Synergistic Inhibition of Human Lung Cancer Cell Growth by Adenovirus-mediated Wild-Type p53 Gene Transfer in Combination with Docetaxel and Radiation Therapeutics in Vitro and in Vivo

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ABSTRACT
Chemotherapy given sequentially or concurrently with external beam radiation therapy has emerged as a standard for the treatment of locally advanced lung cancer. Gene therapy by adenovirus-mediated wild-type p53 gene transfer has been shown to inhibit lung cancer growth in vitro, in animal models, and in human clinical trials. However, no information is available on the combined effects of p53 gene transfer, chemotherapy, and radiation therapy on lung cancer growth in vitro and in vivo. Therefore, we developed two-dimensional and three-dimensional isobologram modeling and statistical methods to evaluate the synergistic, additive, or antagonistic efficacy among these therapeutic agents in human non-small cell lung cancer cell lines A549, H460, H322, and H1299, at the ID50 and ID80 levels. The combination of these three therapeutic agents exhibited synergistic inhibitory effects on tumor cell growth in all four cell lines at both the ID50 and the ID80 levels in vitro. In mouse models with H1299 and A549 xenografts, combined treatment synergistically inhibited tumor growth in the absence of any apparent increase in toxicity, when compared with other treatment and control groups. Together, our findings suggest that a combination of gene therapy, chemotherapy, and radiation therapy may be an effective strategy for human cancer treatment.

INTRODUCTION
Lung cancer is a frequent cause of cancer deaths worldwide (1). Yet, recent advances in understanding lung cancer biology and genetics have suggested new treatment strategies. For example, although the outcome is still poor, the management of locally advanced NSCLC has progressed in recent years with the use of combined modality therapies (2). Optimization of combinations of standard and novel therapeutic agents may improve the outcome of treatment. Synergism (or antagonism) between multiple combined therapeutic agents is usually difficult to assess both experimentally and clinically. Although mathematical synergism in cell lines and in animal models may not directly correlate with clinical response, it could help select combination treatments by predicating therapeutic synergy, warn of the possibility of antagonism, and provide critical data for the optimal timing of combined treatments in human clinical trials.

Docetaxel, a derivative of taxane, is a highly active agent in lung cancer. The taxanes mediate cytotoxicity by stabilizing the microtubules of the cellular mitotic apparatus and preventing their depolymerization into free tubulin (3, 4). Docetaxel in particular, has been shown to inhibit cell replication and be cytotoxic in vitro to various tumor cell lines, to exhibit antitumor activity in different animal tumor systems, and to be effective in the treatment of common human cancers (5–10). Chemotherapeutic agents used as induction therapy may be effective in treating systemic micrometastasis but may also have radiosensitizing effects that increase local tumor control. Improved local tumor control can substantially increase long-term survival in lung cancer (11). Because taxanes have been shown to arrest cells in both G2 and M (the phases in the cell cycle most sensitive to ionizing radiation) their radiosensitizing potential has been explored both in vitro and in vivo, and in clinical trials (12–15).

The tumor suppressor gene p53, which is frequently mutated somatically or deleted in various human cancers, plays an important role in tumorigenesis. The tumor suppressor activity of the gene is mainly through its ability to induce cell growth arrest or apoptosis in response to a variety of stress signals,
whereas a lack of functional p53 usually leads to increased genomic instability, deregulated cell proliferation, accelerated tumor progression, and elevated cellular resistance to anticancer therapy (16–18). Gene therapy by adenovirus-mediated introduction of the wild-type human p53 gene into tumors that are deficient in functional p53 has shown a significant tumor-suppressing efficacy both in animal systems and in human clinical trials (19–25). Furthermore, the tumor-suppressing activities of such therapy have been reported to be enhanced by combination with many chemotherapeutic drugs or ionizing radiation in various human tumors (26–28).

Gene replacement with Ad-p53 could potentially restore chemotherapeutic and radiation therapy sensitivity to primary lung cancers that lack p53 function. However, clinical trials of combination therapy are frequently conducted empirically in the absence of both in vitro and in vivo supporting data (29). A model to investigate complex three-agent interactions may be useful in designing clinical trials. No information is available on the combined effects of the three therapeutic agents Ad-p53, docetaxel, and radiation on tumor growth in vitro and in vivo.

Therefore, in the study described here, we developed mathematical modeling and statistical analysis methods for evaluating (in terms of synergism or antagonism) the therapeutic efficacy of the combination treatment with these three therapeutic agents in vitro and in vivo. Our methodology will provide a valuable tool for presenting critical supporting data for human clinical trials. We also demonstrated for the first time that combination treatment with docetaxel, Ad-p53, and radiation synergistically inhibited the growth of human NSCLC cells in vitro and in vivo (in animal models), which suggests that this three-modality combination treatment may constitute an effective approach to human cancer therapy.

MATERIALS AND METHODS

Cell Lines and Cell Culture. Four human NSCLC cell lines, A549, H1299, H322, and H460, of varying p53 gene status were used for in vitro and in vivo experiments. The A549 line, which contains wild-type p53, was maintained in Ham’s F12 medium supplemented with 10% FCS. The H1299, H322, and H460 lines, which have an internal homozygous deletion of the p53 gene, a mutated p53 gene, or the wild-type p53 gene, respectively, were maintained in RPMI 1640 supplemented with 10% FCS and 5% glutamine. All of the cells were incubated in a humidified incubator supplied with 5% carbon dioxide. All of the cell cultures were tested regularly for the presence of Mycoplasma.

Agents. Docetaxel (Taxotere) was obtained from Rhône- Poulenc Aventis Pharma (Frankfurt, Germany). Three recombinant adenoviral vectors were used: Ad-p53, which contains a wild-type p53 gene, was used as a gene therapy agent; Ad-Luc, which contains a luciferase gene, was used as a nonspecific negative control; Ad-EV, an empty vector with no transgene insert, was used as a negative control. All three were made replication-deficient by deleting the E1 and E3 regions from the viral genome (30). All three recombinant adenoviral vectors were constructed as described previously (31, 32). Viral stocks were prepared by the Vector Core Facility at M. D. Anderson Cancer Center (Houston, TX). Viral titers were determined by absorption measurement (vp/ml) and plaque assay (plaque-forming units/ml). Potential contamination of the viral preparations by wild-type virus was monitored by PCR analysis.

Growth Inhibition and XTT Assay. Inhibition of tumor cell growth by combination treatment with docetaxel, Ad-p53, and radiation were analyzed by quantitatively determining cell viability using an improved XTT assay (Roche Molecular Biochemicals, Indianapolis, IN; Ref. 33). The XTT assay is based on the cleavage of the yellow tetrazolium salt XTT [sodium 3’-[1-phenylamino-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate] by mitochondrial dehydrogenases of viable cells, which results in the formation of an orange formazan dye. Briefly, cells were plated in 96-well microtiter plates at 1 × 10⁵ cells/well in 100 µl of medium. One day after the cells were plated, 25-µl aliquots of media containing varying concentrations of docetaxel were added into each well. After 24 h of exposure to docetaxel, the medium was removed from each well and replaced with a 100-µl aliquot of medium containing Ad-p53 or Ad-Luc vector at various MOI in units of vp/cell. After 4 h of incubation with adenoviral vectors, cells in 96-well plates were irradiated with various doses of γ-radiation in a 137Cs unit at room temperature. Cells were then incubated at 37°C in a humidified atmosphere under 5% CO₂. Five days after incubation, cell growth and viability were quantified by XTT assay. Briefly, the culture medium was removed, and 50 µl of XTT reaction mixtures were added into each well with fresh medium at a final concentration of 0.3 mg/ml/well. Cells were then incubated for 2 h at 37°C. The absorbance was measured at a wavelength of 450 nm against a reference wavelength of 630 nm in a microplate reader (Model MRX; Dynatech Laboratories, Chantilly, Virginia). Percentage cell viability was calculated in terms of the absorbency in treated cells relative to the absorbency in the untreated control cells. Experiments were repeated at least three times with quadruplicate samples for each treatment in each individual experiment.

The treatment schedule and experimental design were chosen and optimized based on: (a) experimental observations that docetaxel could reduce the number of clonogenic cells in tumors undergoing radiotherapy by its own cytotoxic action and its radiosensitizing ability and that docetaxel given within 24–48 h before irradiation acted as a potent enhancer of tumor radiosensitivity and increased the therapeutic gain of irradiation (14, 34); (b) the clinically relevant treatment schedule in which a single bolus of docetaxel (30–40 mg/kg i.v.) was usually given 24 h before 3–5 daily fractions of radiation (14, 34); and (c) the facts that p53-deficient cells were usually shown to be resistant to radiation therapy and wild-type p53 expression could sensitize the response of tumor cells to irradiation and enhance p53-induced apoptosis through the mechanism of irradiation-induced DNA-damaging repair (28). This schedule takes advantage of reoxygenation of hypoxic tumor cells during the interval between drug treatment, exogenous wild-type p53 gene expression, and radiation delivery, and is in the closest relevance to clinical practice.

Isobolograms Analysis of Two- and Three-Agent Combinations. The effects of two-agent combinations and three-agent combinations on tumor cell growth of four human NSCLC cells (H1299, H460, H322, and A549) in vitro were respectively analyzed by two-dimensional and three-dimen-
sional isobolograms originally described by Steel and Peckham (35) and improved by us. The schematic representations of the two-dimensional and three-dimensional isobologram methods are illustrated in Fig. 1.

The effects of the combined therapeutic agents on tumor cell growth were analyzed quantitatively and statistically by plotting the observed experimental data onto the corresponding isobolograms according to methods described by Kano et al. (36). When the observed data points for a combination fell mainly within the envelope of additivity, the effect of the combination was considered as having an additive effect (Fig. 1, A and D). When the observed data points for a combination fell into the area below the envelope of additivity, the combination effect was regarded as supra-additive (synergism). When the observed data points for a combination fell outside the square or the cube, the effect of the combination was considered protective. Both subadditive and protective interactions were considered as antagonistic.

To determine the significance of synergism in a combination, Wilcoxon’s signed-rank tests were performed to compare the mean values of the observed data with the predicted minimum or maximum values for the additivity. If the mean value of the observed data was equal to, or smaller than, the predicted maximum value but equal to, or larger than, the predicted minimum value, the effect of the combination was considered additive. If the mean value of the observed data was smaller than the predicted minimum value, the effect of the combination was considered synergistic; if larger than the predicted maximum value, antagonistic, \( P \leq 0.05 \) was considered significant. All of the statistical analyses were performed using Statistica software (StatSoft Inc., Tulsa, Oklahoma).

**Efficacy of Combination Treatments in Animal Models.** All of the animals were maintained, and animal experiments were performed, under NIH and institutional guidelines established for the Animal Core Facility at M. D. Anderson Cancer
Synergism of Combination/Ad-p53-Docetaxel-Radiation

RESULTS

Effect of a Single Therapeutic Agent on Tumor Cell Growth. The dose responses of NSCLC cells to the individual therapeutic agents, Ad-p53, docetaxel, and γ-radiation, were analyzed by determining the relative viability of the treated cells. The cytotoxicity of each agent was assayed in vitro against four NSCLC cell lines (A549, H460, H322, and H1299). Ad-Luc was used as a nonspecific control agent for gene transfer. The dose effect of each agent on tumor cell growth was subjected to the median-effect plot (29, 38). The concentrations of agents that caused 50 and 80% inhibition of growth (ID50 and ID80) were determined (Table 1). Ad-p53, docetaxel, and radiation were shown to be potent cytotoxic to all of the cell lines tested. In terms of Ad-p53 treatment, H1299 cells (p53 null) were the most sensitive and H460 cells (p53 wild type) were the most resistant. In terms of docetaxel treatment, the relative potency of docetaxel against tumor cell growth was (in order from highest to lowest) H322>H460>H1299>A549. In terms of radiation treatment, H460 were the most sensitive and H1299 cells the least sensitive. Ad-Luc was poorly cytotoxic to all of the cell lines tested, at dose levels that transduce up to 80% of cells (data not shown). The ID50 and ID80 for Ad-Luc could be determined only at a very high dose.

Isobolograms Analysis of Two-Agent Combination. The effects of two-agent combinations docetaxel + Ad-p53, Ad-p53 + radiation, docetaxel + radiation, docetaxel + Ad-Luc, and Ad-Luc + radiation on four NSCLC cell lines (A549, H460, H322, and H1299; Fig. 2) were analyzed. All of the experiments were repeated at least three times, with quadruplicate samples for each treatment in each experiment. The observed data and the predicted outcomes of treatment with these combinations are summarized in Table 2. The observed data and the predicted minimum or maximum data were compared using Wilcoxon’s signed-rank test; Ps smaller than 0.05 were considered significant (36, 39).

At the ID50 level, the combination of docetaxel + Ad-p53 produced a significant synergistic effect in H1299 cells (P = 0.046), but an additive effect in A549, H322, and H460 cells (Fig. 2A, D+P). In contrast, at the ID50 level, the combination of docetaxel + Ad-p53 produced a significant synergistic effect in H460 (P = 0.0117), H322 (P = 0.0117), and H1299 (P = 0.028) cells, but an additive effect in A549 cells (Fig. 2B, D+P). At both the ID50 and ID80 levels, the combination of Ad-p53 + radiation was significantly synergistic in all of the cell lines tested (P < 0.05; Fig. 2A and B, P+R). At both ID50 and ID80 levels, the combination of docetaxel + radiation produced an additive effect in H460, H322, and A549 cells with the observed data points being distributed either within the additive envelop or scattered around the boundary between the additive and the synergistic area, whereas the synergistic effect was evident only in H1299 cells (Fig. 2, A and B, D+R; Table 2). By comparison, neither the docetaxel + Ad-Luc nor the Ad-Luc + radiation...
combination produced a significant synergistic effect in any cell line regardless of ID level (Fig. 2, A and B, D+L and L+R; Table 2). Together, these data suggested that the observed synergism in tumor cells treated with Ad-p53 in combination with either docetaxel or radiation was specific for the agents tested.

Isobolograms Analysis of Three-Agent Combinations.

The effects of combinations of the three therapeutic agents under study (Ad-p53, docetaxel, and radiation) on tumor cell growth of four human NSCLC cells (H1299, H460, H322, and A549) were analyzed quantitatively and statistically in vitro (Fig. 3). The observed and predicted values for the combined effects are summarized in Table 2. At both the ID50 and ID80 levels, the combination of docetaxel, Ad-p53, and radiation showed synergistic cancer cell cytotoxicity (P < 0.01 at ID50 and P < 0.001 at ID80) in all four cell lines tested. In contrast, the combination of docetaxel, Ad-Luc, and radiation produced no synergistic effects in any of the cell lines tested at either the ID50 or ID80 levels (Fig. 3, A and B, D+L+R). These observations were consistent with those plotted on the two-dimensional isobolograms with Ad-Luc as a control and suggested that Ad-p53 made an important contribution to the observed synergistic inhibition of NSCLC cell growth by the three-agent combination of docetaxel, Ad-p53, and radiation.


To see whether the synergistic effect of the combination of Ad-p53, docetaxel, and radiation observed in vitro would result in a similar response in vivo, we examined the combined effects of these three agents on tumor growth in H1299 and A549 s.c. xenografts in nude mice (Fig. 4). When the average size of xenografted tumors reached 5–8 mm in diameter, mice were first given a single i.v. docetaxel (30 mg/kg); mock treatment with PBS was used as a control. Twenty-four h after docetaxel injection, mice received injections with Ad-p53, Ad-EV, or Ad-Luc vectors at a dose of 5 × 10^10 vp/tumor; again, PBS was used as a control. Twenty-four h after administration of adenoviral vectors, radiation therapy was carried out by locally irradiating tumors with 5 Gy of 60Co-γ-radiation. For each experiment, mice were divided into 10 treatment groups: four control groups (PBS, docetaxel alone, Ad-p53 alone, and radiation alone); three groups treated with two-agent combinations (docetaxel + Ad-p53, docetaxel + radiation, and Ad-p53 + radiation); and three groups treated with three-agent combinations of docetaxel + Ad-p53 + radiation, docetaxel + Ad-Luc + radiation, and docetaxel + Ad-EV + radiation). Each treatment group contained five mice, and all experiments were repeated twice.

Statistical analysis using the ANOVA method revealed that, as a whole, the combination of docetaxel + Ad5CMV-p53 + γ-radiation significantly inhibited tumor growth in both the H1299 (Fig. 4A) and A549 (Fig. 4B) tumor model (P < 0.05), when compared with other treatments and controls groups. We also analyzed the existence of synergistic interaction of the three-agent combinations in these tumors (see “Materials and Methods”). When combined, docetaxel, Ad-p53, and radiation produced significantly synergistic inhibitory effects on tumor growth in both H1299 and A549 tumor models at day 20 (P < 0.05). By comparison, combinations in which a control vector (either Ad-EV or Ad-Luc) was used with docetaxel and radia-
tion produced much less inhibition of tumor growth in both tumor models (Fig. 4, A and B, DER and DLR), thus suggesting that the synergistic effects of the three-agent combination of docetaxel + Ad-p53 + radiation were specific to the inclusion of Ad-p53 in the combination.

DISCUSSION

Although advances in combined chemoradiation therapy have led to incremental improvements of survival in patients with advanced NSCLC, most patients still succumb to their disease (40). Recently, new chemotherapeutic agents such as the taxanes (e.g., paclitaxel, docetaxel), topoisomerase inhibitors (e.g., topotecan, irinotecan), and novel nucleotide analogues (e.g., gemcitabine, vinorelbine) with different mechanisms of action and cytotoxicity profiles have shown promising antitumor activity in lung cancers and other cancers (41). Docetaxel has been shown to be one of the most active single agents against NSCLC in Phase I and II clinical trials (42). Many of these new agents have been used in combined modality trials with radiotherapy because of their different mechanisms of interaction with ionizing radiation and radiosensitizing potential (40). Several Phase II trials have demonstrated increased sur-
vival and acceptable toxicity in NSCLC patients treated with such combined regimens (40).

A major impediment to successful therapy, however, is the high rate of local failure (>40%; Ref. 2). One possible solution to this problem is to increase the radiation dose to the tumor by using radiosensitizing agents and adding an additional local treatment such as gene therapy. The potential radiation sensitizing effects of certain genes that are inactivated during cancer progression make this an attractive approach. Promising therapeutic responses have been demonstrated in advanced NSCLC treated with wild-type p53 delivered locally with recombinant adenoviral vectors alone or in combination with chemotherapy such as the DNA-damaging agent cisplatin (20, 22, 24, 25).

An aggressive treatment approach combining new chemotherapeutic agents such as docetaxel, gene therapy with Ad-p53, and radiotherapy may improve the outcome for patients with advanced NSCLC. However, what is currently lacking is critical information on the cytotoxicity and target response to such a complicated combined-modality therapy. This study was intended to help fill this gap. In brief, we have developed methods for two- and three-dimensional isobologram modeling and for statistical analysis to evaluate triple combined-modality therapy in human NSCLC cell lines in vitro and in animal models in vivo. We used the improved isobologram method of Steel and Peckham for evaluating the interaction of combined treatments in vitro (35). This method, generally, is much more stringent for analyzing synergism or antagonism than other methods because it coordinates a wider area of uncertainty (envelope of additivity) instead of an additive line. Thus, the possibility of predicting false-positive synergistic or antagonistic interactions, a problem inherent in other methods, was eliminated. The original methods of constructing isobolograms were very complicated and usually involved plotting by hand. We improved the method for making isobolograms for this study by using computer-based dose-response curves and fitting them with the median-effect equation (43). This reduced the inaccuracy and inconvenience of the conventional hand-plotting method. We also developed mathematical and statistical analytical methods for producing sophisticated three-dimensional isobolograms that could be used to evaluate the interaction of the three-agent combinations.

In the present study, we evaluated the interaction of three agents in either two-agent or three-agent combinations in four

### Table 2 Combination effects of docetaxel, Ad-p53, and radiation in two- or three-agent combinations

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<td>Additive</td>
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<td>0.455</td>
<td>0.386</td>
<td>0.744</td>
</tr>
</tbody>
</table>

*<sup>a</sup>Mean values of observed data points.<br>**<sup>b</sup>Predicted minimum values.<br>***<sup>c</sup>Predicted maximum values.<br>**<sup>d</sup>D, docetaxel; P, Ad-p53; R, radiation; L, Ad-Luc.<br>**<sup>e</sup>Mean value of observed data was less than predicted minimum data but not significantly different (P > 0.05).<br>**<sup>f</sup>Mean value of observed data was larger than predicted maximum data but not significantly different (P > 0.05).
NSCLC cell lines at both ID_{50} and ID_{80} levels. We used both ID levels because, although isobologram analysis of combined effects is commonly done at the ID_{50} level, isobologram analysis at the ID_{80} level is more relevant to clinical cancer treatment settings. We found that the combination of Ad-p53 + radiation produced significantly synergistic effects in all of the cell lines tested at both the ID_{50} and ID_{80} levels, whereas the combinations of docetaxel + Ad-p53 and docetaxel + radiation produced mixed effects ranging between additive and synergistic. The three-agent combination also produced significantly synergistic effects in all of the cell lines tested at both the ID_{50} and ID_{80} levels. No antagonistic effects were observed for any two- or three-agent combinations. On the other hand, the combinations containing control vector (docetaxel + Ad-Luc, Ad-Luc + radiation, and docetaxel + Ad-Luc + radiation) showed only additive or additive-to-antagonistic effects in all of the cell lines tested, and no synergistic effects, despite doses of Ad-Luc that exceeded Ad-p53. Together, these findings suggest that the adenoviral vector-mediated wild-type p53 gene therapy specifically induced the observed suppression of NSCLC cell growth. Interestingly, although H460 and A549 cells both contain the wild-type p53 gene and showed resistance to single-agent Ad-p53 treatment in our studies, they also showed a significant response to combination treatments that included Ad-p53 with docetaxel and radiation; moreover, the growth of both cell lines was synergistically inhibited. Together, these results imply that the application of this combined modality therapy might be broadened to include cancers that show resistance to a single therapeutic agent.

Cellular responses to ionizing radiation and to many chemotherapeutics are frequently attributed to (a) the cytotoxicity of such agents to actively proliferating cells and (b) the apoptosis they induce (44, 45). Because apoptosis is a genetically programmed cellular response to environmental stresses or stimuli, mutations in apoptotic pathways could theoretically result in cellular resistance to therapeutic drugs
and radiation treatment. For example, the status of the p53 tumor suppressor gene in tumor cells has been shown to be a strong determinant of cellular response to treatment with either radiation or chemotherapy; the vulnerability of tumor cells to radiation or chemotherapy is greatly reduced by mutations that abolish p53-dependent apoptosis (17, 46–48). These results suggested that the inactivation of p53 might produce treatment-resistance of tumor cells to docetaxel chemotherapy and radiotherapy. On the other hand, they suggest that restoration of p53 function in p53-deficient cells or overexpression of exogenous p53 in p53-wild type tumor cells might overcome the cellular resistance and enhance the cellular response to either chemotherapy or radiotherapy via a mechanism leading to p53-dependent apoptosis (17, 46–48). These results suggested that the inactivation of p53 might produce treatment-resistance of tumor cells to docetaxel chemotherapy and radiotherapy. On the other hand, they suggest that restoration of p53 function in p53-deficient cells or overexpression of exogenous p53 in p53-wild type tumor cells might overcome the cellular resistance and enhance the cellular response to either chemotherapy or radiotherapy via a mechanism leading to p53-dependent apoptosis (17, 46–48).

**REFERENCES**


Synergistic Inhibition of Human Lung Cancer Cell Growth by Adenovirus-mediated Wild-Type p53 Gene Transfer in Combination with Docetaxel and Radiation Therapeutics in Vitro and in Vivo

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