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The role of copper in disulfiram-induced toxicity and radiosensitization of cancer cells

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Abstract

Disulfiram has been used for several decades in the treatment of alcoholism. It now shows promise as an anti-cancer drug and radiosensitizer. Proposed mechanisms of action include the induction of oxidative stress and inhibition of proteasome activity. Our purpose was to determine the potential of disulfiram to enhance the anti-tumor efficacy of external beam γ -irradiation and ^{131}I -metaiodobenzylguanidine (^{131}I -MIBG), a radiopharmaceutical used for the therapy of neuroendocrine tumors. **Methods:** The role of copper in disulfiram-induced toxicity was investigated by clonogenic assay after treatment of human SK-N-BE(2c) neuroblastoma and UVW/NAT glioma cells. Synergistic interaction between disulfiram and radiotherapy was evaluated by combination index analysis. Tumor growth delay was determined *in vitro* using multicellular tumor spheroids and *in vivo* using human tumor xenografts in athymic mice. **Results:** Escalating disulfiram dosage caused a biphasic reduction in the surviving fraction of clonogens. Clonogenic cell kill after treatment with disulfiram concentrations less than 4 μM was copper-dependent, whereas cytotoxicity at concentrations greater than 10 μM was caused by oxidative stress. The cytotoxic effect of disulfiram was maximal when administered with equimolar copper. Likewise, disulfiram's radiosensitization of tumor cells was copper-dependent. Furthermore, disulfiram treatment enhanced the toxicity of ^{131}I -MIBG to spheroids and xenografts expressing the noradrenaline transporter. **Conclusions:** The results demonstrate that (i) the cytotoxicity of disulfiram was

copper-dependent; (ii) molar excess of disulfiram relative to copper resulted in attenuation of disulfiram-mediated cytotoxicity; (iii) copper was required for the radiosensitizing activity of disulfiram and (iv) copper-complexed disulfiram enhanced the efficacy not only of external beam radiation but also of targeted radionuclide therapy in the form of ^{131}I -MIBG. Therefore disulfiram may have anti-cancer potential in combination with radiotherapy.

Keywords: disulfiram, copper, ^{131}I -metaiodobenzylguanidine, neuroblastoma, radiosensitizer

INTRODUCTION

Disulfiram (Antabuse), ~~an inhibitor~~ of aldehyde dehydrogenase (1), has been used for the treatment of alcoholism for several decades. This drug also has a variety of other actions which suggest that it has potential as an anti-cancer agent. These include the induction of oxidative stress (2,3), the generation of copper-dependent toxicity (2,4), proteasome inhibition (4-6), and NF- κ B inhibition (7,8). Despite its diverse range of pharmacological activities, prolonged treatment with disulfiram has negligible, reversible, adverse effects and is considered a safe drug (9). Disulfiram is currently undergoing clinical trials for the treatment of various cancers including melanoma, liver, lung and prostate cancer. To determine the potential application of this agent in cancer therapy, it is important to understand its mechanisms of action as well as its effects in combination with other therapeutic modalities.

Disulfiram-induced cytotoxicity has previously been reported to be mediated by oxidative stress (2,3) which may be enhanced by the presence of copper (2). Copper-binding drugs have been shown to inhibit proteasome activity (10) and generate reactive oxygen species (ROS) (11). Disulfiram chelates copper, and it may be the disulfiram-copper complex which is the toxic form of the drug (4). Many tumors contain elevated levels of copper (10) which may render them selectively susceptible to disulfiram-induced toxicity.

A potentially significant mechanism of disulfiram-induced cell death involves inhibition of proteasome activity. The proteasome degrades misfolded, superfluous or damaged proteins and controls many cellular processes involved in differentiation, proliferation, signal transduction, cell cycle progression and apoptosis. Proteasome activity is increased in cancer

cells compared with normal cells (12) and the proteasome inhibitor bortezomib is being used in the treatment of multiple myeloma. Disulfiram has been reported to inhibit proteasome activity and induce apoptosis selectively in cancer cells, but not normal cells (4). The copper-binding activity of disulfiram may also be involved in this mode of action as the formation of organic copper complexes appears to be responsible for pro-apoptotic proteasome inhibition (4). Other proteasome inhibitors (13), including the reduced form of disulfiram (diethyldithiocarbamate, DDC) (14), have radiosensitizing effects, and this may also apply to disulfiram, further increasing its potential as an anti-cancer agent.

For tumor types which are both radiosensitive and chemosensitive, such as neuroblastoma, combined chemotherapy and radiotherapy may offer the best prospects for a positive outcome to treatment. A feature of neuroblastoma is expression of the noradrenaline transporter (NAT), allowing targeted radiotherapy using a radiolabelled structural analogue of noradrenaline, ¹³¹I-metaiodobenzylguanidine (MIBG). This radiopharmaceutical has proven to be an excellent palliative and long-term remissions have been achieved following single-agent treatment (15). However, the optimal way to use ¹³¹I-MIBG has yet to be defined, and increasingly it is administered in combination with other treatments in attempts to exploit the expression of several different targets in the rapidly evolving cells of tumors. Future advances in ¹³¹I-MIBG therapy are expected to derive from concurrent administration of radiosensitizers. Drug resistance is also an important cause of failure in the treatment of neuroblastoma, possibly caused by increased P-glycoprotein expression after chemotherapy (16). Disulfiram inhibits P-glycoprotein-mediated drug

resistance (17), thereby increasing sensitivity to chemotherapeutic agents and this approach has been used successfully for treating pediatric tumors, including retinoblastoma and neuroblastoma (18). Having previously demonstrated synergy between the topoisomerase I inhibitor topotecan and ¹³¹I-MIBG (19,20), we now describe the use of similar methodology to test the potential synergistic effect of disulfiram with ¹³¹I-MIBG treatment.

We report an investigation of the extent to which disulfiram's ability to induce cell death occurred through copper- or ROS-dependent mechanisms. In the SK-N-BE(2c) and UVW/NAT cancer cell lines, clonogenic survival was decreased by disulfiram treatment and the presence of copper was necessary for tumor cell kill at low, clinically achievable, concentrations of disulfiram. Moreover, the molar ratio of disulfiram:copper was critical for cytotoxicity. Finally, a potent radiosensitizing effect of disulfiram was demonstrated *in vitro* and *in vivo* for the first time, and this also was copper-dependent.

MATERIALS AND METHODS

Reagents

Disulfiram (tetraethylthiuram disulfide) was purchased from Sigma-Aldrich (Dorset, UK) and Antabuse obtained from Actavis (Gentofte, Denmark). Stock solutions of both agents were prepared in dimethyl sulfoxide (DMSO), then diluted in culture medium (maximum DMSO concentration 0.1% v/v). All other drugs were dissolved in culture medium. All cell culture media and supplements were purchased from Invitrogen (Paisley, UK) and all other chemicals were from Sigma-Aldrich (Dorset, UK). No-carrier-added ¹³¹I-MIBG was prepared as described previously (20).

Cell Culture

Human neuroblastoma-derived SK-N-BE(2c) cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The UVW cell line was derived from a human glioblastoma (21). Cell lines were authenticated in-house using the AmpF/STR Identifier kit (Applied Biosystems, Warrington, UK). SK-N-BE(2c) cells were maintained in DMEM containing 15% (v/v) fetal calf serum (FCS). UVW cells were transfected to express the NAT gene as previously described (22), and were maintained in MEM, containing 10% (v/v) FCS and 1 mg/ml geneticin at 37°C in a 5% CO₂ atmosphere.

Clonogenic Survival Assay

Cells were seeded in 25 cm² flasks at 10⁵ cells/flask. When cultures were in exponential growth phase, medium was removed and replaced with fresh medium containing disulfiram, the cell impermeable copper chelator bathocuproine disulfonic acid (BCPD, 300 μM), the antioxidant *N*-acetyl-*L*-cysteine (NAC, 1mM), copper (II) chloride or various combinations of these agents were added simultaneously. Cells were incubated with drugs for 24 hours. Cells were γ -irradiated using an Alcyon II ⁶⁰Co-cobalt source at a dose-rate of 1 Gy/min, then incubated for 24 hours. For experiments conducted in serum-free conditions, cells were washed twice with PBS, before adding serum-free medium with or without drugs. After drug treatment, cells were seeded for clonogenic assay as previously described (19,20).

Spheroid Growth Assay

Multicellular spheroids were used for the evaluation of ^{131}I -MIBG treatment because beta-particle cross-fire irradiation makes a considerable contribution to its efficacy. This component of tumor cell kill would be under-represented in cellular monolayers (23). SK-N-BE(2c) and UVW/NAT cells were cultured as multicellular tumor spheroids using the liquid overlay technique (24). Briefly, spheroids were initiated by inoculating cells into an agar-coated flask. After 3 to 4 days, spheroids were retrieved by centrifugation and resuspended in fresh serum-free culture medium containing disulfiram and/or copper for 24 h. Simultaneously, ^{131}I -MIBG was added to the medium at a final concentration of 1 MBq/ml for SK-N-BE(2c) and 0.5 MBq/ml for UVW/NAT spheroids. After treatment, spheroids were washed twice and those of approximately 100 μm in diameter were transferred individually into agar-coated wells of 24-well plates. Individual spheroid growth was monitored twice per week using an inverted phase-contrast microscope connected to an image acquisition system. Two perpendicular diameters, d_{max} and d_{min} , were measured using image analysis software (ImageJ) and the volume, V (μm^3), was calculated using the formula: $V = \pi \times d_{\text{max}} \times d_{\text{min}}^2 / 6$ (25).

Combination Treatments

The cytotoxic interaction between disulfiram and radiation was examined using clonogenic assay, according to the method of Chou and Talalay (26). Initially, exponentially growing cells were treated with each agent alone to determine effective doses. Cells were subsequently treated with

a range of doses of disulfiram and γ -radiation, administered simultaneously, using a fixed dose ratio of disulfiram to γ -radiation, so that the proportional contribution of each agent in the mixtures would be the same at all treatment intensities. The fixed dose ratio was 0.1 μ M disulfiram:0.9 Gy γ -radiation, based on IC_{50} values of 0.34 μ M and 3 Gy, for disulfiram and γ -radiation, respectively. The effectiveness of combinations of disulfiram and γ -radiation was quantified by determining a combination index (CI) at various levels of cytotoxicity. As the method of interaction was unknown, CI values were calculated assuming both mutual exclusivity (where the drugs have similar mechanisms of action) and mutual non-exclusivity (where the drugs have dissimilar mechanisms of action). $CI < 1$, $CI = 1$, and $CI > 1$ indicate synergism, additivity, and antagonism, respectively.

Tumor Xenografts

Six-week-old female, congenitally athymic nude mice of strain CD1 *nu/nu* were obtained from Charles River plc (Kent, UK). *In vivo* experiments were carried out in accordance with the Animals (Scientific Procedures) Act 1986. Tumors in athymic mice formed from SK-N-BE(2c) and UVW/NAT cells express the NAT enabling active uptake of ^{131}I -MIBG. Subcutaneous tumor growth was established as previously described (19). Mice were used for experimental therapy when the SK-N-BE(2c) tumor volumes had reached approximately 500 mm³ or when UVW/NAT tumors had grown to approximately 60 mm³. To monitor potential toxicity, experimental animals were examined daily for signs of distress and weighed weekly. Mice were randomized into treatment groups, each consisting of 6 animals that received:

PBS solution (i.p. injection); 200 mg/kg disulfiram solution (suspension in water, by oral gavage); 10 MBq ^{131}I -MIBG (i.p. injection); 5 Gy γ -radiation or simultaneous administration of disulfiram and ^{131}I -MIBG or disulfiram and γ -radiation. The activity of ^{131}I -MIBG given to the mice was shown previously by us to induce significant delay of growth but incomplete sterilisation of UVW/NAT xenografts (19). Tumors were measured with callipers immediately before treatment and twice weekly thereafter. On the assumption of ellipsoidal geometry, diameter measurements were converted to an approximate tumor volume by multiplying half the longest diameter by the square of the mean of the two shorter diameters. Mice whose xenograft volume reached 1,900 mm³ were euthanized.

Statistics

Data are presented as means \pm standard error of the mean (SEM), unless otherwise stated, with the number of independent repetitions provided in the legend to each figure. Statistical significance was determined using Student's *t* test. A *P* value < 0.05 was considered to be statistically significant and < 0.01 highly significant.

RESULTS

Disulfiram's Cytotoxicity

Escalating concentrations of disulfiram caused a biphasic toxicity profile in SK-N-BE(2c) cells (Figure 1). Initial clonogenic kill was maximal after administration of 1.7 μM disulfiram. Increasing disulfiram beyond this concentration resulted in partial reversal of cytotoxicity, up to 10 μM

disulfiram. Thereafter, cytotoxicity increased and, following the administration of 17 μM disulfiram, clonogenic survival was 10% of untreated control levels (Fig. 1A). The effect on clonogenic survival of the clinical formulation of disulfiram used in animal studies, Antabuse (Actavis), was also evaluated. A concentration-dependency profile similar to that generated by disulfiram was observed (Fig. 1A).

A glioma cell line transfected with the NAT gene, UVW/NAT, responded in an analogous manner with respect to clonogenic cell kill in response to a range of concentrations of disulfiram (Fig. 1B). Again, the initial clonogenic survival nadir was observed following treatment with 1.7 μM disulfiram. A similar biphasic dose-toxicity profile was also observed when UVW/NAT cells were exposed to Antabuse (Fig. 1B).

Disulfiram's Biphasic Dose-Response

Treatment with 1 mM of the antioxidant NAC alone had no significant effect, but prevented the reduction in clonogenic survival of SK-N-BE(2c) cells and UVW/NAT cells induced by concentrations of disulfiram $> 10 \mu\text{M}$, whereas NAC had no significant effect on disulfiram-induced toxicity at concentration $\leq 10 \mu\text{M}$ (Fig. 1). Therefore, a ROS-independent mechanism of cell kill predominated at low concentrations of disulfiram ($\leq 10 \mu\text{M}$).

Although bathocuproine disulfonic acid (BCPD) alone had no significant effect, the disulfiram-induced reduction in clonogenic survival of SK-N-BE(2c) cells was prevented by BCPD, at 300 μM , in the first phase of the dose-response, up to and including 10 μM disulfiram, suggesting a role for copper in disulfiram-induced cytotoxicity at low concentrations. Although

UVW/NAT cells were more resistant to disulfiram, the initial, maximal, clonogenic cell kill observed following treatment with 1.7 μ M disulfiram was also prevented by BCPD (Fig. 2B).

Effect of Copper on Disulfiram's Cytotoxicity

In serum-free medium and in the absence of copper, disulfiram, at concentrations up to 10 μ M, induced no significant reduction in survival of either SK-N-BE(2c) or UUVW/NAT clonogens (Fig. 3), whereas the inclusion of copper chloride in the incubation medium resulted in dose-dependent clonogenic cell kill. This was maximal when disulfiram and copper concentrations were equimolar (Fig. 3). These observations suggest not only that the presence of copper is important for the cytotoxicity of disulfiram, but also that the concentration of copper relative to disulfiram is a major determinant of disulfiram's potency.

Copper-Dependence of Disulfiram's Radiosensitization

To investigate the potential for disulfiram to act as a radiosensitizer, SK-N-BE(2c) cells were γ -irradiated alone or in combination with disulfiram. **A radiation dose-response was clearly demonstrated with enhancement of the radiation-induced clonogenic kill by disulfiram at all radiation doses (Fig. 4). IC₅₀ values of 3.23 or 1.88 Gy, were observed in the absence or presence of disulfiram, respectively.** The effective doses of disulfiram and radiation were estimated from dose-responses of each agent alone. A constant dose ratio of 1:9 disulfiram (μ M): γ -irradiation (Gy) was used in the combination experiments. The combination index values were determined according to

Chou and Talalay (26) and are shown in Table 1. As the manner of the interaction between the agents was uncertain, the formula was solved in two different ways, corresponding to modes of action of the two agents which are similar or distinct. Synergistic kill of SK-N-BE(2c) clonogens after treatment with combined disulfiram and γ -irradiation was manifest by CI values < 1 for all dose intensities examined. For example, at the ED₃₀ (dose required to kill 30% of clonogens), CI values were 0.45 ± 0.12 or 0.68 ± 0.19 assuming mutually exclusive or independent modes of action, respectively. Corresponding CI values at the ED₅₀ were 0.62 ± 0.14 , and 0.70 ± 0.19 and, at the ED₉₀, 0.70 ± 0.15 , and 0.80 ± 0.2 . Increasing CI values suggest decreasing synergism at higher dose intensities. Nonetheless the observation of CI values < 1 throughout the toxicity range of the experimental combination therapy indicated synergy at all strengths of treatment.

The involvement of copper in the radiosensitizing action of disulfiram was investigated by clonogenic assay of cells treated with combinations of disulfiram and γ -radiation in the presence or absence of serum, BCPD or copper chloride. The clonogenic survival of γ -irradiated SK-N-BE(2c) cells was not significantly affected by removal of serum or incubation with BCPD or copper chloride. Clonogenic cell kill resulting from treatment with $0.34 \mu\text{M}$ disulfiram was prevented by removal of serum ($P < 0.05$) or inclusion of BCPD ($P < 0.05$), and enhanced by the addition of copper chloride ($P < 0.05$) (Fig. 5). Furthermore, the enhanced clonogenic kill induced by combined disulfiram and radiation treatment relative to single agent treatment was prevented by serum removal ($P < 0.05$) or addition of BCPD ($P < 0.05$). In

contrast, incubation with copper resulted in potentiation of the cell kill achieved by the disulfiram and radiation combination ($P < 0.05$).

Effect of Disulfiram and ^{131}I -MIBG on Spheroid Growth Delay

Copper chloride alone had no effect on the growth rate of spheroids composed of SK-N-BE(2c) or UVW/NAT cells, whereas disulfiram alone (at 33.7 μM) decreased the growth rate of SK-N-BE(2c) and UVW/NAT spheroids. The evaluation of a range of equimolar doses of disulfiram and copper indicated that treatment of spheroids with 3.37 μM induced growth delay but not sterilisation (data supplied but not shown - Supplemental Figure). Therefore 3.37 μM equimolar disulfiram/copper was the concentration used in subsequent evaluation of enhancement of cell kill by ^{131}I -MIBG targeted radiation.

Using relative spheroid volume on the last day of measurement as an index of efficacy, single agent ^{131}I -MIBG treatment reduced spheroid growth to 54 and 87% of control for SK-N-BE(2c) and UVW/NAT spheroids respectively. Similarly disulfiram/copper reduced growth to 69% and 58% of control volume for SK-N-BE(2c) and UVW/NAT spheroids, respectively. In contrast, combined treatment with ^{131}I -MIBG and disulfiram/copper sterilised all SK-N-BE(2c) and UVW/NAT spheroids – manifest as a failure to increase in size throughout the 20 or 22 days' duration of the experiments respectively (Fig. 6).

Antitumor Activity of Disulfiram and Radiation

The anti-tumor effect of disulfiram alone or in combination with

radiation treatment was studied *in vivo* using subcutaneous tumor xenografts grown in athymic mice. None of the animals in this study showed signs of distress. Alternative radiation modalities were examined – external beam γ -rays from a ^{60}Co -cobalt source and the NAT-seeking radiopharmaceutical ^{131}I -MIBG administered by intraperitoneal injection.

Although disulfiram alone had no significant effect on tumor growth, the growth inhibitory effect of γ -radiation or ^{131}I -MIBG was enhanced by combination treatment with disulfiram in both tumor types (Fig. 7). The times after treatment for 4-fold (SK-N-BE(2c)) or 10-fold (UVW/NAT) increase in tumor volume (τ_4 or τ_{10}) are shown in Table 2. These results indicate that combination treatment with disulfiram and radiation produced a greater tumor growth delay than either agent alone, and this was apparent in 2 different xenograft models, and using 2 different sources of radiation.

DISCUSSION

In response to treatment of neuroblastoma and glioma cells with disulfiram, we observed a biphasic pattern of clonogenic cell kill. Similar dose-response patterns have been reported in disulfiram-treated myeloma cells (27), in murine leukemia cell lines incubated with the reduced form of disulfiram (DDC) (28) and in pheochromocytoma cells treated with the structurally-related compound pyrrolidine dithiocarbamate (PDTC) (29).

It has been suggested that many of the effects of disulfiram are due to its ability to induce oxidative stress. For example, disulfiram depleted antioxidant levels in rat cortical astrocytes (2) and elevated ROS levels in melanoma cells (3). Moreover, disulfiram's toxicity to melanoma cells was

prevented by exogenous antioxidants (3). In the present study, the antioxidant NAC prevented the reduction in clonogenic survival induced only by high concentrations ($> 10 \mu\text{M}$) of disulfiram, but had no effect on disulfiram-induced toxicity at lower concentrations. Clinical studies have indicated that after a single oral dose of 500 mg, or repeated doses of 250 mg, plasma concentrations of disulfiram were less than $2 \mu\text{M}$ (30, 31). Therefore, a ROS-independent mechanism is expected to predominate at clinically achievable concentrations of disulfiram.

PDTC is structurally similar to disulfiram and has been shown to cause serum-dependent apoptosis of breast tumor cells (32). We also observed that the presence of serum was necessary for disulfiram-induced toxicity at low ($< 10 \mu\text{M}$) disulfiram concentrations. The toxicity of some metal-chelating compounds, including disulfiram, may be dependent on the presence of metal ions such as copper (33), and high copper concentrations have been noted in commercially prepared FCS (34). Therefore, it is possible that the presence of copper in serum was, at least partly, responsible for the serum-dependent disulfiram toxicity.

Disulfiram-induced toxicity to rat astrocytes was prevented by the cell-impermeable copper chelator BCPD (2). We also observed the inhibition of clonogenic cell kill, at disulfiram concentrations less than $10 \mu\text{M}$, by the inclusion in the medium of BCPD, suggesting a role for copper in disulfiram-induced cytotoxicity. At concentrations of disulfiram greater than $10 \mu\text{M}$, the ROS-dependent cytotoxic effect predominated. Similarly, PDTC-induced apoptosis of breast cancer cells was also suggested to be a result of its copper binding activity (35).

The reduced potency of disulfiram resulting from BCPD treatment was similar to that observed in response to incubation with serum-free medium, suggesting that serum in the medium was sufficient and necessary for the disulfiram-induced toxicity. The inclusion of exogenous copper in the incubation medium was sufficient to reinstate the toxic effect of disulfiram in the absence of serum, whereas BCPD, in molar excess relative to disulfiram, prevented the toxic effect of copper, presumably by competing with disulfiram for extracellular copper binding.

Clonogenic cell kill was maximal when disulfiram and copper concentrations were equimolar. Increasing concentration of disulfiram in molar excess of copper progressively reduced the cytotoxic effect of the disulfiram-copper complex. This suggests that the concentration of copper relative to disulfiram is crucial with respect to cytotoxicity.

The increased potency of disulfiram observed in the presence of serum may also be explained by copper chelation. Serum contains approximately 15 μM copper (36). Therefore cells cultured in 10 or 15% (v/v) serum would be exposed to 1.5 or 2.3 μM copper. In serum-containing medium, the greatest cell kill resulting from disulfiram treatment occurred at 1.7 μM . This observation is consistent with the requirement for copper chelation for optimal effectiveness of disulfiram. It also suggests that there would be sufficient copper in human and murine serum to allow the formation of effective levels of the disulfiram-copper complex.

Similarly, it has been observed that the toxicity of disulfiram to *Salmonella typhimurium* was increased by the elevation of copper sulphate levels to equimolar concentration, but was decreased in the presence of excess

disulfiram (37). The presence of surplus disulfiram may result in competition between free disulfiram and disulfiram-copper complexes for cellular uptake or intracellular binding. This competition for targets may then explain the effect of increasing survival observed with increasing disulfiram concentration above copper concentration.

The dithiocarbamates disulfiram and PDTC have been reported to be effective inhibitors of proteasome activity (5) especially when complexed with copper (6). Because the proteasome plays a pivotal role in the control of cell cycle progression and survival after potentially toxic insult, proteasome inhibitors are regarded as promising anti-cancer agents. They can induce apoptosis as a consequence of the accumulation of undegraded proteins and the prevention of NF- κ B activation. Disulfiram also facilitates copper uptake (33), increasing its intracellular availability. Therefore, it appears that disulfiram has potential as an anti-cancer agent. In particular, the absence of anti-tumor effect by disulfiram administered as a single agent as opposed to its enhancement of radiotoxicity suggests that disulfiram's efficacy should be characterized by tumor specificity and minimal damage to tissues which fail to accumulate radiopharmaceutical.

It has been suggested that proteasome inhibitors may be effective radiosensitizers (13). Therefore it is possible that disulfiram or its metabolites have radiosensitizing properties. Indeed, DDC has previously been shown to enhance radiation sensitivity in rapidly proliferating cells (14). We observed that disulfiram acted synergistically with γ -radiation to potentiate clonogenic cell kill at all levels of toxicity. The clonogenic survival of cells exposed to γ -radiation was not affected by removal of serum or the addition of BCPD or copper(II) chloride, indicating that radiation-induced clonogenic cell kill was

not copper-dependent. Conversely, clonogenic cell kill by disulfiram at 0.34 μM , a concentration previously shown to inhibit proteasome activity in cell-based assays (5), was prevented by removal of serum or addition of BCPD and enhanced by copper supplementation. Moreover, the enhanced clonogenic cell kill resulting from combined disulfiram and γ -radiation treatment was prevented by serum removal or incubation with BCPD, whereas the addition of copper reinstated the toxicity of γ -radiation combined with disulfiram in serum-free conditions. This suggests that copper was necessary for radiosensitization by disulfiram.

We have demonstrated *in vitro*, and for the first time *in vivo*, that disulfiram acted as a radiosensitizer. The effect of disulfiram alone was less appreciable in spheroids compared with monolayers. Resistance associated with aggregates of cells is a commonly observed effect and has been attributed to intercellular linkage, interactions between cell and matrix and limited penetration resulting in gradients of oxygen, nutrients and drugs (38). Disulfiram did, however, enhance the radiation-induced delay of the growth of spheroids derived from two different tumor cell lines and retarded the growth of two human tumor xenografts in athymic mice. Not only did disulfiram enhance the anti-tumor efficacy of γ -radiation delivered from an external source, but it also potentiated the efficacy of ^{131}I -MIBG targeted radionuclide therapy, in both spheroid and xenograft models. Significantly, the synergistic activity *in vivo* was achieved at a disulfiram dosage which had negligible effect on xenograft tumor growth and which has previously been demonstrated to be associated with insignificant side effects (9). Such features are highly desirable

in a radiosensitizing agent (39). The positive interaction between disulfiram and ^{131}I -MIBG is worthy of further investigation.

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FIGURE LEGENDS

FIGURE 1. Cytotoxicity of disulfiram is biphasic with respect to dose. Clonogenic survival after exposure of (A) SK-N-BE(2c) cells or (B) UVW/NAT cells to disulfiram for 24 h in the absence or presence of NAC, or to the clinical formulation of disulfiram (Antabuse); $n = 4$. Data are means \pm SEM; significance of differences: * $p < 0.05$, ** $p < 0.01$ from untreated control, † $p < 0.05$ from disulfiram alone. Abbreviations: DSF (disulfiram) and NAC (*N*-acetyl-*L*-cysteine).

FIGURE 2. Copper is necessary for toxicity of disulfiram at concentrations $< 4 \mu\text{M}$. Clonogenic survival after exposure of (A) SK-N-BE(2c) cells or (B) UVW/NAT cells to disulfiram for 24 h in the absence or presence of BCPD; $n = 5$. Data are means \pm SEM; significance of differences: * $p < 0.05$, ** $p < 0.01$ from untreated control, † $p < 0.05$ from disulfiram alone. Abbreviation: BCPD (bathocuproine disulfonic acid).

FIGURE 3. Clonogenic cell kill is dependent on the relative concentrations of disulfiram and copper. Clonogenic survival after exposure of (A) SK-N-BE(2c) cells or (B) UVW/NAT cells to disulfiram for 24 h in the absence or presence of CuCl_2 in serum-free medium; $n = 4$. Data are means \pm SEM; significance of differences from untreated controls: * $p < 0.05$, ** $p < 0.01$.

Figure 4. Effect of disulfiram on radiosensitivity of SK-N-BE(2c) cells. Clonogenic survival was determined after exposure of cells to γ -radiation alone or simultaneously with 0.34 mM disulfiram (n=4) after correcting for plating efficiency and disulfiram cytotoxicity alone. Data are means \pm SEM. Abbreviation: DSF (disulfiram).

FIGURE 5. Enhancement of radiation cell kill by disulfiram requires copper.

Clonogenic survival after exposure of SK-N-BE(2c) cells to 0.34 μ M disulfiram, 3 Gy γ -radiation, or a combination of both treatments in (1) serum-containing medium, (2) serum-containing medium with 300 μ M BCPD, (3) serum-free medium, or (4) serum-free medium containing 1 μ M CuCl_2 ; n=3. Data are means \pm SEM; significance of differences: * $p < 0.05$, ** $p < 0.01$ from untreated control, † $p < 0.05$, †† $p < 0.01$ from γ -irradiation alone. Abbreviations: DSF (disulfiram) and BCPD (bathocuproine disulfonic acid).

FIGURE 6. Disulfiram enhances the delay of spheroid growth induced by ^{131}I -MIBG. Multicellular spheroids derived from (A) SK-N-BE(2c) or (B) UVW/NAT cells were treated with disulfiram + CuCl_2 (3.37 μ M), ^{131}I -MIBG (1 MBq/ml for SK-N-BE(2c), 0.5 MBq/ml for UVW/NAT), or a combination of both. Data are expressed as mean spheroid volume at every time point divided by original volume (V/V_0) \pm SD of 13 to 23 spheroids per treatment. Abbreviation: DSF (disulfiram).

FIGURE 7. Disulfiram enhances radiation-induced growth delay of experimental tumors. Growth of human tumor xenografts in athymic mice exposed to PBS, disulfiram alone (200 mg/kg), γ -radiation (5 Gy), ^{131}I -MIBG (10 MBq), or combinations of disulfiram with γ -radiation or ^{131}I -MIBG. Xenografts were derived from (A) SK-N-BE(2c) or (B) UVW/NAT cells. Data are expressed as mean tumor volume at every time point divided by original volume (V/V_0) \pm SD. Abbreviation: DSF (disulfiram).

TABLE 1
The effect of disulfiram and γ -radiation on combination indices

Effect level	Mutually exclusive	Mutually non-exclusive
ED ₃₀	0.45 ± 0.12	0.68 ± 0.19
ED ₄₀	0.61 ± 0.14	0.69 ± 0.19
ED ₅₀	0.62 ± 0.14	0.70 ± 0.19
ED ₇₀	0.64 ± 0.14	0.73 ± 0.20
ED ₉₀	0.70 ± 0.15	0.80 ± 0.21

Combination index values are means ± SEM of 4 experiments.

TABLE 2

The effect of single agent or combination treatment on tumor growth

Treatment	τ_4 - SK-N-BE(2c) (days)	τ_{10} - UVW/NAT (days)
PBS	20.7	17.4
DSF [†]	25.5	18.9
γ -radiation	34.2	27.9
¹³¹ I-MIBG	39.1	31.5
DSF + γ -radiation	54.0	45.3
DSF + ¹³¹ I-MIBG	57.6	47.2

The effect of treatment on the time taken for 4-fold or 10-fold increases (τ_4 or τ_{10}) in volume of tumors derived from SK-N-BE(2c) and UVW/NAT cells respectively. Data are means of 6 xenografts per treatment.

[†]Abbreviation: DSF (disulfiram).

FIGURE 1

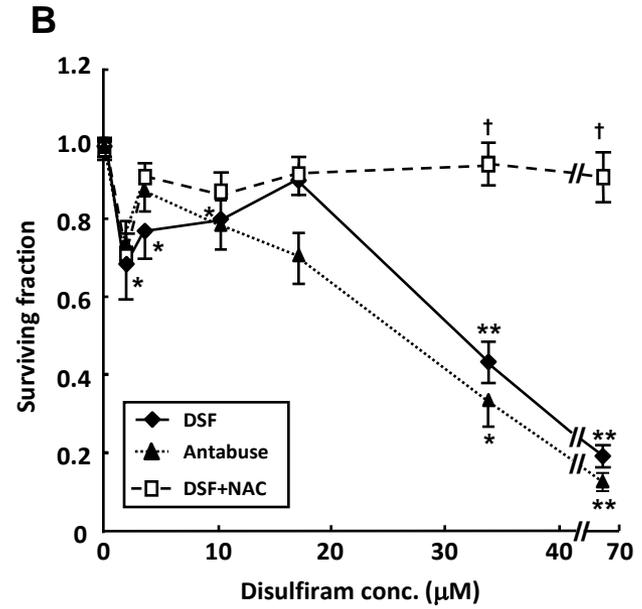
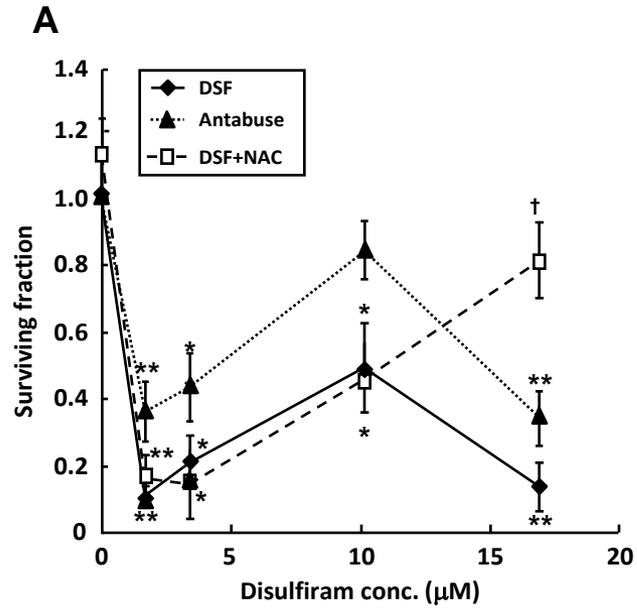
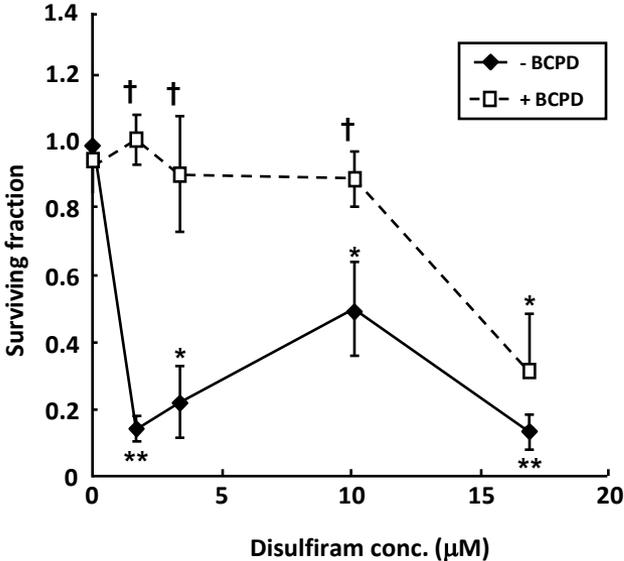


FIGURE 2

A



B

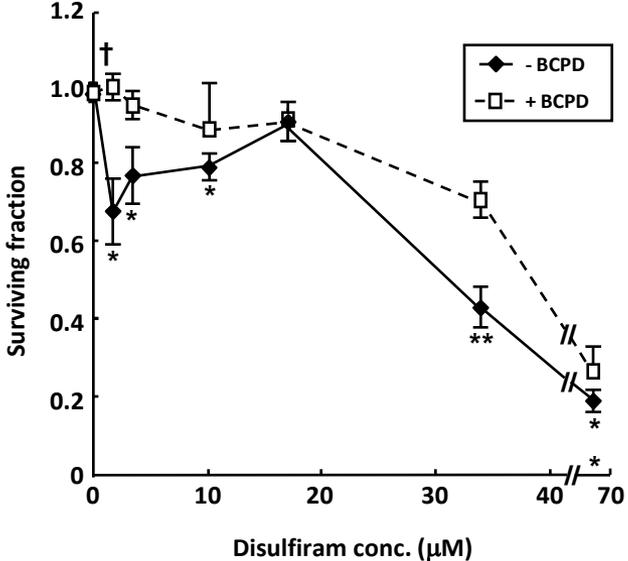


FIGURE 3

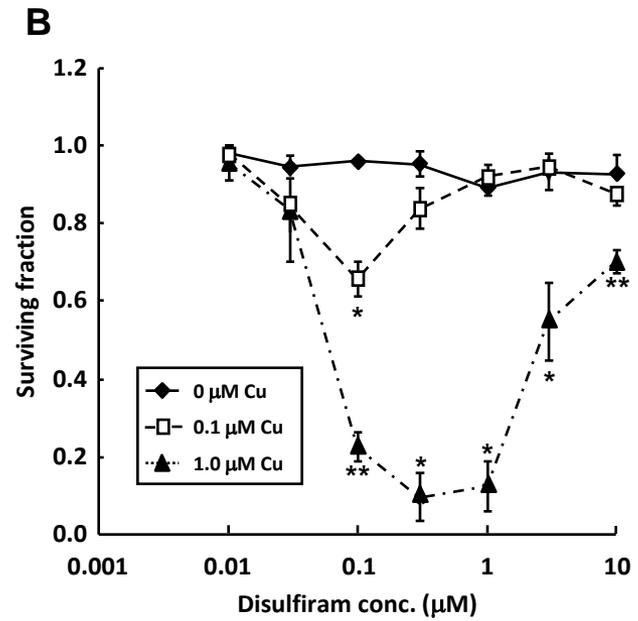
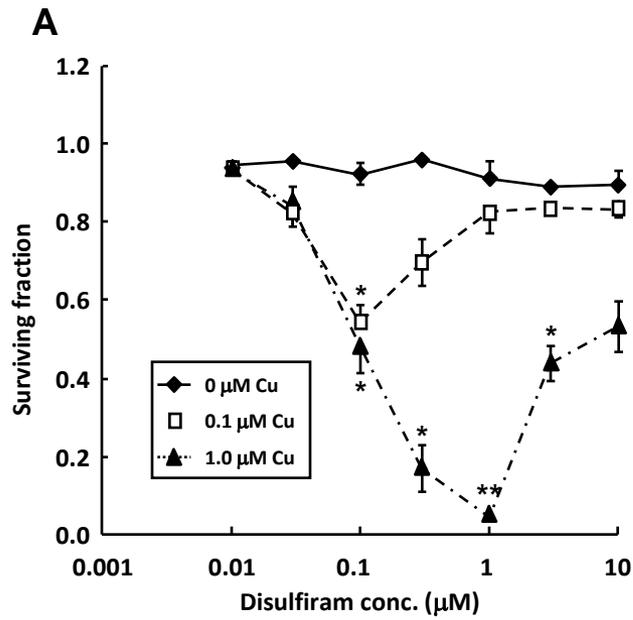


FIGURE 4

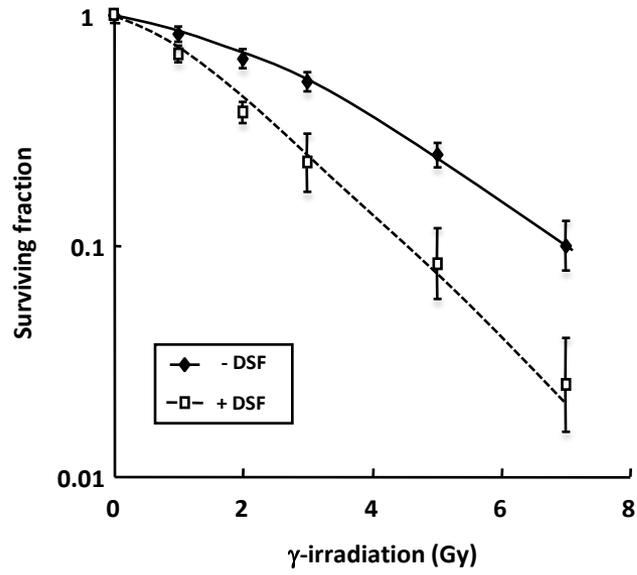


FIGURE 5

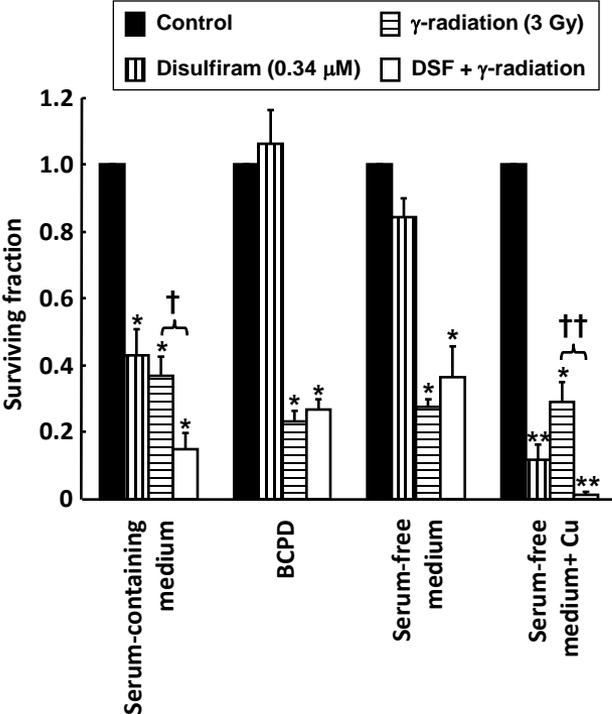


FIGURE 6

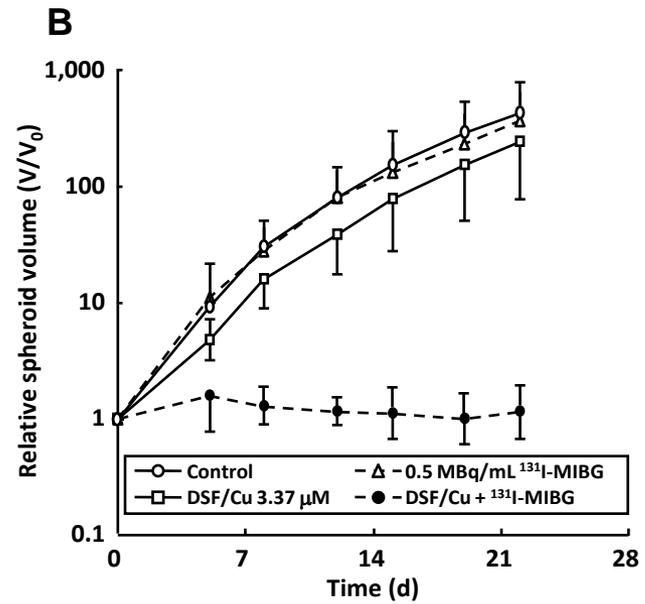
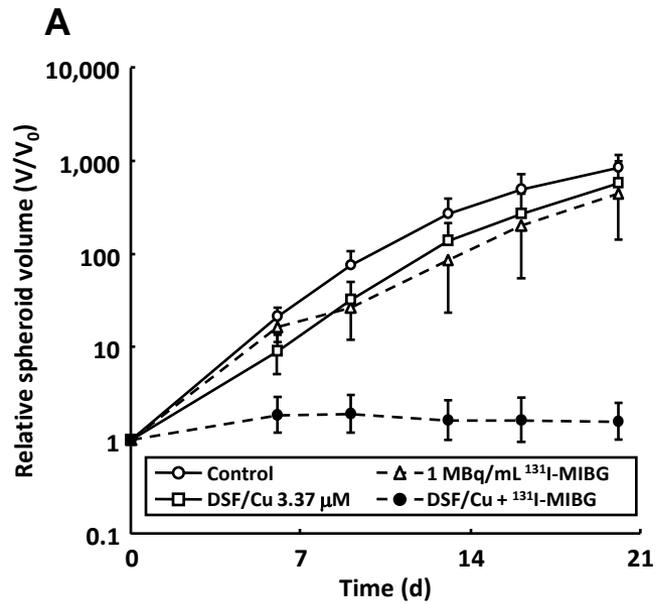


FIGURE 7

