-rabbit horseradish peroxidase-conjugated IgG were obtained from Bio-Rad (Richmond, CA). Mouse anti-β-actin monoclonal antibody, was from Sigma (St. Louis, MO). Hoechst 33258 was from Calbiochem (San Diego, CA). Genejuice was obtained from Novagen (San Diego, CA). ECL Western Blotting Detection Reagents were obtained from Amersham Biosciences (Buckinghamshire, United Kingdom). CaspACE Assay System was obtained from Promega (Madison, WI).

Cell lines

ACHN human RCC cells (CRL-1161) were obtained from ATCC (Manassas, VA), and SN12C cells were a kind gift from Dr. George Thomas. The cells were maintained in MEM 1X media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acid, and 0.75% sodium bicarbonate.

Antisense transfections

Human p21^{Waf1/Cip1} antisense and control ODNs were synthesized by Biognostik (Gottingen, Germany). The human p21 antisense ODN sequence was 5'-ATC-CCC-AGC-CGG-TTC-TGA-CAT-3'. The random scrambled sequence control ODN (SC1) and the FITC-labeled ODN were both 5'-TGG-ATC-CGA-CAT-GTC-AGA-3', and the sequence for the second control ODN (SC2; used for the experiment depicted in Fig. 1) was 5'-TAC-AGT-CTT-GGC-CGA-CCC-CTA-3'. For the transfection procedure, 2×10^6 cells were seeded and grown to 70% confluence and washed with sterile PBS, and the FITC-labeled random ODNs and p21 antisense and scrambled control ODNs (200, 400, or 600 nM) were transfected as previously described⁴, with GeneJuice as transfection reagent. Briefly, after 8 h incubation at 37°C with the appropriate ODN in Genejuice and OptiMEM, the media was withdrawn and complete media was re-added for another 16 h.

Western blotting

Cells were washed with PBS and lysed in lysis buffer (50 mM HEPES, 1% Triton X-100, 10 mM Na Pyrophosphate, 100 mM Na Fluoride, 4 mM EDTA) at °C. Cell lysates were centrifuged ($13,000 \times g$, 4°C, 10 min) and the supernatants were electrophoresed and immunoblotted. The membranes were blocked in 5% non-fat dry milk for 1 h at room temperature, and probed with appropriate antibodies. Membranes were then probed with HRP-tagged anti-mouse or anti-rabbit IgG antibodies diluted 1:5,000 – 1:15,000 in 5% non-fat dry milk for 1 h at room temperature. Chemiluminescence was detected using enhanced ECL.

Caspase assay

The CaspACE assay kit (Promega, Madison, WI) was used to measure caspase-3 activity per the manufacturer's instructions. Briefly, 2×10^6 cells per 10 cm dish were treated as described in the text. Positive control cells were treated with 4 μM Camptothecin. The cells were harvested, washed, and equal protein quantities were incubated with the DEVD-pNA caspase-3 substrate in Caspase Assay Buffer. The plates were covered with parafilm and incubated at 37°C overnight. Color development was measured at 405 nm.

MTT assay

A 200 μ L aliquot of cells (5×10^4 cells in complete media) were added to a 96 well plate and incubated for 24 hr at 37 °C in a humidified incubator containing 5% CO₂ in air. After incubation, each condition of ODNs was transfected into each well for 24 hr and DNA damaging agents dissolved in DMSO were added to each of the 96 wells triply and incubated for 24 hr. Control cultures were treated with DMSO. After incubation, a 20 μ L MTT solution (5 mg/mL in phosphate buffer) was added to each well and the incubation continued for 4 h, after which time the solution in each well was carefully removed. The blue crystalline precipitate in each well was dissolved in DMSO (100 μ L). The visible absorbance at 560 nm of each well was quantified using a microplate reader.

Evaluation of nuclear morphology

Cells were seeded in 6-well culture dishes and treated with ODNs and indicated concentration (s) of cisplatin or doxorubicin. The cells were then immersed in methanol for at least 10 min. Cells were stained in 1 μ g/ml Hoechst 33258 in water with 1% nonfat dry milk. After staining for 10 min, the cells were rinsed in water and dried completely. Nuclear morphology was visually evaluated by fluorescence microscopy.

RESULTS

Antisense ODNs enter cells and attenuate p21

In order to determine the role of p21 in RCC cells, we used antisense techniques. ACHN cells were transfected with a 21-bp antisense ODN which encompassed the ATG start codon of the human *p21* (CDKN1) gene, and which we have previously shown to be effective in reducing p21 protein levels in several cell lines including a human breast cancer cell line⁴, human renal mesangial⁶ cells, and human⁷ and (with a similar ODN) rat⁸ vascular smooth muscle cells. In order to determine transfection efficiency, a FITC-labeled ODN of the same size was transfected into ACHN RCC cells once they were 70% confluent, and 24 h later, these cells were fixed and examined under fluorescence microscopy (Fig. 1a). Comparing the fluorescence to the light microscopy of the same field, it can be seen that transfection efficiency is nearly 100% at 600 nM similar to what we⁸ and others⁹ have previously reported in other cell lines.

To ascertain whether p21 protein is reduced by the antisense ODN after successful transfection, ACHN cells were transfected under similar conditions as above, and the cells were lysed and examined for p21 and β -actin levels (the latter as a loading and specificity control) by immunoblotting. In the presence of antisense ODN, levels of p21 protein were decreased in an ODN concentration-dependent fashion from 200-600 nM, with higher p21 attenuation at 600 nM (Fig. 1b) corresponding to the observed higher transfection efficiency (see Fig. 1a). There was no effect of either antisense or scrambled control ODN on β -actin (later confirmed with p57, see below), supporting specificity of the antisense ODN to p21, and there was minimal effect of two distinct scrambled control ODNs on p21 (Fig. 1b), confirming that the attenuation by antisense was not due to the transfection procedure or to the mere presence of the ODN.

Attenuation of p21 leads to apoptosis

p21 has been shown to be both pro- and anti-apoptotic, likely depending on cell type as well as the molecular context in which it is assayed. It has been previously shown in other cell lines that decreasing p21 using a variety of techniques leads to growth arrest or stimulation, as well as increased or decreased levels of apoptosis chemotherapy sensitization. Thus, we asked what role p21 plays in ACHN cells under conditions of continuous serum incubation.

In order to show that the effects of p21 attenuation are generalizable to RCC, we next examined both ACHN and another RCC cell line, SN12C. Cells were transfected with antisense or scrambled control ODNs at various concentrations and, 24 h after transfection was initiated, were examined for apoptosis using two different assays. PARP is a member of the apoptosis cascade lying downstream of the caspases, and its cleavage represents a distal node in the programmed cell death pathway. PARP cleavage began to occur at 200 and 400 nM and was higher at 600 nM of antisense ODN transfection in both cell lines whereas there was no effect of the control ODN at the same concentrations on PARP cleavage; β -actin level was unchanged, confirming gel loading consistency (Fig. 2a). To confirm specificity of the antisense ODN, we also examined another CKI, p57, and show that its level is unaltered by p21 antisense or control ODNs, further demonstrating specificity of the antisense p21 ODN for p21 (Fig. 2b). To confirm that apoptosis was indeed occurring after p21 attenuation, we examined caspase-3 cleavage. The caspase family of proteases are essential components of an evolutionarily conserved cell death pathway in multicellular eukaryotes and play key roles in inflammation and apoptosis in mammalian cells. Caspase-3 has substrate specificity for the amino acid sequence DEVD (Asp-Glu-Val-Asp), and is inhibited by the tetrapeptide inhibitor Ac-DEVD-CHO; its catalytic activity was assessed colorimetrically using the labeled Ac-DEVD-pNA substrate. After antisense p21 transfection, caspase-3 cleavage was induced in ACHN cells at 400 nM (Fig. 2c), consistent with the PARP data.

Attenuation of p21 sensitizes cells to apoptosis

While, as described above, a decrease in p21 protein alone results in apoptosis, we next asked whether antisense p21 increases the sensitivity of RCC cells to conventional DNA-damaging chemotherapy agents; these agents have not been successful in the past due to minimal efficacy and/or unacceptable adverse effects. It is quite possible that this lack of efficacy is at least in part due to p21 induction by repair pathways, thereby allowing p53-mediated DNA repair to occur unimpeded in the cancer cells¹⁰.

We utilized two common conventional chemotherapeutic agents, each of which functions by causing DNA damage. While no conventional chemotherapeutic agent used alone is currently effective against RCC, many have shown promise *in vitro* when combined with pro-apoptotic agents. For example, cisplatin, when combined with antisense against bcl-2, has shown efficacy in RCC cell lines¹¹, and its apoptotic effect is enhanced by the addition of a rapamycin analog². Doxorubicin, in combination with anti-Fas mAB¹² or TRAIL/Apo2L¹³, showed a synergistic cytotoxic effect in several RCC cell lines.

We first evaluated cytotoxicity of these agents when each was used alone in ACHN cells. As measured by the MTT assay, cisplatin showed approximately 50% cytotoxicity at between 1 and 10 μ M, and doxorubicin at between 0.1 and 0.5 μ M (Fig. 3). Thus, we utilized these concentrations in subsequent studies.

While p21 attenuation alone results in apoptosis (see Fig. 2 and reference ⁴), we next asked whether transfection with antisense p21 ODN potentiates apoptosis induced by the chemotherapeutic agents. ACHN and SN12C cells were incubated in serum-containing media with doxorubicin or cisplatin at the concentrations discussed above, and apoptosis was first assessed qualitatively by PARP cleavage (Fig. 4a). PARP cleavage was seen only at the highest concentration of each chemotherapeutic agents in both control conditions (i.e. without antisense ODN), yet marked PARP cleavage was seen in all cells transfected with p21 antisense ODN with some enhancement in PARP cleavage with addition of these chemotherapeutic agents in SN12C cells. At the highest concentration (10 μ M) of cisplatin, p21 was absent in SN12C cells, consistent with apoptosis under these conditions.

The ultimate purpose of chemotherapy in cancer is to cause death of cancer cells while sparing normal cells. While PARP cleavage indicates that apoptosis is occurring, this is not a quantitative assay. Thus, in order to quantitate cell survival after chemotherapeutic agent exposure in the presence or absence of p21 attenuation, we used the MTT assay. Cells in the presence of antisense p21 but absence of chemotherapy showed a lower survival (Fig. 4b,c). In the presence of doxorubicin at 0.1 and 1 μ M and of cisplatin at 1 and 10 μ M (concentrations which were optimized above; see Fig. 3), there was markedly decreased cell survival in cells transfected with antisense p21 ODN as compared to either no ODN or scrambled control ODN (Fig. 4b,c). Thus, to provide the same degree of cell killing, one would require at least an order of magnitude lower dose of chemotherapeutic agent in the presence of antisense p21 ODN as compared to cells with no antisense or control ODN. Thus, provided there are minimal non-specific effects of p21 attenuation, these DNA-damaging chemotherapeutic agents should be reassessed at lower doses in the presence of p21 attenuation as possible RCC treatment.

To confirm that apoptosis was indeed occurring under conditions of antisense p21 and chemotherapy, appropriately treated cells were fixed and stained with Hoechst stain to visualize nuclear morphology; ACHN cells incubated with antisense p21 ODN resulted in a lower density of cells as well as a higher number of condensed, apoptotic nuclei than cells treated under both control conditions (Fig. 5).

Attenuation of p21 decreases anti-apoptotic proteins and increases phospho-p53

A major strategy for chemotherapeutic success involves channeling malignant cells into apoptotic pathways, and, consequently, a frequent cause of chemotherapeutic failure results from malfunction of apoptosis; we next asked whether p21 attenuation results in decreases in anti-apoptotic proteins. The Bcl-2 family of inhibitors can have both pro- and anti-apoptotic effects, yet Bcl-2 itself is generally anti-apoptotic. Bcl-2 is overexpressed in a wide variety of cancers including RCC, where its inhibition was associated with enhancement of chemotherapy efficacy¹¹. In addition, antisense Bcl-2 is in clinical trials, though the results thus far are not promising. The IAP class of apoptosis inhibitors function by binding to and thus inactivating caspases, and XIAP, one of the most common of this class, inhibits caspases 3, 7, and 9. In RCC, overexpression of XIAP predicts a worse prognosis¹⁴, an expected finding due to its anti-apoptotic effect.

When p21 was attenuated in ACHN cells using our antisense construct, both in the presence and absence of cisplatin or doxorubicin, both XIAP and Bcl-2 were markedly attenuated (Fig. 6a). In addition, examination of phospho-p53 in these p53-wt cells showed an increase in association with p21 attenuation in cells which did not receive chemotherapy as well as in those which received both cisplatin and doxorubicin in the presence of antisense p21 ODN, whereas the levels of non-phosphorylated p53 were unchanged (Fig. 6b). This finding is of considerable interest, as it suggests that an increase in the active form of p53 is involved in the pro-apoptotic function of p21, at least in this cell line. Thus, the mechanism of apoptosis through attenuation of p21 may involve a feedback increase in the pro-apoptotic and pro-senescence “guardian of the genome,” p53.

DISCUSSION

Approximately one-third of patients with kidney cancer present with metastatic disease, and these patients have severely limited treatment options. Despite the recent findings of efficacy of novel targeted therapeutics, such as the kinase inhibitors sorafenib, sunitinib, and the mTOR inhibitors including temsirolimus, new approaches are desperately needed for those patients who do not respond to current treatment. Due to the historically unsatisfactory response of RCC to “standard” immunomodulating agents, DNA damaging chemotherapeutic agents have been investigated, but when used alone these drugs have shown limited success. There are

reports of cytotoxic synergy when standard agents are used in combination with other therapies^{2,3}; along these lines, a recent report of a systematic search for combination therapies of chemosensitizers and standard agents has shown promise¹⁵. In the present study, we have taken another look at the standard agents in RCC and have investigated their use in combination with attenuation of a protein which plays a key role in orchestrating the apoptotic response of the cell after DNA damage. The fact that antisense localizes strongly to the kidney supports the likelihood for success for our method in cancer of this organ. Our findings may result in a novel approach to treatment of this unusually chemotherapy resistant disease by defeating at least one aspect of its resistance armamentarium.

While the initial descriptions of p21 focused on its location in the tumor suppressor pathway downstream of p53 and its function as an inhibitor of G1 cyclin kinases, more recent studies have shown that this protein has multiple and diverse functions, ranging from its role as an assembly factor for cyclin-cdk interaction to its positive effect on cell proliferation, and, more recently, to its pleiotropic functions in apoptosis. In many cancer cell lines (and likely in malignancies *in vivo*), p21 appears to be anti-apoptotic, a fact which may explain its transcriptional activation in the p53 DNA damage repair pathway¹⁰. In previous studies from our and other laboratories in breast cancer, and in this study in kidney cancer, we have exploited this anti-apoptotic function of p21 to potential therapeutic advantage.

It is generally accepted that inactivation of apoptosis is essential for cancer development. Thus, chemotherapy must reinstate such programmed cell death in order to be effective against most cancers, a finding which has consistently been exploited in drug discovery. By using the anti-apoptotic function of p21 to therapeutic advantage by attenuating this protein, we have identified another paradigm with which to attack therapy-resistant cancers at their Achilles' heel. A reassuring ancillary finding in our studies, and a novel aspect of this work, is that activated (i.e. phosphorylated) p53 is increased with p21 attenuation under most conditions. This data suggests not only is there a feedback loop whereby p21 influences p53 levels, but that p53 itself may mediate some of the pro-apoptotic activity associated with reduction of p21. It has been reported that inactivation of p21 can sensitize colon cancer cells to apoptosis *via* an increase in the active form of p53¹⁶. In addition, p53 and p21 show inverse expression levels in esophageal squamous cell carcinoma when examined by immunohistochemistry¹⁷. While not investigated in our study, it is conceivable that, through this mechanism, the recently reported p53-induced senescence¹⁸ occurs in cells whose p21 is attenuated, which would be another salutary effect of antisense p21 on cancer, both with and without supplemental chemotherapy. A caution in this regard is that, although p21 does in fact possess p53-independent effects, whether our p21 inhibitory strategy to promote apoptosis will hold true in p53-mutant cells remains to be seen and is currently under investigation in our laboratory.

Antisense therapy for cancer, while currently in limited use, has shown considerable potential and is close to being of general use in the clinic. Due to the fact that the kidney is among the highest perfused of any organ, intravenously infused ODNs reach high concentrations in this organ; thus of all the cancers for which antisense holds promise, the potential therapeutic utility of this approach is maximal in kidney disease. Phosphorothioate ODN concentration in the kidney and liver is markedly higher than in other tissues after either intravenous or intraperitoneal administration in mice¹⁹, and renal epithelial cells efficiently take up such ODNs without apparent degradation. While plasma clearance of labeled phosphorothioate ODN is relatively rapid ($t_{1/2} \sim 5$ min), the urinary excretion is considerably slower ($t_{1/2} \sim 28$ min), such that it would be conceivable to infuse antisense p21 ODN immediately prior to systemic chemotherapy in order to sensitize renal epithelial cells and, presumably highly vascular RCC tumors, to DNA damaging chemotherapy. Of course, whether ODNs actually enter tumor tissue (quite likely due to the highly vascular nature of RCC) and any toxic effects upon the other highly concentrated tissue, the liver, is not known and is currently being investigated in our

laboratory. The potential issue of long-term p21 attenuation resulting in cancer is controversial, but there is no theoretical or experimental evidence that short-term p21 inhibition (as would be required for tumor sensitization) has any adverse effects.

Other laboratories have shown that attenuation of p21 using antisense ODNs increases radiosensitivity of a variety of tumor cell lines, the mechanism in this case likely being that p21 is blocking apoptosis by interfering with cell death machinery through inhibition of cdk-mediated caspase-9 activation. There are only a few reports using antisense p21 as a sensitizer to chemotherapy, one showing that breast cancer cell have increased sensitivity towards paclitaxel²⁰, and the other (using adenoviral-mediated antisense p21) showing that prostate cancer cells are sensitized to doxorubicin²¹. Techniques for attenuating p21 are of course not limited to antisense or even siRNA methods, and our laboratory is currently evaluating small molecules which act as p21 inhibitors and which could be administered orally. Ours is the first study demonstrating sensitization to heretofore ineffective chemotherapeutic agents in a notoriously chemotherapy-resistant tumor type, and to suggest a mechanism for this effect involving anti-apoptotic proteins as well as p53, thereby offering novel therapeutic paradigms for drug development in this disease.

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Abbreviations

RCC	renal cell carcinoma
ODN	oligodeoxynucleotide
SC	scrambled control
PARP	Poly(ADP-ribose) polymerase

Figure 1. Antisense p21 ODN attenuates p21 in ACHN cells

ACHN cells were grown to confluence and transfected with FITC-labeled random sequence, antisense p21 (AS), or two distinct scrambled sequence control (SC1 and SC2) ODNs at the concentrations indicated as described in Materials and Methods.

a. The cells were transfected with FITC-labeled random ODNs and examined under visible and UV light at 40 X magnification.

b. The cells were lysed, and equal protein quantities were electrophoresed and immunoblotted with p21 antibody. The same lysate was immunoblotted with β -actin antibody as a loading control. CM refers to non-transfected cells in 10% serum-containing (complete) media; GJ refers to cells treated only with the transfection reagent GeneJuice.

Figure 2. Attenuation of p21 causes apoptosis

ACHN and SN12C cells were grown to confluence and transfected with antisense (AS) or scrambled sequence control (SC1) ODN as described in Materials and Methods.

a. The cells were lysed and subjected to immunoblotting with p21, PARP, and β-actin antibodies. Control refers to non-transfected cells in serum-containing media. GJ refers to cells treated only with the transfection reagent GeneJuice. The cleaved product of PARP (M_r 85,000) is indicated.

b. ACHN cells were lysed and subjected to immunoblotting with p21, p57, PARP, and β-actin antibodies. Control refers to non-transfected cells in serum-containing media. The cleaved product of PARP (M_r 85,000) is indicated.

c. ACHN cell lysate in (b) was assessed for caspase-3 activation using a colorimetric method as described in Materials and Methods. CM refers to non-transfected cells in serum-containing (complete) media; GJ refers to cells treated only with transfection reagent Genejuice. CT refers to cells treated with camptothecin (4 μM) as a positive control for apoptosis. AS and SC represents antisense p21 ODN and scrambled control ODN transfected at 400 nM, respectively.

Figure 3. Determination of cytotoxicity of chemotherapy agents in ACHN cells

ACHN cells were grown to confluence and incubated with the indicated concentrations of cisplatin or doxorubicin for 24 h and then subjected to the MTT assay as described in Materials and Methods.

a

b

Figure 4. Attenuation of p21 sensitizes ACHN cells to chemotherapy

ACHN and SN12C cells were grown to confluence and incubated with 400 nM antisense (AS) or scrambled sequence control (SC) ODN as described in Materials and Methods.

- The cells were lysed and subjected to immunoblotting with p21 and PARP antibodies β -actin is a loading control.
- The cells were subjected to the MTT assay as described in Materials and Methods. * $p < 0.01$ as compared to CM and SC, and ** $p < 0.05$ as compared to CM and SC, at the same drug concentrations.

Figure 5. Apoptosis is increased by antisense p21 incubation

ACHN cells were grown to confluence and incubated with 400 nM antisense (AS) or scrambled control (SC) ODN as described in Materials and Methods, followed by application of cisplatin or doxorubicin at the indicated concentrations for 24 h. Cells were fixed and stained with Hoeschst 33258 as described in Materials and Methods. In AS-treated cells, apoptotic cells are indicated with arrows. For a better view of morphology, black arrows are shown magnified in lower panels, indicating apoptotic cells in AS panels and non-apoptotic cells in SC panels.

a

b

Figure 6. Anti-apoptotic proteins are decreased and phospho-p53 is enhanced by p21 attenuation

ACHN cells were grown to confluence and incubated with 400 nM antisense (AS) or scrambled control (SC) ODN as described in Materials and Methods, followed by application of cisplatin or doxorubicin at the indicated concentrations for 24 h. The cells were lysed and subjected to immunoblotting with antibodies to (a) anti-apoptotic proteins and (b) native and phospho-p53. β -actin is a loading control.