Triphendiol (NV-196), development of a novel therapy for pancreatic cancer

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Despite incremental progress in the treatment of pancreatic adenocarcinoma, the prognosis of patients remains poor. Here, we report the preclinical studies in pancreatic cancer cells that demonstrate the efficacy of triphendiol (NV-196, a synthetic isoflavene) both as a monotherapy and as a gemcitabine sensitizer. The in-vitro effects of triphendiol on the pancreatic cancer cell lines HPAC and MIAPaCa-2 were determined using cell proliferation, flow cytometry, and western blot analysis. The antiproliferative activity of triphendiol was also investigated in two xenograft models of pancreatic cancer (HPAC and MIAPaCa-2). As a monotherapy, triphendiol-inhibited cell proliferation-induced p53-independent G_2/M cell cycle arrest and activation of the intrinsic (mitochondrial) apoptosis pathway. Triphendiol-induced apoptosis was caspase independent and death receptor independent, whereas cell necrosis was caspase mediated. Using combination index analysis, we have shown that pretreatment of pancreatic cancer cells with triphendiol enhanced the cytotoxic effect of gemcitabine, the standard of care used to treat advanced pancreatic cancer. In xenograft models of pancreatic cancer, the rate of tumor proliferation on mice coadministered with triphendiol and gemcitabine was significantly reduced when compared with the corresponding tumor proliferation rates from the respective monotherapy-control and vehicle-control groups. Triphendiol was recently granted Investigational New Drug status by the US Food and Drug Administration. These data justify the commencement of clinical studies investigating the utility of combining triphendiol and gemcitabine in patients with early-stage and late-stage pancreatic cancer. *Anti-Cancer Drugs* 22:719–731 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Background

Pancreatic cancer is the fourth most lethal malignancy in the USA. The American Cancer Society has predicted that there will be 42 470 new cases of pancreatic cancer and 35 240 deaths in 2009, giving an incidence-to-death ratio of 83% [1]. The most aggressive malignancy of the pancreas is adenocarcinoma, an exocrine neoplasm of pancreatic ductal epithelial cells that constitutes more than 90% of pancreatic cancer, but is rarely curable and has an extremely poor 5-year survival rate [1]. Hereditary, environmental, occupational, and social factors are recognized as potential contributors to the development of pancreatic cancer [2].

Gemcitabine is the drug of choice for palliative treatment of patients with unresectable ductal pancreatic adenocarcinoma, improving quality of life and increasing survival to about 6–7 months [3,4] when compared with the historical mean survival of 4 months [1] from diagnosis. The relatively low incidence of gemcitabine-associated side effects compared with other treatment options enables additional combination therapy options to be tested [5]. However, although many combinations have been tested, no compelling evidence of clinically relevant improvements in the quality of life and survival has been observed. Thus, it is fair to state that there are still no evidence-based treatment options for gemcitabine-refractory, advanced pancreatic cancer, nor are there any effective additional

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first-line agents superior to gemcitabine [6]. Thus, early diagnostic markers and new therapeutic targets for pancreatic cancer are urgently needed.

Although the mechanism of action of gemcitabine (a fluorinated nucleoside analog) is not fully understood, its metabolites, dFdCDP and dFdCTP, are known to induce forced termination of DNA chain elongation and inhibition of ribonucleotide reductase, respectively, resulting in mitochondrial depolarization and apoptosis induction. The exploitation of increased DNA synthesis in cancer cells over normal cells explains its reduced toxicity profile when compared with other cytotoxic agents [7,8]. The exact mechanism of gemcitabine resistance in pancreatic adenocarcinomas has not be characterized [9]; however, it has been hypothesized that antiapoptotic B cell lymphoma protein (Bcl-2) family members [8,10], elevated PI3K-Akt pathway activity [11], perhaps because of overexpression of HMGA1 [12], elevated NFκB [9], elevated Src tyrosine kinase activity [13], and deoxycytidine kinase inhibition [14] contribute to the resistant phenotype. Aberrant expression of c-Met and c-Src is also implicated in the development of gemcitabine resistance [13].

With only modest clinical benefit, gemcitabine remains the standard of care in adjuvant [15] and palliative chemotherapy [3] of pancreatic cancer, hence there is an urgent need for novel agents that, when used in combination with gemcitabine, can improve its efficacy, and patient outcomes. Synthetic isoflavanoids such as phenoxodiol (NV-06) have been shown to sensitize epithelial ovarian carcinoma cells to gemcitabine, docetaxel, paclitaxel, topotecan, and carboplatin, thus rendering pan-resistant cells sensitive to these chemotherapeutics and lowering the dose of drug necessary to inhibit tumor growth [16–18]. Phenoxodiol degrades Akt, X-linked inhibitor of apoptosis (XIAP), and cFLIP [19] resulting in the activation of the intrinsic caspase-driven (mitochondrial) apoptotic pathway and reactivation of the Fas death receptor pathway in epithelial ovarian carcinoma cells. Although pancreatic cancer shows initial sensitivity to gemcitabine therapy, the onset of resistant disease is rapid and typically fatal. In this study, we have demonstrated that triphendiol (an analog of phenoxodiol) induced both caspase-dependent and caspase-independent apoptosis pathways and caused a reduction in total Akt protein accompanied by XIAP degradation in HPAC and MIAPaCa-2 cell lines, with supporting evidence from PANC-1 cells for some of these findings. These data may account for the ability of triphendiol to sensitize pancreatic cancer xenografts to gemcitabine.

Methods

Cells

Human pancreatic cell lines HPAC, MIAPaCa-2, and PANC-1 were obtained from the American Type Culture Collection (Manassas, Virginia, USA). The cells were maintained at 37°C, 5% CO_2, and cultured in Dulbecco's modified Eagle's medium (DMEM) with Gln and high glucose (Mediatech, Manassas, Virginia, USA) with 10% fetal bovine serum (FBS, HyClone, Logan, Utah, USA) and penicillin/streptomycin.

Antibodies

Antibodies used in this study included p53 and p21 (Calbiochem, EMD, San Diego, California, USA); caspase 9, XIAP, COX IV, Bcl-2, and Akt (Cell Signaling Technology, Danvers, Massachusetts, USA) and caspase 2, cytochrome *c*, and Bid (BD Pharmingen, San Diego, California, USA). GAPDH (RDI, Concord, Massachusetts, USA) or β-actin (Sigma, St. Louis, Missouri, USA) was used as a housekeeping gene to standardize the protein loading on the gels and blots.

Cell viability

Cell viability was measured on 96-well plates using the MTS assay (CellTiter 96 Aqueous One; Promega, Madison, Wisconsin, USA) as described earlier [16].

Fluorescence-activated cell sorting analysis

Apoptosis was measured by fluorescence-activated cell sorting (FACS) analysis using annexin-V–FITC and propidium iodide (PI; Biovision, Mountain View, California, USA) as probes [20]. Mitochondrial membrane potential was measured by FACS analysis using JC-1 (Biovision) as a probe [20]. Cell cycle was analyzed on ethanol-fixed, RNaseA-treated cells with PI staining. In each spectrum, at least 10 000 gated events were recorded and analyzed using FACSCalibur (BD Biosciences, San Jose, California, USA). All experiments were done in triplicate with error bars representing one standard deviation. Caspase inhibitor I (zVAD-fmk) was obtained from Calbiochem.

Western blot analysis

Cells were seeded on 100-mm tissue culture plates and grown overnight to approximately 90% confluence. Cells were then treated with the drug combinations for the indicated time points. After the final treatment step, the cells were lysed in 500 µl of a lysis buffer containing 1% of Nonidet P-40, 0.1% of SDS, 0.5% of deoxycholate, 150 mmol/l of NaCl, 50 mmol/l of Tris–HCl (pH 7.5), 1 mmol/l of EDTA, 1 mmol/l of phenylmethanesulfonyl fluoride, and protease inhibitors (Roche Diagnostics Corporation, Roche Applied Science, Indianapolis, Indiana, USA) on ice for 30 min. Cell lysates were harvested with a sterile cell scraper and centrifuged at 15 000 × g for 15 min to remove cell debris. Protein was assayed by the bicinchoninic acid method (Pierce, Thermo Fisher Scientific, Rockford, Illinois, USA) and 50-µg aliquots were separated on 12%T, 2.6%C SDS-PAGE gels. Proteins were electrophoretically transferred to polyvinylidine fluoride membranes (Immobilon P; Millipore, Billerica, Massachusetts, USA) by the wet transfer method. These membranes were probed with specific antibodies and with anti-GAPDH, which was

used as the housekeeping gene to standardize the protein loading. The blots were developed on X-ray films using horseradish peroxidase-conjugated secondary antibodies (Southern Biotech, Birmingham, Alabama, USA) and a chemiluminescent substrate (ECL; Amersham, New York, USA).

Caspase activity

After a 48-h treatment with 10 μmol/l NV-196, HPAC, MIAPaCa-2, and PANC-1 cells were harvested by trypsinization and resuspended in DMEM supplemented with 10% FBS. The cells were incubated with caspase substrates for 1 h at 37°C, 5% CO_2, and then probed with annexin-V–Cy5 and sytox blue. Caspase-positive cells were gated as shown in the histograms and these cells are indicated on annexin-V–Cy5/sytox blue spectra as blue cells. In-situ caspase activity was assayed using fluorescent substrates. For caspase-2, FITC-VDVAD-fmk (Biovision K182-100); caspase-3, FITC-DEVD-fmk (K183-100), and caspase-9, FITC-LEHD-fmk (K199-100) was used. These substrates bind irreversibly to their respective activated caspases, and caspase activation was quantified by flow cytometry. After 1-h incubation at 37°C in 5% CO_2, the cells were washed with Dulbecco's phosphate buffered saline containing 5% FBS and resuspended in annexin-V binding buffer containing annexin-V–Cy5 (Biovision K103-3) and sytox blue (Invitrogen), a dye that binds DNA. Caspase activation, apoptosis and necrosis were measured simultaneously using a violet, blue, and red laser in a BD LSRII flow cytometer.

Xenograft studies

Five-week-old male Balb/c nude mice were maintained on an isoflavone-free diet to remove background isoflavone levels contributed by standard feed. On the day of inoculation, a suspension of MIAPaCa2 or HPAC cells was prepared in DMEM (–FBS), chilled on ice, and then an equal volume of matrigel (BD Biosciences) was added. Mice were inoculated subcutaneously with 3×10^6 MIAPaCa2 or HPAC cells bilaterally (midway between the axillary and the inguinal region) along the dorsal surface, ensuring that the cells to be injected were growing at the midend log phase. Tumor growth was monitored for more than 10–15 days. Triphendiol was administered daily by oral gavage in 1% carboxymethylcellulose as a vehicle. Gemcitabine was injected intraperitoneally every third day for a total of four doses (q3d × 4) in PBS. For triphendiol–gemcitabine combination groups, animals were first treated with triphendiol and then with gemcitabine. For the respective HPAC and MIAPaCa-2 xenograft studies, the administration schedule, dosage, and route of delivery of gemcitabine and triphendiol when treated as monotherapy or in combination are given in the caption to Fig. 6. Animal studies were conducted under the University of Sydney Animal Ethics Committee protocol number N00/10-2005/3/4209 in accordance with the guidelines of the National Health and Medical Research Council of Australia.

Results

Triphendiol inhibits proliferation and induces apoptosis in pancreatic cell lines

Triphendiol as a monotherapy exhibits marked time-dependent antiproliferative activity against HPAC, PANC-1, and MIAPaCa-2 pancreatic cancer cell lines. Of the pancreatic cancer cell lines used, MIAPaCa-2 was the most sensitive to the antiproliferative effect of triphendiol after 24-h exposure; however, after 48-h exposure, the viability of all cell lines was reduced at 30 μmol/l (Fig. 1a–c). After 120-h exposure, the efficacy of triphendiol was pronounced against all cell lines [inhibitory concentration (IC_{50}) of approximately 8 μmol/l for both HPAC and PANC-1] with MIAPaCa-2 being the most sensitive ($IC_{50} = 0.8$ μmol/l; Fig. 1d). For comparison, under the same culture conditions gemcitabine has an IC_{50} value of between 0.05 and 0.1 μmol/l for MIAPaCa-2 and HPAC, and approximately 4 μmol/l for PANC-1 (data not shown).

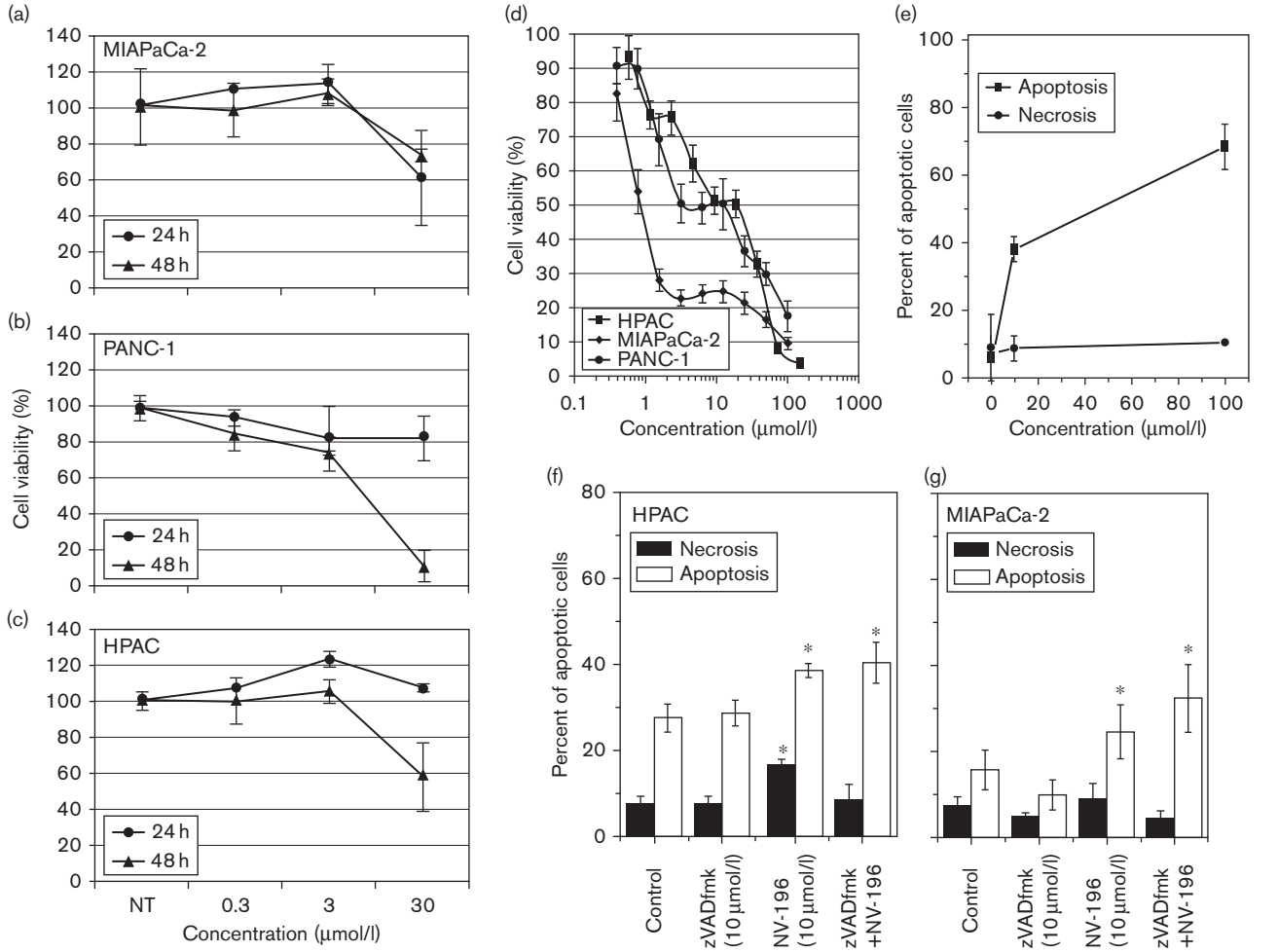
Apoptosis-induction studies in pancreatic cancer cell lines using triphendiol and gemcitabine

To assess the proapoptotic potential of triphendiol in pancreatic cancer, we conducted dose-escalating studies for more than 48 h and used FACS analysis on annexin-V–FITC/PI stained cells. In all pancreatic cancer cell types, triphendiol induced apoptosis in a dose-dependent manner (representative data for MIAPaCa-2 is shown in Fig. 1e). As a monotherapy, triphendiol induced very little necrosis in pancreatic cancer cells (Fig. 1e–g).

Effect of caspase inhibitor I on in-vitro triphendiol-dependent apoptosis in MIAPaCa-2 and HPAC pancreatic cancer cells

HPAC cells are resistant to Apo-2L, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and sensitive to FasL-dependent apoptosis, whereas MIAPaCa-2 cells are sensitive to TRAIL-dependent apoptosis and resistant to FasL-dependent apoptosis, both of which are caspase-dependent apoptosis pathways. As a result, Fas-activating antibody CH11 (Upstate, Millipore, Billerica, Massachusetts, USA) and TRAIL were used as positive controls, respectively, in these cell types (data not shown). In HPAC cells, pretreatment of cells with caspase inhibitor I (zVAD-fmk; 10 μmol/l) for 1 h, followed by 48-h exposure to NV-196 (10 μmol/l) inhibited triphendiol-dependent cell death characterized by cell detachment and PI binding, but did not inhibit triphendiol-induced apoptosis (Fig. 1f). In MIAPaCa-2 cells, pretreatment of cells with zVAD-fmk resulted in an inhibition of triphendiol-dependent necrosis and apparently enhanced triphendiol-induced apoptosis, perhaps as a result of the cells being blocked from advancing to a cell death characterized by cell detachment and PI binding (Fig. 1g). These results show that a large

Fig. 1



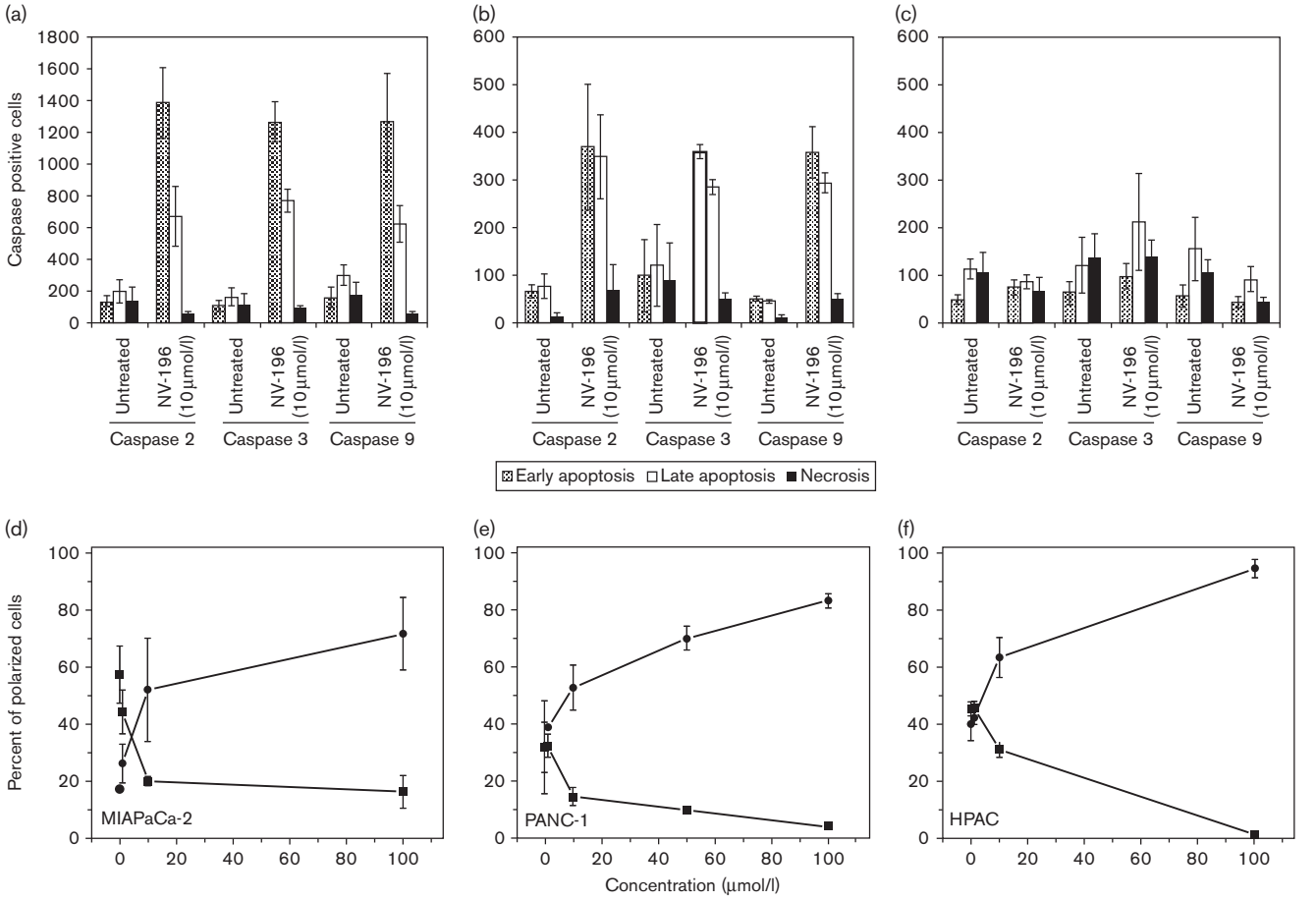
Triphendiol (NV-196) decreases the cell viability of pancreatic cell lines. The viability (in percentage, normalized to untreated cells) of pancreatic cancer cells after treatment with increasing concentrations of NV-196 after 24, 48, and 120-h treatment. Each experiment was done in triplicate. Efficacy of NV-196 against MIAPaCa-2, PANC-1, and HPAC pancreatic cancer cell lines (a–d). (a) MIAPaCa-2 (● 24-h and ▲ 48-h treatment), (b) PANC-1 (● 24-h and ▲ 48-h treatment), (c) HPAC (● 24-h and ▲ 48-h treatment), and (d) 120-h treatment: ▲ PANC-1, ◆ MIAPaCa-2, and ■, HPAC. (e and f) Cells were preincubated with 10 μmol/l of z-VAD-fmk for 1 h before the addition of 10 μmol/l of triphendiol. After 42–46 h, the cells were harvested and assayed for apoptosis by fluorescence-activated cell sorting analysis. In each spectrum, 10 000 gated events were recorded and analyzed. All experiments were done in triplicate; error bars represent one standard deviation. Unpaired *t*-tests were used for statistical analysis; $*P<0.05$. (e) Comparing control apoptosis with treatment on HPAC cells: control versus NV-196 $P=0.003$, control versus NV-196 + zVADfmk $P=0.018$. Comparing control necrosis with treatment on HPAC cells: control versus NV-196 $P=0.001$. (f,g) Comparing control apoptosis with treatment on MIAPaCa-2 cells: control versus NV-196 $P=0.040$, control versus NV-196 + zVADfmk $P=0.008$.

component of triphendiol-induced apoptotic effect uses caspase-independent pathways in pancreatic cancer, whereas the triphendiol-induced pathways resulting in detached, PI-binding cells are caspase dependent.

Effect of triphendiol on caspase activation in pancreatic cancer cell lines

Caspase activation, apoptosis, and necrosis were measured simultaneously in untreated and NV-196-treated pancreatic cancer cells by flow cytometry (Fig. 2). After 48 h, NV-196 induced the activation of caspases 2, 3, and 9 in MIAPaCa-2 and PANC-1 cells (Fig. 2a and b). Caspase-positive cells were also annexin-V-Cy5 positive showing that the NV-196-induced caspase activation was occurring in cells undergoing apoptosis. In both MIAPaCa-2 and PANC-1, approximately 60% of triphendiol-treated, caspase-positive cells were annexin-V positive and sytox-blue negative indicating that they were in the early stages of apoptosis (Fig. 2a and b). Approximately 30% of the caspase-positive cells bound both annexin-V–Cy5 and sytox blue, indicating that these cells were entering the later stages of apoptosis. Less than 5% of the triphendiol-induced, caspase-positive cells were sytox blue positive and annexin-V negative, indicating that caspases do not contribute much to triphendiol-dependent necrosis. Although caspases 2, 3 and, 9 were

Fig. 2



Assessment of in-situ caspase activity and mitochondrial integrity in triphendiol-treated pancreatic cancer cells. Fluorescence-activated cell sorting analysis of caspases 2, 3, and 9 in MIAPaCa-2 : (a) PANC-1 (b), and HPAC (c) cells in response to triphendiol (10 µmol/l, 48 h) compared with vehicle control. For mitochondrial depolarization studies, pancreatic cancer cells were treated with tripendiol (10 µmol/l) for 48 h. Data for MIAPaCa-2 (d), PANC-1 (e), and HPAC (f) are shown. Aggregated JC-1 red fluorescence from intact, polarized mitochondria is displayed on the Y-axis, whereas monomeric JC-1 green fluorescence from apoptotic cells with depolarized mitochondria is displayed on the X-axis. The upper left quadrant is defined as polarized cells. The sum of the upper right and lower right quadrants are defined as depolarized cells. In each spectrum, 10 000 gated events were recorded and analyzed. All experiments were done in triplicate and error bars represent one standard deviation.

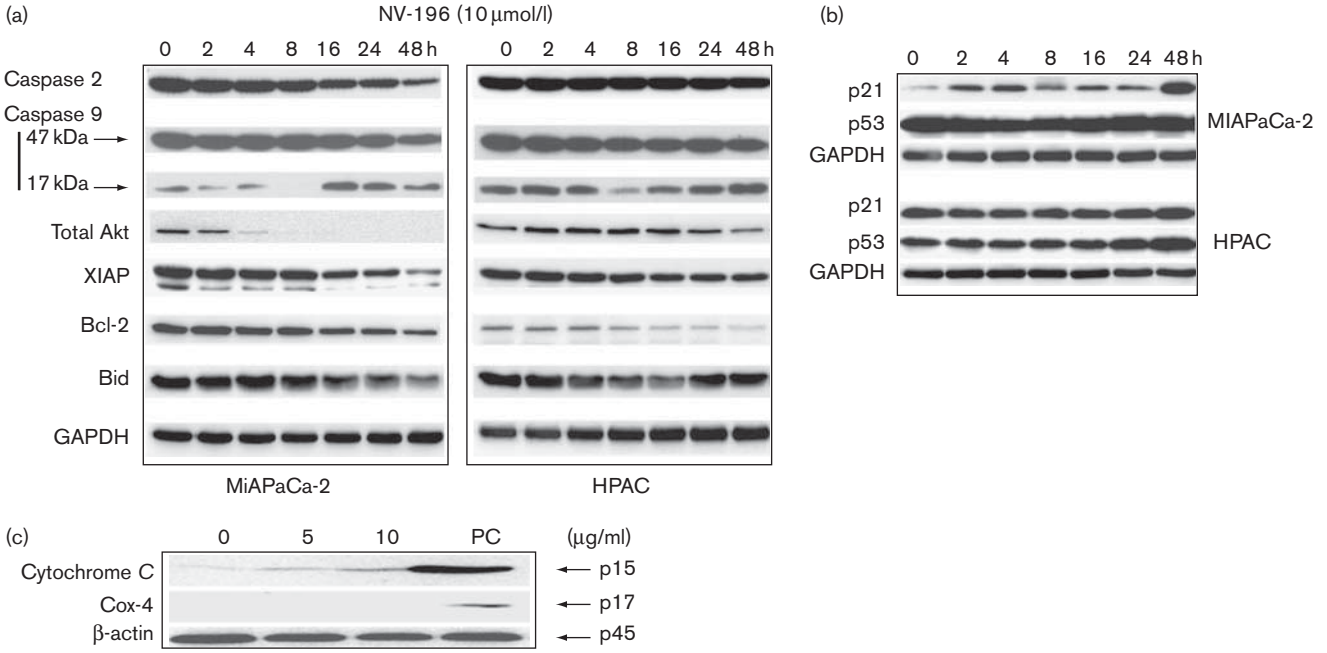
all activated by triphendiol in MIAPaCa-2 and PANC-1 cells (Fig. 2a and b), gemcitabine activated only caspases 3 and 9 (data not shown). Activation of caspase 9 suggests that the intrinsic (mitochondrial) pathway of apoptosis was activated by both the compounds. Activation of caspase 2 by triphendiol could be because of cellular stress or DNA damage but the role of caspase 2 in apoptosis remains controversial [21].

With the exception of caspase 3, which was modestly induced in triphendiol-treated HPAC cells, neither caspase 2 nor caspase 9 was induced in this cell line under the conditions used (Fig. 2c). At a higher concentration (30 µmol/l), triphendiol activated caspase 9 but not caspase 2 in HPAC cells (data not shown). These data suggest that caspase-independent mechanisms of apoptosis are activated in triphendiol-treated HPAC cells.

Western blot analysis showed that full-length caspase 2 (48 kDa) was cleaved in MIAPaCa-2 cells by exposure to triphendiol (10 µmol/l; Fig. 3a). In contrast, caspase 2 was cleaved to a lesser extent by triphendiol in HPAC cells (Fig. 3a). Over the course of the time-course experiment, triphendiol induced an initial drop in cleaved caspase 9 (17 kd), followed by an increase after 16 h in both cell types (Fig. 3a). By western blot analysis, triphendiol did not cause significant cleavage of caspase 3 in MIAPaCa-2 and HPAC cells; however, a longer exposure of the same blots showed minor cleavage of caspase 3 in HPAC cells (data not shown). Taken together, these data suggest that triphendiol affects both caspase activation and synthesis.

Earlier studies on OEC cells had shown that the caspase 3 inhibitor, XIAP is significantly reduced by phenoxodiol and that this isoflavanoid stimulates autodegradation of this baculoviral inhibitor of apoptosis repeat-containing

Fig. 3



Triphendiol regulates both caspase-dependent and caspase-independent death-inducing pathways in pancreatic cancer. (a) Western blot analysis of caspase 2, caspase 9, XIAP, Bcl-2, Bid in MIAPaCa-2, and Akt in MIAPaCa-2 and HPAC cells. (b) Western blot analysis of p21and p53 expression in MIAPaCa-2 and HPAC cells. In both data sets, cells were treated with 10-μmol/l triphendiol for 0, 4, 8, 16, 24, and 48 h. Cells were lysed, preparations centrifuged, and lysates were collected. Respective cell lysate (25 μg) samples from each time point were separated by SDS-PAGE, transferred to polyvinylidine fluoride membranes and probed with antibodies specific to the target antigen. Protein loading was standardized to GAPDH and the GAPDH data shown is representative of replicate studies (RDI, Concord, Massachusetts, USA). (c) Western blot analysis of cytochrome *c* release from PANC-1 cells treated with NV196 (5 and 10 μg/ml) for 48 h. A parallel experiment was performed in which the cytoplasmic and mitochondrial fractions were separated before western blot analyses. Cox-4 was included to show the integrity of the cytoplasmic and mitochondrial preparations and protein loading was standardized to β-actin. XIAP, X-linked inhibitor of apoptosis.

gene family member [16]. In both HPAC and MIAPaCa-2 cells, triphendiol reduced the level of XIAP (Fig. 3a); however, XIAP degradation was more pronounced in MIAPaCa-2 cells. Reduced XIAP levels may explain caspase 3 activation in triphendiol-treated MIAPaCa-2 cells [22]. Significantly, total Akt is also reduced at an early time point (4 h), suggesting that total Akt removal, accompanied by XIAP degradation, are two of the earliest events in the mechanism of action of triphendiol (Fig. 3a). Reduction in Akt expression would also impact the expression of XIAP [23]. Further studies are required to understand the process of XIAP removal caused by triphendiol.

Effect of triphendiol on in-vitro mitochondrial potential in pancreatic cancer

Triphendiol induced mitochondrial depolarization in all three pancreatic cancer cells (MIAPaCa-2, PANC-1, and HPAC) tested, in a dose-dependent manner after 48 h of the treatment, thus confirming that the intrinsic pathway of apoptosis has been activated (Fig. 2d–f). In mitochondrial preparations from triphendiol-treated pancreatic cancer cells, cytochrome *c* was released into the surrounding buffer, further confirming that triphendiol compromises mitochondrial integrity and thereby primes the cell to undergo apoptosis (Fig. 3c).

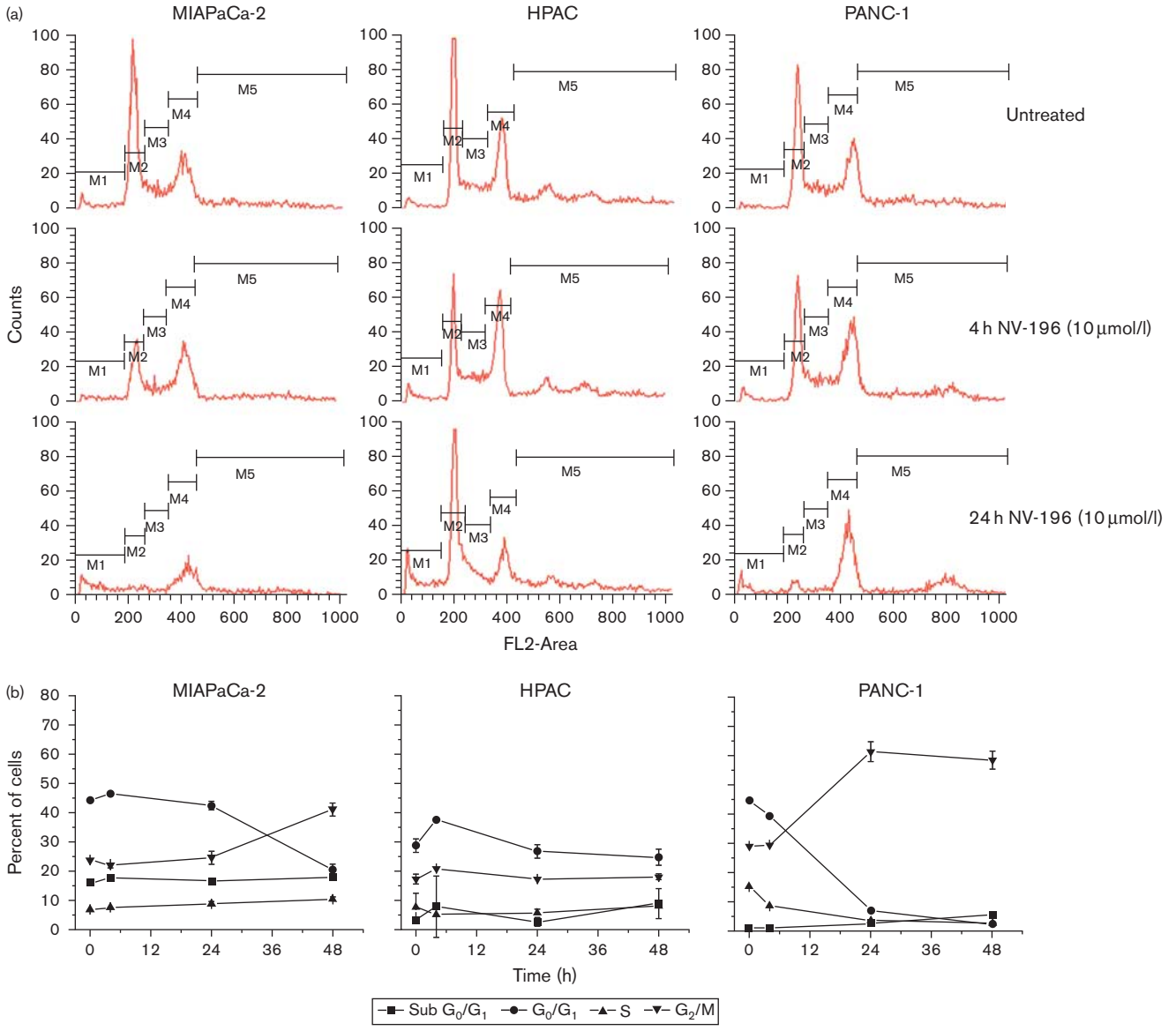
Effect of triphendiol on Bcl-2 family member stoichiometry in pancreatic cancer cell lines

Given the observation that triphendiol was able to induce mitochondrial depolarization, we investigated whether its action may be involved in altering the stoichiometry of proapoptotic (Bid, Bak, Bcl-2-associated X protein) and antiapoptotic (Bcl-2, Bcl-xL) Bcl-2 family members. After triphendiol treatment of MIAPaCa-2 and HPAC cells, Bcl-2 levels were reduced in both cell lines (Fig. 3a). Treatment with triphendiol induced Bid cleavage over time in MIAPaCa-2 cells (Fig. 3a), whereas there seemed to be a transitory increase in Bid cleavage in HPAC cells (Fig. 3a).

Effects of triphendiol on in-vitro cell cycle progression in pancreatic cancer

In all three pancreatic cancer cells assessed (HPAC, MIAPaCa-2, and PANC-1), triphendiol arrested cell cycle in the G_2/M phase to varying degrees, with a loss of G_0/G_1 and an increase in the sub G_0/G_1 phase (Fig. 4). It is worth noting that the timing of triphendiol-induced

Fig. 4



Effect of triphendiol on cell-cycle progression in MIAPaCa-2, HPAC, and PANC-1 pancreatic cancer cells. (a) Representative fluorescence-activated cell sorting (FACS) spectra and (b) histograms of untreated and triphendiol-treated MIAPaCa-2, HPAC, and PANC-1 cells. Gates: M1 = sub-G_0/G_1, M2 = G_0/G_1, M3 = S, M4 = G_2/M, and M5 = polyploidy cells. In each spectrum, 10 000 events were recorded, gated, and analyzed. All experiments were done in triplicate; (b) combined plots of cell cycle distribution. Cells were exposed to 10 µmol/l of triphendiol for 0, 4, 24, and 48 h. Cells were fixed in ethanol, stained with propidium iodide and analyzed by FACS analysis. All experiments were done in triplicate; error bars represent one standard deviation.

G_2/M arrest is different for each cell line. PANC-1 was arrested in G_2/M some 4–24 h after triphendiol treatment and remains in arrest for at least 48 h. MIAPaCa-2 cells enter G_2/M arrest some 24–48 h after triphendiol treatment. Accompanying the arrest is a corresponding decrease in the G_0/G_1 cell population. The G_2/M arrest observed in HPAC cells in response to triphendiol was less pronounced and was accompanied by a G_0/G_1 arrest. However, the arrest induced would seem to be transient (Fig. 4).

The cell cycle is controlled by cyclin-dependent kinases, which in turn are regulated by p21, usually through a p53-dependent pathway. Most pancreatic cancers have mutant p53, which arrests p53-dependent transcription. HPAC cells are unusual in having a wild-type p53, whereas MIAPaCa-2 and PANC-1 have R248W and R273H mutations in p53. These mutations are in loop 3 of p53 and affect the ability of p53 to bind DNA [24]. The basal level of p53 protein is 94× higher in MIAPaCa-2 than HPAC (data not shown). The

expression of p53 in MIAPaCa-2 was unaffected by triphendiol (Fig. 3b), but was elevated by 48 h in HPAC (Fig. 3b). The expression of p21 is vanishingly low in MIAPaCa-2 and is elevated by triphendiol (Fig. 3b), whereas p21 is abundant in HPAC and is essentially unchanged by triphendiol (Fig. 3b).

Evidence of triphendiol and gemcitabine synergy in pancreatic cancer cell lines

Using the multiple drug-effect equation of Chou and Talalay [25], we have demonstrated that triphendiol synergizes the toxic effect of gemcitabine against pancreatic cancer cells. Combination testing of drugs can be done directly (i.e. concurrently exposure), or in sequence (i.e. sequential exposure). We observed that optimal sensitization of HPAC (Fig. 5a) and MIAPaCa-2 (Fig. 5b) pancreatic cancer cells was achieved using a 24-h pretreatment of cells with triphendiol at varying concentrations followed by a 120-h treatment of gemcitabine. Without pretreatment, the evidence of sensitization *in vitro* was less pronounced in both cell lines (not shown). A strong synergy (combination index of < 0.5) was observed using the triphendiol–gemcitabine combination against both pancreatic cancer cell lines; however, the synergy was dependent on the concentration of individual components. The observed synergy in MIAPaCa-2 was apparent across all the IC parameters investigated (i.e. IC_{50}–IC_{99}) with a dose reduction index of between 3-fold and 4-fold (i.e. reduction in the effective dose of gemcitabine as a monotherapy). The observed synergy was less extensive using the HPAC cell line, although a dose-reduction index of approximately 7.8-fold was observed for gemcitabine at the IC_{50} parameter, and no evidence of synergy was observed at IC_{75} and IC_{90}. The data demonstrate that triphendiol effectively sensitizes both pancreatic cancer cell lines to gemcitabine, although when compared with HPAC, the MIAPaCa-2 cell line was more sensitive to the combination.

Efficacy of triphendiol as a monotherapy against HPAC and MIAPaCa-2 xenograft models of pancreatic cancer

In the MIAPaCa-2 model of pancreatic cancer, animals administered the combination of triphendiol and gemcitabine at 50 and 4 mg/kg, respectively, displayed significantly reduced rates of tumor proliferation and terminal tumor burden (% T/C = 38) in comparison with the respective single-agent controls (Fig. 6a and b). Moreover, those animals receiving a combination of triphendiol and gemcitabine at dosages half that of the single-agent controls (25 mg/kg of triphendiol and 2 mg/kg of gemcitabine) also had significantly reduced tumor proliferation kinetics and reduced terminal tumor burden (%T/C = 47.8) when compared with monotherapy controls (Fig. 6a and b). The effectiveness of both triphendiol and gemcitabine dosage combinations was further shown by the observation that tumors on animals administered either combination proliferated at significantly reduced rates in a dose-responsive manner when compared with the respective monotherapy controls (Fig. 6a) Together these data indicate that triphendiol and gemcitabine when administered in combination act synergistically in reducing the overall tumor burden using the MIAPaCa-2 pancreatic cancer tumor model.

In the HPAC model of pancreatic cancer, when compared with vehicle control, orally administered triphendiol [100 mg/kg, quaque die (every day) × 12] retarded the rate of tumor proliferation, at a level similar to that of gemcitabine [20 mg/kg, *quaque die* (every 3 days) × 4; Fig. 6c]. Animals administered with both triphendiol and gemcitabine at 100 and 20 mg/kg, respectively, had significantly reduced tumor growth and terminal tumor burden (%T/C = 51) when compared with vehicle and single-agent controls (Fig. 6c and d). The observed mean terminal tumor burden for the combination was markedly below that of the respective single-agent controls (100 mg/kg of triphendiol, T/C = 94%; for 20 mg/kg of gemcitabine, T/C = 79%; Fig. 6d). These data suggest that when administered in combination, triphendiol and gemcitabine may act synergistically to reduce the overall tumor burden using the HPAC pancreatic cancer xenografts.

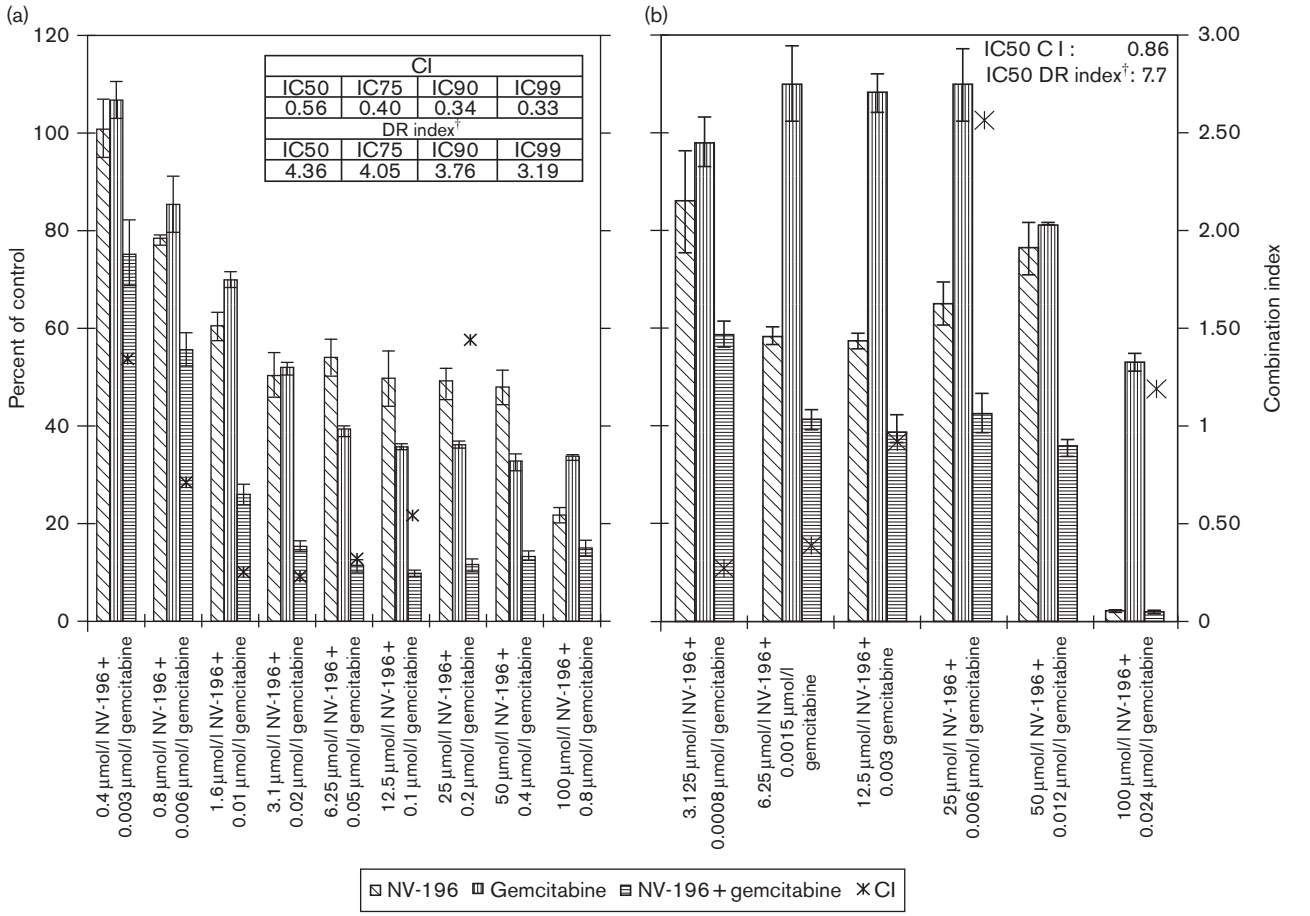
The pronounced antiproliferative effect observed in the combination groups from both studies further supports the intended use of triphendiol as a sensitizer to be used in combination with gemcitabine in the treatment of human pancreatic cancer.

In those animals receiving high-dose and low-dose combinations of gemcitabine and triphendiol, all markers of renal and hepatic function were comparable to that of control animals as were the white blood cell and red blood cell counts (data not shown). Histopathological analysis of vital organs revealed no abnormalities that could be attributed to drug toxicity (not shown). These data demonstrate that triphendiol does not exacerbate gemcitabine toxicity with respect to the parameters measured and can be used safely in combination with gemcitabine at the dosages employed.

Discussion

The results of this study show that triphendiol (NV-196) inhibited cell proliferation, arrested cell cycle, and induced cell death through an intrinsic (mitochondrial) apoptosis pathway in pancreatic adenocarcinoma cell lines. Triphendiol induced dose-dependent mitochondrial depolarization in three pancreatic cancer cell lines (HPAC, MIAPaCa-2, and PANC-1), independent of their p53 status: HPAC have wild-type p53 and MIAPaCa-2, and PANC-1 have mutant p53 [26]. In addition, the data presented demonstrate that triphendiol augments the antiproliferative effect of gemcitabine in two in-vivo models of pancreatic cancer.

Fig. 5



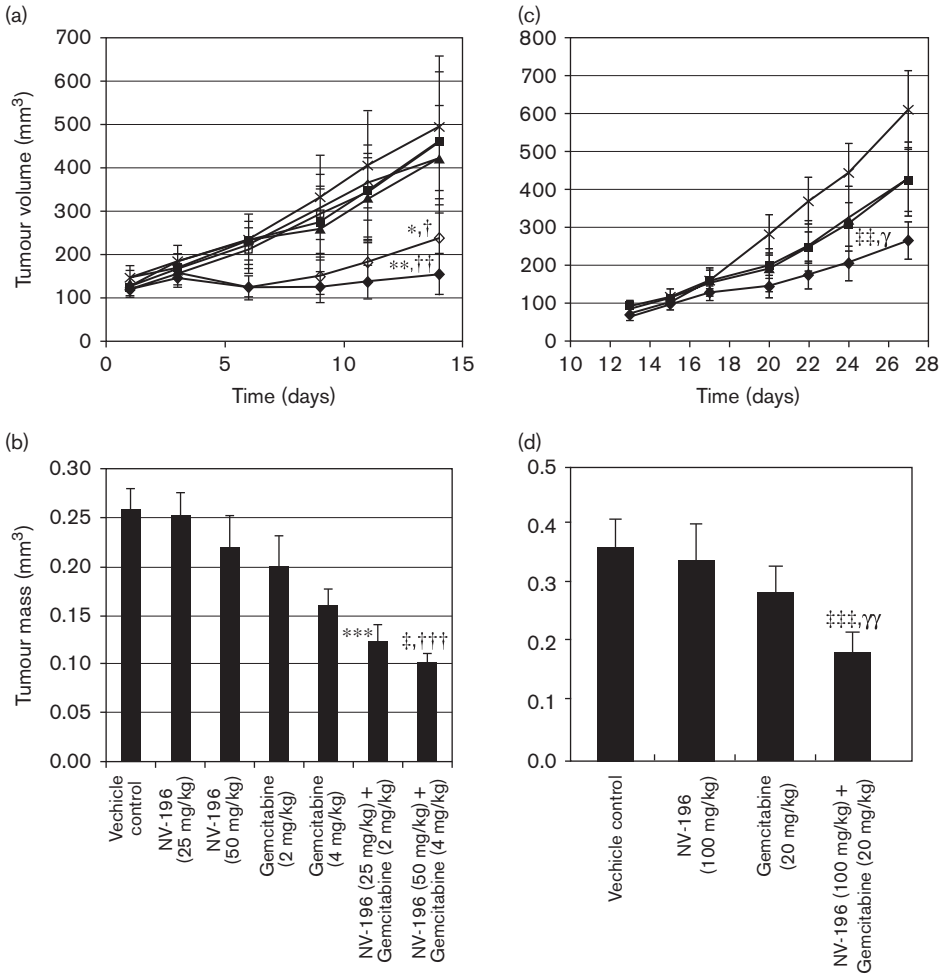
Assessment of triphendiol and gemcitabine synergistic toxicity against pancreatic cancer cell lines. Triphendiol sensitizes pancreatic cancer cell lines [(a) MIAPaCa-2; (b) HPAC] to the cytotoxic effects of gemcitabine and using multiple drug-effect equation of Chou–Talalay and combination index analysis. A 24-h preexposure of the cells to triphendiol was used. Inset shows the combination index analysis and dose reduction index at inhibitory concentrations $(IC)_{50}$, IC_{75}, IC_{90}, and IC_{99} parameters in (a), and IC_{50} parameters in (b). †Fold reduction in the effective concentration of gemcitabine when combined with triphendiol. CI, combination index.

The mechanism of action of triphendiol in pancreatic cancer seems to involve both caspase-dependent and caspase-independent death-inducing pathways, suggesting that its mechanism of action in this type of cancer involves a novel intrinsic (mitochondrial) pathway similar to that described by Kroemer and Martin [27]. Possible mechanisms of how triphendiol activates these pathways are outlined in a review article by Saif *et al.* [28]. In this study, triphendiol induced the downregulation of Bcl-2, an antiapoptotic Bcl-2 family member that protects the integrity of the mitochondria. Reduction of Bcl-2 is likely to allow mitochondrial depolarization, thereby explaining the release of cytochrome *c* and potentially other mitochondrial proteins, including endonuclease G, that stimulate caspase-dependent and caspase-independent cascades, respectively. The structurally related analoge NV-128 has been shown to induce mitochondrial membrane destabilization in chemoresistant ovarian cancer cells, resulting in the translocation of endonuclease G from the mitochondria to the nucleus, where it catalyzes DNA degradation and promotes caspase-independent apoptosis [23]. Similar to the mechanism of action of NV-128, the caspase-independent apoptosis observed in triphendiol-treated cells may occur as a result of translocation of endonuclease G from the mitochondria to the nucleus.

Triphendiol-mediated reduction in total Akt protein levels may explain the reduced levels of XIAP observed because of decreased rates of phosphorylation. Similar to the phenoxodiol (a structurally related isoflavanoid molecule) mechanism of action in ovarian cancer [16], triphendiol may further promote XIAP degradation by mediating the release of mitochondrial Smac/DIABLO and htrA2/Omi, which actively sequester and proteolytically degrade XIAP, respectively.

The reduction in total Akt and XIAP accompanied by the subsequent activation of caspases 9 and 3 after the

Fig. 6



Efficacy of triphendiol and gemcitabine combination therapy in xenograft models of pancreatic cancer. MIAPaCa-2 (a and b). (a) Comparative mean tumor volume data [± standard error of the mean (SEM)] taken from tumor-bearing mice treated with vehicle control (group 1, ×), triphendiol [25 mg/kg, per orally (p.o.), *quaque die* every day (q.d.) × 14; group 2, △], triphendiol (50 mg/kg, p.o., q.d. × 14; group 3, ▲), gemcitabine [2 mg/kg, intraperitoneally (i.p.), *quaque die* every 3 days (q3d) × 4; group 4, ■], gemcitabine (4 mg/kg, q3d × 4; group 5, □), triphendiol (25 mg/kg, p.o. q.d. × 12) and gemcitabine (2 mg/kg, i.p. q3d × 4; group 6, ◇), triphendiol (50 mg/kg, p.o. q.d. × 14) and gemcitabine (4 mg/kg, i.p., q3d × 4; group 7; ◆); *P=0.03 (group 6 vs. 2); †P=0.016 (group 6 vs. 4); **P=0.005 (group 7 vs. 3); ††P=0.0022 (group 7 vs. 5). (b) Comparative mean terminal tumor mass. ***P=0.003 (group 6 vs. 2); ‡P=0.006 (group 7 vs. 3); †††P=0.021 (group 7 vs. 5). Groups 6 and 7 were both significantly different from vehicle control in all the analyses. HPAC (c and d). (c) Comparative mean tumor volume data (± SEM) taken from tumor-bearing mice treated with vehicle control (group 1, ×), triphendiol (100 mg/kg p.o., *quaque die* every day × 12; group 2, ▲), gemcitabine (20 mg/kg i.p., q3d × 4; group 3, ■) and a combination group of triphendiol (100 mg/kg p.o., q.d. × 12) and gemcitabine (20 mg/kg i.p., q3d × 4; group 4; ◆). ‡‡P=0.04 (group 4 vs. 2); γP=0.03 (group 4 vs. 3). (d) Comparative mean terminal tumor mass. ‡‡‡P=0.07 (group 4 vs. 2); γγP=0.043 (group 4 vs. 3). Group 4 was significantly different from vehicle control in all the analyses (P<0.01). Unpaired *t*-tests were used for statistical analysis.

release of mitochondrial cytochrome *c* explains the mechanism by which triphendiol potentiates caspase-mediated apoptosis. In this scheme, it is not known whether caspase 2 directly activates caspase 3 or initiates mitochondrial membrane depolarization by truncation of Bid and subsequent translocation of t-Bid to the mitochondria; however, it is thought to be directly responsible for the cleavage of Bid. The status of p53 expression may also affect the activation of caspase 2, although in the current lines assayed caspase 2 activation was independent of p53.

Another possible mechanism is that triphendiol induces apoptosis through the activation of stress-induced apoptosis by virtue of its ability to stimulate caspase-2, which complexes with p53-induced protein with a death domain (PIDD) and receptor-interacting protein-associated ICH-1/CED-3-homologous protein with a death domain to form the PIDDosome [29]. PIDD is regulated by p53, which is often in the form of dominant-negative mutations in pancreatic cancer [26]. It is possible that p73 can substitute for p53 in cells with the phenotype [30].

We have demonstrated that triphendiol arrested the cell cycle of pancreatic adenocarcinoma cell lines. The cell cycle arrest induced by triphendiol seems to be p53 independent as it is observed in both wild-type p53 cells (HPAC) and in cells with dominant-negative p53 mutations (MIAPaCa-2 and PANC-1). p21, a key regulator of cyclin-dependent protein kinases, is enhanced by triphendiol through a p53-independent pathway in MIAPaCa-2 cells but is essentially unchanged in HPAC cells, which have wild-type p53.

The action of triphendiol on the cell cycle in pancreatic cancer is different from that of phenoxodiol in head and neck squamous cell carcinoma (HNSCC). Phenoxodiol arrested the cell cycle of HNSCC cells at the G_1–S phase, resulting in cells being arrested in the G_0/G_1 phase [31]. In contrast, triphendiol arrests the cell cycle in the G_2/M phase in pancreatic cancer. It seems that both phenoxodiol and triphendiol affect the cell cycle, but the effects seem to be tumor specific. Phenoxodiol also induced a p53-independent enhancement of p21 expression, resulting in a specific loss of cyclin-dependent kinase 2 (cdk2) activity in HNSCC cells. In MIAPaCa-2 cells, which have the dominant-negative R148W p53 mutation, triphendiol induced p21 expression similar to that observed in HNSCC cells, whereas the mechanism by which triphendiol induced cell-cycle arrest in HPAC cells seems to be p53 dependent. p21WAF1/CIP-1 expression in MIAPaCa-2 cells was very low, and triphendiol treatment resulted in its enhanced expression. MIAPaCa-2 cells have a dominant-negative R248W mutation in p53; hence, the triphendiol-induced expression of p21 seems to be p53 independent. Many pancreatic cancer cells have p53 mutations [26]. These mutations are in loop 3 of p53 and affect the ability of p53 to bind to DNA [24]. Expression of p21 seems to correlate with the p53 phenotype in pancreatic cancer [32].

Several p53-independent transcription factors that induce expression of p21 have been described, including Sp1, Sp2, AP2, and STATs [33]. Recent studies in HNSCC cells have shown that in p53-negative cells some of its functions are taken over by p73, a p53-family transcription factor that upregulates the proapoptotic proteins Puma and Noxa, which promote mitochondrial depolarization [34]. Unlike p53, mutations of p73 are less common [34]. The role of p73 in cell cycle arrest in p53-negative or p53-mutant pancreatic cancer cells has not been rigorously characterized [30].

As a monotherapy, triphendiol has multiple anticancer effects on pancreatic adenocarcinoma, including the retardation of cell proliferation, cell cycle arrest and the induction of apoptosis through the intrinsic (mitochondrial) pathway. Triphendiol enhanced the antiproliferative activity of gemcitabine and the mechanism by which this is achieved has been partially characterized by these studies. In addition to gemcitabine's documented inhibition of nucleoside kinases, ribonucleotide reductase and termination of DNA chain synthesis by nuclear DNA polymerase, we have shown that gemcitabine induces apoptosis by the intrinsic pathway and arrests the cell cycle of pancreatic adenocarcinoma cell lines (data not shown). It seems that triphendiol sensitizes pancreatic adenocarcinoma cells to gemcitabine by inducing XIAP and Akt degradation, which is similar to the mechanism by which phenoxodiol sensitizes ovarian cancer cells to the platinums, docetaxel and gemcitabine [16–19] and melanoma to carboplatin [35]. The mechanism of sensitization will be investigated further to determine more precisely those pathways modulated by triphendiol, which result in an increased sensitivity to gemcitabine. For example, in Colo357 pancreatic adenocarcinoma cells, gemcitabine-dependent apoptosis can be regulated by Bcl-2 family members, being inhibited by Bcl-xL and enhanced by Bcl-2-associated X protein overexpression [8]. In addition, Bcl-xL overexpression profoundly inhibited gemcitabine-dependent apoptosis in a nonsmall cell lung cancer cell line KNS62 [36].

Despite advances in our understanding of the molecular and genetic basis of pancreatic cancer, the outcome for this disease remains dismal. Gemcitabine offers only modest improvement of tumor-related symptoms and marginal advantage of survival. Over the last decade, multiple cytotoxic (5-FU, capecitabine, irinotecan, cisplatin, oxaliplatin, etc) [37–40] and targeted agents (bevacizumab, cetuximab) [41,42] have been combined with gemcitabine in randomized phase III trials and none of these combinations showed dramatic superiority over single-agent gemcitabine. Having said that, the combination of gemcitabine and erlotinib is the first combination therapy to demonstrate survival benefits in pancreatic cancer in a phase III study albeit a modest one [43,44]. The median survival was better for the combination treatment: 6.24 versus 5.91 months, although statistically significant, but shows about 12–14 days of gain with erlotinib therapy. Recent Investigational New Drug approval from the Food and Drug Administration will enable us to clinically assess triphendiol in combination with gemcitabine in pancreatic adenocarcinoma patients to determine its efficacy in extending median survival time and time to progression.

Conclusion

Given the poverty of treatment options available especially for the treatment of late-stage pancreatic cancer, there is an urgent need for the development of novel agents that have the potential to impact survival rates and quality of life for the patients. These data provide evidence that triphendiol has potential utility as a gemcitabine chemosensitizer against pancreatic cancer and warrant further evaluation of this agent in clinical studies [28]. Triphendiol was well tolerated in a phase Ia human clinical study conducted in Australia with no

drug-related adverse events noted and adequate levels of triphendiol were achieved in plasma [45]. Triphendiol was granted orphan drug status in January 2008 for late-stage melanoma and pancreatic cancer, and Investigational New Drug status in January 2009 by the US Food and Drug Administration. Clinical investigation will start using triphendiol as an orally delivered drug in combination with gemcitabine on early-stage and/or late-stage patients with pancreatic cancer.

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Conflicts of interest

Competing interests: Xiaohong Wang, Ki H. Kim, Ayesha B. Alvero, John Anthony Thompson, and M. Wasif Saif. have no competing interests. Alan J. Husband and David M. Brown are shareholders and employees of Novogen, the parent company of Marshall Edwards. Robert McKernan is an employee of Novogen. Ainslie Whiting is no longer an employee of Novogen. Gil Mor and Ewan Milne Tytler received research contracts from Marshall Edwards to conduct this research.

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