Molecular Medicine 8(4): 185–199, 2002

Anti-Tumor Efficacy of a Novel Antisense Anti-MDM2 Mixed-Backbone Oligonucleotide in Human Colon Cancer Models: p53-Dependent and p53-Independent Mechanisms

Hui Wang,[1,3] Li Nan,[1] Dong Yu,[5] J. Russell Lindsey,[2,3] Sudhir Agrawal,[5] and Ruiwen Zhang[1,3,4]

[1]Department of Pharmacology and Toxicology, Division of Clinical Pharmacology, University of Alabama at Birmingham, Birmingham, Alabama, USA

[2]Department of Genomics and Pathobiology, University of Alabama at Birmingham, Birmingham, Alabama, USA

[3]Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, Alabama, USA

[4]Gene Therapy Center, University of Alabama at Birmingham, Birmingham, Alabama, USA

[5]Hybridon, Inc., Cambridge, Massachusetts, USA

Accepted March 19, 2002

Abstract

Background: The MDM2 oncogene is amplified or overexpressed in many human cancers and MDM2 levels are associated with poor prognosis. MDM2 not only serves as a negative regulator of p53 but also has p53-independent activities. This study investigates the functions of the MDM2 oncogene in colon cancer growth and the potential value of MDM2 as a drug target for cancer therapy, by inhibiting MDM2 expression with an antisense anti-human–MDM2 oligonucleotide.

Materials and Methods: The selected antisense mixed-backbone oligonucleotide was evaluated for its in vitro and in vivo antitumor activity in human colon cancer models: LS174T cell line containing wild-type p53 and DLD-1 cell line containing mutant p53. The levels of MDM2, p53 and p21 proteins were quantified by Western blot analysis.

Results: In vitro antitumor activity was found in both cell lines, resulting from specific inhibition of MDM2 expression. In vivo antitumor activity of the oligonucleotide occurred in a dose-dependent manner in both models and synergistically or additive therapeutic effects of MDM2 inhibition and the cancer chemotherapeutic agents 10-hydroxycamptothecin and 5-fluorouracil were also observed.

Conclusions: These results suggest that MDM2 have a role in tumor growth through both p53-dependent and p53-independent mechanisms. We speculate that MDM2 inhibitors have a broad spectrum of antitumor activities in human cancers regardless of p53 status. This study should provide a basis for future development of anti-MDM2 antisense oligonucleotides as cancer therapeutic agents used alone or in combination with conventional chemotherapeutics.

Introduction

Human cancer has been increasingly viewed as diseases characterized by loss of cell-cycle control and increased genetic instability. Both oncogenes and tumor suppressor genes play a crucial role in cancer formation, growth, and progression. Activation of proto-oncogenes that promote cell growth in combination with the inactivation of tumor suppressor genes that inhibit cell growth by means of cell growth arrest and induction of programmed cell death or apoptosis leads to tumor progression and malignancy. Perhaps the most important and best-studied tumor suppressor gene is p53 (1). Abnormalities of the p53 tumor suppressor gene are among the most frequent molecular defects in human and animal neoplasia. Nevertheless, studies also have provided increasing evidence that the tumor suppressor function of p53 can be inhibited without mutation. Perhaps one of the most important findings in this area is that the MDM2 oncogene is a negative regulator of wild-type p53 (2,3). The MDM2–p53 autoregulatory feedback loop regulates the intracellular p53 function: the MDM2 gene is a target for direct transcriptional activation of p53 and MDM2 protein is a negative regulator of p53 (3–8). In addition, MDM2 protein interacts with other cellular proteins that are involved in cell-cycle regulation, including pRb, E2F1/DP1, p300, and p19ARF (3–8). Overexpression of MDM2 is demonstrated in a variety of human tumors and may be due to gene amplification (9), increased transcription (10,11), and/or enhanced translation (12,13). Many studies have shown that overexpression of MDM2 is associated with poor prognosis in

Correspondence and reprint requests should be addressed to: Ruiwen Zhang, MD, PhD, DABT, Department of Pharmacology and Toxicology, University of Alabama at Birmingham, VH 113, Box 600, 1670 University Boulevard, Birmingham, AL 35294-0019. Phone: 205-934-8558; fax: 205-975-9330; e-mail: ruiwen.zhang@ccc.uab.edu.

many human malignancies (9–31). Therefore, MDM2 plays a crucial role in cell-cycle control and tumor transformation and growth. Like p53, MDM2 has been suggested as a target for rational drug design for cancer therapy (6–8,32–39).

Gastrointestinal cancers remain a major public health problem both in the United States and worldwide (40). In the United States, colorectal cancer is the second most common cancer in women and the third most common cancer in men (40). Although there has been considerable progress in research on the etiology, prevention, and experimental therapy of gastrointestinal cancers, no fully effective approaches are available currently for the treatment and prevention of this disease. MDM2 overexpression has been observed in human colorectal cancer (25). The objective of the present study was to use both in vitro and in vivo models to evaluate the therapeutic effectiveness of a recently identified anti–human-MDM2 antisense oligonucleotide (38,39) in the treatment of human colorectal cancers when administered alone or in combination with conventional chemotherapeutic agents.

Materials and Methods

Test Oligonucleotides

The test oligonucleotide, Oligo AS, a 20-mer mixed-backbone oligonucleotide (5′**UG**ACACCTGTTCT-CAC**UCAC**-3′) and its mismatched control (Oligo ASM, 5′**UG**T**T**CACC**C**T**T**TT**T**TCA**T**UCAC-3′) were synthesized, purified, and analyzed as previously described (41). Two nucleosides at the 5′end and four nucleosides at the 3′end are 2′*O*- methylribonucleosides (represented by boldface letters); the remaining are deoxynucleosides. The underlined nucleosides of Oligo ASM are the sites of the mismatched controls compared with Oligo AS. For both mixed-backbone oligonucleotides, all internucleotide linkages are phosphorothioate. The purity of the oligonucleotides was shown to be greater than 90% by capillary gel electrophoresis and PAGE, with the remainder being n-1 and n-2 products. The integrity of the internucleotide linkages was confirmed by ^{31}P-NMR.

Chemicals and Reagents

Cell culture media, anti-human β-actin (SC-15) monoclonal antibody, and phosphate-buffered saline (PBS) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS), antibodies against p53 and p21, lipofectin, trypsin, penicillin-streptomycin, and trypan blue stain were purchased from GIBCO-BRL (Grand Island, NY, USA). The anti–human-MDM2 monoclonal antibody (37,38) was kindly provided by J. Chen (Moffitt Cancer Center, Tampa, FL, USA). Chemotherapeutic agents adriamycin and 5-fluorouracil (5-FU) were obtained from Sigma. The natural product topoisomerase I inhibitor 10-hydroxycamptothecin (HCPT) was obtained from the Midwest Co. (Beijing, China) with the purity of the drug being greater than 98% (42).

Cell Culture

The tumor cell lines, LS174T and DLD-1, were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured according to their instructions. LS174T cells were cultured in MEM with 0.1 mM nonessential amino acids and Earle's balanced salt solution containing 10% FBS and DLD-1 cells in RPMI 1640 medium containing 10% FBS. All media included 1% penicillin/streptomycin. The in vitro biological activity of oligonucleotides was determined by using the conditions described earlier (37–39,43). Cells were incubated with Oligos AS or ASM at various concentrations for 72 hr, in the presence of lipofectin (7 μg/ml). The effects on cell growth were analyzed by trypan blue viability staining (43).

Animal Tumor Model

Human cancer xenograft models were established using the methods reported previously (39,43,44). Pathogen-free female nude mice (5 weeks old) were purchased from Frederick Cancer Research and Development Center (Frederick, MD, USA) and accommodated for 5 days for environmental adjustment prior to study. Cultured LS174T or DLD-1 cells were harvested from the monolayer cultures, washed twice with culture medium (without FBS), resuspended in FBS-free culture medium, and injected subcutaneously (2×10^6 cells, total volume 0.2 ml) into the left inguinal area of the mice. The animals were monitored by general clinical observation, determination of body weight, and measurement of tumor growth.

In Vivo Chemotherapy

The animals bearing human cancer xenografts were randomly divided into various treatment groups and a control group (6 mice/group). The control (non–oligo-treated) group received physiologic saline only. The oligonucleotides dissolved in physiological saline (0.9% NaCl) were administered by intraperitoneal injection at various dose levels. The injection volume was based on the body weight (5 μl/g body weight) and the oligonucleotide concentrations were adjusted on the basis of the dose. HCPT was suspended in cottonseed oil and given by gavage (volume; 10 μl/g body weight). The dose was 3 mg/kg/day, 7 consecutive days for the first week of treatment and every other day afterwards. 5-FU was given by intraperitoneally at a dose of 10 mg/kg/day (volume; 5 μl/g body weight). Tumor growth was monitored by the measurement, with calipers, of two perpendicular diameters of the implant every other day. Tumor weight (g) was calculated by the formula

$$1/2a \times b^2$$

where *a* is the long diameter (cm) and *b* is the short diameter (cm). Tumor xenograft pathology was evaluated by using the methods previously reported (43).

Western Blot Analysis

The MDM2, p53, and p21 levels in cultured cells or tumor xenografts were analyzed by using the methods described previously (37–39). In brief, cell lysates or tumor tissue homogenates containing identical amounts of total protein were fractionated by SDS-PAGE and transferred to Bio-Rad Trans-Blot nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). The nitrocellulose membrane was then incubated with blocking buffer (PBS containing 0.1% Tween 20 and 5% nonfat milk) for 1 hr at room temperature and washed with the washing buffer (PBS containing 0.1% Tween 20) for 5 min twice. The membrane was incubated with primary (anti-MDM2, anti-p53, anti-p21, or anti–β-actin) antibody overnight at 4°C or for 1 hr at room temperature with gentle shaking. The membrane was washed with the washing buffer for 15 min and then twice for 5 min, and then incubated with 1:5000 diluted goat anti-mouse IgG-horse radish peroxidase conjugated antibody (Bio-Rad) for 1 hr at room temperature. After washing as described, the protein of interest was detected by ECL reagents from Amersham (Arlington Height, IL, USA). The density of each protein band was analyzed by a densitometry measurement (Bio-Rad Model GS-670 Imaging Densitometer) and expressed as percentage of the controls, normalized by corresponding β-actin level.

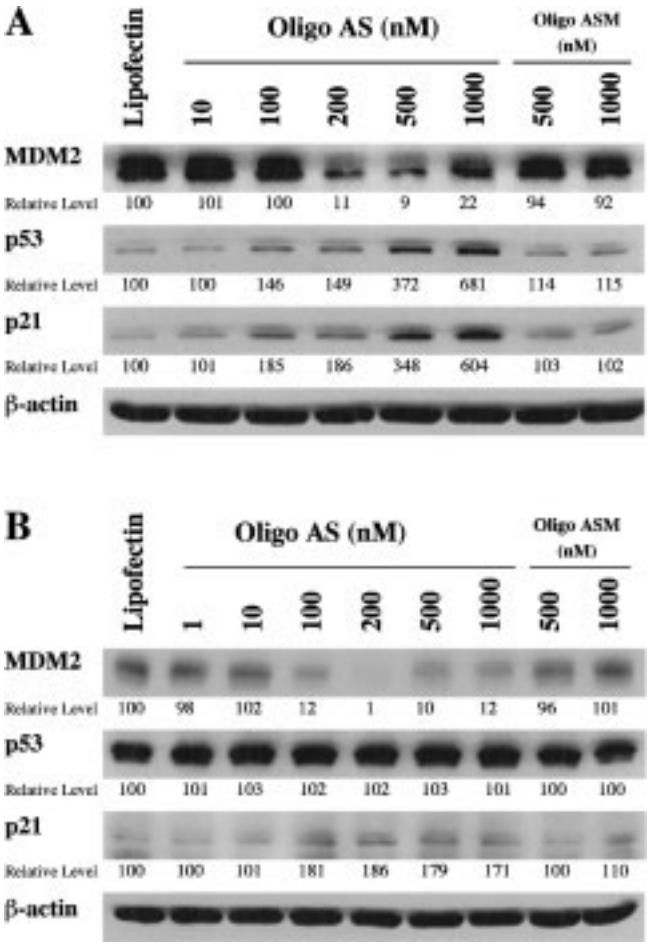

Fig. 1. Effects of anti-MDM2 antisense oligonucleotides on MDM2, p53, and p21 protein levels in LS174T (A) and DLD-1 (B) cells in culture. Cells were incubated with Oligo AS at various concentrations for 24 hr, in the presence of lipofectin (7 μg/ml). Identical total protein (20 μg) was analyzed by SDS-PAGE, followed by Western blotting. Inhibitory effects of Oligo AS on MDM2 expression are shown in a dose-dependent manner, with greater than 90% inhibition be observed at 500 nM of Oligo AS. The protein levels of p53 and/or p21 were increased in a dose-dependent manner. The control mismatched Oligo ASM had minimal effects on the levels of these proteins. Relative levels of each protein were expressed as percentage of control, normalized by corresponding β-actin level.

Results

Antitumor Activity of Antisense Anti-MDM2 Oligo AS in Human Colon Cancer LS174T Model That Contains Wild-Type p53

In Vitro Biological Activity In vitro inhibition of MDM2 expression by Oligo AS was shown in a sequence-specific, dose-dependent manner (Fig. 1A). The anti-MDM2 oligo, Oligo AS, specifically inhibited MDM2 expression in LS174T cells, and p53 and p21 levels were elevated accordingly. The mismatched control oligonucleotide, Oligo ASM, had minimal effects on MDM2, p53, or p21 protein levels at various concentrations up to 1000 nM, the highest concentration tested in the study. Oligo AS inhibited the growth of tumor cell lines in vitro in a dose-dependent manner, with an IC_{50} value of 323 nM for a 72-hr treatment. The mismatched oligonucleotide, Oligo ASM, had no significant effect on tumor cell growth.

Following in vitro exposure to combinations of oligos and the chemotherapeutic agents, HCPT, adriamycin, and 5-FU, the protein levels of MDM2, p53, and p21 were determined in LS174T cells (Fig. 2). Cells were incubated with 400 nM of Oligo in the presence of lipofectin for 24 hr, followed by addition of various concentrations of chemotherapeutic agents and incubation for additional 24 hr. As illustrated in Figure 2 (panel I, lanes A), HCPT induced p53, p21, and MDM2 in a dose-dependent manner, as we reported in an earlier study with human breast cancer cell line MCF-7 (45). Following the treatment with Oligo AS, MDM2 expression was inhibited, resulting in significantly elevated p53 and p21 levels (panel I, lanes B). The mismatched control Oligo ASM showed minimal effect on the protein levels of MDM2, p53, or p21 (panel I, lanes C). Adriamycin slightly induced p53 and p21 in LS174T cells (panel II, lanes A). Following the combination treatment with Oligo AS, MDM2 expression was

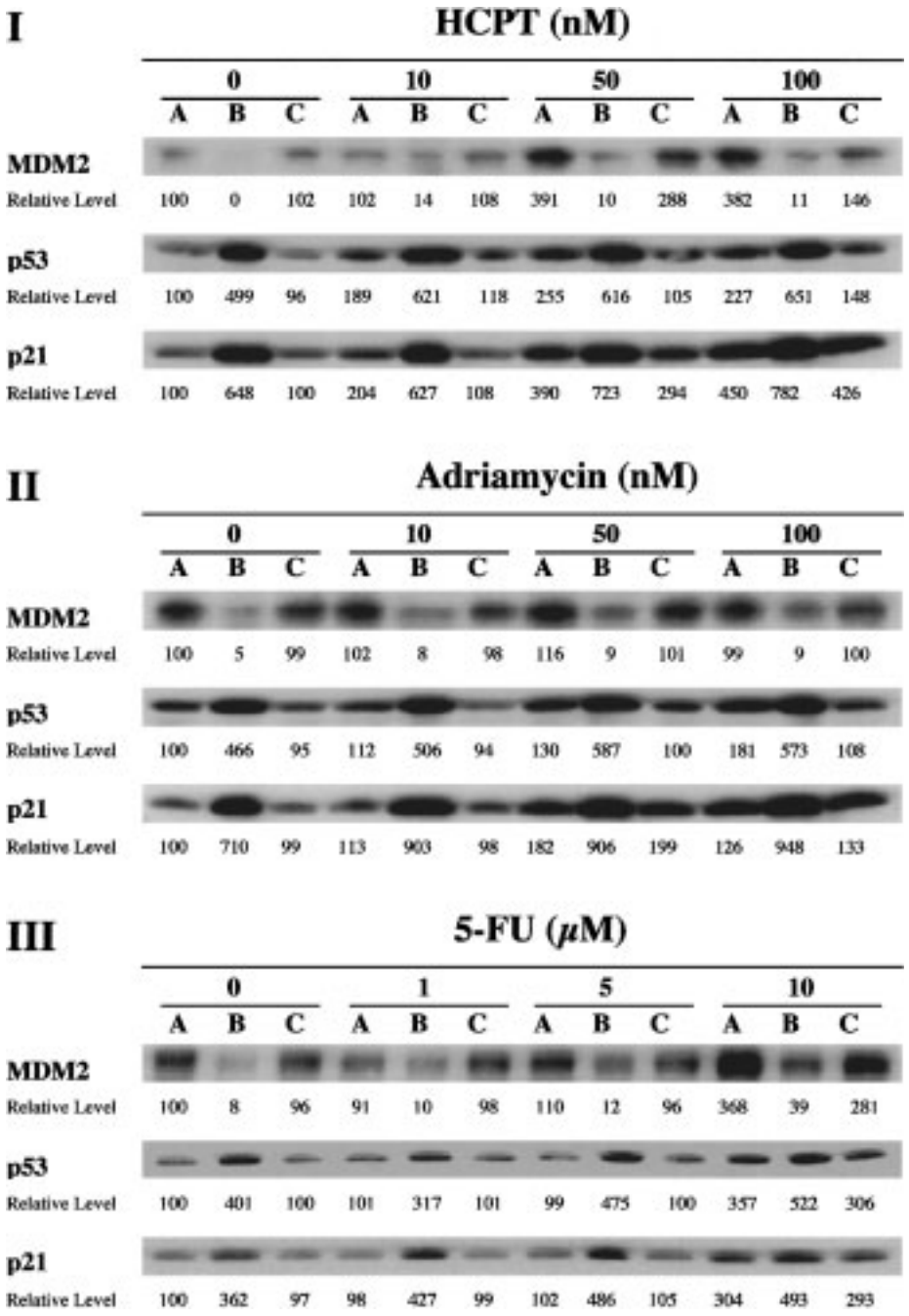

Fig. 2. Synergistic effects of combination treatment of anti-MDM2 oligo AS and the cancer chemotherapeutic agents on MDM2, p53, and p21 protein levels in LS174T cells in culture. Cells were incubated with 400 nM Oligo AS or ASM in the presence of lipofectin for 24 hr, followed by addition of various concentrations of HCPT (panel I), adriamycin (panel II), or 5-FU (panel III) and incubation for additional 24 hr. At various concentrations, the effects on MDM2, p53, and p21 levels were evaluated following treatment with cytotoxic agents alone (lanes A) or pre-treatment with Oligo AS (lanes B) or Oligo ASM (lanes C). Relative levels of each protein were expressed as percentage of control (lipofectin alone), normalized by corresponding β-actin level.

inhibited, resulting in significantly elevated p53 and p21 levels (panel II, lanes B). The mismatched control Oligo ASM showed minimal effect on the protein levels of MDM2, p53, or p21 (panel II, lanes C). The effects of Oligo AS on 5-FU–induced p53, p21, and MDM2 levels were also evaluated (Fig. 2, panel III). 5-FU induced p53, p21, and MDM2 in a dose-dependent manner (panel III, lanes A). Following the treatment with Oligo AS, MDM2 expression was inhibited, resulting in significantly elevated p53 and p21 levels (panel III, lanes B). The mismatched control Oligo ASM showed minimal effect on the protein levels of MDM2, p53, or p21 (panel III, lanes C).

In Vivo Biological Activity

Antitumor Activity Based on previous studies with cell lines that contain wild-type p53 (39), the effect of Oligo AS on in vivo tumor growth was first evaluated in LS174T xenograft model at a daily intraperitoneal dose of 20 mg/kg. Oligo AS showed significant inhibitory effect on tumor growth (Fig. 3 and Table 1). The mismatched control Oligo ASM showed minimal effect (Fig. 3A and Table 1). Following HCPT treatment (3 mg/kg/day for the first week and 3 mg/kg every other day for remaining treatment period), tumor growth was inhibited by approximately 50% (Fig. 3A and Table 1). Following the combination treatment of Oligo AS and HCPT, significant synergistic effects were observed (Fig. 3A and Table 1). At the end of the experiment, the mean tumor size of the animals treated with HCPT and Oligo AS was 12% of that of the control animals treated with saline, whereas the mean tumor sizes for animals treated with Oligo AS or HCPT alone were 37.3% and 50.8% of that of the controls, respectively (Table 1). Similar significantly synergistic effects were observed following the combination treatment of Oligo AS and 5-FU (Fig. 3B and Table 1). At the end of the experiment, the mean tumor size of the animals treated with 5-FU and Oligo AS was 10.9% of that of the control animals treated with saline, whereas the mean tumor sizes for animals treated with Oligo AS or HCPT alone were 37.3% and 45.3% of that of the controls, respectively (Table 1). The mismatched control Oligo ASM showed no significant effect on HCPT or 5-FU–associated tumor growth inhibition (Fig. 3 and Table 1).

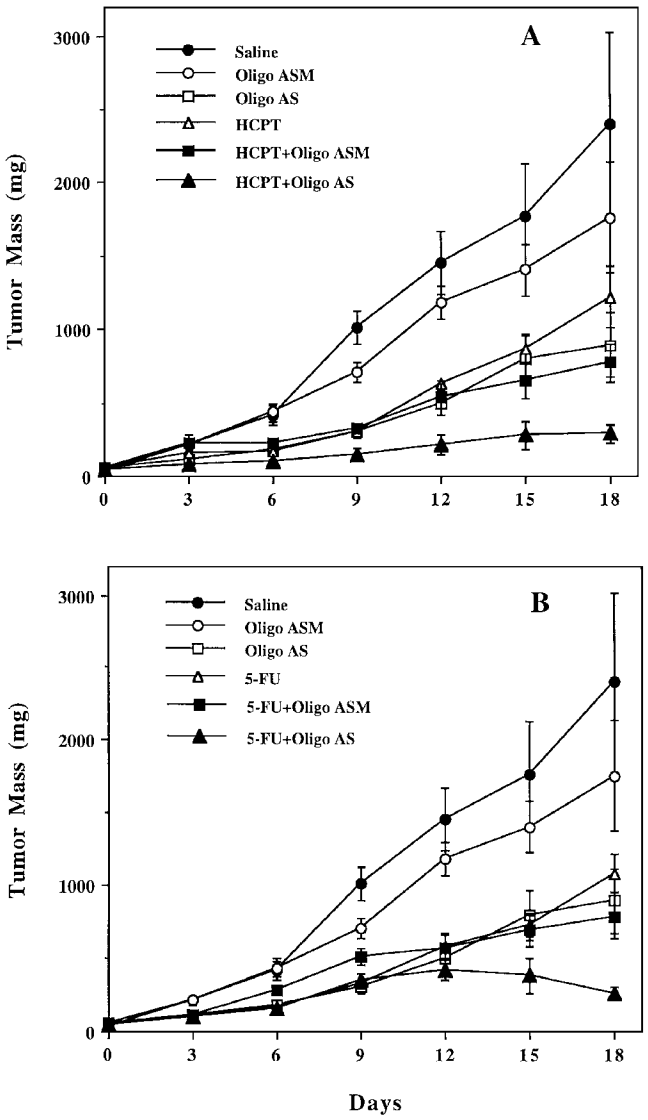

Fig. 3. In vivo synergistic effects between Anti-MDM2 oligonucleotide, Oligo AS, and chemotherapeutic agents HCPT and 5-FU in mice bearing human colon cancer LS174T xenografts. Doses for drugs alone or in combinations are Oligo AS and ASM, 20 mg/kg/day; HCPT, 3 mg/kg/day for the first week and 3 mg/kg every other day for remaining treatment period; and 5-FU, 10 mg/kg/day. The therapeutic effects for each treatment are listed in Table 1. The host toxicity (survival rate) is listed in Table 2. Synergistic or additive effects on tumor growth were noted in combination therapy.

Xenograft Pathology Tumors from control mice and mice given Oligo ASM were indistinguishable histologically. Cellularity of the tumors was typical of anaplastic colon cancer as evidenced by disorderly gland formation, oval to round open-faced nuclei, some degree of polarity of cells with apical clear zones and basilar nuclei, modest to abundant mucous production, and numerous mitotic figures. Tumor tissues from mice treated with Oligo AS contained patches of collagenous connective tissues replacing tumor and necropurulent, ulcerative changes. No significant histologic changes were found in tumors of mice treated with 5-FU alone. Tissues from mice treated with combination of 5-FU and Oligo AS were small nodules of scar tissue containing scattered islands of tumor cells. Tumor tissues from mice treated with HCPT alone or in combination with Oligo ASM contained large areas of necropurulent degeneration with ulceration of adjacent skin. Tumor tissues from mice treated with HCPT in combination with Oligo AS were characterized by marked tumor atrophy.

In Vivo Inhibition of MDM2 Expression and Activation of p53 Western blot analyses of pooled LS174T xenograft tissues indicated that Oligo AS specifically inhibited MDM2 expression and activated p53 in vivo (Fig. 4A). The control Oligo ASM had no effect on the protein levels, further demonstrating the specificity of Oligo AS.

Host Toxicity One of the concerns of combination therapy is the potential risk of increased host toxicity. In the present study, no significant increase in host toxicity, in terms of body weight gain/loss or survival rates, was observed with combination treatment (Table 2).

Table 1. Therapeutic effectiveness of Anti-MDM2 oligonucleotide administered alone or in combination with cytotoxic agents in animals bearing LS174T xenografts

Day		No Oligo [1] (% T:C)	+ASM [2] (% T:C)	+ASM Ratio (%) ([2]/[1])	+Anti-MDM2 AS [3] (% T:C)	+Anti-MDM2 AS Ratio (%) ([3])/[1])
3	Oligo alone	100	99	99	51	51
	HCPT	74	103	138	37	49
	5-FU	50	51	102	47	94
6	Oligo alone	100	105	105	44	44
	HCPT	41	55	134	24	58
	5-FU	40	67	167	38	96
9	Oligo alone	100	70	70	30	30
	HCPT	30	32	107	15	50
	5-FU	33	51	156	35	108
12	Oligo alone	100	82	82	34	34
	HCPT	44	37	85	15	34
	5-FU	40	39	99	29	73
15	Oligo alone	100	79	79	45	45
	HCPT	49	37	75	16	32
	5-FU	41	39	95	22	53
18	Oligo alone	100	73	73	37	37
	HCPT	51	32	63	12	24
	5-FU	45	33	72	11	24

The ratio ([2])/[1] or [3]/[1]) can be used to illustrate the potential additive or synergistic effects when the oligos were given in combination with cytotoxic agents. When the ratio for combination therapy is less thatn 100% (compared to cytotoxic agents alone), an effect of antisense oligo is indicated. If the ratio for combination therapy is the same as that of oligo treatment alone, an additive effect is indicated. If the ratio for combination therapy is significantly less than that of oligo treatment alone, a synergistic effect is indicated. For example, at the end of the experiment (day 18), the ratio for 5-FU + AS/5-FU is 24% (10.9%/45%) and less than 100%, indicating an effect of oligo AS, and, in addition, this ratio is less than the ratio for oligo AS alone (37%; AS/Saline), indicating a synergistic effect between 5-FU and Oligo AS. The ratio for 5-FU + ASM/5-FU is 72% (33%/45%) and less than 100%, indicating an effect of oligo ASM; however, this ratio is almost the same as the ratio for oligo ASM alone (73%), indicating no synergistic effect, but an additive effect, between 5-FU and Oligo ASM. In conclusion, additive or synergistic effects between HCPT and Oligo AS were found throughout the treatment period. Additive or synergistic effects between 5-FU and Oligo AS were found only on days 15 and 18. No additive or synergistic effects between Oligo ASM and HCPT or 5-FU were found, except for HCPT + ASM on days 15 and 18 and 5-FU + ASM on day 18. An additive effect between HCPT and ASM was found on these days.

Dose-Dependent Effects of Oligo AS on Chemotherapeutic Effects of HCPT To confirm the above results, in vivo synergistic effects between HCPT and Oligo AS were further determined in LS174T model, in a separate study using three different doses of Oligo AS (1, 10, 20 mg/kg/day, 5 days/week for 2 weeks). As illustrated in Figure 5A, at the 1 mg/kg level, Oligo AS showed no effect on HCPT therapeutic efficacy. At the 10 and 20 mg/kg levels, Oligo AS significantly increased HCPT efficacy.

Antitumor Activity of Antisense Anti-MDM2 Oligo AS in Human Colon Cancer DLD-1 Model That Contains Mutant p53

In Vitro Biological Activity In vitro inhibition of MDM2 expression by Oligo AS occurred in a sequence-specific, dose-dependent manner (Fig. 1B). The best inhibitory effect (>95%) on MDM2 expression in DLD-1 cells was observed with 200 nM Oligo AS in the presence of lipofectin, whereas the inhibitory effects were approximately 90% at higher concentrations (500 and 1000 nM). No significant changes in the protein levels of the mutant p53 were observed after Oligo AS treatment. The p21 levels were elevated at the concentration of 100 nM or higher, which is independent of p53. Control oligonucleotide, Oligo ASM, had no effect on MDM2, p53, or p21 protein levels following treatment of the cells with various concentrations of ASM up to 1000 nM. Oligo AS inhibited the growth of tumor cell lines in vitro in a dose-dependent manner, with an IC_{50} value of 140 nM for a 72-hr treatment. The mismatched oligonucleotide, Oligo ASM, had no significant effect on tumor cell growth.

The protein levels of MDM2, p53, and p21 were determined in DLD-1 cells following in vitro combination treatment with Oligos (200 nM) and

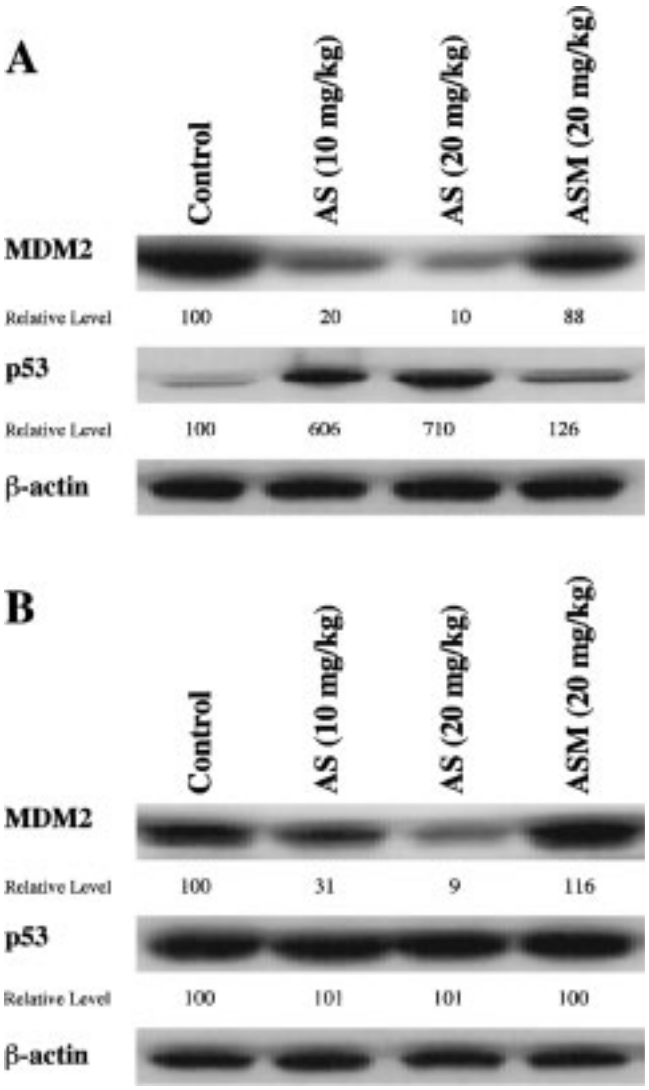

Fig. 4. In vivo inhibition of MDM2 expression by Oligos. Oligo AS inhibited the MDM2 expression in LS174T xenograft **(A)** and DLD-1 xenograft **(B)**. Identical amounts of total protein from the tumor homogenates (100 μg) were analyzed by Western blot using a monoclonal anti-MDM2 or anti-p53 antibody. The mismatch oligonucleotide, Oligo ASM, showed no effect. The p53 levels were elevated in LS174T model and no change in p53 levels was observed in DLD-1 model. Relative levels of each protein were expressed as percentage of control (saline), normalized by corresponding β-actin level.

the chemotherapeutic agents, HCPT, adriamycin, and 5-FU (Fig. 6). Cells were incubated with 200 nM Oligo in the presence of lipofectin for 24 hr, followed by addition of various concentrations of chemotherapeutic agents and incubation for additional 24 hr. As illustrated in Figure 6 (panel I, lanes A), HCPT slightly induced MDM2 but increased p21 levels in a dose-dependent manner as we reported in an earlier study with human breast cancer cell line MDA-MB-468, which contains mutant p53 (45). Following the treatment with Oligo AS, MDM2 expression was inhibited, resulting in significantly elevated p21 levels (panel I, lanes B). The mismatched control Oligo ASM showed minimal effects on the protein levels of MDM2 and p21 (panel I, lanes C). No changes in p53 levels were observed in cells untreated or treated with HCPT in presence of lipofectin, Oligo AS or ASM, indicating that the changes in MDM2 and p21 levels were independent of p53.

Also shown in Figure 6, adriamycin slightly induced p21 in DLD-1 cells (panel II, lanes A). Following the treatment with Oligo AS, MDM2 expression was inhibited, resulting in significantly elevated p21 levels (panel II, lanes B). The mismatched control Oligo ASM showed minimal effects on the protein levels of MDM2 and p21 (panel II, lanes C). No changes in p53 levels were observed in cells untreated or treated with adriamycin, Oligo AS or ASM, further indicating that the changes in MDM2 and p21 levels were independent of p53. 5-FU–induced MDM2 (panel III, lanes A). Following the combination treatment with Oligo AS, MDM2 expression was inhibited, but no changes in p21 levels were observed (panel III, lanes B). The mismatched control Oligo ASM showed minimal effects on the protein levels of MDM2, p53 or p21 (panel III, lanes C).

Table 2. Survival rates of animals bearing LS174T xenografts and treated with anti-MDM2 oligonucleotide administered alone or in combination with cytotoxic agents

Day AS		No Oligo (%)	+ASM (%)	+Anti-MDM2 (%)
3	Oligo alone	100	100	100
	HCPT	100	100	100
	5-FU	100	100	100
6	Oligo alone	100	100	100
	HCPT	100	100	100
	5-FU	100	100	100
9	Oligo alone	100	100	100
	HCPT	67	83	83
	5-FU	100	100	83
12	Oligo alone	100	100	100
	HCPT	50	83	83
	5-FU	100	100	83
15	Oligo alone	100	100	100
	HCPT	50	67	83
	5-FU	100	100	83
18	Oligo alone	100	100	100
	HCPT	50	67	67
	5-FU	100	100	83

In Vivo Biological Activity The effect of Oligo AS on in vivo tumor growth was evaluated in the DLD-1 xenograft model at various daily intraperitoneal doses (1, 10, and 20 mg/kg); a dose-dependent

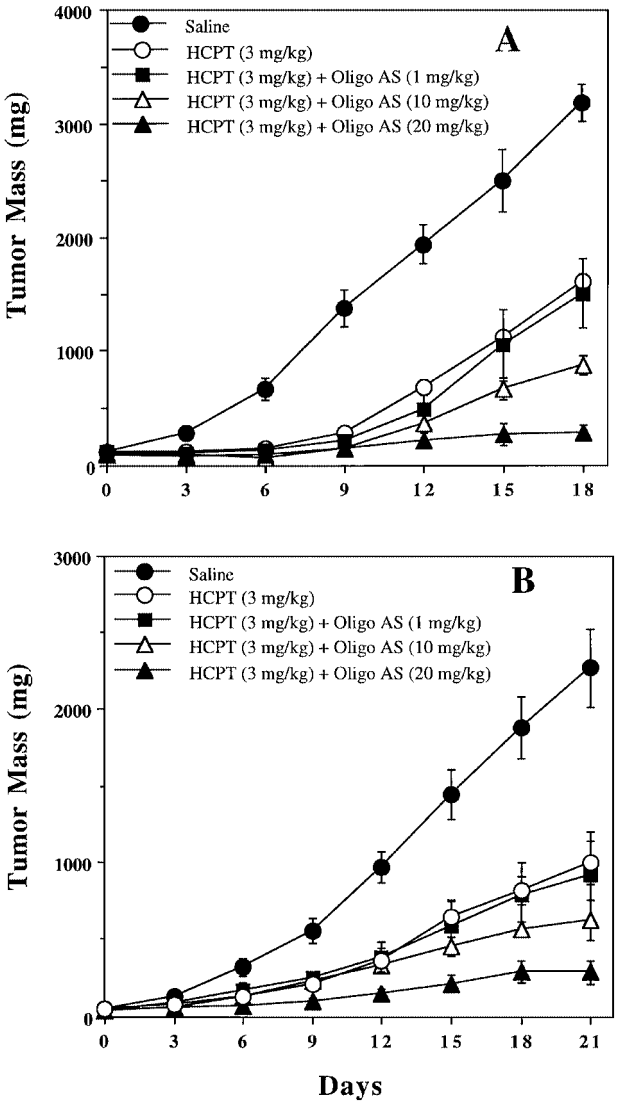

Fig. 5. Dose-dependent effects of Oligo AS on HCPT efficacy in LS174T (A) and DLD-1 (B) models. Doses for drugs alone or in combinations are Oligo AS, 1, 10, and 20 mg/kg/day 5 days/week for 2 weeks; and HCPT, 3 mg/kg/day for the first week and 3 mg/kg every other day for remaining treatment period.

response was observed (Fig. 7A). At 1 mg/kg/day, Oligo AS showed minimal effects on tumor growth. The mismatched control Oligo ASM showed minimal effects at 20 mg/kg/day (Fig. 7B, Table 3). Following treatment with HCPT (3 mg/kg/day for the first week and 3 mg/kg every other day for remaining treatment period), tumor growth was inhibited by approximately 51% at the end of the experiment (day 21) (Fig. 7B and Table 3). Following combined treatment with Oligo AS and HCPT, significant additive or synergistic effects were observed (Fig. 7B and Table 3). At the end of the experiment, the mean tumor size of the animals treated with HCPT and Oligo AS was 20% of that of the control animals treated with saline, whereas the mean tumor sizes for animals treated with Oligo AS or HCPT alone were 36.8% and 51.0% of that of the controls, respectively (Table 3). Similar significantly additive or synergistic effects were observed following combined treatment with Oligo AS and 5-FU (Fig. 7C and Table 3). At the end of the experiment, the mean tumor size of the animals treated with 5-FU and Oligo AS was 23.1% of that of the control animals treated with saline, whereas the mean tumor sizes for animals treated with Oligo AS or HCPT alone were 36.8% and 44% of that of the controls, respectively (Table 3). The mismatched control Oligo ASM showed no substantial effects on HCPT– or 5-FU–associated tumor growth inhibition (Fig. 7B and 7C, Table 3), further confirming the specificity of Oligo AS as an antisense agent.

Xenograft Pathology Tumors from control mice (saline or Oligo ASM) were composed of very tightly packed cells with round to oval, open-faced nuclei and modest amounts of cytoplasm, forming sheets, and cords of cells separated by very thin zones of fibrous connective tissue. Although the tumor masses from mice treated with chemotherapeutic agents HCPT and 5-FU alone or in combination with Oligo AS were significantly smaller than control tumors, they were histologically similar to the control tumors from mice. Tumors from mice treated with Oligo AS were histologically similar to control tumors except for modest increases in connective tissue with necrotic changes in tumor tissues.

Host Toxicity No significant increase in host toxicity, in terms of body weight gain/loss and survival rates, was observed with combination treatment of Oligo AS and HCPT or 5-FU (Table 4). Of note, in the HCPT and Oligo ASM combination, survival rates were lower than the controls, which, however, is not appreciably different from that of the control groups in the study of LS174T model (Table 2), suggesting that the host toxicity observed in the groups treated with the combination protocol (HCPT plus Oligo ASM or AS) be associated with HCPT (Tables 2 and 4).

In vivo Inhibition of MDM2 Expression Western blot analyses of pooled DLD-1 xenograft tissues indicated that Oligo AS specifically inhibited MDM2 expression in a dose-dependent manner in vivo (Fig. 4 B). The control Oligo ASM had no effect on the protein levels, further demonstrating the specificity of Oligo AS. No changes in p53 levels were observed, indicating that in vivo antitumor activity of Oligo AS is independent of p53.

Dose-dependent Effects of Oligo AS on Chemotherapeutic Effects of HCPT in DLD-1 Model To confirm these results, in vivo synergistic effects between HCPT and Oligo AS were further determined in DLD-1 model, in a separate study using three different doses of

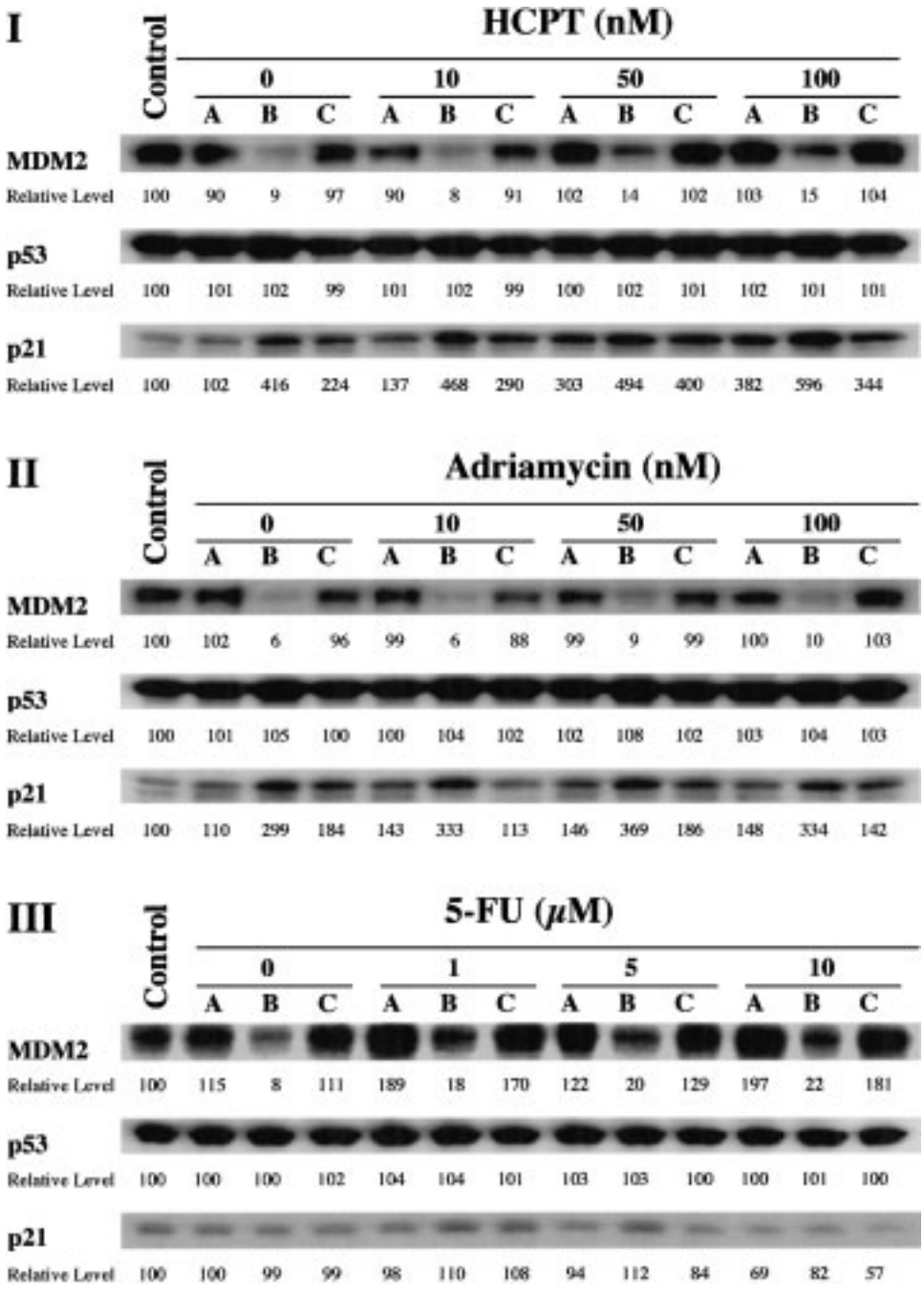

Fig. 6. Effects of combination treatment of Anti-MDM2 Oligo AS and the cancer chemotherapeutic agents on MDM2, p53, and p21 protein levels in DLD-1 cells in culture. Cells were incubated with 200 nM Oligo AS or ASM in the presence of lipofectin for 24 hr, followed by addition of various concentrations of HCPT (panel I), adriamycin (panel II), or 5-FU (panel III) and incubation for additional 24 hr. At various concentrations, the effects on MDM2, p53, and p21 levels were evaluated following treatment with cytotoxic agents alone (lanes A) or pre-treatment with Oligo AS (lanes B) or Oligo ASM (lanes C). Relative levels of each protein were expressed as percentage of control (no treatment), normalized by corresponding β-actin level.

Oligo AS (1, 10, 20 mg/kg/day, 5 days/week for 2 weeks). As illustrated in Figure 5B, at the 1 mg/kg level, Oligo AS showed no effect on HCPT therapeutic efficacy. At the 10 and 20 mg/kg levels, Oligo AS significantly increased HCPT efficacy in a dose-dependent manner.

Discussion

The MDM2 oncogene has been suggested as a novel target for cancer therapy, especially the p53–MDM2 interaction (6–8,32–39,46,47). The rationale for this is based on the following observations: a) MDM2 amplification and overexpression occur in many types of

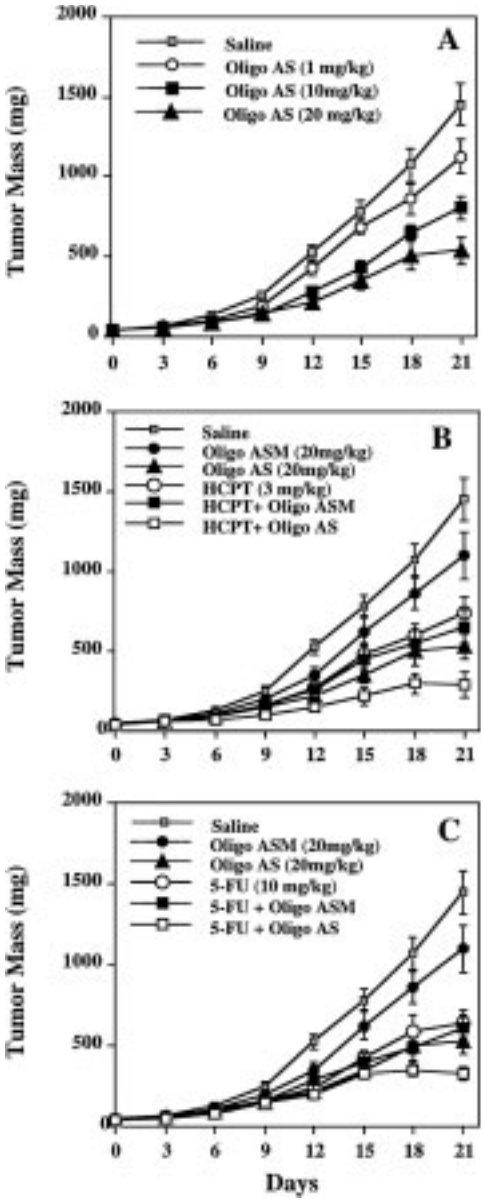

Fig. 7. In vivo antitumor activities of Oligo AS administered alone or in combination with HCPT and 5-Fu in mice bearing human colon cancer DLD-1 xenografts. Doses for Oligo AS alone were 1, 10, and 20 mg/kg/day (panel A). Doses in combination therapy were the same as that with LS174T model. In a dose-dependent manner, Oligo AS inhibited DLD-1 tumor growth (panel A). Additive or synergistic therapeutic effects were observed in combination therapy with Oligo AS and HCPT (panel B) or 5-FU (panel C). The therapeutic effectiveness for each treatment is summarized in Table 3. The host toxicity (survival rate) is summarized in Table 4.

human cancers and the MDM2 levels correlate with poor prognosis in some cancers; b) p53, which is negatively regulated by MDM2, plays a major role in tumor growth; c) p53-mediated growth arrest and/or apoptosis have been suggested to be major mechanisms for currently used cancer therapy such as DNA-damaging chemotherapeutics and radiation therapy; d) loss of p53 function and/or overexpression of MDM2 is believed to correlate with tumor resistance to conventional therapy; and e) MDM2 has displayed both p53-dependent and p53-independent activities in connection with its tumorigenic properties. In the past few years, several strategies have been used to test the hypothesis that, by disrupting p53–MDM2 interaction, the negative regulation of p53 by MDM2 is diminished and the cellular functional p53 level is increased, particularly following DNA-damaging treatment, resulting in tumor growth arrest and/or apoptosis that leads to better therapeutic responses. These approaches include the use of polypeptides (33), antibodies (34,35), and antisense oligonucleotides (37–39).

Recently, we identified an anti-MDM2 antisense PS-oligo that effectively inhibits MDM2 expression in tumor cells containing MDM2 gene amplifications (37). Anti–human-MDM2 antisense PS-oligos were initially screened in two cell lines, JAR (choriocarcinoma) and SJSA (osteosarcoma), which contain wild-type p53, amplified MDM2 genes, and overexpression of MDM2 oncoprotein. Of nine PS-oligos screened, Oligo AS5 (5′-GATCACTCCCAC-CTTCAAGG-3′), which can hybridize to a position ~360 bp downstream of the translation start codon, reproducibly decreased MDM2 protein levels in both cell lines by 3- to 5-fold at concentrations of 100–400 nM in the presence of lipofectin (37). Oligo AS5 also induced RNase H cleavage of the target MDM2 mRNA, resulting in truncation and degradation of the target (37). Following AS5 treatment, the p53 protein level was elevated and its activity was increased as evidenced by induction of p21 expression. JAR cells treated with AS5 showed a substantial increase in the levels of apoptosis (37). AS5 did not cause visible apoptosis in the H1299 cells that lack p53 (37). These results suggest that apoptosis induced by AS5 is due to activation of p53 following MDM2 inhibition by the oligo.

After publishing the above encouraging observations with Oligo AS5 (37), we continued the screening project with the primary goals being to a) obtain new oligos with better in vivo stability, b) determine the effects of anti-MDM2 oligos on human tumor cells with varying status of p53 and/or MDM2 expression, and c) identify candidate cell lines that can be used in future in vivo studies. In tested cell lines, PS-oligo AS5-2 (5′-TGACACCT-GTTCTCACTCAC-3′) had the highest activity and were used in further studies. Among 26 cell lines (16 types of human cancers) tested, Oligo AS5-2 significantly activated p53 in all cells with low levels of wild-type p53, even in those with very low levels of MDM2 (38). AS5-2 has no effect on p53 levels in cells with null p53, H1299, and SK-N-MC, or those with mutant p53 (38). Based on the above screening, a modified analog of AS5-2 with advanced antisense chemistry, Oligo AS, was designed and evaluated in subsequent studies. In cell lines that contain wild-type p53 and amplified

Table 3. Therapeutic effectiveness of anti-MDM2 oligonucleotide administered alone or in combination with cytotoxic agents in mice bearing human colon cancer DLD-1 xenografts

Day		No Oligo [1] (% T:C)	+ASM [2] (% T:C)	+ASM Ratio (%) ([2]/[1])	+Anti-MDM2 AS [3] (% T:C)	+Anti-MDM2 AS Ratio (%) ([3]/[1])
3	Oligo alone	100	82	82	77	77
	HCPT	88	93	106	80	91
	5-FU	79	85	108	74	93
6	Oligo alone	100	88	88	72	72
	HCPT	74	69	93	56	76
	5-FU	76	86	113	65	85
9	Oligo alone	100	79	70	59	59
	HCPT	62	62	99	41	65
	5-FU	61	65	106	58	95
12	Oligo alone	100	66	66	41	41
	HCPT	51	49	96	28	54
	5-FU	46	54	118	38	83
15	Oligo alone	100	79	79	44	44
	HCPT	61	58	95	28	45
	5-FU	54	51	93	42	78
18	Oligo alone	100	81	81	47	47
	HCPT	56	52	92	27	49
	5-FU	56	46	83	33	60
21	Oligo alone	100	76	76	37	37
	HCPT	51	45	88	20	39
	5-FU	44	43	97	23	53

MDM2 gene, SJSA and JAR, Oligo AS specifically inhibited MDM2 expression and p53 levels were elevated accordingly (39). This oligo has been shown to be active in in vitro and in vivo breast cancer models (48).

The purpose of the present study was to investigate further the role of MDM2 in human colon cancer using in vitro and in vivo models that contain wild-type p53 (LS174T) or mutant p53 (DLD-1) but with MDM2 expression. We demonstrated at least five significant results. First, the novel anti-MDM2 mixed-backbone oligonucleotide Oligo AS specifically inhibited MDM2 expression in both LS174T and DLD-1 cells, with dose-dependent inhibitory effects on cell growth, regardless of p53 status. Second, in a dose-dependent manner, the in vivo antitumor effects of Oligo AS were observed in LS174T and DLD-1 tumor models in nude mice, following sequence-specific, dose-dependent inhibition of MDM2 expression in vivo. Third, after combination therapy with Oligo AS and conventional cancer chemotherapeutic agents HCPT and 5-FU, in vivo synergistic or additive therapeutic effects were found in both LS174T and DLD-1 models. Fourth, in LS174T cells, combination treatment with Oligo AS and cancer chemotherapeutic agents HCPT, adriamycin and 5-FU significantly elevated chemotherapeutic agent-induced p53 and p21 levels, resulting from inhibition of chemotherapeutic agent-induced MDM2 expression, indicating that the in vivo synergistic effects between Oligo AS and conventional chemotherapeutic agents are associated with a p53-dependent pathway in cancers containing wild-type p53 expression. Finally, in DLD-1 cells, combination treatment with Oligo AS and the cancer chemotherapeutic agents HCPT, adriamycin, and 5-FU had no effect on the mutant p53 levels. Oligo AS specifically inhibited the chemotherapeutic agent-induced MDM2 expression and increased p21 levels, indicating that the in vivo synergistic or additive effects between Oligo AS and the chemotherapeutic agents are independent of p53 but associated with MDM2 and possibly with p21.

In general, human cancer cell lines or tumor tissues with MDM2 gene amplifications or overexpression often have wild-type p53 (4–9), presumably inactivated by MDM2. Several studies have now shown that overexpression of MDM2 is associated with poor prognosis in human malignancies, including osteosarcoma (14), soft tissue sarcoma (15–17,49), breast cancer (18), ovarian cancer (19), cervical cancer (20), oral squamous cell carcinoma (21,22), brain tumor (23,50), esophageal cancer (24), colorectal carcinoma (25), bladder cancer (26–28, 51), urothelial carcinoma (29), leukemia (30), and large B-cell lymphoma (31). These studies suggest

Table 4. Survival rates of animals bearing DLD-1 xenografts and treated with anti-MDM2 oligonucleotide administered alone or in combination with cytotoxic agents

Day		No Oligo	+ ASM	+ Anti-MDM2
AS		(%)	(%)	(%)
3	Oligo alone	100	100	100
	HCPT	100	100	100
	5-FU	100	100	100
6	Oligo alone	100	100	100
	HCPT	100	100	83
	5-FU	100	100	100
9	Oligo alone	100	100	100
	HCPT	100	67	83
	5-FU	100	100	100
12	Oligo alone	100	100	100
	HCPT	100	50	83
	5-FU	100	100	100
15	Oligo alone	100	100	100
	HCPT	100	50	83
	5-FU	100	100	100
18	Oligo alone	100	100	100
	HCPT	100	50	83
	5-FU	100	100	100
21	Oligo alone	100	67	100
	HCPT	100	50	83
	5-FU	100	100	100

that overexpression of MDM2 may be associated with inactivation of wild-type p53, and inhibiting MDM2 expression in these tumors may lead to re-activation of p53 and induction of cell growth arrest or apoptosis. Many cancer therapeutic agents exert their cytotoxic effects through activation of wild-type p53, and the restoration of wild-type p53 can increase the sensitivity of tumors to DNA-damaging agents (52). Restoration of wild-type p53 may also overcome the drug resistance of human cancers associated with p53 dysfunction (53). Activation of p53 by DNA damage such as cancer chemotherapy and radiation treatment may, however, be limited in cancers with MDM2 expression, especially those with MDM2 overexpression. Therefore, inactivation of the MDM2 negative feed-back loop may increase the magnitude of p53 activation following DNA damage, thus enhancing the therapeutic effectiveness of DNA-damaging drugs. In the present study, we provide experimental evidence supporting this hypothesis. In LS174T cells that contain wild-type p53, the cancer chemotherapeutic agents HCPT, adriamycin, and 5-FU induced p53 levels, but this was limited owing to MDM2 overexpression. Following treatment with antisense anti-MDM2 Oligo AS, MDM2 expression was specifically inhibited, resulting in significantly increases in cytotoxic agent-induced p53 and p21 levels. These findings are consistent with the in vivo synergistic effects following combination treatment of Oligo AS and the cytotoxic agents HCPT or 5-FU. These results further confirm our earlier findings with cell lines that contain amplified MDM2 gene and overexpressed MDM2 protein (37–39). Therefore, we conclude that the MDM2–p53 interaction can serve as a novel drug target, even if MDM2 and/or p53 are expressed at basal levels.

In addition to its interaction with p53, MDM2 binds to and interacts with other cellular proteins such as the pRB (54), E2F1 (55), p300 (56), ARF (57–59), p73 (60), Numb (61), and ribosomal protein L5 (62), and RNA (63), and regulates the MyoD transcription factor (64). The biological consequences of these activities are not fully understood, but may be associated with transforming properties of MDM2 that may be p53 independent (6–9). MDM2 gene products include several forms of polypeptide, representing alternatively spliced MDM2 variants (65). Various alternatively spliced MDM2 polypeptides are present in several human tumors (66–68). Of the five forms of MDM2 analogs, only one retains p53-binding capacity. However, cDNAs coding for all five forms of alternatively spliced MDM2 independently transform NIH3T3 cells, indicating that these MDM2 transcripts have the p53-independent transforming ability (66,67). The effects of MDM2 overexpression on mammary tumorigenicity are seen in p53-null mice (69), indicating that MDM2 can cause transformation and tumor formation via a p53-independent mechanism. Furthermore, overexpression of MDM2 is associated with resistance to the antiproliferative effects of transforming growth factor β (TGF-β), which is p53 independent (70).

In the present study, we provide direct evidence supporting the possibility of p53-independent activity of MDM2. In DLD-1 cells that contain mutant p53, Oligo AS and the cancer chemotherapeutic agents HCPT, adriamycin, and 5-FU had no effect on the mutant p53 levels. These cytotoxic agents, however, induced MDM2 levels and p21 levels. The mechanisms are not clear. Following treatment with Oligo AS, MDM2 expression was specifically inhibited, resulting in a significant increase in cytotoxic agent (HCPT and adriamycin)-induced p21 levels. More important, the in vivo antitumor activity of Oligo AS was observed in the DLD-1 model following administration alone or in combination with the cytotoxic agents HCPT or 5-FU, which is independent of p53 status.

Although the mechanisms responsible for increasing p21 levels following MDM2 inhibition were not determined in the present study, the interaction between MDM2 and p21 is indicated. In human tumor cells treated with HCPT, up-regulation of p21 has been suggested to be both p53 dependent and p53 independent (45). Further study should elucidate the potential interaction between MDM2 and p21 and its role in tumor transformation and growth.

One of the advantages of using antisense oligonucleotides or MDM2-specific antibodies is that these agents may exert their effects in all MDM2-expressing tumors regardless of p53 status. This is important because the p53-independent activity of MDM2 may play a role in MDM2 tumorigenicity. Inhibition of MDM2 expression will ultimately prevent the interaction of MDM2 and other cellular proteins.

One potential drawback is that these agents may have similar effects on normal host tissues, resulting in activation of endogenous p53 (71). The tolerance of increased p53 levels in normal tissues will be the key for the success of approaches aimed at eliminating MDM2 from cells. The activation of p53 in normal tissues following DNA-damaging treatment and the resultant cell growth arrest and apoptosis are believed to be associated with side toxicities of conventional therapy. A recent study demonstrated that inhibition of p53 function can in fact prevent host toxicity associated with DNA-damaging treatment (72). In the present study, no significant changes were observed in host toxicity following combination therapy with Oligo AS and cytotoxic agents in either tumor models. However, this does not rule out the possibility of increased host toxicity, especially through the p53-dependent pathway; the antisense oligonucleotide used is human-MDM2–specific and, therefore, may have little effect on mouse normal tissues. Further study examining the potential host toxicity following treatment of anti–mouse-MDM2 oligonucleotides is underway.

In conclusion, we demonstrated that the selected specific anti–human-MDM2 mixed-backbone oligonucleotide has significant anti-tumor activity in vitro and in vivo, regardless of p53 status. These results suggest that MDM2 have a role in tumor growth through both p53-dependent and p53-independent mechanisms. We speculate that MDM2 inhibitors such as antisense anti-MDM2 oligonucleotides have a broad spectrum of antitumor activities in human cancers regardless of p53 status. Therefore, this study should provide a basis for future development of anti-MDM2 antisense oligonucleotides as cancer therapeutic agents used alone or in combination with conventional chemotherapeutics (73).

Acknowledgments

This study was supported by National Institute of Health grant R01 CA 80698 (to R.Z.). We thank Dr. J. Chen for providing anti-MDM2 antibodies, L. P. Le, J. Sutton, M. Shackelford, and J. Hosmer for their excellent technical assistance.

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