Antisense Anti-MDM2 Oligonucleotides As a Novel Therapeutic Approach to Human Breast Cancer: In Vitro and in Vivo Activities and Mechanisms

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ABSTRACT

The mouse double minute 2 (MDM2) oncogene has been suggested as a target for cancer therapy. It is amplified or overexpressed in many human cancers, including breast cancer, and MDM2 levels are associated with poor prognosis of several human cancers, including breast cancer, ovarian cancer, osteosarcoma, and lymphoma. In the present study, we investigated the functions of MDM2 oncogene in the growth of breast cancer and the potential value of MDM2 as a drug target for cancer therapy by inhibiting MDM2 expression with a specific antisense antihuman-MDM2 oligonucleotide (oligo). The selected antisense mixed-backbone oligo was evaluated for its in vitro and in vivo antitumor activity in human breast cancer models: MCF-7 cell line containing wild-type p53 and MDA-MB-468 cell line containing mutant p53. In MCF-7 cells, p53 and p21 levels were elevated, resulting from specific inhibition of MDM2 expression by the antisense oligo (AS). In MDA-MB-468 cells, after inhibition of MDM2 expression, p21 levels were elevated, although p53 levels remained unchanged. After i.p. administration of the antisense anti-MDM2 oligo, in vivo antitumor activity occurred in a dose-dependent manner in nude mice bearing MCF-7 or MDA-MB-468 xenografts. In both models, in vivo synergistically or additive therapeutic effects of MDM2 inhibition and the clinically used cancer chemotherapeutic agents irinotecan, 5-fluorouracil, and paclitaxel (Taxol) were observed. 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 ASs, have a broad spectrum of antitumor activities in human breast cancers, regardless of p53 status. This study should provide a basis for future development of anti-MDM2 ASs as cancer therapeutic agents used alone or in combination with conventional chemotherapeutics.

INTRODUCTION

The tumor suppressor gene TP53/P53 product p53 (1) regulates the normal cell cycle by activating the transcription of genes that control progression through the cycle and of other genes that help maintain the genomic integrity of the cells as it coordinates the cellular response to DNA damage by inducing cell cycle arrest or apoptosis (1–6). Alterations of the p53 gene are the most frequent genetic abnormalities in human malignancies (3, 4, 7–17). Mutations in p53 arise with an average frequency of 50%, but the incidence varies from zero in carcinoid lung tumor (13) through 30–86% in breast cancers (14–16) to 97% in primary melanomas (17). Mutations in p53 correlate strongly with a poor prognosis in breast cancer (18), and the level of p53 protein expression may be a predictor of distant metastasis of human breast cancers (19).

p53 also plays an important role in cancer therapy. DNA damaging treatments, such as chemotherapy and radiation therapy, increase p53 levels, leading to cell growth arrest or apoptosis (20–23). p53 induces the expression of many genes, including MDM2 (24), GADD45 (25), and CIP1/WAF1 (26). It has been suggested that modulating p53-mediated cell arrest and/or apoptosis may lead to the sensitization of tumor cells to DNA damaging chemotherapeutic agents and radiation therapy. Human cancer cell lines with mutant p53 tend to exhibit less growth inhibition than wild-type p53 lines after treatment with DNA damaging treatments, such as chemotherapy and radiation therapy, showing an increase in G1 arrest and in expression of p53 reporter genes MDM2, CIP1/WAF1, and GADD45 (27). These studies suggest that p53 may be a target for improving therapeutic effects of cancer therapy and radiation therapy. P53 gene therapy has also been suggested as a new approach to human cancer treatment (28–32). The transfection of human cancer cell lines (including breast carcinoma) that have mutant p53 with a wild-type P53 gene significantly inhibits tumor cell growth (28,
29). The tumorigenicity of breast cancer cell lines with mutations in both \(P53\) and \(RB1\) (retinoblastoma gene) is reduced by the expression of wild-type forms of either \(P53\) or \(RB1\) (30). Extensive preclinical studies of \(p53\) gene therapy have now been carried out (32). However, studies have suggested that the effectiveness of \(p53\)-mediated therapy may be hindered because of the impact of MDM2, a negative regulator of \(p53\).

The MDM2 oncogene was first cloned as an amplified gene on a murine double-minute chromosome in the 3T3DM cell line, a spontaneously transformed derivative of BALB/c 3T3 cells (33). Overexpression of the MDM2 gene in NIH 3T3 cells confers the tumorigenicity (33). Additional evidence supporting MDM2 as an oncogene comes from a study of mice with targeted overexpression of MDM2 in the mammary epithelium during lactation (34). In the mammary glands with the high levels of MDM2, normal mammary development and terminal differentiation were inhibited, with many cells becoming multinucleated and polyploid that are typical phenotypes of cells with inactive \(p53\) and high incidence of mammary gland tumor being observed (34), indicating that overexpression of MDM2 is directly associated with the mammary tumorigenesis. The connection between MDM2 and cancer is also shown in human cancers (reviewed in Ref. 35). The MDM2 gene is amplified in a number of human tumors, including breast cancer (36–41). Studies analyzing both MDM2 amplification and \(p53\) mutations demonstrate that mutations in MDM2 and \(p53\) genes do not generally occur within the same tumor and that 29 of 33 MDM2 amplification-positive tumors had wild-type \(p53\), indicating overexpression of MDM2 is a means of inactivation of \(p53\). Using immunohistochemical techniques, overexpression of MDM2 protein (with or without gene amplification) has now been reported in various human cancers, including breast cancer (36, 37, 41–45).

The expression of MDM2 is induced by \(p53\) (24, 46), and \(mdm2\) oncprotein binds to \(p53\) with high affinity, inhibiting its ability to act as a transcription factor (47), indicating that MDM2 functions as a negative regulator of \(p53\). Studies have shown that MDM2 knockout mouse embryos die shortly after implantation; however, mice carrying both inactivated MDM2 and \(p53\) genes are viable (48, 49), additionally demonstrating the important function of MDM2 that negatively regulates \(p53\).

In cell culture studies, MDM2 overexpression abrogates the ability of \(p53\) to induce cell cycle arrest and apoptosis (50–52). In addition, MDM2 has also been shown to enhance the degradation of \(p53\) (53, 54), suggesting that it can regulate \(p53\) functions through multiple mechanisms. In addition, MDM2 has been shown to bind to the \(pRB\) (55), \(E2F-1\) (56), ribosomal protein L5 (57), and RNA (58), suggesting that MDM2 has \(p53\)-independent activities that may be associated with transforming properties of MDM2.

Many studies suggest that overexpression of MDM2 is associated with inactivation of wild-type \(p53\) (reviewed in Ref. 35). It has also been demonstrated that many cancer therapeutic agents exert their cytotoxic effects through activation of wild-type \(p53\). However, the activation of \(p53\) by DNA damaging treatments, such as cancer chemotherapy and radiation, may be limited in cancers with MDM2 expression, especially those with MDM2 overexpression. Therefore, inactivation of the MDM2–\(p53\) negative feedback loop may increase the magnitude of \(p53\) activation after DNA damaging treatments, thus enhancing their therapeutic effectiveness. It is also possible to overcome some drug resistance in tumors with dysfunctional \(p53\) and overexpression of MDM2.

In our laboratory, we have been interested in developing novel genetically based cancer therapy, with an emphasis on antisense approach. Antisense oligos have been shown to be unique research tools in the study of the regulation of gene expression and gene functions. They are also potential therapeutic agents based on rational gene-based drug design. Antisense oligos may achieve their effects by targeting mRNA with which they can hybridize and specifically block protein expression (59, 60). Recently, we et al. (61–63) have developed anti-MDM2 oligos that showed antitumor activities both in vitro and in vivo. In the present study, we used an anti-MDM2 oligo designed with an advanced chemistry (mixed-backbone oligos) as a research tool to investigate the role of MDM2 in the development and treatment of human breast cancer and, by using in vivo approaches, to systematically evaluate the antisense oligo as a therapeutic agent used alone or in combination with cancer chemotherapeutics. These studies will not only provide the proof of principle for anti-MDM2 oligo itself but also contribute to the evaluation of the usefulness of antisense therapy in general.

**MATERIALS AND METHODS**

**Test Oligos.** The anti-MDM2 AS, Oligo AS, a 20-mer mixed-backbone oligo (5′-UGACACCTTGTTCTACUCAC-3′) and its mismatch control (Oligo ASM, 5′-UGTCAC-CCTTTTTCATUCAC-3′) were synthesized, purified, and analyzed as described previously (62, 64). Two nucleosides at the 5′-end and four nucleosides at the 3′-end were 2′-O-methylribonucleosides (represented by boldface letters); the remaining are deoxyribonucleosides. The italicized nucleosides of Oligo ASM are the sites of the mismatch controls compared with Oligo AS. For both mixed-backbone oligos, all internucleotide linkages are phosphorothioate. The purity of the oligos was shown to be >90% by capillary gel electrophoresis and PAGE, with the remainder being n-1 and n-2 products (62). The integrity of the internucleotide linkages was confirmed by \(^{31}\)P NMR.

**Chemicals and Reagents.** Cell culture media, PBS, 5-FU, and antihuman β-actin (SC-15) monoclonal antibody were obtained from Sigma Chemical Co. (St. Louis, MO). FBS, Lipofectin, trypsin, penicillin-streptomycin, and trypan blue stain were purchased from Life Technologies, Inc. (Grand Island, NY). The antihuman MDM2 (SMP-14) monoclonal antibody was purchased from Santa Cruz Biotechnology, Inc. The monoclonal antibodies against \(p53\) (Ab-6) and p21 (Ab-1) were purchased from Oncogene Research Products (Boston, MA). Irinotecan was obtained from Pharmacia (Kalamazoo, MI). Paclitaxel (Taxol) was obtained from Mead Johnson Oncology Products (Princeton, NJ).

**Cell Culture.** The tumor cell lines MCF-7 and MDA-MB-468 were obtained from the American Type Culture Collection (Rockville, MD) and cultured following their instruction. MCF-7 cells were grown in MEM media containing 10% FBS, 1 mM nonessential amino acids and Earle’s balanced salt solution, 1 mM sodium pyruvate, and 10 mg/liter bovine insulin.
MDA-MB-468 cells were grown in DMEM/Ham’s F-12 medium (DMEM/F-12 1:1 mixture) containing 10% FBS. In vitro studies, cells were incubated with Oligos AS or ASM at various concentrations for 24 h 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. The protein levels of p53 and/or p21 were increased in a dose-dependent manner. Oligo ASM had no effect on the levels of these proteins. Relative levels of each protein were expressed as a percentage of control, normalized by corresponding β-actin level.

Animal Tumor Model. Human cancer xenograft models were established using the methods reported previously (62, 65–67). The protocol for animal use and care was approved by the Institutional Animal Care and Use Committee in the University of Alabama at Birmingham. Female athymic nude mice or C.B-17-scid/scid mice (4–6 weeks old) were purchased from Frederick Cancer Research and Development Center (Frederick, MD) and accommodated for 5 days for environmental adjustment before study. To establish MCF-7 human breast xenografts, each of the nude mice was implanted a 60-day s.c. slow release estrogen pellet (SE-121, 1.7 mg of 17β-estradiol/pellet; Innovative Research of America, Sarasota, FL). Cultured MCF-7 cells were harvested from the monolayer cultures, washed twice with MEM medium, resuspended in MEM, and injected s.c. (10 × 10^6 cells, total volume 0.2 ml) into the left inguinal area of the mice. To establish MDA-MB-468 human breast xenografts, cultured MDA-MB-468 cells were harvested from the monolayer cultures, washed twice with 10% DMEM Ham’s F-12 medium, resuspended in serum-free DMEM Ham’s F-12 medium:Matrigel basement membrane matrix (2:1), and injected s.c. (10 × 10^6 cells, total volume 0.2 ml) into the left inguinal area of the C.B-17-scid/scid mice.

The animals were monitored by general clinical observation, body weight, and tumor growth. Tumor growth was monitored by the measurement, with calipers, of two perpendicular diameters of the implant. Tumor size (weight in grams) was calculated by the formula, \(1/2a^2b\), where “a” is the long diameter (cm) and “b” is the short diameter (cm). The animals were used in the chemotherapy study when the tumor size reached 50–150 mg.

In Vivo Chemotherapy. The animals bearing human cancer xenografts were randomly divided into various treatment groups and a control group (six mice per group). The control (nonoligo treated) group received physiological saline only. The oligos dissolved in physiological saline (0.9% NaCl) were administered by i.p. injection at a dose of 25 mg/kg/day. 5 days/
week for 3 weeks in MCF-7 model and 5 weeks in MDA-MB-468 model. 5-FU was given i.p. at 10 mg/kg/day, 5 days/week for 3 weeks in MCF-7 model and 5 weeks in MDA-MB-468 model. Irinotecan (50 mg/kg/day) was given i.p. on days 0, 7, and 14 in MCF-7 model or on days 0, 14, and 28 in MDA-MB-468 model. Paclitaxel (10 mg/kg/day) was given i.p. on days 1, 4, 8, 11, and 15 in MCF-7 model and on days 1, 4, 14, 18, 29, and 32 in MDA-MB-468 model.

**Western Blot Analysis.** The MDM2, p53, and p21 levels in cultured cells or tumor xenografts were analyzed by using the methods described previously (61–63, 67). 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). The nitrocellulose membrane was then incubated with blocking buffer (PBS containing 0.1% Tween 20 and 5% nonfat milk) for 1 h at room temperature and washed twice with the washing buffer (PBS containing 0.1% Tween 20) for 5 min. The membrane was incubated with primary (anti-MDM2, anti-p53, anti-p21, or anti-β-actin) antibody overnight at 4°C or for 1 h at room temperature with gentle shaking. The membrane was washed with the washing buffer for 15 min, twice for 5 min, and then incubated with a 3000 diluted goat antmouse IgG-horse radish peroxidase conjugated antibody.
RESULTS

In Vitro Inhibition of MDM2 Expression and Activation of p53 and p21 in Human Breast Cancer MCF-7 Cells That Contain Wild-Type p53 by Anti-MDM2 Oligo Administered Alone or in Combination with Cancer Chemotherapeutics.

In vitro inhibition of MDM2 expression by Oligo AS was shown in a sequence-specific, dose-dependent manner, and p53 and p21 levels were elevated accordingly (Fig. 1A). Oligo ASM had little or no effect on MDM2, p53, or p21 protein levels at 500 and 1000 nM, the highest concentrations tested in the study. Oligo AS inhibited the growth of tumor cell lines in vitro in a dose-dependent manner, with a mean IC50 being 67 nM for a 72-h treatment. Oligo ASM had minimal effect on tumor cell growth.

After 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 MCF-7 cells. Cells were incubated with 200 nM Oligo AS or ASM in the presence of Lipofectin for 24 h, followed by an addition of various concentrations of chemotherapeutic agents and an incubation for an additional 24 h. As illustrated in Fig. 2, panel I, Lanes A, HCPT induced p53 and p21 in a dose-dependent manner, as we reported in an early study with MCF-7 cells (67). After the treatment with Oligo AS, MDM2 expression was inhibited, resulting in a significant elevation in p53 and p21 levels by 3–8-fold (panel I, Lanes C). The mismatch control Oligo ASM showed minimal effect on the protein levels of MDM2, p53, or p21 (panel I, Lanes B). Adriamycin slightly induced p53 and p21 in MCF-7 cells (panel II, Lanes A). After the combination treatment with Oligo AS, MDM2 expression was inhibited, resulting in a significant elevation in p53 and p21 levels by 5–9-fold (panel II, Lanes C). The mismatch control Oligo ASM showed minimal effect on the protein levels of MDM2, p53, or p21 (panel II, Lanes B). After the treatment with Oligo AS, MDM2 expression was inhibited, resulting in a significant elevation in p53 and p21 levels by 3–8-fold (panel III, Lanes C). The mismatch control Oligo ASM showed minimal effect on the protein levels of MDM2, p53, or p21 (panel III, Lanes B).

In Vivo Antitumor Activity of Anti-MDM2 Oligo in Human Breast Cancer MCF-7 Xenograft Model That Contains Wild-Type p53. On the basis of previous studies with cell lines that contain wild-type p53 (62), the effect of Oligo AS on in vivo tumor growth was evaluated in the MCF-7 xenograft model at a daily i.p. dose of 25 mg/kg. Oligo AS showed significant inhibitory effects on tumor growth (Fig. 3A; Table 1). After 5-FU treatment (10 mg/kg/day), tumor growth was inhibited by ~50% on day 18 (Fig. 3A). After the combination treatment of Oligo AS and 5-FU, significant additive effects were observed (Fig. 3A; Table 1). Similar significant additive effects were observed after the combination treatment of Oligo AS and paclitaxel (Fig. 3B; Table 1). The mismatch control Oligo ASM showed no effect on tumor growth when administered alone and no effect on 5-FU- or paclitaxel-associ-
Table 1  Therapeutic effectiveness of Anti-MDM2 oligonucleotide administered alone or in combination with chemotherapeutic agents (mean growth ratio: % T:C)\(^a\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No Oligo</th>
<th>+ Oligo ASM</th>
<th>Ratio (%)</th>
<th>+ Oligo AS</th>
<th>Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7 xenograft model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligo alone</td>
<td>100</td>
<td>89</td>
<td>89</td>
<td>55(^b)</td>
<td>55</td>
</tr>
<tr>
<td>5-FU</td>
<td>57(^b)</td>
<td>49(^c)</td>
<td>85</td>
<td>29(^b)</td>
<td>51(^c)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>52(^b)</td>
<td>49(^c)</td>
<td>94</td>
<td>32(^b)</td>
<td>62(^c)</td>
</tr>
<tr>
<td>Irinotecan</td>
<td>41(^b)</td>
<td>16(^c)</td>
<td>37</td>
<td>15(^b)</td>
<td>36</td>
</tr>
<tr>
<td>MDA-MB-468 xenograft model</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oligo alone</td>
<td>100</td>
<td>96</td>
<td>96</td>
<td>60(^b)</td>
<td>60</td>
</tr>
<tr>
<td>5-FU</td>
<td>78(^b)</td>
<td>72(^c)</td>
<td>92</td>
<td>31(^b)</td>
<td>40(^c)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>70(^b)</td>
<td>52(^c)</td>
<td>74(^c)</td>
<td>29(^b)</td>
<td>41(^c)</td>
</tr>
<tr>
<td>Irinotecan</td>
<td>46(^b)</td>
<td>18(^c)</td>
<td>39</td>
<td>4(^b)</td>
<td>9</td>
</tr>
</tbody>
</table>

\(\)\(^a\)  % T:C, percentage of mean tumor mass of treated group compared with the control group treated with saline.
\(\)\(^b\)  \(P < 0.01\) when compared with saline control.
\(\)\(^c\)  \(P < 0.01\) when compared with corresponding chemotherapeutics.

The relative 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 (AS or ASM + chemotherapeutic agent) is <100% (compared with cytotoxic agents alone), an effect of 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 smaller than that of oligo treatment alone, a synergistic effect is demonstrated (e.g., at the end of the experiment [day 18] with MCF-7 model, the ratio for 5-FU + AS/5-FU is 51% (29.57%) and <100%, indicating an effect of oligo AS, and, in addition, this ratio is similar to that for oligo AS alone (55%; AS/saline), indicating an additive effect between 5-FU and Oligo AS. However, the ratio for irinotecan + AS/irinotecan is 36% (15.41%) and <100%, indicating an effect of oligo AS, and this ratio is significantly less than the ratio for oligo ASM alone (72%), indicating no synergistic effect but an additive effect, than that Oligo AS alone (55%), indicating a synergistic effect between irinotecan and Oligo AS. In conclusion, additive or synergistic effects between Oligo AS 5-FU, paclitaxel, or irinotecan and ASM were found throughout the treatment period. Synergistic effects between Oligo ASM and irinotecan were also found.

In Vitro Activity of Antisense Anti-MDM2 Oligo in Human Breast Cancer MDA-MB-468 Model That Contains Mutant p53. In vitro inhibition of MDM2 expression by Oligo AS occurred in a sequence-specific, dose-dependent manner (Fig. 1B). No significant changes in the protein levels of the mutant p53 were observed after Oligo AS treatment (Fig. 1B). The p21 levels were elevated, which is independent of p53 (Fig. 1B). The control Oligo ASM had significantly less effect on MDM2, p53, or p21 protein levels at 500 and 1000 nm concentrations. Oligo AS inhibited the growth of MDA-MB-468 cells in vitro in a dose-dependent manner, with a mean IC\(_{50}\) being 150 nm for a 72-h treatment. Oligo ASM had minimal effect on tumor cell growth.

The protein levels of MDM2, p53, and p21 were determined in MDA-MB-468 cells after in vitro combination treatment with Oligos and the chemotherapeutic agents HCPT, adriamycin, and 5-FU (Fig. 5). Cells were incubated with 200 nm Oligo AS in the presence of Lipofectin for 24 h, followed by an addition of various concentrations of chemotherapeutic agents and incubation for an additional 24 h. As shown in Fig. 5, after the treatment with Oligo AS, MDM2 expression was inhibited (panels I–III, Lanes C). The mismatch control Oligo AS showed minimal effects on MDM2 levels (panels I–III, Lanes B). As shown in Fig. 5 (panels II and III, Lanes C), after the combination treatment with Oligo AS and adriamycin or 5-FU, p21 levels were elevated. The mismatch control Oligo AS showed minimal effects on the p21 levels (Lanes B). No significant changes in p21 levels were observed with HCPT treatment (panel I). No changes in p53 levels in cells untreated or treated with HCPT, adriamycin, or 5-FU were observed, additionally indicating that the changes in MDM2 and p21 levels were independent of p53.

In Vivo Antitumor Activity of Anti-MDM2 Oligo in Human Breast Cancer MDA-MB-468 Model That Contains Mutant p53 after the Treatment of Oligo Alone or in Combination with Cancer Chemotherapeutics. The effect of Oligo AS on in vivo tumor growth was demonstrated in the MDA-MB-468 xenograft model (Fig. 6A; Table 1). After the treatment of 5-FU alone (10 mg/kg/day, 5 days/week), limited tumor growth inhibition was observed (Fig. 6A; Table 1). After the treatment of combination with Oligo AS and 5-FU, however, significant synergistic effects on tumor growth were observed.

\(^a\) Wang, H. et al., unpublished data.
Similar significant synergistic effects were observed after the combination treatment of Oligo AS and paclitaxel (Fig. 6B; Table 1). The mismatch control Oligo ASM showed no effect. The p53 levels were elevated in MCF-7 model, and no change in p53 levels was observed in MDA-MB-468 model. Relative levels of each protein were expressed as a percentage of control (Saline), normalized by corresponding β-actin level.

DISCUSSION

MDM2 oncogene has been suggested as a novel target for cancer therapy, especially the p53-MDM2 interaction pathway. 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 after DNA damaging treatment, resulting in tumor growth arrest and/or apoptosis that leads to better therapeutic responses. These approaches include the use of polypeptides (68), antibodies (69–71), and antisense oligos (61–63). The purpose of the present study was to investigate additionally the role of MDM2 in human breast cancer by using in vitro and in vivo models that contain wild-type p53 (MCF-7) or mutant p53 (MDA-MB-468) but with MDM2 expression. In the present study, we have demonstrated at least five significant results: (a) the novel anti-MDM2 mixed-backbone oligo, Oligo AS, specifically inhibited MDM2 expression in both MCF-7 and MDA-MB-468 cells in a dose-dependent manner, regardless of p53 status; (b) in vivo antitumor effects of Oligo AS were observed in both tumor xenograft models; (c) after combination therapy with Oligo AS and conventional cancer chemotherapeutic agents 5-FU, paclitaxel, and irinotecan, in vivo synergistic or additive therapeutic effects were found in both models, regardless of p53 status; (d) in MCF-7 cells, combination treatment with Oligo AS and cancer chemotherapeutic agents HCPT, adriamycin, and 5-FU significantly diminished tumor growth, and (e) in MDA-MB-468 cells, combination treatment with Oligo AS and cancer chemotherapeutic agents HCPT, adriamycin, and 5-FU significantly inhibited tumor growth.
elevated chemotherapeutic agent-induced p53 and p21 levels, resulting from MDM2 expression, indicating that the in vivo synergistic effects between Oligo AS and conventional chemotherapeutic agents be associated with a p53-dependent pathway in cancers containing wild-type p53 expression; and (e) in MDA-MB-468 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 MDM2 expression and increased p21 levels, indicating that the in vivo synergistic or additive effects between Oligo AS and conventional chemotherapeutic agents are independent of p53 but associated with MDM2 and possibly with p21.

The role of MDM2 in human cancer has been extensively studied. Overexpression of MDM2 is associated with poor prognosis in human malignancies, including breast cancer. Studies suggest that overexpression of MDM2 be associated with inactivation of wild-type p53, and inhibiting MDM2 expression in these tumors may lead to reactivation of p53 and induction of cell growth arrest or apoptosis. The majority of clinically used cancer therapeutic agents exert their cytotoxic effects through activation of wild-type p53, and the restoration of wild-type p53

Fig. 5 Synergistic effects of combination treatment of Oligo AS and the cancer chemotherapeutic agents on MDM2, p53, and p21 protein levels in MDA-MB-468 cells in culture. Cells were incubated with 200 nM Oligo AS or ASM in the presence of Lipofectin for 24 h, followed by an addition of various concentrations of HCPT (I), adriamycin (II), or 5-FU (III) and incubation for an additional 24 h. At various concentrations, the effects on MDM2, p53, and p21 levels were evaluated after treatment with cytotoxic agents alone (Lanes A) or pretreatment with Oligo ASM (Lanes B) or Oligo AS (Lanes C). Relative levels of each protein were expressed as a percentage of control, normalized by corresponding β-actin level.
can increase the sensitivity of tumors to DNA-damaging agents. Restoration of wild-type p53 may also overcome the drug resistance of human cancers associated with dysfunction of p53. Activation of p53 by DNA damage, such as cancer chemotherapy and radiation treatment, may, however, be limited in cancers with MDM2 expression. Therefore, inactivation of the MDM2 negative feedback loop may increase the magnitude of p53 activation after DNA damage, thus enhancing the therapeutic effectiveness of DNA damaging drugs. In the present study, we provided experimental evidence supporting this hypothesis. In MCF-7 cells that contain wild-type p53, the cancer chemotherapeutic agents HCPT, adriamycin, and 5-FU induced p53 levels, which, however, was limited because of MDM2 expression. After treatment with Oligo AS, MDM2 expression was specifically inhibited, resulting in significant increases in cytotoxic agent-induced p53 and p21 levels. These findings are consistent with the in vivo synergistic effects after combination treatment of Oligo AS and cytotoxic agents 5-FU, paclitaxel, and irinotecan. These results additionally confirm our earlier findings with cell lines that contain amplified MDM2 gene and overexpressed MDM2 protein (61–63). 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.

p53-independent activity of MDM2 has also been suggested (72–75). MDM2 gene products include at least five forms of polypeptide, representing alternatively spliced MDM2 variants (76). Various alternatively spliced MDM2 polypeptides are present in several human tumors (77–79). Of the five forms of MDM2 analogues, only one retains p53 binding capacity. However, cDNAs coding for all five forms of alternatively spliced MDM2 independently transform NIH 3T3 cells, indicating that these MDM2 transcripts have the p53-independent transforming ability (78, 79). The effects of MDM2 overexpression on mammary tumorigenicity are seen in p53-null mice (34), 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, which is p53 independent (80).

In the present study, we have provided direct evidence supporting the possibility of p53-independent activity of MDM2. In MDA-MB-468 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. After treatment with Oligo AS, MDM2 expression was specifically inhibited, resulting in a significant increase in cytotoxic agent-induced p21 levels. More important, in vivo antitumor activity of Oligo AS was observed in the MDA-MB-468 model after administration alone or in combination with cytotoxic agents 5-FU, paclitaxel, and irinotecan, which is independent of p53 status. Although the mechanisms responsible for increasing p21 levels after MDM2 inhibition were not determined in the present study, the interaction between MDM2 and p21 is indicated. Our earlier studies indicated that in human tumor cells treated with HCPT, up-regulation of p21 is both p53 dependent and p53 independent (67). Additional 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 oligos 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, and ~50% of human cancers have mutant p53 expression. Inhibition of MDM2 expression will ultimately prevent the interaction of MDM2 and other cellular proteins, e.g., the antisense anti-DM2 oligo we
developed (61–63) increases E2F-1 levels after microinjection, as demonstrated by others (81).

To our surprise, both Oligo AS and ASM showed synergistic effects on tumor growth inhibition when used in combination with irinotecan. Although the exact mechanisms are not determined in the present study, both pharmacokinetic and pharmacodynamic mechanisms may be involved, including uptake and retention of the parent drug (irinotecan) and its active form (SN-38), plasma protein binding of the drug, conversion of irinotecan to SN-38, or interaction of oligo with the drug target (topoisomerase I) and DNA. Nevertheless, the finding that oligos significantly increase the therapeutic effectiveness of irinotecan, a clinically used cancer therapeutic agent, will provide a new means to best use this agent and its analogues. More recently, Oligo AS, but not ASM, has been shown to specifically increase therapeutic effects of two analogues of irinotecan, HCPT (62) and topotecan (82), indicating a unique interaction between oligos and irinotecan.

In conclusion, we have demonstrated that the selected specific antihuman-MDM2 mixed-backbone oligo had significant antitumor activity in both in vitro and in vivo breast cancer models, regardless of p53 status, suggesting that MDM2 has a role in tumor growth through both p53-dependent and p53-independent mechanisms. We speculate that MDM2 inhibitors, such as antisense anti-MDM2 oligos, 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 oligos as cancer therapeutic agents used alone or in combination with conventional chemotherapeutics.

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REFERENCES


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