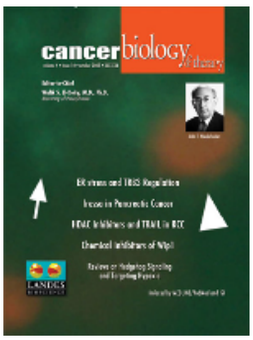

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Research Paper

Synergistic Interaction of Hyperthermia and Gemcitabine in Lung Cancer

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KEY WORDS

hyperthermia, gemcitabine, lung cancer, apoptosis, caspase-3, synergistic

ABBREVIATIONS

NSCLC	non-small cell lung cancer
H&E	hematoxylin and eosin
AI	apoptotic index
MI	mitotic index

ACKNOWLEDGMENTS

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ABSTRACT

Hyperthermia increases cytotoxicity of various antineoplastic agents. We investigated the cytotoxic effects of Gemcitabine and/or hyperthermia on BZR-T33 (human non-small-cell lung cancer cells) in vitro and in immune-suppressed athymic nude mice. Isobologram analysis of monolayer cell cultures for cytotoxicity demonstrates a synergistic interaction between hyperthermia and Gemcitabine. Clonogenic results show significant reductions in surviving fractions and colony size for both therapies; greatest reduction was for the combined therapy group. Using cell cycle analysis, hyperthermia enhanced Gemcitabine-induced G_2-M arrest resulting in destruction of 3.5 log cells. Apoptotic studies (Annexin-V FITC staining) showed that hyperthermia augmented Gemcitabine-induced apoptosis. Transmission electron microscopy demonstrated pathology observed in cultures exposed to either therapy present in cultures exposed to both therapies. Studies in nude mice show that the combination therapy group had both an initial decrease in tumor size, and a significantly delayed rate of growth. Additionally, using tumor material harvested from nude mice two days after end to treatment reveals a significantly greater apoptotic index and significantly smaller mitotic index for the combined therapy group. Western blots of the same tumor material, showed that heat shock protein 70 was not significantly increased, however, caspase-3 activity of was significantly increased because of the combined therapy. In conclusion, the combined therapy is synergistic in effect because of hyperthermia enhancing Gemcitabine-induced apoptosis.

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths in both men and women in the US today. Last year lung cancer was responsible for 28% of all cancer-related deaths (186,550 patients).[1] Standard-of-care for limited stage NSCLC (I/II) is surgical resection, and for locally advanced NSCLC (IIIa), multi-modality therapy (chemotherapy, radiation treatment and surgery) is being evaluated.[2] Although there have been highly advertised improvements in cancer therapies, long-term prognosis for patients with advanced stage (IIIB/IV) non-small cell lung cancer (NSCLC) which is present in 75% of new cases is dismal. In this group, systemic chemotherapy regimens are "standard" with 9–12 month median survival.[3]

Future improvements are likely to come from newer 'directed' agents, concurrent combined or adjunctive modality treatments, but so far, none have shown more than a few months improvement in survival.[4] Examples of 'directed' agents are IRESSA® and Tarceva™ (erlotinib). The IRESSA® ISEL study, a double blind, placebo controlled, parallel group, multicenter, randomized Phase III, survival study in 1692 patients with advanced NSCLC was suspended because it did not significantly prolong life, median 5.6 vs. 5.1 months for the control group.[5] The Tarceva™ study, a randomized, double blind, placebo-controlled trial in 731 patients with stage IIIB/IV NSCLC resulted in a significant ($p < 0.001$) increase in overall survival, Tarceva™ achieved only a median survival of 6.7 months vs. 4.7 months for the placebo-controlled group.[6] An example of combined therapy trial, recently published, is a multi-institutional phase II trial in 56 patients with stage IIIB/IV NSCLC employing cisplatin and S-1 (tegafur, 5-chloro-2, 4-dihydropyridine, and potassium oxonate) that revealed an apparent increased median survival time of 11 months.[7]

An example of an adjunctive modality treatment is hyperthermia which has demonstrated potential as a treatment for metastatic lung cancer.[8] Several investigators have shown that hyperthermia can either sensitize cancer cells to subsequent chemo/radiation therapy[9,10] or may enhance the cytotoxic effect of these interventions.[9,11] Human lung cancer cells have an increased thermosensitivity when compared to normal lung cells.[9,12]

Hyperthermia resulted in arrest of cells in the G_2-M phase of the cell cycle.[13] In contrast, normal cells, when exposed to hyperthermia show a substantial G_1 arrest and S-phase delay with an even longer G_2 arrest. The S-phase and M-phase are the most sensitive to cell killing.[14] Cancer cells when exposed to hyperthermia show a selectively induced apoptosis during the S-phase in lung cancer cells, but, the exact mechanism is unknown.[15] In another study, we demonstrate that hyperthermia induces apoptosis in lung cancer cells by activation of cell-death membrane receptors of the tumor-necrosis-factor family or extrinsic pathway.[16] Hyperthermia has been shown to be safely tolerated in patients with advanced lung cancer historically,[17] and more recently in a phase I clinical trial of whole-body hyperthermia[18] making this an attractive potential therapeutic adjunct.

Gemcitabine, a chemotherapeutic agent with proven efficacy in the treatment of lung cancer, is a deoxycytidine nucleoside analogue that affects several enzymes involved in DNA synthesis and repair. Once transported into the cell, Gemcitabine (dFdCyd) must be phosphorylated in order to become activated. The triphosphate form, dFdCTP, can directly inhibit DNA synthesis or inhibit replication by addition of dFdCMP into the new DNA strand. The diphosphate form, dFdCDP, inhibits the formation of deoxynucleoside triphosphates needed for DNA synthesis by blocking the activity of ribonucleotide reductase.[19,20] Gemcitabine has been shown to cause cell cycle arrest in S-phase leading to apoptosis,[21] or be incorporated in RNA thereby inducing apoptosis due to poisoning of topoisomerase I in lung cancer cells.[22] In a recent review article, Fennel[23] discusses how Gemcitabine reduces the splice variant CASP9b and thus facilitates apoptosome formation and apoptosis via the intrinsic pathway.

Because both Gemcitabine and hyperthermia cause apoptosis of lung cancer cells, the combination may result in enhanced cell kill and has shown promising results in cell culture experiments from our lab,[12] and others.[24,25] In preliminary studies, we compared the effect of Gemcitabine and hyperthermia administered either simultaneously or sequentially. Our studies confirmed those of Van Bree et al.[24] and Haveman et al.,[26] that when both Gemcitabine and hyperthermia were administered simultaneously a reduced cytotoxicity occurred. Based on these studies and the fact that heat could inhibit further cytotoxic effects of dFdC-metabolites, we elected to study the effect of different temporal combinations of dFdC and hyperthermia on malignant-transformed, immortalized human bronchial epithelial cell line.

The purpose of this series of experiments, using a translational model, was to assess which temporal combination was the most effective treatment for lung cancer, and to gain an insight into the mechanisms involved. We evaluated hyperthermia, Gemcitabine, and various temporal combinations in both 2-D cell culture, and in nude mice experimental models. The most effective in reducing the amount of viable cells was further analyzed by growth curves in 3-D, transmission electron microscopy, apoptotic index, mitotic index, and Western blotting of both HSP 70 and caspase-3 from tumor tissue. Our results demonstrate that the combination treatment of hyperthermia followed 24 h later by Gemcitabine in lung cancer xenografts is more effective in reducing tumor size and inducing apoptosis in cancer cells than either treatment modality used alone, or other temporal combinations.

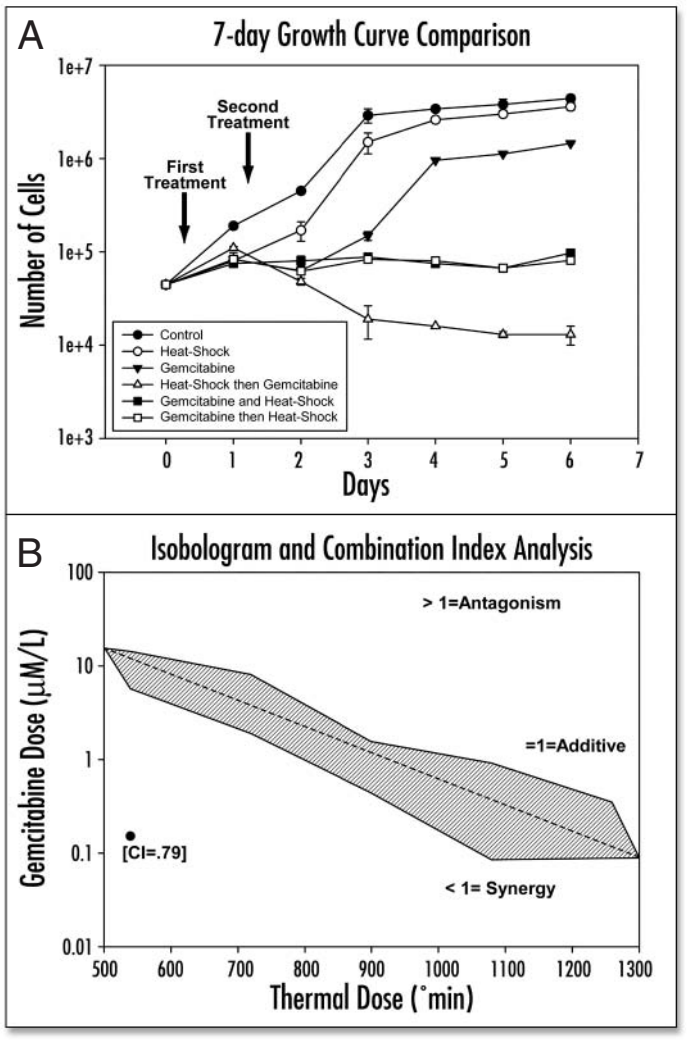

Figure 1. (A) Seven-day growth curves for all groups. Thermal dose was 43˚C for 180 min and Gemcitabine dose was 0.15 mM for 180 min. Data show that by the end of the experiment, a significant reduction of cell numbers by hyperthermia (24%), Gemcitabine (33%), and combined therapies (97%) has occurred. Cell growth slowed significantly for all groups as confluence is reached. Additionally, it appears as though the combination of heat followed 24 h later by Gemcitabine was the most effective in reducing the number of viable cells. (B) The isobologram and envelope of additivity were constructed using the method of Steel and Peckham (1979). The envelope of additivity defines the confidence limits for a simple additive effect of two independent variables. The (•) represents the concentration of both therapies that results in a 50% cell-kill. The boxed value is the combination index (C.I.) and implies a synergistic relationship.

MATERIALS AND METHODS

General methods. Experimental design. All studies were conducted with the human lung cancer cell line-BZR-T33 (generously donated by Curtis C. Harris, MD, National Cancer Institute, Bethesda, MD). BZR-T33 is a Ha-ras transfectant of an immortalized human bronchial epithelial cell line and is malignant in both soft agar and nude mice.[27,28] Both cell lines have doubling times of approximately 24 h. In a preliminary experiment, we studied the effect of various temporal relationships on the cytotoxic effect of hyperthermia and Gemcitabine in 2-dimensional cell culture in order to determine if there was a most 'efficient' combination. Study groups were: no treatment (C), hyperthermia (H), Gemcitabine (G), Gemcitabine followed 24 h later by hyperthermia (G/H), Gemcitabine and hyperthermia administered

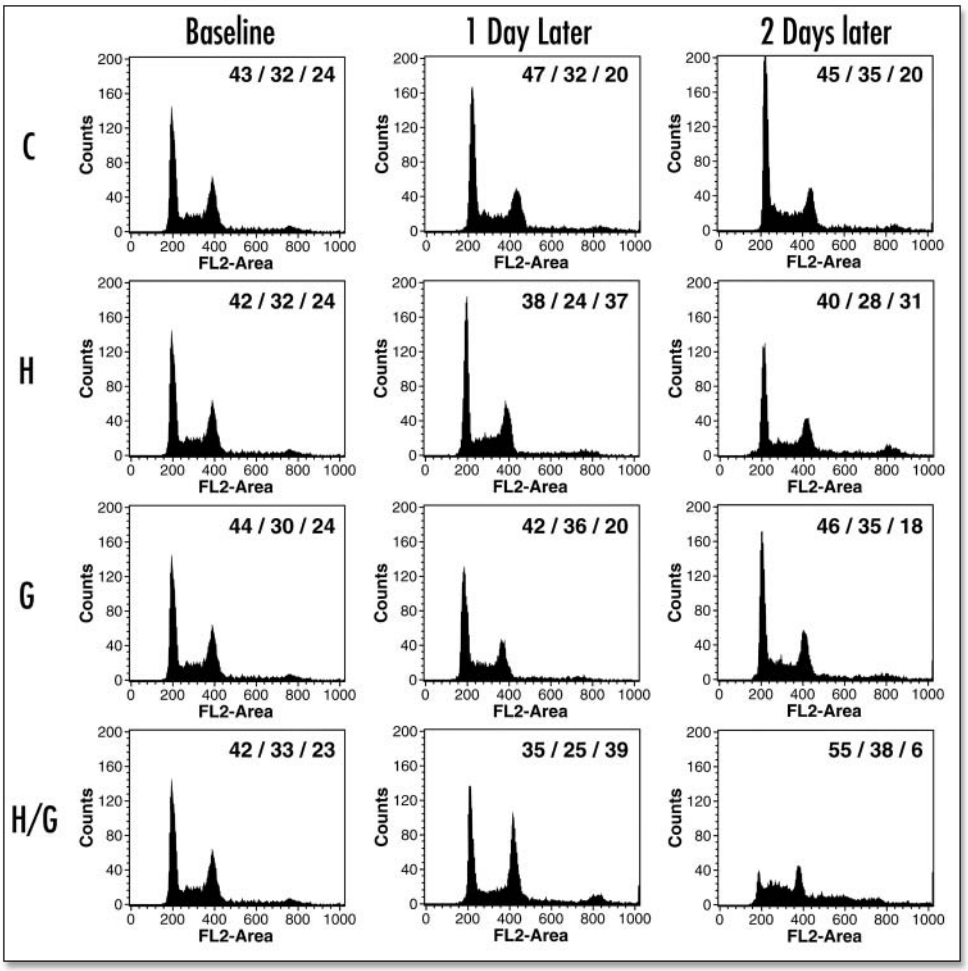

Figure 2. DNA histograms of BZR-T33 cells after removal of culture medium and cellular debris by three HBSS washes. Data shows progressive changes observed on days 1 and 2 after treatments. Values in upper right are percentages in G_0/G_1-, S-, G_2/M-phases of the cell cycle. Hyperthermia (H) induced a decrease in S-phase and an increase in G_2-M-phase cells. Gemcitabine (G) induced a decrease in G_2-M-phase cells. The combined therapy (H/G) depicts a decrease in S-phase cells and increase in G_2/M cells on day 1. These cells were subsequently destroyed by day 2 by Gemcitabine.

at the same time (G and H) and hyperthermia followed 24 h later by Gemcitabine (H/G).

Once a most efficient combination was determined, we studied its effect in more detail in 2- and 3-dimensional cell culture and in nude mice. Study groups were: no treatment (C), hyperthermia (H), Gemcitabine (G), and hyperthermia followed 24 h later by Gemcitabine (H/G). For 2-dimensional cultures, the cells were maintained in humidified 95% air, 5% CO_2 incubators in RPMI 1640 (catalogue no. 12–702Q, BioWhitaker, Walkerville, MD) 90%; fetal bovine serum 10%, at 37°C. For 3-dimensional cultures, cells were added to a 55 ml rotating wall vessel (RWV, Synthecon, catalogue no. RCCS) containing 2 mg/mL CultisphereGL microcarriers (Sigma, cat# M9293) and GTSF-2 media (Hyclone, cat#SH3A099.01) with 10% Fetal Bovine Serum (Hyclone, catalogue no. SH30070.03). Cells were cultured for fifteen days prior to experimentation; this allows the cells to aggregate to the microcarriers and develop into tissue-like assemblies (TLA's). TLA's from the single bioreactor were divided evenly into four separate 10 ml disposable RWV's, and prepared for the experiment.[29]

In vitro cell culture studies. Hyperthermia exposure was accomplished by preheating an incubator to 43°C (verified with a mercury thermometer). For exposure, culture vials were placed into the preheated incubator and maintained there for the specified duration (43°C, 95% RH and 5% CO_2). Two-dimensional cell culture studies included seven-day growth curves, clonogenic assay, and isobologram analysis. Three-dimensional cell culture studies included effects on cell cycle as determined by flow cytometry and ultrastructural effects as shown by transmission electron microscopy.

Animal model. Animal experiments were approved by the Animal Care and Use Committee of the University of Texas Medical Branch, Galveston TX. All animals received humane care in compliance with the University guidelines, state and federal regulations, and the standards of the "Guide for the Care and Use of Laboratory Animals," published by the National Institutes of Health (Publication No. 86–23, revised 1985). Congenitally athymic nude mice (BALB-C) purchased from Harlan-Sprague Dawley (Indianapolis, IN) were used for these experiments (total n = 50). The mice were matched by sex (male) and were acclimatized for at least two weeks prior to use in a limited access facility that housed them in laminar-flow racks. At the time of the experiments, animals were approximately six weeks of age. Nude mice were injected subcutaneously in the supraclavicular region with 100 µL of 10^6 BZR-T33 cells. These studies included effects of each regimen on tumor size, viability, growth, and protein expression of HSP 70 and caspase-3.

Pharmaceuticals. Gemcitabine (Gemzar™, Eli Lilly, Indianapolis, IN) was obtained as a powder and reconstituted with HBSS for injection. HBSS (injectable) was used to maintain hydration in the mice.

Immunodetection of proteins. Tumors were harvested 2 days after the end of therapy and homogenized in 5 mL of RIPA buffer [1 mL lysis buffer, 30 µL of PMSF and 10 pL of aprotinin (1.9 mg/mL)] then sonicated on ice 3 times for 10 sec each. The mixture was then centrifuged at 15000 RPM for 5 min; the supernatant was collected and stored at -80°C. Protein concentration in the lysate was determined by Bradford method[30] and fractionated on a 10% SDS-PAGE at 100 V for 60 min. Proteins were then transferred to a nitrocellulose membrane, blocked in 10% Blotto A (10 grams of non-fat powered milk, 100 mL of TBS [tris-buffered saline] and 0.02–0.05% Tween 20), incubated with the primary antibody at 1:100 to 1:500 in 1% Blotto A, washed with TBST (100 mL of TBS and 0.05% Tween-20), incubated at room temperature for 2 h with a CruzMarker™ compatible secondary antibody (1:1000). We used anti-HSP70 monoclonal antibody (catalogue no. SPA-810, StressGen, Victoria, B.C. Canada) at 1:500. Also, we used rabbit anti-caspase-3 polyclonal antibody (catalogue no. AAP-113, StressGen, Victoria, B.C. Canada) at 1:100. The blot was probed with polyclonal antibody using the manufacturer's protocol. The bands were visualized by fluoro-luminescence and analyzed by UN-SCAN-IT software (version 5.1, Silk Scientific, Orem, UT). This software provides a densimetric analysis and comparison between lanes of the relative intensity of the bands formed. Results are displayed as both bands and graphed data.

Specific research methods. In vitro studies. *Preliminary comparison.*[31] Determination of LD_{33} for Gemcitabine required a dose-response study in triplicate, with each data point in triplicate. The cells were exposed to Gemcitabine in the following concentrations (0.1, 0.5, 1.0, 5.0 and 10 µM) for 30, 60, 120 and 180 min. After exposure, cells were washed with HBSS,

and reincubated with fresh media. After 24 h in fresh media, cells were harvested by trypsinization and counted by Coulter Counter. Results were averaged and expressed as mean ± SEM and compared to untreated controls. Next, we needed to determine the hyperthermia dose-response range for these cells. Three separate trials were conducted with cells exposed to the following temperatures: 40, 41, 42, 43, 43.5, 44°C for 60, 120 and 180 min. Three hours after hyperthermia exposure, cells were washed with HBSS, and reincubated with fresh media. After 24 h, cells were harvested by trypsinization and counted by Coulter Counter, with results expressed as mean ± SEM. Once the LD_{33} for both therapies was determined, they were combined in an experiment to determine which was most effective in reducing the number of viable cells. In order to determine if a specific temporal relationship was most efficient in reducing the amount of viable cells the following groups were developed and studied in a simple experiment that quantitated the amount of cells that survived the combined therapy. Groups were: no treatment (C), hyperthermia (H), Gemcitabine (G), Gemcitabine followed 24 h later by hyperthermia (G/H), Gemcitabine and hyperthermia administered at the same time (G and H) and hyperthermia followed 24 h later by Gemcitabine (H/G).

Isobologram analysis for degree of interaction. To determine if the cytotoxic effects of hyperthermia and Gemcitabine were antagonistic, additive, or synergistic, an isobologram analysis was performed according to Steel and Peckham[32] as previously applied to cisplatin cytotoxicity. Isobologram analysis is a statistical means of determining confidence limits for the additive effects of two independent variables. Briefly, effect of either hyperthermia or Gemcitabine was determined by counting the number of cells after exposure. The study groups in these in vitro studies included: hyperthermia (H, 3 h at 43°C), Gemcitabine (G, 0.15 μM for 3 h in normal cell culture media), and combined (H/G) hyperthermia (3 h at 43°C) followed 24 h later by Gemcitabine (0.15 μM for 3 h in normal cell culture media). An isobologram was constructed on a Cartesian plane with the values for hyperthermia and Gemcitabine plotted on the *x* and *y* -axes respectively, at equal distances from the origin. The envelope of additivity (Peckham) is defined by the dotted lines.[33]

Combination index. The combination index (CI) is defined by the following equation:

$$CI = (D)1/(Dx)1 + (D)2/(Dx)2 + (D)1(D)2/(Dx)1(Dx)2,$$

where (D)1 is the dose necessary for a particular effect in the combination, (Dx)1 is the dose of the same drug which will produce the same level of effect by itself, (D)2 is the same for the second drug. The third term of the equation is added because we assume that the effects of the drugs are mutually exclusive, e.g., not the same mode of action, and thus adds additional rigor to the calculations. A CI of > 1 implies antagonism, = 1 is additivity, and < 1 synergy.[34]

Clonogenic assay. Aliquots of cells were harvested 4 h after each intervention and plated at low concentrations (10^2 cells per 100-mm plate); multiplicity was determined and then cells were allowed to grow for seven days without being disturbed; cells (colonies) were then fixed with Carnoy's fixative and stained with methyl blue. Colonies of more than 50 cells were determined to represent unhindered growth and were counted; surviving fraction was then determined (SF).[35] Additionally, each of these colonies was measured at least in two dimensions, and surface area was determined (size).

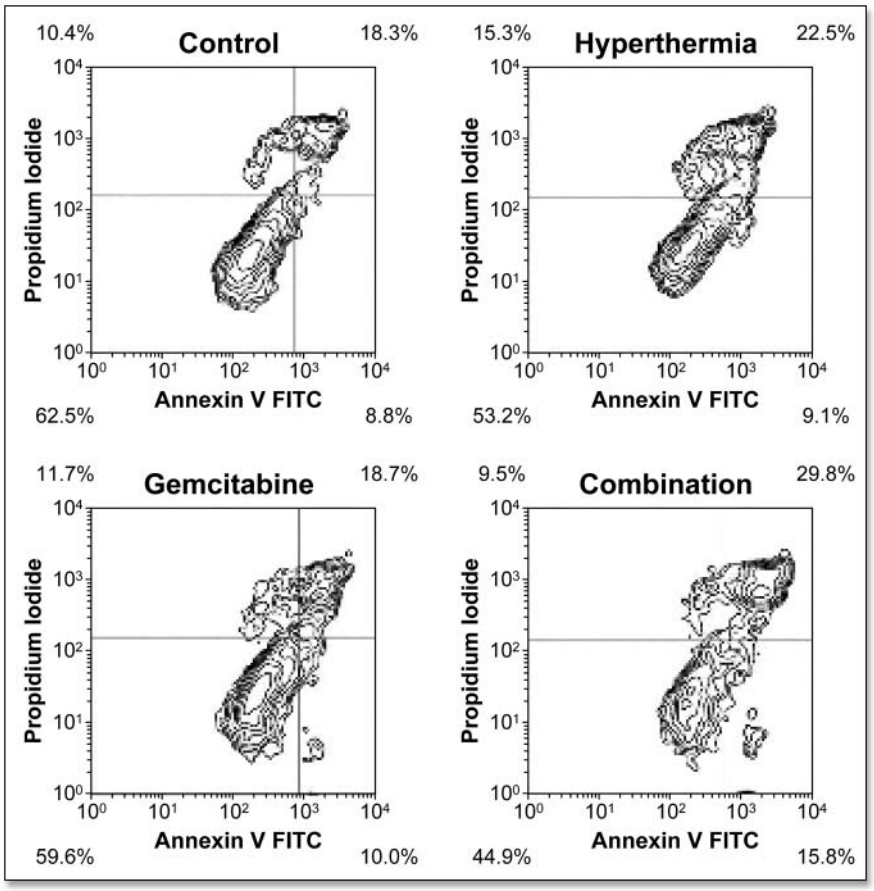

Figure 3. Topographical maps of DNA showing an increase in Annexin V-FITC positive cells after the combination therapy (45%).

Cell cycle evaluation. Aliquots of cells from each group were collected at 0 and 24 h after each intervention and 24 h following the last intervention. Single treatments were done at 0 h, combined treatments at 0 and 24 h. Cell volumes were washed with HBSS three times (to remove cellular debris leaving only attached cells). Cells were fixed, and DNA labeled with propidium iodide prior to analysis by flow cytometry.

In vitro studies-3-D. 4 x 10^5 cells/ml of BZR-T33 cells were added to a 55 ml rotating walled vessel (RWV) cultured in the reduced-gravity environment for 15 days prior to experimentation. Then TLAs from the single bioreactor were divided evenly into four separate 10 ml disposable RWVs, and labeled as control-no treatment (C), hyperthermia only (H), Gemcitabine only (G), or hyperthermia then Gemcitabine (H/G) and then allowed to recover overnight at 37°C. On experimental day 0, the RWV labeled for combination therapy received exposure to 43°C for 3 h; media was changed and cells then placed back in 37°C incubator. Media was changed in the remaining three RWV's to be consistent and to maintain cultures. On day 2, the RWV labeled for drug treatment only and the RWV labeled for combination therapy received 1 mL of 0.15 μM of Gemcitabine directly into the media in the RWV using a standard luer-lock 1 cc syringe. The culture was returned to the incubator and allowed to rotate 3 h at 37°C before it was removed and media was changed (to remove the drug from the TLA's and as a part of normal culture maintenance) and was returned to the incubator and allowed to rotate at 37°C. The RWV labeled for hyperthermia treatment only was placed in a prewarmed incubator set to 43°C and allowed to rotate 3 h. At the end of the 3-h time course, all treated RWV's and the control RWV were pulled from the incubator and the media changed/replaced. All RWV's were returned to a 37°C incubator and allowed to recover over night. Twenty-four hours later, tissue-like aggregates from each group were saved in separate tubes. A portion of the aggregates

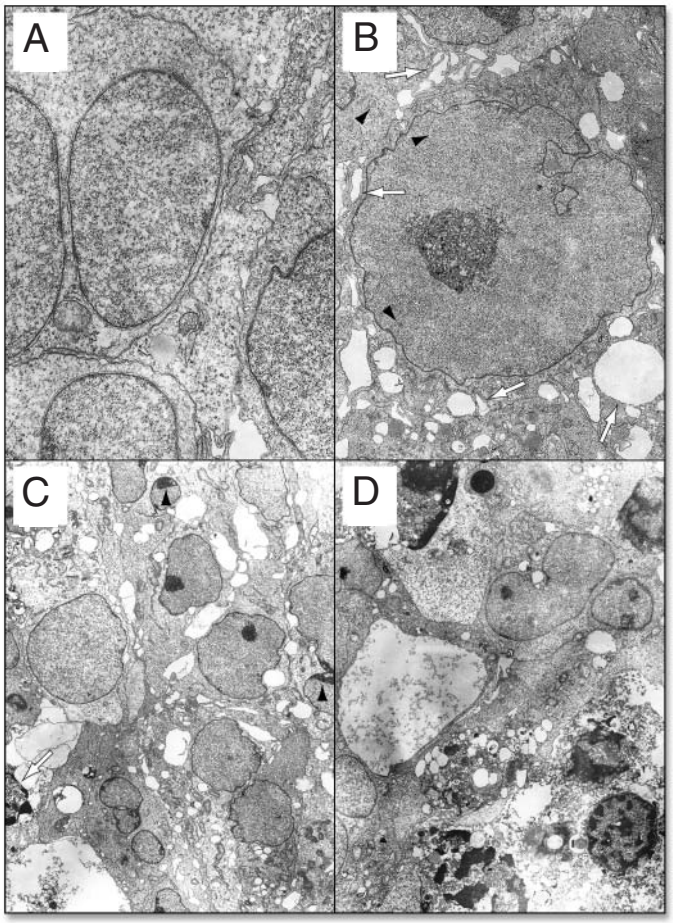

Figure 4. Transmission electron micrographs. (A) Control culture. Cells are tightly apposed to each other, they have large nuclei with even contours and cytoplasm filled mostly with ribosomes. Bar = 1 µm. (B) In hyperthermia-treated culture, cells are separated by large intercellular spaces, they have numerous microvilli (arrows), their nuclei have irregular outlines (arrowheads), and cytoplasm is vacuolated (thick arrows). Bar = 3 µm. (C) Gemcitabine-treated culture. Cells are separated with large intercellular spaces, some nuclei have irregular outlines, and cytoplasm is vacuolated. Peripheral condensation of chromatin is evident in some cells (arrowheads). Arrow points to a nucleus with peripherally condensed chromatin and disintegrated structure. Asterisks indicated disintegrated cytoplasm. Bar = 3 µm. (D) Cell culture treated with a combination of hyperthermia and Gemcitabine. Many destroyed cells are in the field, their nuclei displaying different degrees of chromatin condensation. Arrow indicates an apoptotic body within a cell. Bar = 2 µm.

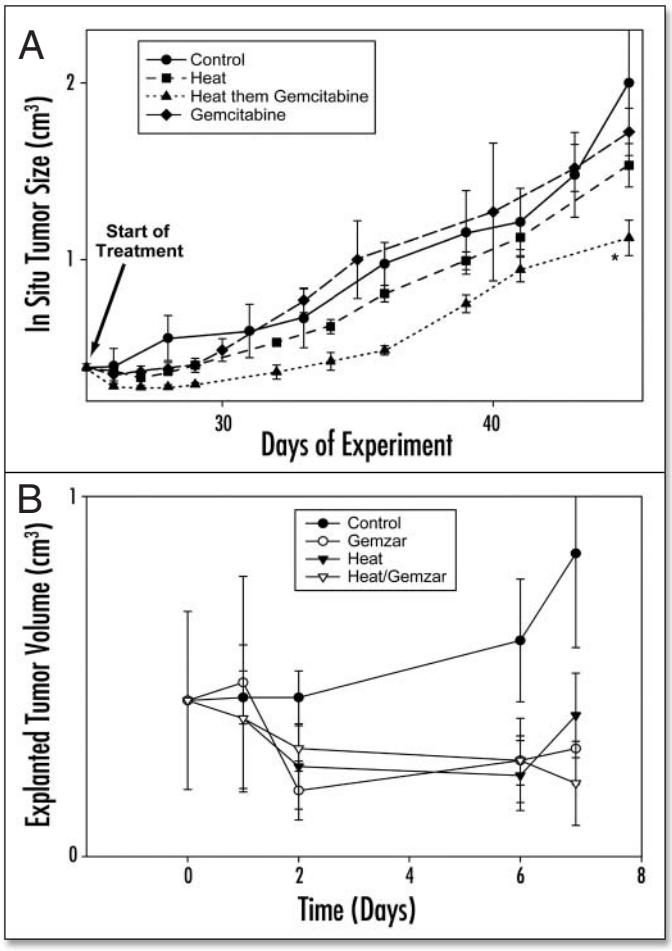

Figure 5. Tumor-growth curve for BZR-T33 tumors subjected to each intervention. (A) Both single modality intervention groups responded to therapy by not increasing in size for three days following treatment. The combined therapy group decreased in size by 30% within two days following treatment (ANOVA, $p < 0.05$). (B) Explanted tumor volumes were smaller for all treated groups when compared to control. The tumors that received the combination therapy had the smallest tumors (Tukey 1-way ANOVA, $p < 0.05$).

was assayed for viability, cell count and observed by transmission electron microscopy.

Assay of cell number. Briefly, the TLA's were removed from the RWV's and placed into 15 ml conical tubes labeled to match the treatment they received. The cells were dissociated from the culti-sphere beads using 2 mM disodium EDTA for 5 min at 37°C. The tube was gently rocked to further release the cells. The beads were then allowed to settle by gravity, and the suspended cells collected by pipette. Cells were washed in warm 1X DPBS twice, then counted using trypan exclusion and a standard hemocytometer.

Assay for viability. Viability was assessed using the BioVision Annexin-V FITC apoptosis Detection Kit (#K101-25, BioVision, Mountain View CA 94043). All tubes were double stained with both Annexin V-FITC and Propidium Iodide (Biovision Labs, Kit #K101-25). Briefly, cells were disaggregated by collagenase, collected by centrifugation, and resuspended in 1 X binding buffer. 5 x 10^5 cells from each experimental group were placed in tubes labeled the same as the treatment groups. To this, 5 µL of Annexin V-FITC and 5 µL of propidium iodide (PI) were added, and the samples were stored in the dark at room temperature. Analysis was by flow cytometry for detection of Annexin V-FITC (Ex = 488 nm, Em = 530 nm) with the FITC signal detector, and PI detection using the phycoerythrin emission signal detector.

Transmission electron microscopy. Other aliquots were removed for transmission electron microscopy, cell cultures were fixed in a mixture of 2% formaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer, post-fixed in 1% OsO_4, stained en bloc in 2% aqueous uranyl acetate, dehydrated in ethanol and embedded in epoxy resin Poly/Bed 812 (Polysciences, Warrington, PA). Ultrathin sections were cut on a Reichert Ultracut S ultramicrotome, stained with lead citrate, and examined in a Philips 201 electron microscope at 60 kV. Interpretation was by an electron microscopist (SP) blinded as to study group or hypothesis.

Establishment of tumors in nude mice. BZR-T33 cells were grown to 100% confluence in a T-175 flask, harvested in trypsin, and collected in 10 ml of media. Cell count was determined by Coulter counter in triplicate. Cells were then centrifuged at 5°C x 1100 rpm x 5 min. Concentrated cells were resuspended and divided to achieve 10^6 cells/100 µL that was then injected subcutaneously in the supraclavicular area.

Treatment. All mice were treated between days 21 and 35 following cell injection and when tumor volume was between 0.2 and 0.5 cm^3. If tumors

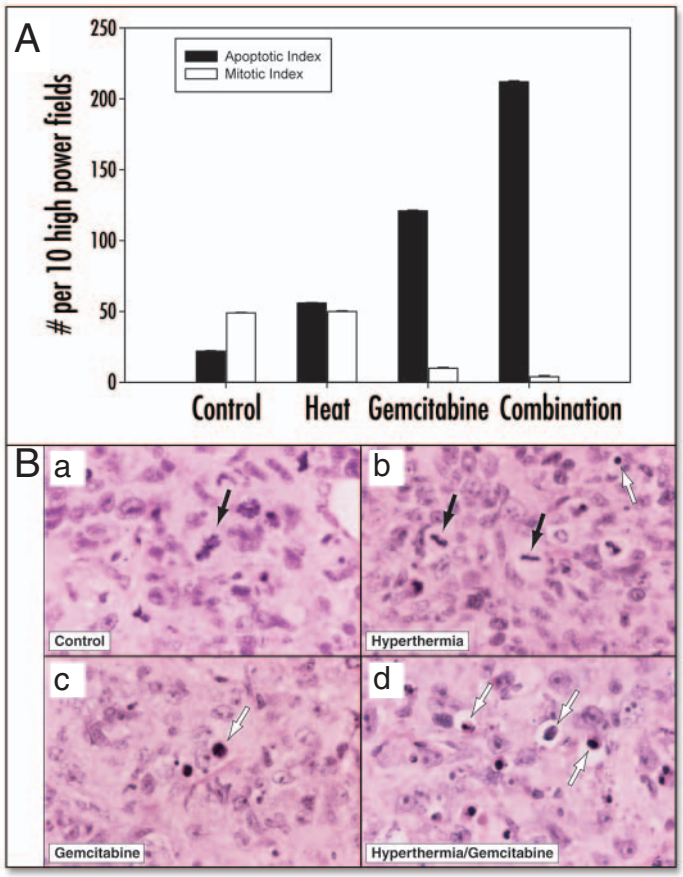

Figure 6. (A) Apoptotic (AI) and mitotic index (MI) comparison 2 days after final treatment. AI was found to be least for control, increasing with both single modality therapies, and maximum in combination therapy (hyperthermia/Gemcitabine), in contrast the MI was found to be similar in control and hyperthermia, decreased 5-fold in Gemcitabine, and decreased more than 10-fold in combination (hyperthermia/Gemcitabine). (B) Representative photomicrographs of sections from all four groups. In hyperthermia, we observed both the apoptotic and mitotic phenotypes present. Less mitosis was observed in the Gemcitabine teated tissue. The tissue receiving combination therapy showed a distinct phenotype where it appears as thought he nucleus has 'pulled' away from the cytoplasm. This phenotype was quite numerous in these sections and represents cells undergoing apoptosis.

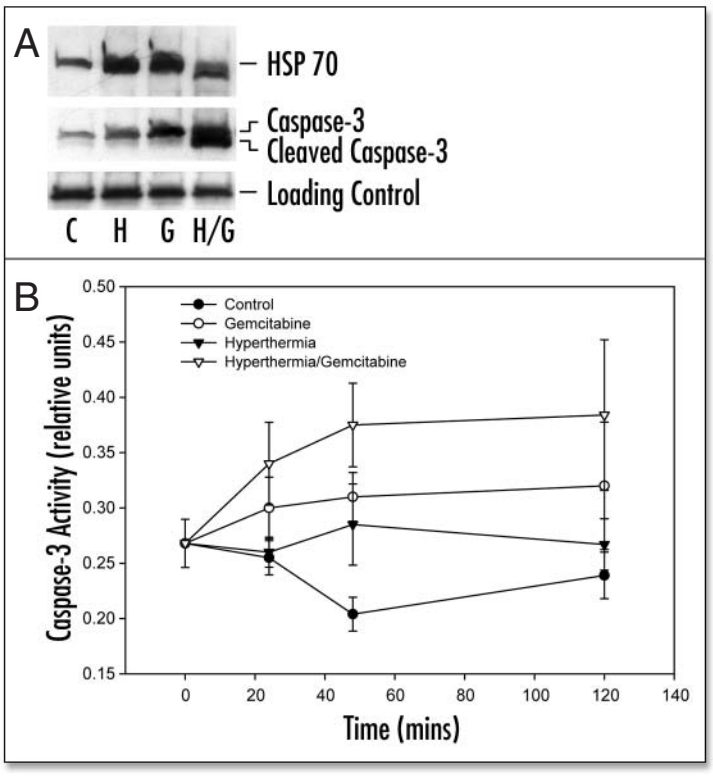

Figure 7. Western blots. (A) Heat shock protein 70 (HSP70) is compared between groups of explanted tumor tissue 2-days after the end of therapy. There is a 42% increase (sig, $p < 0.05$) in HSP70 expression after hyperthermia, 32% after Gemcitabine (sig, $p < 0.05$), but only a 2.4% increase (not sig, $p > 0.05$) in the H/G group. Caspase-3 shows a significant increase in all therapies with the highest expression (2-fold. $p < 0.05$) seen in the H/G group. (B) Caspase-3 activity assay. Tumor material from each group 2-days after the end of therapy was assayed for caspase-3 activity. Data reveals that there is significantly more caspase-3 activity found in the cells being treated with the combination therapy than in any other group (ANOVA, $p < 0.05$).

did not fall into this volume range during the predetermined days, the mice were not used in this study. Study was terminated when mass of tumor was calculated to be >10% of the animals' weight as required by our ACUC. All hyperthermia treatments were conducted in a preheated (40°C) incubator specifically set aside for this use. Mice received an ip injection of 0.5 mL of HBSS prior to heating. The heated control group received the same ip injection and was housed in a different incubation maintained at 37°C. During the heat or control treatment, animals were supplied with water (40° or 37°C), and temperature of the incubator was verified every 10 min by two thermometers and recorded.[36] Mice were given another 0.5 mL HBSS injection post treatment, and then returned to the step-down facility. All Gemcitabine treatments were by ip injection of 250 mg of Gemcitabine per Kg of animal body weight, following which the mice were placed in the step-down facility. Gemcitabine control animals received an ip injection of an equal volume of HBSS with Gemcitabine. For all mice receiving combination treatments, the Gemcitabine portion of the treatment was given 24 h following the end of hyperthermia.

Determination of LD_{33} for combination therapy in nude mice. For the nude mouse experiments, adjustments of both Gemcitabine and hyperthermia dose were required to sustain a viable population of animals. The combination intervention was compared to two positive controls, e.g., Gemcitabine alone and hyperthermia alone. Negative control animals received the same inoculum of cells but had no treatment. Nude mice with an inoculum of 10^6 BZR-T33 cells subcutaneously in the supraclavicular region were: control (C, no treatment), Gemcitabine (G, 250 mg/Kg ip), hyperthermia (H, 90 min x 40°C), and the combination group (H/G) hyperthermia (90 min x 40°C) then followed 24 h later with Gemcitabine (250 mg/Kg, ip). Previously, during model development, animals had been monitored to determine core body temperature at these preselected incubator temperatures. Additionally, two animals were subjected to the treatment for determination of core body temperature only and were not part of the experimental data reported. Upon completion of the treatment, the animals were returned to a limited-access step-down unit for the remainder of the experiment. There were eight mice in each group, one mouse from each of these groups was sacrificed 24 h after treatment for tumor harvest analysis and not included in the growth curve.

Tumor growth curves. Tumors were measured three times per week except for the initial three days after treatment when daily measurements were taken. Measurements were made using a caliper for three perpendicular dimensions [a (shortest diameter), b, c (longest)]. From these measurements, tumor volume and weight were calculated as follows:

tumor volume (V mm^3) = $(4/3\pi)$ a x b x c, and tumor weight (W mg) = a^2 x c/2.[36]

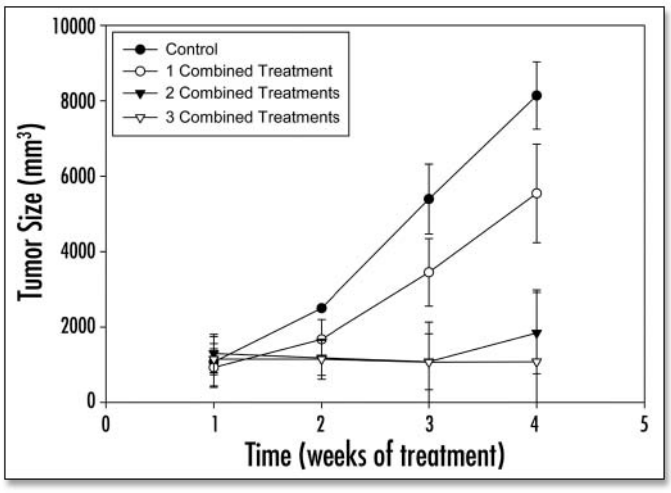

Figure 8. Mice with BZR-T33 tumors were divided into four groups and received no treatment (Control), or, one, two or three combined treatments each pair separated by seven days. Results show that tumor growth appears to be ablated as long as therapy is continued. Tumor size for the three-treatment group was significantly smaller (ANOVA, $p < 0.05$) than either the Control or One-treatment group.

Values for each mouse were logged in a spreadsheet, and the averages of tumor volume for each treatment group were plotted as growth curves. Tumor weight was monitored closely, and mice were sacrificed when tumor weight became 10% of their body weight.

In the next growth-curve experiment, the same four groups (C, G, H, H/G) were developed. Each major group contained six-subgroups of six individual mice. After tumors were developed, one subgroup from each group was sacrificed at each of the following time-points 24 and 48 h and six and seven days after treatments; the tumor was excised and volume determined by displacement. Tumors were immediately frozen at -70°C.

Indices of growth and viability. Tumors were harvested 24 h after final treatment from one mouse in each group x three runs. Both apoptotic and mitotic indices were determined after staining with hematoxylin and eosin (H & E). Apoptotic index (AI) was determined as number of apoptotic cells observed per 10 random high power fields; mitotic index (MI) was determined as number of mitotic cells observed per ten random high power fields by a pathologist (RL) blinded to group and experimental hypothesis. Criteria for AI included pyknotic nucleus, small nucleus and cell size, and reduced nucleus to cytoplasm ratio (N:C). Criteria for MI included irregular condensation of chromatin with unipolar, bipolar, and even tri-polar mitoses.

Caspase-3 activity assay. This assay measures caspase-3 activity in cell extracts. Briefly, cells were lysed and TWEEN added. Assay volume of 100 µL was used and caspase-3 activity determined using a commercially available assay kit (cat # 235419, Calbiochem, San Diego, CA). Absorbance was read at 405 nm in a microtiter plate-reader at 30-min intervals for 120 min.

Multiple dose combination therapy. These experiments were carried out in both 2-D cell cultures and nude mouse models using the following four groups: control (no-treatment), group 1 (one combination dose), group 2 (two combination doses seven days apart), and group 3 (three combination doses seven days apart). For the animal studies, tumor size was measured twice weekly and recorded. Data was expressed as group mean ± SEM.

Statistical analysis. Values are expressed as the mean ± standard error of the mean (SEM). For growth curve experiments, growth rates after treatment were compared between groups as slopes of their curves. Comparison of growth curves is by one-way analysis of variance (ANOVA). Significant differences were detected at $p < 0.05$; each experiment was repeated at least three times.

Table 1 **Clonogenic assay**

Sample	Surviving Fraction (%)	p*	Ave Size mm²	p*
Control	72	-	16.5	-
Hyperthermia	29	<0.05	10.1	<0.05
Gemcitabine	39	<0.05	9.2	<0.05
Combination	12	<0.05	2.7	<0.05

Compared to control.

EXPERIMENTAL RESULTS

In Vitro 2-D studies. Dose-response curves demonstrate hyperthermia followed by Gemcitabine most effective. A dose-response study to determine LD_{33} for Gemcitabine showed that, for escalating doses ranging in concentration from 0.1 µM to 10 µM; 0.15 µM of Gemcitabine maintained for 3 h, killed 33% of these cells and was therefore the LD_{33}. This will be the dose used for these experiments unless otherwise noted. A dose response study for hyperthermia was reported earlier;[12] 43°C for 180 min in the BZR-T33 cell line was the LD_{33}.

Figure 1A shows the number of cells plotted against days in growth medium. Control cells demonstrated the usual growth curve. Heat-treated cells showed a significant three-day delay, then the familiar growth pattern. Gemcitabine treatment caused a significantly reduced number of cells; normal growth characteristics resumed 48 h after removal of Gemcitabine. The combined treatment (H/G) caused a significant decrease in the total number of cells in the population, which remained static. Other combined treatments that were not as effective were, simultaneous administration and the sequence of Gemcitabine followed 24 h later by heat. These curves that were very similar suggesting that heat may have had an adverse effect on either Gemcitabine itself or its affect on the cell. Therefore, the rest of the study will focus on the combination of hyperthermia administered first followed 24 h later by Gemcitabine. This result suggests that hyperthermia when applied in conjunction with Gemcitabine exhibits a pronounced effect on the number of cells surviving this exposure.

A means of quantifying this effect is with the isobologram analysis of the cytotoxic effect (Fig. 1B), which showed a synergistic effect between Gemcitabine and hyperthermia. In addition, the CI was determined to be 0.79, which implies a synergistic effect observed when hyperthermia precedes Gemcitabine by 24 h.

Clonogenic assay reveals hyperthermia followed by Gemcitabine most effective. Long-term survival potential of cells after treatment was studied by clonogenic survival assay. Results summarized in Table 1, show that the surviving fraction of control cells were 72% with an average colony size of 16.5 mm. This value was reduced to 29 and 39% for heat and Gemcitabine treated samples, respectively, with a concomitant decrease in average colony size to 10.1 and 9.2 sq mm. When cells were treated first with heat followed by Gemcitabine, the surviving fraction was reduced to 12%. This characteristic was also reflected in the average size of the clones reduced to 2 to 7 sq mm.

Cell cycle analysis reveals cell killing occurs after G_2/M arrest. Control cells maintained approximately 45% of cells in G_0/G_1, 22% in G_2/M, and 33% in S-phase throughout the experimental period. Data (Fig. 2) show that hyperthermia reduced the percentage of cells in S-phase and increased the percentage in G_2/M, thus imposing a G_2/M block after 24 h that returned to normal on day 2. Gemcitabine decreased the percentage of cells in G_2/M and increased the percentage of cells in S-phase. The combined modality shows on day-1 the effect of hyperthermia in that cells are shifted from S- to G_2/M. On the second day, Gemcitabine reduced the total number of cells to 7% of the starting number with only 6% of these remaining in G_2/M. The cells in G_2/M as a result of hyperthermia were nearly all destroyed by Gemcitabine and cells in both G_0/G_1 and S-phase are greatly

reduced in number. Thus, it appears that hyperthermia 'sensitized' the cells to the effect of Gemcitabine.

In vitro 3-D studies. Viability assay shows combination most effective in reducing number of viable cells. While there are many more issues to consider when using 3-D cell cultures, the major reason we included them in this study was to determine if the degree and type of effect from treatments was similar to that seen in either 2-D cell cultures or nude mice. The number of cells that survived treatment in each of the four groups, while more than for comparable groups in 2-D, shows the same trends as in 2-D in that the combined therapy significantly reduced the number of surviving cells when compared to the other treatment groups. Compared to control, hyperthermia reduced the population by 9%, Gemcitabine by 3%, and the combination by 17.6%. Flow cytometry results (Fig. 3) reveal that for the combined therapy group, 55.1% SEM ± 8 of cells were propidium iodide and/or Annexin V positive which indicates that apoptosis should be considered as the major cell-death factor present in this population.

Transmission electron microscopy shows combination-treated cells revealed pathology representative of both treatment groups. In control cultures, cells formed tight aggregates on the substrate (Fig. 4A). They had large nuclei with smooth contours, cytoplasm full of ribosomes with a few cisterns of granular endoplasmic reticulum, and some mitochondria.

In heat-treated cultures, cells were separated with large intercellular spaces; they had numerous microvilli; the outlines of the nuclei became irregular; and, the cytoplasm of many cells was heavily vacuolated (Fig. 4B).

Drug-treated cultures also displayed large intercellular spaces, irregular outlines of many nuclei, and cytoplasmic vacuolation (Fig. 4C). Different stages of apoptosis could be observed in these cultures: from peripheral condensation of chromatin characteristic for the early steps to apoptotic bodies in between the cells and cells displaying more progressive stages of degeneration of their nuclei and cytoplasm.

In cultures treated with combination of heat and drug, we observed many degenerated cells with their nuclei having peripherally condensed chromatin, apoptotic bodies and degenerated cytoplasm (Fig. 4D).

In vivo studies. Application of combined therapy to nude mice reveals smallest tumors. The dose of heat (43°C for 3 h) and the effective dose of Gemcitabine (0.l5 μM for 3 h) were lethal to a large majority of the nude mice studied. Therefore, alterations in both dosages occurred. The maximum tolerated heat dose was determined to be 40°C for 90 min. The Gemcitabine dosage presented other difficulties, because unlike cell culture experiments the drug cannot be removed after the 3-h exposure period. We determined that the maximum effective dose of Gemcitabine as part of the combined therapy was 250 mg/Kg, ip.

Tumors produced in mice by inoculating BZR-T33 cells exhibited differential growth property in response to different treatment such as heat, drug, and combination. The results are summarized in Figure 5A, which shows that until the day of treatment, the growth curve of all groups was superimposable on the control growth curve and had a tumor volume of 0.01 ± 0.005 mm^3 per day. Changes that differed significantly from the control group were not observed until after treatment had been initiated. In both single modality treatment groups for hyperthermia or for Gemcitabine, tumor size did not change significantly during the first three days following treatment (tumor size 0.028 ± 0.004 mm^3 and 0.026 ± 0.001 mm^3/day, respectively). After this time period, both single modality groups resumed growth at the same rate as the control group evidenced by similar curve slopes ($p > 0.05$). Tumor developed by cancer cells treated with both hyperthermia and Gemcitabine (H/G) decreased by 30% on day 2 following treatment (0.003. ± 0.002 mm ^{3}/day) was significantly less ($p < 0.001$) than that of C (0.06 ± 0.001) for nine days following the initiation of treatment.

The second growth-curve 5B, compares actual tumor volumes of explanted tumors. Six animals in each group (C, G, H, H/G) were sacrificed at each time-point and tumor volume determined by the displacement method. Tumor volume in all three treatment groups is significantly smaller (ANOVA, $p < 0.05$) than control with the H/G group tumors being the smallest.

Light microscopy demonstrates higher AI and Lower MI in combination treatment group. AI and MI of tumor cells harvested from mice was determined 24 h after the final treatment. We measured the AI for individual treatments as well as for combinations. As summarized in Figure 6A, AI was found to be least for control, increasing with single modality therapy, and maximum in combination therapy (H/G). Both G and H/G were significantly greater than H; H/G was significantly greater than G. In H/G we observed mitotic cells undergoing apoptosis. The MI was found to be similar in C and H, decreased 5-fold in G, and decreased more than 10-fold in H/G. This value was least with the combination therapy (Fig. 6B). Interestingly, there is a clear inverse relationship between the AI and the MI with these treatments.

HSP 70 expression less in combination therapy cells. Since hyperthermia induces heat shock protein and apoptosis is mediated through caspase-3, we measured the level of these proteins in the tumors on day 2 after treatment. We observed that heat shock protein *HSP 70* was elevated from control in all groups, but to a lesser extent in H/G than in any other group (Fig. 7A). Since the antibody used is specific for inducible HSP70 (HSP72) and does not cross react with the constitutive HGC 70 (HSP73), the increased expression represents the effects of the therapy. In single treatment groups, HSP 70 was elevated 42% in H and 32% in G. In H/G, however, there was only a 2.4% increase in HSP 70 over C.

Caspase-3 studies reveal both cleavage and activity greatest in combination therapy group. Cleaved caspase-3 is greatest in the combination therapy (Fig. 7B). Cleaved caspase-3 increases 50% in H and 80% in G. Combination therapy resulted in a 2-fold increase in caspase-3. Evidence of caspase-3 cleavage is observed in H/G only results of the caspase-3 activity assay (Fig. 7C) clearly show significantly increased activity for the H/G group when compared to control (ANOVA, $p < 0.05$), whereas neither the G nor H group's level of activity was significantly different.

Multiple treatments of combined therapy most effective. We evaluated how effective multiple treatments of hyperthermia/Gemcitabine combination is in reducing tumor growth. For this experiment, three groups were utilized: 1 group had 1 combination therapy, 2nd group had two combination therapies 7-days apart, and the 3rd group had three combination therapies, seven days apart. Tumor volume was measured at the end of each week over four weeks. The results presented in Figure 8, show that in the first week tumor development in the control was slow but increased steadily after the second week. Tumor volume changed from 2200 mm^3 to 8000 mm^3 in this period. After the first combined treatment, the growth rate was reduced which attained a value of 4500 mm^3. In subsequent second and third treatments, the tumor volume attained a value of about 2000 mm^3 and 1800 mm^3, respectively.

DISCUSSION

The major objective of this study was to determine whether the combination of hyperthermia followed by Gemcitabine 24 h later was more effective in in vitro and in vivo preparations of human lung cancer than either treatment alone. The study compared the effects of hyperthermia, Gemcitabine, and the combination on both 2- and 3-dimensional cell culture and in nude mice. We demonstrate in vitro: (1) an isobologram analysis reveals a synergistic interaction; (2) hyperthermia resulted in an accumulation of cells in G_2/M, (3) Gemcitabine preferentially kills cells in G_2/M; (4) combination therapy increased apoptosis; and (5) transmission electron microscopy shows an accumulation of effects. In vivo experiments reveal: (1) combination therapy significantly delays tumor growth, (2) that extracted tissue had a greater presence of apoptosis, lesser presence of mitosis, less HSP70 and more activated caspase-3; and, (3) multiple combination therapies prevented tumor growth during the experimental period. The results of these data reveal that the combination of hyperthermia followed by Gemcitabine is more effective in reducing the number of viable lung cancer cells both in vitro and in nude mice, and suggests that this may be an effective combination for treating patients.

Our data demonstrated that treatment of lung cancer cells in vitro and in vivo with hyperthermia followed by Gemcitabine results in greater cytotoxicity than either treatment alone and as such our findings are in agreement with Van Bree et al[24] and Haveman et al.[26] In contrast to these aforementioned studies however, we elected to first treat with hyperthermia followed by Gemcitabine, in addition, our hyperthermia interval was 180 min and 90 min (in vitro and in vivo, respectively). In vitro data revealed that the remaining viable cells have decreased ability to form colonies and slower rate of growth as evidenced by the clonogenic assay and 7-day growth curves. Similar results were seen in our nude mouse experiments when the same lung cancer cells are treated with hyperthermia followed by Gemcitabine. Evidence of increased cell kill includes an apoptotic index that is higher in combination therapy treated tumors than in tumors treated with either hyperthermia or Gemcitabine. Evidence of slower rate of growth includes lower mitotic index in combination therapy treated tumors than either single treatment as well as decreased tumor growth on the tumor growth curve in mice treated with combination therapy.

Heat shock protein 70 is elevated in cells as a response to stress and serves a protective role to the cell.[37] HSP70 plays a physiological role in tumour cells as an inhibitor of apoptosis occurring both spontaneously and after stress.[38] The response of lung cancer cells to stress is altered when compared to normal bronchial epithelial cells.[12,39] It is likely because of this difference that lung cancer cells are susceptible to death by hyperthermia. HSP 70 levels in treated human lung cancer both in vitro and in nude mice were studied as an index of the cells' ability to mount a protective response to the various treatments. Because the group treated with hyperthermia followed by Gemcitabine had much less increase in HSP 70 levels from control when compared to either hyperthermia or Gemcitabine alone, the decrease in cell proliferation of these cells is further supported.

Caspase-3 is an enzyme involved in apoptosis.[40] Increased levels of caspase-3 suggest apoptosis as the mechanism for cell death.[41] Reconstitution of MCF-7 cell extracts with procaspase-3 resulted in an efficient and complete processing of procaspase-9 and reveal an essential function of caspase-3 for procaspase-9 processing leading to apoptosis.[42] The levels of caspase-3 in samples from combination therapy treated lung cancer cells both in vitro and in nude mice show markedly more caspase-3 than in groups treated with either therapy alone. In addition, the activated caspase-3 assay displays significantly more caspase-3 activity in cells treated with the combination therapy. This evidence supports the decreased cell viability seen in cell culture as well as the higher apoptotic index seen in nude mice that were treated with combination therapy.

Recent studies have shown that various chemotherapeutic agents kill cancer cells at specific points in the cell cycle.[43,44] It has been shown that multiple chemotherapeutic agent that target cancer cells at different stages of the cell cycle are more likely to have synergistic properties when used in combination than agents that target cancer cells at the same point in the cell cycle.[45] In our experiments following hyperthermia, lung cancer cells had a dramatic decrease in cells in the S-phase with a corresponding increase in cells in G_2/M. Gemcitabine therapy alone in our experiments reduced the number of cells in the G_2/M phase, suggesting that Gemcitabine preferentially kills cells in previous S-phase supporting the work by Mose et al.[46] When cells are subjected to hyperthermia followed by Gemcitabine 24 h later, flow cytometry demonstrates an initial reduction in S-phase and an increase in G_2/M phase cells on day 1. On day 2, the total number of cells was significantly decreased with only 6% in G_2/M suggesting that nearly all S-phase cells were destroyed. This augmented cell destruction is a result of cell cycle synchronization by the previous hyperthermia, followed by the timely administration of Gemcitabine.

Hyperthermia and Gemcitabine have each previously been used in the treatment of lung cancer, but the combination has yet to be used in patients. Our work is significant because: (1) we correlated findings in three different models—2- and 3-dimensional cell cultures and in nude mice; (2) we showed that effects of the combination therapy is greater than predicted by each therapy alone; and (3) we demonstrated that the synergistic effect is the result of an increased rate of apoptosis caused by separate effects on the cell cycle.

We are encouraged by the fact that results in 3-dimensions were representative of both that from 2-dimensions and in vivo experiments and are hopeful that through further validation we can establish this as an acceptable model in which to study cancer-tissue's response to therapy. Four reasons for the difference between 2-D and 3-D: (1) it may be more difficult for either or both therapies to penetrate a tumor mass; (2) 3-D induced cell differentiation may offer a protective state against one or both of these therapies; (3) additional cell death may be occurring at later times-outside the experimental interval; and, (4) the central core may be hypoxic and therefore protective against these interventions. A shortcoming of this experimental model occurred when we were unable to apply the LD_{33}s for both therapies to nude mice. Although outcomes of each type of experiment supported each other, in reality, we did end up studying different therapeutic doses in each model system. An additional shortcoming is in the use of malignantly transformed immortalized human bronchial cells, rather than a 'true' human lung cancer cell line. We selected this cell line to study for a number of reasons: (1) its parent non-transfected cell line serves as its non-malignant control which we have extensively studied,[12] (2) it displays characteristics thought to be predictive of malignancy and (3) it is clonal in origin, allowing for a less complicated 'picture' to interpret.

Although this combined therapy intervention may not result in a cure or long-term decrease in tumor growth, it may lead to a decrease in undesirable side effects from each of the therapies because of lower dosages, as we observed in our animal studies. Future studies will focus on the mechanisms and effects of multiple doses of hyperthermia and Gemcitabine as well as further development of the 3-dimensional tissue model. The results seen in cell culture and in nude mice treated with combination hyperthermia and Gemcitabine are suggestive of clinical benefit in the use of the combination of both modalities.

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