Therapeutic interactions between stathmin inhibition and chemotherapeutic agents in prostate cancer

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Abstract

Limitations of prostate cancer therapy may be overcome by combinations of chemotherapeutic agents with gene therapy directed against specific proteins critical for disease progression. Stathmin is overexpressed in many types of human cancer, including prostate cancer. Stathmin is one of the key regulators of the microtubule network and the mitotic spindle and provides an attractive therapeutic target in cancer therapy. We recently showed that adenovirus-mediated gene transfer of anti-stathmin ribozyme could suppress the malignant phenotype of prostate cancer cells in vitro. In the current studies, we asked whether the therapeutic effects of stathmin inhibition could be further enhanced by exposure to different chemotherapeutic agents. Exposure of uninfected LNCaP human prostate cancer cells or cells infected with a control adenovirus to Taxol, etoposide, 5-fluorouracil (5-FU), or Adriamycin resulted in modest decrease in proliferation and clonogenicity. Interestingly, exposure of cells infected with an anti-stathmin adenovirus to Taxol or etoposide resulted in a complete loss of proliferation and clonogenicity, whereas exposure of the same cells to 5-FU or Adriamycin potentiated the growth-inhibitory effects of the anti-stathmin ribozyme, but the cells continued to proliferate. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling analysis of uninfected cells or cells infected with a control adenovirus showed modest induction of apoptosis in the presence of different drugs. In contrast, cells infected with the anti-stathmin adenovirus showed a marked increase in apoptosis on exposure to Taxol or etoposide and a modest increase on exposure to 5-FU or Adriamycin. Overall, the effects of combinations of anti-stathmin ribozyme with Taxol or etoposide were synergistic, whereas the effects of combinations of anti-stathmin ribozyme with 5-FU or Adriamycin were additive. Moreover, triple combination of anti-stathmin ribozyme with low noninhibitory concentrations of Taxol and etoposide resulted in a profound synergistic inhibition of proliferation, clonogenicity, and marked induction of apoptosis. This synergy might be very relevant for the treatment of prostate cancer because Taxol and etoposide are two of the most effective agents in this disease. Thus, this combination may provide a novel form of prostate cancer therapy that would avoid toxicities associated with the use of multiple chemotherapeutic agents at full therapeutic doses. [Mol Cancer Ther 2006;5(12):3248–57]

Introduction

Prostate cancer is the most frequently diagnosed malignancy and the second leading cause of cancer-related deaths among men in the United States. In the early stage of the disease, the treatments of choice are radical surgery or radiation therapy. Both treatment modalities are associated with significant morbidity and mortality. Although, in advanced prostate cancer, systemic androgen ablation frequently leads to tumor regression, the disease usually progresses to an androgen-independent state that does not respond to endocrine manipulations. Although chemotherapy can have some therapeutic benefits, prostate cancer is widely viewed as a chemoresistant neoplasm. Thus, novel therapeutic approaches are needed to improve the outlook for patients with advanced prostate cancer.

Stathmin is the founding member of a family of microtubule–destabilizing proteins that play a critically important role in the assembly and disassembly of the mitotic spindle (1–4). It regulates the mitotic spindle through cell cycle–dependent changes in its state of phosphorylation that are mediated by p34cdc2 kinase, mitogen-activated protein kinase, and other kinases (2, 5–8). Stathmin is expressed at high levels in a wide variety of human malignancies, including prostate carcinoma (9–12), and provides an attractive target for cancer therapy (13). High levels of stathmin expression in cancer cells were shown to correlate with their proliferative potential and seem to be necessary for the maintenance of their malignant phenotype (13–15). Interestingly, when biopsy specimens from human prostate cancers were immunostained with an anti-stathmin antibody, immunoreactivity was seen in poorly differentiated tumors but not in hyperplastic prostate or highly differentiated tumors (12). More importantly, the level of expression of stathmin was shown to correlate with the malignant behavior of prostate cancer (12). Hence, it was proposed...
that the level of expression of stathmin may serve as a prognostic marker in prostate cancer (12).

We recently generated replication-deficient bicistronic adenoviral vectors that carry an anti-stathmin ribozyme that targets stathmin mRNA in prostate cancer cells (16, 17). We showed that adenovirus-mediated gene transfer of anti-stathmin ribozyme can suppress the malignant phenotype of prostate cancer cells in vitro (17). In this study, we asked whether the therapeutic effects of anti-stathmin ribozyme in prostate cancer could be further enhanced by exposure to chemotherapeutic agents. Thus, we evaluated the effects of combinations of anti-stathmin adenovirus with four different chemotherapeutic agents on proliferation, clonogenicity, and apoptosis in human prostate cancer cells.

Materials and Methods

Reagents

Taxol (paclitaxel), etoposide, 5-fluorouracil (5-FU), and Adriamycin were purchased from Sigma (St. Louis, MO). All four drugs were dissolved in DMSO as a 10 mmol/L stock solution and stored at −20°C in aliquots.

Cell Lines

The human androgen-independent LNCaP prostate cancer cell line that we used in this study was described previously (18). LNCaP cells were grown in RPMI 1640 supplemented with 10% charcoal-stripped fetal bovine serum, 5 μg/mL human insulin, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained at 37°C in a humidified 5% CO2 environment.

Production of Recombinant Adenoviruses

The recombinant adenoviruses that we used in this study were previously described in detail (17). Briefly, a replication-deficient bicistronic adenoviral vector (Ad.Rz.GFP) that coexpresses green fluorescent protein (GFP) and an anti-stathmin ribozyme (Rz305) was constructed for targeting human stathmin mRNA (16, 17). The control vector contained the GFP reporter gene under the control of the CMV5 promoter without the anti-stathmin ribozyme sequences (17). Each recombinant transfer plasmid was cotransfected with part of the Ad5 genome selected to promote in vivo homologous recombination between the two DNA molecules, resulting in infectious adenoviruses. The recombinant adenoviruses were propagated in 293 cells, purified by cesium chloride gradient ultracentrifugation, dialyzed, and stored at −80°C (17, 19). The infectious viral titers were determined by plaque assays in 293 cells (17, 19).

Adenoviral Infections in vitro

Cells were seeded in six-well culture plates 24 h before virus infection. For all experiments described below, cells were infected with either control Ad.GFP or anti-stathmin Ad.Rz.GFP adenoviruses at a multiplicity of infection (MOI) of 5 in 2% reduced serum medium for 3 h. After infection, the virus was removed and the cells were further incubated in complete growth medium. The efficiency of transduction by the recombinant adenoviruses was confirmed by measuring the fraction of cells that expressed GFP by flow cytometry or fluorescence microscopy as described previously (17).

Cell Proliferation Assays

To assess the rate of proliferation, equal numbers of cells (2 × 10^5) were infected with either control or anti-stathmin adenoviruses in triplicates and the cells were exposed to different chemotherapeutic agents at various concentrations (Taxol, 1–6 nmol/L; etoposide, 0.5–2 μmol/L; 5-FU, 1–5 μmol/L; or Adriamycin, 2–10 nmol/L). The cells (floating and detached) were harvested and stained with trypan blue to determine cell viability. Viable cells were counted in a hemocytometer on alternate days, and growth curves were generated using the means of triplicate alternate day cell counts.

To assess the effects of triple combination of anti-stathmin ribozyme, Taxol, and etoposide, uninfected cells and cells infected with either Ad.GFP or Ad.Rz.GFP were incubated in the presence or absence of two different groups of noninhibitory concentrations of Taxol and etoposide: (a) 1 nmol/L Taxol and 0.25 μmol/L etoposide or 1 nmol/L Taxol and 0.5 μmol/L etoposide and (b) 2 nmol/L Taxol and 0.25 μmol/L etoposide or 2 nmol/L Taxol and 0.5 μmol/L etoposide. Cells were counted on alternate days, and growth curves were generated as above.

Clonogenic Assays

Anchorage-independent growth was assessed by colony formation in a methylcellulose semisolid medium (17). Equal numbers of cells were infected with either control or anti-stathmin adenoviruses in triplicates as above, and the cells were incubated in growth medium containing different chemotherapeutic agents (Taxol, 4 nmol/L; etoposide, 2 μmol/L; 5-FU, 5 μmol/L; or Adriamycin, 10 nmol/L) for 3 days. The cells were then washed in PBS, and equal numbers of cells (1 × 10^5) were resuspended in 5 mL methylcellulose-based semisolid medium (0.9% methylcellulose, 1% bovine serum albumin, and 0.1 mmol/L β-mercaptoethanol prepared in RPMI 1640 containing 30% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin; ref. 17). The cells were plated in six-well tissue culture plates in triplicates and incubated at 37°C in 5% CO2. The colonies that formed were counted after 2 weeks. For triple combination studies, uninfected cells or cells infected with either control Ad.GFP or Ad.Rz.GFP were incubated in growth medium with or without Taxol (1 nmol/L) and etoposide (0.5 μmol/L) for 3 days. The cells were plated in methylcellulose as above.

Cell Cycle Analysis

We used propidium iodide staining of fixed whole cells to analyze the distribution of cells in the different phases of the cell cycle as described previously (17). LNCaP cells were infected either with control or anti-stathmin adenoviruses at a MOI of 5 as above. Three hours after infection, the virus was removed and the cells were incubated in growth medium without drugs or in growth medium containing 1 nmol/L Taxol and 0.5 μmol/L etoposide. After 48 h, the cells were harvested, fixed in 70% ethanol, washed in PBS, and resuspended for 30 min at 37°C in 1 mL propidium iodide solution (PBS containing 0.05 mg/mL, 0.1% sodium citrate, and 1 μg/mL RNase; ref. 17). DNA
content was analyzed within 2 h in a Becton Dickinson (Bedford, MA) FACStar Plus flow cytometer at 488-nm single laser excitation. The cell cycle distribution was analyzed using WinList software.

**Evaluation of Therapeutic Interactions**

The therapeutic interactions between the anti-stathmin adenovirus and the different drugs were analyzed according to the method of Chou and Talalay (20) with the help of the Calcusyn software suite (Biosoft, Cambridge, United Kingdom). Median effect plots were determined by generating dose-response curves for anti-stathmin adenovirus, Taxol, etoposide, Adriamycin, and 5-FU. Combination index (CI) values were then calculated at different drug concentrations, and the Fa-CI plots were generated by Calcusyn. According to Chou and Talalay, a CI of <1 indicates a synergistic interaction, a CI of 1 indicates an additive interaction, and a CI of >1 indicates an antagonistic interaction (20).

**Apoptosis Assays**

To evaluate the effects of the combinations of anti-stathmin ribozyme with different chemotherapeutic agents on apoptosis, we used terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (17). Cells infected with either control or anti-stathmin adenoviruses were incubated in growth medium containing different chemotherapeutic agents (Taxol, 4 nmol/L; etoposide, 2 μmol/L; 5-FU, 5 μmol/L; or Adriamycin, 10 nmol/L). Both floating and attached cells were harvested 5 days later, and the effect of ribozyme-mediated stathmin inhibition on apoptosis was quantified. DNA fragmentation was assessed by TUNEL assay using a cell death detection kit (Roche Applied Science, Indianapolis, IN) according to the instructions of the manufacturer. Briefly, the cells were fixed, permeabilized, and incubated with the TUNEL reaction mixture. The free 3’ OH groups of fragmented DNA labeled with tetramethylrhodamine-labeled nucleotides were quantified by flow cytometry as described (17). For triple combination studies, cells infected with either Ad.GFP or Ad.Rz.GFP were incubated in the presence or absence of Taxol (1 nmol/L) and etoposide (0.5 μmol/L) for 5 days and analyzed by the TUNEL assay as above.

**Statistical Analysis**

The data are expressed as mean ± SD. The data were analyzed for statistical significance using the two-tailed Student’s t test. *P* < 0.05 were considered statistically significant.

**Results**

We evaluated the effects of anti-stathmin ribozyme in combination with different chemotherapeutic drugs on the growth rates of LNCaP cells. Figure 1 illustrates the effects of different concentrations of four chemotherapeutic drugs on the growth of LNCaP cells. Figure 1A shows the effects of different concentrations of Taxol or etoposide, and Figure 1B shows the effects of different concentrations of 5-FU or Adriamycin. Points, mean of triplicate alternate day cell counts.

![Figure 1](attachment:image_url)

**Figure 1.** Effects of combination of chemotherapeutic agents and anti-stathmin adenovirus on the rate of proliferation of LNCaP cells. A, growth curves of uninfected cells and cells infected with either control Ad.GFP or Ad.Rz.GFP adenoviruses in the presence and absence of different concentrations of Taxol or etoposide. B, growth curves of uninfected cells and cells infected with either control Ad.GFP or Ad.Rz.GFP adenoviruses in the presence and absence of different concentrations of 5-FU or Adriamycin. Points, mean of triplicate alternate day cell counts.
on the rate of proliferation of LNCaP cells in the presence and absence of anti-stathmin ribozyme. Interactions between different therapeutic agents are best evaluated at low subtherapeutic concentrations to avoid saturation artifacts. In the experiment illustrated in Fig. 1, we used a low MOI of the recombinant adenoviruses (i.e., MOI of 5) and low concentrations of the different chemotherapeutic drugs. Transduction efficiencies in cells infected with the recombinant adenovirus ranged from 65% to 75% in the experiments described. The concentrations of the different drugs that we used were selected in pilot experiments and were significantly below the IC50 (data not shown). The proliferative rates of uninfected cells or cells infected with the control Ad.GFP virus were not affected by the lower concentrations of Taxol (1–2 nmol/L) and were modestly inhibited at increasing concentrations of Taxol (4–6 nmol/L; Fig. 1A). Similarly, exposure of uninfected cells or cells infected with the control Ad.GFP virus to etoposide resulted in modest dose-dependent decrease in growth rates (Fig. 1A). In contrast, exposure of cells infected with Ad.Rz.GFP virus to the same concentrations of Taxol or etoposide resulted in marked growth inhibition at the lower concentrations and a virtually complete loss of proliferation at the higher concentrations of Taxol or etoposide (Fig. 1A). Exposure of cells to different concentrations of 5-FU (1–5 μmol/L) or Adriamycin (2–10 nmol/L) also resulted in a modest dose-dependent decrease in proliferation of uninfected cells and cells infected with the control Ad.GFP virus (Fig. 1B). Although exposure of Ad.Rz.GFP-infected cells to 5-FU or Adriamycin resulted in further growth inhibition, the cells continued to proliferate (Fig. 1B). Thus, stathmin inhibition seemed to result in greater sensitization of prostate cancer cells to the growth-inhibitory effects of Taxol and etoposide than to those of 5-FU or Adriamycin.

We also evaluated the effects of the combinations of anti-stathmin ribozyme with different chemotherapeutic agents on apoptosis in LNCaP cells (Fig. 2). TUNEL analysis of cells infected with the control Ad.GFP adenovirus showed very little apoptosis (0.4%; Fig. 2A). When the control Ad.GFP-infected cells were exposed to Taxol, etoposide, Adriamycin, or 5-FU, the fraction of TUNEL-positive cells increased modestly to 2.8%, 3.5%, 1.1%, and 1.7%, respectively (Fig. 2B–E). Infection of cells with Ad.Rz.GFP adenovirus alone at a low MOI resulted in a slightly larger fraction of TUNEL-positive cells (3.7%) than in cells infected with the control Ad.GFP virus (0.4%; Fig. 2A). The fraction of TUNEL-positive cells were also slightly higher when Ad.Rz.GFP-infected cells were exposed to Adriamycin and 5-FU (6.6% and 7.2%, respectively; Fig. 2D and E). However, a much larger increase in the fraction of apoptotic cells was observed when Ad.Rz.GFP-infected cells were exposed to Taxol (25.8%) or etoposide (22.2%; Fig. 2A and B). Thus, when anti-stathmin adenovirus is used in combination with chemotherapeutic agents, a much larger increase in apoptosis was seen in the presence of etoposide and Taxol than in the presence of Adriamycin and 5-FU.

We then examined the effects of combinations of anti-stathmin ribozyme with different chemotherapeutic agents on the clonogenic potential of LNCaP cells. The clonogenic assay is the *in vitro* assay that correlates best with *in vivo* assays of tumorigenicity (21, 22). Figure 3 illustrates the effects of combinations of Ad.GFP or Ad.Rz.GFP adenoviruses with different drugs on the clonogenicity of LNCaP cells. Clonogenicity of cells infected with the control Ad.GFP virus was reduced by 35%, 39%, 31%, and 32% on exposure to Taxol (Fig. 3B), etoposide (Fig. 3C), Adriamycin (Fig. 3D), and 5-FU (Fig. 3E), respectively.
The decrease in the clonogenic potential of Ad.GFP-infected cells on exposure to the same drugs was similar to that seen in uninfected cells (Fig. 3B–E). Moreover, stathmin inhibition alone resulted in a 42% decrease in clonogenicity in Ad.Rz.GFP-infected cells relative to control Ad.GFP-infected cells (P = 0.003; Fig. 3A). When Ad.Rz.GFP-infected cells were exposed to Taxol or etoposide, clonogenicity was profoundly decreased by 96% (P = 0.0005; Fig. 3B) and 98% (P = 0.0003; Fig. 3C), respectively. In comparison, clonogenicity of the same cells was only moderately reduced to 40% (P = 0.006) and 38% (P = 0.003) on exposure to Adriamycin (Fig. 3D) or 5-FU (Fig. 3E). Thus, the observed decrease in clonogenicity following exposure to anti-stathmin adenovirus with Taxol (Fig. 3B) or etoposide (Fig. 3C) is much greater than the decrease in clonogenicity following exposure to anti-stathmin adenovirus with Adriamycin (Fig. 3D) or 5-FU (Fig. 3E). The same combinations were tested at different drug concentrations, and the observed antilognomous effects in Ad.Rz.GFP-infected cells were always more potent in cells exposed to Taxol or etoposide than in cells exposed to 5-FU or Adriamycin (data not shown).

We used the median effect analysis method of Chou and Talalay (20) to determine whether the observed effects on clonogenicity were additive or synergistic. This method identifies an interaction as synergistic, additive, or antagonistic by determining the difference between the observed combination effect and the expected additive effect. We used the CalcuSyn software that uses the equations of Chou and Talalay to assess the therapeutic interactions between the anti-stathmin adenovirus and different chemotherapeutic agents. This software takes into account both the potency (Dm values) and the shapes of the dose-effect curves (m values) to precisely analyze the combination effect of two agents. The CI values and the fraction affected (Fa) for each dose was used to generate the Fa-CI plots (Fig. 4). The CI values were determined to be <1 when the anti-stathmin adenovirus was combined with different concentrations of either Taxol (1–6 nmol/L; Fig. 4A) or etoposide (0.25–2 μmol/L; Fig. 4B). In contrast, the CI values were ~1 when the anti-stathmin adenovirus was combined with different concentrations of either Adriamycin (1–10 nmol/L; Fig. 4C) or 5-FU (0.5–5 μmol/L; Fig. 4D). Thus, these data are indicative of a synergistic interaction when the anti-stathmin adenovirus is combined with either Taxol or etoposide and an additive interaction when the anti-stathmin adenovirus is combined with either Adriamycin or 5-FU.

Because taxanes and etoposide are frequently used together in combination therapy in prostate cancer, we tested the combination of anti-stathmin ribozyme with Taxol and etoposide on proliferation, clonogenicity, and apoptosis in LNCaP cells. In these studies, we used much lower concentrations of Taxol and etoposide that had no significant growth-inhibitory effects in uninfected cells or cells infected with the control Ad.GFP virus. These concentrations were also noninhibitory when used in cells infected with Ad.Rz.GFP adenovirus as shown in Fig. 1. Figure 5 illustrates the effects of the triple combination of anti-stathmin ribozyme, Taxol, and etoposide on the rate of proliferation of LNCaP cells. Exposure of uninfected cells or cells infected with control Ad.GFP virus to low noninhibitory concentrations of either Taxol (1 nmol/L) or etoposide (0.25 or 0.5 μmol/L) resulted in a modest but reproducible decrease in the rate of proliferation (Fig. 5A). In contrast, exposure of Ad.Rz.GFP-infected cells to 1 nmol/L Taxol and 0.25 μmol/L etoposide resulted in a more marked decrease in the rate of proliferation, whereas a complete loss of proliferation was observed at 1 nmol/L Taxol and 0.5 μmol/L etoposide (Fig. 5A). When uninfected cells or cells infected with control Ad.GFP virus were exposed to higher but also noninhibitory concentrations (2 nmol/L Taxol and 0.25 μmol/L etoposide or 2 nmol/L Taxol and 0.5 μmol/L etoposide), there was a slight

![Figure 3. Effects of combination of anti-stathmin adenovirus and chemotherapeutic agents on the clonogenic potential of LNCaP cells.](image-url)
increase in inhibition of proliferation (Fig. 5B). However, exposure of cells infected with Ad.Rz.GFP adenovirus to the same concentrations resulted in complete cessation of growth (Fig. 5B). Similarly, in vitro clonogenic assays, the observed anticlonogenic effects in Ad.Rz.GFP-infected cells at different noninhibitory drug concentrations were always more potent than the effects of anti-stathmin ribozyme alone or the drugs alone. Figure 6 is a representative illustration of the effects of low noninhibitory concentrations of Taxol (1 nmol/L) and etoposide (0.5 μmol/L) on the clonogenic potential of LNCaP cells in the presence and absence of stathmin inhibition. Clonogenicity of uninfected cells or cells infected with control Ad.GFP virus was modestly reduced by 20% following exposure to low concentrations of Taxol and etoposide (Fig. 6B) relative to the clonogenicity of the same cells in the absence of the drugs (Fig. 6A). In contrast, stathmin inhibition alone decreased the clonogenicity of the Ad.Rz.GFP-infected cells by 42% ($P = 0.003$; Fig. 6A). More strikingly, when Ad.Rz.GFP-infected cells were exposed to Taxol and etoposide, clonogenicity was drastically reduced by >98% ($P = 0.0006$; Fig. 6B).

To gain insights into the mechanism of synergistic inhibition of growth on exposure of LNCaP cells to anti-stathmin ribozyme, Taxol, and etoposide, we compared the cell cycle distribution of cells that were exposed to Taxol and etoposide in the presence and absence of stathmin inhibition (Fig. 7). Figure 7A shows the cell cycle distribution of uninfected cells, cells infected with the control Ad.GFP adenovirus, and cells infected with Ad.Rz.GFP adenovirus in the absence of the drugs. Exposure of uninfected cells or control Ad.GFP-infected cells to low noninhibitory concentrations of Taxol (1 nmol/L) and etoposide (0.5 μmol/L) resulted in a modest increase in accumulation of cells in the G2-M phases, with a corresponding decrease in the fraction of cells in the G0-G1 phases of the cell cycle (Fig. 7B). In contrast, a much more profound mitotic arrest occurred when the cells were exposed to the same concentrations of Taxol and etoposide in the presence of stathmin inhibition (Fig. 7B).

We also analyzed the effects of the triple combination on apoptosis by TUNEL assay. The cells infected with the control Ad.GFP virus showed a very small fraction of TUNEL-positive cells (0.3%), whereas infection with the Ad.Rz.GFP virus resulted in slightly more TUNEL-positive cells (3.6%; Fig. 8A). After exposure to low noninhibitory concentrations of Taxol (1 nmol/L) and etoposide (0.5 μmol/L), the fraction of TUNEL-positive cells in Ad.Rz.GFP-infected cells increased to 37.9% (Fig. 8B). Thus, in this assay too, exposure to low noninhibitory concentrations of Taxol and etoposide resulted in a much greater increase in the fraction of TUNEL positivity in cells infected with Ad.Rz.GFP adenovirus compared with uninfected cells or cells infected with the control Ad.GFP adenovirus (data not shown). Thus, stathmin inhibition can enhance the effects of very low concentrations of Taxol and etoposide to result in very potent inhibition of proliferation, clonogenicity, and marked induction of apoptosis.

Discussion
Prostate cancer is generally considered a chemotherapy-insensitive disease. Conventional chemotherapy in advanced prostate cancer has modest effects on survival and is not curative. Although Taxol is one of a few chemotherapeutic agents that have some activity against
prostate cancer cells in vitro, the results of clinical studies in which Taxol was used as a single agent in prostate cancer have been generally disappointing (23, 24). Its clinical use is further reduced due to toxicity associated with its long-term administration at high doses. Thus, the mitotic spindle and the strategies that target mitosis may provide an attractive therapeutic strategy for advanced prostate cancer.

Stathmin may provide an excellent molecular target for prostate cancer therapy (12, 17). We had previously described the design and testing of several anti-stathmin hammerhead ribozymes that cleave stathmin mRNA catalytically (16). More recently, we incorporated these ribozymes into adenoviral gene transfer vectors for targeting stathmin in prostate cancer cells (17). Our studies showed that the anti-stathmin adenoviruses can suppress the malignant phenotype of prostate cancer cells (17). In this report, we examined the hypothesis that an anti-stathmin ribozyme may be of greater therapeutic benefit if combined with chemotherapeutic agents, especially ones that target the mitotic spindle. Thus, we evaluated the therapeutic interactions between an anti-stathmin ribozyme and four different chemotherapeutic agents in assays of proliferation, clonogenicity, and apoptosis in human prostate cancer cells. We examined the effects of combination of anti-stathmin adenovirus with a microtubule-interfering drug (Taxol), a topoisomerase inhibitor (etoposide), an antimetabolite (5-FU), and an anthracycline (Adriamycin). Although the anti-stathmin ribozyme chemosensitized LNCaP cells to all four chemotherapeutic agents, the therapeutic interactions with the different agents were significantly different. In all three assays, exposure of Ad.Rz.GFP-infected cells to either Taxol or etoposide resulted in striking growth-inhibitory effects, marked inhibition of clonogenic potential, and profound induction of apoptosis. These observations are of considerable clinical interest because complete inhibition of growth could be achieved at concentrations that resulted in <50% inhibition when these chemotherapeutic agents were used as single agents. Just as importantly, profound inhibitory effects were seen at a relatively low MOI of the anti-stathmin adenovirus and at subtherapeutic concentrations of the drugs. In comparison, although exposure of the same cells to 5-FU or Adriamycin potentiated the growth-inhibitory effects of the anti-stathmin ribozyme, the LNCaP cells continued to proliferate. When these interactions were analyzed further by the method of Chou and Talalay, these interactions between anti-stathmin therapy with Taxol or etoposide were clearly synergistic. In contrast, the interaction of 5-FU or Adriamycin with stathmin inhibition was additive. These observations are particularly relevant because prostate cancer is more sensitive to Taxol and etoposide than to 5-FU and Adriamycin. Although most of the cells were infected by the recombinant adenoviruses in the experiments described, we cannot rule out the existence of bystander effects. As seen in Figs. 2 and 8, a small fraction of cells were observed to be GFP negative and were still TUNEL positive (<5%), which is compatible with a bystander effect. Alternatively, it is also possible that the small fraction of GFP-negative cells may have been transduced by very low copy of the virus. Consequently, they may have seemed uninfected by flow cytometry but were phenotypically affected by stathmin inhibition.
Because both Taxol and stathmin inhibition interfere with the regulation of the microtubules that make up the mitotic spindle, it is not surprising that the combination of the two interventions would result in a synergistic interaction. These findings are consistent with our previous study that showed synergistic inhibition of growth of K562 leukemic cells on stathmin inhibition and Taxol exposure (25). Nonetheless, the exact molecular mechanism that is responsible for the observed synergistic interaction between stathmin inhibition and Taxol is not clear. Numerous lines of evidence have shown that a deficiency in stathmin decreases the rate of catastrophes and sequestration of tubulin molecules, thereby shifting the equilibrium between the polymerized and unpolymerized tubulin in favor of polymerized tubulin (1, 3, 26, 27). Taxol, on the other hand, stabilizes microtubules by binding to polymerized tubulin (28). Hence, when stathmin-inhibited cells are exposed to Taxol, the cells will have difficulty depolymerizing the microtubules due to stathmin deficiency and the polymerized microtubules will be further stabilized by Taxol binding. This may explain, at least in part, the observed synergy between stathmin inhibition and Taxol exposure.

As we had previously seen with Taxol exposure (25), exposure of cells to etoposide also arrests cells in the G2-M phases of the cell cycle, eventually leading to apoptotic cell death (data not shown). However, unlike Taxol, microtubule staining of etoposide-treated LNCaP cells revealed

**Figure 6.** Effects of triple combination of anti-stathmin adenovirus, Taxol, and etoposide on the clonogenic potential of LNCaP cells. A, clonogenicity of uninfected, control Ad.GFP-infected, and Ad.Rz.GFP-infected cells at baseline in the absence of Taxol and etoposide. B, clonogenicity of uninfected, control Ad.GFP-infected, and Ad.Rz.GFP-infected cells in the presence of noninhibitory concentrations of Taxol (1 nmol/L) and etoposide (0.5 μmol/L). Columns, mean of three different experiments; bars, SD. Statistical significance was determined using Student’s t test. Asterisks, statistically significant inhibition of clonogenicity.

**Figure 7.** Effects of triple combination of anti-stathmin adenovirus, Taxol, and etoposide on the cell cycle distribution of LNCaP cells. A, DNA histograms of uninfected cells, cells infected with the control Ad.GFP, and cells infected with Ad.Rz.GFP adenovirus in the absence of drug exposure. B, DNA histograms of uninfected cells, cells infected with the control Ad.GFP, and cells infected with Ad.Rz.GFP adenovirus after 48 h of exposure to 1 nmol/L Taxol and 0.5 μmol/L etoposide.
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Very rare mitotic figures (data not shown). This suggests that exposure to etoposide blocks cells in the G2 phase of the cell cycle. These observations agree with the studies of Lock and Ross (29) and Lock (30) who showed that exposure of Chinese hamster ovary cells to etoposide results in a decline in the mitotic index and the arrest of cells in late G2 phase. They also showed that the etoposide-induced G2 arrest results from the rapid inhibition of the activity of p34<sup>cdc2</sup>, a protein kinase that is critical for the transition from the G2 phase into mitosis in eukaryotic cells (29, 30). p34<sup>cdc2</sup> kinase is known to phosphorylate a variety of cellular proteins, including Rb and p53, in a cell cycle–dependent manner (31, 32). Previous studies from our own laboratory had shown a cell cycle–dependent increase in the level of phosphorylation of stathmin in the G2-M phases that is mediated by p34<sup>cdc2</sup> kinase (6). When stathmin is phosphorylated by p34<sup>cdc2</sup> kinase as cells enter mitosis, its microtubule-depolymerizing activity is lost, tubulin is polymerized, and the mitotic spindle is formed (2). In subsequent studies, we had also shown that dephosphorylation of stathmin late in mitosis is necessary for spindle disassembly and the exit from mitosis (3, 4). Thus, in cells exposed to etoposide in which stathmin is inhibited, the cells might have difficulty entering mitosis due to inhibition of p34<sup>cdc2</sup> kinase and difficulty exiting mitosis due to stathmin deficiency.

We believe that the effects of the triple combination of anti-stathmin therapy with Taxol and etoposide at low noninhibitory concentrations may be important for the design of effective therapies in the future. In all three assays, the observed effects were much greater than the effects of ribosome with Taxol or ribosome with etoposide. Although synergistic interactions were observed when anti-stathmin therapy was combined with either Taxol or etoposide at subtherapeutic concentrations, the triple combination resulted in complete inhibition of growth and clonogenicity at low noninhibitory concentrations of the drugs. We hypothesize that, when the prostate cancer cells in which stathmin is inhibited are exposed to Taxol and etoposide simultaneously, cells will have difficulty entering mitosis, depolymerizing their spindles, and exiting mitosis. This hypothesis is supported by a more profound G2-M arrest in cells exposed to Taxol and etoposide in the presence of stathmin inhibition than in the absence of stathmin inhibition (Fig. 7). Thus, anti-stathmin ribozyme can markedly enhance the effects of low noninhibitory (and probably nontoxic) concentrations of Taxol and etoposide and may lead to profound inhibition of tumor growth and marked induction of apoptosis. Because Taxol and etoposide are two of the most active chemotherapeutic agents in prostate cancer, combination of these agents with stathmin inhibition may provide a superior form of combination therapy that would also avoid toxicities associated with the use of multiple chemotherapeutic agents at their maximally tolerated doses.

Figure 8. Effects of triple combination of anti-stathmin adenovirus, Taxol, and etoposide on apoptosis in LNCaP cells. A, dot plot showing the fraction of TUNEL positivity in Ad.GFP- and Ad.Rz.GFP-infected cells in the absence of Taxol and etoposide. B, dot plot showing the fraction of TUNEL positivity in Ad.GFP- and Ad.Rz.GFP-infected cells in the presence of noninhibitory concentrations of Taxol (1 nmol/L) and etoposide (0.5 nmol/L). The experiment is a representative of three independent experiments.

References


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