Published in final edited form as:

Free Radic Biol Med. 2008 March 1; 44(5): 856–867. doi:10.1016/j.freeradbiomed.2007.11.009.

Increased Oxidative Stress Created by Adenoviral MnSOD or CuZnSOD plus BCNU Inhibits Breast Cancer Cell Growth

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Abstract

Superoxide dismutases (SODs) have been found to decrease tumor formation and angiogenesis. SOD gene therapy, as with many other gene transfer strategies, may not completely inhibit tumor growth on its own. Thus, concomitant therapies are necessary to completely control the spread of this disease. We hypothesized that intratumoral injection of AdSOD in combination with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) chemotherapy would synergistically inhibit breast cancer growth.

Our data indicate that BCNU when combined with SOD overexpression increased oxidative stress as suggested by elevated glutathione disulfide (GSSG) production in one of three breast cancer cell lines tested, at least in part due to glutathione reductase (GR) inactivation. The increased oxidative stress caused by BCNU combined with adenovirally-expressed SODs, manganese or copper zinc SOD, decreased growth and survival in the three cell lines tested *in vitro*, but had the largest effect in the MDA-MB231 cell line, which showed the largest amount of oxidative stress.

Delivery of MnSOD and BCNU intratumorally completely inhibited MDA-MB231 xenograft growth and increased nude mouse survival *in vivo*. Intravenous (IV) BCNU, recapitulating clinical usage, and intratumoral AdMnSOD delivery, to provide tumor specificity, provided similar decreased growth and survival in our nude mouse model. This cancer therapy produced impressive results suggesting the potential use of oxidative stress induced growth inhibitory treatments for breast cancer patients.

Keywords

superoxide dismutase; adenovirus; xenografts; BCNU; breast cancer

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Introduction

Superoxide dismutase (SOD) is an antioxidant protein that converts superoxide anion radical into hydrogen peroxide plus molecular oxygen. The SOD gene has been successfully delivered by a range of methods increasing the amount of SOD protein within cells and xenografts in various model systems [1]. The increase in the mitochondrial-targeted SOD, MnSOD, or the cytosolic SOD, CuZnSOD, resulted in decreased cancer cell growth but in general did not completely eliminate breast tumor growth [1]. Consequently, additional treatment strategies are necessary to enhance animal survival and decrease or inhibit breast cancer growth.

The treatment strategies utilized here focused on modulating the redox environment of the cancer cell. 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), a FDA approved chemotherapeutic agent, was utilized due to the ability of this drug to react not only with DNA but more importantly for our studies showing an effect with the antioxidant protein glutathione reductase (GR) and other redox regulators including thioredoxin reductase (TR) [2]. BCNU has been shown to inactivate GR stoichiometrically through isocyanate reactions most likely with lysine in the binding site of the enzyme [3]. This inactivation causes glutathione depletion and a more oxidizing cellular environment [4]. Reduction in GR enzymatic activity, GR being a co-enzyme for the peroxide removing enzyme glutathione peroxidase (GPx), likely reduces the effectiveness of GPx contributing to elevation of oxidative stress.

Combining the two strategies, SOD gene therapy and BCNU chemotherapy, was a logical step in increasing the overall cellular oxidation; in theory, SOD produces hydrogen peroxide, while BCNU inhibits hydrogen peroxide removal. Also, previous clinical trials have utilized BCNU for the treatment of breast cancers with a patient response of about 20%, which is much lower than the response of brain tumors at almost 50% [5]. Combining the two treatments, AdSOD and BCNU, should cause a much better response than either treatment alone in breast cancer. We hypothesized that AdMnSOD or AdCuZnSOD gene therapy combined with BCNU chemotherapy would synergistically inhibit breast cancer cell growth. Since normal tissues already in general contain high amounts of SOD compared to cancer cells, the treatment should also have specificity as well.

In the present study, we found that the human breast cancer cell line, MDA-MB231, responded to BCNU therapy, while MCF 10A and MCF-7 breast cell lines were less affected. BCNU inhibited glutathione reductase (GR) activity but did not effect GPx. Decreased GR resulted in increased oxidative stress, as measured by GSSG/GSH ratios, in MDA-MB231 cells, but had little effect in MCF 10A or MCF-7 cells. Combined with SOD overexpression, BCNU inhibited GR activity without altering SOD or thioredoxin reductase (TR) activity. Growth and survival of MCF 10A and MCF-7 cells decreased with SOD plus BCNU compared to the empty vector plus BCNU treated cells. MDA-MB231 cells treated *in vitro* with AdMnSOD plus BCNU or AdCuZnSOD plus BCNU resulted in a synergistic decrease in growth and survival. We have shown for the first time that MDA-MB231 xenograft growth was completely inhibited with combined AdMnSOD plus BCNU therapy and this treatment prolonged animal survival.

These data suggest that combining BCNU chemotherapy with SOD gene therapy could be extremely successful in the treatment of breast cancer. To our knowledge, this is the first time such an approach has been utilized in breast cancer.

Methods

Cell Culture

All cells were grown in the same media in order to directly compare treatments outcomes. Immortalized, non-malignant breast epithelial MCF 10A cells, human mammary adenocarcinoma, MDA-MB231 (MB231) cells, and human mammary adenocarcinoma MCF-7 cells (American Type Culture Collection (ATCC), Rockville, Maryland) were grown in 95% DMEM-F12 media (Mediatech Inc., Herndon, VA), 5% horse serum (HyClone, Logan, Utah) and supplemented with 20 ng/ml epidermal growth factor, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone, (Sigma Chemical Co., St. Louis, MO), 0.1% gentamicin, and 0.25 µg/ml amphotericin B (Mediatech Inc.). Cultures were incubated at 37°C in 95% air and 5% CO₂. Medium was changed every 3–5 days. MCF-7 and MDA-MB231 cells were subcultured with 0.25% trypsin and 0.1% EDTA. Trypsin-sensitive MCF 10A cells were placed in the above described media plus 20% FBS after trypsinization in order to inactivate the trypsin enzyme activity (HyClone). Cells were collected at 500 × g for 5 min, and re-suspended in the above described complete media.

Adenoviral Transduction and BCNU Treatment of Cultured Cells

Viral vectors utilized for these studies include AdCMVEmpty (AdEmpty), AdCMVMnSOD (AdMnSOD), and AdCMVCuZnSOD (AdCuZnSOD) manufactured by Viraquest, Inc. (North Liberty, IA) as previously described [1, 6, 7]. The CMV promoter was employed to drive gene expression. The MOI for all experiments were calculated from the plaque forming units (pfu, or active viral particles) as measured by plaque assay by Viraquest, Inc. for each vector preparation.

For viral transduction, MCF 10A, MCF-7, or MDA-MB231 cells were plated into 100 mm dishes (Nalge Nunc International, Rochester, NY) at a density of 5×10^5 cells/plate. The next day, 50 multiplicity of infection (MOI) of adenoviral constructs were added into 5 mL serum-free media followed in two hours by the addition of 5% horse serum. Media was changed after 24 hours of viral exposure. BCNU (0 or 5 µM) exposure began at 42 hours post transduction for 6 hours. Forty-eight hours after infection, cells were scrape harvested or trypsinized and collected for further experimentation. For clonogenic survival and growth curves, cells were collected after BCNU treatment and plated into 6- or 24-well dishes, respectively (Nunc).

Cell Homogenization and Protein Quantitation

For antioxidant enzyme analysis, cells were washed in phosphate-buffered saline (PBS: KCl 2.7 mM, KH₂PO₄ 1.5 mM, NaHPO₄ 8 mM and NaCl 136.9 mM, pH 7.0), scrape harvested, and pelleted at 500 × g for 5 min in 1.5 ml microfuge tubes. Following centrifugation, the cell pellet was resuspended in 50 mM phosphate buffer (pH 7.8). The resuspended cells were then sonicated on ice for 30 seconds using a Vibra Cell cup horn sonicator (Sonics and Materials, Inc., Danbury, CT) at 30% amplitude. The Bradford method was utilized to determine protein concentrations (Bio-Rad Laboratories, Hercules, CA) and standardized with bovine serum albumin (BSA).

Western Blot Analysis

PAGE gel analysis was performed on equally loaded protein samples and transferred onto nitrocellulose membrane as previously described [1]. Blots were incubated in primary antibodies to human GR diluted 1:4000 for 1 h at RT (Lab Frontier, Seoul, Korea) followed by goat anti-rabbit IgG conjugated with horseradish peroxidase (Millipore, Billerica, MA) at a 1:10,000 dilution. All blots were repeated at least twice on separately prepared samples and subjected to Ponceau S staining before antibody exposure to ensure even protein loading

following transfer. Anti-actin antibody was diluted 1:200 (Sigma Chemical Co.) and utilized as another method for determining even protein loading following stripping of the GR primary antibody.

Activity Gel Analysis

For native gel analysis of GR activity, total cellular protein (100 µg) was separated on a native 8% polyacrylamide gel. The gel was stained with 3.4 mM GSSG, 0.36 mM NADPH, 0.052 mM dichlorophenol-indophenol, and 1.1 mM 3(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium (MTT; Sigma). GR activity was indicated by the presence of a blue precipitate band in the gel [8, 9]. Confirmation of GR activity band location was determined by loading bovine GR (1 U, Sigma) to each gel as the standard for comparison (band not shown).

In the GPx activity gel assay, total cellular protein (400 µg) was separated on an 8% native polyacrylamide gel. The gel was soaked in 1 mM glutathione (GSH) for three 10 min washes. Gels were then exposed to 0.008% cumene hydroperoxide (Sigma) for 10 min. GPx was visualized by reaction of the ferrous compounds with the peroxide turning the gel blue-green [8, 10]. Achromatic bands signify the removal of hydroperoxides where the GPx protein was active. Bovine GPx (50 mU, Sigma) was utilized as the protein control.

The SOD activity gel assay is based on the inhibition of the reduction of nitroblue tetrazolium (NBT, Fisher Scientific International Inc., Hampton, NH) by SOD [8, 11]. SOD activity of total cellular protein (150 µg) was observed following electrophoresis on a 12% native polyacrylamide gel. MnSOD expression was visualized by the addition of 0.75 mM NaCN that inhibits CuZnSOD activity.

Spectrophotometric Antioxidant Assays

GR activity assay—To determine glutathione reductase (GR) activity, the assay buffer (100 mM PB, 30 mM GSSG, 0.8 mM NADPH and 1% BSA), was first prepared and incubated for about 5 min. until the NADPH disappearance was constant [12]. Once the NADPH absorbance was constant, 500 µg sample was added to 3 ml reaction mixture in a 3 ml quartz cuvette. The disappearance of NADPH was monitored for 5 min at 340 mM. One unit will reduce 1.0 µmole of oxidized glutathione per minute at pH 7.6 and 25°C.

Glutathione assay—Cell pellets were resuspended in 50 mM KPO₄ buffer pH 7.8 containing 1.34 mM DETAPAC (Sigma). Total glutathione content was determined utilizing the Anderson methodology [13]. Reduced glutathione (GSH, Sigma) and glutathione disulfide (GSSG) were distinguished by the addition of 2 µl of a 1:1 mixture of 2-vinylpyridine (Sigma) and ethanol per 30 µl of sample followed by incubation for 2 hrs. The assay was then performed as previously described [14]. All glutathione determinations were normalized to the protein content of whole homogenates determined with Lowry analysis [15].

Measurement of reactive oxygen species (ROS) levels

The level of intracellular peroxide was determined using DCFH-DA (Molecular Probes, Inc., Eugene, OR). Briefly, cells were incubated with 10 µM of DCFH-DA in PBS for 40 min at 37°C with 5% CO₂. Cells were then harvested and lysed with 1% NP-40. The fluorescent intensity of DCFH in the lysate was assessed using a microplate reader (SpectraFluor Plus, Tecan, Durham, NC) with an excitation wavelength of 485 nm and emission wavelength of 590 nm. The protein concentration of the lysate was determined by using the DC Protein assay (Bio-Rad) and the ROS levels were expressed as mean fluorescent intensity/mg protein. The oxidation insensitive probe was utilized as a control for efflux and uptake.

Protein oxidation (carbonyl) assay

Cells were lysed in 25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM MgCl₂, 1 mM EDTA, 2% glycerol. Cell extracts were monitored for their oxidized protein content by measuring the soluble protein fraction for dinitrophenol (DNP) binding, using a protein carbonyl ELISA kit as recommended by the manufacturer (Cell Biolabs Inc., San Diego, CA). Briefly, 100 µg of protein samples were adsorbed onto a 96-well plate overnight at 4°C. The protein carbonyls present in the sample or standard were derivatized to DNP hydrazone and probed with an anti-DNP antibody, followed by an HRP conjugated secondary antibody. The absorbance of each well was read using a microplate reader with 450 nm as the primary wavelength. The protein carbonyl content in an unknown sample was determined by comparing with a standard curve that was prepared from predetermined reduced and oxidized BSA standards.

Cell Population Doubling Time

Forty-two hours after adenoviral transduction, cells were treated with BCNU for 6 h and then collected. Cells were plated into 24 well plates at a density of 5,000 cells per well and grown for 24 h, which was designated day 1. Cells were trypsinized and counted on alternate days for 9 or 11 days using a Coulter Counter. The doubling time for each cell population was determined in hours (*DT*) using the following equation: $DT(\text{hours}) = 0.693(t - t_0)/\ln(N_t/N_0)$, where t_0 = time at which exponential growth began, t = time in hours, N_t = cell number at time t , and N_0 = initial cell number [16–19] from growth curves (data not shown).

Clonogenic Survival

Six hours after BCNU treatment and 48 h after adenoviral transduction, cells were harvested and plated into 6 well plates at a density of 100 (ethanol treatment groups) or 1000 cells (BCNU treatment groups) per well and allowed to attach overnight; this was day 1. Following 10–14 days of growth, survival of single cells was determined by a defined colony outgrowth. The colonies were fixed and stained with 0.1% crystal violet and 2.1% citric acid. Those colonies containing greater than 50 cells were scored. Cellular surviving fraction (SF) was calculated as follows: $SF = \text{colonies formed}/(\text{cells seeded} \times PE)$, where PE is the plating efficiency. PE was calculated from the parental untreated cell lines as the number of colonies formed divided by the number of cells seeded multiplied by 100 [16–18].

In Vivo Tumor Xenografts

With approval of the University of Iowa's Office of Animal Resources following standards set forth by the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health, female nude mice (5–8 per group, Harlan, Indianapolis, IN), were injected subcutaneously with MDA-MB231 cells (4×10^6) as described previously [1]. Xenografts were grown to approximately 5 mm × 5 mm as measured using a vernier caliper. The tumor volume was determined utilizing the following equation: $TV(\text{mm}^3) = (L \times W^2)/2$, where TV = tumor volume, L = length and W = width [16–18, 20]. Once tumors reached about 70 mm³, a 100 µl volume of 1×10^9 plaque forming units (pfu) of *AdEmpty*, *AdMnSOD*, or *AdCuZnSOD* prepared in 3% sucrose PBS solution, was delivered directly into the tumor using a 25 gauge needle attached to a 1 cc tuberculin syringe. Two days later, upon maximal protein expression, either 40% ethanol or 15 mg/kg dose of BCNU (in 40% ethanol plus 60% saline in 50 µl, ~45 mg/m², 375 µg total) was injected directly into the tumor mass. For tail vein injections a 25 mg/kg dose was delivered (10 µg/µl, 2% ethanol in ddH₂O, 50–80 µl, 625 µg total). This IV BCNU dose was approximately 75 mg/m² which is about 1/2 the single dose given to human patients (150 mg/m²).

Adenoviral and BCNU injections were given intratumorally to avoid systemic effects and for even distribution of the drug into the tumor. Injections of BCNU were also given IV to mimic clinical delivery of this chemotherapeutic. Tumors were subsequently measured every 2–4 days using a vernier caliper [16–18, 20]. In late stages of the experiments, disease free animals and animal survival was monitored every week. At the conclusion of the experiment or when tumor volumes were greater than about 10×10 mm, animals were euthanized by CO₂ asphyxiation. Animal response to treatment was determined as either: 1) a complete response: the tumor completely regressed; 2) a partial response: growth was delayed; or 3) no response: the tumor continued to grow at the same rate.

Statistics

For *in vitro* data, statistical analysis was determined using the GraphPad Prism 3.03 program by ANOVA followed by a post-hoc Tukey test. *In vivo* survival curves were estimated using SAS version 9.0 [21] with the Kaplan-Meier method [22]. The log-rank statistic [23] was used to test the hypothesis of equality of survival curves across the different groups. To determine statistical differences in tumor volumes over time, the linear mixed model analysis with either a AR(1) or compound symmetry within-animal covariance structure was used [24]. Akaike's information criterion [25] was used to determine the approximate covariance structure. Pairwise comparisons were adjusted by Bonferroni's multiple comparison procedure. Error bars represent the mean $\pm$ SEM of $n = 3$ separate experiments. Immunoblot and activity gel analyses were repeated at least twice on separately collected samples to show reproducibility.

Results

BCNU Inhibited GR Activity in Breast Cell Lines

The antioxidant enzyme glutathione reductase (GR) has been determined in previous experimentation to be irreversibly inactivated by BCNU [26, 27]. To test the effectiveness of BCNU as a treatment that would change the redox status of cells, the GR enzyme activity was first determined.

The human breast cell lines, MCF 10A, MCF-7, and MDA-MB231, were given increasing doses of BCNU. After treatment with BCNU all three cell lines responded with decreased GR activity as measured by activity gel analysis (Fig. 1A). The GR activity bands (100 kDa) were undetectable in the presence of 5 μ M BCNU in the MCF 10A and MDA-MB231 cells, while 10 μ M BCNU almost completely inhibited GR activity in the MCF-7 cells (Fig. 1A) consistent with published data [27]. Glutathione reductase (GR) protein levels were relatively unchanged following dose escalation of BCNU (Fig. 1B, GR 50 kDa, actin 42 kDa). MCF 10A and MDAMB231 GPx activity (84 kDa) was unchanged after BCNU exposure (Fig. 1C). The MCF-7 cells had undetectable GPx activity with or without BCNU in gels loaded with 400 μ g protein.

Utilizing quantitative spectrophotometric analysis, GR enzymatic activity was decreased in MCF10A, MCF-7, and MDA-MB231 cells following BCNU exposure (Fig. 1D). MCF 10A and MCF-7 cells contained similar GR activity levels with 22 ± 5 and 20 ± 5 mU/mg protein (Fig. 1D, left and middle panel), respectively. MDA-MB231 had less GR activity, starting with about half, 10 ± 3 mU/mg protein (Fig. 1D, right panel). The addition of increasing amounts of BCNU decreased GR activity in all three cell lines. GR was virtually undetectable by as little as 0.625 μ M BCNU in MCF 10A and MDA-MB231 cells and by 10 μ M BCNU in MCF-7 cells (* $p < 0.05$ vs. 0 μ M BCNU, # $p < 0.01$ vs. 0 μ M BCNU, Fig. 1D). The qualitative activity gel analysis paralleled GR quantitative spectrophotometric activity assay values showing greatly reduced activity bands in MCF 10A and MDA-MB231 as

early as the 0.625 μ M BCNU dose (Fig. 1A). By 5 μ M BCNU, MCF 10A and MCF-7 cells had nearly undetectable GR activity bands.

Breast Cell Lines Had Increased Oxidative Stress After BCNU treatment

The effect of GR depletion by BCNU on the glutathione (GSH) system was then analyzed. Since the GSH system is an important redox buffer, measuring GSH, GSSG, and %GSSG should provide a good indication of the amount of oxidative stress produced following a dose escalation of BCNU (Fig. 1E).

Measurements of GSH suggested that MCF 10A cells contained lower basal levels of GSH compared to the MCF-7 and MDA-MB231 cells (Fig. 1E, left panel). After BCNU treatment, MCF 10A cells (squares) and MCF-7 cells (circles) displayed little change in GSH following increasing doses of BCNU (Fig. 1E, left panel). GSH levels in MDA-MB231 cells (triangles) increased about 60%. The increased GSH suggested that MDA-MB231 cells were trying to compensate for the reduction in GR activity and the inability to reduce GSSG to GSH after BCNU treatment by inducing GSH production.

The amount of glutathione disulfide (GSSG) in breast cells was then analyzed. Data suggested that in the MCF 10A cells (squares) and MCF-7 cells (circles), the GSSG seemed to be relatively constant. MDA-MB231 cells (triangles) had a 4-fold increase in GSSG in response to increasing amounts of BCNU (Fig. 1E, middle panel). The increased amounts of GSSG within the MDA-MB231 cell line suggested that GSH was utilized by GPx and other enzymes to reduce H₂O₂ and other oxidants.

The increase in the %GSSG indicates that the MDA-MB231 cells were under increased oxidative stress after BCNU treatment. In contrast, MCF 10A cells (squares) and MCF-7 cells (circles) did not have an increased %GSSG (Fig. 1E, right panel). MDA-MB231 cells treated with BCNU increased the %GSSG by 3-fold over the dose range of BCNU tested (Fig. 1E, right panel). Overall, the increased GSH, GSSG, and %GSSG in the malignant breast cell line MDA-MB231, suggested that these cells were under increased oxidative stress after BCNU treatment. The GSH system in MCF-7 cells did not respond to BCNU treatment likely due to their low levels of GPx. The data suggested that the malignant MDA-MB231 cells were much more sensitive to BCNU treatment than the non-malignant MCF 10A cells.

Cellular response to combined SOD and BCNU treatment *in vitro*

The interaction between BCNU and SOD adenoviral delivery in breast cells was assessed following treatment by determining GR, TR, and SOD activity utilizing an in-gel assay for activity. Breast cells treated with 50 MOI AdCuZnSOD or AdMnSOD, a dose determined from previous experiments [1], effectively increased SOD activity as measured by activity gel (Fig. 2A, lanes 1–4). BCNU treatment of human breast cells after either AdSOD transduction (50 MOI) had little effect on MnSOD or CuZnSOD enzymatic activity as determined by activity gel analysis (Fig. 2A, lanes 5–8). Similar to results in Fig. 1, BCNU treatment (5 μ M for 6 h) greatly reduced GR activity in all cell lines (data not shown). In contrast, thioredoxin reductase (TR), another antioxidant enzyme known to be inhibited by BCNU, seemed to be unaffected at this low dose of BCNU as determined by in-gel enzymatic activity (data not shown). Thus, BCNU treatment of human breast cells effectively decreased GR activity without compromising SOD or TR activity.

Adenoviral SOD Transduction Plus BCNU Inhibited Malignant Breast Cell Growth Providing Additional Therapeutic Gain Over AdSOD Alone

After determining that Ad*MnSOD* and Ad*CuZnSOD* increased respective SOD protein and activity (4- to 8-fold) following a 50 MOI dose as previously reported [1] and the appropriate dose for BCNU (5 μ M, Fig. 1) administration was ascertained, breast cells were assayed for cell growth *in vitro*. Note that an increase in *DT* indicates decreased cell growth calculated from cell growth data.

MCF 10A cells had decreased growth with SOD plus BCNU treatment compared to control treatments. As depicted in Table 1, *DT* of cells receiving Ad*Empty*, Ad*MnSOD*, or Ad*CuZnSOD* increased at most by 1, 4, and 5 h, respectively (column 2, Table 1). Control and Ad*Empty* groups treated with BCNU for 6 h grew at nearly the same rate as untreated control cells with a doubling time (*DT*) of 27 and 28 h, respectively. MCF 10A cells that received BCNU as well as Ad*MnSOD* or Ad*CuZnSOD* increased the doubling time over BCNU alone by about 5 h (column 2, Table 1). These data suggest that at this dose of BCNU, MCF 10A cell growth was only moderately affected.

The cellular response of malignant MCF-7 cells to BCNU plus Ad*SOD* treatment was then analyzed. The treatment of MCF-7 cells with Ad*Empty*, Ad*MnSOD*, or Ad*CuZnSOD* moderately increased *DT* compared to the untreated control cells by about 4 or 6 h (column 3, Table 1). BCNU treatment of MCF-7 cells increased *DT* versus control cells by 7 h, while the *DT* of Ad*Empty* plus BCNU treated cells did not change over Ad*Empty* alone (column 3, Table 1). Ad*MnSOD* plus BCNU and Ad*CuZnSOD* plus BCNU increased *DT* by 12 and 11 h versus the control plus BCNU and 15 and 14 h versus the Ad*Empty* plus BCNU treatment group (column 3, Table 1). MCF-7 cell growth was monitored for 11 days due to a slower doubling time (*DT*), about 40 h, compared to the MCF 10A or MDA-MB231 cell lines, about 25 h (Table 1). Thus, growth of MCF-7 cells was additively decreased when treated with Ad*SOD* plus BCNU.

Finally, MDA-MB231 malignant cell growth was assessed following treatment strategies aimed at decreasing cell growth. Growth analysis suggested that MDA-MB231 cells were sensitive to Ad*CuZnSOD* plus BCNU and Ad*MnSOD* plus BCNU treatment. MDA-MB231 cells transduced with Ad*MnSOD* or Ad*CuZnSOD* produced a 4 or 9 h increase in doubling time (*DT*), respectively, compared to the control untreated group (column 4, Table 1). Ad*Empty* gene therapy had no effect on MDA-MB231 cell growth. When MDA-MB231 cells were treated with BCNU, a 9 h increase in *DT* was achieved compared to the control. An 11 h increase in *DT* was achieved when MDA-MB231 cells were treated with Ad*Empty* and BCNU, compared to Ad*Empty* treatment group. MnSOD and CuZnSOD overexpressing MDA-MB231 cells plus BCNU increased *DT* by 26 and 30 h, respectively, compared to untreated control cells (column 4, Table 1). An apparently greater than additive increase in *DT* was achieved with Ad*MnSOD* plus BCNU (* p <0.05 vs. control) when compared to the growth of Ad*MnSOD* or BCNU alone by increasing *DT* by 22h or 17h, respectively. MDA-MB231 cell growth was significantly decreased with Ad*CuZnSOD* plus BCNU treatment vs. Ad*CuZnSOD* alone or BCNU alone (# p <0.05 vs. control+BCNU or Ad*CuZnSOD*), increasing *DT* by 21 h.

Combining Ad*MnSOD* or Ad*CuZnSOD* with BCNU greatly decreased malignant breast cell growth with the greatest effect in the MDA-MB231 cell line. These data suggested that combining multiple treatment modalities could be extremely useful in controlling tumor cell growth.

Clonogenic Cell Survival Was Decreased and Oxidative Stress Increased Following AdMnSOD plus BCNU or AdCuZnSOD plus BCNU

After determining that necrotic and apoptotic death, as assayed with annexin V/PI by flow cytometry, was not increased following combined treatment (data not shown), we utilized clonogenic survival as a measurement of replicative cell death. MCF 10A cells transduced with AdEmpty (50 MOI, 77% survival vs. control) decreased clonogenic cell survival by about 20%, while AdMnSOD (40% survival) or AdCuZnSOD (49% survival) transduction reduced survival by nearly 2-fold (Fig. 2B, black bars, upper panel). The addition of BCNU to control (63% survival) and AdEmpty (47% survival) treated cells decreased MCF 10A survival by nearly 2-fold (Fig. 2B, open bars). AdMnSOD plus BCNU (17% survival, * $p < 0.001$ vs. control) or AdCuZnSOD plus BCNU (19% survival, # $p < 0.001$ vs. AdCuZnSOD, * $p < 0.001$ vs. control) combined with BCNU resulted in a 4-fold decrease in survival versus untreated control (black bar) and 2-fold decrease compared to the BCNU only treatment (Fig. 2B, open bar). The clonogenic survival results indicate that either form of SOD plus BCNU significantly decreased survival.

Similar to cellular growth as determined by *DT* analysis, adenoviral SOD transduction had a moderate effect on clonogenic cell survival in MCF-7 cells (black bars, Fig. 2B, middle panel). Since clonogenic survival was only slightly affected by AdSOD gene transfer, the measured decrease in cell growth was likely due to a decrease in cell proliferation (mitosis). There is a possibility in the MCF-7 cells, that AdSOD plus BCNU decreased growth by inducing cell cycle checkpoint arrest in order to repair damage to cellular macromolecules, such as DNA. Once repaired, the cell viability might be preserved. MCF-7 cells treated with BCNU (open bars, 57%, † $p < 0.05$ vs. control) resulted in a 2-fold decrease in survival (Fig. 2B). AdEmpty (60%, † $p < 0.01$ vs. control), AdMnSOD (42%, † $p < 0.01$ vs. AdMnSOD, * $p < 0.001$ vs. control), or AdCuZnSOD (40% survival, # $p < 0.001$ vs. AdCuZnSOD, * $p < 0.001$ vs. control) combined with BCNU significantly decreased survival compared to untreated control MCF-7 cells (open bars, Fig. 2B). The survival rate was only moderately reduced when BCNU was delivered in combination with AdMnSOD or AdCuZnSOD compared to MCF-7 cells treated with BCNU alone (open bars, Fig. 2D).

Similar to the growth curve analysis, AdMnSOD (50 MOI, 53% survival, * $p < 0.001$ vs. control) and AdCuZnSOD (50 MOI, 47% survival, * $p < 0.001$ vs. control) transduction decreased MDA-MB231 cell survival by 2-fold compared to the control untreated cells (black bars, Fig 2B, lower panel). BCNU (5 μ M, 6 h) treatment of MDA-MB231 control (open bars, 20% survival, * $p < 0.001$ vs. control) and AdEmpty (open bars, 18% survival, * $p < 0.001$ vs. control) groups diminished survival by 4-fold (Fig. 2B). The addition of BCNU to the AdMnSOD (5% survival, * $p < 0.001$ vs. control, # $p < 0.001$ vs. AdMnSOD) or the AdCuZnSOD groups (5% survival, * $p < 0.001$ vs. control, # $p < 0.001$ vs. AdCuZnSOD) produced more than a 10-fold apparently greater than additive cell killing compared to the control or AdEmpty treated cells (Fig. 2B). Thus the treatment of MDA-MB231 cells with AdSOD plus BCNU was far more effective than the other two breast cell lines and suggested that MDA-MB231 breast cancer cells could be successfully treated with this therapy.

The inhibition of cell survival is linked to increased oxidative stress in the MDA-MB231 cells after combined SOD plus BCNU. Three methods were utilized to determine the role of oxidative stress in this system: decreased GSH buffering capacity, increased peroxide formation, and increased cellular protein damage. All three methods showed that combined SOD and BCNU increased oxidative stress. Figure 3A depicts an increase in oxidative stress due to the significant increase in the %GSSG in MDA-MB231 cells (Fig. 3A, lower panel) with little change in MCF 10A or MCF-7 cells. Specifically, in MDA-MB231 cells AdMnSOD plus BCNU significantly increased the %GSSG compared to control or

AdEmpty (* $p < 0.01$), while *AdCuZnSOD* plus BCNU also had significantly higher %GSSG compared to *AdEmpty* or control groups ($^{+}p < 0.001$). Similarly, an increase in DCFH fluorescence suggested that combined *AdMnSOD* plus BCNU treatment created an increase in oxidative stress due to increased peroxide in MDA-MB231 cells (Fig 3B, * $p < 0.01$ vs. *AdEmpty* plus BCNU, $p < 0.001$ vs *AdMnSOD*). As a control, we determined that there were no changes in the oxidative insensitive probe between treatments (data not shown). Finally, the formation of protein carbonyl adducts indicates peroxide damage to proteins increased significantly in MCF-7 cells treated with *AdCuZnSOD* plus BCNU (Fig. 3C, middle panel, * $p < 0.01$ vs. *AdEmpty* plus BCNU). *AdMnSOD* plus BCNU or *AdCuZnSOD* plus BCNU also significantly increased cellular protein damage in MDA-MB231 cells (Fig. 3C, lower panel, # $p < 0.05$ vs. *AdMnSOD*; $^{+}p < 0.001$ vs *AdCuZnSOD*). Together, these results suggest that as oxidative stress increases, cellular survival is decreased following this treatment regimen.

MDA-MB231 Xenografts Treated with *AdSOD* Combined with BCNU Inhibited Tumor Growth and Prolonged Animal Survival

To test whether *AdSOD* plus BCNU could indeed act as a good breast cancer therapy modality, MDA-MB231 xenografts were injected intratumorally with *AdMnSOD* plus BCNU or *AdCuZnSOD* plus BCNU as well as the proper controls (Fig. 4). The tumors injected with *AdMnSOD* (1×10^9 pfu) in combination with BCNU (15 mg/kg, $\diamond 0 \pm 0$ mm³, # $p < 0.0001$ vs. BCNU, $n = 7$) showed significantly decreased growth compared to the BCNU treatment group ($\square$, 245.6 ± 62.5 mm³, $n = 8$) and greatly reduced growth compared to the *AdEmpty* plus BCNU group ($\circ$, 93 ± 71.9 mm³, $n = 8$). Also, *AdEmpty* plus BCNU significantly decreased growth ($^{+}p < 0.001$ vs. control plus BCNU, Fig. 4A). *AdCuZnSOD* plus BCNU ($\circ$, 154.3 ± 109.2 mm³, $n = 7$) decreased growth but not as well as *AdMnSOD* plus BCNU (Fig. 4A). MDA-MB231 xenografts completely responded, 100%, to *AdMnSOD* plus BCNU therapy (* $p < 0.001$ vs. control), while treatment with *AdCuZnSOD* plus BCNU (# $p < 0.05$ vs. control) only had 71.4% of the tumors completely regress (Table 2, column 2). Tumors that received only *AdMnSOD* ($\blacklozenge$, 121.7 ± 67.2 mm³, * $p < 0.0001$ vs. control, $n = 6$) responded with significantly decreased growth at day 26 (Fig. 4A) with 67% of the animals completely responding to treatment (Table 2, column 2). *AdEmpty* plus BCNU decreased growth compared to tumors only receiving BCNU and had complete response to therapy in 62.5% of mice (Fig. 4A; Table 2, column 2). BCNU treatment alone also decreased growth compared to control treated tumors at day 26 (Fig. 4A). BCNU treated tumors had 37.5% of animals completely responding to treatment (Table 2, column 2). *AdEmpty* ($\bullet$, 255.2 ± 83.5 mm³, $n = 8$) and *AdCuZnSOD* ($\blacktriangle$, 210.8 ± 96.0 mm³, $n = 5$) treated xenografts did not have significantly different growth compared to control treated xenografts ($\blacksquare$, 409.8 ± 72.5 mm³, $n = 7$ Fig. 4A). Important to note is that BCNU given directly into tumors did cause a short-term necrosis of the skin. This quickly healed as seen in human use and did not become a vesicant, meaning the cell damage did not continue to worsen. Dashed lines indicated one or more animals within that group were sacrificed when tumors reached 10×10 mm. The final tumor volume of the animal was carried throughout the rest of the experiment and thus the tumor volume did not increase greatly after this point.

Mouse survival increased with 85.7% surviving after *AdMnSOD* plus BCNU ($\diamond$) compared to 75% in *AdEmpty* plus BCNU ($\circ$) or 62.5% in control plus BCNU ($\square$) treatment groups at day 166 (Fig. 4B). Intratumoral injection of only *AdMnSOD* also prolonged animal survival compared to untreated control tumors with 50% survival at day 166 ($\blacklozenge$, Fig. 4B). *AdCuZnSOD* plus BCNU survived similarly to BCNU treated xenografts with 71.4% of the mice surviving at day 166 ($\circ$, Fig. 4B). Mice bearing control ($\blacksquare$), *AdEmpty* ($\bullet$), *AdCuZnSOD* ($\blacktriangle$) treated xenografts had 28.6, 25, and 60 percent survival respectively at day 166 (Fig. 4B).

In order to recapitulate clinical delivery of BCNU, the above experiment was also done utilizing IV administration of the drug. Thus, following MDA-MB231 xenograft growth and *AdMnSOD* (1×10^9 pfu, day 1) delivery intratumorally, BCNU (25 mg/kg, day 3) was given by tail vein injection (Fig. 4C). Similar to tumor growth after intratumor drug, *AdMnSOD* and IV BCNU tumor growth was significantly decreased (○, * $p < 0.05$ vs. control, $n = 7$, $3.5 \pm 2.4 \text{ mm}^3$, day 46) compared to control tumors (■, $n = 8$, $228.8 \pm 71.0 \text{ mm}^3$, day 46, Fig. 4C). Either treatment alone, *AdMnSOD* (●, $n = 8$, $73.3 \pm 27.0 \text{ mm}^3$, day 46) or IV BCNU (□, $n = 8$, $109.9 \pm 61.5 \text{ mm}^3$, day 46) also decreased growth, but not to the extent of the combined therapy, *AdMnSOD* plus BCNU (Fig. 4C).

Animal survival was also extended following the combined *AdMnSOD* and IV BCNU (○) increasing survival by at the least 35.7% over IV BCNU only (□, 50% survival) with 85.7% of the animals surviving at day 156 (Fig. 4D). *AdMnSOD* alone (●) and control (■) treated xenografts had 62.5 and 37.5 % of animals surviving at day 156, respectively (Fig. 4D). The increase in survival and decrease in tumor growth in mouse xenografts following treatment suggests that IV delivery of BCNU and intratumoral injection of *AdMnSOD* to aid in tumor delivery could greatly control breast cancer growth in humans.

Discussion

In order to control tumor growth and decrease the number of cells surviving, a single therapeutic agent will likely not be sufficient. Thus, the knowledge that SOD gene therapy can decrease growth and survival of breast cancer cells, although incompletely, suggests a need for concomitant therapies aimed at greater control of tumorigenesis. With this in mind, the goal of this study was to determine if *AdMnSOD* or *AdCuZnSOD* gene therapy combined with BCNU chemotherapy would synergistically inhibit breast cancer cell growth.

The studies presented here showed that BCNU treatment effectively decreased GR activity in the breast cells tested. Whether the effects of BCNU in our system are solely due to GR inhibition are also currently under investigation by specifically inhibiting GR with siRNA. In unpublished work, we found that siRNA to GR decreased breast cancer cell viability when given with *AdMnSOD* (Dr. Wenqing Sun, manuscript under review). Thus, at least part of the breast cell response to *AdMnSOD* plus BCNU was due to GR inhibition. Secondly, since BCNU is known to be a strong alkylating agent, some of the growth inhibitory effects could be directly related to DNA damage. If this is the case, the production of hydrogen peroxide following SOD dismutation of superoxide in proximity to mitochondrial or nuclear DNA likely enhances DNA double strand breaks only adding to the oxidative stress related growth and viability inhibition [28].

We also determined that oxidative stress was increased in MDA-MB231 cells by BCNU administration, but not in the other two cell lines examined. The effective GPx activity was likely decreased by inhibiting GR, an essential antioxidant enzyme in the peroxide detoxification system of GPx, the recycling of GSSG to GSH. The addition of SOD overexpression to BCNU treatment inhibited cell growth to the greatest extent in the malignant breast cell lines, MCF-7 and MDA-MB231. Non-malignant MCF 10A cells treated with *AdSOD* plus BCNU resulted in a minimal decrease in cell growth and clonogenic survival. The reduction in cell survival may be from the greater increase in %GSSG within the MDA-MB231 cells versus the MCF 10A and MCF-7 cell lines, 4-fold vs. a minimal change in %GSSG.

When BCNU was combined with *AdSOD*, oxidative stress as measured by %GSSG and downstream indicators, DCFH oxidation sensitive fluorescent probe and protein carbonyl formation, statistically significant increased oxidative stress in MDA-MB231 cells was

observed. The production of oxidants upstream of antioxidant scavenging available for conversion by SOD to hydrogen peroxide may also be different between these cell lines. Preliminary findings have suggested that superoxide production was the same between the cell lines as determined by EPR analysis of cellular and mitochondrial superoxide production (data not shown). Since the basal superoxide levels do not seem to be different between these lines, artificially increasing the superoxide production with superoxide generators such as ionizing radiation, Adriamycin, or antimycin could be useful. Combining increased superoxide with increased SOD expression and decreased peroxide removal with BCNU should pull the dismutation reaction of superoxide towards theoretically generating a greater amount of H₂O₂ within cancer cells and reducing the growth with fewer side effects. Taken together, these results shown here suggest that the functional consequence of increased SOD and BCNU treatment is increased oxidative stress and decreased cancer growth.

Finally, *in vivo* delivery of the combined treatment, Ad*MnSOD* and BCNU, into MDA-MB231 xenografts showed complete inhibition of tumor growth and prolonged survival. In contrast, Ad*CuZnSOD* plus BCNU treatment of tumors resulted in only 71.4% of the animals completely responding to this therapy. The reason why MnSOD had better efficacy than CuZnSOD when combined with BCNU is unclear. One possible explanation may be the localization of MnSOD to the mitochondria where superoxide is produced from the electron transport chains, whereas CuZnSOD may not be as effective in increasing local oxidative stress in the cytosol or nucleus due to fewer superoxide anions present to combine with the chemotherapeutic effects of BCNU. It is also important to point out that only one dose of SOD and BCNU was delivered, whereas patients may receive 4 doses [29]. The resultant decrease in tumor growth compared to either BCNU or Ad*MnSOD* alone further suggests the usefulness of this combination in human tumor treatment. There is the possibility that combining the two treatments, Ad*SOD* and BCNU, as well as delivering multiple doses, as done in our oral cancer model, would be an even greater success [30]. Given the success of Ad*MnSOD* plus BCNU and the moderate response when utilizing the CuZnSOD adenoviral vector, it would be important to determine the possible mechanisms for this difference in response.

Besides the difference in the location of the enzymatic reaction of the two SODs tested, MnSOD and CuZnSOD, researchers have shown that the type of SOD utilized may affect the nutrients available to the tumor by modulating microvessel formation [31]. Wheeler *et al.* has shown that adenoviral delivery of EcSOD, but not CuZnSOD, decreased vessel formation in melanoma xenografts. This decreased vessel formation correlated with a decrease in tumor growth. They point out that VEGF, vascular endothelial growth factor, was also decreased by EcSOD overexpression and may be a mechanism for tumor inhibition.

Another possible reason why CuZnSOD was not as effective in decreasing tumor growth when combined with BCNU as MnSOD overexpression could be the removal of superoxide by MnSOD in the mitochondria may affect nitric oxide (NO[•]) [32]. NO[•] readily reacts with O₂^{•-} to form peroxynitrite (ONOO⁻). The peroxynitrite reacts with nearby proteins and inhibits the SOD enzymatic function by nitration. In the mitochondria, MnSOD and NO[•] are both competing for superoxide. Investigators hypothesize that increasing MnSOD would allow NO[•] to remain, reducing peroxynitrite formation, leaving NO[•] available to react with other macromolecules within the mitochondria [32]. Not only is more NO[•] available, Pacelli *et al.* has shown that NO[•] can enhance H₂O₂-mediated cell-killing as well as induce DNA double strand breaks [33]. Thus increasing amounts of NO[•], by NO[•] donor or AdiNOS (adenoviral inducible nitric oxide synthase), combined with MnSOD overexpression, or increased peroxide, could synergistically inhibit cell growth. The combination of increased

NO[•] and H₂O₂ may then play a key role in modulating cell signaling pathways as well as by the deleterious effects both could have when reacting with mitochondrial proteins. This reaction of NO[•] and H₂O₂ in the mitochondria may be responsible for the differences we have observed *in vivo* between MnSOD and CuZnSOD overexpression when combined with BCNU.

Keeping these results in mind, the combination of MnSOD with the chemotherapeutic agent, BCNU, to treat human breast cancer could be extremely successful in a clinical setting. These data suggest that utilizing MnSOD as a cancer treatment adjuvant in combination with treatment modalities that increase oxidative stress could greatly enhance tumor response in malignant cancers.

Acknowledgments

This research was supported by grants from the National Institute of Health, P01 CA66081 and T32 CA78586, and the Holden Comprehensive Cancer Center at the University of Iowa. We would like to thank the following for their generous contributions to our project: Viraquest, Inc., personnel in the Office of Animal Resources at the University of Iowa, Dr. Sujatha Venkataraman and Susan Walsh.

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List of Abbreviations

Ad	adenovirus
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea or Carmustine
CAT	catalase
CDNB	1-chloro-2,4-dinitrobenzene
CuZnSOD	copper-zinc superoxide dismutase
DETAPAC	diethylenetriaminepentaacetic acid
DT	doubling time
FBS	fetal bovine serum
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	glutathione
GSSG	glutathione disulfide
GST	glutathione-S-transferase
H₂O₂	hydrogen peroxide
MCF-7	human mammary adenocarcinoma cells
MCF 10A	immortalized, non-malignant breast epithelial cells
MDA-MB231	human mammary adenocarcinoma cells
MnSOD	manganese superoxide dismutase
MOI	multiplicity of infection
MTT	3(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium
PB	phosphate buffer
pfu	plaque forming units
Prx	peroxiredoxin
ROH	alcohols
ROS	reactive oxygen species
ROOH	alkyl hydroperoxides
RT	room temperature
SEM	standard error of the mean
O₂^{•-}	superoxide radical anion
SOD	superoxide dismutase
TR	thioredoxin reductase
Trx	thioredoxin

Figure 1.

Glutathione (GR) activity was inhibited and oxidative stress increased following exposure to BCNU in human breast cells. A) GR activity bands (100 μ g protein per well) were decreased almost completely by 5 μ M BCNU after 6 h in the MCF 10A and MDA-MB231 cells and by 10 μ M BCNU after 6 h in the MCF-7 cells with increasing BCNU levels. B) GR protein in MCF10A, MCF-7, and MDA-MB231 cells remained relatively unchanged following BCNU treatment. C) MCF 10A and MDA-MB231 cells displayed no decreased GPx activity with increasing BCNU levels (400 μ g protein), while MCF-7 cells had undetectable GPx activity (400 μ g protein). D) Spectrophotometric analysis of GR activity following BCNU treatment demonstrated significantly decreased breast cell GR activity. MCF 10A (left panel) and

MB-231 cell (right panel) GR enzymatic function was inhibited even at the lowest dosage of BCNU studied ($\#p<0.01$, $*p<0.05$ vs. 0 BCNU). MCF-7 cells were affected at higher concentrations ($*p<0.05$). E) MDA-MB231 breast cancer cells treated with increasing amounts of BCNU resulted in a more oxidized environment as indicated by an increase in GSH and GSSG, and the %GSSG. Left panel: MCF 10A (squares) contained 50% less GSH than the malignant breast lines, MCF-7 (circles) and MDA-MB231 (triangles). As the BCNU concentration increased, the MDA-MB231 cells (triangles) increased the amount of intracellular GSH in a dose-dependent manner. The GSH levels in MCF 10A and MCF-7 cells did not respond to BCNU treatment. Middle panel: Glutathione disulfide (GSSG) increased as the concentration of BCNU increased in MDA-MB231 cells. This indicates that GSSG was not efficiently recycled to GSH upon BCNU exposure. Right panel: MDA-MB231 cells treated with BCNU resulted in a more oxidizing environment as shown by a 4-fold increase in the %GSSG. No statistical differences were achieved due to the large variability between data of the three separate experiments.

Figure 2.

Increased AdSOD plus BCNU decreased breast cell replicative survival. A) MCF 10A (upper panels), MCF-7 (middle panels), and MB-231 cells (lower panels) treated with AdMnSOD or AdCuZnSOD (50 MOI) effectively increased SOD activity as measured by activity gel (lanes 3, 7; 4, 8, respectively). BCNU (5 μ M, 6 h) treatment combined with either AdSOD transgene expression had little effect on MnSOD or CuZnSOD enzymatic activity (lanes 5–8). B) Top panel: Clonogenic cell survival following AdMnSOD or AdCuZnSOD infection was decreased about 2-fold compared to control untreated and AdEmpty treated MCF 10A cells (black bars). BCNU also diminished survival of control and AdEmpty treated cells (open bars). AdMnSOD or AdCuZnSOD combined with BCNU produced a decrease in clonogenic survival by 4-fold versus untreated control (black bar) and 2-fold compared to BCNU only treated MCF 10A cells (open bars, * p <0.001 vs. control, # p <0.001 vs. AdCuZnSOD, † p <0.05 vs. control, ‡ p <0.01 vs. AdMnSOD). Middle panel: Comparable to growth results, adenoviral transduction had little effect on cell survival in MCF-7 cells. BCNU treated MCF-7 cells (open bars) resulted in a 2-fold decrease in survival († p <0.05 vs. control). BCNU combined with AdEmpty († p <0.01 vs. control), AdMnSOD (* p <0.001 vs. control, ‡ p <0.01 vs. AdMnSOD), or AdCuZnSOD (* p <0.001 vs. control, # p <0.001 vs. AdCuZnSOD) significantly decreased cell survival compared to untreated controls, while there was a moderate change in the survival rate over BCNU alone treated MCF-7 cells (open bars). Lower panel: Similar to the growth curve analysis, AdMnSOD and

AdCuZnSOD transduced MDA-MB231 cells diminished survival diminished survival by 4-fold (* $p < 0.001$ vs. control, black bars). BCNU treatment of MDA-MB231 cells and combined with *AdEmpty* diminished survival by 4-fold (* $p < 0.001$ vs. control, open bars). The addition of BCNU to *AdMnSOD* (* $p < 0.001$ vs. control, # $p < 0.001$ vs. *AdMnSOD*) or *AdCuZnSOD* (* $p < 0.001$ vs. control, # $p < 0.001$ vs. *AdCuZnSOD*) treatment produced more than a 10-fold apparently greater than additive cell killing compared to the control and *AdEmpty* treated cells and about a 3-fold decrease in survival versus control plus BCNU and *AdEmpty* plus BCNU groups. Error bars represent mean $\pm$ SEM of $n = 3$.

Figure 3.

Increased oxidative stress after SOD plus BCNU increased cellular protein damage. A) The increased percentage of glutathione disulfide (GSSG) in AdMnSOD plus BCNU indicates increased oxidative stress in MDA-MB231 cells, $10 \pm 1.0\%$, compared to control $3 \pm 0.7\%$, and AdEmpty treated cells, $3 \pm 0.6\%$ (* $p < 0.01$). Similarly, AdCuZnSOD plus BCNU had increased %GSSG, $9 \pm 0.6\%$, compared to control and AdEmpty with $^+p < 0.001$. No significant changes in glutathione system were found in MCF 10A or MCF-7 cells following drug delivery B) Peroxide as measured by ester cleavage of DCFH was significantly increased in AdMnSOD plus BCNU, 470 ± 11 mean intensity/mg protein, compared to AdEmpty plus BCNU, 391 ± 6 mean intensity/mg protein (* $p < 0.01$ vs AdEmpty plus BCNU, $p < 0.001$ vs AdMnSOD). Nonsignificant changes were seen in the MCF 10A and MCF-7 cell lines. C) Protein carbonyl formation was also utilized as a marker of oxidative stress. Again, SOD plus BCNU treatment had little effect on MCF 10A cell damage. AdCuZnSOD (15 ± 1.6 nmol/g) or AdCuZnSOD plus BCNU (15 ± 0.5 nmol/g) produced increased carbonyl formation in MCF-7 compared to control (8.8 ± 0.2 nmol/g) and AdEmpty plus BCNU (8.9 ± 0.3 nmol/g), * $p < 0.01$ vs. AdEmpty plus BCNU. Again, MDA-MB231 cells were significantly affected by SOD plus BCNU treatment. Protein carbonyl formation was significantly increased from 7.9 ± 0.1 nmol/g in AdMnSOD treated cells to 10 ± 0.4 nmol/g in AdMnSOD plus BCNU, $^{\#}p < 0.05$ vs. AdMnSOD. Similar results were produced in AdCuZnSOD plus BCNU 13 ± 0.4 nmol/g treated cells compared to AdCuZnSOD treatment (8.7 ± 0.2 nmol/g), $^+p < 0.001$ vs. AdCuZnSOD.

Figure 4.

MDA-MB231 xenografts treated with Ad*MnSOD* combined with BCNU inhibited tumor growth completely and greatly prolonged nude mouse survival. A) MDA-MB231 xenografts were injected intratumorally with Ad*Empty* (●), Ad*MnSOD* (◆), or Ad*CuZnSOD* (▲, 1×10^9 pfu) alone (day 1) or in combination with BCNU (□, ◇, 15 mg/kg, day 3). Tumors that received only Ad*MnSOD* (◆, * $p < 0.0001$ vs. control) responded with significantly decreased growth at day 26. Ad*MnSOD* plus BCNU (◇, # $p < 0.0001$ vs. control plus BCNU) completely inhibited tumor growth compared to BCNU (□) and Ad*Empty* plus BCNU (○) groups at day 26. Ad*Empty* plus BCNU and the Ad*CuZnSOD* plus BCNU () groups similarly decreased xenograft growth with Ad*Empty* plus BCNU significantly inhibiting tumor formation compared the BCNU group (+ $p < 0.001$ vs. BCNU) at day 26. BCNU treatment alone also decreased growth compared to control treated tumors (■) at day 26. Dashed lines in Figure 4A indicate that animals were sacrificed once the tumor load was 10×10 mm. The final tumor volume was carried throughout the rest of the experiment. Error bars on the control tumor xenograft data are representative of the error within the experiment and are calculated from SEM. B) Mouse survival increased after Ad*MnSOD* plus BCNU treatment compared to Ad*Empty* plus BCNU or control plus BCNU treatment groups at day 36. Intratumoral injection of only Ad*MnSOD* also prolonged animal survival compared to untreated control tumors at day 36. C) IV BCNU and intratumoral injection of Ad*MnSOD* controlled MDA-MB231 malignant cell growth and greatly increased animal survival. MDA-MB231 xenografts treated with Ad*MnSOD* (○, 1×10^9 pfu, day 1, $n = 8$) intratumorally and IV BCNU (25 mg/kg, day 3) tumor growth was significantly decreased to 3.5 ± 2.4 mm³ on day 46 with a * p -value < 0.05 vs. control treated tumors. Control tumors

(■, n = 8) grew to $228.8 \pm 71.0 \text{ mm}^3$ by day 46. Ad*MnSOD* only (●, n = 8) decreased growth to $73.3 \pm 27.0 \text{ mm}^3$ on day 46. IV BCNU (□, n = 8) alone measured on day 46 had decreased growth to $109.9 \pm 61.5 \text{ mm}^3$ compared to control xenografts. D) Animal survival was increased following the combined Ad*MnSOD* and IV BCNU with 85.7% animal survival at day 156. IV BCNU only (□) had 50% animal survival at day 156. Ad*MnSOD* alone had 62.5% animal survival; while control xenografts had 41.7% of animals surviving at day 156.

Table 1

Doubling time (*DT*) was greatly increased after BCNU and Ad*SOD* treatment in the malignant breast cancer cell lines, MCF-7 and MDA-MB231 cells.

Treatment groups	MCF 10A	MCF-7	MDA-MB231
	Doubling Time (hours)	Doubling Time (hours)	Doubling Time (hours)
Control	25 ± 3	40 ± 3	24 ± 1
AdEmpty (50 MOI)	26 ± 5	44 ± 3	23 ± 1
AdMnSOD (50 MOI)	29 ± 6	44 ± 3	28 ± 2
AdCuZnSOD (50 MOI)	30 ± 5	46 ± 5	33 ± 5
Control + BCNU (5 µM)	27 ± 2	47 ± 5	33 ± 3
AdEmpty (50 MOI) + BCNU (5 µM)	28 ± 4	44 ± 2	35 ± 10
AdMnSOD (50 MOI) + BCNU (5 µM)	32 ± 1	59 ± 9	50 ± 11 [*]
AdCuZnSOD (50 MOI) + BCNU (5 µM)	32 ± 9	58 ± 5	54 ± 20 [#]

Data represents the mean ± SEM.

*
p<0.05 vs. control

p<0.05 vs. control+BCNU or AdCuZnSOD

Table 2

MDA-MB231 xenografts completely responded to Ad*MnSOD* plus BCNU therapy.

MDA-MB231 Treatment Groups	Complete Response (percent)	Partial Response (percent)	No Response (percent)
Control	0 (0/7)	71.4 (5/7)	28.6 (2/7)
AdEmpty	25 (2/8)	62.5 (5/8)	12.5 (1/8)
Ad<i>MnSOD</i>	66.7 (4/6)	33.3 (2/6)	0 (0/8)
AdCuZn<i>SOD</i>	20 (1/5)	60 (3/5)	20 (1/5)
BCNU	37.5 (3/8)	37.5 (3/8)	25 (2/8)
AdEmpty plus BCNU	62.5 (5/8)	25 (2/8)	12.5 (1/8)
Ad<i>MnSOD</i> plus BCNU	100 (7/7) *	0 (0/7)	0 (0/7)
AdCuZn<i>SOD</i> plus BCNU	71.4 (5/7) #	28.6 (2/7)	0 (0/7)

*
p<0.001 vs. control

p<0.05 vs. control