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Altered Expression of a Metformin-Mediated Radiation Response in SA-NH and FSa Tumor Cells Treated Under *In Vitro* and *In Vivo* Growth Conditions

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Abstract

Purpose—To assess the radio-sensitizing effect of the biguanide drug Metformin used alone or in combination with reactive oxygen species (ROS) modifying agents *N*-acetyl-L-cysteine (NAC) or emodin, and contrasted to the mitochondrial complex 1 inhibitor rotenone in altering the radiation responses of the p53 wild type SA-NH and p53 mutant FSa mouse tumor lines grown either *in vitro* or *in vivo*.

Materials and Methods—Tumor cells were grown to confluence *in vitro* and exposed to a single 4 Gy dose in the presence or absence of Metformin (5 mM) and ROS modifiers or to 10 Gy with or without Metformin as tumors in the flanks of C3H mice using a tumor growth delay assay.

Results—Both Metformin and rotenone protected SA-NH ($P<0.001$) while sensitizing FSa ($P<0.001$) to 4 Gy. Neither NAC nor emodin altered Metformin's action. Metformin was also directly toxic to FSa cells ($P=0.002$). In contrast, *in vivo* Metformin (250 mg/kg) sensitized both SA-NH (9 day growth delay, $P<0.05$) and FSa (4 day growth delay, $P<0.05$) tumors if administered 1 h before irradiation.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

Dr. David J. Grdina and Dr. Jeffrey S. Murley are minority equity partners in Pinnacle Oncology LLC regarding the potential novel uses of amifostine. Dr. Richard C. Miller has no conflict of interest to disclose. Ms. Raziye Rana Senlik has no conflict of interest to disclose. Dr. Alfred W. Rademaker has no conflict of interest to disclose. The authors alone are responsible for the content and writing of the paper.

Conclusion—Metformin effects on tumor cells measured under *in vitro* conditions may differ from those determined *in vivo* due to p53 and heterogeneous environmental factors.

Keywords

Metformin; p53; *N*-acetyl-L-cysteine; Emodin; Rotenone

INTRODUCTION

Metformin, 1,1-dimethylbiguanide hydrochloride, is the first line drug used in the treatment of type II diabetes (Khouri et al. 2004). It is currently invoking considerable interest for repurposing as a potential anti-cancer agent for use in radiation therapy (Song et al. 2012, Zhang T et al. 2014, Zhang Y et al. 2014, Cheng et al. 2015, Koritzinsky 2015). Metformin has been reported to moderately protect mouse embryo fibroblasts from cytotoxicity induced by ionizing radiation while being directly cytotoxic to human cancer cells (Muaddi et al. 2013). It has been reported to induce a direct cytotoxic response in malignant cells and impair tumor growth (Song et al. 2012, Muaddi et al. 2013, Buzzai et al. 2007, Storozhuk et al. 2013, Zannella et al. 2013, Skinner et al. 2013). These properties offer the excellent potential for the exploitation of metformin to enhance the therapeutic ratio when combined with radiation therapy (Song et al. 2012, Koritzinsky 2015).

Numerous studies conducted to characterize metformin's properties as a potential anti-cancer agent have, however, produced at times contradictory results. While metformin was observed to radio-sensitize various tumor cell lines to ionizing radiation (Song et al. 2012, Zhang T et al. 2014, Zhang Y et al. 2014, Cheng et al. 2015, Koritzinsky 2015) it has also been reported to protect human HCT116 colorectal cancer (Muaddi et al. 2013, Skinner et al. 2013) and A549 lung cancer cells (Skinner et al. 2013) along with SA-NH murine sarcoma cells (Miller et al. 2014). Its actions modifying radiation response have been linked to its ability to modify mitochondrial metabolism and associated reactive oxygen species (ROS) production (Wheaton et al. 2014, Andrzejewski et al. 2014, Weinberg et al. 2015). In particular, metformin can inhibit mitochondrial complex I activity which in turn affects intracellular ROS production. In non-malignant cells it is proposed that this leads to an inhibition of the entry of electrons into the electron transport chain resulting in reduced ROS production, a protective mechanism (Algire et al. 2012). Inhibition of mitochondrial complex I in cancer cells, in contrast, appears to result in elevated ROS production presumably leading to sensitization and cell death. This has been observed following exposure of cancer cells to the mitochondrial complex I inhibitors BAY 87-2243 and rotenone (Wheaton et al. 2014, Andrzejewski et al. 2014, Weinberg et al. 2015, Algire et al. 2012, Schockel et al. 2015, Cheng et al. 2016). The role of metformin in affecting ROS production in cells is further complicated by observations in some cell systems that metformin can act as an anti-oxidant by inhibiting or repressing ROS production by the NADPH enzymes NOX 2 and 4 (Cheng et al. 2015, Bost et al. 2012), while elevating ROS production in other cell lines through the elevation of NOX 2 and 5 (Zhang Y et al. 2014, Picone et al. 2015). Caution is therefore required in interpreting metformin's effects on modifying the intracellular redox environment of individual normal and malignant cell systems as it relates to expected changes in its use for affecting therapeutic efficacy.

Another important factor in assessing metformin's ability to modify intracellular ROS is the p53 mutational status of cells (Buzzai et al. 2007, Skinner et al. 2013). While the effects of metformin on cancer cell viability have been identified as being mainly related to modulation of AMPK activity and autophagy, p53 is known to be a downstream effector of AMPK (Sui et al. 2015). Since it is one of the more frequently mutated genes in cancer, we chose to exam the effects of metformin on both a p53 wild type (WT) and a mutant (Mut) murine tumor model, e.g. SA-NH WT and FSa Mut sarcoma mouse tumors. The transcription factor p53 is known to play an important role in regulating mitochondrial respiration by modulating the balance between the utilization of aerobic respiratory and glycolytic pathways (Matoba et al. 2006, Sinthupibulyakit et al. 2010). Oxidative stress in p53 wild type cells can be protected against through the up-regulation of oxidative phosphorylation along with modulation of anti-oxidants in contrast to p53 mutant cells which are solely dependent upon glycolysis (Sinthupibulyakit et al. 2010). In addition to effects on mitochondrial complex I, production of ROS via NOX 4 and overall intracellular oxidative stress are recognized as being differentially affected by the p53 mutational status of cells, with p53 mutant as compared to functional p53 wild type cells exhibiting an elevated level of oxidative stress (Boudreau et al. 2014, Pervin et al. 2013). Metformin's ability to affect intracellular ROS production in cells and modify their responses to exposure to ionizing radiation should therefore also be affected by the p53 mutational status of cells.

In our previous report we demonstrated the radio-protective properties of metformin on SA-NH sarcoma cells, human microvascular endothelial cells, and mouse embryo fibroblasts grown and treated *in vitro*, and mouse bone marrow progenitor cells exposed under *in vivo* conditions (Miller et al. 2014). Each of these cell systems are characterized as having functional wild type p53, and each was observed to be significantly protected by metformin against radiation-induced cell killing. In the present study we expand our investigation of metformin as a radiation response modifier by including two murine tumor model systems differing in p53 mutational status. Metformin's effect on radiation response was investigated under *in vitro* conditions utilizing a colony forming assay and compared to *in vivo* responses using a standard tumor growth delay method. The mitochondrial complex I inhibitor rotenone was also investigated as a radiation response modifier for comparison with metformin (Goncalves et al. 2011, Green et al. 2011). The free radical scavenger *N*-acetyl-L-cysteine (NAC) and free radical generating drug emodin were previously successfully used to modify the very low radiation dose adaptive response in both SA-NH and FSa sarcoma tumor models (Miller et al. 2016). Utilizing these same drug concentrations and exposure times found to be previously effective we expanded our investigation to determine their effects, if any, on metformin's ability to modify radiation response in these tumor model systems (Srinivas et al. 2007, Miller et al. 2016).

MATERIALS AND METHODS

Cells and Culture Conditions

SA-NH (p53 wild-type) cells derived from a spontaneously arisen SA-NH murine sarcoma tumor and FSa (p53 mutant) cells from a methylcholanthrene-induced C3H female mouse fibrosarcoma and adapted for *in vitro* growth were supplied by Dr. Luka Milas, the

University of Texas MD Anderson Cancer Institute. SA-NH cells stably transfected with a pcDNA3 plasmid containing a mutant I κ B α gene under the control of a CMV promoter in which serine's 32 and 36 are mutated and a neomycin resistance gene to allow the selection of stable transfectants using G-418 and designated SA-NH+mI κ B α 1 were developed as described in detail elsewhere (Murley et al. 2004) and were used to assess the role of NF κ B activation in the ability of metformin to protect p53 WT cells. Growth conditions for SA-NH +mI κ B α 1 cells were identical to those of SA-NH cells. All three cell lines were routinely checked for mycoplasma contamination and were used at passages 5–10. SA-NH and SA-NH+mI κ B α 1 cells were cultured in McCoy's 5A medium, and FSa cells in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Denville Scientific Inc., Metuchen, NJ), penicillin (100 units/ml) and streptomycin (100 μ g/ml) (Invitrogen Life Technologies) as described in detail elsewhere (Miller et al. 2016). All cell cultures were maintained at 37 °C in a humidified environment containing 5% CO₂. For all cell survival experiments, cells were grown to confluence and maintained in confluence for three days. One day prior to all experiments the cells were refed with fresh medium.

Drugs

Metformin (1,1-Dimethylbiguanide hydrochloride) and *N*-acetyl-L-cysteine (NAC) were dissolved in phosphate-buffered saline (PBS) immediately prior to use at concentrations of 500 mM and 100 mM respectively, and sterilized using a 0.22 μ m syringe filter according to the methods described elsewhere (Miller et al. 2014; Miller et al. 2016). Emodin (1,3,8-Trihydroxy-6-methylantraquinone) and Rotenone were dissolved in DMSO at concentrations of 100 mM immediately prior to use. Each drug was obtained from Sigma-Aldrich, St. Louis, MO. Cells were treated with 5 mM metformin for 30 min or 1 h, 5 mM metformin for 30 min and then 10 mM NAC or 50 μ M emodin were added for an additional 30 min as previously described (Miller et al. 2016), or 1 μ M rotenone for 1 h prior to radiation exposure.

Irradiation Conditions

For *in vitro* studies, tumor cells were irradiated with a single 4 Gy dose. *In vivo* irradiations of SA-NH and FSa tumors were performed on tumor-bearing mice that were placed in lead-shielded cylindrical clear-plastic holders. Their unshielded tumor-bearing right hind legs were secured in place with surgical tape during irradiation and exposed to a single 10 Gy dose. All irradiations were performed using a Philips RT250 x-ray generator with a 1mm copper filter operating at 15 mA and 250 kVp at a dose rate of 1.33 Gy/min.

Cell Survival Assay

Immediately following each treatment protocol, cell survival was determined using an *in vitro* colony forming assay. Cells were counted, diluted, and known numbers seeded into 100-mm tissue culture dishes to allow the development of 100–200 colonies per dish. Colonies of SA-NH and SA-NH+mI κ B α 1 or FSa cells were fixed and stained 10 and 14 days later, respectively. Each experiment was repeated three times.

Animals and Tumor Models

Female C3H mice, 8–11 weeks old (Harlan Laboratories, Indianapolis, IN), were housed in a specific pathogen free barrier facility at The University of Chicago. Experiments were carried out with The University of Chicago Animal Care and Use Committee (IACUC) approval, ACUP number 70672. The University of Chicago has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (A3523-01) and conducts its reviews in accordance with United States Public Health Service (USPHS) regulations and applicable federal and local laws. For the tumor growth delay experiments, mice were housed up to five per cage under standard conditions (12 h light and 12 h dark at 48% relative humidity and a constant temperature of 22 °C). The right hind legs of mice were injected subcutaneously with either 5×10^6 SA-NH or 3×10^6 FSa cells in a volume of 0.1 ml and were monitored daily. Total numbers of mice in each experimental group from two separate experiments ranged from 7 to 16 animals.

Tumor Growth Delay Measurements

Treatment and measurements began when tumors grew to a minimum volume of 250 mm³ as measured in three dimensions (length along the right hind leg, width at its widest point, and the height of the tumor measured from the femur to the highest point of the tumor). Tumor-bearing mice in the control group were loaded into the animal holding chamber with their hind legs taped to the chamber for the same amount of time that the tumor-bearing mice in the radiation groups were secured. Animals in all experimental groups were sacrificed when tumors exceeded an approximate volume of 1000 mm³.

Statistical Analysis

Means and standard errors were calculated for all data points from at least three independent experiments. Pairwise comparisons of cell survival between each of the experimental conditions were performed using a Student's two-tailed *t*-test (SigmaPlot software 11.0, SPSS, Chicago, IL). Mean tumor volumes over time from 7 days on were compared among groups using repeated measures analysis of covariance (ANCOVA) assuming a quadratic model and accounting for separate variances in each group. Post-hoc pairwise comparisons among groups were also done using the Tukey-Kramer method (Kramer 1956). Absolute tumor growth delay values in the metformin treated groups was defined as the median time in days for the tumors to grow to greater than 1000 mm³ minus the median time in days for 10 Gy only tumors to grow to this size (Schimming et al. 1999). Since some tumors did not reach 1000 mm³, time to reach 1000 mm³ was compared between groups using a Kaplan-Meier curve and the log rank test.

RESULTS

In Vitro Toxicity of Metformin

Presented in Fig. 1 is a toxicity assessment for a 1 h exposure of metformin on p53 wild type SA-NH and p53 mutant FSa cells. Metformin at a dose of 5 mM exhibited no toxic effect on SA-NH cells, but reduced cell survival of FSa cells by over 20%.

Effects of Metformin on Radiation Response of SA-NH and FSa under *In Vitro* Conditions

SA-NH and FSa cells were exposed to a dose of 4 Gy alone or following a 30 min or 1 h treatment with 5 mM of metformin. As described in Fig. 2, metformin significantly enhanced resistance of SA-NH cells to ionizing radiation following either a 30 min or 1 h exposure ($P < 0.001$). In contrast, a 5 mM dose of metformin significantly reduced survival of FSa cells following both a 30 min and 1 h exposure ($P < 0.001$). The overall reduction in survival of FSa cells induced by metformin following irradiation presumably reflects both a direct cytotoxicity as described in Fig. 1, as well as a radiation sensitizing component.

Effect of NF κ B Activation on Metformin's Modification of Radiation Response

To assess the potential role of NF κ B activation on metformin's ability to affect radiation response, p53 wild type cells were stably transfected with a mutant I κ B α vector and a resulting clone was isolated and designated SA-NH+mI κ B α 1. The effects of radiation with and without exposure to metformin on this clone are presented in Fig. 3. Under conditions under which NF κ B activation is inhibited, metformin was ineffective in altering radiation response indicating the requirement for an intact NF κ B signaling pathway for this process to occur. Metformin exposure times of 30 min and 1 h were equally ineffective in altering radiation response.

Effects of Metformin in Combination with NAC or Emodin as Compared to Rotenone on Radiation Response

Presented in Fig. 4 is a comparison of metformin administered in combination with the free radical scavenger NAC or the free radical generator emodin along with the mitochondrial complex I inhibitor rotenone on affecting radiation response of SA-NH and FSa tumor cells. Neither NAC nor emodin altered the magnitude of the protective effect exerted by metformin on SA-NH cells. Rotenone's effects on enhancing SA-NH cell survival following 4 Gy was essentially of the same magnitude as described for metformin alone in Fig. 2. In contrast, metformin combined with NAC or emodin and rotenone alone each sensitized FSa cells by a similar magnitude as evidenced by the comparable survival levels.

Effect of Metformin on the Radiation Response of SA-NH Assayed by Tumor Growth Delay

In contrast to metformin's enhancement of radiation resistance of SA-NH treated under *in vitro* conditions, SA-NH tumors were significantly sensitized to a 10 Gy dose of ionizing radiation when tumor bearing mice were injected with a 250 mg/kg dose of metformin either 1 h before ($P < 0.05$), or following irradiation ($P = 0.05$ at day 10). Presented in Fig. 5 are tumor growth delay plots describing the increased sensitivity of SA-NH tumors to ionizing radiation when treated with metformin. Administering metformin 1 h prior to exposure to ionizing radiation was more effective, a delay of 9 days ($P = 0.001$), than if given 1 h afterwards, a delay of 3.5 days ($P = 0.015$), in extending the time required for tumors in each of the groups to achieve a comparable size.

Effect of Metformin on the Radiation Response of FSa Assayed by Tumor Growth Delay

As described in Fig. 6, FSa tumors exposed to metformin at a dose of 250 mg/kg 1 h before radiation exposure continued to exhibit a sensitizing response consistent with that observed

under *in vitro* conditions ($P < 0.05$ comparing curves over time and at days 7–14), and an increase of 4 days of growth ($P = 0.013$) to achieve a comparable size with tumors only exposed to 10 Gy. When exposed to metformin following exposure to ionizing radiation, FSa tumors failed to exhibit a significant sensitization response, $P > 0.05$. Only the pre-irradiation treatment with metformin was observed to be effective in significantly sensitizing FSa tumors to the effects of a 10 Gy exposure.

DISCUSSION

The transcription factor p53 is known to be an important mediator in the regulation of reactive oxygen species (ROS), antioxidant status, and mitochondrial respiration in cells (Sablina et al. 2005, Milicevic et al. 2014, Budanov et al. 2014). It facilitates protection against oxidative stress through the upregulation and expression of anti-oxidant genes including copper zinc superoxide dismutase (Sod1), manganese superoxide dismutase (Sod2), glutathione peroxidase (GPx1), aldehyde dehydrogenase 4 and catalase (Sinthupibulyakit et al. 2010, Sablina et al. 2005, Milicevic et al. 2014, Budanov et al. 2014). Malignant cells, in contrast to normal cells, are characterized by an elevated level of ROS which has been implicated in the process of carcinogenesis and which are kept under control through the action of these anti-oxidant proteins activated by wild type p53 (Weinberg et al. 2015, Turrens 2003, Solaini et al. 2011). This element of protection from the effects of elevated ROS, however, is compromised and absent in p53 mutant tumor cells, making them more susceptible to the therapeutic effects of agents that can exacerbate ROS production and elevate the oxidative environment of the cell. Also linked to deficits in p53 function is the impairment of the cytochrome c oxidase, e.g. mitochondrial complex IV, which is important in mitochondrial respiration (Matoba et al. 2006, Zhou et al. 2003).

Metformin is an inhibitor of mitochondrial complex I, which not only affects the production of ROS, but also inhibits mitochondrial respiration (Wheaton et al. 2014, Andrzejewski et al. 2014, Weinberg et al. 2015, Algire et al. 2012, Schockel et al. 2015, Cheng et al. 2016). It is not surprising therefore, that metformin would express a direct toxic effect on p53 mutant cells such as demonstrated in Fig. 1 for FSa fibrosarcoma cells. In contrast to the isogenic p53 null human colon carcinoma cell line HCT116 (–/–), which was earlier reported to be sensitive to the toxicity of metformin, p53 mutant FSa cells carry only a substitution of methionine for valine at position 169 suggesting that complete loss of function of p53 is not required for this toxic effect to be expressed (Buzzai et al. 2007, Storozhuk et al. 2013, Schimming et al. 1999). If both complex I and IV are compromised through the action of metformin and p53 mutational status, mitochondrial respiration would be significantly inhibited leading to cell death and an associated reduction in cell survival as described by the response of FSa as compared to SA-NH to metformin in Fig. 1.

Metformin's ability to affect radiation response in cells reflects complex processes that are modified directly or indirectly as a result of p53 mutational status. As discussed above, malignant as compared to normal cells express an elevated intracellular ROS environment that is controlled through the action of elevated anti-oxidant enzymes such as the superoxide dismutases, glutathione peroxidase and catalase. This balance in p53 wild type malignant cells allows for cell growth and a control of the oxidative environment. Metformin, through

its inhibition of mitochondrial complex I and associated mitigation of ROS production, as well as its anti-oxidant effects on NADPH enzymes Nox 2 and 4 (Cheng et al. 2015, Bost et al. 2012), has exhibited radio-protective effects on a variety of normal and p53 wild type tumor model systems irradiated under *in vitro* conditions. Consistent with our earlier report, metformin administered 30 min or 1 h prior to radiation exposure significantly protected p53 wild type SA-NH tumor cells (see Fig. 2) (Miller et al. 2014). FSA cells exposed under these same conditions were sensitized rather than protected by metformin. The overall response of FSA reflects both the direct toxicity of metformin on these cells, as well as an enhanced sensitization to 4 Gy of ionizing radiation. Metformin's effects on mitigating radiation sensitivity are not limited solely to its anti-oxidant properties, but appears to also be affected by NF κ B-mediated survival pathways as evidenced by the lack of a radiation sensitivity modifying effect in SA-NH cells stably transfected with a mutant I κ B α vector that inhibits NF κ B activation (Murley et al. 2004). Since p53 mutations are known to promote cancer progression by augmenting NF κ B activation in the context of chronic inflammation and enhance both proinvasive and prosurvival activities, inhibition of NF κ B activity in p53 mutant tumor cells should exhibit as profound effect or more than was observed in the p53 WT SA-NH tumor model (Cooks et al. 2013; Bellazzo et al., 2015). Prosurvival pathways associated with p53 mutational status and NF κ B activation play a determinant role in metformin's effects on radiation response.

The association of anti-oxidant properties exhibited by metformin and its subsequent modifying effects on radiation response prompted the use of other ROS modifying agents, the free radical scavenger NAC and the free radical generator drug emodin, to be administered in conjunction with metformin. Rotenone, a potent inhibitor of mitochondrial complex I, was used to investigate its comparative effectiveness with metformin as a radiation modifying agent. Surprisingly, neither NAC nor emodin under conditions shown to be effective in altering the very low radiation dose adaptive response in SA-NH and FSA tumor models (Miller et al. 2016) were observed to affect metformin's radiation sensitivity modifying effectiveness (see Fig. 4). Furthermore, rotenone treatment effectively mimicked the effectiveness of metformin used alone in protecting SA-NH but sensitizing FSA tumor cells. Neither NAC nor emodin has been reported to adversely affect mitochondrial complex I, while rotenone's effects are primarily attributed to its ability to inhibit this process (Green et al. 2011, Srinivas et al. 2007, Miller et al. 2016). These data suggest that NAC and emodin treatment are insufficient to alter significantly metformin's effectiveness by enhancing or inhibiting ROS production that results following the inhibition of mitochondrial complex I. This may reflect the inherent efficiency and levels of anti-oxidant enzymes present in the p53 wild type SA-NH cells, along with the associated prosurvival pathways that results in enhanced radiation resistance as compared to the deficit of these processes expressed in the p53 mutant FSA tumor cells. It has been reported that while metformin and rotenone both are effective in inhibiting mitochondrial complex I, they do so by acting on different sites of the complex (Wheaton et al. 2014). Mitochondrial complex I produces superoxide which is then converted quickly to hydrogen peroxide by superoxide dismutase activity. Metformin, in contrast to rotenone, has been reported not to increase these ROS species, while rotenone stimulates such ROS production. The lack of modifying effects of NAC and emodin on metformin's ability to alter radiation response, along with the similarity between rotenone

and metformin as radiation response modifiers for tumor cells differing in p53 status irradiated under relative homogenous aerated conditions *in vitro*, suggests that the primary process being affected is mitochondrial metabolism along with its downstream consequences.

Tumor growth delay studies were performed to further characterize the radiation survival modifying effects of metformin under more environmentally complex conditions. Metformin was observed to significantly sensitize SA-NH cells regardless if it was administered 1 h prior to or following exposure to ionizing radiation (See Fig. 5). This is in contrast to the protective properties expressed when irradiation and metformin exposure was performed under *in vitro* conditions as shown in Fig. 2. While SA-NH cells grown under *in vitro* conditions were uniformly well oxygenated and supplied with ample nutrients for growth, these cells grown in the flank of mice as solid tumors were exposed to heterogeneous environmental conditions including both oxygen and nutrient deprivation. Impairment of mitochondrial respiration by metformin under these conditions would result in greater stress and enhanced metabolically associated cell killing. In particular, metformin is effective in decreasing blood glucose levels. Consequently, as a result of limited glucose availability, mitochondrial ATP production would be inhibited resulting in subsequent cell sensitization and death (Weinberg et al. 2015). An alternative but not mutually exclusive hypothesis has proposed that inhibition of mitochondrial complex I would also result in a reduced rate of oxygen consumption in these cells resulting in an overall increase in oxygen diffusion and subsequent tumor oxygenation, e.g. a reduction in the degree of heterogeneity in oxygen tension experienced by a greater proportion of the tumor cells during irradiation (Zannella et al. 2013). This in turn could also result in an overall sensitization of the tumor to ionizing radiation. Under these *in vivo* conditions of heterogeneous environmental factors, metformin was also found to significantly sensitize FSa tumors when administered prior to exposure to ionizing radiation (see Fig. 6). Surprisingly, metformin was relatively ineffective in sensitizing FSa cells when administered following irradiation. Since defects in the cytochrome c oxidase mitochondrial complex IV have been associated with p53 mutant malignant cells, it may be that the metabolic stress affecting mitochondrial respiration in FSa cells immediately following irradiation was too severe to observe additional damage induced by the post-radiation treatment with metformin.

Metformin offers the promise of not only selectively sensitizing malignant cells to the toxic effects of radiation and chemotherapy, but also of serving as an effective chemopreventive and radio-protective agent in protecting dose limiting normal tissues from tumorigenesis and cell killing (Song et al. 2012, Miller et al. 2014). Complicating its repositioning as an effective cancer therapeutic agent is the diversity of effects attributed to its use. It has been described as both a ROS inducer and scavenger (Cheng et al. 2015, Algire et al. 2012, Picone et al. 2015), and its properties appear to be significantly affected by the p53 mutational status of the target cells (Muaddi et al. 2013, Buzzai et al. 2007). At the core of its activity is its ability to inhibit mitochondrial complex I and alter glucose availability for glycolytic respiration, thus altering downstream metabolic processes associated with subsequent cell growth, survival and death in both malignant and non-malignant cells.

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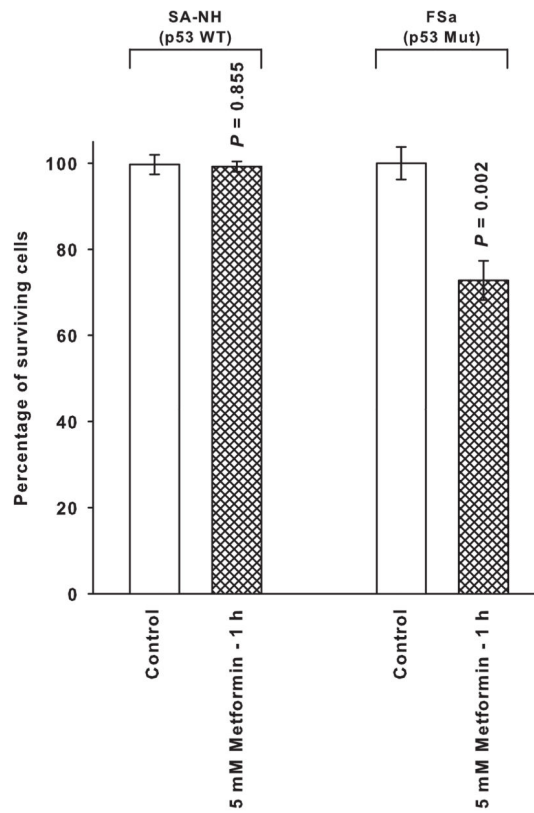
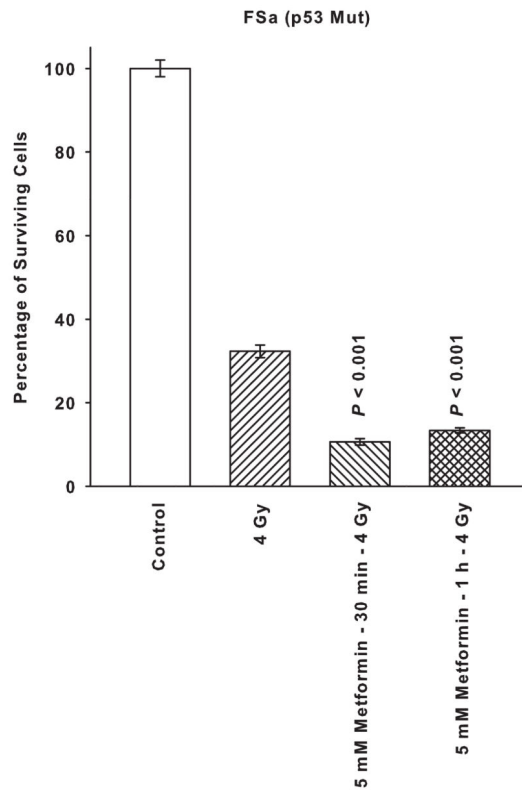


Figure 1.

The toxicity profile for metformin in p53 wild type (WT) SA-NH murine sarcoma and p53 mutant (Mut) FSa fibrosarcoma cells exposed to a 5 mM concentration for 1 h. Each experiment was performed three times and error bars represent the standard error of the mean (SEM). *P* values comparing the survival of metformin treated cells with those of their corresponding untreated controls are presented for comparison.



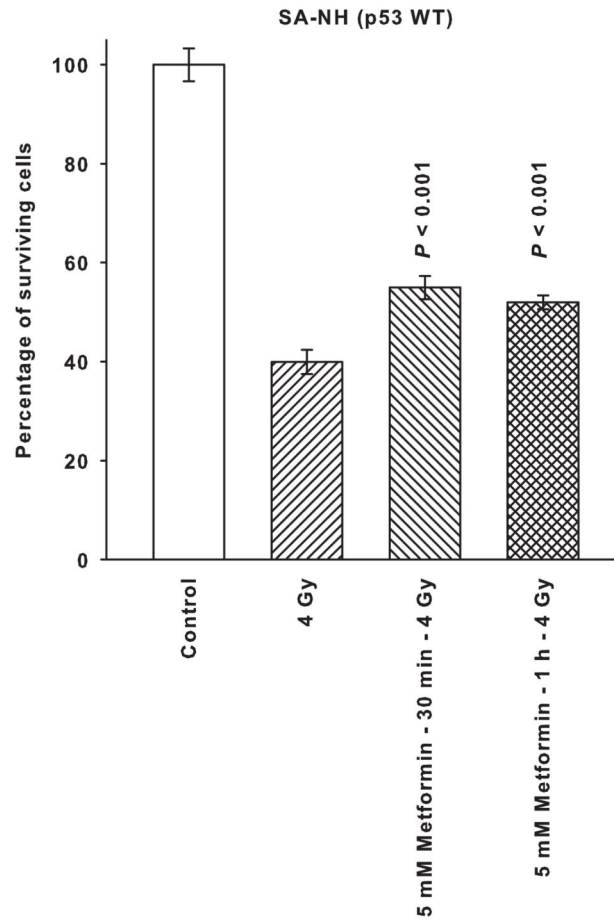


Figure 2.

The radiation response modifying effects of a 5 mM dose of metformin on SA-NH and FSa tumor cells following an exposure of 30 min or 1 h administered prior to irradiation with a single 4 Gy dose. Each experiment was repeated three times and error bars represent the SEM. *P* values comparing cell survival at 4 Gy only to those following treatment with metformin are presented for comparison.

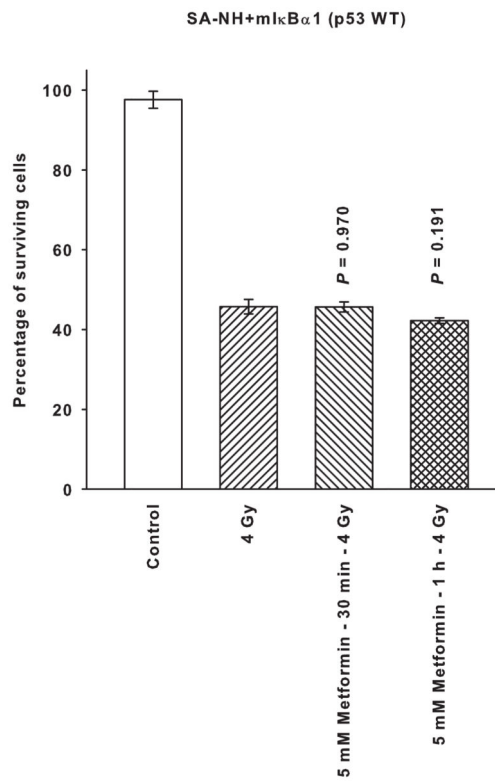
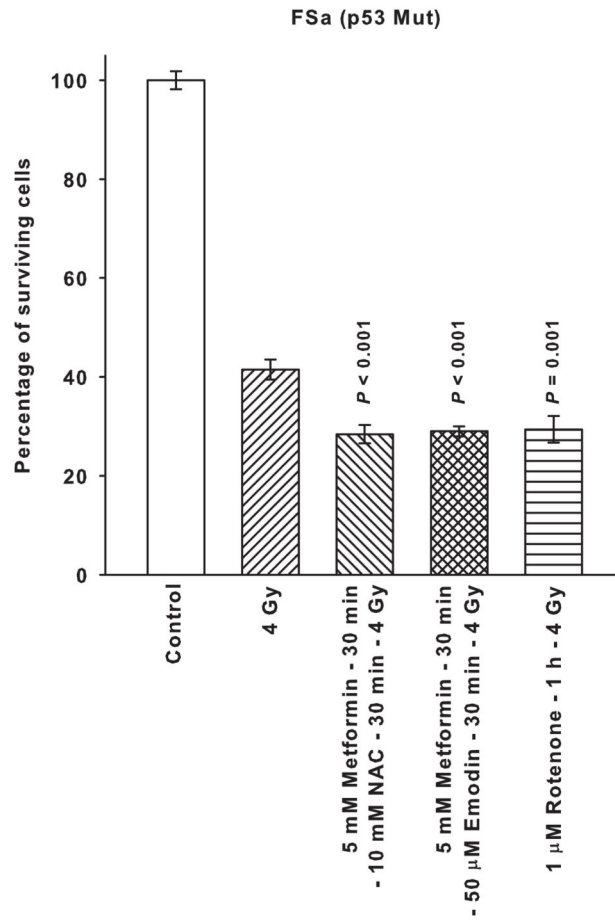


Figure 3.

The absence of a radiation response modifying effect following a 30 min or 1 h exposure of SA-NH cells stably transfected with a mutant I κ B α (SA-NH+mI κ B α .1) to a 5 mM dose of metformin is described. Each experiment was repeated three times and error bars represent the SEM. *P* values comparing cells treated with 5 mM of metformin for 30 min or 1 h prior to irradiation and cells exposed to 4 Gy only are presented for comparison.



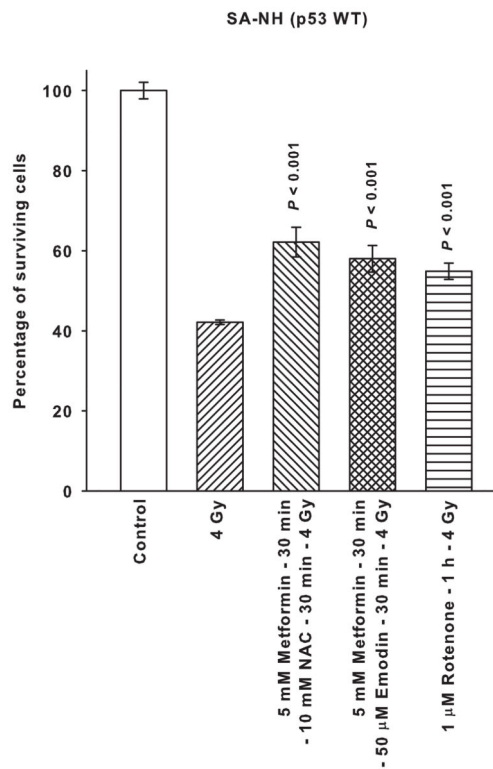


Figure 4.

The radiation response modifying effects of 5 mM metformin administered for 30 min followed by a 30 min exposure to 10 mM of the free radical scavenger N-acetyl-cysteine (NAC) or 50 μ M of the free radical generating drug Emodin are contrasted to those exerted by a 1 h exposure to the mitochondrial complex I inhibitor Rotenone (1 μ M) using p53 WT SA-NH and p53 Mut FSa. Surviving fractions were compared to corresponding radiation only treated cells. Each experiment was repeated three times and error bars represent the SEM. *P* values comparing radiation with metformin plus NAC, metformin plus Emodin, and Rotenone to that of radiation only are presented for comparison.

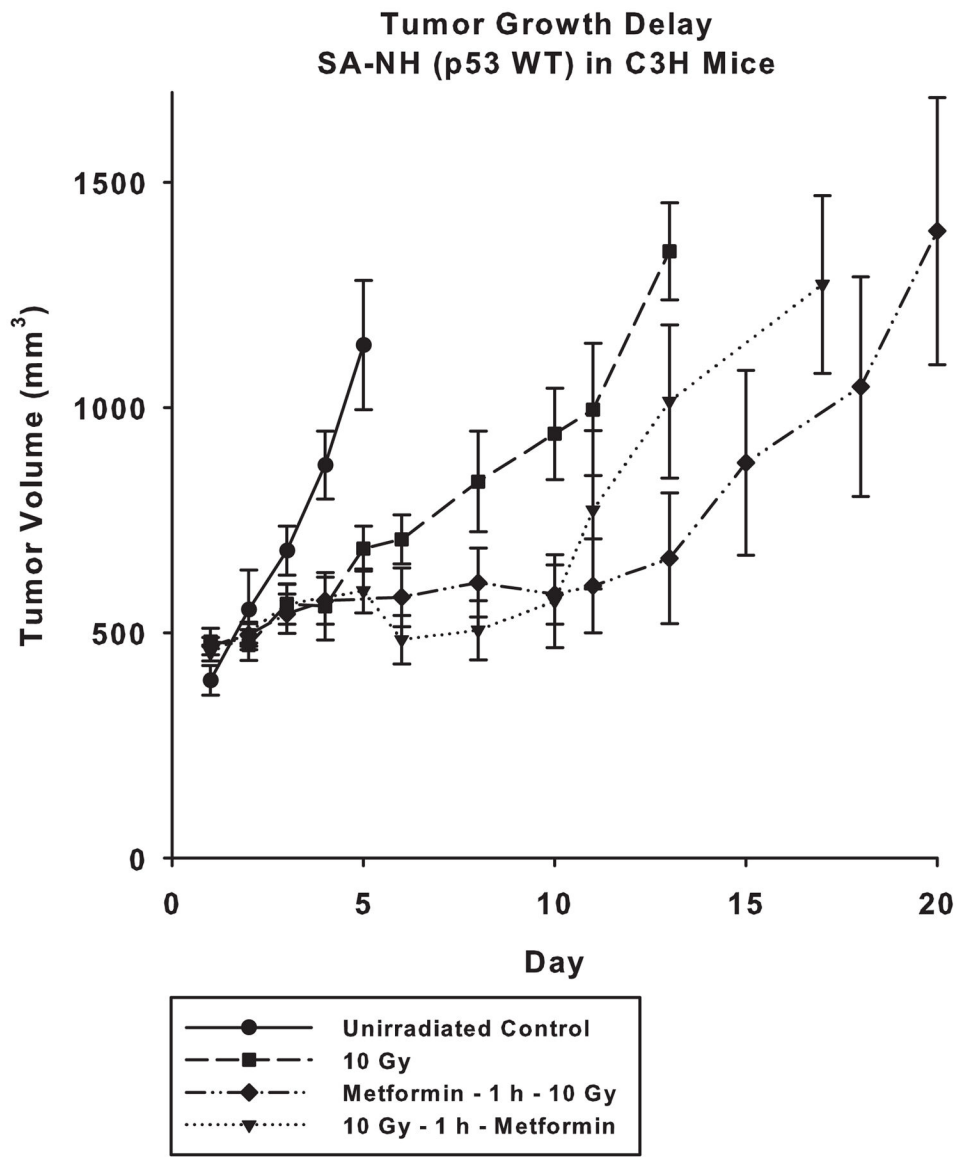


Figure 5. Tumor growth delay studies demonstrating the effects of a single 10 Gy dose alone or in combination with a 250 mg/kg dose of metformin administered i.p. either 1 h prior to or following irradiation of SA-NH tumors grown in the flanks of C3H mice. Treatment of tumors began when they reached an average size of approximately 250 mm³ and growth measurements ended after they exceeded 1000 mm³ in size. An untreated control group was also monitored for comparison. Each metformin plus radiation treated tumor group is compared to the radiation only group. Mean growth times and SEM were determined. Mean tumor volumes over time were compared among groups using repeated measures analysis of covariance (ANCOVA) assuming a quadratic model and accounting for separate variances in each group. A post-hoc pairwise comparison among groups was performed using the Tukey-Kramer method. Time to reach 1000 mm³ in size in each group was estimated using a Kaplan-Meier curve and compared between groups using the log rank test.

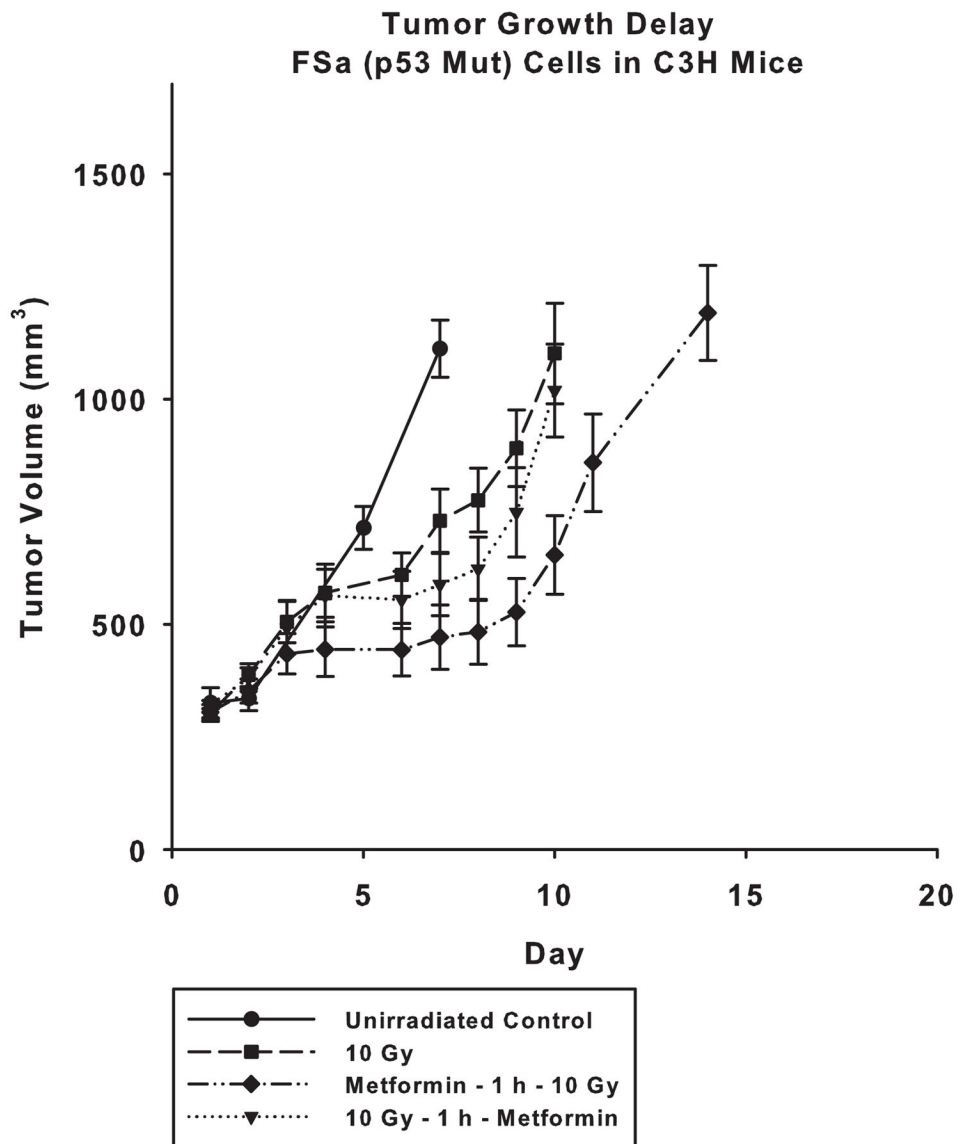


Figure 6. Tumor growth delay studies demonstrating the effects of a single 10 Gy dose alone or in combination with a 250 mg/kg dose of metformin administered i.p. either 1 h prior to or following irradiation of FSa tumors grown in the flanks of C3H mice. Treatment of tumors began when they reached an average size of approximately 250 mm³ and growth measurements ended after they exceeded 1000 mm³ in size. An untreated control group was also monitored for comparison. Each metformin plus radiation treated tumor group is compared to the radiation only group. Mean growth times and SEM were determined. Mean tumor volumes over time were compared among groups using repeated measures analysis of covariance (ANCOVA) assuming a quadratic model and accounting for separate variances in each group. A post-hoc pairwise comparison among groups was performed using the Tukey-

Kramer method. Time to reach 1000 mm³ in size in each group was estimated using a Kaplan-Meier curve and compared between groups using the log rank test.

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