Sulindac enhances adenoviral vector expressing mda-7/IL-24-mediated apoptosis in human lung cancer

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Abstract
Several studies have shown antitumor activities of the melanoma differentiation–associated gene 7 (mda-7) and the nonsteroidal anti-inflammatory drug sulindac when used as a monotherapies against a wide variety of human cancers. However, the combined effects of mda-7 and sulindac have not previously been tested. Therefore, we tested the antitumor activity of an adenoviral vector expressing mda-7 (Ad-mda7) in combination with sulindac against non–small cell lung cancer cells in vitro and in vivo. When treated with Ad-mda7 in combination with sulindac, human lung cancer cells (A549 and H1299) underwent growth suppression resulting in apoptosis. The growth inhibition induced by Ad-mda7 in combination with sulindac was significantly greater than that observed with Ad-mda7 or sulindac alone. Furthermore, the degree of growth inhibition induced using this combination was dose-dependent for sulindac. Treatment with Ad-mda7 in combination with sulindac had no growth inhibitory effects on human normal lung (CCD-16) fibroblasts. We then investigated the mechanism by which sulindac enhances Ad-mda7-mediated apoptosis. Sulindac increased expression of ectopic MDA-7 protein in tumor cells, thereby increasing the expression of downstream effectors RNA-dependent protein kinase, p38MAPK, caspase-9, and caspase-3 and enhancing apoptosis of non–small cell lung cancer cells. Pulse-chase experiments showed that the increased expression of MDA-7 protein in sulindac-treated cells was due to increased half-life of the MDA-7 protein. Finally, treatment of human lung tumor xenografts in nude mice with Ad-mda7 plus sulindac significantly suppressed growth (P = 0.001) compared with Ad-mda7 or sulindac alone. Our results show for the first time that combined treatment with Ad-mda7 plus sulindac enhances growth inhibition and apoptosis of human lung cancer cells. The increased antitumor activity observed with the combination treatment is a result of increased half-life of MDA-7 protein. Regulation of protein turnover is a heretofore-unrecognized mechanism of this nonsteroidal anti-inflammatory drug. [Mol Cancer Ther 2005;4(2):291–304]

Introduction
The novel gene mda-7, also known as IL-24, belongs to the interleukin (IL)-10 cytokine family (1, 2). We and others have previously shown that mda-7/IL-24, when expressed ectopically, exhibits potent tumor suppressive activity against a variety of human cancer cells with minimal or no toxic effects to normal cells (3–12). Additionally, an adenoviral vector expressing mda-7 (Ad-mda7) administered intratumorally to human lung tumor xenografts has shown antitumor and antiangiogenic activities (13). The mechanism by which mda-7 exerts its cytotoxic effects varies and is cell-type dependent. A number of molecular effectors have been shown to play a role in mda-7-mediated apoptosis including activation of the caspase cascade (5, 6), activation of RNA-dependent protein kinase (PKR; ref. 14), activation of c-Jun-NH2-kinase (JNK; ref. 15), inhibition of phosphoinositide-3 kinase (16), activation of the stress response known as the unfolded protein response (17), activation of p38MAPK (18), and activation of Fas-FasL.5 However, although the upstream initiator effectors differ among the tumor cell types tested, the downstream pathways seem to converge at the level of mitochondrial disruption and activation of the caspase cascade and culminating in apoptosis. In addition to its antitumor activity, we recently showed antimetastatic activity of MDA-7 against human lung tumor cells both in vitro and in vivo (19). The results of these studies strongly indicate that mda-7/IL-24 is a potent tumor suppressor gene. Additional evidence in support of this hypothesis is the

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5 B. Gopalan, S. Sharma, A. Litvak, S. Chada, and R. Ramesh, unpublished data.
finding of Ellerhorst et al. (20) who showed an inverse correlation between MDA-7 expression and melanoma progression. In recent studies, we showed that the MDA-7 protein is secreted and that the secreted protein, sMDA-7, has potent antiangiogenic activity (21). Caudell et al. (22) further showed that SMAD-7 functions as a Th1 cytokine. Collectively, these studies show that the mda-7 gene encodes multiple functions and holds considerable promise for cancer therapy. On the basis of its unique properties, a phase I trial of an adenoviral vector expressing mda-7 (Ad-mda7/INGN-241) for treatment of patients with solid tumors has been completed. In this study, Ad-mda7/INGN-241 was administered intratumorally to 22 patients. Preliminary results indicated that Ad-mda7 was well tolerated with no unusual toxicities and induced high levels of apoptosis in tumors (23, 24). Although Ad-mda7 is being tested as a monotherapy, combination of Ad-mda7 with conventional therapies, such as radiotherapy and chemotherapies, and other novel therapies, such as steroids, antisense, oligonucleotides, and nonsteroidal anti-inflammatory drugs (NSAID), will likely prove even more effective. Treatment of lung cancer cells and gliomas with Ad-mda7 in combination with radiotherapy enhanced growth suppression both in vitro and in vivo (15, 25, 26). In addition, Ad-mda7 combined with antisense oligonucleotides against ras inhibited the growth of pancreatic cancer cells (27). Ad-mda7 combination with Herceptin exhibited enhanced activity against Her-2/neu–positive breast cancer cell in vitro and in vivo (28). Sulindac, a NSAID family member, is well known for its anti-inflammatory activity, which derives from its ability to inhibit the cyclooxygenase (COX) enzymes (29). Recent studies have revealed a link between COX-2 expression and carcinogenesis, suggesting that inhibiting COX-2 can prevent cancer growth or progression (30). In fact, NSAID treatment of patients with adenomatous polyposis coli resulted in regression of colonic adenoma thereby reducing the risk of colon cancer (31–33). Based on the findings of the above studies, the beneficial effects of NSAIDs have also been tested in the prevention of lung, esophageal, prostate, pancreatic, and gastric cancers, in which COX-2 is constitutively expressed (30, 33–37). The results of these studies have clearly shown that the antitumor activity of sulindac or other NSAID occurs by inhibiting COX-2. However, sulindac sulfone, a metabolite of sulindac lacking the inhibitory effects on COX-2, reduced the incidence of tumors in animal models of breast and colon cancer (35, 38). Subsequent studies have shown that sulindac and its metabolites induce apoptosis of various cultured tumor cell lines including a lung cancer cell line through COX-independent pathways (34, 39). The mechanism by which sulindac sulfone exerts its antiproliferative and antineoplastic activity is by inhibition of cyclic guanosine 3′,5′-monophosphate phosphodiesterase, activation of protein kinase G, and phosphorylation of selective substrates, such as β-catenin and JNK (40). From these studies, it is now evident that sulindac and its metabolites exert potent antitumor activity against a broad spectrum of human cancer cells by inhibiting various signaling pathways. Recent in vitro and in vivo studies have shown that anticancer drugs, such as cisplatin, paclitaxel, or docetaxel, in combination with sulindac metabolites, had synergistic inhibitory effects in lung cancer cells (31, 42). Similarly, combined treatment of sulindac plus epidermal growth factor receptor inhibitor or sulindac plus lactacystin enhanced antitumor activity against colorectal cancer cells (43). The mechanism by which sulindac and its metabolites increased antitumor activity of lactacystin was by affecting the proteasome activity. Previous studies using chemotherapies and gene therapy treatment strategies have shown that combination with sulindac and its metabolites enhances the drug’s potency (41–44). Studies have also shown that combining gene therapy with various drugs enhanced transduction efficiency resulting in enhanced tumor cell killing (45). However, the combination of gene therapy with sulindac has not been previously reported. We therefore examined the effect of sulindac in combination with Ad-mda7 against human lung cancer cells. Our results show, for the first time, that treatment of human lung cancer cells with sulindac in combination with Ad-mda7 leads to enhanced additive to synergistic growth inhibition and cell killing. Furthermore, we show that enhanced killing is due increased half-life of MDA-7 protein and not due to increased transduction efficiency. Additionally, the enhanced killing is selective for tumor cells with minimal effect on normal cells. The findings of the present study provide a basis for combining Ad-mda7 with sulindac for lung cancer treatment.

**Materials and Methods**

**Cell Lines and Cell Culture**

Human non–small cell lung cancer cell lines A549 (adenocarcinoma, wild type for p53) and H1299 (large cell carcinoma, null type for p53) were obtained from Drs. A. Gazdar and J.D. Minna (University of Texas Southwestern Medical Center, Dallas, TX). The normal lung fibroblast cell line CCD-16 was obtained from American Type Culture Collection (Rockville, MD). A549 and H1299 cells were maintained in appropriate medium as previously described (5). CCD-16 cells were cultured in α media supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY) and maintained at 37°C in a humidified 5% CO2 plus 95% air atmosphere.

**Agents**

Sulindac, sulindac sulfone, MG132 (a proteasome inhibitor), and cycloheximide (a protein synthesis inhibitor) were obtained from Sigma Chemical Co. (St. Louis, MO). Sulindac was dissolved in 1 mol/L Tris-HCl (pH 8.0), to make 100 mmol/L stock solution. MG132 was dissolved in DMSO to make 10 mmol/L stock solution. These stock solutions were stored frozen at −20°C.

**Recombinant Adenoviral Vector**

Ad-mda7 and Ad-luc vectors were constructed and purified as previously reported (5, 6). The transduction efficiencies for the cell lines were determined with an
adenoviral vector carrying green fluorescent protein (Ad-GFP). Transduction efficiency was >80% when infected with 3,000 viral particles per cell (vp/cell; data not shown). On the basis of these results, cells were treated with 3,000 vp/cell in all subsequent experiments. To determine the effect of sulindac on adenovirus transduction, tumor and normal cells were infected with Ad-GFP at 100 vp/cell and analyzed for GFP expression at 24 hours by fluorescence-activated cell sorting (FACS; Table 1).

**Cell Proliferation Assay**

All three cell lines (A549, H1299, and CCD-16) were seeded in 60-mm-diameter tissue culture dishes at a density of 1 × 10^5 cells/dish in triplicate. The next day, cells were treated with PBS (control), Ad-luc (3,000 vp/cell; control), Ad-mda7 (3,000 vp/cell; control), sulindac, or a combination of PBS plus sulindac, Ad-luc plus sulindac, or Ad-mda7 plus sulindac. The concentrations of sulindac tested were 0.125, 0.25, and 0.5 mmol/L. At 72 hours after the start of treatment, the cells were harvested by trypsinization, washed, and subjected to trypan blue exclusion assay as previously described (5). Cell growth was determined by calculating the mean of the cell counts for each treatment group and expressed as a percentage of the total number of cells treated with PBS, Ad-luc, or Ad-mda7 treatment alone (set to 100%).

**Cell Cycle Distribution and Apoptosis**

Cells (5 × 10^5) were seeded in a 10-cm-diameter tissue culture dish and treated with PBS, Ad-luc (3,000 vp/cell), Ad-mda7 (3,000 vp/cell), sulindac, or a combination of PBS plus sulindac, Ad-luc plus sulindac, or Ad-mda7 plus sulindac. Each treatment group was tested in triplicate. The concentrations of sulindac used were the same as those for the cell proliferation assay. At 72 hours after the start of the treatment, cells were harvested, washed, and analyzed for cell cycle phases and apoptotic fraction as previously described (5). Cell growth was determined by calculating the mean of the cell counts for each treatment group and expressed as a percentage of the total number of cells treated with PBS, Ad-luc, or Ad-mda7 treatment alone (set to 100%).

**Table 1. Transduction efficiency in lung cancer (A549 and H1299) and normal (CCD-16) cells treated with Ad-GFP and sulindac**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Sulindac (mmol/L)</th>
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<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>A549</td>
<td>48.8 ± 6.5</td>
</tr>
<tr>
<td>H1299</td>
<td>79.5 ± 2.2</td>
</tr>
<tr>
<td>CCD-16</td>
<td>15.7 ± 0.2</td>
</tr>
</tbody>
</table>

NOTE: Cells were treated with Ad-GFP (100 vp/cell) for 3 hours, followed by sulindac at the indicated concentrations for 24 hours. Percentages of transduction efficiency were determined by flow cytometry.

*P < 0.05 compared with Ad-GFP treatment alone. No other differences between the groups shown was significant.

**Immunofluorescence Assay**

Cells (1 × 10^6) were seeded in two-well chamber slides (Fisher Scientific, Pittsburgh, PA) and treated with PBS, Ad-mda7 (3,000 vp/cell), PBS plus sulindac (0.5 mmol/L), or Ad-mda7 plus sulindac (0.5 mmol/L). At 48 hours after the start of treatment, the cells were washed with PBS and fixed in PBS-buffered 4% paraformaldehyde for 30 minutes at room temperature. The cells were then permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate for 10 minutes at room temperature, followed by incubation with normal goat serum. At 30 minutes after the start of the incubation, cells were washed with PBS and incubated with rabbit polyclonal anti-human MDA7 antibody (Introgen Therapeutics Inc., Houston, TX) for 1 hour at 37°C. The cells were then washed thrice with PBS and incubated for 1 hour with goat anti-rabbit FITC-tagged secondary antibody (Vector Laboratories, Burlingame, CA), washed three times in PBS, mounted with a coverslip, and observed for MDA-7 protein expression using a Nikon fluorescence microscope (Nikon, Melville, NY). Photomicrographs were obtained at high-power magnification.

**Proteasome Activity Assay**

Proteasome activity assays were done as previously described (43). Briefly, H1299 cells were seeded in six-well plates (2 × 10^5 cells/well) and treated with Ad-mda7, Ad-mda7 plus sulindac, or Ad-mda7 plus MG132 (5 μm). The sulindac concentrations tested were the same as those in the other assays. At 24 hours after the start of treatment, the cells were lysed in proteasome buffer [10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA, 20% glycerol, 5 mmol/L ATP, and 4 mmol/L DTT], sonicated, and then centrifuged at 1,300 × g at 4°C for 10 minutes. The upper supernatant was collected and the protein concentration of cell lysates was determined as previously described (5). To assay the chymotrypsin-like activity of the proteasome, the fluorogenic substrate succinyl–leucyl–leucyl–valine–tyrosine–7-amino-4-methylcoumarin (Chemicon International, Inc., Temecula, CA) was used. Twenty-microgram total protein from each treatment group described above was diluted to 100 μL in reaction buffer [25 mmol/L HEPES (pH 7.5), 0.5 mmol/L EDTA, 0.05% NP40, and 0.001% SDS]. Fluorogenic substrate was added to each sample and incubated at 37°C for 1 hour. The intensity of fluorescence in each sample solution was measured using a fluorescence plate reader (Dynatech Laboratories, Chantilly, VA) at 360 nm excitatory and 460 nm emission wavelengths. All readings were standardized using the fluorescence intensity of an equal volume of free 7-amino-4-methylcoumarin solution (50 μm). The values were expressed as percentages for control greater than the internal positive control percentages provided by the supplier.

**Real-time Quantitative Reverse Transcription-PCR**

H1299 cells seeded in six-well plates (5 × 10^5/well) were treated with Ad-mda7 (3,000 vp/cell) or Ad-mda7 plus sulindac (0.125, 0.25, or 0.5 mmol/L). Untreated cells served as controls in these experiments. At 48 hours after the start of treatment, the cells were washed in PBS, trypsinized, and resuspended in 1.0 mL PBS. The cell
suspension was transferred into 1.5 mL Eppendorf tubes and centrifuged for 5 minutes at 10,000 rpm at 4°C. The supernatant was discarded and total RNA from the cell pellet was extracted using an RNA isolation kit as described by the manufacturer (Ambion Corp., Austin, TX). The isolated RNA was then treated with DNase I to remove residual DNA and subsequently quantitated using a spectrophotometer at 260 and 280 nm wavelengths. Total RNA (0.1 μg) from each sample was reverse transcribed using a SuperScript reverse transcriptase kit (Invitrogen, Carlsbad, CA). Quantification of mda-7 mRNA was done using real-time quantitative reverse transcription-PCR. Briefly, quantitative PCR was done in 20 μL volumes consisting of 1 μL total RNA, 10 μL PCR Supermix (PE Applied Biosystems, Foster City, CA), 0.2 μm mda-7-specific primers, and 0.1 μm fluorescent probe. The resulting relative increase in reporter and quencher fluorescent dye emission was monitored in real-time during PCR amplification using a 7,700 sequence detector (PE Applied Biosystems). The two-step PCR cycling was carried out as follows: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 minutes at 95°C, and 1 minute at 60°C. The human GAPDH housekeeping gene was used as internal control in the amplification reactions and the primers provided by the supplier (PE Applied Biosystems).

The oligonucleotide sequences used in the assays described above are as follows: mda-7 5′-primer, CCC-GTAATAAGCTTGGTACCG; and mda-7 3′-primer, TAATAAGCCGAAGCAGCTC; probe, FAM-TGGAAATTCGGCTTACAAGACATGACTGTG-TAMRA.

All reactions were done in triplicate. After the cycling reaction was complete, a standard curve, the threshold cycling (Ct) value of each sample, and its corresponding starting quantity based on the standard curve were determined using the 7,700 sequence detector system software (PE Applied Biosystems). The differences in mda-7 mRNA expression among various treatment groups were expressed as the change in values over glyceraldehyde-3-phosphate dehydrogenase.

**Half-Life Assay**

H1299 cells were seeded at a density of 2 × 10⁵ cells in a 60-mm-diameter tissue culture dish. The next day, the cells were infected with Ad-mda7 (3,000 vp/cell). At 48 hours after infection, sulindac (1 mmol/L) was either added or not added and the incubation continued. Two hours later, the protein synthesis inhibitor cycloheximide (10 μg/mL) was added to the cells and the incubation continued. Cells were harvested at 0, 3, 6, 9, 11, and 13 hours after cycloheximide treatment; cell lysates were then prepared and analyzed for MDA-7 protein expression by Western blot analysis as previously described (5, 6).

**Western Blot Analysis**

Cells treated with PBS, Ad-mda7, Ad-p53, Ad-luc, sulindac, sulindac sulfone, or a combination of Ad-luc, Ad-p53, or Ad-mda7 with sulindac or sulindac sulfone was subjected to Western blot analyses as previously described (5, 6). The following primary antibodies were used for detection: caspase-3 and poly (ADP-ribose) polymerase (PARP, BD Pharmingen, San Diego, CA); caspase-9, pJNK, and pp38 MAPK (Cell Signaling Technology, Inc., Beverly, CA); PKR, Bax, Bak, Bel-2, Bcl-XL, COX-2, p53, and ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA); β-actin (Sigma); and MDA-7 (Introgen Therapeutics). The proteins were detected using appropriate horseradish peroxidase–conjugated secondary antibodies, and visualized on enhanced chemiluminescence film (Hyperfilm, Amersham, Arlington Heights, IL) by application of Amersham’s enhanced chemiluminescence Western blotting detection system. The results were verified by repeating the experiments at least twice. The protein band intensity was semiquantitatively determined as previously described (5).

**In vivo Analysis**

To determine whether sulindac enhances Ad-mda7-mediated tumor growth inhibition of xenograft tumors in vivo, H1299 lung tumor cells (5 × 10⁵) were injected s.c. into the lower right flank of athymic BALB/c female nude mice (n = 48). When the tumor reached 50 to 100 mm³, the animals were divided into groups and treated as follows: PBS (n = 8), sulindac (n = 8), Ad-luc (n = 8), Ad-mda7 (n = 8), Ad-luc plus sulindac (n = 8), or Ad-mda7 plus sulindac (n = 8). The mice were treated with Ad-luc or Ad-mda7 intratumorally (3 × 10⁵ vp/dose) thrice a week. In mice receiving sulindac, 40 mg/kg was given i.p. daily. Animals were weighed weekly to determine the body weight. Tumor growth was monitored and measured thrice a week as described previously (13, 21). At 22 to 25 days after initiation of the treatment, all animals were killed via CO₂ inhalation, and the tumors were removed for histopathologic examination and Western blot analysis. Experiments were done twice for reproducibility and twice for statistical significance.

**Statistical Analysis**

Student’s t test and ANOVA were used to calculate the statistical significance of the experimental results. A value of P < 0.05 was considered statistically significant.

**Results**

**Sulindac Enhances Ad-mda7-Mediated Growth Inhibition in Lung Cancer Cells**

Because previous studies have shown that sulindac exerts cytotoxic effects on cancer cells (33), we conducted preliminary experiments to determine the minimum cytotoxic dose of sulindac against non–small cell lung cancer (A549 and H1299) cells and normal (CCD-16) cells. Treatment of these cells with sulindac at various concentrations (0.062, 0.12, 0.25, 0.5, 1, and 2 mmol/L) showed growth inhibition, with an IC50 of 0.58 ± 0.01, 0.61 ± 0.07, and 0.94 ± 0.04 mmol/L in A549, H1299, and CCD-16 cells, respectively (data not shown). At higher doses (1 and 2 mmol/L), cell proliferation was inhibited in both tumor and normal cells resulting in apoptosis (data not shown). However, the inhibitory effect on tumor cell proliferation was higher (>90%) than in normal cells (60%). On the basis of these results, we tested sulindac at concentrations of...
<0.5 mmol/L in subsequent experiments. To investigate whether sulindac in combination with Ad-mda7 can inhibit cell proliferation and induce apoptosis, A549, H1299, and CCD-16 were treated with PBS, Ad-luc, and Ad-mda7, alone and in combination with sulindac (0.125, 0.25, or 0.5 mmol/L). Analysis of cells 72 hours after treatment showed that the combination of sulindac and Ad-mda7 significantly inhibited tumor cell proliferation compared with cells that were treated with Ad-mda7 or sulindac alone ($P = 0.001$; Fig. 1A). The growth inhibitory effects produced by this combination therapy were also significant compared with the other treatment groups and were sulindac dose dependent. In contrast, no significant growth inhibitory effects were observed in normal fibroblast cells treated with Ad-mda7 plus sulindac at any concentration tested compared with the other treatment groups. These results indicate that sulindac enhances Ad-mda7-mediated inhibitory activity in tumor cells but not in normal cells.

To further evaluate whether treatment with Ad-mda7 plus sulindac induces apoptosis, tumor and normal cells were analyzed 72 hours after treatment for apoptotic changes by FACS analysis. The number of cells in sub-G0-G1 phase, an indicator of apoptotic changes, was determined by flow cytometry. No significant difference in the transduction results were observed in at least two independent experiments. The ability of therapeutic agents to enhance adenovirus transduction has been reported (45). Based on this report, we determined whether sulindac enhances adenoviral transduction. For this purpose, tumor and normal cells were infected with Ad-GFP at 100 vp/cell and treated with sulindac at various concentrations (Table 1). Cells were transduced with Ad-GFP at low particle numbers because >80% of cells are transduced at a higher virus-particle number, making it difficult to determine the effects of sulindac on transduction. At 24 hours after treatment, the cells were analyzed by flow cytometry. No significant difference in the transduction effects were observed when lung tumor cancer cells were treated with Ad-mda7 in combination with sulindac sulfone (data not shown). These results show that lung tumor but not normal cells undergo apoptosis when treated with Ad-mda7 and sulindac or sulindac sulfone. Furthermore, the growth inhibitory effects mediated by Ad-mda7 and sulindac are independent of the p53 status given that they occurred in p53-null H1299 and p53 wild-type A549 tumor cell lines. However, the contribution of other genetic factors in the sensitization of these two cell lines to Ad-mda7 and sulindac is not known now and is beyond the scope of the present study.

**Sulindac Does Not Increase Adenoviral Transduction**

The ability of therapeutic agents to enhance adenovirus transduction efficiency has previously been reported (45). Based on this report, we determined whether sulindac
efficiency was observed between cells treated with Ad-GFP plus sulindac and cells treated with Ad-GFP alone (Table 1). However, transduction was increased in A549 cells that had been treated with 0.5 mmol/L sulindac compared with other groups treated with sulindac at lower concentrations ($P = 0.001$).

**Sulindac Increases Ectopic MDA-7 Expression**

To identify the mechanism by which sulindac enhances Ad-md7-mediated growth inhibition and apoptosis in lung cancer cells, we first examined ectopic MDA-7 protein expression by Western blotting. All three cell lines (H1299, A549, and CCD-16) were treated with Ad-md7 and sulindac for 48 hours and analyzed for MDA-7 expression. In Ad-md7-treated A549 and H1299 cells, sulindac markedly increased the levels of ectopic MDA-7 in a dose-dependent manner (Fig. 2A); endogenous MDA-7 expression was not detected in cells treated with either PBS or sulindac alone. MDA-7 protein expression was increased 2 to 4.5 times in the tumor cells treated with Ad-md7 plus sulindac compared with cells treated with Ad-md7 alone. In contrast, in normal CCD-16 cells treated with Ad-md7, sulindac only slightly increased ectopic MDA-7 protein expression. The level of MDA-7 protein increase in CCD-16 cells after Ad-md7 plus sulindac treatment was <2-fold compared with cells treated with Ad-md7 alone.

To evaluate subcellular localization of MDA-7 protein, immunofluorescence studies were done. Consistent with our Western blot data, MDA-7 expression was significantly elevated in cells treated with Ad-md7 and sulindac compared with cells treated with Ad-md7 alone (Fig. 2B). Furthermore, the subcellular localization of MDA-7 was not altered by sulindac treatment (Fig. 2B). MDA-7 expression was not detectable in cells treated with PBS or sulindac alone. Expression was also not detectable when cells were stained with an isotypic FITC-labeled antibody, indicating specificity. These results show that sulindac increases ectopic MDA-7 expression in a dose-dependent manner and suggest that this increase contributes to increased apoptotic activity.

To test whether the ability to increase ectopic protein expression was unique to sulindac, experiments were also carried out using sulindac sulfone. Treatment of H1299 cells with Ad-md7 and sulindac sulfone showed increased ectopic MDA-7 expression compared with cells treated with Ad-md7 alone (Fig. 2C). The level of induction, however, was observed to be less than that seen with sulindac. MDA-7 expression was not detected in cells that were treated with PBS or sulindac sulfone. These results show the ability of sulindac and its metabolites to increase ectopic protein expression. We next tested whether sulindac specifically increased only MDA-7 protein expression: For this purpose, H1299 tumor cells were treated with Ad-p53 (50 vp/cell) or Ad-p53 and sulindac and analyzed for p53 protein expression and apoptotic cells. Note that cells were treated with 50 vp/cell as these cells are very sensitive to Ad-p53 at higher concentrations. Sulindac increased the levels of ectopic p53 protein expression in tumor cells treated with Ad-p53 that resulted in increased cell killing (Fig. 2D). Sulindac also increased ectopic GFP protein expression. However, increased GFP protein expression did not result in cell killing (data not shown). These results show that sulindac enhanced ectopic expression of a wide-variety of transgenes (MDA-7, p53, and GFP) and is not specific to Ad-md7. However, the enhanced killing effect is only observed when sulindac is combined with Ad-md7 or Ad-p53.

**Sulindac Enhances Ad-md7-Mediated Apoptotic Signaling**

We previously reported that induction of Ad-md7-mediated apoptosis in lung cancer cells was associated with activation of the caspase cascade, including cleavage of caspase-9, caspase-3, and PARP (5, 6). To determine whether treatment with Ad-md7 and sulindac affects the caspase cascade, we analyzed tumor and normal cells for these molecular markers. Tumor (A549 and H1299) cells treated with Ad-md7 alone or in combination with sulindac showed cleavage of capase-9, caspase-3, and PARP, which are indicators of activation of the caspase cascade (Fig. 3A). The expression of cleaved caspase-9, caspase-3, and PARP corresponded to both the concentration of sulindac and the level of MDA-7 expression. Activation of caspase-9, caspase-3, and PARP was also observed in A549 (but not H1299) cells that had been treated with Ad-luc plus the highest concentration of sulindac (0.5 mmol/L) and was consistent with the increased apoptotic fraction revealed in these cells by FACS analysis (Fig. 1B). However, the level of activation was significantly lower than that in A549 cells treated with Ad-md7 plus sulindac. The caspase cascade was not activated in either A549 or H1299 cells that were untreated or treated with sulindac alone. In CCD-16 cells, the caspase cascade was not activated in cells that were treated with Ad-md7 alone or in combination with sulindac compared with cells that were untreated or treated with sulindac alone, Ad-luc alone, or a combination of Ad-luc plus sulindac (Fig. 3B). These results show that sulindac selectively enhances caspase cascade activation in tumor but not normal cells. We next examined additional effector molecules upstream of the caspase cascade that are modulated by Ad-md7 and sulindac treatments. Previous studies have shown PKR, p38MAPK, and pJNK to be important in Ad-md7-induced apoptosis in lung cancer cells (14, 15, 18). Similarly, regulation of the Bcl-2 family (Bax, Bak, Bcl-2, and Bcl-xL) proteins has been shown to be critical for sulindac-induced apoptosis and independent of p53 status (46, 47). On the basis of these reports, we evaluated the expression of PKR, pJNK, pp38MAPK, and pJNK to be important in Ad-md7-induced apoptosis in lung cancer cells (14, 15, 18). Similarly, regulation of the Bcl-2 family (Bax, Bak, Bcl-2, and Bcl-xL) proteins has been shown to be critical for sulindac-induced apoptosis and independent of p53 status (46, 47). On the basis of these reports, we evaluated the expression of PKR, pJNK, pp38MAPK, and pJNK to be important in Ad-md7-induced apoptosis in lung cancer cells (14, 15, 18). Similarly, regulation of the Bcl-2 family (Bax, Bak, Bcl-2, and Bcl-xL) proteins has been shown to be critical for sulindac-induced apoptosis and independent of p53 status (46, 47). On the basis of these reports, we evaluated the expression of PKR, pJNK, pp38MAPK, and pJNK to be important in Ad-md7-induced apoptosis in lung cancer cells (14, 15, 18). Similarly, regulation of the Bcl-2 family (Bax, Bak, Bcl-2, and Bcl-xL) proteins has been shown to be critical for sulindac-induced apoptosis and independent of p53 status (46, 47).
Figure 2. Sulindac increases MDA-7 protein expression. A, tumor (A549 and H1299) and normal (CCD-16) cells were treated with PBS or Ad-mda7, followed by treatment with sulindac at the indicated concentrations. At 48 h after infection, the cells were harvested and immunoblotted for MDA-7. Increased MDA-7 protein expression was observed in cells that were treated with Ad-mda7 and sulindac compared with cells that were treated with Ad-mda7. MDA-7 expression was not observed in untreated or sulindac-treated cells. The difference in the level of MDA-7 protein expression was semiquantitatively determined by densitometry and expressed as a ratio. β-actin was used as a protein loading control. B, H1299 cells were treated with PBS (a), sulindac (b), Ad-mda7 (c), or with a combination of Ad-mda7 plus sulindac (d). At 48 h after treatment, cells were subjected to immunofluorescence staining for MDA-7 protein as described in Materials and Methods. Cytoplasmic MDA-7 expression was observed in cells treated with Ad-mda7 or Ad-mda7 plus sulindac. However, MDA-7 protein expression was significantly increased in cells treated with Ad-mda7 plus sulindac compared with Ad-mda7-treated cells. MDA-7 protein expression was not detected in cells treated with PBS or sulindac. Original magnification, ×400. C, A549 and H1299 cells were treated with PBS or Ad-mda7 in the presence or absence of sulindac sulfone and analyzed for MDA-7 expression. Increased MDA-7 expression was observed in cells treated with Ad-mda7 and sulindac sulfone compared with cells that were treated with Ad-mda7. MDA-7 expression was not observed in PBS or sulindac sulfone–treated cells. The difference in the level of MDA-7 protein expression was semiquantitatively determined by densitometry and expressed as a ratio. β-actin was used as a protein loading control. D, H1299 cells were treated with low dose of Ad-p53 (50 vp/cell) or Ad-p53 and sulindac, and analyzed for p53 protein expression and apoptotic cells as described in Materials and Methods. Increased p53 protein was observed in Ad-p53- and sulindac-treated cells that correlated with increased apoptotic cells compared with cells that were not treated or treated with Ad-p53. Bars, SE.
treated with Ad-luc plus sulindac compared with untreated, Ad-luc-treated, and sulindac-treated cells. However, the PKR levels in the cells treated with Ad-luc plus sulindac were lower than those observed in cells treated with Ad-mda7 plus sulindac. The increase in PKR, pJNK, and pp38MAPK was associated with the expression levels of MDA-7 induced by sulindac. No change in the expression levels of Bax, an inducer of apoptosis, was detected in any of the treatment groups (Fig. 3C). The expression level of Bcl-2 or Bcl-xL, two inhibitors of apoptosis, was also not significantly changed in any of the treatment groups (data not shown). These

Figure 3. Sulindac increases Ad-mda7-mediated apoptotic signaling. Tumor A549 and H1299 cells (A) and normal (CCD-16) cells (B) were either untreated or treated with Ad-luc or Ad-mda7, alone or in combination with sulindac, at the indicated concentrations. At 72 h after treatment, cells were harvested and lysed, and activation of caspase-9 and caspase-3 and cleavage of PARP was determined by Western blot analysis. The difference in the level of protein expression was semiquantitatively determined by densitometry and expressed as a ratio. β-actin was used as a protein loading control. C, A549 and H1299 cells were treated as described above for 48 h and analyzed by Western blot analysis for various molecular effectors of apoptosis. The difference in the level of protein expression was semiquantitatively determined as described above. D, analysis for COX-2 expression in A549 and H1299 cells treated with Ad-mda7 or Ad-mda7 and sulindac. COX-2 expression was increased in Ad-mda7 and Ad-mda7 plus sulindac–treated cells compared with cells that were treated with PBS or sulindac. However, no significant change in COX-2 expression was observed in Ad-mda7-treated cells compared with Ad-mda7– and sulindac-treated cells. Changes in protein levels were determined semiquantitatively as described above. β-actin served as internal loading control.
Table 2. Cell cycle distribution in lung cancer cells treated with sulindac, Ad-mda 7, or both

<table>
<thead>
<tr>
<th>Treatment</th>
<th>A549 (%)</th>
<th>H1299 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G1</td>
<td>S</td>
</tr>
<tr>
<td>Untreated control medium</td>
<td>68.6 ± 0.7</td>
<td>26.4 ± 0.21</td>
</tr>
<tr>
<td>Control + sulindac 0.125 mmol/L</td>
<td>64.8 ± 0.14</td>
<td>28.9 ± 0.35</td>
</tr>
<tr>
<td>Control + sulindac 0.5 mmol/L</td>
<td>75.6 ± 0.14</td>
<td>18.5 ± 0.14</td>
</tr>
<tr>
<td>Ad-mda7</td>
<td>41.3 ± 0.63</td>
<td>31.6 ± 0.63</td>
</tr>
<tr>
<td>Ad-mda7 + sulindac 0.125 mmol/L</td>
<td>35.6 ± 0.14</td>
<td>31.8 ± 0.7</td>
</tr>
<tr>
<td>Ad-mda7 + sulindac 0.5 mmol/L</td>
<td>43.4 ± 0.21</td>
<td>42.6 ± 0.07</td>
</tr>
<tr>
<td>Ad-luc</td>
<td>73.8 ± 1.5</td>
<td>17.1 ± 1.9</td>
</tr>
<tr>
<td>Ad-luc + sulindac 0.5 mmol/L</td>
<td>70.0 ± 8.1</td>
<td>18.5 ± 6.3</td>
</tr>
</tbody>
</table>

NOTE: Cells were treated with PBS, Ad-luc, or Ad-mda7 alone, or in combination with 0.5 mmol/L sulindac for 72 hours followed by FACS analysis. The percentage of cells in each cell cycle phase was determined by analysis of the DNA content histogram. Values are the means of duplicate samples ± SD. Similar results were observed in at least two independent experiments.

results support the idea that the induction of apoptosis by Ad-mda7 plus sulindac primarily depends on the ability of sulindac to enhance ectopic MDA-7 expression.

The possibility that enhanced tumor cell killing with Ad-mda7 plus sulindac treatment was due to COX-2 inhibition was next investigated in A549 and H1299 cells. Increased COX-2 expression was observed in cells treated with Ad-mda7 and Ad-mda7 plus sulindac (Fig. 3D). However, there was no significant difference in COX-2 expression levels between the two treatment groups. COX-2 expression was not observed in cells that were treated with PBS or sulindac.

Effects of Sulindac and Ad-mda7 Treatment on Cell Cycle

Previous studies have shown that sulindac induces cell cycle arrest at G1 (48), and that Ad-mda7 induces cell cycle arrest at G2-M (5–7). On the basis of these reports, we investigated by FACS analysis the combined effects of sulindac and Ad-mda7 treatment on cell cycle regulation. Tumor cells were either untreated or treated with sulindac, Ad-luc, Ad-mda7, or Ad-mda7 plus sulindac for 72 hours. As previously reported, Ad-mda7 but not Ad-luc treatment increased the number among the G2-M phase of cell cycle in both A549 (27.2%) and H1299 (42.5%) cells (Table 2). Sulindac treatment alone increased the number of cells in the G1 phase. In both tumor cell lines, the number of G1-phase cells was markedly increased at 0.5 mmol/L compared with 0.125 mmol/L sulindac (75.6% versus 64.8% in A549 and 74.6% versus 66.4% in H1299 cells, respectively). Treatment with sulindac and Ad-mda7 abrogated Ad-mda7-induced G2-M arrest. The effect was more pronounced among cells treated with 0.5 mmol/L sulindac in combination of Ad-mda7, resulting in a decrease in the number of G2-M-phase cells, from 27.2% to 12.3% in A549 and from 42.5% to 32.4% in H1299 cells, respectively. Abrogation of Ad-mda7-induced G2-M-phase arrest by sulindac was also observed at 48 hours after treatment (data not shown). These results show that sulindac and Ad-mda7 affect different phases of the cell cycle and that sulindac-enhanced Ad-mda7 tumor cell killing does not occur via increased G2-M arrest.

Because the killing effect and regulation of proapoptotic molecules in Ad-mda7- and sulindac-treated H1299 and A549 tumor cells were similar, we narrowed our focus in studying the underlying mechanism in only one cell line. Subsequent studies were carried out in H1299 cells as described below.

Sulindac Delays Ectopic MDA-7 Protein Degradation

To assess the mechanism by which sulindac increases ectopic MDA-7 protein, we examined the effect of sulindac on transcriptional activity and MDA-7 protein degradation in H1299 cells. To determine the effect of sulindac on the transcriptional activity of Ad-mda7, we did quantitative real-time PCR analysis of RNA samples extracted from cells that were untreated or that were treated with Ad-mda7 alone or with sulindac at various concentrations. No significant difference in mRNA levels was observed in the cells treated with Ad-mda7 plus sulindac compared with untreated and Ad-mda7-treated cells (Fig. 4A). To evaluate whether sulindac treatment regulates MDA-7 protein degradation, H1299 cells were treated with Ad-mda7 alone or in combination with sulindac for various durations, and the half-life of MDA-7 protein was determined. The MDA-7 protein levels in the Ad-mda7-treated control cells decreased over time (Fig. 4B); protein degradation was complete at 11 hours. In contrast, the degradation of MDA-7 protein in the cells treated with Ad-mda7 plus sulindac was delayed as shown by substantial levels of detectable protein at 13 hours. Semiquantitative analysis of the protein levels indicated that at 0 to 13 hours, the MDA-7 protein level was 8 to 10 times higher in the cells treated with Ad-mda7 plus sulindac than in the Ad-mda7-treated cells (Fig. 4C). These results show that the increase in ectopic MDA-7 protein expression in cells treated with Ad-mda7 plus sulindac is a result of a sulindac-mediated delay of MDA-7 protein degradation.

Sulindac-Enhanced MDA-7 Expression Is Not Due to Inhibition of Proteasome Activity

Given that recent studies have shown that some NSAIDs inhibit proteasome activity (44, 49), we sought...
to determine whether the enhanced MDA-7 protein expression mediated by sulindac is due to its ability to inhibit proteasome activity. For this purpose, the effects of sulindac were compared with those of MG132, a known proteasome inhibitor (50) by Western blotting, ubiquitin degradation assay, and proteasome enzymatic activity assay. Western blotting showed that sulindac or MG132 treatment for 12 hours in combination with Ad-mda7 enhanced MDA-7 protein expression compared with cells treated with Ad-mda7 alone (Fig. 5A). However, sulindac enhanced total MDA-7 protein levels, which included both nascent unglycosylated protein and MDA-7 proteins that were glycosylated at different levels, as indicated by multiple bands. In contrast, MG132 enhanced the level of nascent MDA-7 protein, albeit less strongly than did sulindac, and one glycosylated form of MDA-7 protein. Similar results were obtained at 6 and 24 hours after treatment (data not shown). Thus, the mechanisms by which sulindac and MG132 enhance MDA-7 protein seem to differ.

We next determined the ability of sulindac to inhibit proteasome activity. Western blot analysis for total ubiquitinated proteins, an indicator of inhibition of the proteasome pathway, showed ubiquitinated proteins in MG132-treated cells but not in sulindac-treated cells (Fig. 5B). These results show that sulindac, unlike MG132, does not inhibit proteasome activity or the proteasome pathway. Consistent with these findings are the results of the proteasome activity assay in which treatment with Ad-mda7 alone or with sulindac did not inhibit the proteasome activity compared with untreated control cells (Fig. 5C). In contrast, treatment with Ad-mda7 plus MG132 resulted in significant inhibition of proteasome activity ($P = 0.01$). These results suggest that sulindac-enhanced MDA-7 protein expression is not due to inhibition of proteasome activity.

**Sulindac Enhances Ad-mda7-Mediated Lung Tumor Growth Suppression In vivo**

To determine whether Ad-mda7 plus sulindac treatment enhances tumor growth suppression, pilot in vivo experiments were conducted using a lung tumor xenograft model. Compared with mice treated with PBS, sulindac, Ad-luc, Ad-mda7, or Ad-luc plus sulindac, mice treated with Ad-mda7 plus sulindac showed a significant growth suppression ($P < 0.001$; Fig. 6A). A significant tumor inhibition was also observed in mice that were treated with Ad-mda7 alone or Ad-luc plus sulindac compared with PBS- and Ad-luc-treated mice ($P = 0.03$). No significant growth inhibition was observed in sulindac-treated mice compared with PBS-treated mice. Furthermore, treatment-related toxicity as evidenced by morbidity, loss of body weight, and death was not observed in mice treated with Ad-mda7 plus sulindac, suggesting that the treatments were well tolerated (data not shown).

Analysis of s.c. tumors 24 hours after the last treatment with sulindac revealed that MDA-7 protein levels were 3 to 12 times higher in the tumors from mice treated with Ad-mda7 plus sulindac than in the tumors from the mice treated with Ad-mda7 (Fig. 6B). Some faint protein bands

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**Figure 4.** Sulindac delays MDA-7 protein degradation. A, H1299 cells were treated with PBS (control), Ad-mda7 alone, or Ad-mda7 in combination with sulindac at the indicated concentrations for 48 h. RNA extraction and quantitative real-time PCR were done as described in Materials and Methods. The results were expressed as a ratio of mda-7 mRNA/glyceraldehyde-3-phosphate dehydrogenase. No substantial difference in the mda-7 mRNA levels was observed between the cells treated with Ad-mda7 plus sulindac and the cells treated with Ad-mda7 alone. B, H1299 cells were treated with Ad-mda7 for 48 h. After a 2-h incubation in medium without sulindac (----) or with 1 mmol/L sulindac (+), cells were incubated for the indicated periods with 10 μg/mL cycloheximide, a known protein synthesis inhibitor. Cell lysates were prepared at the indicated time points and analyzed by Western blot analysis for MDA-7 protein levels. Treatment with sulindac delayed the degradation of MDA-7 protein. β-actin was used as an internal loading control. C, MDA-7 protein expression levels between H1299 cells not treated with sulindac and sulindac-treated H1299 cells were semiquantitatively determined using ImageQuant software (Molecular Dynamics/Amersham Pharmacia Biotech, Piscataway, NJ). Columns, mean of two independent experiments; bars, SE.
were also observed in the tumors of mice treated with PBS, sulindac, and Ad-luc plus sulindac. However, we believe that these bands were nonspecific or cross-reacting with mouse tissue proteins because the corresponding assay using anti-MDA-7 antibody revealed no endogenous MDA-7 protein in the parental H1299 tumors and murine normal cells (data not shown). These results show that treatment of lung tumors with Ad-md7 plus sulindac enhances growth suppression in parallel to enhanced MDA-7 protein expression, a finding consistent with our in vitro results.

**Discussion**

We found that treatment of human non–small cell lung cancer cells with Ad-md7 in combination with sulindac results in significant growth suppression and apoptosis both in vitro and in vivo. Furthermore, the inhibitory effects are selective for tumor but not normal cells. The ability to inhibit growth of both H1299 (p53-null) and A549 (p53 wild type) cells suggests that this treatment was also observed in the tumors of mice treated with PBS, sulindac, and Ad-luc plus sulindac. However, we believe that these bands were nonspecific or cross-reacting with mouse tissue proteins because the corresponding assay using anti-MDA-7 antibody revealed no endogenous MDA-7 protein in the parental H1299 tumors and murine normal cells (data not shown). These results show that treatment of lung tumors with Ad-md7 plus sulindac enhances growth suppression in parallel to enhanced MDA-7 protein expression, a finding consistent with our in vitro results.
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combination strategy is applicable for human cancers that are defective in p53, which account for >50% of human cancers (51). However, the efficacy of treatment with Ad-mda7 plus sulindac on tumor cells that have defects in other genes or pathways is not known and needs to be tested. Our results also reveal a mechanism by which sulindac enhances Ad-mda7-mediated tumor killing. Preliminary experiments showed that sulindac, in a dose-dependent manner, increased the expression of ectopic MDA-7 protein in Ad-mda7-treated cells, suggesting that the enhanced killing was due to increased MDA-7 protein expression. However, the mechanism by which sulindac increased ectopic protein expression is not known. Previous studies from several laboratories have shown the ability of various drugs to enhance adenoviral transduction efficiency (45). Such findings suggested that the increased MDA-7 protein expression observed in cells treated with Ad-mda7 plus sulindac was due to increased Ad-mda7 transduction that was mediated by sulindac. However, sulindac, unlike the various other drugs, did not enhance adenovirus transduction efficiency. Sulindac also increased ectopic p53 protein expression in cells that were treated with Ad-p53 plus sulindac that resulted in enhanced cell killing. These results suggest that the ability of sulindac to increase ectopic protein expression is not restricted to MDA-7 alone but is applicable to other adenoviral-encoded proteins. Similarly, the ability to increase MDA-7 protein was not unique to sulindac as increased protein expression was also observed in sulindac sulfone–treated cells. Although sulindac also increased MDA-7 protein expression in normal cells, the expression levels were significantly lower than those in the tumor cells; the reason for this difference in normal cells is unclear and should be investigated. We also assessed, after treatment, the molecular effectors that are known to mediate tumor cell inhibition and apoptosis. We and others have previously shown that Ad-mda7 treatment of lung cancer cells results in a G2-M-phase arrest and induces apoptosis via activation of the caspase cascade and PKR (5–7, 14). Furthermore, Ad-mda7-mediated apoptosis in lung cancer cells is independent of Bax, Bak, and Bcl-2 (5, 6). A requirement for Bax in Ad-mda7-mediated killing, however, has been shown in mesothelioma cells (9). Sulindac, unlike mda-7, induces G1-phase arrest, up-regulates Bax and Bak expression, and inhibits COX-2 expression (46). More recently, Bax was shown to be necessary for sulindac-induced apoptosis in Bcl-deficient colorectal cancer cells (52). Another study showed sequential decrease in Bcl-2 expression and increased apoptosis in an intestinal tumor model after treatment with sulindac (47). In the present study, sulindac abrogated Ad-mda7-mediated G2-M-phase arrest and this abrogation was more pronounced in A549 than in H1299 cells. Additionally, increased expression of PKR, pJNK, and p38MAPK were the increase in caspase-9 and caspase-3 expression and activation. Previous studies in colon cancer cells have shown regulation of Bak, Bax, Bcl-2, and COX-2 by sulindac (46–52). Interestingly, in lung cancer cells, we did not find any significant change in the expression levels of these effector molecules by sulindac or Ad-mda7 or the combination. The increased levels of effector molecules that are regulated by Ad-mda7 (PKR, p38MAPK, and pJNK) but not by sulindac suggests that the increased tumor cell killing is merely due to a sulindac-mediated increase in ectopic MDA-7 protein expression. Additional evidence for this claim comes from our study where cells treated with increasing the dose of Ad-mda7 viral particles such that the endogenous MDA-7 protein levels equaled to the levels achieved with Ad-mda7 and sulindac resulted in similar level of tumor cell killing. However, additional molecules/pathways (cyclic guanosine 3',5'-monophosphate–phosphodiesterase, protein kinase G, and β-catenin) that have previously been reported to be affected by sulindac and its metabolites were not examined in the present study. It is possible that sulindac, when used at high concentrations that induce apoptosis, regulates the expression of some of these molecules. However, in the present study, we used sulindac at concentrations below its IC50 value that does not induce apoptosis. In support of these observations are the findings that treatment of H1299 cells with Ad-p53 plus sulindac resulted in increased ectopic p53 expression and enhanced cell death. Because the effect of Ad-mda7 and sulindac on H1299 and A549 tumor cells were the same, we narrowed our focus on studying the underlying mechanism for enhanced killing using only H1299 cells as described below. We next investigated the mechanism by which sulindac increased MDA-7 protein expression. As described above, the possibility that sulindac increases transduction efficiency was ruled out by the results of flow cytometry assays using GFP reporter. Alternative explanations for increased MDA-7 protein levels included increased transcriptional activity, increased protein half-life, and increased antiproteasome activity. That sulindac did not affect transcriptional activity was shown by real-time quantitative reverse transcription-PCR. The major determinant for protein half-life is the presence of degradation signals contained in the protein. Degradation of most cellular proteins is rapidly done by the proteasome/ubiquitination system, both to eliminate misfolded or denatured polypeptides and to regulate the concentration of components critical for control of cell cycle and metabolism (53–55). Furthermore, recent studies have shown that NSAIDs can inhibit proteasome activity (44). On the basis of these studies, we examined the effect of sulindac on protein half-life and proteasome activity. Sulindac increased the half-life of ectopic MDA-7

6 Unpublished data.
protein, as well as the half-life of endogenous proteins, such as p53 (data not shown). The ability of sulindac to increase protein half-life is an unexpected and novel finding. Although the half-life of MDA-7 protein was increased, it is still possible that increased MDA-7 protein levels is partly due to an effect of sulindac on the proteasome/ubiquitin pathway. To test this possibility, we compared sulindac with the proteasome inhibitor MG132 (50). Sulindac, unlike MG132, did not inhibit the proteasome pathway directly or indirectly. Lack of direct inhibitory activity of sulindac on proteasomes was shown by the proteasome activity assay; on the other hand, lack of indirect inhibitory activity was shown by the absence of total ubiquitinated proteins in sulindac-treated cells. The discrepancy between our results from those previously reported is not clear (43, 44). One possibility is that the concentration of sulindac used in the present study was much lower than that used in the studies previously reported. Alternatively, the inhibition of proteasome activity by sulindac may be tumor-type dependent. We are currently investigating these possibilities in the laboratory.

The ability of sulindac and its metabolites to increase the half-life of therapeutic proteins, such as MDA-7 or p53, has immediate clinical implications in that repeated vector injections may be minimized but still achieve the same therapeutic effect. Phase I clinical trials have been completed for Ad-mda7 and for sulindac and its metabolites; these studies have indicated that these agents have no significant toxic effects and are well tolerated when used alone (23, 24, 56). However, combination therapy using Ad-mda7 plus sulindac and its metabolites has not yet been tested; our animal study did not show any enhanced toxicity due to the combination of Ad-mda7 plus sulindac compared with individual treatments. Therefore, additional preclinical studies evaluating the therapeutic effect of Ad-mda7 and sulindac or its metabolites on tumor cells are warranted before testing of this therapy in humans.

Finally, our animal experiments, which were to test whether sulindac enhances Ad-mda7-mediated tumor cell killing in vivo, revealed significant tumor growth inhibition in the mice treated with Ad-mda7 plus sulindac compared with the control treatment groups. These inhibitory effects were due to increased MDA-7 protein expression in the tumors consistent with our in vitro results. Although we have shown proof-of-principle using s.c. tumor xenografts, additional studies evaluating the therapeutic effects on a spectrum of human xenograft tumors are warranted. Furthermore, the effect of Ad-mda7 and sulindac combination therapy on disseminated lung cancer has not been tested and, compared with localized disease, is more relevant to cancer therapy in clinical settings. We are currently conducting these experiments in our laboratory. In conclusion, we have identified a novel mechanism of action of sulindac. Our results form a basis for additional preclinical studies using Ad-mda7 and sulindac for treatment of human lung cancer.

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References

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Molecular Cancer Therapeutics

Sulindac enhances adenoviral vector expressing mda-7/IL-24–mediated apoptosis in human lung cancer

Yasuhisa Oida, Began Gopalan, Ryo Miyahara, et al.


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