Abstract. The highly metastatic human pancreatic cell line L3.6 was used to study mechanisms for antitumor activity with various chemotherapeutic drug combinations. The most effective drugs were daunorubicin (IC_{50} 0.4 ÌM), doxorubicin (IC_{50} 2.2 ÌM), paclitaxel (IC_{50} 5.3 ÌM) and 5-fluorouracil (IC_{50} 5.4 ÌM). The most effective drug combination was equitoxic concentrations of paclitaxel and daunorubicin. Kinetic analysis demonstrated that both paclitaxel and daunorubicin had to be added simultaneously for maximum cytotoxicity. Daunorubicin treatment alone demonstrated ROS (reactive oxygen species) induction and cellular morphological changes more consistent with chemical damage in a total of 93% of the cells and apoptotic changes in 20% of the cell population. The apoptosis induced by daunorubicin does not appear to be caspase-dependent, as demonstrated by the lack of conversion of the procaspases 8 and 3. Within 24 h of treatment with paclitaxel, Bcl-2 formed a doublet at 26 kilodaltons and the expression was abrogated with daunorubicin and the combination of the two drugs as determined by Western blots. These data suggest that the human pancreatic cell line L3.6 is more effectively killed following treatment with chemotherapeutic agents that cause death through at least two pathways, a caspase-dependent and caspase-independent apoptosis and necrosis.

Adenocarcinoma of the pancreas is the fourth leading cause of cancer death in the United States, with an average median survival of 6 months (1,2). Presently, the treatment options include surgical resection and chemoradiotherapy. These treatment modalities have not had a significant impact on the overall 5-year survival rate, which remains less than 2-5% (2). Recently, gemcitabine has shown antitumor activity against a variety of solid tumors, including pancreatic carcinoma (3,4). Gemcitabine has had a modest effect on improving the quality of life and a marginal effect on improved survival by 2 months, becoming the new standard of care in the treatment of pancreatic carcinoma (5). There have been several studies analyzing several other chemotherapeutic agents alone and in combination. These drugs include 5-fluorouracil, gemcitabine, paclitaxel, cisplatin, ifosfamide, mitomycin C, streptozotocin, the nitroureas and the anthracyclines (5).

There have been several recent studies which analysed the in vitro cytotoxicity of various chemotherapeutic agents using a panel of human pancreatic cell lines (6). These studies concluded that pancreatic cells have a low sensitivity to single-agent treatment but, when multiple drug combinations are used, the sensitivity of these cells to the antitumor agents is significantly increased.

**Abbreviations:** MTT, 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide; PI, propidium iodide; ROS, reactive oxygen species; DMSO, dimethyl sulfoxide; carboxy-DCFDA-5- (and-6)-Carboxy-2’,-7-dichlorofluorescin diacetate; IC_{50}, Inhibitory concentration 50%; DNR, daunorubicin.

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**Key Words:** Pancreatic carcinoma cells, paclitaxel, daunorubicin, apoptosis, necrosis.
Most of the aforementioned chemotherapeutic agents induce programmed cell death or apoptosis as their primary mechanism of cell destruction and involve a well-defined cascade of proteolytic cleavage events (7,8). In addition to this caspase-dependent pathway of cell death, there is now sufficient evidence to suggest that apoptosis can occur in the absence of active caspases. This caspase-independent cell death has been shown to be initiated by a range of stimuli, including tumor necrosis factor (TNF) (9), FasL (10), ultraviolet irradiation (11), granzyme B (12) and chemotherapeutic agents (13). In the caspase-dependent pathway, both the proximal caspase 8 and the downstream effector caspases such as caspase 3 are activated (13). In the caspase-independent pathway, a signal is transmitted from the plasma membrane to the mitochondria resulting in a decreased expression of Bcl-2, cytochrome c release and mitochondrial membrane disruption (14). In addition, ROS are most probably generated, causing an additive cytotoxic effect on living tissue. It is not known whether the cells that have undergone caspase-independent cell death have morphological features similar to apoptosis or necrosis, or both.

In this recent study we chose to analyze several chemotherapeutic agents for their activity against the well-characterized human pancreatic adenocarcinoma cell line, L3.6 (15). The most active drugs were further analyzed for Bcl-2 activation, procaspases 8 and 3 activation, and ROS generation. These cytotoxic and biochemical events were correlated to morphological changes exhibited in the L3.6 cell line following drug exposures.

Materials and Methods

Drugs and chemicals. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide (MTT), dimethylsulfoxide (DMSO), EDTA, phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin, bovine serum albumin (BSA), chicken egg albumin (CEA) and b-mercaptoethanol were purchased from Sigma Chemical Co. (St Louis, MO, USA). 5-(and-6)-Carboxy-2',7'-dichlorofluorescin diacetate (carboxy-DCFDA) was from Molecular Probes, Inc., Eugene, OR, USA. The Annexin-V FITC conjugate (recombinant) was obtained from Biosource International, (Camarillo, CA, USA). The following was purchased from Gibco Laboratories (Grand Island, NY, USA): RPMI-1640 medium, fetal calf serum (FCS), L-glutamine, penicillin, streptomycin and trypsin-EDTA. Doxorubicin was purchased from Gensin Laboratories, LTD. (Irvine, CA, USA), daunorubicin was purchased from Bedford Laboratories, LTD. (Bedford, Ohio), paclitaxel and cisplatin were obtained from Bristol-Myers Squibb, Co. (Princeton, NJ, USA), gemcitabine was purchased from Eli Lilly, Co. (Indianapolis, IN, USA), and 5-fluorouracil was purchased from Roche Laboratories, Inc, (Nutley, NJ, USA). Mouse monoclonal antibodies against human procaspase 8, procaspase 3 and Bcl-2 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Protein quantitation reagents were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

Cell line and culture. The human pancreatic cell line (L3.6) was a gift from Dr. Isaiah J. Fidler (The University of Texas M.D. Anderson Cancer Center, Houston, TX, USA). These cells were originally subclones derived from the L3.3 variant by Dr. Michael P. Vezeridis (Roger Williams Medical Center, Providence, RI, USA). The L3.6 cell line was maintained as monolayer culture in RPMI-1640 medium, supplemented with 10% FCS, 1% L-glutamine, 1% penicillin and 1% streptomycin. The cultures were incubated at 37°C in a mixture of 5% carbon dioxide and 95% oxygen. The cultures were tested and found to be free of Mycoplasma. The cultures were passed every 4 days by trypsin-EDTA treatment for 10 min at 37°C and passed at 5 x 10^6 cells/10ml.

In vitro cytotoxicity assay. For cytotoxicity assays, exponentially growing cells were washed twice and resuspended in complete RPMI-1640 medium at a density of 0.5 x 10^5 cells/ml in a final volume of 0.2 ml per well in a 96-well microtitrter plate. Various concentrations of the specific chemotherapeutic agent were added in each well. The cells were then incubated for various times at 37°C in a humidified atmosphere of 95% oxygen and 5% carbon dioxide. At the end of the incubation period, the media was removed from each well and 50 µl of MTT dye (20 mg/ml in saline) was added and incubated for 6 h at 37°C. After this 6-h incubation, 150 µl/well dimethyl sulfoxide was added to each plate. The optical density of the solution was read on a microplate ELISA autoreader (Bio-Tek Instruments, Winooski, VT, USA) at a wavelength of 540 nanometers. The rate of growth inhibition was calculated as (1-mean absorbency of treated wells/mean absorbency of control wells) X 100. Inhibitory concentration (IC50) is the concentration (mM) that inhibits cell growth by 50% at 72 h. All experiments were performed in triplicate.

In the remainder of the experiments presented, the concentrations used were 0.4 µM daunorubicin and 5.3 µM paclitaxel, which are equitoxic concentrations of these drugs. Large cell numbers were used to allow for the various histological and biochemical analyses.

Histological evaluation. Cytospins (Shandon Incorporation, Pittsburgh, PA, USA) of L3.6 pancreatic cells treated with 0.4 µM daunorubicin and 5.3 µM paclitaxel alone or in combination for 48 h were generated by centrifuging the cells onto glass slides (200 ml aliquot/slide) for 3 min at 750 rpm. Wright’s stains of all slides were performed using an automated Hemastainer (Geometric Data, Wayne, PA, USA). Slides were analyzed microscopically, photographed (Olympus BH 2, Tokyo, Japan) and evaluated for morphological changes.

Apoptosis and necrosis detection. The L3.6 cells were grown as a monolayer and untreated or treated with 5.3 µM paclitaxel alone, 0.4 µM daunorubicin alone, or a combination of paclitaxel (5.3 mM) and daunorubicin (0.4 µM) for 24 h or 48 h. The cells were then treated with trypsin-EDTA and washed twice and resuspended at 2-3 x 10^6 cells/ml in Annexin-V binding buffer containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2. Aliquots of cells (100 µl/tube) were incubated with 5 µl of Annexin-V FITC, mixed and incubated for 15 min at room temperature in the dark. At this step, propidium iodide (PI) at a concentration of 5 µg/ml was added to distinguish necrotic cells. Binding buffer (400 µl) was added to each tube and the cells were analyzed by flow cytometry within 1 h of staining (16). For these studies the samples were analyzed on a FACScalibur flow cytometer (BD, St. Jose, CA,
USA). The instrument is calibrated daily utilizing Calibrite beads (BD). Cells treated with chemotherapeutic agents were compared to untreated cells. Cells that were labeled with annexin V were considered apoptotic and those labeled with PI were considered necrotic (16).

**Determination of ROS.** Production of ROS was detected by use of carboxy-DCFDA (17). A stock solution of carboxy-DCFDA (15mM) was prepared in DMSO and stored over nitrogen vapor. The L3.6 cells (1 x 10^6 cell in 1 ml) were incubated with 50 mM carboxy-DCFDA for 20 min at 37°C. The cells were then centrifuged and resuspended in 1 ml of RPMI-1040 media in the presence of 5.3 μM paclitaxel alone, 0.4 μM daunorubicin alone or paclitaxel (5.3 μM) and daunorubicin (0.4 μM) together for 120 min. Following the specific incubation period, the cells were analyzed by flow cytometry.

**Western blot analysis.** Cell lysates were made from 5 x 10^6 L3.6 cells grown in the presence of daunorubicin, paclitaxel, or the combination of both drugs at a concentration of 0.4 mM daunorubicin and 5.3 μM paclitaxel following a 6-h incubation or with 0.4 μM daunorubicin and 5.3 μM paclitaxel concentrations following a 24-h or 48-h incubation. Following the various incubation periods (6-h, 24-h and 48-h), the cells were harvested and washed 3 times in phosphate-buffered saline (PBS). Cells were resuspended in lysis buffer [0.1 M Tris-HCl (pH 8.0), 1% (w/v) Triton-100, 1 mg/ml phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin and 5 mg/ml pepstatin], vortexed and placed on ice for 30 min. After centrifugation at 1,000 x g for 20 min, an aliquot of the supernatant containing 25 mg of protein was resuspended in sample loading buffer [2.5 M Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromphenol blue], boiled for 3 min and electrophoresed on a 15% polyacrylamide gel. The proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked with a 0.05% PBS-Tween-5% low-fat dry milk solution at 4°C overnight. The membrane was subsequently incubated with mouse monoclonal antibodies directed against human procaspase 8 (2 μg/ml), human procaspase 3 (1 μg/ml), and human Bcl-2 (1 μg/ml) and incubated for 2 h at room temperature. The blots were washed in PBS-Tween solution and incubated with rabbit anti-mouse antibodies conjugated to horseradish peroxidase (1:150,000 dilution; Oxford, Oxford, MI, USA) or with rabbit anti-mouse antibodies conjugated to horseradish peroxidase (1:5,000 dilution: Oxford) for 30 min at room temperature. After three washes with PBS-Tween solution, immunoreactive proteins were detected using the enhanced chemiluminescence system (Amersham, Arlington Heights, IL, USA) and recorded by fluorography on Hyperfilm, according to the manufacturer’s instructions. Fluorograms were quantitated by image densitometry using the Molecular Analyst program for data acquisition and analysis (Bio-Rad).

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC50 (μM)*</th>
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<tbody>
<tr>
<td>Daunorubicin</td>
<td>0.4</td>
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<tr>
<td>Doxorubicin</td>
<td>2.2</td>
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<tr>
<td>Paclitaxel</td>
<td>5.3</td>
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<tr>
<td>5-Fluorouracil</td>
<td>5.4</td>
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<tr>
<td>Cisplatin</td>
<td>6.3</td>
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<tr>
<td>Gemcitabine</td>
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*IC50 calculation as described in “Materials and Methods”.

**Table I. Cytotoxicity for L3.6.**

**Figure 1.** Cytotoxicity assay to analyze the combinations of paclitaxel and daunorubicin using the IC50 and IC25 concentrations. The data is expressed as optical density measurements at 540 nM of the MTT dye (O.D. 540 nM). The L3.6 cells were incubated with either media (Control), paclitaxel at the IC50 (T50) or IC25 (T25) concentration alone, daunorubicin at the IC50 (D50) or IC25 (D25) concentration alone or various combinations for 72 h. See "Materials and Methods" section. Values shown are the means of six experiments; bars, SD.**

**Figure 2.** Cytotoxicity assay to determine the optimal incubation period for paclitaxel and daunorubicin using the IC50 concentration. The data is expressed as optical density measurements at 540 nM of MTT dye (O.D. 540 nM). The L3.6 cells were incubated with either media (Control), paclitaxel IC50 for 72 h (T72), daunorubicin IC50 for 72 h (D72), daunorubicin IC50 and paclitaxel IC50 in combination for 72 h (D72 + T72), paclitaxel IC50 72 h and then the addition of daunorubicin IC50 after the first 24 h for the remaining 48 h (D48), and paclitaxel IC50 72 h and then the addition of daunorubicin IC50 after the first 48 h for the remaining 24 h (D24). See "Materials and Methods" section. Values shown are the means of six experiments; bars, SD.
Cytotoxicity assay. The IC\textsubscript{50}s for paclitaxel, daunorubicin, doxorubicin, cisplatin, 5-fluorouracil and gemcitabine are shown in Table I. As demonstrated, daunorubicin is the most active cytotoxic drug against the L3.6 cells, with an IC\textsubscript{50} of 0.4 µM, followed by doxorubicin with an IC\textsubscript{50} of 2.2 µM. The next active cytotoxic drugs are paclitaxel with an IC\textsubscript{50} of 5.3 µM and 5-fluorouracil with an IC\textsubscript{50} of 5.4 µM. Cisplatin and gemcitabine are the least active drugs of the six-chemotherapeutic agents tested against the L3.6 cell line with IC\textsubscript{50} values of 6.3 µM and 9.3 µM, respectively.

Daunorubicin and paclitaxel-induced cytotoxicity. Various combinations of the six-chemotherapeutic agents were tested for activity against the L3.6 cell line. The best combination to induce maximum cytotoxicity was daunorubicin and paclitaxel. As shown in Figure 1, daunorubicin and paclitaxel added together at their equitoxic IC\textsubscript{50} concentrations for 72 h caused greater than a 90% growth inhibition of the L3.6 cell line as compared to the control cells (no drug added). When either paclitaxel or daunorubicin were used at their equitoxic IC\textsubscript{25} drug concentrations alone there was a 25% growth inhibition as compared to control cells. The combination of paclitaxel and daunorubicin at their equitoxic IC\textsubscript{25} concentrations demonstrated a 50% growth inhibition as compared to control cells. When the equitoxic IC\textsubscript{50} concentrations of paclitaxel or daunorubicin was combined with the equitoxic IC\textsubscript{25} concentration of daunorubicin or paclitaxel, respectively, only 50% growth inhibition was observed, as compared to the control cells (data not shown). In addition, all other combinations of the doxorubicin, cisplatin, 5-fluorouracil and gemcitabine only showed a 50%-75% growth inhibition at their equitoxic IC\textsubscript{50} concentrations of the L3.6 cells (data not shown). The results indicate that the most effective drug combination was paclitaxel and daunorubicin at their equitoxic IC\textsubscript{50} concentrations.

Figure 3. Morphological characteristics as determined by light microscopy and Wright’s stain. The L3.6 cells were incubated for 48 h with media alone (panel A), 5.3 µM paclitaxel (panel B), 0.4 µM daunorubicin (panel C), or 5.3 µM paclitaxel and 0.4 µM daunorubicin (panel D) in combination. The cells were cytopun, stained with Wright’s stain and analyzed by light microscopy. Panel A is consistent with normal cells, panel B is consistent with apoptosis, panel C is consistent with necrosis and panel D is consistent with necrosis.
Figure 4. Determination of the percentage of L3.6 cells undergoing apoptosis and necrosis as assessed by FITC-annexin V (AV) and PI uptake following a 24-h exposure to chemotherapeutic agents paclitaxel and daunorubicin using flow cytometry. The lower case (a) are untreated cells (control) and (b) are drug-treated cells. Panel A is paclitaxel (5.3 mM) alone (Top: FITC-annexin V; Bottom: PI); Panel B is daunorubicin (0.4 mM) alone; Panel C is both paclitaxel (5.3 mM) and daunorubicin (0.4 mM) together; Panel D is the actual percentages of cells stained with FITC-annexin V and PI for each condition. The flow cytometric graphs illustrate control cells as the overlay in the graph. Positive results represent a shift to the right of the treated cells as compared to the control cells. These control cells are treated the same way but were not incubated in the presence of drug.

Figure 5. Determination of the percentage of L3.6 cells undergoing apoptosis and necrosis as assessed by FITC-annexin V and PI uptake following a 48-h exposure to chemotherapeutic agents paclitaxel and daunorubicin using flow cytometry. The lower case (a) are untreated cells (control) and (b) are drug-treated cells. Panel A is paclitaxel (5.3 µM) alone (Top: FITC-annexin V; Bottom: PI); Panel B is daunorubicin (0.4 µM) alone; Panel C is both paclitaxel (5.3 µM) and daunorubicin (0.4 µM) together; Panel D is the actual percentages of cells stained with FITC-annexin V and PI for each condition. The flow cytometric graphs illustrate control cells as the overlay in the graph. Positive results represent a shift to the right of the treated cells as compared to the control cells. These control cells are treated the same way but were not incubated in the presence of drug.
Kinetic analysis of daunorubicin and paclitaxel. The IC$_{50}$ concentrations of daunorubicin and paclitaxel (equitoxic concentrations) were used to determine the optimal sequence of addition and time period for maximum cytotoxicity. As shown in Figure 2, the optimal sequence and incubation period for greater than 90% cell growth inhibition occurred when daunorubicin was added at the same time as the paclitaxel and incubated with the L3.6 cells for 72 h. When paclitaxel was added first followed by addition of daunorubicin after 24 h of incubation, then allowed to incubate for an additional 48 h together (D48+T72), there was an approximately 80% cell growth inhibition, as compared to the control cells. When equitoxic IC$_{50}$ concentrations of daunorubicin were added 48 h after equitoxic IC$_{50}$ concentrations of paclitaxel (D24+T72), there was approximately a 50% cell growth inhibition as compared to the control cells. If conversely, daunorubicin was incubated with the L3.6 cell line for the entire 72 h and paclitaxel was added either 24 h or 48 h after daunorubicin, 80% and 50% growth inhibition was observed, respectively. In each variable the cytotoxicity was less than the addition of both drugs added at the same time for 72 h (~90% growth inhibition). These results suggest that paclitaxel and daunorubicin are more effective when added simultaneously and incubated for 72 h with the L3.6 cell line.

Cell morphology. The cells were stained with Wright’s stain following a 48-h incubation of the L3.6 cell line with paclitaxel (5.3 μM), daunorubicin (0.4 μM), or both drugs at equitoxic concentrations of paclitaxel (5.3 μM) and daunorubicin (0.4 μM) in combination.

As shown in Figure 3, when the L3.6 cells are incubated with no drug, shown in panel A, the cells are viable and appear normal with a high nucleo-cytoplasmic ratio, all cell membranes are intact with abundant cytoplasmic granules. As shown in Figure 3, panel B, paclitaxel-treated cells demonstrate apoptotic bodies with cell shrinkage, nuclear and membrane blebbing, and nuclear chromatin breaks with irregular crescentic beading and nodular masses. With daunorubicin treatment alone, as shown in Figure 3, panel C, the cells have no features typical of apoptosis as demonstrated in paclitaxel-treated cells shown in panel B. These cells in panel C demonstrate absence of normal cellular detail and nuclear uptake of trypan blue, both indicative of necrosis. Finally, in panel D, the combination of both paclitaxel and daunorubicin demonstrate similar cellular features as daunorubicin alone, including cytoplasmic vacuolization, nuclear pyknosis, lack of cellular detail and 100% of nuclear uptake of trypan blue. These results in panel D demonstrate that, with the combination of both paclitaxel and daunorubicin, the cells appear to have more features consistent with necrosis as opposed to apoptosis.
Figure 7. Western blot analysis of procaspase 8, procaspase 3 and Bcl-2 levels in L3.6 cell lysates following exposure of intact cells with paclitaxel and daunorubicin for 24 h. lane 1=media alone, lane 2=paclitaxel (5.3 μM) alone, lane 3=daunorubicin (0.4 μM) alone, lane 4=paclitaxel (5.3 μM) and daunorubicin (0.4 μM) together. Panel A is L3.6 cell lysates analyzed for procaspase 8 levels; Panel B is L3.6 cell lysates analyzed for procaspase 3 levels; Panel C is L3.6 cell lysates analyzed for Bcl-2 levels; Equal amount of protein (25 μg) was analyzed per lane. See "Materials and Methods".

Figure 8. Western blot analysis of procaspase 8, procaspase 3 and Bcl-2 levels in L3.6 cell lysates following exposure of intact cells with paclitaxel and daunorubicin for 48 h. lane 1=media alone, lane 2=paclitaxel (5.3 μM) alone, lane 3=daunorubicin (0.4 μM) alone, lane 4=paclitaxel (5.3 μM) and daunorubicin (0.4 μM) together. Panel A is L3.6 cell lysates analyzed for procaspase 8 levels; Panel B is L3.6 cell lysates analyzed for procaspase 3 levels; Panel C is L3.6 cell lysates analyzed for Bcl-2 levels; Equal amount of protein (25 μg) was analyzed per lane. See "Materials and Methods".
Analysis of L3.6 cells for apoptosis and necrosis by flow cytometry following paclitaxel and daunorubicin treatment for 24-h and 48-h exposure. L3.6 cells were exposed to paclitaxel, daunorubicin, or both drugs at equitoxic concentrations for either 24 h (Figure 4) or 48 h (Figure 5), then analyzed by flow cytometry for annexin V and PI staining. Untreated cells demonstrated no uptake of annexin V or PI following a 24-h or 48-h incubation period indicating no apoptosis or necrosis of these cells. These results are indicated on each cytometric graph as the overlay curve. As shown in Figure 4, panel A, L3.6 cells treated with 5.3 μM paclitaxel showed no annexin V staining or PI uptake after 24-h exposure. The L3.6 cells exposed to 0.4 μM daunorubicin alone (Figure 4, panel B) demonstrated no annexin V staining but did show PI uptake (96% of the cells), consistent with no detectable apoptotic activity but extensive necrotic activity (15). As shown in Figure 4, panel C, the combination of both paclitaxel and daunorubicin at 24 h did not demonstrate annexin V staining but did demonstrate PI uptake in 99% of the cells, again consistent with high necrotic activity and no apoptosis. In panel D, the actual percentages of cells stained with annexin V and PI obtained from the cytometric graphs are illustrated.

When the 48-h exposure time is analyzed for annexin V and PI staining under the same conditions and drug concentrations, the L3.6 cells with paclitaxel alone (Figure 5, panel A) treatment demonstrate that approximately 30% (panel D) of the cells stain with annexin V but not PI. Daunorubicin treatment alone following a 48-h exposure demonstrated approximately 19% (panel D) annexin V staining and 93% PI uptake (Figure 5, panel B). Panel C illustrates the combination of paclitaxel and daunorubicin showing 24% (illustrated in panel D) of the cells staining with annexin V and 99% (illustrated in panel D) of the cells staining with PI. These data demonstrate that incubation with paclitaxel alone induces apoptosis but not necrosis, at 48 h. In contrast, daunorubicin alone induces necrosis without apoptosis at 24 h. At 48 h both an apoptotic and extensive necrotic response is observed. The combination of the two drugs induces a necrotic response by 24 h with no detectable apoptotic response. The addition of daunorubicin does not enhance the apoptotic response to paclitaxel.

Determination of ROS. L3.6 cells were incubated with carboxy-DCFDA then exposed to 100 μM H2O2, 5.3 μM paclitaxel, 0.4 μM daunorubicin, or 5.3 μM paclitaxel and 0.4 μM daunorubicin at equitoxic concentrations in combination for 120 min. Analysis of these cells by flow cytometry is shown in Figure 6. As demonstrated in panel A, cells treated with H2O2 exhibit a shift in the curve as compared to untreated cells consistent with the induction of ROS. Paclitaxel treatment of the L3.6 cells did not generate ROS, as demonstrated by no shift in the curve as compared to untreated cells shown in panel B. In contrast, daunorubicin treatment did induce the generation of ROS within 15 min (data not shown), which continued for 120 min as shown in panel C. When daunorubicin was incubated with L3.6 cells in the presence of paclitaxel these cells also generated ROS as shown in panel D. In panel E the actual percentages of cells that exhibited carboxy-DCFDA fluorescence following the treatment of paclitaxel and daunorubicin are shown. The percentage of cells that demonstrate carboxy-DCFDA fluorescence following H2O2 treatment was nearly 90%, with paclitaxel it was 0%, whereas following daunorubicin treatment it was approximately 50%, and with the combination it was nearly 80%. This demonstrates that daunorubicin alone induces the generation of ROS and is enhanced in the presence of paclitaxel. However, paclitaxel alone failed to induce the generation of ROS in the L3.6 cell line.

Activation of Bcl-2 and procaspases 8 and 3. The L3.6 cells were exposed to paclitaxel, daunorubicin, or equitoxic concentrations of both for 6 h, 24 h and 48 h. The cells were then analyzed for activation of procaspase 8, 3 and Bcl-2. There were no differences between the untreated cells and the drug exposed cells at 6 h (data not shown). As shown in Figure 7, paclitaxel, daunorubicin, and the combination of these drugs incubated for 24 h demonstrated no changes in the activation status of procaspase 8 (panel A) and procaspase 3 (panel B). As shown in panel C of Figure 7, the Bcl-2 protein following paclitaxel incubation for 24 h (lane 2) demonstrates a doublet at approximately 26 kilodaltons. Lanes 3 and 4 of panel C, Figure 7, show decreased expression of Bcl-2 as compared to the control (lane 1) and no doublet was visualized following incubation with either daunorubicin alone (lane 3) or daunorubicin plus paclitaxel together (lane 4) for 24 h.

As shown in Figure 8, panel A, following a 48-h incubation with paclitaxel, there was proteolytic cleavage of procaspase 8 into smaller fragments (lane 2) in comparison to untreated cells (lane 1). Daunorubicin treatment of L3.6 cells for 48 h (lane 3) showed no cleavage of procaspase 8. The combination of daunorubicin and paclitaxel for 48 h (lane 4) demonstrates cleavage of procaspase 8 indicating activation (lane 3). As shown in Figure 8, panel B, paclitaxel incubation with L3.6 cells for 48 h demonstrate cleavage of procaspase 3 (lane 2) as compared to untreated cells (lane 1). When the L3.6 cells were incubated with daunorubicin (lane 3) or daunorubicin plus paclitaxel (lane 4) for 48 h, the procaspase 3 is not cleaved as compared to the untreated cells (lane 1). These results indicate that paclitaxel induces cell death through the activation of procaspases 8 and 3. In contrast, daunorubicin alone does not appear to cause cell death through the activation of procaspases 8 and 3. The combination of paclitaxel and daunorubicin appear to activate procaspase 8 but to a lesser extent than paclitaxel alone with no significant activation of
pro caspase 3. When Bcl-2 expression was analyzed following exposure of drug for 48 h, paclitaxel (lane 2), daunorubicin (lane 3) or both (lane 4) showed a decreased expression of Bcl-2, as compared to untreated cells (lane 1).

These results are consistent with down-regulation of Bel-2 by paclitaxel, daunorubicin and the combination of the two drugs in the L3.6 pancreatic cell line. In addition, the data suggests that paclitaxel alone causes apoptosis through the caspase 8 and 3 cascade, whereas daunorubicin does not. The combination of paclitaxel and daunorubicin appears to be partially activating procaspase 8 but not procaspase 3, causing apoptosis, but not to the same extent as paclitaxel alone.

Discussion

In this study we demonstrate that the most effective agent used singly is daunorubicin, and the most effective combination is paclitaxel and daunorubicin (Table I and Figure 1). The kinetic experiments demonstrate that equitoxic concentrations of both paclitaxel and daunorubicin need to be added together at the initiation of culture to the L3.6 cell line in order to achieve optimal cytotoxicity (Figure 2).

The mechanisms of action for paclitaxel and daunorubicin are very well established (17-19). In the case of paclitaxel, microtubular disruption and G2/M cell arrest is the basis of its antitumor activity (18). Paclitaxel has also been shown to induce inhibition of the antiapoptotic molecule Bcl-2 via phosphorylation (20). Daunorubicin, on the other hand, has been shown to induce apoptosis through a separate mechanism with the generation of ceramide (21), inhibition of topoisomerase II activity (19), and to stimulate the generation of ROS (22). In the present study, we demonstrate that paclitaxel and, to a lesser extent, daunorubicin induce apoptosis in the L3.6 human pancreatic cell line (Figures 3, 4, 5). Paclitaxel demonstrates a caspase-dependent apoptotic cascade, which appears to be different from the caspase-independent cascade induced by daunorubicin (Figures 7 and 8). Paclitaxel causes doublet formation at 26 kilodaltons consistent with phosphorylation of the Bcl-2 protein, possibly inducing inhibition of Bcl-2 antiapoptotic activity (Figure 7, panel C, lane 2). In contrast, daunorubicin does not appear to induce the early (24 h) doublet formation of Bcl-2 but does cause the down-regulation of this molecule at 24 h and 48 h. Furthermore, daunorubicin does not appear to activate the proximal caspase 8 (Figure 8, panel A) or the executioner caspase 3 (Figure 8, panel B) as shown with paclitaxel treatment.

In addition, the data demonstrates that, unlike paclitaxel, daunorubicin causes the generation of ROS (Figure 6) resulting in the induction of the necrotic pathway. This is also observed morphologically when the L3.6 cells are treated with daunorubicin and daunorubicin plus paclitaxel for 48 h (Figure 3).

Taken together, both the morphological changes and the enzymatic conversion of procaspases 8 and 3 with paclitaxel and not daunorubicin suggest a caspase-independent apoptotic pathway for daunorubicin. This is also confirmed by the cytomeric analysis of the annexin V staining of 20% of the cells following daunorubicin treatment alone.

Daunorubicin causes generation of ROS very early, within 15 min of exposure to the L3.6 cells. It is likely that daunorubicin induces an early necrotic pathway. The majority of the cells in both the daunorubicin-treated and the combination with paclitaxel demonstrates necrosis as shown by PI uptake. The optimal cytotoxicity was observed when equitoxic concentrations of both daunorubicin and paclitaxel were added to the L3.6 cells at the same time, suggesting that both the necrotic pathway and the apoptotic pathway, both caspase-dependent and -independent apoptosis, effectively cross-talk to cause a maximum antitumor effect.

The combination of anthracyclines and the taxanes is not a novel concept; it was first investigated in breast cancer, both in vitro and in vivo (23,24). Hahn and co-workers using several different cell lines, reported that doxorubicin and taxol were less than additive in in vitro cytotoxic assays (23). The incubation period was only 24 h in their experiments, which was not long enough as suggested by our data from this manuscript. Clinical studies suggest that paclitaxel and doxorubicin in breast cancer patients is highly active, which again is not consistent with the findings of Hahn and coworkers (25). Syrigos and co-workers have recently demonstrated in a phase-II study that liposomal doxorubicin in combination with docetaxel in advanced pancreatic cancer was well-tolerated with longer than usual median survival (8.5 months) in this poor prognosis group (26).

The importance of these findings reported in our study is that we show two conventional chemotherapeutic agents with known activity inducing tumor death via three distinct mechanisms; caspase-dependent apoptosis, caspase-independent apoptosis and necrosis, that appear to enhance each other's activity. Moreover, the future of cancer treatment may be in combining chemotherapeutic agents that induce not only apoptosis using both the caspase-dependent and caspase-independent pathways, but also drugs that induce necrosis as well.

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