Abrogation of Estrogen Receptor Signaling Augments Cytotoxicity of Anticancer Drugs on CaSki Cervical Cancer Cells

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Abstract. *Background: We have reported that a gene therapy approach, using a dominant-negative estrogen receptor blocker (DN) gene, can cause cell death in cervical cancer cells in vitro. We investigated the mechanisms for enhanced cell killing when DN was combined with cisplatin (CP) and paclitaxel (TX). Materials and Methods: Cells were transduced with DN at 24 h and/or treated with drugs at 48 h, and harvested at 48 and 72 h after transduction. Effects were determined using the MTT cytotoxic, and TUNEL and caspase-3 activity apoptotic assays. Results: Each agent induced cytotoxic and apoptotic effects, and activated caspase-3. In the combined treatments, significant synergistic effects were observed based on the MTT and TUNEL assays, but with antagonistic caspase-3 activation effect. Conclusion: The enhanced cell killing effect was mediated by the initiation of new and multiple mechanisms, particularly via caspase-independent pathways.*

Cancer is a complex disease that frequently involves the accumulation of multiple genetic/cellular abnormalities in a multistep process. However, many of these diverse and complex activities may be controlled by only a limited number of signature mechanisms. For example, inactivation of the p53 tumor suppressor gene is a key event for lung and a variety of other types of cancer. Estrogen imbalance is a critical factor in the development of a variety of gynecological cancers including cervical cancer (1-3). The latter is strongly supported by several cervical cancer mouse models which indicate that estrogen is required for the initiation and maintenance of cervical cancer (4, 5). These signature pathways have become prime targets for the development of precise intervention and therapeutic protocols for cancer. One of these approaches is focused on the development of targeted gene therapy protocols (6-8).

We have been using a gene therapy approach in cervical cancer cells (9). Specifically, a transduction of a low dose of an adenovirus which expresses a dominant negative estrogen receptor gene (DN) into CaSki cervical cancer cells reduced the expression of HPV E6 and E7 oncogenes, blocked cell proliferation at the G1- and S-phases, inhibited expression of cyclin D1 and caused apoptosis in approximately 15% of the cancer cells. It appears that the transduction caused the restoration of apoptosis-inducing mechanisms, a highly relevant mechanism in cancer therapy strategy (9).

The use of adenovirus, or other viruses, as a delivery mechanism for gene therapy can produce some undesirable effects (10), *e.g.* induction of the host immune response and systemic toxicity. In addition, gene therapy alone may not deliver complete therapeutic success, as cancer cells can develop resistance mechanisms (11) and host toxicity limits the use of high doses of gene therapy vectors. Therefore, the use of therapeutic protocols that combine different therapeutic mechanisms may achieve more desirable efficacy while minimizing side-effects (12, 13). The potential benefits of gene in combination with drugs for combined therapy are beginning to gain recognition (14, 15). We investigated the potential therapeutic effect of DN in combination with the conventional chemotherapeutic drugs, cisplatin (CP) and paclitaxel (TX), in the same cervical cancer cell line, CaSki, which we have studied before (9). The two drugs were selected because they have been used to treat cervical cancer and have independent mechanisms of action for the induction of cytotoxicity (16-18). The interactive effects between the gene and chemotherapy were

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Key Words: Cervical cancer, estrogen receptor, gene therapy, chemotherapy, cytotoxicity, apoptosis, MTT, caspase-3, CaSki.

0250-7005/2008 $2.00+.40

investigated based on their induction of cytotoxicity (MTT assay) and, specifically, of apoptosis (TUNEL and caspase 3 activity assays).

Materials and Methods

Cell line, chemical supplies and vectors. Briefly, CaSki cells contain approximately 600 copies of the HPV-16 genome per cell and express estrogen receptor (19). DN produces dominant negative estrogen receptor proteins intracellularly that bind and inhibit cellular estrogen receptor proteins (20).

CP and TX were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other laboratory supplies were purchased from Sigma-Aldrich (St. Louis, MO, USA), Fisher (Houston, TX, USA), *etc*.

Transduction and treatment protocols. For culture and transduction of CaSki cells with DN, the procedures have been described in our previous publication (9). Briefly, exponentially growing cells at 24 h after seeding were transduced with DN of different plague forming units (pfu) per cell for 4 h at 37˚C, with slow rocking of the culture plates. For 96-well plate cultures, 50 µl of DN was added to each well and 500 µl was added for the 6-well-plate cultures. An appropriate amount of excess complete medium was added to each well. The plates were left in the incubator until the time of harvest.

For treatment with CP and TX, exponentially growing cells, at 48 h after seeding, were treated with different concentrations of each of the two chemotherapeutic drugs. The drugs were dissolved in 10% ethanol. Untreated cells and cells treated with 1% ethanol were used as negative and solvent controls. Cells were harvested at 24, 48 and 72 h after treatment.

For the combined treatment protocol, cells were transduced with DN at 24 h after seeding and with each of the drugs at 48 h after seeding. Treated cells were harvested at 24 and 48 h after the drug treatment.

MTT assay for cytotoxicity. The assay kit was purchased from Sigma-Aldrich. The assay is based on the color change from cleavage of the yellow MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) by mitochondrial and cytosolic dehydrogenases of living cells to the purple formazan dye. Dead cells would have zero to low concentrations of the dehydrogenases. The change in color was determined quantitatively using a spectrophotometer with reading at 570 nm wavelength (Spectra Max model 190; Molecular Devices, Sunnyvale, CA, USA). These experiments were run with triplicate wells and three independent experiments were conducted.

At the beginning of our investigation, the MTT assay was used for determining dose-dependent cytotoxicity of each agent: DN, CP and TX. CaSki cells were plated into 96-well plates with 20,000 cells per well for each treatment condition and 15,000 for each control condition. At 24 h after plating, cells were transduced with different pfu/cell of DN (9) and then harvested at 48 and 72 h later. With CP and TX, cells were treated with different doses of each drug at 48 h after the plating of cells. Treated cells were harvested at 24, 48 and 72 h later. Doses of agents that induced 10-30% cytotoxicity were selected for the investigation of combined effect of DN and drugs on cytotoxicity.

For the determination of combined effects, 96-well plates were used. These wells were organized into different groups: control (1% ethanol), DN (1 pfu), CP (3.0 µM), CP (7.0 µM), TX (0.005 µM), TX (0.03 µM), and the different combinations of DN with each of the drugs. As before, the transduction was carried out at 24 h after the plating of cells and chemical treatment 48 h. Cells were harvested at 24 and 48 h after the drug treatment (*i.e.* 48 and 72 h after the transduction). The collected cells were evaluated using spectrophotometer.

TUNEL assay for the determination of apoptosis. The assay kit was purchased from Roche (Indianapolis, IN, USA) and used according to instructions. The assay is based on the determination of 3'-OH DNA ends in low molecular weight DNA fragments or in single strand breaks of high molecular weight DNA. This DNA damage, especially the former type, is believed to be the hallmark of cells that undergo apoptosis. This specific assay was used in the investigation to compliment the MTT assay. The TUNEL assay determined cell death from a self-initiated cell death pathway (apoptosis) as opposed to cytotoxicity which is due to a combination of both apoptosis and necrosis (MTT assay).

The assay protocol as provided by the supplier was used without modification. Positive and negative controls were prepared according to instructions. Cells treated with different conditions were harvested and spread onto lysine–coated slides and stained. The stained slides were coded and analyzed under a light microscope. Positive and negative control slides were used for standardization of the scoring criteria. From each treatment, 100 cells were randomly selected and evaluated for the presence of brown staining nuclei (apoptosis) or for the absence of staining (live cells). Two independent experiments were performed.

Caspase-3 activity assay. The CaspACE™ assay that detects the caspase-3 class of protease activity at the single cell level was purchased from Promega (Madison, WI, USA). Increased protease activity is indicative of apoptosis *via* the caspase-dependent pathway, the last crucial step of the apoptotic process. Cells were plated into 6-well plates and treated as described above. Cells were then harvested at 48 and 72 h after transduction and processed according to the instructions with the assay. Culture plates were read by our Spectra Max Gemini (Molecular Devices) for fluorescence at 405(ex)/510(em) using microplate readers. Sample protease activities on substrate were expressed as pmol of 7-amino-4-trifluoromethyl coumarin.

Statistical evaluation of the data. Data were collected and organized for the determination of significant differences using the standard Student's *t*-test.

Since the MTT assay has a more extensive collection of dose–response data, the data were used for the determination of interactions between the gene and chemotherapy (synergism, additive, and antagonism) based on the isobologram determination (21). Briefly, the interaction was determined by calculating the combination index (CI) using the equation:
$CIx=(D_1/Dx_1)+(D_2/Dx_2)+\alpha(D_1)(D_2)/(Dx_1)(Dx_2)$, where CIx represents the CI value for x% effect, Dx_1 and Dx_2 represent the doses of agents 1 and 2 required to exert x% effect alone, and D_1 and D_2 represent the doses of agents 1 and 2 that elicit the same x% effect in combination with the other agent, respectively. The factor α indicates the type of interaction: α=0 for mutually exclusive drugs (similar mechanisms of action), and α=1 for mutually non-exclusive drugs (independent modes of action) (22, 23); the equation was resolved for α=1. A CI of 1 indicates additivity, a CI of <1 synergism and a CI of >1 antagonism.

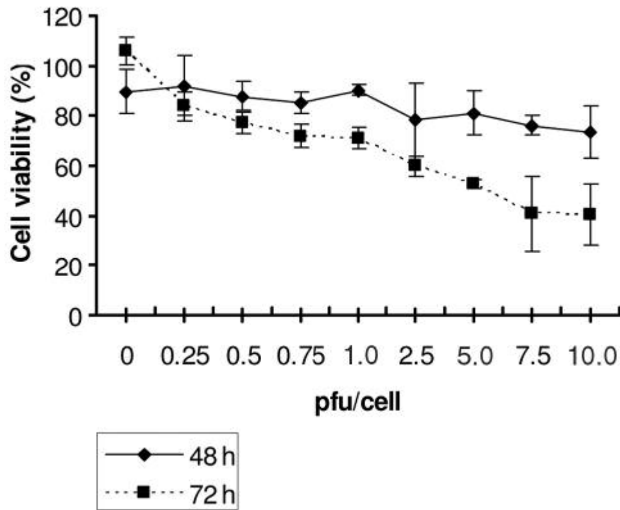

Figure 1. *A total of 20,000 cells/well were seeded in each well of 96-well plates. MTT assay was performed in triplicate experiments. The control of the first experiment for 72 h was considered to represent 100% cell viability and used as the basis for the remaining two experiments. Results are expressed as mean±SD.*

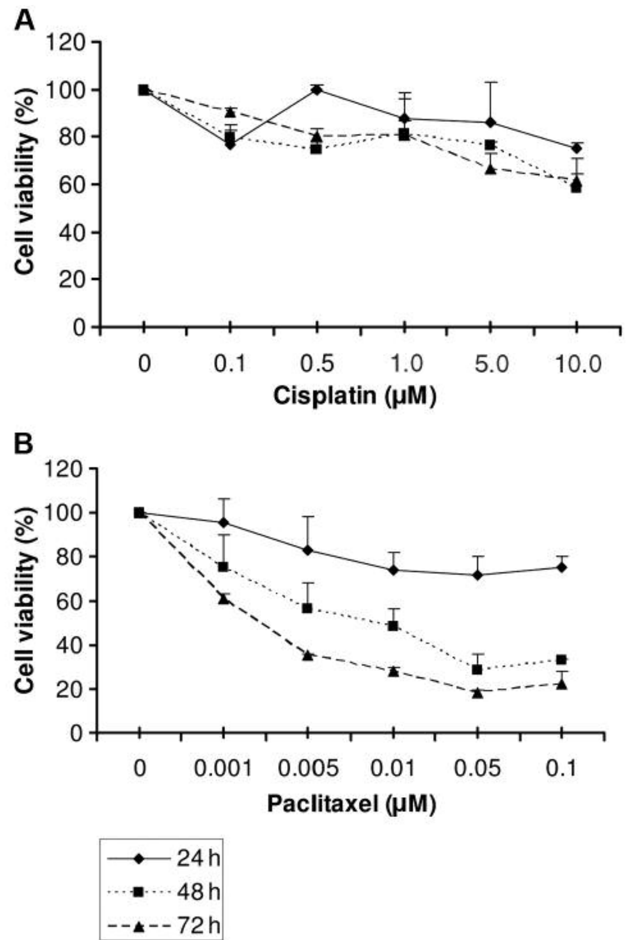

Figure 2. *MTT assay was performed in two experiments. Controls were considered to represent 100% cell viability. Results are expressed as mean±SD.*

Additional interactive effects were determined based on the following equation. For the TUNEL assay, the equation is: Expected effect (%) = Observed effect (%) from gene-therapy + Observed effect (%) from drug-therapy – Observed effect (%) from one untreated control.

Results

Dose selection. The DN, CP and TX were tested individually for their dose-dependent induction of cytotoxicity using the MTT assay. Results from the investigation are presented in Figures 1 and 2. As shown in Figure 1, DN induced a dose-dependent expression of cytotoxicity from 0.25 to 10 pfu/cell concentration, with maximum effect at 72 h after transduction. Since our plan was to select a low dose that would cause about 10-20% cytotoxicity for subsequent combined therapy experiments, we chose the 1 pfu/cell concentration for additional investigations.

CP showed low cytotoxicity in CaSki cells, based on the test doses from 0.1 to 10 μM (Figure 2A). The effect is consistent with other reports indicating that CaSki cells are resistant to CP treatment (24). For our purpose, we chose the CP concentrations that caused about 10-30% cytotoxicity. Therefore, 3.0 and 7.0 μM of CP were selected for further investigations and they are below the therapeutic serum concentrations in cervical cancer patients (25; and as per supplier's brochure).

Treatment of cells with TX from 0.001-0.1 μM caused high cytotoxicity in CaSki cells. For TX, the two selected concentrations of 0.005 and 0.03 μM for further investigations are below the therapeutic serum concentrations (as per supplier's brochure).

Cytotoxicity of combined gene and chemotherapy – MTT assay. The MTT assay, which is based on enzyme activities, is designed to provide an estimate of viability in the entire population. The results of the investigation from single and combined treatments are summarized in Figure 3. The data show that cells treated with low doses of single and combined agents exhibited, in general, significantly higher cytotoxicity than did the untreated control. In addition, cells treated with the combined DN and TX 0.03 μM therapy exhibited significantly more cytotoxicity than did the DN-treated cells.

Based on the isobologram analysis, the combined treatment between DN and CP showed synergistic effects at the 48 hour harvest (Table I). However, at the 72 h harvest,

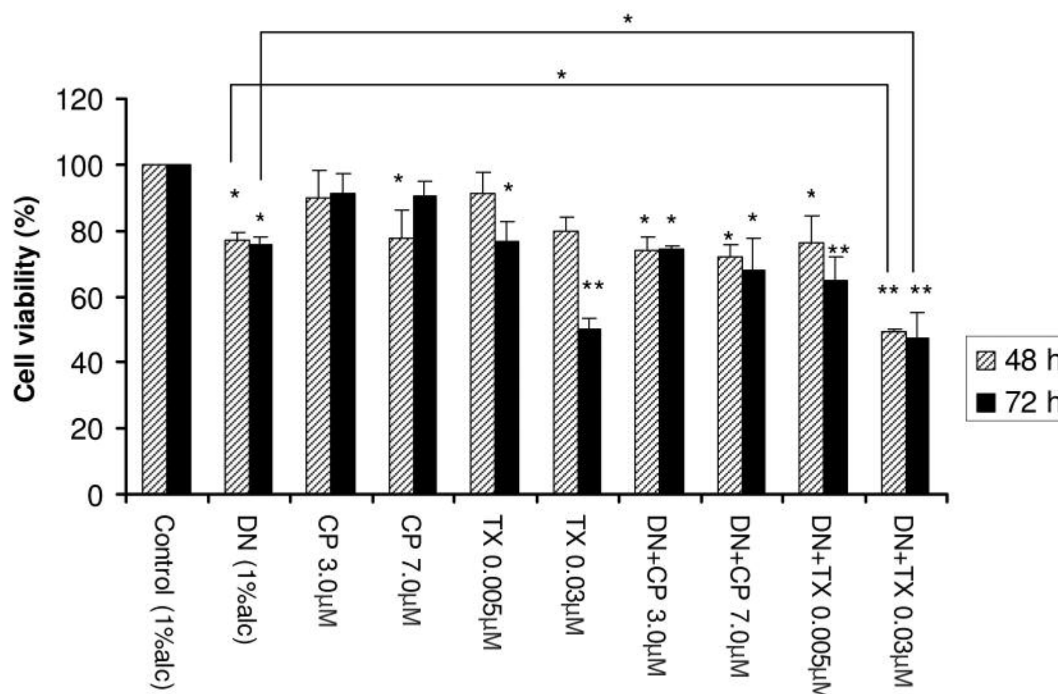

Figure 3. *MTT assay was performed in triplicate experiments. Each control for 48 h and 72 h was considered to represent 100% cell viability. Results are expressed as mean±SD; *p<0.05, **p<0.01 (control vs. treatment).*

Table I. *Viability after combined treatments with gene and chemotherapeutic agents in CaSki cells: MTT assay.*

Treatment duration	Dose for x% effect				Viability[a]	Combination index (CI)[b]	Interaction
	Combination	Alone					
	CP or TX (D1)	DN (D2)	CP(Dx1), μM	DN(Dx2), pfu			
48 h	CP 3.0 μM	1 pfu	12.16	8.57	73.9	0.39	Syn
	CP 7.0 μM	1 pfu	12.61	9.46	72	0.72	Syn
	TX 0.005 μM	1 pfu	0.08	7.53	76.1	0.20	Syn
	TX 0.03 μM	1 pfu	0.13	18.49	49.2	0.31	Syn
72 h	CP 3.0 μM	1 pfu	5.61	2.95	74.2	1.05	Ant
	CP 7.0 μM	1 pfu	6.80	3.80	68.1	1.56	Ant
	TX 0.005 μM	1 pfu	0.03	4.20	65	0.48	Syn
	TX 0.03 μM	1 pfu	0.05	6.40	47.5	0.84	Syn

[a]Data are from mean values of three independent experiments performed in triplicate. [b]CI=1: Additivity; CI<1: synergism; CI>1: antagonism (by the isobologram method, see text for explanation).

an antagonistic effect was observed. In the DN and TX-treatment groups, synergistic effects were consistently observed for both time points.

TUNEL assay for apoptosis. The results as shown in Table II indicate that the single and combined treatment conditions induced apoptosis, especially in the 72 h groups. The expected effect from the combined treatment was calculated based on the addition of the effects from the single treatment conditions (see formula for the calculation in the Materials and Methods section).

The combined effect on apoptosis is consistent with that on cytotoxicity as determined by the MTT assay (Table I). For example, the treatment with DN and CP produced less effect at 72 compared with 48 h, whereas the effect is consistent using DN and TX.

Table II. *Apoptosis after combined treatment with gene and chemo-therapeutic agents in CaSki cells: TUNEL assay.*

Treatment (48 h)	% Apoptotic cells		Significance		Apoptosis (%)	
	mean±SD		*p* (*vs.* con)	*p* (*vs.* DN)	Expected	Increase (fold)
Control (1% alc)	9	3.1				
DN (1% alc)	11	5.6				
CP 3.0 μM	10	7.2				
CP 7.0 μM	8	2.3				
TX 0.005 μM	12	7.2				
TX 0.03 μM	11	6.4				
DN+CP 3.0 μM	14	1.0	*		12	1.17
DN+CP 7.0 μM	19	3.1	**		10	1.90
DN+TX 0.005 μM	13	1.0			14	0.93
DN+TX 0.03 μM	24	2.1	**	*	13	1.84
Treatment (72 h)	**% Apoptotic cells**		**Significance**		**Apoptosis (%)**	
	mean±SD		*p* (*vs.* con)	*p* (*vs.* DN)	Expected	Increase (fold)
Control (1% alc)	4	0.6				
DN (1% alc)	16	1.5	**			
CP 3.0 μM	12	1.5	**			
CP 7.0 μM	7	2.6				
TX 0.005 μM	8	0.6	**			
TX 0.03 μM	10	1.5	*			
DN+CP 3.0 μM	17	4.7	*		24	0.71
DN+CP 7.0 μM	25	9.5	*		19	1.32
DN+TX 0.005 μM	21	7.6	*		20	1.05
DN+TX 0.03 μM[a]	26	-			22	1.18

A total of 180,000 cells were seeded in 6 well-plates. TUNEL assay was used in triplicate experiments. Each control for 48 h and 72 h was expressed as apoptotic cells/100 cells. Results are expressed as mean ±SD. **p*<0.05, ***p*<0.01 (Student's *t*-test). Expected effect(%) = Observed effect (%) from gene-therapy + Observed effect (%) from drug-therapy – Observed effect (%) from the untreated control. [a]One of the experiments had poor cell morphology due to extensive toxicity therefore no data were collected.

Caspase-3 activity assay for apoptosis. The results as shown in Table III clearly indicate that treatment of the CaSki cells with either DN, CP or TX induced more than a 3-fold increase in caspase-3 activity. From the combined treatment conditions, the calculation for expected results was performed similar to that for the TUNEL assay. The data show that the results, under any of the combined treatment conditions, were less than additive but may be antagonistic, compared with the expected increase in caspase-3 activity.

Table III. *Caspase-3 activity after treatment with gene and chemo-therapeutic agents in CaSki cells.*

Treatment (48 h)	pmol of AFC/mg protein/60 min	Significance		Caspase-3 activity	
	mean±SD	*p* (*vs.* con)	*p* (*vs.* DN)	Expected	Increase (fold)
Control (1% alc)	33.4±47.23				
DN (1% alc)	150.2±72.12				
CP 3.0 μM	168.8±238.72				
CP 7.0 μM	313.8±83.72	*			
TX 0.005 μM	737.8±193.68	*			
TX 0.03 μM	1,124.9±135.41	*			
DN+CP 3.0 μM	43.8±62.01			285.6	0.15
DN+CP 7.0 μM	229.9±70.22	*		430.6	0.53
DN+TX 0.005 μM	406.1±42.43	**	*	854.6	0.48
DN+TX 0.03 μM	197.6±131.52			1241.7	0.16
Treatment (72 h)	**pmol of AFC/mg protein/60 min**	**Significance**		**Caspase-3 activity**	
	mean±SD	*p* (*vs.* con)	*p* (*vs.* DN)	Expected	Increase (fold)
Control (1% alc)	69.4±0.35				
DN (1% alc)	191.6±64.56				
CP 3.0 μM	125.0±34.93				
CP 7.0 μM	143.6±28.43				
TX 0.005 μM	834.8±67.03	*			
TX 0.03 μM	1,118.6±42.6	**			
DN+CP 3.0 μM	71.8±2.76			247.2	0.29
DN+CP 7.0 μM	159.2±4.45	**		265.8	0.60
DN+TX 0.005 μM	251.6±45.96	*		957.0	0.26
DN+TX 0.03 μM	296.1±41.01	*		1240.8	0.24

A total of 200,000 cells were seeded in 6 well plates. Caspase-3 activity assay was conducted in two independent experiments. Results are expressed as mean±SD. **p*<0.05, ***p*<0.01 (Student's *t*-test). Expected effect(%) = Observed effect (%) from gene-therapy + Observed effect (%) from drug-therapy – Observed effect (%) from one untreated control.

Discussion

With the advancement of molecular technology and better understanding of carcinogenesis, highly specific procedures are being developed for targeted cancer therapy. We reported previously that the transduction of CaSki cells by DN caused serious cellular crisis by reducing the expression of HPV *E6* and *E7* genes, blockage of cell proliferation at the G1- and S-phases, suppression of cyclin D1 protein and induction of apoptosis (9). The response to DN is consistent with the expression of estrogen receptors in CaSki cells (19) and the blockage by DN (20). Our current report indicates that the induction of apoptosis (confirmed by the TUNEL assay) by DN was mediated by activation of caspase-3 enzyme. Together, these results indicate that the blockage of the

estrogen receptor in CaSki initiated a cascade of cellular events that are consistent with the reactivation of p53 and Rb, and the subsequent activation of the apoptotic machinery for the self-destruction of the cancer cells (9).

Despite the specificity of gene therapy procedure, it becomes clear that the efficacy can be enhanced by using combined therapy protocols (6). Such combined protocols using agents with distinctly different anticancer mechanisms are more likely to overcome the plasticity of tumor cell populations and to achieve significantly better therapeutic outcome with lower therapeutic doses of each agent (8, 14, 15). In this report, we have shown for the first time that the combination of DN and conventional chemotherapeutic drugs, especially TX, caused significant enhancement of cytotoxicity, and specifically apoptosis, in the CaSki cervical cancer cells.

Among the tested agents, the mechanisms of cytotoxicity for CP and TX are completely independent of that of DN. CP induces intrastrand and interstrand DNA cross-links that are difficult for cells to repair (16). TX interferes with tubulin formation, therefore treated cells undergo grossly aberrant mitosis (17) which was not observed after treatment of CaSki cells with DN (Au, unpublished data). In addition, TX-treated cervical cancer cells undergo cell death *via* the TRAIL-dependent as well as the mitochondrial-mediated apoptotic pathways (18). We observed that each agent induced cytotoxic and apoptotic effects in CaSki cells. In the combined treatment of DN with CP, synergistic effect was observed at 48 h and then antagonistic effect at 72 h, based on the MTT assay (total enzyme-based assay for cytotoxicity). The reduced effect was supported by the TUNEL assay (individual cell-based assays for apoptosis). The non-additive effect at 72 h is consistent with the knowledge that CaSki cells are resistant to CP (24). In the combined treatment of DN with TX, synergistic effects were observed in both 48 and 72 h based on the MTT and the TUNEL assays. On the other hand, the caspase-3 activity (functional) assay indicated that the combined treatments caused a less than additive effect and may actually have an antagonistic effect although each agent clearly induced caspase-3 activity individually. The data suggest that the combined treatments induced cell death *via* new and multiple mechanisms which may involve caspase-independent pathways such as apoptosis (Type I), autophagy (Type II) and necrosis (Type III) (25-27). Indeed, cervical cancer cells can perform caspase-independent apoptosis (28) and TX can induce damage that participates in such process (29). In future studies, other biomarkers can be investigated to confirm the mechanisms of cell death, *e.g.* $p15^{INK4b}$, $p16^{INK4a}$ and $p14^{ARF}$ for non-apoptotic cell death (30) or FasL-mediated necrosis (31). In addition, other cervical cancer cell lines and animal models need to be used for investigation.

The broad-based induction of cell death by the combined treatment that utilizes distinct mechanism of actions is a highly preferable feature for cancer therapy. In addition, the synergistic effect from using low doses of both DN and TX is highly encouraging because the low doses will minimize the occurrence of serious side-effects that are frequently observed in patients treated with high doses of therapeutic agents. The use of DN in an animal model indicates that it is effective in eradicating cancer cells *in vivo* with no detectable side-effects in normal cells (Hassan M, unpublished observations). For cervical cancer, such a treatment protocol with low dose regimens of therapeutic agents may allow the successfully treated patients to retain fertility, a highly desirable feature. Our observation confirms reports from others regarding the promise of combined therapy protocols (14, 15). Furthermore, the adenovirus is a promising vector for use in gene therapy because the virus can be modified to enhance tropism and delivery of specialized products for significant anti tumor effect (10). The described gene and chemotherapy approach is potentially useful for the development of treatment protocols for cervical and other types of estrogen-dependent cancer.

Acknowledgements

The study is partially supported by a Visiting Professor Fellowship to MYH from the Kangwon National University, Korea; John Sealy Memorial Foundation Grant to WWA; National Institute of Health (NIH) grants HD046639, and M.D. Anderson SPORE Career Development award CA098258 to SAS; NICHD grant R01-HD 46228 to AA. The authors express their appreciation to Dr Memy Hassan for his advice on the caspase-3 activity assay.

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Received March 14, 2008
Revised May 19, 2008
Accepted May 23, 2008